Propagation of *Eucalyptus grandis* In Vitro

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Nodular tissue formed on cotyledonary explants derived from *Eucalyptus grandis* seedlings developed on NZFRI *Eucalyptus* Initiation Medium. If transferred frequently (7 days or less) on NZFRI *Eucalyptus* Proliferation Medium, somatic embryos formed on the nodular tissue. Infrequent transfer of tissue on the same medium tended to give rise to adventitious shoots. Variations of a somatic embryo germination protocol (Watt et al., 1991) gave rise to plants capable of transfer to the field.

INTRODUCTION

Eucalyptus species are amongst the most productive and fastest growing hardwood plantation species. In South Africa, *E. grandis* is the most important and commonly grown species, comprising 80% of the total planted area of *Eucalyptus* (Malan et al., 1995). *Eucalyptus* species are grown mainly for pulp for high quality paper and other forest products. Increasing demand worldwide for these products has created a demand for new methods for increasing the productivity of *Eucalyptus* species and for increasing the plantation area of eucalypts. *Eucalyptus* plantations are established by seedlings, cuttings, or coppice. There has been a trend towards clonal forestry using cuttings because of the potential gains in productivity and in selection for desirable wood properties (Campinhos, 1980; Chaperon et al., 1978; Easley, 1989; Lambeth, 1988). However, not all genotypes are amenable to propagation by cuttings (Davidson, 1978) and multiplication rates may also be limiting. Micropropagation techniques offer the potential for higher multiplication rates of some genotypes. Micropropagation systems will also be necessary if genetic transformation of genotypes is desired.

Somatic embryogenesis has the potential to provide high multiplication rates of uniform genotypes. Plants of *E. citriodora* (Muralidharan et al., 1989) and *E. grandis* (Watt et al., 1991) have been successfully regenerated via somatic embryogenesis. The New Zealand Forest Research Institute (FRI) has developed successful somatic embryogenesis protocols for pines, and a pilot project was initiated to develop similar techniques for *Eucalyptus* species. *Eucalyptus grandis* was chosen as a model system to establish somatic embryogenesis techniques, as the species is easily propagated by cuttings and also by tissue culture.

The aim of this study was to evaluate and develop techniques for production of somatic embryos from hypocotyl and cotyledon explants in *E. grandis*. Experiments were initiated to produce embryogenic tissue, to see if nodular tissue would produce somatic embryos, and to determine conditions required for the growth, development, and germination of somatic embryos.

MATERIALS AND METHODS

Seed Germination. *Eucalyptus grandis* seeds (Tanzania batch P8/0/77/17, Proseed, Rotorua) were sterilised for 5 min in 50 ml of mercuric chloride (270 mg litre^{-1}) with three drops of Silwet L77 added as a wetting agent. Seeds were then washed twice in sterile distilled water. Ten seeds per dish were placed on 1/2-strength MS germination medium (Murashige and Skoog, 1962) in sterile petri dishes (90 mm × 12 mm). Seeds were incubated under 16-h photoperiod (5 μEm^{-2}sec^{-1}) at a temperature of 21C day/17C night. After 5 days on germination medium, seedcoats were removed using sterile forceps and scalpel blades. With this procedure, seeds germinated in approximately 15 days and 90% to 100% germination was achieved.

Callus Initiation. The cotyledons and hypocotyls (2 to 10 mm in length) of germinated seedlings were cut into pieces 1 mm long. The cotyledon and hypocotyl pieces were placed on NZFRI *Eucalyptus* Initiation Medium (EIM) (Smith and Cranshaw, unpublished) in a line so that the origin of each explant could be easily identified. The explants were incubated under 16-h photoperiod, at a light intensity less than 5 μEm^{-2} sec^{-1} at a temperature of 21C day/17C night.

