SHOOT FORMATION IN EUCALYPTUS GLOBULUS HYPOCOTYL EXPLANTS

SEIBI OKA*, EDWARD C. YEUNG, and TREVOR A. THORPE†

Department of Biology, University of Calgary, Calgary, Alberta, Canada T2N 1N4

(Received for publication 3 March 1982; revision 18 August 1982)

ABSTRACT

Hypocotyl segments of **Eucalyptus globulus** Labill., when cultured on a defined Schenk & Hildebrandt medium with auxin and cytokinin, produced shoots and roots, with or without callus formation. Shoot development was best on transfer to medium lacking phytohormones. Histological examination of shoot formation revealed that initiation of the process began early in culture by division of epidermal and subepidermal cells. This led to the formation of nodular tissue (meristemoids), some of which developed subsequently into leafy vegetative shoots.

INTRODUCTION

Eucalyptus species are fast-growing trees whose timber is dense, hard, and durable (Goncalves *et al.* 1979). In addition, the sizes attained by some eucalypts and their regenerative capacity have contributed to their widespread use in commercial plantations.

There are some 445 species and 115 interspecific hybrids of *Eucalyptus* recognised (Chippendale 1976). Of these, about 30 have been reported to form callus, and even fewer to undergo some organised development *in vitro* (Goncalves *et al.* 1979; Hartney & Barker 1980). There are apparently no published reports on tissue culture studies of *E. globulus,* a large tree that is used ornamentally as well as for lumber.

In this study we have looked at the morphogenetic behaviour of hypocotyl segments *in vitro,* and followed the histology of shoot formation.

MATERIALS AND METHODS

Seeds of *E. globulus* were sterilised with 10% commercial bleach (5.25% NaC10) for 10 min and germinated aseptically on a 0.6% water-agar medium containing 2% sucrose at 28° ± 2°C under 16-h photoperiod. Germination began within 2 to 3 days after sowing. In initial studies various parts of the seedlings were placed in culture on Murashige & Skoog (1962), and Schenk & Hildebrandt (SH) (1972) mineral salts and their organics, with 2% sucrose and containing varying levels of auxin and cytokinin.

* Permanent address: Sericultural Experiment Station, Yatabe, Ibaraki 305, Japan.
† To whom reprint requests should be addressed.

The organic additives used in the SH medium were myo-inositol 100 mg/*l*, thiamine hydrochloride 5.0 mg/*l*, nicotinic acid 5.0 mg/*l*, and pyridoxine hydrochloride 0.5 mg/*l*. Fifty-millimetre Erlenmeyer flasks containing 20 ml of medium (pH 5.6), which was solidified with 0.8% agar and autoclaved at 121°C for 15 min, were used. Culture was carried out in a Percival Incubator (Model No. 1-35LLVL), at a light intensity of *c.* 50 $\mu E/m^2/s$ and a 16-h photoperiod and thermoperiods of 25°C (16 h/day) and 20°C (8 h/night).

For histological examination, 1-mm hypocotyl segments were collected at 2-day intervals for 22 days and fixed in 3% glutaraldehyde in 0.05M phosphate buffer (pH 6.9) for 24 h. The segments were then dehydrated in a tertiary butyl alcohol series, embedded in Paraplast, and sectioned at 7 μm using an American Optical rotary microtome. The sections were stained with a solution containing Safranin 0, basic fuchsin, and crystal violet (0.5%, 0.2%, 0.2% respectively in 50% EtOH) and counter-stained with fast green (0.5% in 100% EtOH).

RESULTS

Morphogenetic Responses of Hypocotyl Segments

Initial experiments indicated that the best callus initiation and growth in culture occurred on hypocotyl segments from 6-day-old seedlings, placed on SH mineral salts. Further work was done with segments 3–4 mm long taken from the middle region of the hypocotyls (Fig. 1), and placed horizontally in culture on SH medium. Using this explant system and varying concentrations of naphthalene acetic acid (NAA) and benzylaminopurine (BAP) from 10^{-5} to 10^{-7} M, it was found that auxin at a concentration greater than 10^{-7} M was necessary for tissue survival. In the absence of BAP, but with 10^{-5} to 10^{-6} M auxin, some callus was formed at the ends of segment (Fig. 2), and roots were formed either in the callus or directly from the hypocotyls. Auxin-induced rooting was partly inhibited by BAP at 10^{-7} M. Growth and callus formation were enhanced by higher concentrations of BAP. Compact callus was formed and its colour varied from red to yellow to light-green.

