TISSUE CULTURE PROPAGATION OF *EUCALYPTUS FICIFOLIA* F. MUELL.

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Abstract. Cultures of seedling material on a rooting-medium develop one type of root system in the absence of riboflavin and another type in the presence of this growth factor; these effects appear to depend on either the light intensity or quality during incubation.

The interactions of IBA, BAP, gibberellic acid (GA_3), riboflavin, and sucrose in the culture medium and their effects on the multiplication and rooting of adult material are described. Riboflavin and GA_3 inhibit callus and rooting; GA_3 is antagonistic to BAP; IBA is essential for callus and rooting and these effects are enhanced by a low contentration of BAP. High concentrations of sucrose impair the health of cultures. More callus and rooting is induced on media with low concentrations of nitrate.

INTRODUCTION

Our previous papers on tissue culture propagation of the red-flowering gum, *Eucalyptus ficifolia*, described the use of nodes from aseptic seedlings to select culture media for their multiplication and rooting (2,8,9,10). This work with seedling material could be valuable for the rapid multiplication of progeny from hybridizations and from elite seed orchards; it might also be useful in cases where seeds of a species are rare, difficult to collect and/or expensive. However the main objective of this work with seedling material was to find cultural conditions suitable for the commencement of research to achieve clonal propagation of adult material. In fact, one of the four media selected from our first screening experiment with seedling material was found suitable as a basal medium for adult *E. ficifolia* material. The medium selected,[1] medium - MHMH (19), was modified with subsequent experimentation to: $[MH_{Fe}]$ $IBA_{10\mu M}MH$ (9) and then to: $[MH_{Fe}]$ $IBA_{5\mu M}MH$ (3), and then to: $[MH_{Fe}]$ $IBA_{5\mu M}BAP_{2\mu M}H$ (8). During this period, large clonal populations of adult material were being built-up through repeated subculture of shoots obtained from buds in early 1977. This paper describes the research which has been done on this subcultured adult material in our attempts to achieve satisfactory growth, multiplication rates and rooting.

The research on seedling cultured material was in its final stages in 1978 when, in large experiments with individual growth factors, the significance of riboflavin was first discovered. This paper not only describes the interesting interac-

[1] The coding system describing culture media is described under *Materials and Methods*.

tion of riboflavin in the culture medium with type of incubation using seedling material, it also describes some of the effects of riboflavin with adult cultures.

MATERIALS AND METHODS

Plant material: Seedling material used had been repeatedly subcultured at approximately 2-month intervals over a 3-year period. Adult material (originating from 25 year old trees) had been repeatedly subcultured over an 18-month period.

Culture media: Specific details of basal media used at various stages of experimentation are described in the *Results* section. The majority of the experiments had a factorial design. All culture media was dispensed into UC3OP polycarbonate tubes fitted with polypropylene screw-on lids (Disposable Products Pty. Ltd., Paget Street, Ridleyton, S. Australia). The culture media were sterilized in an autoclave. The gibberellic acid treatments were applied by dissolving in ethanol, applying 10μl amounts to sterile filter paper squares and, on evaporation of the ethanol, adding the squares to already sterilized media.

Incubation: Best growth and multiplication occurred with incubation in low intensity light (10μEm^{-2}s^{1}) at approximately 25°C; an 8/16 h light/dark regimen was used.

Coding of media and treatments: The basic 4-letter code of culture media relates to four categories of ingredients, namely, (1) minerals, (2) auxins, (3) cytokinins, and (4) growth factors, amino acids and sucrose. The concentration in each category is described as low, medium or high and abbreviated as L, M and H respectively. Thus, medium-MHMH consists of the medium (M) concentration of minerals and cytokinins and the high concentration (H) of auxins, growth factors, amino acids and sucrose. Occasionally, Z(zero) is used in the code to mean absence of a category.

[MH_{Fe}] means the medium concentration of minerals except for a subcategory which includes $FeSO_4$, NA_2EDTA and Na_2SO_4 at the high concentration. The specific concentrations of each ingredient are described in (7).

Substitution of the fourth letter of the code by two letters in parentheses means different concentrations of growth factors and amino acids on the one hand and of sucrose on the other hand. For example, [MH] would mean the medium (M) concentration of growth factors and amino acids and the high (H) concentration of sucrose.