The cotyledon and hypocotyl explants were transferred after 2 weeks from initiation medium onto NZFRI *Eucalyptus* Proliferation Medium (EPM) (Smith and Cranshaw, unpublished). Explants were transferred to a fresh spot on this medium at 14 days, and placed on fresh medium every 28 days. Explants were incubated under 16-h photoperiod, at a light intensity less than 5 μEm^{-2} sec^{-1} at a temperature of 21C day/17C night.

Table 1. Response (percentage) of explants on *Eucalyptus* proliferation medium after 3 months.

Explant	Mucilaginous tissue	Nodular tissue	Nodular tissue with roots	No response
Cotyledon	0	50	15	35
Hypocotyl	15	15	10	70

In one experiment, after 10 weeks on EPM, nine clones which formed nodular tissue (Fig.1) (originating from both cotyledons and hypocotyls) were transferred to regeneration medium (Table 1) to see if nodular tissue would produce somatic embryos. The nodular tissue from either cotyledon or hypocotyl explants was placed onto separate dishes so that each explant could be traced back to its origin. Tissue was moved to a fresh spot on the medium at 14 days, and transferred onto fresh medium every 28 days. Tissue was maintained under 16-h photoperiod (light intensity less than 5 μEm^{-2} sec^{-1}) at a temperature of 21Cday/17C night for approximately 16 weeks. Somatic embryos formed on the nodules if subculture was frequent (7 days), and adventitious shoots formed with less frequent subculture (14 days or more).

The nodular tissue was split into 3- to 5-mm-diameter pieces and eight pieces per plate were placed onto 1/2 MS media containing different concentrations of growth

