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High frequency plant regeneration from *Eucalyptus globulus* Labill. hypocotyls: Ontogenesis and ploidy level of the regenerants

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Abstract

Up to 70% regenerating hypocotyls provided with 5 to 20 buds were obtained on MS medium containing 0.01 or 1 mg l^{-1} NAA and 0.2 mg l^{-1} BA or 0.2 mg l^{-1} BA and 0.2 mg l^{-1} TDZ. The ability to regenerate buds was correlated with the presence of oil glands at a stage in germination when oil secretion was not yet occurring. The regeneration of shoot meristems took place from the cells involved in the differentiation of these glands. Such glands could also appear during callus redifferentiation, giving rise to indirect regeneration. Rooting of the regenerants was efficient using a two-step procedure of induction under darkness in the presence of 3 mg l^{-1} IBA, followed by root development on medium devoid of growth regulators under a 16-h photoperiod, the medium being solidified with Gelrite. Regenerated plants showed no phenotypic alterations. Nuclear DNA contents in mother-plant material and regenerants were analysed using flow cytometry. There was no evidence of polyploidy in any of the samples, indicating the absence of polyploidisation during cell differentiation and under *in vitro* conditions. No regeneration was obtained from leaf or stem explants from micropropagated plantlets.

Abbreviations: BA – benzyladenine; CH – casein hydrolysate; IBA – indole butyric acid; MS – Murashige and Skoog; NAA – α-naphthalene acetic acid; TDZ – thidiazuron; WPM – Woody Plant Medium

Introduction

Eucalyptus are fast-growing trees originating from Australia and are represented by about 800 species and hybrids, some of them being commercially important for timber, essential oil and pulp (Gupta and Mascarenhas, 1983). Several biotechnological approaches have been developed to circumvent the difficulties of eucalypt breeding due to variability of propagation by seeds and lack of extensive genetic selection combined with the long juvenile period to reach the flowering period (Gupta and Mascarenhas, 1987; McComb, 1994). Although successful results for *in vitro* cloning have been reported for some species (McComb and Bennett, 1986), the rooting phase is frequently a critical and limiting step (Hartney, 1980). In addition, bud regeneration through *in vitro* culture of excised tissues or organs is also difficult to control for most species of eucalypts. Except for the few cases in which regeneration was achieved from mature organs such as *E. citriodora* leaves (Muralidharan and Mascarenhas, 1987), leaves and stems excised from *E. gunnii* micropropagated plantlets (Teulières and Boudet, 1992), stem and leaf callus of *E. tereticornis* (Subbaiah and Minocha, 1990) and leaves of micropropagated *E. grandis* (Lainé and David, 1994), most of the successful results were obtained from very juvenile organs such as excised hypocotyls or cotyledons. Bud regeneration from juvenile organs was reported for *E. gunnii* (Adam et al., 1992), *E. marginata*, *E. diversicolor* (Bennett and McComb, 1982), *E. camaldulensis* (Diallo and Duhoux, 1984) and *E. nova-anglica* (Mehra-Palta, 1982) cotyledons and *E. alba* hypocotyls (Kitahara and Caldas, 1975). Very few regenerants were also described from *E. globulus* hypocotyls (Oka et al., 1982).

It appears from the literature that important differences in the ability of the various eucalypt species to regenerate buds were observed and that this ability remained very limited, according to most of the published reports. In this paper, we present a rapid and efficient procedure for regeneration of *E. globulus*, a species particularly interesting for the pulp industry in Southern Europe, Australia, China, and so on. Starting from excised hypocotyls, the best conditions leading rapidly to numerous regenerants were defined. Protocols for efficient rooting and acclimatization were also established. In addition, the competent cells for regeneration were identified and the ploidy levels of the regenerants were determined using flow cytometry.

