The Role of the Phytohormone Auxin in Adventitious Rhizogenesis in *Grevillea*

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Abstract

Grevillea (Proteaceae) is one of the woody Australian native species that have high commercial value in the floriculture market. *Grevillea* spp. vary greatly in their ease of propagation from cuttings, and some *Grevillea* cultivars are generally considered to be difficult-to root. Efforts to improve propagation success will be essential in the development of these species for commercial production. The objectives in this study were to study the response of *Grevillea* cuttings to auxin application, and to determine whether or not rooting differences between difficult and easy-to-root cultivars can be explained by differences in the endogenous auxin levels, or the capability to take up and transport applied auxin.

GC-MS analysis was used to measure the levels of auxin in the rooting zones. The level of auxin in the endogenous pool was measured by adding ^{13}C-labelled IAA ($^{13}C_6$-IAA) and ^{13}C-labelled IBA ($^{13}C_6$-IBA) as internal standards for quantitative mass-spectral analysis. The cultivars *G*. 'Royal Mantle' (easy-to-root) and *G*. 'Coastal Dawn' (difficult-to-root) were used for the comparisons. The capacity for auxin transport in cuttings of the two *Grevillea* cultivars was estimated by applying labeled auxin with high specific activity (^{3}H-IBA, *Bioscientific Pty*) to the cuttings. Distribution of radioactivity was determined in the base of the cuttings, their leaves and the rest of the stems harvested at various periods after applying auxin.

Apical auxin application to the difficult-to-root cultivar 'Coastal Dawn' resulted in a higher or comparable percentage of rooting compared to basal application and might potentially reduce the amount of auxin used to induce rooting. Rooting differences between the difficult and easy-to-root *Grevillea* cultivars did not appear to be related to their endogenous auxin levels or their ability to take up the applied auxin. There were no significant differences in the endogenous IAA and IBA levels between the easy and difficult-to-root cultivars, and both cultivars demonstrated an increase in endogenous IAA and IBA levels following IBA application. However, a different distribution pattern of the top applied [^{3}H]-IBA was noted between the difficult- and easy-to-root cultivars. The applied IBA in the difficult-to-root cultivar resided in the leaf whereas in the easy-to-root cultivar in the stem base. These studies suggest auxin distribution patterns may be an important factor in adventitious root formation.

1. Introduction

Grevillea (Proteaceae) is one of the woody Australian native genera that have high commercial value in the floriculture market. Woody plants are generally more difficult to propagate vegetatively than herbaceous species (Lovell and White, 1986; Thorpe and Harry, 1990), and very limited research has been done on the propagation of various Australian native species. *Grevillea* are popular as landscape plants and are high value ornamental plant. The inflorescences of most *Grevillea* hybrids are large, terminal, and colourful and have long stems, and have a growing potential as cut flowers (Joyce and Beal, 1999). *Grevillea* spp. vary greatly in their ease of propagation from cuttings, and some *Grevillea* cultivars are quite difficult-to root. *Grevillea* 'Coastal Dawn' semi hard wood cuttings produced roots after 12 weeks with a rooting percentage of less than 30 % (unpublished report).

Vegetative propagation by cuttings is the most preferred tool for mass production, since it is relatively easy, does not require sophisticated facilities and expertises, and is cheap compared to propagation through tissue culture. Efforts to improve propagation success, i.e. to induce a higher percentage of cuttings to produce adventitious roots, and to promote earlier rooting, will be essential in the development of *Grevillea* 'Coastal Dawn' for commercial production.

1.1. Determination of Auxin Levels in Grevillea *Plants and Cuttings*

The hypothesis that adventitious root formation is under the control of auxin has come from studies that showed that the application of exogenous auxin to cuttings, normally to the basal parts, promotes rooting. In order to get a better understanding on the role of auxin in rooting, it is important to measure the absolute amount of endogenous auxin at the time of taking the cuttings, as well as the changes in the level of endogenous auxin during the process of root initiation. Quantification of endogenous hormone levels has been rarely carried out because it requires sophisticated techniques and equipment for measuring the very low quantities of auxin in plants (nanograms per gm fresh weight of tissue). Techniques for measuring levels of hormones have been discussed by Beveridge *et al* (1997).