Nodular tissue (NT) was produced within 2–3 weeks in culture in the presence of 10^{-6} M NAA and 10^{-7} M BAP on about 15% of the cultures (Fig. 3). These nodules were small and globular, and apparently arose at the boundary between the cut ends of the initial explants and the induced callus. While most of the NT was green, some was red-pigmented.

To determine if there was a positional response in respect to NT formation, 2- to 2.5-mm-long segments from various regions of the hypocotyl were cultured on the latter medium. The highest frequency of NT formation (32%) was obtained from segments taken from the uppermost portion of the hypocotyl (Table 1). Furthermore, while the minimum size of the hypocotyl segment to survive *in vitro* was 0.5 mm in length, segments more than 1 mm were preferred as these suffered less dehydration and consequently almost all segments survived in culture.

The influence of polarity on NT formation was examined by placing the segments vertically, inverted, or horizontal in culture (Table 2). Nodular tissue formation occurred if segments were placed vertically or horizontally, and the basal portion of these segments produced more NT.

TABLE 1—Positional effect on nodular tissue (NT) formation in hypocotyl segments (each 2–2.5 mm) of **E. globulus** after 3 weeks in culture. Values represent the average of two experiments

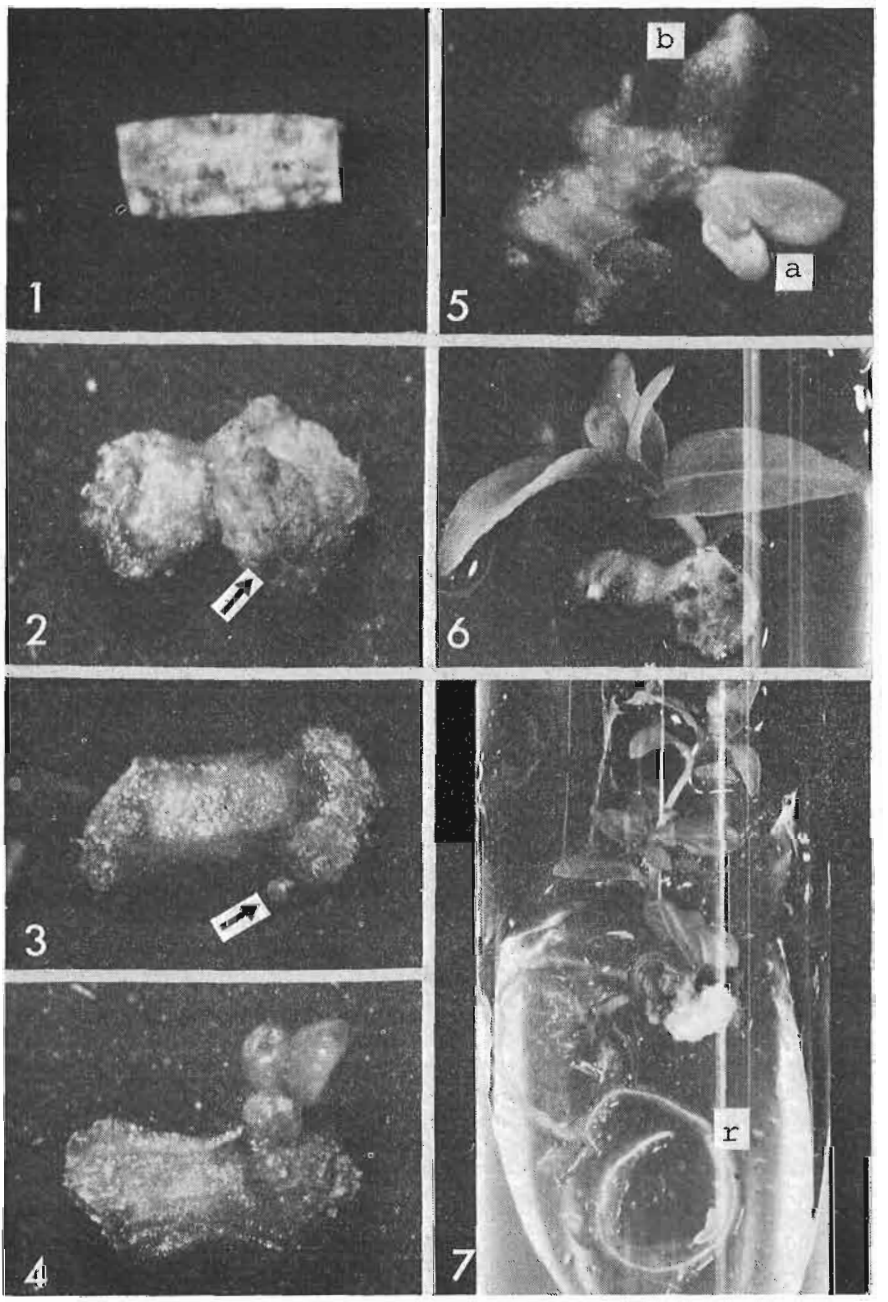
Zone No.	No. of explants	Explants forming NT	
		No.	%
1 (uppermost)	28	9.0	32.1
2	28	6.0	21.4
3	28	4.5	16.1
4	28	3.0	10.7
5	28	1.5	5.4
6 (lowermost)	28	0.5	1.8