Materials and methods

Plant material and in vitro *culture*

Seeds and clones of micropropagated plantlets of *Eucalyptus globulus* Labill. were provided by J. Oller and G. Toval (E.N.C.E., Pontevedra, Spain). Sterile seedlings were obtained from seeds sterilized with 70% ethanol (v:v) for 5 min and subsequently in a 7% (w:v) calcium hypochlorite (70% active chlorine) solution containing 0.1% Teepol (v:v) for 20 min. They were then rinsed several times in sterile distilled water. The seeds were germinated on Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962; SIGMA, St Louis, USA, ref. M-5519) supplemented with 3% sucrose (w:v) and solidified with 0.7% agar (w:v) (SIGMA, ref. A-1296). The micropropagated clones (327-20-GR and 334-I-AR) were established from apical internodes excised from plants grown under greenhouse conditions. They were subcultured every three weeks on multiplication medium [MS salts added with White's vitamins (White, 1943), 2% sucrose (w:v), 0.01 mg l^{-1} NAA and 0.1 mg l^{-1} BA] and had been grown *in vitro* for one year at the beginning of our experiments. All the media were adjusted to pH 5.6 $\pm$ 0.1 with 1N KOH before autoclaving for 20 min at 120 °C. The seedlings and vitroplantlets were grown in Petri dishes (100 $\times$ 15 mm) and in jars (120 $\times$ 120 mm), respectively, at 25 ° $\pm$ 1 °C day and 21 ° $\pm$ 1 °C night temperatures under a 16 h daily photoperiod. Irradiance was 70 μmol m^{-2} s^{-1} from Sylvania Grolux F36W tubes.

For bud regeneration, cotyledons and hypocotyls excised from seedlings and leaves and internodes excised from the micropropagated clones were laid onto MS or WPM (Woody Plant Medium, Lloyd and McCown 1981; SIGMA, ref. M-6774) media supplemented with various combinations of NAA (0.01 to 1 mg l^{-1}) and/or BA (0.2 to 1 mg l^{-1}). Thidiazuron (TDZ; Schering SA, Berlin) was also used alone (0.05 to 5 mg l^{-1}) or combined with 0.2 mg l^{-1}BA. Casein hydrolysate (CH) (0.1 to 10 g l^{-1}) was added in some experiments. Each Petri dish contained 40 explants and the conditions of culture were as described above. For rooting, individual regenerants were isolated and transferred to tubes containing 15 ml of half strength MS basal salts supplemented with White's vitamins, 2% (w:v) sucrose, 1 to 3 mg l^{-1} IBA and either 0.6% agar (w:v) or 0.3% Gelrite (w:v) (Phytagel, SIGMA, ref. P-8169). After 3 to 7 days of induction under darkness, the microcuttings were transferred under a 16-h photoperiod on the same basal medium without growth regulators for root elongation. Acclimatization of the rooted microcuttings was performed in a greenhouse (16-h photoperiod with a 25/18 $\pm$ 1 °C day/night temperatures), using sterlux compost (Barbin S A, Rungis, France; ref. 2304) and covering the plantlets with plastic tunnels for two weeks.

Histological analyses

Samples for paraffin embedding were fixed for 24 h with a 95 ° ethanol:35% formalin:acetic acid mixture (F. A. A. 85:10:5, v:v:v), then washed, dehydrated and embedded into paraffin. The 6 μm sections were stained with methyl green-pyronin (Lison, 1960) in order to detect areas with intense meristematic activity. Semi-thin sections were also prepared from material fixed with 4% glutaraldehyde in Na cacodylate buffer (pH 7) for 2 h, then washed in the same buffer, post-fixed with 1% OsO_4-cacodylate buffer and dehydrated for Araldite embedding. Semi-thin sections were cut with a LKB ultramicrotome fitted with a Diatom diamond knife, then stained with 27% basic fuchsin - 73% toluidine blue dissolved in 30% ethanol. All the sections were finally examined with an Axioscop Zeiss light microscope.

Flow cytometry

Quantification of nuclear DNA contents in the various parts of the mother plants and in the regenerants was assessed by flow cytometry (Service of Cytometry, CNRS, Gif-sur-Yvette, France). Isolation of nuclei was carried out by mechanical chopping with a razor blade into Bergounioux's et al. buffer (1988) with the addi-

tion of 1 μl ml^{-1} ß-mercaptoethanol and 5 μg ml^{-1} bisbenzimide (Hœchst 33342, Sigma) for ploidy analysis or ethidium bromide for genome size determination. The extracted nuclei were then filtered through a 30 μm nylon mesh and analyzed with an Epics V cytofluorimeter (Coulter, Hilaeah, FL, USA) fitted with an argon ion laser (Spectra-Physics, Les Ulis, France) using 60 mW at 351–364 nm. The blue emission of the DNA-fluorochrome complex was collected through 418 nm long wave pass and 530 nm short wave filters. Chicken erythrocytes (2C = 2.33 pg) were used as internal reference (Marie and Brown, 1993). Ten thousand nuclei were analyzed per sample. Genome size estimation was based on the fluorescence histogram by comparing the mean position of the peak of plant nuclei with the mean peak position of the internal reference.