Our preliminary studies have demonstrated two *Grevillea* cultivars that have differing rooting ability. *G.* 'Royal Mantle' **(Figure 1 A)** is relatively easy-to-root and more responsive to applied auxin than *G.* 'Coastal Dawn' **(Figure 1 B).** It has also been demonstrated that rooting in these cultivars is seasonal**.**

1.2. Determination of the Pattern of IAA Transport

The traditional method of applying auxin to the bases of the cuttings has not been always successful in inducing rooting. It has been hypothesized that difficult to root species, as opposed to the easy-to-root species, require higher amounts of applied auxin to induce rooting.

A **B**

Figure 1 (A). *Grevillea* 'Coastal Dawn' and **(B)**'Royal Mantle'

This project aimed to answer the following questions:

(1) Does differing rooting ability between cultivars correlate with the endogenous levels of auxin within the plant tissue?

(2) Does the applied auxin change endogenous auxin levels within the plant tissue, particularly at the bases of the cutting, where roots are normally formed? Do these changes relate to the percentage of rooting in the two cultivars of *Grevillea?*

(3) Are there any differences in auxin uptake between these two cultivars of *Grevillea* which have different rooting abilities?

(4) Do differences in rooting capacity of both cultivars of *Grevillea* correlate with differences in the capacity to transport auxin?

(5) How is top and basally applied auxin transported within the cuttings?

2. Materials and Method

2.1. Propagation

Winter experiments were conducted between July-September and summer experiments between December-February. *Grevillea* cuttings were taken from container-grown, mature stock plants kept in a greenhouse at The University of Queensland, Gatton (UQ Gatton) Nursery southern Queensland. Tip cuttings **(Figure 2 A)** were three nodes long with one mature, fully-developed leaf. Stem cuttings **(Figure 2 B)** were two nodes-long, collected from the fourth to the sixth nodes below the tips with the shoot tip removed.

A **B**

Figure 2. (A) *Grevillea* 'Coastal Dawn'and **(B)** 'Royal Mantle' stem (left) and tip (right) cuttings

The base of the cutting was trimmed and the leaves on the lower node were removed, leaving only one fully-developed leaf on top. The cuttings were rinsed thoroughly in tap water before being treated with IBA.

IBA used was in the form of powder, dissolved in 50 % ethanol and diluted with distilled water. Concentrations used were 16 g L^{-1}, 8 g L^{-1} and 4 g L-1. With the basal application, the basal 10-mm of each cutting was dipped for 5 seconds in IBA. With the top application cuttings, the top 10-mm part of the cuttings was dipped in IBA solution (1 g L^{-1}) for 5 seconds.

Cuttings were then planted in 4.5 x 4.5 x 7.5 cm tubes with peat, perlite and vermiculite 1:1:1 and 120 g mini Osmocote (N : P : K : Mg = 16 : 3.5 : 9.1 : 1.2) per 60-L medium. Planted cuttings were placed in a mist propagation house on a heated bench. Bench temperature ranges were 21-27 °C in summer, 17-26 °C in spring, 10-25 °C in winter and 17-26 °C in autumn. Light

intensity in the propagation house was maintained around 440 µE m^2 s^{-1}. Shade cloth was put on the propagation house during summer.

All experiments were conducted in a randomised block design. Each treatment was replicated 3 times with 10 cuttings per experimental unit. Observations were made at weekly intervals, beginning 3 weeks after the cuttings were planted, by carefully lifting the cuttings from the medium, checking for roots and replacing the cuttings into the medium. Root number was evaluated two weeks after the first root initiation was observed, and roots > 2 mm length, were recorded. Data on rooting success were analysed using logistic regression (Collett, 1991) followed by Genmode analysis, whereas data on root number and time to root were analysed using analysis of variance (ANOVA) followed by LSD tests when appropriate using Minitab 14.

2.2. Auxin extraction and purification

Immediately following harvest, plant tissues were weighed and frozen in liquid N_2 and stored at -80 ºC prior to analysis. Individual samples were then ground under liquid N_2 and extracted with 10 mL of 50% methanol with 250 mg L^{-1} butylated-hydroxytoluene (BHT). Labelled internal standards $^{13}C_6$-IAA and $^{13}C_6$-IBA were added to each sample in varying amounts according to the treatment (with and without IBA application) and the fresh weight of the harvested plant material. The extracts were centrifuged at 10,000 rpm for 10 minutes at 5 °C. The supernatant was evaporated to dryness then purified on Sep-pack cartridges (Waters ®) after preconditioning with 10 mL of MeOH followed by 10 mL of 0.4 % (v/v) acetic acid in distilled water. The IAA was eluted with 10 mL of 80 % MeOH (v/v). The eluate was reduced to dryness under vacuum at 35 ºC and taken up in 2 mL of 100 % MeOH. Then it was reduced to dryness under vacuum. For each sample three to four independent extractions were performed**.**

2.3. Methylation of auxins

The auxins under investigation (IAA and IBA) were converted to their methyl esters by resuspending the samples in 150 µL MeOH and 600 µL ethereal diazomethane. They were left at room temperature for 10 minutes. Samples were then dried under a stream of nitrogen gas. As a final purification step, auxin-methyl esters were resuspended in 1 mL distilled water then partitioned against 3 x 200 µL diethylether. The hormone-containing ether fraction was then reduced to dryness (as above).