TABLE 2—Influence of explant orientation on nodular tissue (NT) formation in uppermost hypocotyl segments of **E. globulus** after 3 weeks in culture. Values represent the average of two experiments

Orientation of explant	No. of explants	Explants forming NT	
		No.	%
Apical end up	48	10.5	21.9
Basal end up	48	0	0
Horizontal	48	8.5	17.7

When segments with NT were subcultured in the presence of 10^{-6} M NAA and 10^{-7} M BAP, no further growth or differentiation occurred. However, when explants were transferred after 3 weeks in culture to medium lacking auxin with or without cytokinin for a further 3 weeks, three kinds of morphological development were observed. Most of the NT increased in size without any discernible differentiation. Some developed into a single or a few leaves. Some of these leaves were abnormally thickened or had teratoma-like blades with less chlorophyll (Figs 4 and 5). Some NT developed into one or more shoots reaching 0.5–1.0 cm (Fig. 5). Such shoots developed into normal leafy vegetative shoots with further time in culture (Fig. 6). Excision of such shoots and transfer to auxin-containing medium did not generally lead to rooting. However, some shoots left on the original explant and kept on the same auxin/cytokinin medium for about 3 months produced roots and the shoots subsequently elongated to produce plantlets (Fig. 7).

Other manipulative treatments were carried out in an effort to increase the production of NT and subsequent shoot formation. Formation of NT was found to be independent of sucrose concentration from 1 to 5%. Activated charcoal even as low as 0.2% (w/v) inhibited NT formation and did not appreciably increase NT development in subcultured segments. Further tests with auxin and cytokinin concentrations showed that the maximum amount of NT was produced with 2×10^{-7} M NAA and 5×10–^{7} M


1
2
3
4
5
b
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6
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r

FIGS 1–7 (left)—External morphogenetic development of cultured hypocotyl segments of **Eucalyptus globulus**.

FIG. 1—Initial explant, day 0 (× 10). FIG. 2—Explant showing callus development after 7 days in culture. More callus was formed on the basal end of explant (arrow) (× 10). FIG. 3—Appearance of nodular tissue (NT) (arrow) after 14 days in culture (× 10). FIG. 4—Leaf-like structure arising from NT after 30 days in culture (× 10). FIG. 5—Shoot bud (a) and abnormal leaf-like structure (b) after 42 days in culture (× 10). FIG. 6—Leafy vegetative shoot development after 60 days in culture (× 3.4). FIG. 7—Plantlet showing root (r) and further shoot development after 85 days in culture (× 2).

BAP and with uppermost portions of the hypocotyl segment of 6-day-old seedlings. Under these conditions, not only was more NT formed but a much higher proportion of these developed into leafy vegetative shoots when transferred to phytohormone-free medium.

