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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.

INTRODUCTION

Grevillea cv Robyn Gordon is a spontaneous hybrid of *Grevillea bipinnatifida* and *Grevillea banksii*. It was released commercially at the end of 1975 and its large attractive blooms and deeply cut lacy foliage have made it a very popular garden plant (1). *Grevillea* cv Crosbie Morrison, is an eye-catching shrub with red and cream flowers and hairy grey-green foliage. Its parents were *Grevillea lanigera* and *Grevillea lavandulacea* (2).

Unlike the parents which have ample viable pollen, hybrids have practically no pollen. Further, if grown from seed there is a high probability that the seedlings will be different from the original hybrid. Propagation, therefore, has to be from cuttings. The cuttings of 'Robyn Gordon' are fairly difficult to root and, in most cases, growers achieve between 10% and 40% strike. There is more success with 'Crosbie Morrison', but because of the large demand on the hybrid, it would be desirable to achieve higher levels of production. For both hybrids, tissue culture propagation offers the potential for this increased production.

MATERIALS AND METHODS

Disinfestation: This procedure involves the removal of micro-organisms from the surface of the explant. The suppression of the micro-organisms is essential since they are strong competitors with the explant when it is placed on a culture medium.

Stem lengths from the two hybrids were washed under running tap water for 60 minutes as a preliminary procedure. They were then disinfested in a 5% (w/v) solution of calcium hypochlorite made up in 0.1% (v/v) 7X detergent for 20 minutes, and rinsed in two changes of sterile water. The 'Robyn Gordon' plants had been kept in pots in a glasshouse and were maintained in a fairly clean state. The 'Crosbie Morrison' plants were growing in the field. In the case of field grown material, which is vulnerable to insects and disease, disinfestation can be an enormous problem and contamination can occur in as many as 100% of cultures. It is therefore desirable to keep stock plants under glass.

Explant Source: For 'Robyn Gordon', buds were aseptically dissected out from the stem and transferred (one bud/culture tube) to a holding medium consisting of minerals and sucrose (Table 1). The purpose of this holding medium is to allow contaminated cultures to be screened out before they are placed onto more complex media. The explants generally remain on the holding medium for 7-10 days.

Table 1. Constituents and concentrations of the holding medium.

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), $FeSO_4$ (50), Na_2 EDTA (50), Na_2SO_4 (450).
Main carbon source (mM)
Sucrose (60)
Agar ('Fluka')
9g/litre

In the case of 'Crosbie Morrison', the explants taken, consisted of stem pieces, one to two nodes in length. Leaves were cut back to 1 mm. The nodes were transferred aseptically to the holding medium.

Culture Media: For the purposes of plant propagation, the ideal culturing sequence is firstly to find a medium which will induce multiple shoot formation and secondly to find a medium which will induce rooting. Unfortunately, for most species, there is not a single medium which will combine both functions. The use of the multiple shoot medium allows a rapid build up of small shoots and the single shoots can either be induced to proliferate further, or be placed onto a rooting medium to form complete plants which can then be grown on in soil.

In all cases, explants were grown in transparent polycarbonate tubes (with screw caps), containing 10 ml of culture media. All culture media were adjusted to pH 5.5 prior to autoclaving.

Multiplication.

a. *'Robyn Gordon'*: There was about 30% loss of explants from contamination. Explants which were apparently aseptic were transferred to seven replicates of a mini Broad Spectrum experiment. The full Broad Spectrum experiment (3) consists of combinations of four broad categories of constituents, namely (1) minerals, (2) auxins, (3) cytokinins, (4) sucrose plus growth factors plus amino acids, each at three concentrations, 'low', 'medium' and 'high'. This gives an experiment with 81 treatments (media). The mini Broad Spectrum consists of 18 treatments in which the minerals are used only at 'medium' concentration, the auxins and cytokinins are used at all three concentrations and the sucrose, growth factor, amino acid group is used at 'medium' and 'high' concentrations. The full Broad Spectrum is only suitable when large numbers of aseptic explants are available, but it allows a more rigorous testing of media than the mini Broad Spectrum.