When a category has been reduced to a single ingredient, the ingredient and its concentration are stated. For example, IBA $_{10\mu M}$ means that only one auxin is used and that auxin is IBA at 10μM.

Abbreviations of chemical names: IBA (indolebutyric acid), BAP (benzyl amino purine), GA (gibberellic acid), R (riboflavin), GF (growth factors and amino acid category).

RESULTS

Seedling Cultures

The interaction of riboflavin and incubation conditions on the induction of rooting in subcultured shoots: Twenty replicates of uniform shoots from repeatedly subcultured material of seedling origins were placed on medium (Table 1) containing either (1) all growth factors (GF), (2) all GF except riboflavin (R), (3) no GF, or (4) riboflavin alone at 10μM; there were three types of incubation, namely (1) 300μEm^{-2}s^{-1}, (2) 10μEm^{-2}s^{-1}, and (3) in darkness. Light quality was also different but was not quantified. The temperature of incubation was 25°C. There was a total of 240 cultures in the experiment and the results, after 4 weeks incubation, are described schematically in Figure 1.

The high intensity light incubation was clearly inhibitory to rooting in general, except for cultures on medium without growth factors which developed large calluses with root-like teratomas; some of these teratomas burst at their apices and developed strong normal-looking roots.

Dark incubation favoured rooting irrespective of growth factor additions to the culture media. At low light intensity, there was a high percentage of rooting, but the effect of riboflavin was on the morphology of these roots. Its presence produced the development of one or two strong, normal-looking roots per culture in contrast to the almost fibrous root systems that developed on media without riboflavin.

The effect of riboflavin on shoot growth (on a multiplication medium) was beneficial.

Adult Cultures

Multiplication and shoot growth: The multiplication medium at the start of this series of experiments was [MH_{Fe}] $IBA_{5\mu M}BAP_{2\mu M}H(8)$. This medium, and its forerunners, permitted a slow but steady build-up of clonal populations of adult cultures. The growth of the shoots on this medium was apically dominant and compact, that is, with short internodes and little or no development of axillary buds. However, the growth rate was slow and was accompanied with the development of excessive red-coloured basal callus and a dark exudate with extended culture periods.

A large number of experiments was done with this material, particularly with IBA, BAP, gibberellic acid — GA_3, riboflavin and sucrose. GA_3 had striking effects on these cultures

