authority to make sure that this system of heating is acceptable to them. It is a tried and proven method and certainly works well. It is widely used in Queensland and northern new South Wales.

VEGETATIVE PROPAGATION OF *EUCALYPTUS FICIFOLIA* F. MUELL BY NODAL CULTURE *IN VITRO*

R.A. de FOSSARD and R.A. BOURNE

Department of Botany
University of New England
Armidale, N.S.W. 2351, Australia

Abstract. The red-flowering gum, *Eucalyptus ficifolia*, is a very attractive ornamental tree which is propagated by seed because, like many tree species, it is difficult to propagate by cuttings, budding and other classical methods of vegetative propagation. Although the flower colour on individual trees is the same, it is highly variable on different trees reared from seed and can be white, pink, orange, scarlet red or maroon. A method for the clonal propagation of *E. ficifolia* using nodal culture has been developed which involves first the culture of nodes, second the subculture of nodes excised from shoots on the primary cultures and finally the initiation of roots on subcultured nodes.

REVIEW OF LITERATURE

There have been several attempts to regenerate plants from callus of various species of *Eucalyptus* (4,7,9) and successful regeneration has been reported for *E. citriodora* (1) and *E. alba* (8). Organ culture of nodes has also been successful with *E. grandis* (2,3,7). The research with *E. ficifolia* was done using the Broad Spectrum approach which was tested first with tobacco (6) and later with strawberry, and led to the development of a very high multiplication rate (10).

MATERIALS AND METHODS

Culture of aseptic seedlings of *E. ficifolia:* Seeds were treated with 0.1% (v/v) 7X detergent and 5% (w/v) calcium hypochlorite for 20 min, followed by several rinses in sterile water and were then planted on sterile medium-B (5).

Culture of nodes from aseptic seedlings: When the aseptic seedlings had developed six nodes (node 1 — cotyledonary

node), each node was excised and, without defoliation, was planted on one of the 81 media of the Broad Spectrum experiment (6); there were thus 486 cultures, that is six node-replicates of 81 treatments. The Broad Spectrum experiment consisted of combinations of four broad categories of constituents, namely: (1) minerals, (2) auxins, (3) cytokinins and (4) sucrose plus growth factors plus amino acids, each at three concentrations, low, medium and high. This experiment with seedling nodes indicated that four of the 81 media tested might be suitable for nodal culture, and these four (Broad Spectrum codes*: LLLM, LHLH, MMHH and MHMH) were tested using nodes from adult trees.

Disinfestation of nodes from adult trees: The *E. ficifolia* trees used in this study were selected in Sydney and Melbourne, 560 and 1600 km, respectively, from the tissue culture laboratory in Armidale. Several, approximately 6 cm long, branch tips of new growth were cut from each tree and were defoliated down to about half their petioles. The cut end of each branch was sealed with adhesive paper and the branches were placed in plastic bags, closed and transported by air to Armidale (occasionally, the bagged branch tips were stored in a refrigerator at about 4°C overnight prior to air shipment). Between 24 and 48 hours after picking, the branches were treated as follows: (1) Running tap water (town supply with possibly some residual chlorine in it) for 1 hour; (2) 0.1% (v/v) 7X-detergent for 5 min; (3) individual branch tips were then treated with 5% (w/v) calcium hypochlorite (freshly prepared and filtered); (4) three rinses in sterile water; (5) 10 μM ascorbic acid for 2 hours; (6) stored in sterile water until removed for excision of the nodes.

Subculture of nodes: Primary cultures gave rise to small shoots and often multiple buds. Nodes and buds were excised and placed on fresh medium (Broad Spectrum, MHMH) for further growth; these were later subdivided and placed on fresh medium, and the process repeated to obtain clonal populations.

Induction of roots from nodes and nodal subcultures: Broad Spectrum medium MHMH was used as a basal medium to test several combinations of factors to obtain root formation on previously rootless nodal explants and subcultured nodes.

Incubation conditions: 12/12 (hours light/hours dark) at 25°C has been used throughout this research, illumination coming from GRO-LUX fluorescent lights; no other temperature or

---

* The first letter (of the 4-letter code) is for the first category, minerals, the second letter for auxins and so on; L, M, H are abbreviations for low, medium and high concentrations, respectively.

light/dark regimes have been tested, nor have any combinations of an initial period of dark incubation followed by light/dark incubation, been tested, as done for *E. grandis* (3,7).

