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Ultrastructural Studies of Somatic Embryos of *Eucalyptus nitens* and Comparisons with Zygotic Embryos Found in Mature Seeds

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Although somatic embryogenesis has been observed in tissues from a limited number of *Eucalyptus* speices cultured *in vitro*, no comparisons have been made of the morphology and structure of eucalypt somatic embryos and zygotic embryos found in mature seeds. We used scanning and transmission electron microscopy, in conjunction with histological analysis, to compare mature zygotic embryos with somatic embryos of the commercially-important temperate eucalypt *Eucalyptus nitens*. Apart from differences in the nature of the outer coating enclosing both embryo types, somatic embryos of *E. nitens* were observed to have strong similarities with zygotic embryos in seeds in terms of their overall size, morphology and internal cellular organization. Many cells in both sexually-produced and somatic embryos contained numerous lipid-rich globular bodies. The wider significance of these observations is discussed with regard to their potential applications in eucalypt plantation biotechnology programmes.
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Key words: *Eucalyptus nitens*, shining gum, somatic embryo, tissue culture, ultrastructure, zygotic embryo.

INTRODUCTION

Somatic embryogenesis has been documented in tissue cultures of a wide range of higher plants, including both angiosperms and gymnosperms, usually resulting from pre-embryogenic clusters of cells which develop into embryos as a result of alterations in the hormonal and nutritional status of the culture media (Halperin, 1995). From a developmental perspective, somatic embryos have strong similarities to embryos that develop within the embryo sac (Raghavan, 1976) and there is increasing interest in the molecular mechanisms which are closely associated with the formation of somatic and zygotic embryos (Dodeman *et al.*, 1997). Somatic embryogenesis is a valuable tool in plant biotechnology for clonal propagation of elite genotypes via the development of synthetic seed technology (Gray and Purohit, 1991; Redenbaugh, 1993). In the field of tree biotechnology, the production of somatic embryos offers an alternative approach to conventional micropropagation procedures for the establishment of plantations of elite genotypes. The genus *Eucalyptus* contains many commercially-important species (Eldridge *et al.*, 1994) and the development of embryogenic cultures has the potential to provide effective mass propagation procedures and also to assist in the application of gene transfer techniques for further genetic improvement (Teulieres *et al.*, 1994). The formation of somatic embryos or embryo-like structures has been reported for a small number of eucalypt species, including *E. citriodora* (Muralidharan *et al.*, 1989), *E. grandis* (Watt *et al.*, 1991), *E. urophylla* (Tilbok *et al.*, 1995) and, recently in this laboratory, the temperate eucalypts *E. nitens* (shining gum) and *E. globulus* (blue gum) (Bandopadhyay *et al.*, 1999). Somatic embryogenesis may be particularly relevant to biotechnology programmes involving temperate eucalypts such as *E. nitens* and *E. globulus* because conventional micropropagation procedures involving these species can suffer from the problem of low or variable rates of function root formation in micropropagated clones (McComb and Bennett, 1986; MacRae and van Staden, 1993; Bennett *et al.*, 1994). Although detailed ultrastructural studies have been undertaken to compare somatic embryos and their sexual counterparts in some crop species [e.g. rice (Jones and Rost, 1989*a*,*b*) and pearl millet (Taylor and Vasil, 1995, 1996*a*,*b*)], no studies have been reported that have compared the structure of somatic embryos with their zygotic counterparts in any *Eucalyptus* species. In this study we examine the ultrastructure of somatic embryos of *E. nitens* and compare them with mature zygotic embryos found in seeds of this important temperate eucalypt species.

MATERIALS AND METHODS

Source of embryos

The detailed methodology for induction of organogenic cultures of *E. nitens* which also produced somatic embryos has been described elsewhere (Bandyopadhyay *et al.*, 1999). Briefly, a two-step method was developed for efficient shoot regeneration from seedling-derived explants of *E. nitens* involving initiation and growth of callus *in vitro* on MS (Murashige and Skoog, 1962) basal nutrient medium supplemented with 3 % sucrose, 1·0 mg l^{-1} NAA (α-napthalene–acetic acid) and 0·5 mg l^{-1} BAP

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(N^6 benzylaminopurine), followed by their transfer to shoot regeneration medium containing 3 % sucrose, 0·5 mg l^{-1} NAA and 1·0 mg l^{-1} BAP. Shoots from highly regenerative calli were excised and placed in rooting medium containing 2 % sucrose and 3 mg l^{-1} IBA (indole butryic acid), and were incubated in the dark at 21 ± 1°C. In a few of these shoots, wound callus that was produced at the base prior to the root formation also gave rise to a proliferation of new green shoot buds together with the production of several oval-shaped, white somatic embryos. These somatic embryos were used as experimental material for comparison with sexually-produced embryos present in seeds of the same batch of open-pollinated *E. nitens* that was used for initiation of the organogenic cultures (seeds obtained from the Tasmanian Seed Centre, Tasmania, Australia). Seeds were treated with 40 % v/v Domestos (Lever Rexona, Australia; commercial bleach containing 5 % w/v available chlorine) for 30 min in a rotary suspension mixer, followed by several rinsings in sterile distilled water to remove all traces of bleach prior to treatment for microscopic examination.