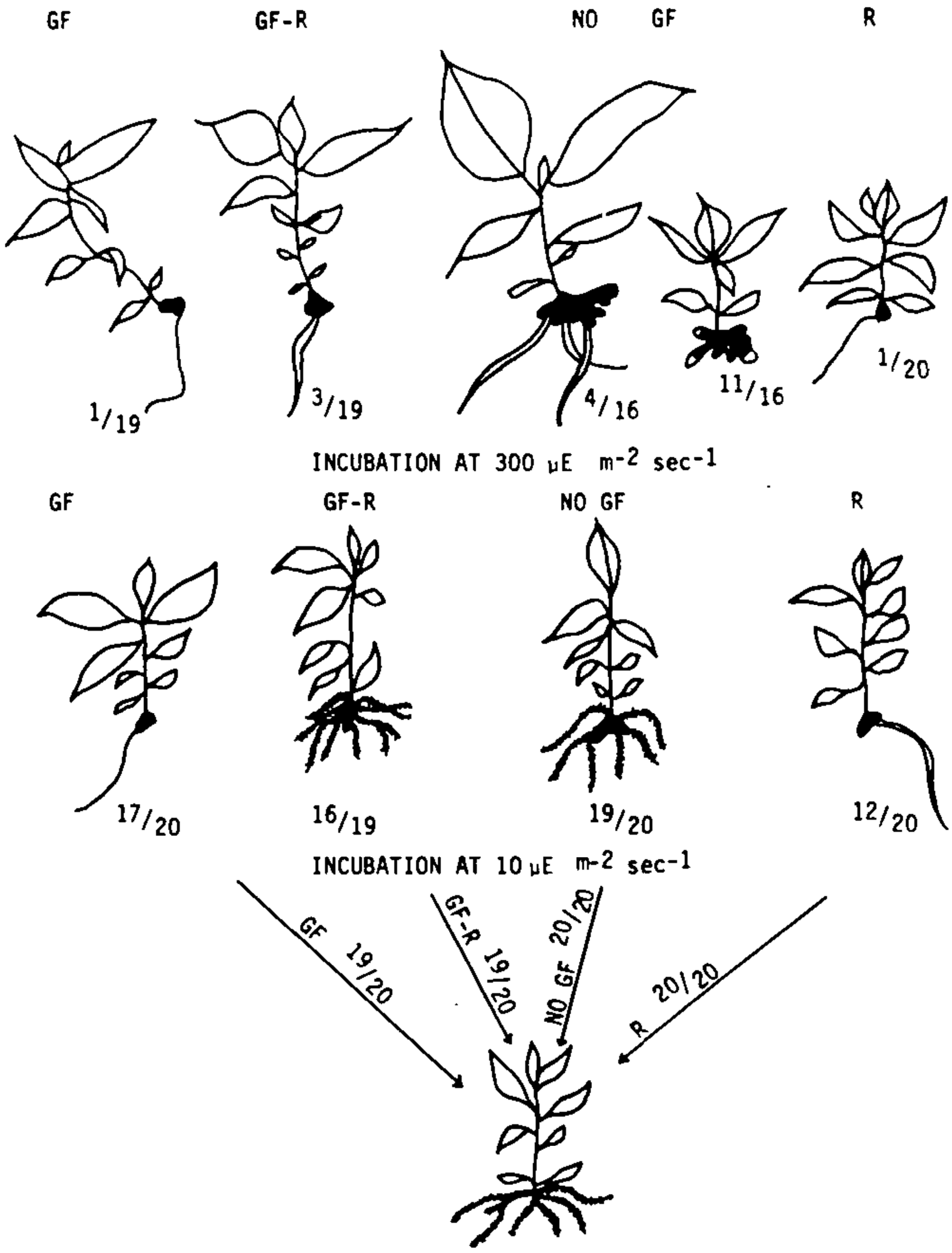

Figure 1. The effect on root formation of media with and without riboflavin, under different light intensities, after four weeks incubation; GF = all growth factors; R = 10 μM riboflavin; fraction under each sketch indicates number of cultures over the total number of cultures, exhibiting illustrated morphogenesis.

and, in particular, stimulated the growth of axillary buds. Both 10μM GA_3 (applied aseptically in alcohol to sterile pieces of filter paper which were then added, after evaporation of the alcohol, to sterile media) and 100μM GA_3 (autoclaved with the culture medium) had this effect; the latter treatment having a far greater effect. However, these GA_3 additions also caused internodal elongation, the formation of abnormal leaves, and eventually callus formation at the base of axillary branches. As a result of this callusing, the axillary branches fall off. The combination of constituents inducing axillary branch formation and least leaf abnormality was:

$$[MH_{Fe}]\ IBA_{5\mu M}BAP_{0.2\mu M}GA_{10\mu M}\ [R_{10\mu M}H_{sucrose}]$$

Table 1. Composition of culture medium for the induction of root formation in cultures of seedling origin.

Macronutrient elements (mM): NH_4NO_3 (5), $MgSO_4$ (0.5), KCl (1.9), $CaCl_2$ (1), NaH_2PO_4 (1).

Micronutrient elements (μM): H_3BO_3 (150), $MnSO_4$ (100), $ZnSO_4$ (40), $CuSO_4$ (1.5), Na_2MoO_4 (1), $CoCl_2$ (1), KI (5), $FeSO_4$ (100), Na_2EDTA (100), Na_2SO_4 (650).

Auxins (μM): IBA (5).

Main Carbon Source (mM): Sucrose (120).

Agar (g/l): Fluka agar (9).

The pH of all culture media was altered to 5.5 with 1N NaOH prior to autoclaving.