2.4. GC-MS-SIM quantification of auxin

Auxins methyl esters were trimethylsilylated with 3 µL of dry pyridine and 10 µL N_2O-bis(trimethylsilyl)trifluoro-acetamide (BSTFA) at 80ºC for 15-20 minutes prior to GC-MS analysis.

GC-selected ion monitoring was performed using a Varian 3800 gas chromatograph. A 25 m x 0.32 mm i.d. x 0.52 µm film HP5 fused silica column was coupled to the mass-selective detector via an open split interface. Helium was used as the carrier gas at an initial flow of 2 mL/min at 60 °C under a pressure of 190 kPa. Samples were injected in the splitless mode. The temperature of the column oven was increased from 50º to 190°C at 30°C per minute, and then to 270°C at 10°C per minute. IAA and IBA eluted under these conditions at 9.6 and 11.0 min, respectively. Full scan spectra were recorded for the peaks co-chromatographing with authentic methylated standards and the mass spectra compared to spectra obtained form authentic standards.

Quantification of endogenous auxins was performed by the isotope dilution method. Peak areas at the correct retention time of an ion pair derived from an endogenous auxins, i.e. 208/202 (ions deriving from $^{13}C_6$-IAA and endogenous IAA) and 203/202 (ions deriving from $^{13}C_1$-IBA and endogenous IBA) and a corresponding stable-isotope-labelled internal standard were determined by GC-MS-SIM. The amount of IAA and IBA was calculated using the formula:

$$\frac{\text{peak area of the endogenous auxin}}{\text{peak area of the internal standard}} \times \frac{\text{the amount of internal standard added (ng)}}{\text{fresh weight of tissues (g)}}$$

The auxin levels are expressed as the amount of hormone (in ng) per g of fresh weight (ng g^{-1} FW).

2.5. IBA transport Pattern

Auxin transport was measured in plants supplied with [^{3}H]-IBA; specific activity 20 µCi mol^{-1} (American Radiochemicals). Plants used for the experiment were between 115-140 mm in height with 6-8 leaves expanded. Each plant was treated with 7.4 kBq of [^{3}H]-IBA (4.4 x 10^5 DPM) in 4 µL of ethanol, applied to the apical bud of each plant.

Six hours after treatment with [^{3}H]-IBA, the shoot tip and leaves were removed, and the remaining stem was cut into 10-mm sections that were then placed into labeled vials according to

the segment numbers. Each vial consists of 3 segments from 4 different plants to obtain a large quantity of radioactivity. Segments were numbered down the stem from top to bottom.

2.6. Incubation of cuttings for distribution of ^{3}H-IBA experiment

Cuttings were incubated with their bases in 1.5 mL eppendorfs, one cutting per vial. 7.4 kBq of [^{3}H]-IBA (4.4 x 10^{5} DPM) in 6 µL of ethanol was added to each vial for the basal application. For top application the same amount was applied using a microsyringe to the shoot tip of each 'Coastal Dawn' cutting and to the top cut stump of each 'Royal Mantle' stem cutting. This volume was tested earlier and was readily taken up by the cuttings. For each top and basal application four replicates of one cutting each was used. Treated cuttings were then planted and kept in a mist propagation unit. The cuttings were removed after 5 and 24 hour and washed under running water, then cut into leaf and equal segments of base and upper-stem. The segments and leaves were crushed using a mortar and pestle separately. The remaining leaf tissue in the mortar was washed with 2 mL of 80% MeOH. Two mL of Ultima Gold LSC scintillant (Packard Packard BioScience B.V., Groningen, The Netherlands) was added to each sample then shaken overnight prior to counting with Packard Tricarb 1600 scintillation counter (Packard Instrument Co., Meriden, USA). For the leaf samples, only 250 µL aliquot was used for counting to avoid colour quenching which can result in a reduction of the scintillation count rate.