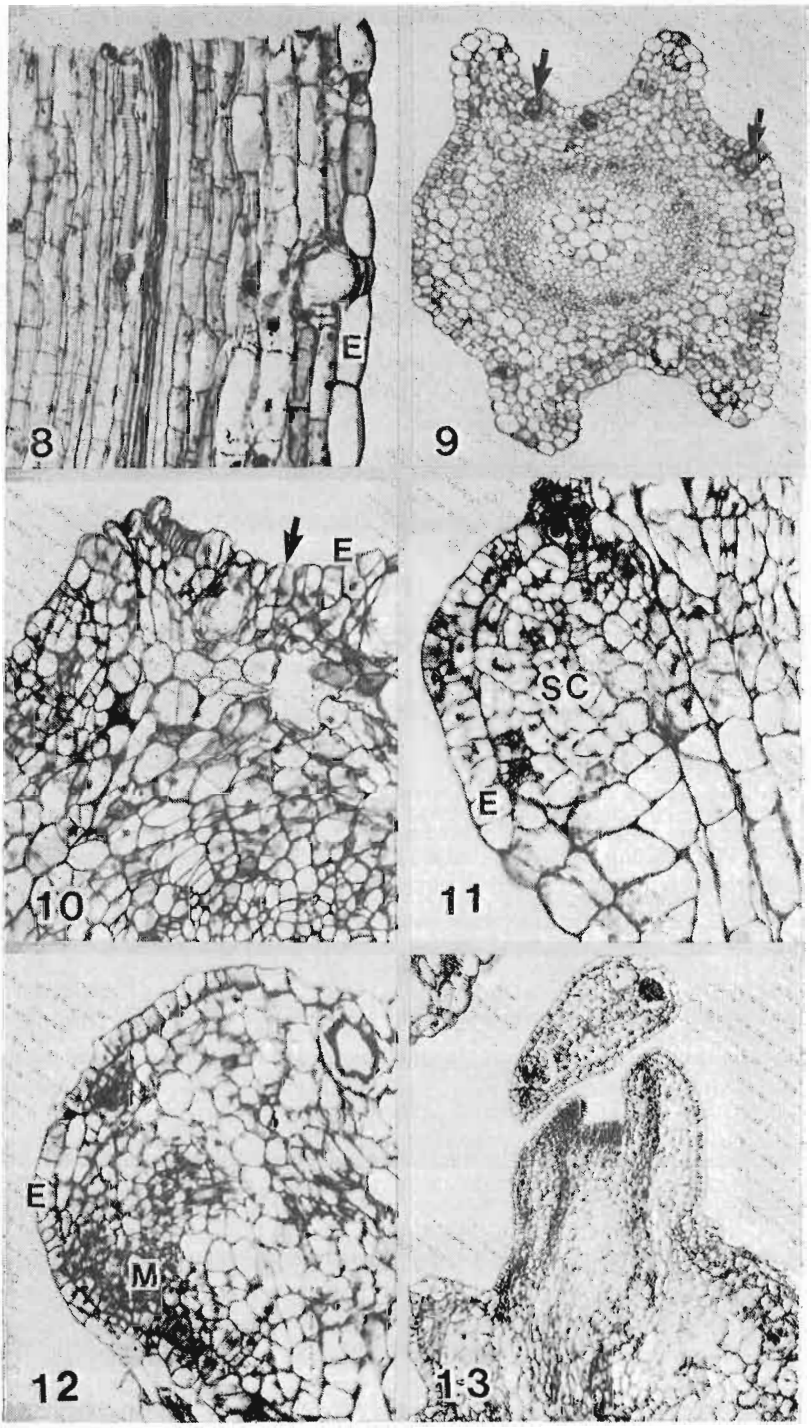
Developmental Sequence

The histological sequence leading to shoot primordium formation in upper portions of the hypocotyl was compared with changes which occurred in the basal portions when cultured under the same conditions. The material was obtained from 8-day-old seedlings, and cultured under the optimal levels of auxin and cytokinin (2×10^{-7} M and 5×10^{-7} M.

When the longitudinal sections of the upper and lower regions of the initial explants were compared, there was no substantial difference in structure except that the length of cells of the epidermis and cortex was greater in the lower segments. Internal secretory cells, which stained red with safranin, appeared very often beneath the epidermis in both regions (Figs 8 and 9). The cross-sections of the upper segments showed typical features of the stem (Fig. 9). In the NT-forming explants from upper segments, the first histological response of the tissue was observed on the fourth day, when the epidermal cells just below the apical cut end began to divide anticlinally, and the cortical parenchyma cells also began to divide randomly (Fig. 10). These divisions were preceded by divisions in the procambium.

During the sixth to eighth day in the culture, the epidermal and cortical cells continued to divide, giving rise to a protuberance which could be observed only microscopically (Fig. 11). The protuberance developed into a prominent nodule (NT) during the next 2 days. Nodular tissue consisted of an epidermal layer one cell thick and a mass of meristematic cells, which were distinguished by their dense staining and small cell size (Fig. 12).

During the fourteenth to sixteenth days in culture, leaf primordia and/or leaf-like structures could be observed on the surface of the NT. Although procambium cell strands were found in the leaf-like tissues, they were sometimes abnormally swollen with some secretory cavities scattered between them. Organised shoot apices were usually observed on the fairly enlarged NT at a later stage (Fig. 13). When NT remained undifferentiated, whorls of cells were sometimes formed inside the partially organised structures, and these nodules were often lignified.


8
E
9
10
E
11
SC
E
12
E
M
13

FIGS 8–13 (left)—Histological sequence of development of nodular tissue (NT) and leaf initiation in upper segments of cultured hypocotyls of **E. globulus**.