Histology

Four somatic embryos and approx. ten bleached seeds of *E. nitens* were fixed in 0·03 M PIPES buffer (pH 6·8) containing 5 % glutaraldehyde and 1 % caffeine for 2–4 h at room temperature (RT) and then gently washed three times (for 10 min each time) in the same buffer. Specimens were dehydrated through a graded ethanol series, then taken through a graded series of ethanol/LR (London Resin) White medium grade acrylic resin and finally placed in LR White acrylic resin at 25 ± 2°C. It was important to ensure that resin infiltrated the specimens sufficiently (typically approx. 10 d with three–four fresh changes of LR White resin) before specimens were polymerized in blocks under UV light in an oxygen-free environment. Sections were cut using a Reichert Ultracut E ultramicrotome, 1–2 μm thick, and stained with 0·05 % (w/v) aqueous Toluidene Blue (pH 4·5) (Gurr, BDH, England).

Scanning electron microscopy

Four somatic embryos and approx. ten bleached seeds of *E. nitens* were fixed in 5 % glutaraldehyde in 0·03 M PIPES buffer (pH 6·8) for 48 h, dehydrated through a graded ethanol series, critically point dried and finally sputter-coated with gold (Bal-Tec SCD005 Sputter Coater, 180 secs at 25 mA, gold coating approx. 10 nm). Specimens were examined in a scanning electron microscope (SEM) (Hitachi S-570) operating at 10 kV.

Two somatic embryos and approx. ten bleached seeds, soaked overnight in water at 25 ± 2°C, were fixed in 5 % (w/v) glutaraldehyde and 1 % (w/v) caffeine in 0·03 M PIPES buffer for 48 h at 25 ± 2°C, and rinsed three times (5 min each rinse) in 0·03 M PIPES buffer (pH 6·8). Tissues were placed in 1 % (w/v) osmium tetroxide in 0·03 M PIPES buffer (pH 6·8) at 25 ± 2°C for 2 h, rinsed in distilled water and dehydrated through a graded ethanol series and infiltrated with medium grade Spurr's resin (Proscitech, Thuringowa, Australia). Specimens were stained with saturated uranyl acetate in 50 % (v/v) methanol and lead citrate as described by Venable and Coggeshall (1965). Sections were cut at 100 nm thickness and placed on 300# copper grids or formvar coated copper slot grids and examined under a JEOL 200 CX transmission electron microscope operating at 100 kV.

Fluorescence microscopy

Sections of somatic embryo and bleached seeds of *E. nitens* were stained to detect lipids with 0·1 % (w/v) aqueous neutral red adjusted to pH 6·5 and mixed with artificial seawater (1 : 1) following the method of Kirk (1970). Sections were immediately examined using UV light under a Carl Zeiss fluorescence microscope with a blue exciter filter (KP 450-490), FT 510 beam splitter and LP 520 barrier filters.

RESULTS

Somatic embryo of *E. nitens* formed sporadically on the underside of wound callus that developed at the base of regenerated shoots (Fig. 1A). None of the somatic embryos had any obvious physical connections to the progenitor tissue and could easily be detached from the callus. Typically, they were shiny, white and slightly soft to the touch, varied in shape from elliptical to ovoid and were 1·0–1·5 mm in length and approx. 0·5 mm in breadth at their mid-point (Fig. 1A, B). Overall, their morphology was similar to that of mature seeds, although the latter were slightly larger (1·5–2·0 mm in length; Fig. 1B). Normal seeds of *E. nitens* have a reddish-brown circular or ovoid pigmented zone, approx. 250 μm in diameter, on their ventral surface which is visible after discolouration of the seed coat following treatment with bleach (Fig. 1B). Interestingly, somatic embryos also had a reddish-brown spot on their ventral surface which, although it was slightly smaller in size and less intense in colour, did bear a strong similarity to the pigmented zone observed on the surface of the bleached seed (Fig. 1B).

SEM studies showed the morphology of *E. nitens* seeds to be reticulate, heart shaped or irregular structures (Fig. 2A, B) with a rough surface (Fig. 2C). These features are characteristic of this group of eucalypts which belong to the subgenus Symphyomyrtus, section Maidenaria, and series Viminales (Pryor and Johnson, 1971; Boland *et al.*, 1980). In comparison, the somatic embryos were cuboid in shape (Fig. 2D, E), and were slightly smaller than seed-derived embryos. They possessed a relatively smooth surface, which was shallowly reticulate on both dorsal and ventral sides (e.g. Fig. 2F).