3. Results and Discussion

3.1. Rooting studies

Previous studies conducted on rooting of the two *Grevillea* cultivars demonstrated that 'Royal Mantle' rooted better than 'Coastal Dawn', and 'Coastal Dawn' had a reduced response to applied IBA **(Table 1).**

Application of IBA did not promote rooting of 'Coastal Dawn' in winter and summer (Table 1). In contrast rooting in 'Royal Mantle' was promoted with application of IBA at higher IBA concentrations (> 4 g L^{-1}) **(Table 1).**

Rooting in summer was higher than in winter for both cultivars. 'Royal Mantle' cuttings can root without IBA treatment in summer **(Table 1).**

Table 1. Influence of IBA on rooting of G.'Royal Mantle' and 'Coastal Dawn' cuttings in winter and summer.

IBA Concentration (g L^{-1})	Rooting percentage *)			
	G. 'Coastal Dawn'		**G. 'Royal Mantle'**	
	Winter	Summer	Winter	Summer
0	0 a	5 a	5 a	41 a
4	0 a	12 a	12 a	33 a
8	20 a	13 a	30 b	60 b
16	0 a	12 a	38 b	72 b

*) Means in the same column followed by the same letters are not significantly different according to Genmode analysis

3.2. Effect of method of IBA application

Top auxin application was examined since basal auxin application in 'Coastal Dawn' cuttings only resulted in low rooting percentages (<20 %) in most seasons (Table 1). The rooting percentage obtained using top application of IBA at 1 g L^{-1} in both cultivars was higher or comparable to that of industry standard for *Grevillea* at 16 g kg^{-1} (Table 2). Therefore, top application of auxin has potential to be used for commercial propagation. Top application might also allow the use of less auxin.

Table 2. Effect of method of auxin application on rooting of *G.* 'Coastal Dawn' Cuttings

Treatment	Percentage of cuttings rooted		
	Autumn	Spring	Summer
Basal IBA 16 (Control)	59	17	12
Top IBA 1	90* (P=0.029)	20 (ns, P=0.58)	27 (ns, P=0.21)
Basal No IBA	0	2	10
Top No IBA	0	9	2

* significantly higher than the control (basal IBA at 16 g kg^{-1}).

Increasing the IBA concentration applied to the top from 1 to 4 g L^{-1} did not increase the rooting percentage in'Coastal Dawn' (data not shown). Top auxin application, however, resulted in some of the roots being formed aerially. These aerial roots were formed directly from the stem (not through callus), below but close to the point of application, i.e. between the shoot tip and the first

mature leaf **(Figure 3).** Only occasionally did aerial roots form below the mature leaf. The tendency to form aerial roots increased with higher IBA concentration, i.e. from 15 % at 1 g L^{-1} IBA to 70 % at 4 g L^{-1} IBA.

Figure 3. Aerial roots formed in G. 'Coastal Dawn' cuttings (arrows) following top IBA application at 4 g L^{-1}

3.3. Endogenous IAA and IBA levels

IAA and IBA were present in the leaves and stems of both *Grevillea* cultivars, and the levels in the easy and difficult-to-root cultivars were similar in both seasons **(Figure 4).** The endogenous levels of IAA were generally higher than IBA, e.g. in winter the stem IAA of 'Royal Mantle' was 3 times the stem IBA **(Figure 4).** These IAA levels in *Grevillea* stems are similar to those reported in previous studies, e.g. 30 – 40 ng/g in pea shoots (Beveridge *et al.*, 1994; Beveridge *et al.*, 1997)

Differences in the auxin levels between seasons were noted. Summer IAA levels were higher and winter IBA levels were lower **(Figure 4).** Some leaf samples taken in summer had IBA levels below the detection limit.