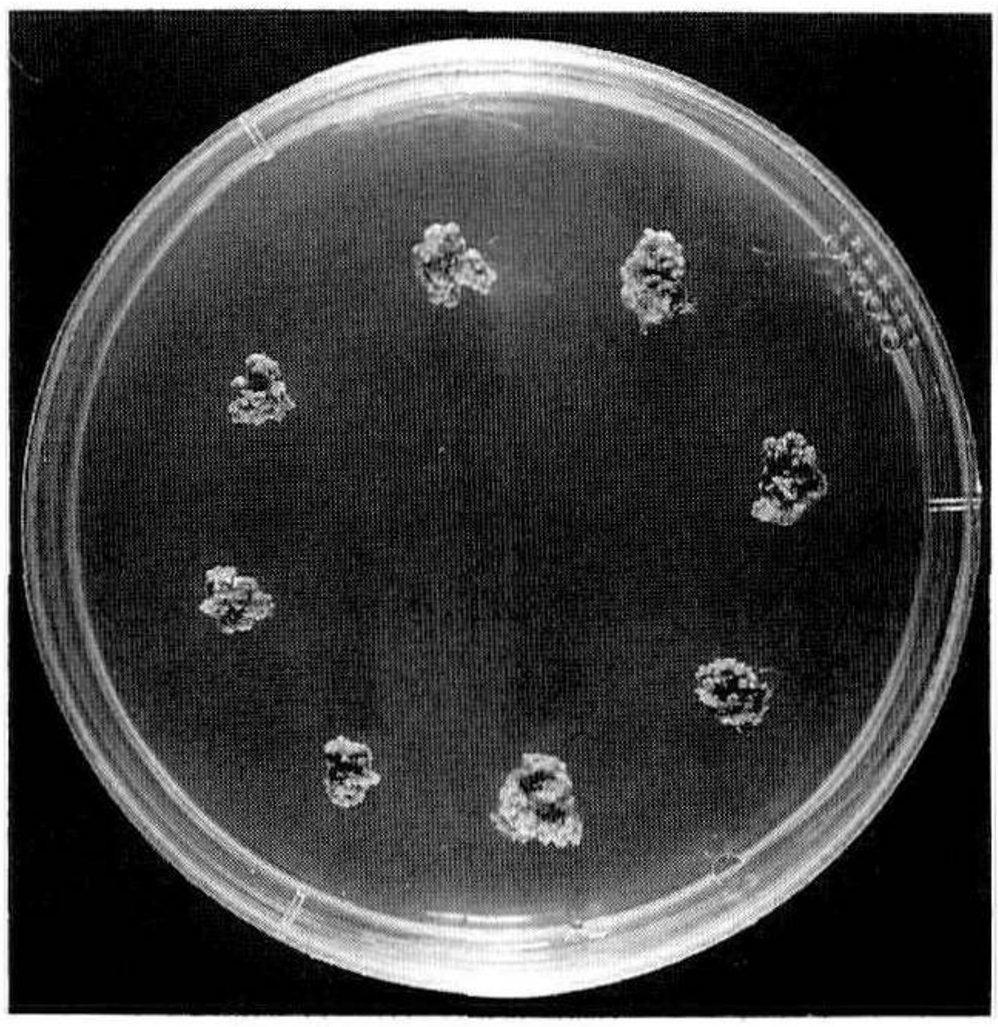

Figure 1. Nodular tissue of *Eucalyptus grandis* on regeneration medium.

