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PROGRESS TOWARD CLONAL PROPAGATION OF EUCALYPTUS SPECIES BY TISSUE CULTURE TECHNIQUES

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Abstract. Large numbers of clonal trees of eucalyptus species have been obtained by culturing nodes of seedling or coppice material. Adult nodes of two species have successfully produced multiple buds. Shoot systems have been established from such buds and research is being directed towards the establishment of healthy plants by inducing these shoots to form roots.

Culture media are discussed and the composition of the most successful media for the production of multiple buds and for rooting are given.

Problems concerning microbial contamination of field collected material are discussed and methods for reducing consequent losses are suggested.

A routine for the establishment of test tube plants in soil is described.

REVIEW OF LITERATURE

A number of attempts have been made to propagate *Eucalyptus* species by tissue culture techniques (4,7,8,9,10). Successful regeneration from lignotuber material has been reported for *E. citriodora* (1) and from seedling hypocotyl callus for *E. alba* (11). Two approaches have been used to propagate from nodal cultures; one is to produce multiple buds and shoots in aseptic cultures and then to induce these shoots to form roots (8,9,10). The other approach is the direct induction of roots and shoots on an initial nodal explant (2,3,7,10). Seedling nodes of *E. ficifolia* have been induced to form multiple buds and subsequently roots (8,9,10). Adult nodes of *E. ficifolia* and *E. polybractea* and seedling nodes of *E. regnans* have produced multiple buds (10). Suitable concentrations of chemical constituents of multiple bud media have been established by the broad spectrum approach (5,6). *E. grandis* plants have been successfully established from cultured nodes of seedlings, coppice and young trees (2,3,7,10). Microbial contamination has been a serious problem with field-collected material (8,9,10).

MATERIALS AND METHODS

Culture Media. *Multiple Bud Medium.* A suitable medium for the production of multiple buds and the growth of shoots from *E. ficifolia* seedling nodes was derived from Broad Spectrum medium MHMH (5,6,8). This medium (Medium A, Table 1) differed from MHMH in having 100μM Fe instead of 50μM and in having IBA as the sole auxin at a concentration of 5μM instead of 10μM. Medium A is the same as Medium 1 reported in (10) except that the concentration of IBA has been changed from 10μM to 5μM.

Table 1. Medium A. General Multiple Bud Medium.

MINERALS		
Macronutrients	(mM)	NH_4NO_3 (10); KNO_3 (10); NaH_2PO_4 (1); $CaCl_2$ (2); $MgSO_4$ (1.5).
Micronutrients	(mM)	H_3BO_3 (50); $MnSO_4$ (50); $ZnSO_4$ (20); $CuSO_4$ (0.1); Na_2MoO_4 (0.1); $CoCl_2$ (0.5); $FeSO_4$ (100); Na_2EDTA (100); Na_2SO_4 (650).
AUXINS	(μM)	IBA (Indole butyric acid) (5).
CYTOKININS	(μM)	Kinetin (1); BAP (Benzylaminopurine) (1).
CARBON SOURCE	(μM)	Sucrose (120).
GROWTH FACTORS AND AMINO ACIDS		
	(μM)	Inositol (600); Nicotinic acid (40); Pyridoxine HCl (6); Thiamine HCl (40); Biotin (1); D-Ca-Pantothenate (5); Riboflavin (10); Ascorbic acid (10); Choline Chloride (10); L-Cysteine-HCl (120); Glycine (50).
AGAR (g/l)		Difco Bacto-Agar (8)

Rooting Medium: Medium B (Table 2) was selected by combining the medium of Cresswell and Nitsch (3) with Broad Spectrum medium MHZH to promote root and shoot growth in *E. grandis* nodes. Medium B is a simplified form of the combination medium reported in (10). As Medium B also induced excellent roots on *E. ficifolia* seedling shoots it was therefore used in experiments with four species.

Table 2. Medium B. Rooting Medium.