Results

Determination of efficient procedures for bud regeneration

Attempts to regenerate buds from either excised hypocotyls or cotyledons of 2 to 21 days-old seedlings or from mature leaf or stem explants excised from *in vitro* plantlets grown on multiplication medium were performed on a range of MS or WPM media containing various combinations of growth regulators.

Conditions leading to regeneration of at least one bud from either hypocotyls or cotyledons are presented in Table 1. All the other conditions tested yielded no buds. Only 8 to 15-days-old cotyledons or hypocotyls were competent for regeneration; younger and older organs were less able or unable to give rise to buds. The effective stage corresponded to seedlings having elongated hypocotyls and fully expanded cotyledons but no leaves. The best medium leading to more than 50% explants (around 70% for hypocotyls and 55% for cotyledons) producing more than 5 buds (up to 20 for hypocotyls and to 10 for cotyledons) after 3 weeks consisted of MS medium containing 0.2 mg l^{-1} BA and 0.2 mg l^{-1} TDZ (Figure 1 a–e). In this condition, bud initiation on hypocotyls occurred directly without extensive callus formation at the distal and proximal ends, with most buds at the distal end (Figure 1b–d). In the presence of 0.2 mg l^{-1} BA and 0.01 mg l^{-1} NAA, regeneration occurred in a more indirect pathway through callus formation. Neoformed buds from excised cotyledons appeared mainly at the wound region (Figure 1a). In both hypocotyls and cotyledons, the development of buds became rapidly heterogeneous, one of them being dominant over the others (Figure 1d). Suppression of the dominant bud activated the development of the others, including the very small ones. Caulogenic calluses excised and transferred on medium without growth regulators gave rise to new generations of buds (Figure 1e). Explants excised from either leaf pieces or internodes of *in vitro* plantlets were totally incompetent for bud regeneration, whatever the conditions of culture experimented.

Rooting and acclimatization

Media used for root induction included half-strength MS inorganic salts with 2% sucrose and White's vitamins supplemented with 0.5 to 5 mg l^{-1} IAA or IBA, and agar or Gelrite. The best rooting efficiency (up to 70% regenerants rooted) was obtained following a one-week induction under darkness on a medium containing 3 mg l^{-1} IBA and solidified with 0.3% Gelrite followed by transfer to the same medium without growth regulators under a 16-h photoperiod. The rooted plantlets produced 1 to 9 roots from a small callus (Figure 1f). The first root primordia were evident after the dark induction period and elongated after transfer. These regenerants were transferred to potting mix without significant loss and did not display any morphological modification when compared to micropropagated plantlets of similar age (Figure 1g).

Identification of competent cells for bud regeneration

The anatomy of the hypocotyls changed during germination and 8–15 day-old hypocotyls, i.e. those giving most efficient bud regeneration, were characterized by a tetrarch primary structure and the presence of differentiating oil glands in a subepidermal position (Figure 1h). These structures were weakly pyroninophilic at the time of excision. The cavity of the oil glands occurred according to a schizogenous pattern as described for other eucalypt species (Carr and Carr, 1970) so that in 2 to 8-day-old seedlings, only the gland initials were observed, firstly at the epidermis level then in the subepidermal position (data not shown). Following excision and transfer of 8–15-day-old hypocotyls, unorganized cell proliferation occurred at both wounded extremities, giving rise to small calluses. Increased meristematic activity leading to direct shoot meristem initiation occurred only superficially, in cells located at the vicinity of differentiating