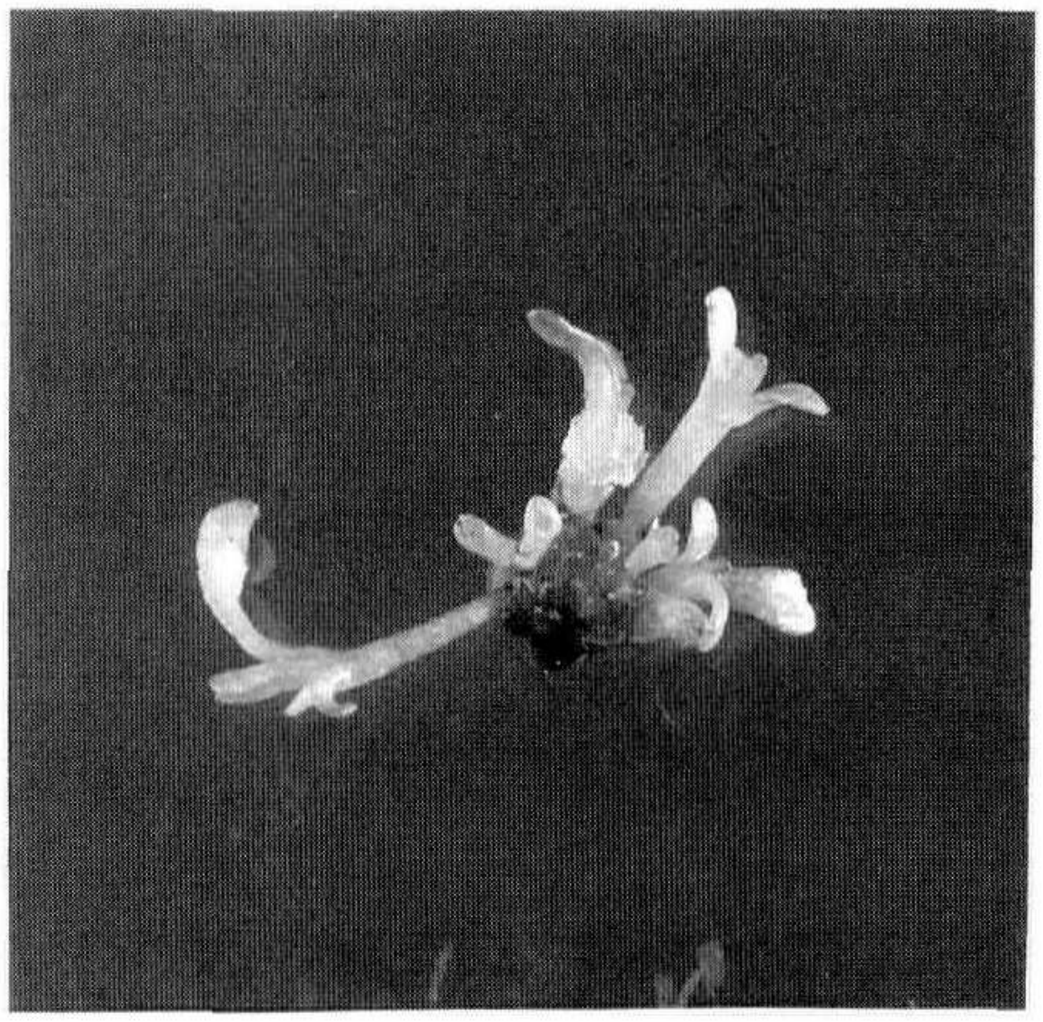

Figure 2. Nodular tissue of *Eucalyptus grandis* simultaneously forming somatic embryos and adventitious shoots. SE, somatic embryos; AS, adventitious shoots.

regulators as described by Watt et al., (1991). The medium was gelled with Gelrite at various concentrations (Table 2). The plates were kept in the dark (covered with black cloth) at 24C for 9 weeks.

Somatic embryos and adventitious shoots (Fig. 2) produced from the nodular tissue were counted under sterile conditions using a dissecting microscope. All somatic embryos were separated from the callus tissue and maintained on R9 medium (Watt et al.,1991) to enable elongation of the hypocotyl and cotyledons. Embryos were moved to a fresh spot on the medium at 14 days, and transferred onto fresh medium every 28 days. The somatic embryos were maintained under 16-h photoperiod, with low light intensity (5 μEm^{-2} sec^{-1}) at a temperature of 21C day/17C night. After 8 weeks on R9 medium, embryos were transferred to 1/2 MS medium with 4 g $litre^{-1}$

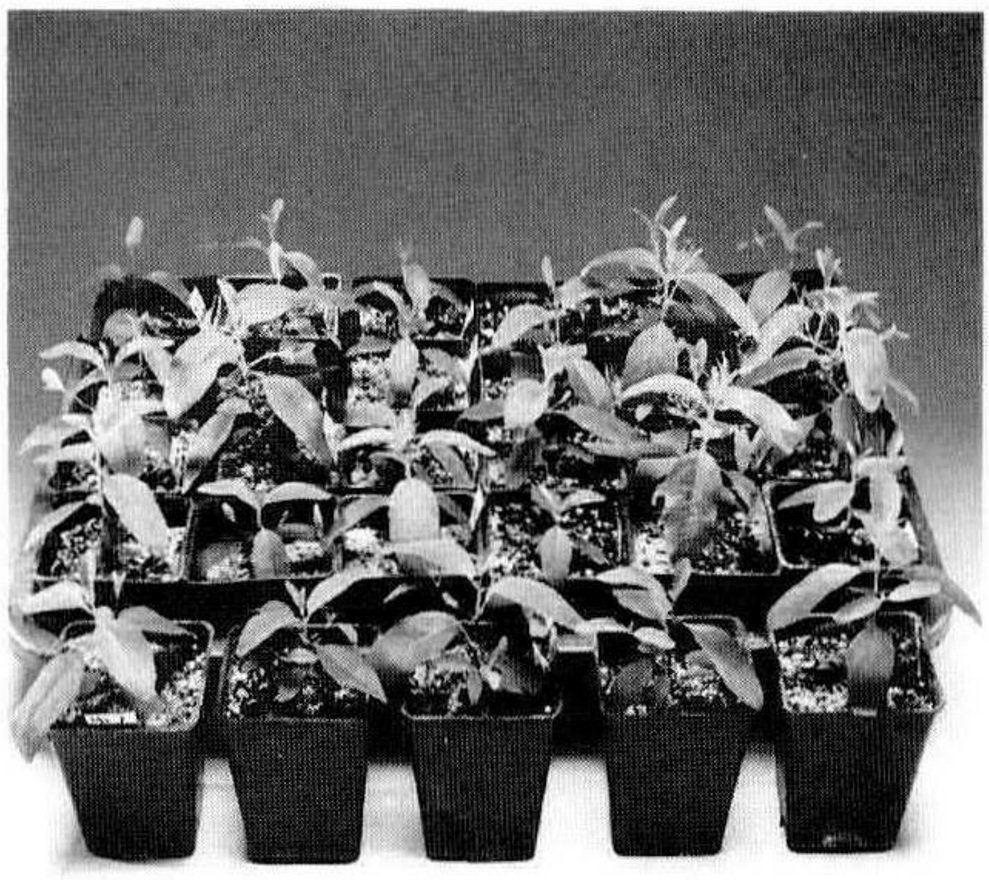

Figure 3. *Eucalyptus grandis* plants regenerated from somatic embryos, after 8 weeks in potting medium.