MINERALS		
Macronutrients	(mM)	$Ca(NO_3)_2$ (2.1); KNO_3 (2.2); KH_2PO_4 (0.92); NH_4NO_3 (1.0); $MgSO_4$ (0.66).
Micronutrients	(μM)	H_3BO_3 (160); $MnSO_4$ (110); $ZnSO_4$ (43); $CuSO_4$ (0.1); Na_2MoO_4 (1); $FeSO_4$ (100); Na_2EDTA (100).
AUXIN	(μM)	IBA (5).
CARBON SOURCE	(mM)	Sucrose (60).
GROWTH FACTORS AND AMINO ACIDS		
	(μM)	Inositol (620); Nicotinic acid (45); Pyridoxine HCl (3.0); Thiamine.HCl (5.5); Biotin (0.3); Folic Acid (1.1); D-Ca-Pantothenate (0.5); Riboflavin (1); Ascorbic Acid (1); Choline Chloride (1); L-Cysteine.HCl (12); Glycine (32).
AGAR (g/l)		Difco Bacto-Agar (8).

Culture Tubes. Aliquots (10 ml) of culture media were dispensed and autoclaved in transparent polycarbonate tubes with screw caps.

Plant Material. Seedling nodes were either prepared aseptically (8) or taken from young potted plants and disinfested in the usual manner (5,8,10). Adult nodes gave best response in culture when selected from healthy, well watered, well fertilized trees.

Species. Seedling nodes of *E. ficifolia*, *E. grandis* and *E. regnans* were planted under aseptic conditions on Medium A or Medium B. Field collected adult nodes of these three species and also *E. polybractea* were treated similarly.

Incubation. Explants were incubated in the dark for at least 24 hours, as this appeared to eliminate or reduce brown exudate formation in the culture medium (3). All cultures were then incubated in a room containing banks of fluorescent lights. Light intensity was about 300 microeinsteins/m^2/sec. The cultures received 12 hours light/12 hours dark at approximately 25°C.

Dissection and Subculture. The successful establishment of multiple buds and shoots required that most explants were dissected and subcultured a number of times on Medium A (Figure 1, Figure 2, Figure 4).

Establishment. Where complete regeneration was achieved, the plants were established in pots.

RESULTS

Progress with Individual Species:

E. ficifolia

Clonal propagation from seedlings. Aseptic seedling nodes produced multiple buds on Broad Spectrum Medium MHMH (8). These buds were subcultured onto Medium A where they proliferated with great vigour. After three passages on this medium the number of cultures had increased from 20 to over three hundred. The multiple buds developed from a mass of green basal tissue and formed a large cluster on the medium. When a cluster of buds was dissected and small pieces containing several buds transferred to fresh medium, further buds developed. Usually, one bud in the new cluster developed into a leading shoot with very small green leaves (Figure 2). Leading shoots were removed and placed on rooting medium (Medium B, Table 2) and the remaining buds were returned to fresh Medium A. In two to three weeks vigorous roots with abundant root hairs developed on most explants on Medium B. Sometimes poor roots and/or teratomas (callus-like projections) developed. When these occurred, the explants were removed