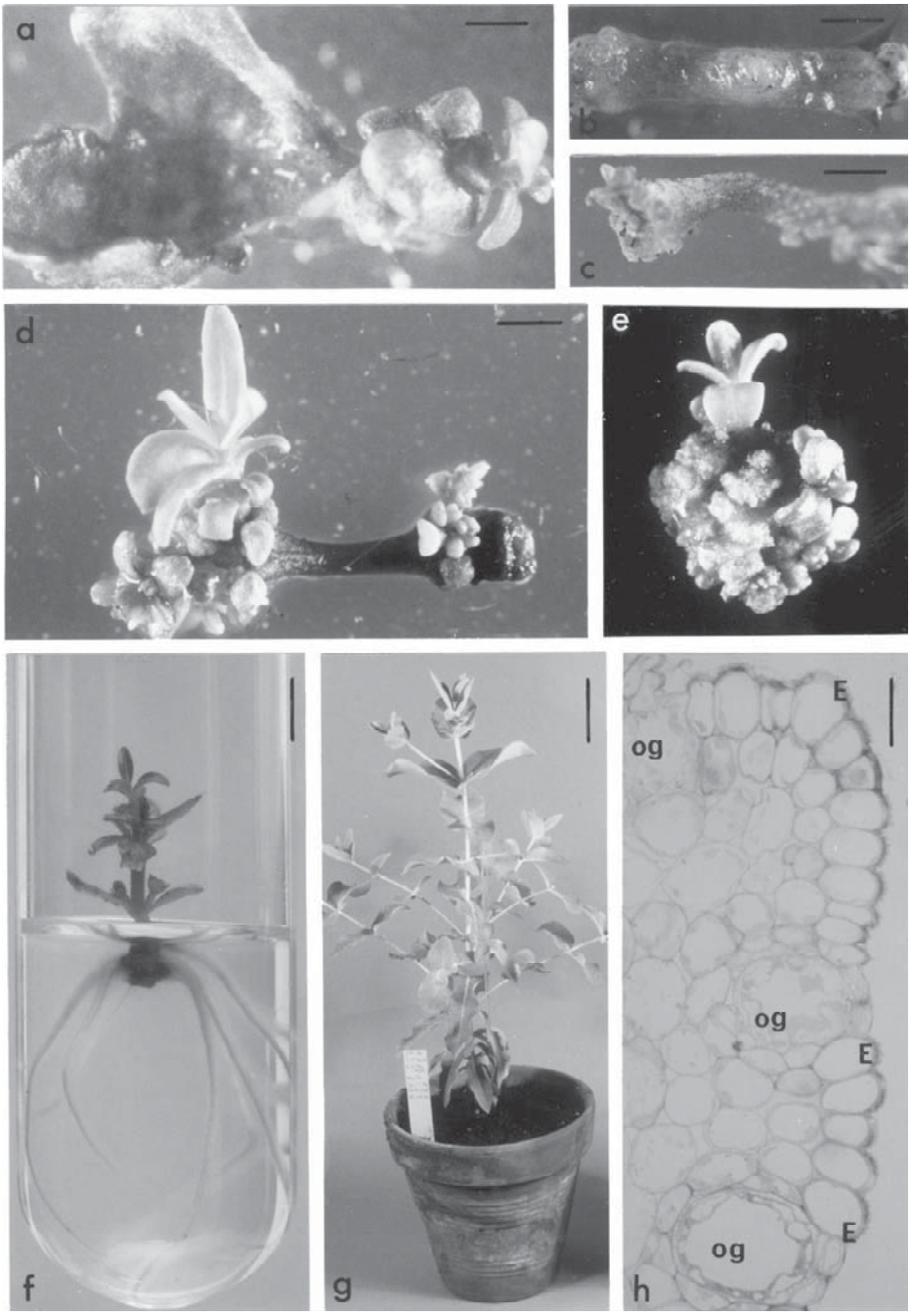

Figure 1. (a–d): Bud regeneration on 10-day-old excised cotyledon (*a*) and hypocotyls (*b–e*) cultivated on MS medium with 0.2 mg l^{-1} BA and 0.2 mg l^{-1} TDZ. (*a–b*) 8 days of culture, (*c*) 12 days of culture, (*d*) 21 days of culture. Bars: 0.2 cm (*a* and *b*), 0.15 cm (*c* and *d*). (*e*) New regenerated buds on a caulogenetic callus subcultured on medium devoid of growth regulators. Bar: 0.2 cm. (*f*) Isolated regenerant rooted after one-week induction under darkness in presence of 2 mg l^{-1} IBA and two weeks on medium without growth regulators under a 16-h photoperiod. Bar: 0.55 cm. (*g*) Rooted regenerant after 3 months in potting mix. Bar: 8.8 cm. (*h*) Transverse section of hypocotyl from a 15-day old seedling. E: epidermis; og: oil gland. Bar: 22 μm.