Figure 4. *Eucalyptus grandis* somatic seedlings in a "starved" state ready for field planting.

charcoal and were maintained under the same lighting conditions for a further 2 days. This procedure was to remove any phenolic substances which were exuded by the germinating somatic embryos.

Somatic embryos were then planted in a mix of perlite and commercial potting mix (1 : 2, v/v) in plastic propagation trays (29 cm × 36 cm) with transparent plastic lids. The propagation trays were placed in a greenhouse and watered every day. After 8 weeks, the plantlets (Fig. 3) which had germinated were transplanted into potting mix in (5 cm × 9 cm) plastic pots. Plantlets were retained in plastic pots for 3 months in a "starved" state as recommended by Faulds and van Dorsser (1987). The leaves turned red, increased in thickness and stems became woody (Fig. 4). This starvation regime is preferred because seedlings become hardier and more drought resistant and, therefore, are more suitable for planting on drier sites or during the drier parts of the growing season. Somatic seedlings of *E. grandis* were planted in the field near Whangarei in late August 1994.

RESULTS

The explants were monitored and scored for tissue development after 3 months (Table 1). A much larger percentage (65%) of cotyledon explants responded on EPM than did hypocotyl explants (30%). The predominant type of tissue produced by both types of explant was a green nodular tissue (Fig. 1), a small percentage of which also produced roots (10% to 15%). The hypocotyl explants which did not respond either died or became translucent.

Somatic embryos were produced on all media tested (Table 2). The largest numbers of somatic embryos were produced on the media with higher levels of growth regulators (media 4, 5, and 6). The concentration of Gelrite in the medium influenced the number of somatic embryos produced, with higher concentrations of gelrite increasing the number of somatic embryos produced regardless of the concentration of growth regulators.

Table 2. Effect of 1/2 MS-based media on somatic embryo production and subsequent conversion.

Media	NAA (mg l^{-1})	BAP (mg l^{-1})	GA_3 (mg l^{-1})	Gelrite (g l^{-1})	No. of somatic embryos produced	No. of somatic established transferred to soil	No. of plants
1	0.01	0.1	0.1	2.0	87	10	1
2	"	"	"	3.0	138	27	7
3	"	"	"	4.0	148	15	4
4	0.04	0.4	0.4	2.0	154	35	11
5	"	"	"	3.0	213	18	5
6	"	"	"	4.0	334	13	2

However, the quality of the somatic embryos was adversely affected by high Gelrite concentration in combination with high growth regulator concentration. Although the greatest number of somatic embryos were produced on medium 6, less than 4% of these survived to be transferred to potting mix. The best quality somatic embryos were produced on medium 4, for over 22% of these embryos were transferred to potting mix and 31% of these grew (which equals 6.8% of initial embryos) and were established in the field. These plants are now 1 year old, over 1 m in height, and phenotypically normal.

DISCUSSION AND CONCLUSION

Cotyledon explants of *E. grandis* were the best tissue for regenerating nodular organogenic and embryogenic tissue. Although medium 6 produced the highest number of somatic embryos, medium 4 was the preferred medium, as better quality embryos were produced and subsequently these converted to plants more successfully.

Somatic embryos were successfully produced from cotyledon explants of*E. grandis* and somatic seedlings have been planted in the field. With the present protocol, both the yield of somatic embryos and the number that successfully converted to plants was relatively low. Further studies are needed to improve the methods so that larger numbers of high quality embryos can be produced and converted to plants.

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