Longitudinal sections through somatic embryos of *E. nitens* showed them to be bipolar structures, completely enclosed in a thin, stratified, translucent coating. Unlike the outer coating of seeds, the outer coat of somatic embryos appeared blackish-blue after staining with Toluidene blue and did not appear to be cellular in nature. However, the overall morphology and internal cellular organization of somatic embryos was strikingly similar to that of mature

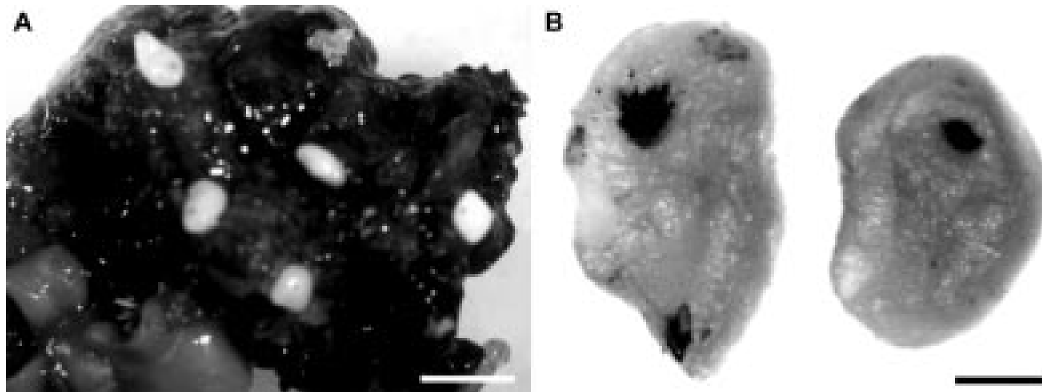

FIG. 1. A, Pale coloured somatic embryos that formed on callus which developed at the base of regenerated shoots of *E. nitens* (part of the shoot is visible in the lower left of the picture). The callus was brown in colour and leached exudates (probably phenolics) into the medium. Somatic embryos did not have any obvious connection to the progenitor callus. Bar = 0·7 mm. B, Enlarged view of a mature seed derived embryo of *E. nitens* after being discoloured by treatment with bleach (left) compared to a somatic embryo formed *in vitro* (right). Note the presence of a deeply pigmented area on the ventral surface of the bleached seed and a similar pigmented region, slightly smaller in area, on the surface of the somatic embryo. Bar = 0·33 mm.

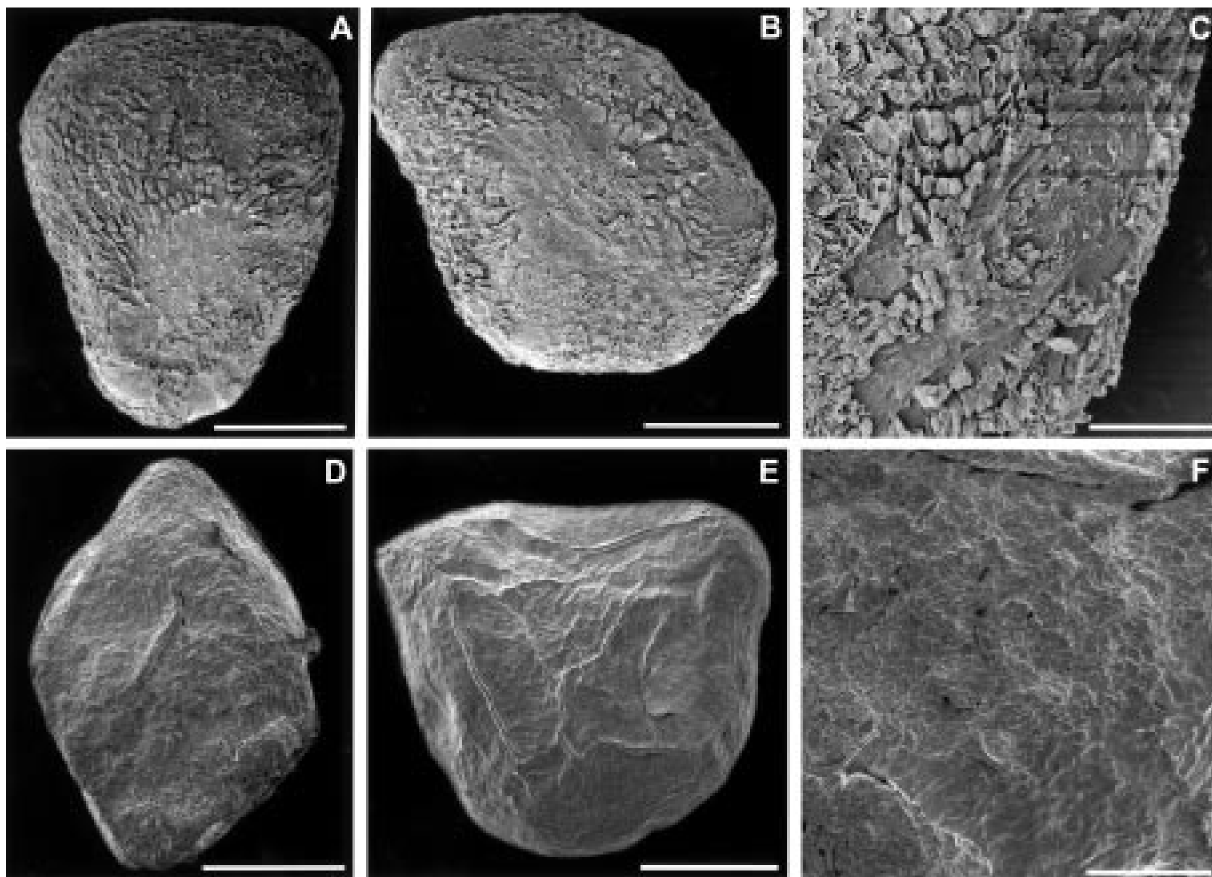

FIG. 2. Scanning electron photomicrographs (SEM) of mature seed-derived embryos and somatic embryos of *E. nitens*. A, SEM showing the ventral and (B) dorsal surface patterns typical of mature seeds after bleach treatment. Bar = 0·43 mm. C, Closer view of the rough, cellular structures comprising the outer surface of the seed coat. B = 0·23 mm. D and E, SEM showing the surfaces typical of somatic embryos possessing a relatively flat surface without reticulation. Bars = 0·38 mm. F, Closer view of surface of a somatic embryo showing the non-cellular nature of the outer surface. Bar = 0·12 mm.