To facilitate medium identification, a specific four letter coding is used, e.g. MLHM. The first letter represents the min-

eral component — in this case at 'medium' (M) concentration, the second letter represents the auxin component in this case at 'low' (L) concentration, the third letter represents the cytokinin component — in this case at 'high' (H) concentration and the fourth letter represents the growth factor/amino acid/sucrose component — in this case at 'medium' (M) concentration. In some experiments, the sucrose concentration is varied independently of the growth factors and amino acids and in this case, a five-letter coding is used, e.g. MLH[MH]. The medium represented by this code is identical to medium MLHM, except that whilst growth factors and amino acids are at 'medium' concentration, sucrose is at 'high' (H) concentration. Where a constituent is not present in the medium it is designated by the letter Z, e.g. MMZM indicates that there are no cytokinins present. The coding convention described will be used to express media constituents in this paper.

From the mini Broad Spectrum experiment, seven media were chosen as having good potential as multiplication media. Further tests were done on these to choose the best and medium-MMLH was selected. Then followed a number of experiments aimed at refining this medium: —

i. a cytokinin experiment testing PBA, BAP, Kinetin, 2iP and Zeatin (see Appendix I) at various concentrations, whilst keeping the other constituents at the original level.

ii. an auxin experiment testing IAA, IBA, NAA, NOA, pCPA and 2,4-D (see Appendix I) at various concentrations whilst keeping the other constituents at the original level.

iii. experiments testing combinations of auxins and cytokinins, whilst keeping the other constituents at the original level.

The experiments on 'Robyn Gordon' were commenced in December 1977 and initially explants were incubated under two conditions: a) a growth cabinet giving (8/16 h light/h dark) at 25°C, and b) a shadehouse covered with 50% shadecloth where the culture tubes were hung in clear plastic 'sausages'. At this time of year, growth was better in the shadehouse. However, as winter came round and the shadehouse was covered with polythene, but unheated, the cultures grew better in the growth cabinet. Some cultures were incubated in a heated glasshouse in winter, but did not grow as well as those in the growth cabinet, probably because the light intensity was too high.

b. *'Crosbie Morrison'*: There was about 85% loss of explants from contamination and many hundreds of explants had to be

cultured before enough could be obtained for transfer to complex media.

Apparently aseptic explants were transferred to three replicates of the full Broad Spectrum experiment. The cultures were initially incubated under fluorescent light with 8/16 (h light/h dark) in a room without temperature control. The growth cabinet and shade house were unavailable for incubation at the time, but both later proved to be more suitable.

From the Broad Spectrum experiment, medium-MLHM was selected as being the best for multiplication. This was further refined after an auxin experiment testing IAA, IBA, NAA, NOA, pCPA and 2,4-D at various concentrations, with the other constituents being kept at the original level. Incubation of this experiment (done during winter) was in the growth cabinet and in a heated glasshouse.

The fact that the two hybrids belong to the same genus would suggest that they may have similar medium requirements for multiplication. Although two different media were chosen, this may have been due to chance experimental error and therefore as a comparison, cultures of each hybrid were tested on the medium selected for multiplication of the other hybrid, i.e. 'Robyn Gordon' was tested on MLHM and 'Crosbie Morrison' was tested on MMLH.

Rooting. None of the cultures from the Broad Spectrum experiments done with the two hybrids developed roots. A series of likely media was therefore tested using complete shoots rather than nodal explants. Basically, this series consisted of 'medium' concentration minerals, IBA at various concentrations, cytokinins at zero and 'low' concentrations and growth factors and sucrose at concentrations varying from zero to 'high'. The series was developed from experience, it being assumed that IBA would be the best auxin for promotion of rooting (since it is used in commercial rooting powders) and that the use of low or no cytokinins would also be likely to encourage rooting. Incubation conditions were the same as for the multiplication experiments. Culture medium - $MIBA_{10\mu M}Z$ [ZM] proved to be very successful for both hybrids.