Experiments with different concentrations (60, 90 and 120mM) of sucrose led to the use of the medium concentration (60mM) in preference to the higher concentrations of sucrose. The higher concentrations not only induced red-coloured basal callus and exudate formation, they markedly increased the tendency for a shoot to dieback when transferred to a 'rooting' medium. The medium most suitable at present for the multiplication of adult material is:

$$[MH_{Fe}]\ IBA_{5\mu M}BAP_{0.2\mu M}[HM]\ \text{(Table 2)}$$

This medium not only eliminates the callus and exudate problem, but also produces a strongly apically-dominant plant which is not compact in form; it induces faster growth of cultures than on media with 2μM BAP.

Table 2. Composition of multiplication medium $[MH_{Fe}]\ IBA_{5\mu M}BAP_{0.2\mu M}$ [HM] for adult *Eucalyptus ficifolia* cultures.

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), $FeSO_4$ (100), Na_2EDTA (100), Na_2SO_4 (650).

Auxins (μM): IBA (5).

Cytokinins (μM): BAP (0.2).

Growth Factors (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).

Amino acids (μM): L-Cysteine HCl (120), Glycine (50).

Main carbon source (mM): Sucrose (60).

Agar (g/l): Fluka agar (9).

A new cycle of experimentation is about to begin using this basal medium, and re-testing the effects of various concentrations of IBA, BAP, GA_3 and riboflavin (all of which were previ-

ously tested on a different more complex basal medium). The aim of this new series of experiments is to achieve the same healthiness of the cultures as on the most recent basal medium but with higher rates of multiplication either of the apically-dominant or the axillary branch type.

Rooting: In our earlier papers, we reported the successful rooting of seedling cultured material, and failure of the same techniques to induce rooting in adult cultured material.

Again, a large number of experiments have been done to try to induce root formation in adult material. The majority of roots that have been induced to form have come after the formation of very large basal callus exhibiting root-like teratomas. In our earliest attempts at rooting, no such root-like teratomas were induced but instead, the roots were thin with no development of laterals and were associated with dark granular callus. Now, strong roots with some laterals arise from the teratomas and in some cases (for example, on media with 0.02μM BAP) thin roots arise from between the teratomas. Ideally, we would like to have root formation arising directly from the shoot without the prior formation of callus. However, it may be necessary to settle for a small amount of callus prior to rooting as occurs in many rooted cuttings.

The presence of IBA (e.g. 5 or 10μM) in the culture medium was essential for the formation of callus and then of roots. However, 10μM IBA in a "rooting" medium, for example $[MH_{Fe}]$ $IBA_{10\mu M}BAP_{0.02\mu M}$ [ZM], led to rapid deterioration of the shoots.

The presence of either 10μM riboflavin or 10μM GA_3 in the "rooting" medium completely inhibited callus development (and thus rooting) even in the presence of IBA; carry-over effects of GA_3 were also apparent since shoots from GA_3 multiplication media did not form any basal callus on IBA-containing 'rooting' medium. In general, GA_3 and BAP had antagonistic effects.

Rooting in cultures of many species often occurs in the absence of a cytokinin but, with adult *E. ficifolia* cultures, improved callusing and rooting occurred in media containing 0.02μM BAP. Cultures on 0.2μM BAP medium produced callus but no roots. Calluses on 0.02μM BAP had a healthier appearance than those on BAP-free media.

Reports of research with the rooting of some species have indicated a preference for particular K^+/NH_4^+ ratios, NO_3^- concentrations, and for lower concentrations of minerals (1,5,6,11, 12); other workers have reported improved rooting when shoots have been placed upside-down in the culture medium (1).

K^+/NH_4^+ ratios in the culture medium ranging from 20/0, 16/4, 12/8 through to 4/16, 0/20 (mM) have been tested in a factorial experiment with IBA (0,10μM) and BAP (0, 0.02μM).

K^+/NH_4^+ ratios ranging from 8/0, 6/2 through to 2/6, 0/8 (mM) have also been tested in all combinations of Ca^{2+}/NO_3^- ranging from 0/0, 1.25/2.5 through to 5/10 (mM) in the presence of 10μM IBA and 0.02μM BAP.

The results of these experiments indicated that both potassium and calcium were essential for callus formation and rooting, and most rooting occurred on cultures on media containing lower concentrations of NH_4^+, NO_3^-, and K^+ and with a higher concentration of Ca^{2+}, than in [MH_{Fe}] of the basal "rooting" medium.