zygotic embryos found in seeds (Fig. 3A–D). As in mature seeds, two cotyledons were present in each somatic embryo, being folded around the root-shoot axis. Each cotyledon in both types of embryo was composed of tightly packed cells and consisted of a single outer layer of epidermal cells enclosing a palisade layer (one cell thick in the somatic embryo, one–two cells thick in the zygotic embryo) and several layers of parenchyma cells (three–four cells thick in

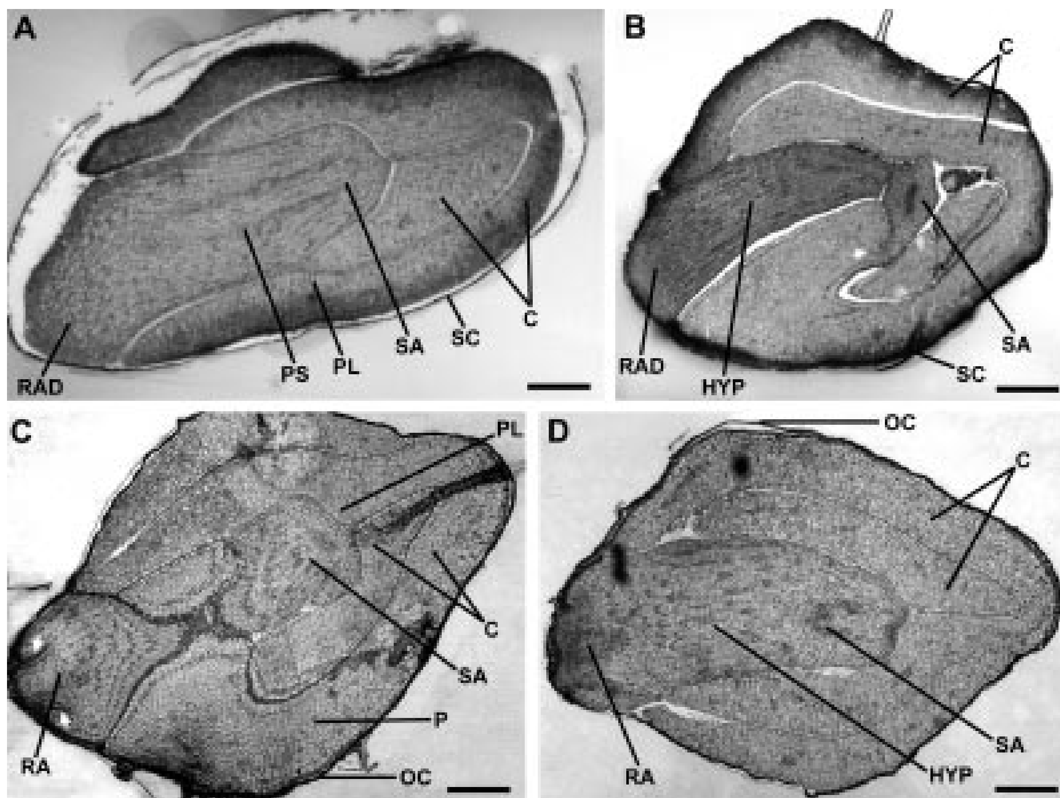

FIG. 3. Light photomicrographs of mature seed derived embryo and somatic embryo of *E. nitens*. A and B, Longitudinal sections of separate seeds showing two cotyledons folded around the root-shoot axis of the embryo. Note the procambial strands in the hypocotyl region and the connection between the cotyledon and the shoot apical meristem area (arrowhead) in Fig. 3B. Bars = 0·2 mm. C and D, Sub-median and median longitudinal sections through separate somatic embryos. Each somatic embryo contains two cotyledons, possessing an upper and lower epidermal layer, a palisade layer and three–four layers of parenchymatous cells. The root and shoot apical meristems are quite conspicuous and consist of compact, thin-walled cells. The arrows in Fig. 3C indicate the presence of a cell layer partially enclosing the root meristem region which is continuous with the cortical region of the embryo. This structure was not observed in the root apical meristem region of the seed-derived embryos. Bars = 0·15 mm. C, Cotyledon; HYP, hypocotyl; SC, seed coat; OC, outer coating; P, palisade layer; P, parenchyma; PS, procambial stands; RAD, radicle; RA, root apical meristem; SA, shoot apical meristem.

the somatic embryo and four–five cells thick in the zygotic embryo). The shoot apex and the radicle end of the somatic embryo were distinct, each containing densely cytoplasmic cells with deeply-stained nuclei. In these respects, the somatic embryos also closely resembled mature seed-derived embryos (Fig. 3A–D).