Further experiments were done to refine this medium for 'Robyn Gordon'. These experiments involved: i. use of a liquid medium on shakers, ii. testing of individual growth factors in combination with the other constituents, iii. testing of other individual auxins, and iv. testing of sucrose levels.

Growing On. Facilities for the care of rooted explants, have, until recently been fairly limited and initial failures to grow on the plants in soil can be attributed to this lack of facilities. Many plants produced in culture tend to be very 'soft', and the

transfer from a sterile, non-stressed, fully supporting environment to one in which factors such as temperature, moisture and nutrient status are constantly varying, must be a great shock. Experiments on soil mixes, types of containers for growing on, and general environmental conditions were done.

RESULTS

Multiplication.

a. *'Robyn Gordon'*: The type of multiplication obtained was not a multiple shoot system. Rather, single apically dominant shoots were produced. The shoots developed an average of six nodes over a four week period and when subculturing it was possible to take a single node as an explant. Application of a 'high' concentration of cytokinin did not overcome the apical dominance. The auxin experiment indicated that a 'low' concentration of NOA as the only auxin gave the best type of growth and the cytokinin experiment indicated that a 'low' concentration of 2iP as the only cytokinin would give best results. However, when a medium was used which combined low NOA and low 2iP the growth of the cultures was not as favourable as expected. Further experimentation has suggested that medium — $MM2iP_{0.1\mu M}H$ (Table 2) may be the most suitable. Still more work needs to be done on testing the growth factor and the mineral groups. About 20% of cultures on this multiplication medium also developed roots.

Associated with the effect of the media on multiplication, there also appears to be an incubation influence. Generally, the 'Robyn Gordon' cultures preferred the conditions in the shadehouse during the late summer and early autumn. They did, however, grow fairly well in the growth cabinet.

Many explants have shown signs of a brown coloration of the older leaves, after several weeks in culture. This coloration begins as isolated spots but eventually spreads to the whole leaf. If affected explants remain too long in culture, they eventually die. Those which have been rooted and planted out in soil appear not to suffer and new growth is free of the browning. If affected explants are subcultured, the subsequent explants may or may not develop the coloration. Microscopic investigation of an affected leaf showed that it was not caused by a fungus. It is probably some physiological disturbance which may be due to inappropriate media conditions.

'Robyn Gordon' explants transferred to the 'Crosbie Morrison' multiplication medium (MLHM), did not grow very well.

b. *'Crosbie Morrison'*: The system of multiplication obtained was of the multiple shoot type, but there have been variations. In initial experiments about four shoots arose from the base of

Table 2. Constituents and concentrations of multiplication media for the two *Grevillea* hybrids.

Constituents	'Robyn Gordon' (MM2iP$_{0.1\mu M}$H)	'Crosbie Morrison' (MZHM)
Macronutrient elements (mM)	NH_4NO_3 (10), KNO_3 (10) NaH_2PO_4 (1), $CaCl_2$ (2) $MgSO_4$ (1.5)	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), $FeSO_4$ (50), Na_2 EDTA (50), Na_2SO_4 (450)	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), $FeSO_4$ (50), Na_2 EDTA (50), Na_2SO_4 (450)
Auxins (μM)	1 μM of each of the following auxins: IAA, IBA, NAA, NOA, 2,4-D, pCPA (see Appendix I for full names)	None
Cytokinins (μM)	2iP (see Appendix I) 0.1μM	10 μM of each of the following cytokinins: Kinetin, BAP, (see Appendix I)
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).	Inositol (300), Nicotinic Acid (20), Pyridoxine. HCl (3), Thiamine. HCl (2), Biotin (0.2), D-Ca-Pantothenate (1), Riboflavin (1), Ascorbic Acid (1), Choline Chloride (1).
Amino acids (μM)	L-Cysteine. HCl (120), Glycine (50)	L-Cysteine. HCl (60), Glycine (5)
Main Carbon Source (mM)	Sucrose (120)	Sucrose (60)
Agar ('Fluka')	9g/litre	9g/litre

the explant. These had fairly long internodes, and generally, from 4-5 nodes/shoot developed over a four week period. Single nodes could be taken as explants. In the most recent experiment however, the multiple shoots which developed had much shorter internodes and there were more shoots (Figure 1). No obvious reason for this could be seen especially as the type of explants taken and the incubation conditions were unchanged. The cultures from which the explants came were about two months older than usual, and it is possible that this may have had an effect although why it should have is not clear. Further experiments need to be done to confirm and to clarify this observation.