Table 1. Conditions leading to bud regeneration from *E. globulus* hypocotyls and cotyledons excised from 8 to 15 day old seedlings (40 samples for each condition, each condition being replicated 2 or 3 times).

	Hypocotyls		Cotyledons	
	Explants with buds*	No. buds per explant	Explants with buds*	No. buds per explant
MS + 0.2 BA + 0.01 NAA	+++	>5	++	>5
MS + 0.2 BA + 0.2 NAA	+++	>5	++	>5
MS + 0.2 BA + 1 NAA	0	0	++	1 to 2
MS + 0.5 BA + 0.05 NAA	+	2 to 5	0	0
MS + 1 BA + 0.05 NAA	0	0	+	2 to 5
MS + 0.2 BA + 0.2 TDZ	+++	>5	++	>5
WPM + 0.1 BA + 1 NAA + 1 CH	0	0	+	1 to 2

* 0, + (< 5%), ++ (5 - 50%), +++ (> 50%)
MS = Murashige and Skoog's basal medium added with 3% sucrose; WPM = Woody Plant Medium containing 2% sucrose. Concentrations of growth regulators are expressed in mg l^{-1} excepted for CH which is expressed in g l^{-1}.

oil glands, as evidenced by their strong staining with pyronin (Figure. 2a and b, arrows). Also, the calluses were able to redifferentiate new oil glands at their periphery and indirect shoot meristems were initiated in their vicinity (Figure 2c, arrows). Later, most of the meristems initiated directly from the hypocotyl or indirectly from the callus became organized and gave rise to leaflets (Figure 2d). These meristems remained in a subepidermal position in the early stages (Figure 2d), then the new shoots elongated and initiated new leaves while the epidermis became disrupted (Figure 2e). The axillary buds of the regenerated shoots developed giving rise to multiple shoots.

Ploidy levels

Flow cytometric analysis of nuclear DNA contents was performed on seedlings, micropropagated plantlets and individual regenerants. In shoot apices of seedlings (Figure 3a) and cultured plants (Figure 3d) as in differentiated cotyledons (Figure 3b), hypocotyls (Figure 3c), leaves (Figure 3e) and stems (Figure 3f), the DNA contents were remarkably stable, with a main peak at the 2C value (2C = 1.13 pg DNA; 40.1% GC) and a very small nuclear population at the 4C position. This indicated that cell differentiation occurred without endopolyploidisation. Series of 10 to 15 plantlets regenerated either from cotyledons or hypocotyls, in presence or absence of TDZ were also analysed; each of them contained the same diploid nuclear DNA content as the initial explants (Figure 3g–j).

Discussion

Although previously reported as recalcitrant to manipulate through *in vitro* culture (Hartney, 1980), *E. globulus* is shown in this paper to be able to regenerate buds in a very efficient manner, provided young organs excised from seedlings at a particular developmental stage are used. Regeneration from excised cotyledons was possible, but the percentages of budding explants were lower than from excised hypocotyls. The efficiency of regeneration was considerably improved compared to previous studies (Oka et al., 1982), both in the extent and the time taken for bud regeneration. Hypocotyls were particularly reactive when excised at the specific stage at which oil glands were not fully differentiated and prior to the initiation of the first leaves by the seedlings, i.e. between 8 and 15 days after sowing.

The reactive cells were always located in the vicinity of the oil glands that were differentiating in subepidermal and epidermal locations. The involvement of superficial tissues in bud formation from embryonic or seedling organs of woody species has been reported for *Picea abies* (Bornman, 1983), *Pinus pinaster* (David et al., 1982), *Pinus sylvestris* (Monnier et al., 1983), *Pseudotsuga* (Cheah and Cheng, 1978) and *Pinus radiata* (Yeung et al., 1981). However, the ability of the juvenile organs to regenerate buds has not been correlated with the presence of specific structures such as the juvenile oil glands of *E. globulus* hypocotyls and callus. In this case, the capacity for bud regen-