Table 1. Constituents and concentrations of medium suitable for the induction of roots from seedling nodes and nodal subcultures of *Eucalyptus ficifolia*.

Macronutrient elements (mM): NH_4NO_3(10), KNO_3(10), NaH_2PO_4(1), $CaCl_2$(2), $MgSO_4$(1.5)
Micronutrient elements (μM): H_3BO_3(50), $MnSO_4$(50), $ZnSO_4$(20), $CuSO_4$(0.1), Na_2MoO_4(0.1), $CoCl_2$(0.5), KI(2.5), $FeSO_4$(10), Na_2EDTA(10), Na_2SO_4(40).
Main carbon source (mM): sucrose (either 60 or 120)
Growth factors (μM): inositol (600), nicotinic acid (40), pyridoxine.HCl(6); thiamine.HCl(40)
Auxins (μM): IBA (indolebutyric acid)(10)
Agar (g/l): Difco Bacto-Agar(8)

RESULTS

Culture of nodes from aseptic seedlings: The experiment with seedling nodes on the 81 media of the Broad Spectrum experiment revealed many different types of responses, including five main types of callus, ten or more types of organized growth and several abnormal morphological responses. In general, combinations with the high concentration of sucrose + growth factors + amino acids favored organized normal development whereas combinations with the low and medium concentrations of this category of constituents led to unorganized (callus) growth. Particularly interesting was the formation of multiple buds on medium MMHH and MHMH; multiple bud formation is the development of axillary buds on the shoots that develop on the initial nodal cultures.

Culture of nodes from adult trees: Microbial contamination losses averaged 30% of cultures for most nodes and about 6% for stem tip cultures (these explants were obtained by removal of the leaves protecting the stem tip). Trees up to 36 years old were used in this study and some nodes from all trees sampled grew on the four media. Some nodal cultures on MMHH produced multiple buds. Many cultures were growing well on these media two months after planting and then, unexpectedly and quite rapidly, deteriorated and died. This response had also been noted with some seedling nodal cultures in the Broad Spectrum experiment. This decline and death of healthy cultures can be avoided by subculture to fresh medium, and possibly by reducing the desiccation of the medium by using

Parafilm closures, an idea presently being tested.

Subculture of nodes and buds: Nodes and buds were excised from primary cultures of seedling nodes and were tested on the same four media used for the nodes from adult trees; best growth of subcultured nodes occurred on medium MHMH. Tissue in contact with this medium became black and warty in appearance, whereas tissue not in touch developed in an apparently normal manner.

The induction of roots on seedling nodes and nodal subcultures: Medium MHMH was used as a basal medium in this work and initially three ideas were tested in an attempt to induce root formation in these rootless explants and cultures. These ideas were: (1) that liquid medium with filter paper wick support for the culture might favor root formation more than agar-solidified medium; (2) that cytokinins might inhibit root formation; and (3) that specific auxins might promote root formation. Tests were done with seedling nodes and with subcultured nodes and involved more than 500 cultures of each type. The results showed clearly that agar-based media were totally superior to liquid media both for shoot development and root formation. Cytokinin-free media were much better than cytokinin-containing media for root formation. Of the six auxins tested, IBA (indolebutyric acid) was the best. Several seedling nodes formed roots on cytokinin-free agar-based media in the presence of the high concentration either of IBA or of a few other auxins; but only one subcultured node produced roots and this was on an agar-based cytokinin-free, auxin-free medium, that is MZZH (where Z = zero). These results led to the concurrent testing of (1) various concentrations of IBA (namely: 0, 0.1, 0.3, 1, 3, 10, 30, 100 μM) on M-ZH, and (2) 36 combinations of iron, IBA, sucrose and growth factors. The second of these two experiments revealed two combinations of the factors tested capable of inducing root formation in nearly all of the seedling nodes and subcultured nodes; the two combinations differed only in concentration of sucrose, and are listed in Table 1.

DISCUSSION

This paper is a progress report on work started in November 1975, and thus covers about 10 months of intermittent research. The problems in trying to find methods for the clonal propagation of adult trees are easy to state in general terms. First, a method for ridding the microbes from the surface of the tissue to be explanted must be devised, and the disinfestation treatment should not be so severe as to harm the tissue. Second, cultural conditions suitable to promote and sustain the

healthy growth of the explanted material must be found; these include finding a suitable culture medium and conditions of incubation. Next, an optional but highly desirable stage, is to find ways to multiply and to sustain the healthy growth of the cultured material; ideally, there should be no need to initiate fresh material from the tree at intervals since this will always be attended by some contamination losses and is also likely to be influenced by seasonal conditions. Multiplication of desirable material in the culture tube offers the nurseryman year-round propagation. Fourth, the cultured material must be induced to form roots and, finally, the rooted material must be established in soil.