TEM examination of the cotyledons from somatic embryos and seed-derived embryos showed them to be composed of highly vacuolated, densely packed cells with prominent cytoplasmic strands (Fig. 4A–C). Cells in the meristematic zone of both embryo types were small, with prominent nuclei and nucleoli (Fig. 4D–F). Apart from the meristematic zones and some epidermal cells, most cells in the cotyledons and the body of the embryo contained numerous, densely staining globular bodies (Fig. 4A–F). To investigate the chemical nature of these globular bodies, sections from somatic and seed-derived embryos were stained with the lipid-detecting fluorochrome, neutral red. This treatment detected high quantities of lipid in the globular bodies as was evident from their bright lemon yellow fluorescence when viewed under UV illumination (Fig. 5A–D). The outer coating enclosing the somatic embryo also exhibited a bright lemon yellow fluorescence after treatment with neutral red (Fig. 5C) indicating a high lipid content (Kirk, 1970). The outer coating could be removed from the embryo as a continuous, semi opaque membrane-like structure. Together with a positive reaction with neutral red, this is consistent with the hypothesis that it had a waxy composition. The outer coat of *E. nitens* also stained lemon yellow after treatment with neutral red (Fig. 5A) but also had an additional layer of lipid deposition which fluoresced blue green under UV illumination (not shown). This is indicative of differences in lipid composition between the coating surrounding the somatic embryo and that of the mature seed (Kirk, 1970).

DISCUSSION

The present study represents the first detailed report, to our knowledge, of the morphology and ultrastructure of fully-formed somatic embryos of any eucalypt species. SEM and TEM studies, coupled with histological analysis, have shown that somatic embryos of *E. nitens* [reported previously by Bandyopadhyay *et al.* (1999)], had strong similarities to zygotic embryos found in seeds. Somatic embryos were slightly smaller than their zygotic