FIG. 8—Median longitudinal section of an initial explant from 8-day-old seedling showing the cut upper end and a one-cell-layered epidermis (E) (× 580). FIG. 9—Transverse section of an initial explant, showing the tissue arrangement and the presence of several secretory cells (arrow) beneath the epidermis (× 220). FIG. 10—Transverse section of 4-day-old explant showing anticlinal divisions (arrow) in the epidermis (E) (× 580). FIG. 11—Longitudinal section of a 6-day-old explant showing anticlinal divisions in the epidermis (E) near the cut end of segment, randomly dividing sub-epidermal cells (SC), and the beginning of NT appearance (× 600). FIG. 12—Longitudinal section through a 12-day-old globular NT, composed of a one-cell-layered epidermis (E) and small, densely staining, meristem-like cells (M) (× 600). FIG. 13—Longitudinal section through a 22-day-old upper segment showing a young shoot primordium (× 250).

By comparison, segments taken from the lower region of the hypocotyl developed callus from the procambial tissues, but division of the epidermal cells was never observed.

DISCUSSION

The eucalypts as a group are extremely important angiosperm hardwoods, being used for lumber, pulp, and paper, as well as ornamentals and shade and shelter trees. As with many trees of commercial interest, vegetative propagation using mature material is very difficult because the classical techniques of grafting and rooting of cuttings have not been generally satisfactory (Hartney 1980). For this reason, vegetative propagation through tissue culture techniques has been tried as a possible alternative (de Fossard *et al.* 1974; Kitahara & Caldas 1975; Sita 1979). The importance of the application of tissue culture methodology to vegetative propagation and tree improvement is widely acknowledged, and has been discussed several times recently (e.g., Rediske 1979; Sommer & Brown 1979; Mott 1981).

While callus formation is apparently fairly easy to induce from various plant parts of juvenile and mature eucalypts, reorganisation of that callus in shoots, roots, and plantlets has not been very successful, and thus attempts to use organ culture have been made (Goncalves *et al.* 1979). Nodal cultures of *E. grandis* Hill ex Maid., and *E. ficifolia* F. Muell. have produced plantlets (de Fossard *et al.* 1974; de Fossard 1978). Adventitious shoot formation has been achieved in hypocotyl callus cultures of *E. alba* Reinw. ex Bl.* (Kitahara & Caldas 1975) and cotyledon callus cultures of *E. citriodora* Hook. (Sita 1979).

In the present study with *E. globulus,* hypocotyl cultures, with or without callus development, could form shoots and roots. In terms of plantlet formation, shoots are usually induced to form roots. In this study this was not very successful although some plantlets were formed.

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* Probably **E. urophylla** S.T. Blake or hybrids thereof [Ed.]

The importance of selecting the most suitable inoculum for *in vitro* culture was again demonstrated in this study. Not only was the hypocotyl the best seedling part, but the uppermost portion of this tissue produced the most nodular tissue and subsequently shoots. However, within the segment the basal portion produced more nodular tissue indicating the effects of polarity both with the excised segments, as well as the entire tissue. In addition, the induction of nodular tissue formation resulted from a critical balance of auxin and cytokinin in the medium. Further development of the nodular tissue required lower levels of the phytohormones, as was demonstrated by transfer to phytohormone-free medium. This phenomenon has been observed in several *in vitro* systems, including shoot-forming conifer explants (*see* Thorpe 1980).

Histologically, the origin of nodular tissue was in the explant and was independent of any callus formation. Unlike some explant systems, e.g., *Convolvulus* (Bonnett & Torrey 1966; *see also* Thorpe 1980), the position of nodular tissue initiation was also independent of any preformed structures, e.g., position of cotyledons or primary leaves, in the explant. Nodular tissue was initiated by division of both epidermal and subepidermal cells. This led to the formation of a meristemoid, i.e., a spherical mass of smaller isodiametric cells with prominent nuclei and densely staining cytoplasm, which preceded primordium formation in *E. globulus*. Some of the nodules formed were lignified and did not produce shoots. These latter findings have all been observed in other tissue culture systems and may well be general features of *in vitro* organogenesis (Thorpe 1980).

ACKNOWLEDGMENTS

S. Oka acknowledges with gratitude the award of a study grant by the Government of Japan, which allowed him to spend 1 year in Calgary. This research was supported by NSERC of Canada grants No. A6704 and No. A6467 to E. C. Yeung and T. A. Thorpe, respectively.

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