The first two problems were tackled simultaneously. Satisfactory disinfestation of new growth was achieved by the methods described in the section on Materials and Methods. The Broad Spectrum approach was used to find suitable media to promote and sustain the growth of nodal explants in culture and, in the first experiment, nodes from aseptic seedlings were used. This strategy permited the testing of many factors without contamination losses, but had the possible disadvantage that the media selected for seedling nodes might not be suitable for the culture of adult tree nodes in the event the four media selected were suitable for tree nodes. One of the four media was also found to be suitable for the subculture of seedling nodes thus laying the foundation for a method for multiplication in the culture tube.

By this time, it was winter and no longer possible to obtain new growth from trees. Research continued with seedling nodes and subcultured nodes to find ways of inducing these materials to form roots. Up until this stage, very few of the several thousand *E. ficifolia* cultures had produced roots, and there was little point in perfecting multiplication techniques until it could be determined whether induction of root formation was possible.

Root formation was induced in all but one of the 20 cultures on the two media described in Table 1 (the two media differ only with respect to the concentration of sucrose). These media, when supplemented with kinetin and BAP (benzyl amino purine), both at 1μM, appear to be suitable for the subculture of nodes, and are an improvement on MHMH-medium used up to this stage, since the part of the culture in touch with the medium did not become black and warty in appearance.

The research programme is continuing with emphasis on defining the factors most important to the initial culture of the seedling nodes and to their subculture, so that by the time new growth is available on selected trees both MHMH-medium and

its experimentally-determined modifications can be tested with tree nodes. It is planned to subculture these tree nodes at monthly intervals both to multiplication-medium and to root-inducing-medium to find out, first, whether these techniques will result in rooted clonal plants suitable for establishment in soil and, second, whether year-round propagation is possible with *E. ficifolia*.

Acknowledgements. The authors gratefully acknowledge the help of Mrs. Natalie Peate and Mrs. Marion Meldrum for selecting suitable adult trees of *E. ficifolia* in the Melbourne and Sydney areas, respectively, and for their help in the collection and airfreighting of samples to Armidale.

LITERATURE CITED

1. Aneja, S., and C.K. Atal. 1969. Plantlet formation in tissue cultures from lignotubers of *Eucalyptus citriodora* Hook. *Current Science* (India) 38:69.
2. Cresswell, R., and R.A. de Fossard. 1974. Organ culture of *Eucalyptus grandis*. *Australian Forestry* 37:55-69.
3. Cresswell, R., and C. Nitsch. 1975. Organ culture of *Eucalyptus grandis* L. *Planta* (Berl.) 125:87-90.
4. de Fossard, R.A. 1974. Tissue culture of *Eucalyptus*. *Australian Forestry* 37:43-54.
5. de Fossard, R.A. 1976. *Tissue Culture for Plant Propagators*. University of New England, Australia.
6. de Fossard, R.A., Aung Myint and E.C.M. Lee. 1974. A broad spectrum tissue culture experiment with tobacco (*Nicotiana tabacum* L.) pith tissue callus. *Physiol. Plantarum* 31:125-130.
7. de Fossard, R.A., C. Nitsch, R. Cresswell and E.C.M. Lee. 1974. Tissue and organ culture of *Eucalyptus*. *New Zealand Jour. Forestry Sci.* 4:267-278.
8. Kitahara, E.H., and L.S. Caldàs. 1975. Shoot and root formation in hypocotyl callus cultures of *Eucalyptus*. *Forest Sci.* 22:242-243.
9. Lee, E.C.M., and R.A. de Fossard. 1974. The effects of various auxins and cytokinins on the *in vitro* culture of stem and lignotuber tissues of *Eucalyptus bancroftii* Maiden. *New Phytologist* 73:707-717.
10. Lee, E.C.M., and R.A. de Fossard, 1976. Regeneration of strawberry plants from tissue cultures. *Proc. International Plant Propagators, Soc. 1975* 25:277-285.