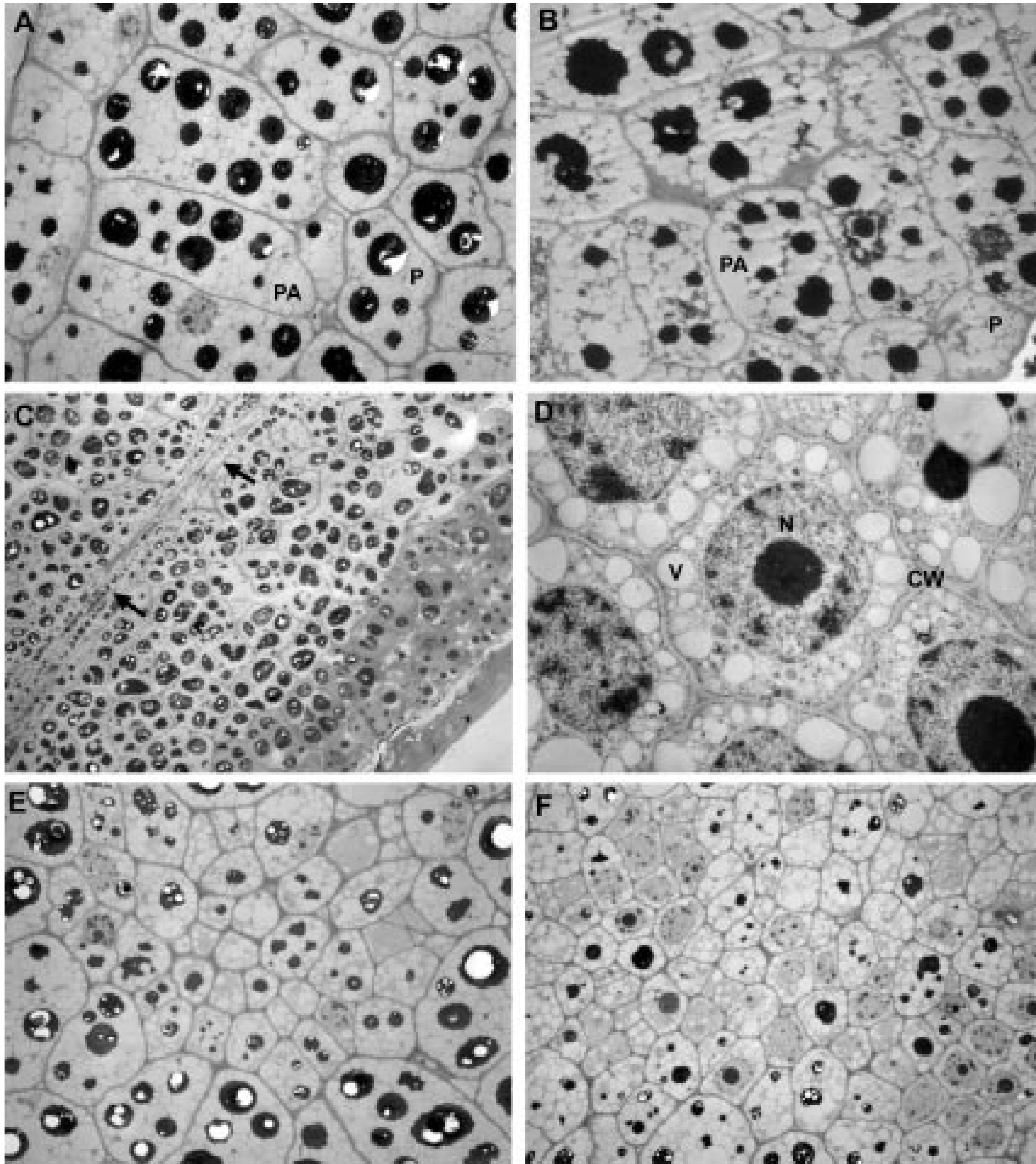

FIG. 4. Transmission electron micrographs of sections through a mature seed and a somatic embryo of *E. nitens*. A and B, Section of a cotyledon from mature seed (A, magnification × 1800) and somatic embryo (B, magnification × 2430) showing palisade layer (PA) and parenchymatous cells (P) containing numerous small vacuoles and several globular bodies (see also Fig. 5). C, Outer cotyledon in somatic embryo showing the development of procambial strands (arrows) composed of elongated, thin-walled cells (magnification × 540). D. A closer view of cells in the meristematic region of a somatic embryo, each possessing a dense cytoplasm and prominent nuclei (N), relatively thin cell walls (CW) and numerous small vacuoles (V) × 9000. E and F, A lower magnification of shoot apical meristem regions in a seed-derived embryo (E) and in a somatic embryo (F) × 1800. The central apical meristem region in embryos of both types consists of small, cytoplasmic, thin-walled cells with prominent nuclei, and which are relatively devoid of globular bodies (see also Fig. 5).

counterparts, but otherwise they were very similar in their overall morphology and internal cellular organization. In particular, fully-formed somatic and zygotic embryos were enclosed by a pair of folded cotyledons, and had a hypocotyl region and the cellular characteristics of root and shoot apical meristems. Somatic and zygotic embryos had similar lipid-rich globular bodies in their cells, except in the meristematic regions, although the somatic embryos had slightly lower levels of lipid reserves in their cotyledonary cells compared to the zygotic embryos. Somatic embryos were surrounded by a thin, waxy coating rather than the seed coat which is derived from ovular integuments. The presence of a waxy coating surrounding the *E. nitens* somatic embryos in noteworthy as most previous reports