The medium chosen for multiplication was MIBA$_{0.1\mu M}$ [HM], but the most recent experiment has indicated that equally good multiplications can be obtained by leaving out IBA altogether (Table 2). The incubation condition which best complemented the effect of the medium was found to be that in the shade house during late summer and early autumn. The growth cabinet gave the next best incubation conditions.

'Crosbie Morrison' explants transferred to the 'Robyn Gordon' multiplication medium (MMLM) did not grow very well.

Figure 1. An unusual 'Crosbie Morrison' culture showing development of both multiple shoots and an apically dominant shoot, on the same medium. More usually only one type of development occurs.

Rooting.

a. *'Robyn Gordon'*: Roots developed after about three weeks on rooting medium. The roots obtained were generally brown in colour, thickened and were associated with some degree of callusing (Figure 2). The root system was considered to be fairly massive for the type of shoot development. It was hoped to reduce the callusing and root thickness by experimenting with auxins and growth factors and also by using liquid media, but there was little success. The medium $MIBA_{10\mu M}Z$ [ZH] (Table 3) was chosen as being the best and about 60% of cultures on this medium developed roots. Results from the growth factor experiment indicated that the inclusion of Biotin in the rooting medium allowed better shoot development but a lower rooting percentage was obtained. Cultures on media without growth factors tended to be small and development of shoots was slow. When the cultures were planted out in soil, however, these smaller ones grew just as well as the larger plants. Incubation conditions did not seem to affect types of roots or rooting percentage.

Figure 2. 'Robyn Gordon' culture showing root development on rooting medium — $MIBA_{10\mu M}Z$ [ZH].

Table 3: Constituents and concentrations of rooting media for the two *Grevillea* hybrids.
'Robyn Gordon' ($MIBA_{10\mu M}Z[ZH]$)
'Crosbie Morrison' ($MIBA_{10\mu M}Z[ZM]$)

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), $FeSO_4$ (50), Na_2 EDTA (50), Na_2SO_4 (450).
Auxins (μM)	
	IBA (see Appendix I) 10 μM.
Main Carbon Source (mM)	
	'Robyn Gordon' — Sucrose (120)
	'Crosbie Morrison' — Sucrose (60)
Agar ('Fluka')	
	9g/litre

b. *'Crosbie Morrison'*: The best rooting medium was found to be $MIBA_{10\mu M}Z[ZM]$ (Table 3) and 98% of cultures formed roots. The roots were slightly thickened and there were more roots/culture than occurred with 'Robyn Gordon'. There was some callusing. As with 'Robyn Gordon', it took about three weeks for roots to appear on cultures; shoot growth was good on the rooting medium. Incubation conditions did not seem to affect types of roots or rooting percentages.

Growing On. As mentioned earlier, there were problems with this stage mainly because of poor facilities. The acquisition of a misting system in recent months has helped considerably.

The 'Robyn Gordon' plants seem particularly sensitive to growing on conditions. Of the rooted plants which have been transferred to soil, only about 40% have survived (Figures 3 and 4) whilst the success rate with 'Crosbie Morrison' has been 80%.

The best procedure so far, for planting out and growing on of both hybrids has been as follows:

1. Remove the lids from the culture tubes and leave the tubes standing in the glasshouse for several days. Water, if the plants become stressed.
2. Take the plant from the culture tube and gently wash off as much agar as possible from around the roots. It has been found that leaving too much agar on the roots may lead to problems with fungal growth.
3. Transfer the plant to a soil mix which is fairly sandy. Good drainage is essential. Plants seem to require heavy shading initially and the light intensity is increased gradually. Polystyrene seedling containers have been the most successful type of container used to date.