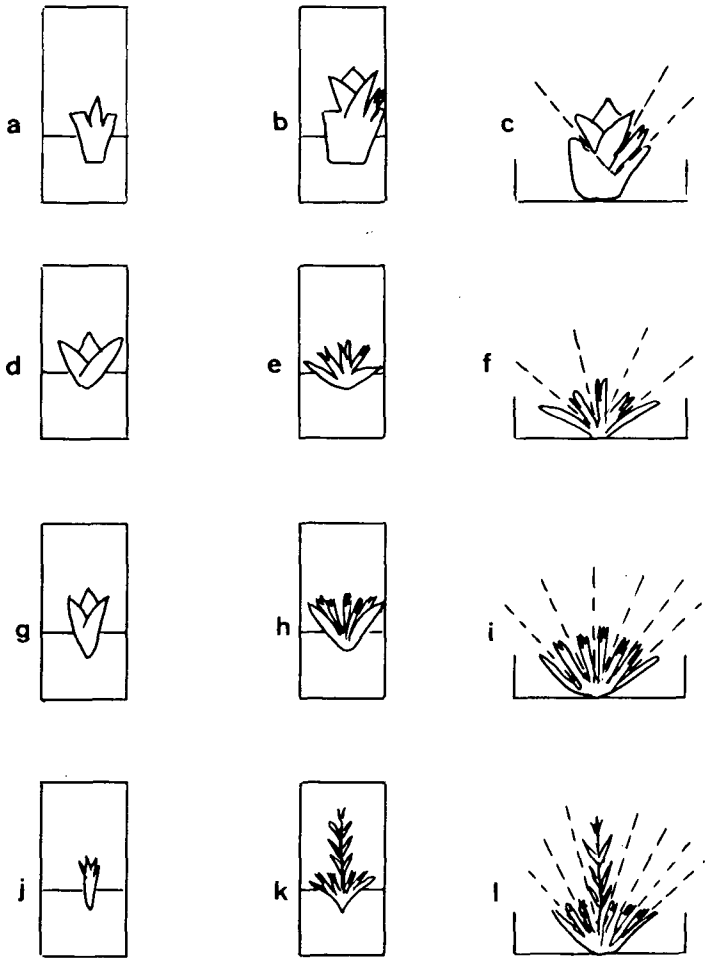

Figure 1. (a-l). Stages in the development of multiple buds and shoots from nodes of adult *E. ficifolia*.
(a) Freshly excised node on medium A. **(b)** Main bud opened. Accessory bud developed. Any other buds which appeared at this stage were very small. Stem base made callus and became swollen. **(c)** Explant was removed to sterile Petri dish and buds dissected. Broken lines show where incisions were made. Most of the stem base was removed. **(d)** Each bud was planted on fresh medium A. **(e)** Two or three buds developed. **(f)** Buds were dissected. Outer leaves were discarded. **(g)** Each bud was planted on fresh medium A. **(h)** Several buds developed. **(i)** Buds were again dissected; outer leaves discarded. **(j)** Each bud was planted on fresh medium A. **(k)** A number of buds and a leading shoot with very small green leaves developed. **(l)** The leading shoot was removed and planted on medium B (rooting medium). The remaining buds were dissected and planted on fresh medium A.

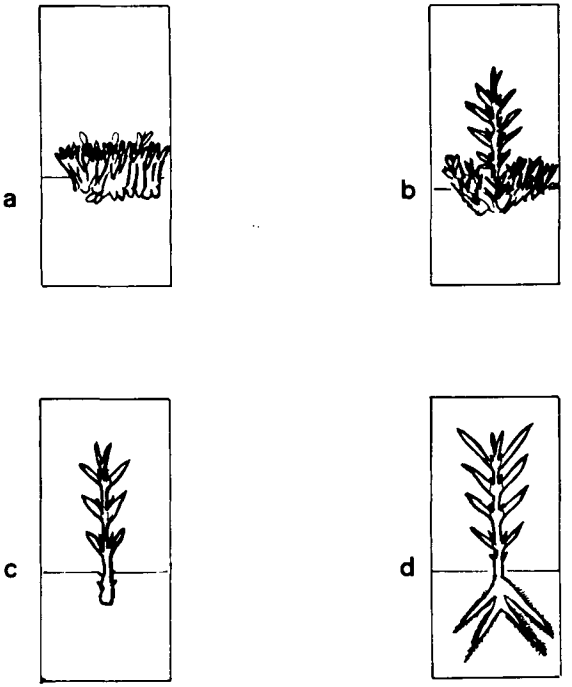

Figure 2. (a-d). Development of whole plant from shoots of seedling *E. ficifolia*.
(a) Multiple buds grown on medium A. **(b)** A leading shoot developed. **(c)** The leading shoot was excised and planted on medium B. Leaves at the base of the stem were trimmed away. ***(d)** Vigorous roots with abundant root hairs appeared in 2-3 weeks.