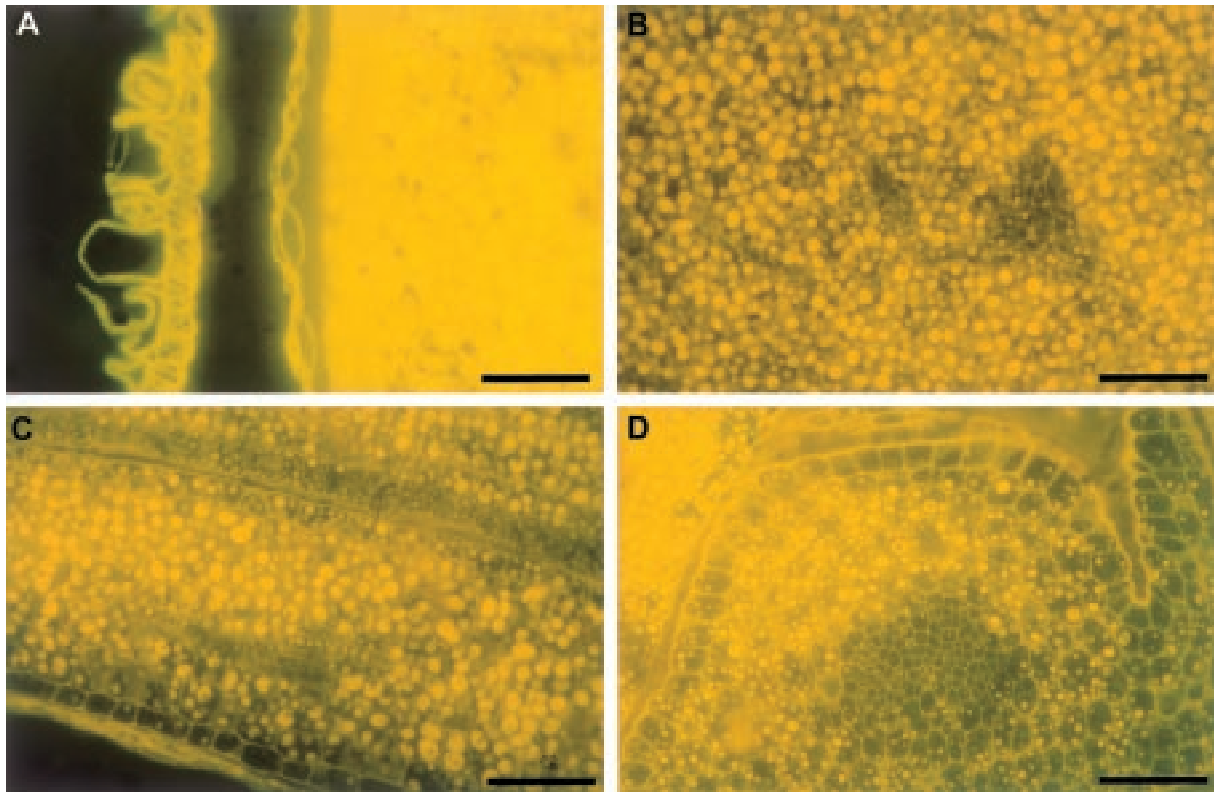

FIG. 5. Photomicrographs of longitudinal sections through a mature seed and a somatic embryo of *E. nitens* stained with neutral red and showing the presence of brightly fluorescing lipid-rich components. A, Section of seed embryo showing intense bright lemon-yellow fluorescence of the globular bodies in cells of the cotyledon. The seed coat exhibited a blue green fluorescing layer (not visible in the photograph) in addition to the bright lemon-yellow fluorescence which is clearly visible on the left of Fig. 5A. Bar = 58 μm. B, Additional view of a section from the cotyledon of a mature seed showing numerous fluorescing globular lipid-rich bodies present in each cell. Bar = 52 μm. C and D, Sections of cotyledon and shoot apical meristem areas of somatic embryos showing fluorescing lipid bodies present in most cells. Note that lipid-rich globular bodies are reduced in size or are absent in epidermal cells (Fig. 5C; bar = 52 μm) and in cells of the apical meristem region (Fig. 5D, bar = 58 μm). The outer coating surrounding the somatic embryo also fluoresced bright yellow (visible in the lower left of Fig. 5C) consistent with the hypothesis that it has a waxy composition.

have indicated that they are naked (Gray and Purohit, 1991; Thorpe, 1995). It is possible that the high levels of brown exudates (probably phenolics), which were evident in cultures of *E. nitens* that produced somatic embryos, facilitated the production of this coating which may have formed to give additional protection to the developing embryo from an otherwise unfavourable culture environment.

From a practical viewpoint, the formation of somatic embryos which are similar in structure to mature zygotic embryos found in seeds and which contain comparable lipid-rich reserves, may be important for commercial biotechnology programmes aimed at mass propagation of elite clones of *E. nitens*. The existence of a waxy coating surrounding the fully-formed embryo may also be of significance as encapsulation of somatic embryos in a synthetic coat is an important step in the production of viable synthetic seeds (Gray and Purohit, 1991; Friend, 1993; Redenbaugh *et al*., 1993). The observation that somatic embryos of *E. nitens* were easily detached from the callus on which they formed, as was the case for somatic embryos of *E. citriodora* (Muralidharan and Mascadenhas, 1987), may be of value for the development of effective procedures for automated harvesting and encapsulation of embryos (Garrett, 1993). However, before applications of this technology can be considered, further work is required to understand the developmental process leading to the production of somatic embryos. To date, we have observed that fully-formed somatic embryos of *E. nitens* develop somewhat sporadically and this has precluded comparative studies with mature seeds to determine germination frequencies and vigour of young plants. As has been noted in other studies involving crop plants, conversion rates of somatic embryos into plants can be variable, with the cellular organization of meristem regions and the sizes of vacuoles in embryo cells identified as factors which may affect the germination capacity of somatic embryos (Nickle and Yeung, 1993; Taylor and Vasil, 1996*b*). In the present study, strong similarities were observed in the cellular patterning of apical meristems in both somatic and zygotic embryos, and also in the extent of vacuolation of cells throughout both embryo types. This may suggest that the developmental sequence of events that led to somatic embryo formation was similar to the sequence of events which leads to zygotic embryo formation during the process of seed development in *E. nitens*. Thus these observations are encouraging for ongoing investigations towards the goal

of developing cultures of *E. nitens* that are capable of producing fully-developed somatic embryos at high frequencies. The availability of such cultures would enable detailed experiments to be undertaken to determine the respective importance of many parameters that may affect the capacity of somatic embryos to convert to intact plants and also the efficacy of various encapsulation strategies to produce viable synthetic seeds. This goal should be achievable as high frequencies of somatic embryogenesis in eucalypt tissues cultured *in vitro* has been reported for *E. citriodora* (Muralidharan *et al.*, 1989). Here, the authors initiated embryogenic cultures from somatic embryos that developed from cotyledonary tissues and they were able to increase substantially the frequency of embryos formed by these cultures by ensuring the selective culture of pre-embryogenically determined cell clusters (PEDCs) (Muralidharan *et al.*, 1989). Manipulation of the culture conditions and microenvironment may also lead to increased frequencies of somatic embryogenesis in *E. nitens* as it is likely that changes in the environment of cells may influence the expression of one or more key genes which play an important role in triggering embryo formation (Dodeman *et al.*, 1997). One such factor may be oxygen supply as growth of carrot tissues in oxygen-depleted media was reported to lead to the production of cultures with a high potential for somatic embryogenesis (Kessell and Carr, 1972). With respect to the present study, it may be noteworthy that somatic embryos of *E. nitens* were produced at the base of callus that was partially engulfed by agar-solidified medium, which may be suggestive of an oxygen-limited microenvironment. Experiments are underway currently to investigate the effects of altering oxygen levels, and other components of the microenvironment of tissues cultured *in vitro* with respect to their effects upon the embryogenic potential of cultures of this commercially-important temperate eucalypt.

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LITERATURE CITED

Bandyopadhyay S, Cane K, Rasmussen G, Hamill JD. 1999. Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species—*Eucalyptus nitens* and *E. globulus*. *Plant Science* **140**: 189–198.

Bennett IJ, McComb JA, Tonkin CM, McDavid DAJ. 1994. Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. *Annals of Botany* **74**: 53–58.