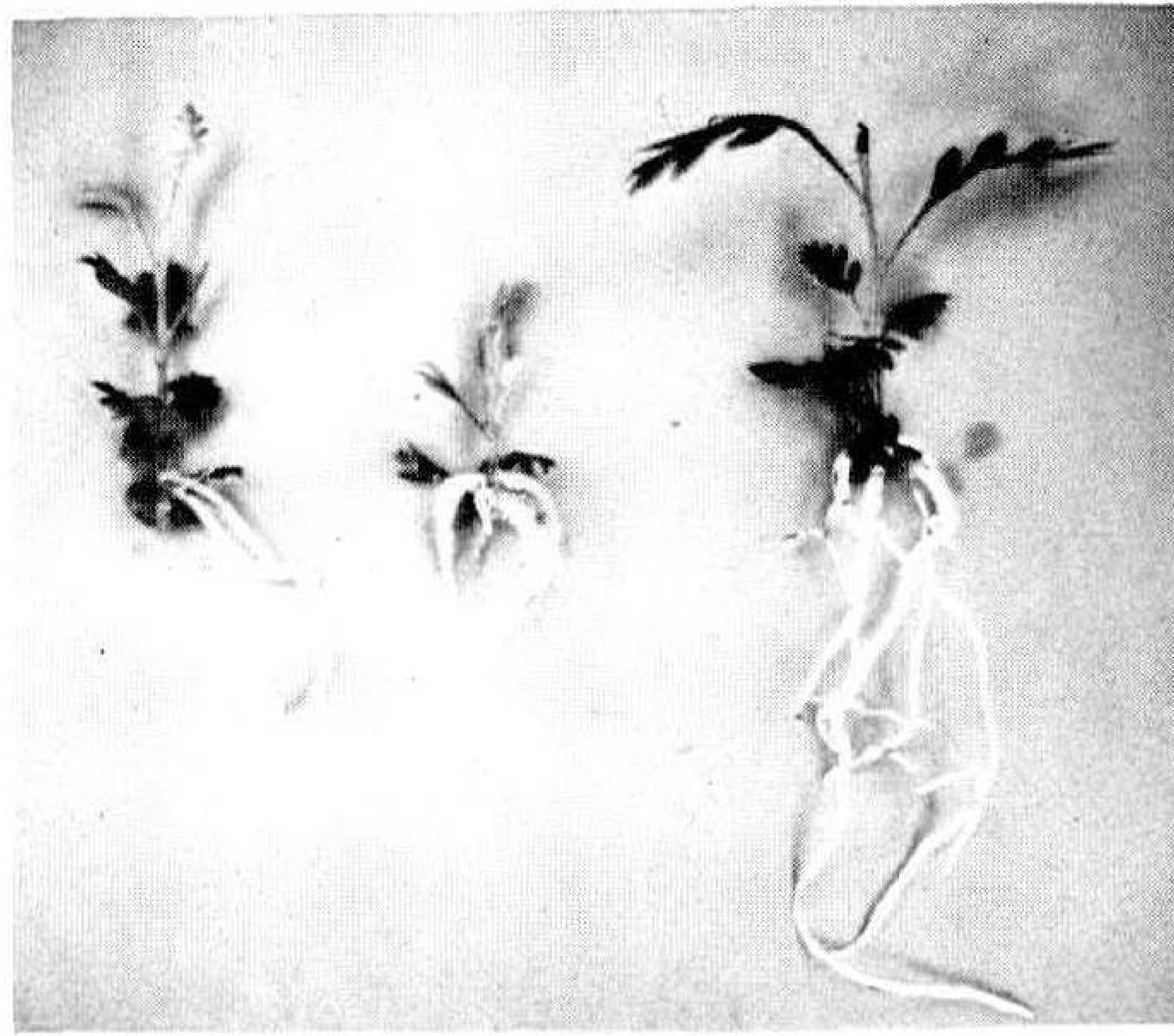

Figure 3. Development of roots in 'Robyn Gordon'. All plants are the same age. The two plants on the left have remained in culture on rooting medium for eight weeks. The plant on the right was transferred to soil after four weeks on rooting medium.