* Sometimes poor roots and/or teratomas developed. This condition was corrected by removing the explant, trimming the base of the stem with a scalpel and replanting on fresh medium B. Good roots generally appeared in about 10 days.

from culture, the stem bases surgically trimmed and the explants placed on fresh rooting medium. Vigorous roots then usually appeared in about ten days. If buds were placed on Medium B without forming good shoots, roots were generally obtained, but the shoots remained too small for the successful establishment of the plants. Many rooted plants with several leaves were successfully established in pots.

Clonal propagation from adults. Nodes taken from 25 year old Melbourne trees were successfully cultured on Broad Spectrum Medium MHMH. Growth was very slow. These nodes were transferred to Medium A where faster, more vigorous growth and multiple buds were observed. Newly cultured nodes were placed directly on Medium A, where they produced multiple buds. The production of shoots from adult nodes required the repeated dissection and subculturing of buds (Figure 1) until all traces of original adult tissue had been discarded. The small leaved shoots which then emerged were identical in appearance with the shoots produced by seedling cultures. The only shoot which has so far been placed on Medium B produced a root after two weeks. Adult nodes placed directly on Medium B without prior subculturing on Medium A failed to root.

E. grandis

Clonal propagation from seedlings, coppice and young trees. Nodes from glasshouse grown seedlings, coppice and young trees successfully formed plants when placed directly on Medium B. Buds started to open in one to two weeks and roots formed in the third and fourth weeks. *E. grandis* roots had fewer and smaller root hairs than *E. ficifolia* rooted on the same medium. *E. grandis* nodes cultured on Medium A developed leaf callus, and the shoots eventually abscissed.

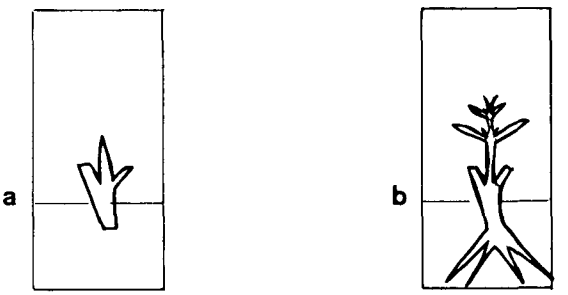

Figure 3. (a-b). Development of whole plant from node of seedling, coppice or young tree of *E. grandis*.
(a) Freshly planted node on medium B. **(b)** A single shoot developed in about 2 weeks and roots developed from 3-4 weeks.