Boland DJ, Brooker MIH, Turnbull JW, Kleinig DA. 1980. *Eucalyptus seeds*. Canberra, Australia: CSIRO.

Dodeman VL, Ducreux G, Kreis M. 1997. Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany* **48**: 1493–1509.

Eldridge K, Davidson C, Harwood C, Van Wyk G. 1994. *Eucalypt domestication and breeding*. New York: Oxford University Press Inc.

Friend DR. 1993. Hydrophobic coatings for synthetic seeds. In: Redenbaugh K, ed. *Synseeds: applications of synthetic seeds to crop improvement*. Boca Raton: CRC Press, 35–46.

Garrett RE. 1993. Encapsulation machinery. In: Redenbaugh K, ed. *Synseeds: applications of synthetic seeds to crop improvement*. Boca Raton: CRC Press, 203–214.

Gray DJ, Purohit A. 1991. Somatic embryogenesis and development of synthetic seed technology. *Critical Reviews in Plant Sciences* **10**: 33–61.

Halperin W. 1995. *In vitro* embryogenesis: some historical issues and unresolved problems. In: Thorpe TA, ed. In vitro *embryogenesis in plants*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 1–16.

Jones TJ, Rost TL. 1989*a*. Histochemistry and ultrastructure of rice (*Oryza sativa* L.) zygotic embryogenesis. *American Journal of Botany* **76**: 504–520.

Jones TJ, Rost TL. 1989*b*. The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. *Botanical Gazzette* **150**: 41–49.

Kessell RHJ, Carr AH. 1972. The effect of dissolved oxygen concentration on growth and differentiation of carrot (*Daucus carota*) tissues. *Journal of Experimental Biology* **23**: 996–1007.

Kirk PW Jr. 1970. Neutral red as a lipid fluorochrome. *Stain Technology* **45**: 1–4.

MacRae S, van Staden J. 1993. *Agrobacterium rhizogenes*-mediated transformation to improve rooting ability of eucalypts. *Tree Physiology* **12**: 411–418.

McComb JA, Bennett IJ. 1986. Eucalypts (*Eucalyptus* spp.). In: Bajaj YPS, ed. *Biotechnology in agriculture and forestry*, *Vol 1*: *Trees 1*. Berlin: Springer Verlag, 340–362.

Muralidharan EM, Mascarenhas AF. 1987. *In vitro* plantlet formation by organogenesis in *Eucalyptus camaldulensis* and by somatic embryogenesis in *E. citriodora*. *Plant Cell Reports* **6**: 256–259.

Muralidharan EM, Gupta PK, Mascarenhas AF. 1989. Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus citriodora*. *Plant Cell Reports* **8**: 41–43.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay of tobacco tissue cultures. *Physiologica Plantarum* **15**: 473–497.

Nickle TC, Yeung EC. 1993. Failure to establish a functional shoot meristem may be a cause of conversion failure in somatic embryos of *Daucus carota* (Apiceae). *American Journal of Botany* **80**: 1284–1291.

Pryor LD, Johnson LAS. 1971. *A classification of the Eucalypts*. Canberra: Australian National University Press.

Raghavan V. 1976. *Experimental embryogenesis in vascular plants*. London, New York, San Francisco: Academic Press, 349–381.

Redenbaugh K. 1993. *Synseeds: applications of synthetic seeds to crop improvement*. Boca Raton: CRC Press.

Redenbaugh K, Fujii JAA, Slade D. 1993. Hydrated coatings for synthetic seeds. In: Redenbaugh K, ed. *Synseeds: applications of synthetic seeds to crop improvement*. Boca Raton: CRC Press, 35–46.

Taylor MG, Vasil IK. 1995. The ultrastructure of zygotic embryo development in pearl millet (*Pennisetum glaucum*: Poaceae). *American Journal of Botany* **82**: 28–44.

Taylor MG, Vasil IK. 1996*a*. The ultrastructure of somatic embryo development in pearl millet (*Pennisetum glaucum*: Poaceae). *American Journal of Botany* **83**: 205–219.

Taylor MG, Vasil IK. 1996*b*. Quantitative analysis of ultrastructural changes during zygotic and somatic embryogenesis in pearl millet (*Pennisetum glaucum* [L.] R. Br.). *Sexual Plant Reproduction* **9**: 286–298.

Teulieres C, Marque C, Boutet A-M. 1994. Genetic transformation of *Eucalyptus*. In: Bajaj TPS, ed. *Plant protoplasts and genetic engineering V*. Berlin, Heidelberg: Springer-Verlag, 289–307.

Thorpe TA. 1995. In vitro *embryogenesis in plants*. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Tibok A, Blackhall NW, Power JB, Davey MR. 1995. Optimized plant regeneration from callus derived from seedling hypocotyls of *Eucalyptus urophylla*. *Plant Science* **110**: 139–145.

Venable JH, Coggeshall R. 1965. A simplified lead citrate stain for use in electron microscopy. *Journal of Cell Biology* **25**: 407–408.

Watt MP, Blakeway F, Cresswell CF, Herman B. 1991. Somatic embryogenesis in *Eucalyptus grandis*. *South African Forestry Journal* **157**: 59–64.

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