Figure 4. A tissue cultured 'Robyn Gordon' plant, eight weeks after planting out.

4. Leave the plants under fairly frequent mist until new root and shoot growth has occurred. Gradually decrease the misting frequency. Both hybrids need to be left for several weeks under mist; new roots develop quickly, but shoots take longer and the foliage is susceptible to wilting.

DISCUSSION

The level of production so far achieved with the two hybrids has been promising and further experimentation aimed at refining the existing media and improving the growing on stage, should result in a very favourable propagation system.

One of the factors which has been limiting is incubation of cultures. The use of 'natural' incubation, i.e. incubation in

glasshouses and shadehouses has been favoured and it is felt that this may be more relevant to nurserymen who wish to try tissue culture techniques and who would not have the artificial incubation facilities. However, there are obviously problems with maintaining continuity of conditions as seasons change, and even from day to day there are fluctuations. Further, particular cultures have particular requirements and it is therefore important to have some degree of flexibility in the 'natural' incubation, e.g. temperature control, control of light intensity through use of shadecloth. Both *Grevillea* hybrids grew best under the conditions found in the shade house during summer and autumn. Unfortunately, the lack of heating in the shade house prevented its use during the colder months.

The rates of multiplication achieved so far have been good. For 'Robyn Gordon' in which six explants can be subcultured every month, one single explant could yield 6^{12} (2,000,000,000) plants in a year. The yield is even higher for 'Crosbie Morrison' for which at least ten explants can be taken from a single culture each month. The percentage of 'Robyn Gordon' cultures to form roots on a rooting medium was not as good as hoped for and it seems that species which are hard to root by normal nursery methods, may also present a problem in tissue culture propagation.

The growing on stage is the one which will prove critical if tissue culture is to be used commercially. It is useless to be able to obtain millions of rooted cultures if they cannot be successfully transferred to soil. The *Grevillea* plants grown in culture are very 'soft' and require more care than would probably be given to a cutting. They are susceptible to moisture stress and take several weeks to establish and start producing new shoots.

The problem of heavy contamination losses with 'Crosbie Morrison' as compared with 'Robyn Gordon' in the initial culturing, highlights the need for explants to be taken from stock plants which are kept under cover and which are routinely sprayed for insects and disease. Contamination losses represent a waste of labour, money and time and careful plant hygiene can generally overcome the problem.

A very interesting point to emerge from the work done on the two hybrids is the fact that their media requirements for multiplication are so different. It was assumed that because they belonged to the same genus they would have similar nutritional and hormonal requirements. A possible reason for the difference between them may lie in the fact that morphologically they are very different (Figure 5) and therefore, by inference, their morphological attributes are associated with physiological differences at the cellular and biochemical level.