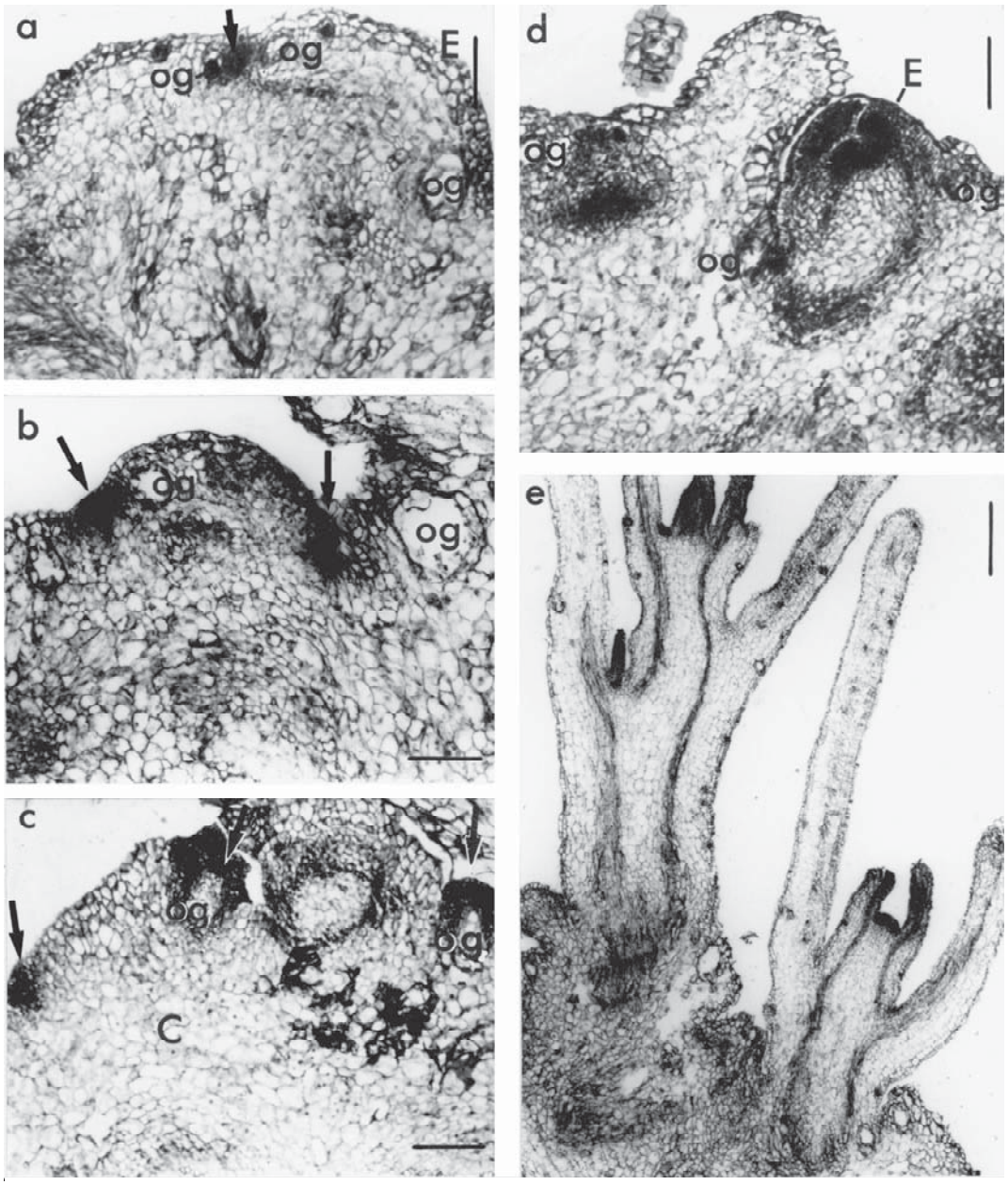

Figure 2. (*a–e*) Successive stages of bud regeneration from excised hypocotyls. (*a* and *b*) Increased pyroninophily and proliferation (arrows) of cells located at the vicinity of oil glands after 6 (*a*) and 8 (*b*) days. Bars: 35 μm (*a*) and 50 μm (*b*). (*c*) Redifferentiation of oil glands at the periphery of a 10-day old callus and cell reactivation (arrows) in the vicinity of these glands. Bar: 50 μm. (*d*) Shoot apical meristem organization below the epidermis of an excised hypocotyl grown *in vitro* for 12 days. Bar: 35 μm. (*e*) Developing regenerated buds after 18 days *in vitro*. Bar: 170 μm. C: callus; E: epidermis; og, oil gland.