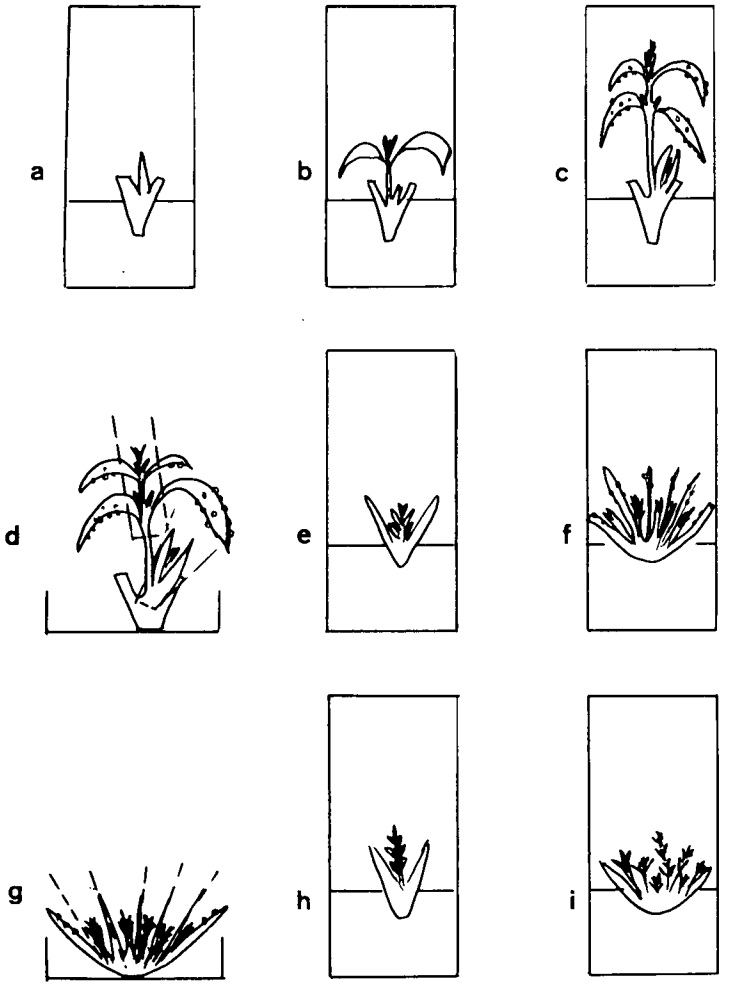

Figure 4. (a-i). Development of multiple buds from nodes of seedling or young tree of *E. regnans*.
(a) Freshly planted node on medium A. **(b)** Bud opened. Leaves were relatively large. Accessory bud appeared. **(c)** Shoot developed on long stalk. Accessory bud developed. Largest leaves developed a white sugary type of callus. **(d)** Callused leaves were removed and remaining apical shoot dissected. Accessory bud with some basal tissue was also removed. **(e)** Each shoot was transferred to fresh medium A. **(f)** Accessory bud gave rise to more new buds than the apical shoot. Some leaves continued to form callus. **(g)** Callused leaves were removed and developing buds were dissected. **(h)** Buds were placed on fresh medium A. **(i)** Multiple buds appeared without large callused leaves.

Clonal propagation from adult trees. No success has been achieved with adult material. Nodes were obtained from forest trees near Coffs Harbour, and invariably the cultures had to be discarded because of microbial contamination.

E. regnans

Clonal propagation from seedlings and young trees. Nodes from large seedlings (50-150 cm high) produced shoots on a number of media including Medium A and Medium B. Leaf callus was common on all cultures. However, by dissecting and subculturing on Medium A (Figure 4) multiple buds free from callus were obtained. In contrast with *E. grandis* no roots developed when nodes were placed directly on Medium B. In one nodal replicate out of ten a shoot with large leaves and a small root formed on broad spectrum Medium MHZH.

Clonal propagation from adult trees. Some nodes from adult forest trees produced shoots on a number of media including Medium A and Medium B but all cultures were eventually lost as a result of microbial contamination.

E. polybractea

Clonal propagation from adult trees. Nodes from adult trees have formed small shoots and multiple buds on Medium A.

Establishment of Cultured Plants in Soil. "Hardening off" was a critical period in the propagation of plants by tissue culture techniques and a considerable amount of intensive care was necessary. When the plants had developed vigorous shoots and roots and a total length of approximately 6-10 cm, the lids were removed from the culture tubes. The plants remained in the open tubes for several days. a few drops of water were added daily to each tube to prevent desiccation of the media and water stress to the plants. The possible introduction of microbial contaminants from the air at this stage was considered to be unimportant.

The plants were then removed from the tubes. Excess media was washed off the roots and the plants were placed in a sand/loam mixture (approximately 1 part sand and 1 part loam) in small pots and thoroughly wetted with a solution of commercial nutrients such as "THRIVE" or "ZEST". A covering of transparent glass or plastic was placed over the plants to maintain humidity and thus to prevent wilting. The covered plants remained for 24 hours in a shaded position and were then transferred to a shade house which screened 80% of light. The covers were removed after about two days but were replaced again for a further day or two if any plants were seen to wilt. New leaves which developed after potting were less prone to wilting than test tube leaves. When plants were established and

making visible growth under sheltered conditions, they were gradually removed to positions of increasing light intensity. Leaf scorch was observed in cases where plants were removed from the shade house directly to positions of full, open sunlight.