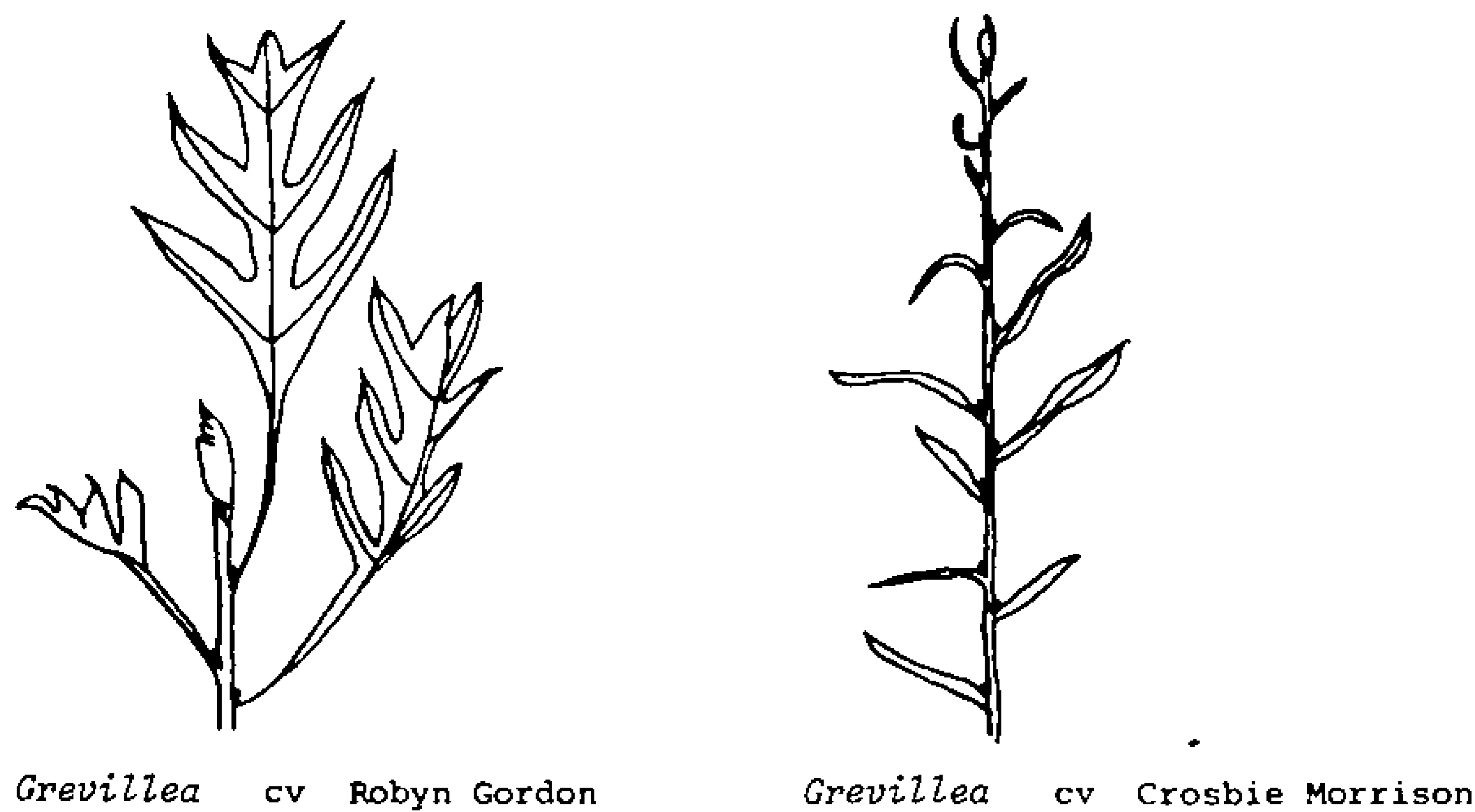

Figure 5. Morphology of the two *Grevillea* hybrids.

For both *Grevillea* hybrids, tissue culture propagation offers the potential for increased production. The numbers of plants which can be grown in culture and transferred to soil, is very high. The time intervals for one complete cycle, i.e. from the taking of an explant to planting out, for both hybrids are about 4-5 weeks on multiplication medium, 3-4 weeks on rooting medium, 1 week for 'hardening off' in the culture tube and 4-6 weeks in soil under mist.

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Appendix I. Full names of the auxin and cytokinin components used in experiments.

Auxins:

IAA (Indoleacetic acid), IBA (Indolebutyric acid), NAA (α-naphthalene - acetic acid), NOA (2-naphthoxyacetic acid), 2,4-D (2,4-dichlorophenoxy - acetic Acid), pCPA (para-chlorophenoxyacetic acid).

Cytokinins:

KINETIN (6-Furfuryl amino purine), BAP (N^6-Benzyl amino purine), PBA (6-(benzylamino) - 9 - (2-tetra hydropyranyl) - 9H - purine), 2iP (N^6 - iso pentenyl amino purine), ZEATIN (6 - (4 - hydroxy - 3 methylbut - 2 - enyl) - amino purine).