eration seems to be the result of combination of adequate and transient stages of differentiation leading to competent cells and of medium composition allowing callus formation and redifferentiation of new oil glands at the periphery of the callus. It could be concluded that these cells were not fully determined at the

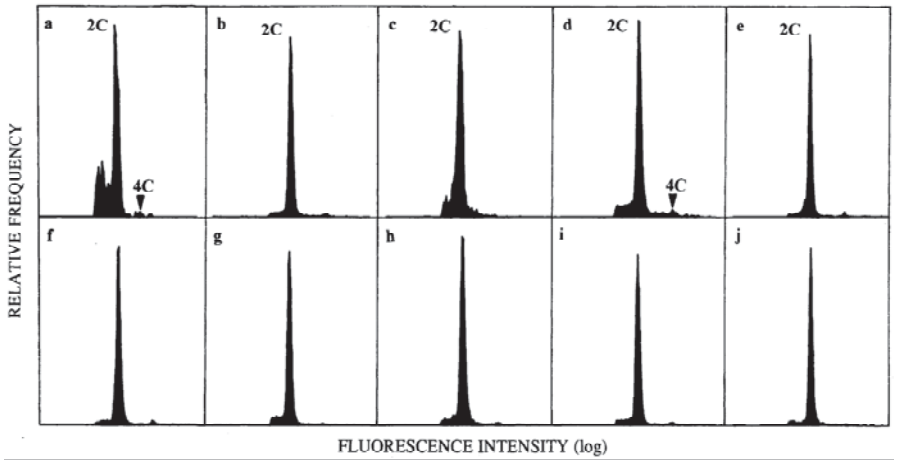

Figure 3. Histograms of distribution of nuclear DNA contents (semi-log scale) in initial organs and regenerants. Apices (*a*), cotyledons (*b*) and hypocotyls (*c*) of 10 days old seedlings; apices (*d*), leaves (*e*) and internodes (*f*) of micropropagated plantlets; leaves of shoots regenerated from excised cotyledons (*g* and *h*) and hypocotyls d (*i* and *j*) grown in presence of 0.01 mg l^{-1} NAA and 0.2 mg l^{-1} BA or 0.2 mg l^{-1} BA and 0.2 mg l^{-1} TDZ.

time of excision, but still able to deviate their developmental pathway and to dedifferentiate under appropriate conditions. Occurrence of secretory structures in otherwise dedifferentiated cell cultures is uncommon. One exception is *Ruta graveolens* where secretory glands towards the periphery of the callus have been described (Peterson et al., 1978). Also proliferation of the epithelial cells surrounding the resin ducts of *Pinus sylvestris* has been reported (Kondrasheva and Yatsenko-Khmelevsky, 1974).

Addition of BA and TDZ was the more efficient combination. TDZ, a substituted urea (N-phenyl-N′-1,2,3-thidiazol-5-ylurea) has been found to mimic cytokinin-like activity in various morphogenetic responses including stimulation of shoot formation on a range of recalcitrant species (Huetteman and Preece, 1993). Although the mode of action of this compound is not yet fully understood (Hare and Van Staden, 1994), it appears that it triggers, quite specifically, the reactivation of cells identified as competent for regeneration in *E. globulus* hypocotyls. Furthermore, contrasting with some other models, the presence of TDZ in the regeneration medium did not limit the further ability to root of the regenerants.

E. globulus regenerated shoots were genetically stable with respect to ploidy levels both *in vivo* and under *in vitro* conditions, without any gross chromosomal abnormalities during regeneration. Although it cannot be excluded that more cryptic DNA modifications could have occurred, all the regenerants displayed normal phenotype after three years of vegetative growth, opening the possibility of large scale and true to type production as well as transformation experiments. In addition, the analysis revealed for *E. globulus* a small genome size (2C = 1.13 pg). Compared to other eucalypt genome sizes (0.77 pg/2C for *E. citriodora*; 1.47 pg/2C for *E. saligna*; Grattapaglia and Bradshaw, 1994), *E. globulus* displays a nuclear DNA content an intermediate range.

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