The highest K^+/NH_4^+ ratio tested (that is 20/0 mM) induced large calluses in the presence of 10μM IBA with either 0 or 0.02μM BAP, whereas no callus formed on media with all other K^+/NH^+ ratios. This suggests that NH_4^+ ions inhibit callus formation. Shoot growth was healthiest on media with the highest K^+ ratio, and on cultures with the highest CA_2^+/NO_3^- ratio.

The testing of full strength, half strength and no minerals of [MH_{Fe}] showed that minerals were necessary for callus and rooting, but there were no significant differences between shoots on full and half-strength minerals.

The inversion of shoots in cultures resulted in much more rooting — but all from callus developed at the apical end; none of the basal ends of upside-down shoots produced callus or roots.

The next series of experiments will be based on these previous experiences. Strong shoots will be selected from cultures grown on media *with* 60mM sucrose and 0.2μM BAP and *without* riboflavin and GA_3. "Rooting" media will include 0.02μM BAP, but other cytokinins and other concentrations need to be re-tested. The mineral constitution of the "rooting" media will be re-tested, particularly with an overall lower level of NO_3^-. IBA and other auxins need to be re-tested at different concentrations, and it might be possible to induce rooting with certain auxin concentrations but avoid their deleterious effects on shoot health by the addition of low concentrations of riboflavin or GA_3.

DISCUSSION

For logistical reasons and by mischance, we have not yet tested one potentially important factor in our study of the tissue culture propagation of *Eucalyptus* (as envisaged in (3) and (8)). This involves the wounding of a tree at its base to induce shoot formation. Such shoots can be induced to form roots in some

cases and, if buds from such rooted cuttings were used to initiate a tissue culture programme, it seems highly probable that they might respond in the same way as seedling material to the multiplication and rooting systems already developed for such material. It is hoped that rooted shoots from the base of adult trees will be available soon for the testing of this idea.

The work described in this paper has confirmed the difficulties involved in the rooting of adult woody material. The successful completion of this research would be valuable because of the abundance of buds on an adult tree and because with some species there is no other alternative, for example, where shoots from the base of the tree cannot be induced to form or, if they are formed, are as difficult to root as from any other part of the tree.

The inclusion of riboflavin alone (that is, with no other growth factors and amino acids) in the multiplication medium (Table 2) will be a great simplification of the medium and is likely to give as good shoot growth and health of cultures as for those grown in the presence of all growth factors and amino acids in this category. Further investigation of the IBA-BAP-GA_3-riboflavin interaction will no doubt result in a programme with higher multiplication rates.

The induction of rooting in such adult cultures remains the big difficulty and, though the effects of many factors are now known, less optimism can be expressed at this time for the successful induction of rooting. The picture will be clearer after we have tested some of the numerous suggestions discussed in this paper. There are difficulties associated with the interpretation of experimental results in rooting research when *one* of the effects of a chemical, such as IBA, is "good", e.g. in producing callus and roots, but *another* effect is "bad", e.g. shoot dieback and death. A shorter duration of exposure to the chemical or the modifying influence of another chemical in the culture medium might lead to a suitable balance between root and shoot growth and health. One other possible method to induce rooting of cultured adult roots is to attempt this outside of the culture tube, using standard propagating procedures. This approach was used successfully for the rooting of tissue-cultured rhododendron cultivars (2), and for cultured Douglas fir shoots (4). Treating the base of cultured shoots of *E. ficifolia* seedling material with 10μM IBA for 1-2 days prior to planting out was successful in this work.

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TISSUE CULTURE PROPAGATION OF TWO *GREVILLEA* HYBRIDS

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Abstract. Favourable results have been achieved in the tissue culture propagation of *Grevillea* cv Robyn Gordon and *Grevillea* cv Crosbie Morrison. Multiplication rates have been fairly high, despite the fact that both hybrids have tended towards single rather than multiple shoot development. Success with rooting cultures has differed, 'Crosbie Morrison', giving 98% success and 'Robyn Gordon' about 60%.

The growing on of cultured plants in soil has presented some problems, and it is obvious that they require more careful attention than normal cuttings.

The methods of propagation are described and successful media for the multiplication and rooting stages are given.