DISCUSSION

The original objectives of our research with *E. ficifolia*. *E. grandis* and *E. regnans* was not only to clonally-propagate selected individuals by tissue culture (TC) but also to produce plants which would flower earlier than seed-propagated material. In the case of *E. ficifolia*, the hope was that TC-propagated plants for sale in nurseries could be seen to have red flowers or orange flowers or some other color of flower at the time of their purchase. In the cases of *E. grandis* and *E. regnans*, the reason for TC-propagation was to provide clonal material from superior trees for early seed production in isolated seed orchards. The fulfilment of these aspirations appeared to be dependent on the successful induction of root formation on material from adult trees, along with the maintenance of the adult physiological state in the rooted material. Our research in 1977 revealed that although seedling material could be used to select cultural conditions suitable for shoot and multiple bud development on nodes from adult trees, the result from experiments with seedling material relative to the induction of rooting could not be extrapolated to adult material. A culture medium that induced root formation on all seedling cultures and subcultures of *E. ficifolia* induced basal callus with adult nodal cultures. These findings led to research directed at two methods for the achievement of clonal propagation, both involving techniques to obtain juvenile material.

The first method involved the mutilation of adult trees at their base to induce the development of lignotuber or epicormic buds, that is to obtain shoots or coppice with juvenile characteristics. This seems to be a worthwhile approach with many species of Eucalyptus (including *E. ficifolia* and *E. polybractea*) but is less effective with non-lignotuberous species such as *E. grandis* and *E. regnans*. Shoots or coppice thus formed would then be used as sources of explants and, because of their juvenile character, might more easily be induced to form multiple buds or roots in culture by using media which induces these organs on seedling material.

The second method involves the culture and repeated subculture of nodes from adult trees on multiple bud inducing medium in the hope that this would lead to a change in their physiology, so that at some point in their subculture the buds would respond to rooting medium (developed for seedling cul-

tures) by forming roots. If this happened it might be because the physiology of the buds had changed from their initial adult status to one similar to that in seedlings; if this were the case, then our original hope of achieving earlier flowering might be a forlorn one, but at least clonal propagation and multiplication would have been achieved.

Both of the above methods are showing signs of success but both are being hampered by the serious microbial contamination problem associated with numerous insect pests which attack Eucalyptus trees growing in the open in Australia. Microbial contamination is greatly reduced when nodes from greenhouse grown Eucalyptus trees are excised and cultured. A protocol for minimizing the field contamination problem is being tested which, in essence, involves putting a "greenhouse" around the material to be excised for culture. The "greenhouses" being tested are the glacine bags and sausage-skins used by plant hybridizers. The procedure is to cut the tree to induce new growth and to enclose this new growth in a bag; the parts of the tree treated are first sprayed with insecticides, such as Rogor, and fungicides, such as Captan. The new growth is later excised and cultured following a standard disinfestation treatment (see Methods) and all non-contaminated cultures will be put on to multiplication medium so as to obviate the need to return to the tree for further material. In other words, in Australia, because of the big insect and associated microbial problem, a lot of time and preparation has to be put into obtaining aseptic Eucalyptus cultures, but that once these have been obtained they become the nuclear stocks for particular genotypes, and can be maintained as such in culture tubes.

A great deal of work remains to be done to test and perfect these techniques, and special difficulties are associated with non-lignotuberous species of Eucalyptus such as *E. grandis* and even more so with *E. regnans*. The signs are that research on the four species described in this paper will be applicable to many species of Eucalyptus and, with some modifications, to many species of shrubs and trees.

One part of this research which might be put into practice immediately is the TC-propagation of seedling material. Seedling clones, even though their potentials are initially unknown, could be valuable in the following circumstances:

(a) Progeny from valuable controlled cross experiments and from elite seed orchards could be multiplied rapidly for commercial purposes or for further experimental work;

(b) Seeds of some species are rare, difficult to collect and/or expensive; unlimited numbers of plants can be obtained by TC-propagation of seedlings from very few seeds.

(c) Representatives of a number of seedling clones could be tested at a particular site and characters such a vigor, pest resistance, suitability to soil type and climate could be noted; further large numbers of well suited plants could later be obtained from the original TC "clone bank".

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