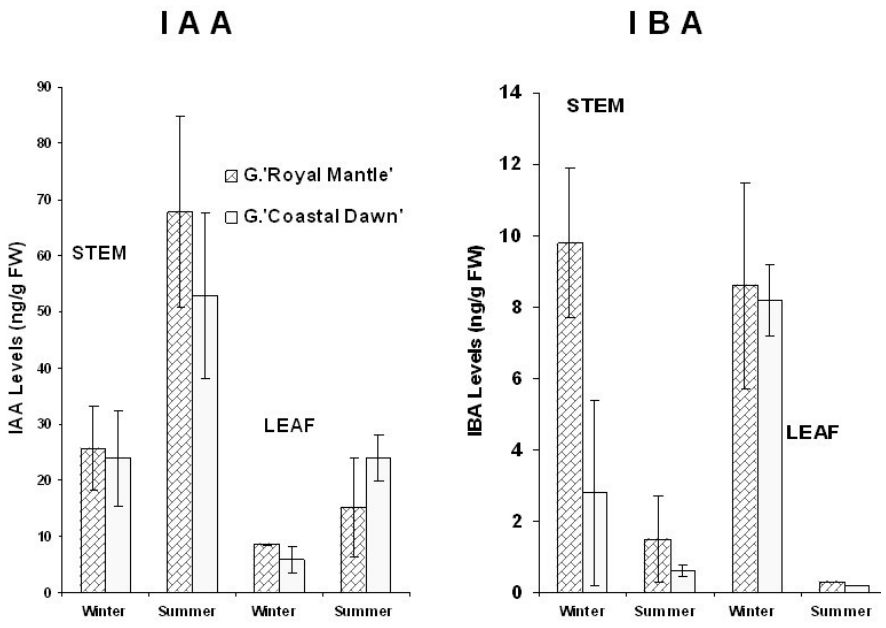

Figure 4. Endogenous IAA (left) and IBA (right) levels (ng/g FW) in *Grevillea* stems and leaf in summer and winter. Results are the mean ± SE of two to four replicates. Each replicate was obtained from 5 plants of each cultivar.

The results of the IAA and IBA measurements in this study demonstrate that there are no significant differences in the endogenous IAA and IBA levels between the easy and difficult-to-root cultivars, both in the stem and the leaves taken in summer and winter **(Figure 4).** Therefore no evidence was obtained to suggest that the difficult-to-root cultivar might have been deficient in IAA or IBA. These results contrast with previous reports in *Cotinus coggyria* (Blakesley *et al*, 1991) and apple (Alvarez *et al.*, 1989) that higher rooting ability correlated with higher endogenous auxin levels.

However, differences in the auxin levels between seasons were noted. In summer when rooting percentage was higher, stem and leaf IAA levels were much higher than in winter. In contrast IBA levels were much lower in summer than in winter **(Figure 6).**

Differences in endogenous IAA levels between seasons have been reported in *Pinus sylvestris* (Sandberg and Ericssson, 1987) and *Abies balsamea* (Sundberg *et al.*, 1987). However, the endogenous IBA levels in plant tissues were not measured in these studies.

3.4. Changes in the IAA and IBA levels in the stem base after IBA application

IBA treatment to cuttings resulted in increases in endogenous levels of IAA **(Figure 5)** and IBA **(Figure 6)** in stems of both cultivars. The peak IAA levels occurred at day 1 for stems **(Figure 5).** The increases in stem IAA levels in IBA-treated 'Coastal Dawn' were more pronounced than

in IBA-treated ‘Royal Mantle’ **(Figure 5).** In both cultivars, the IAA content rapidly returned to near control levels after 7 days **(Figure 5).**

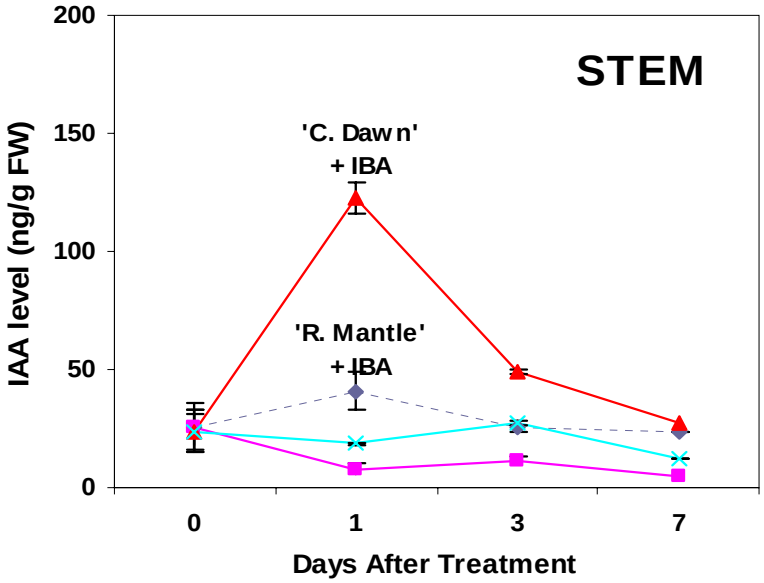

Figure 5. Time course changes in endogenous IAA levels (ng/g FW) in stem bases of ‘Coastal Dawn’ and ‘Royal Mantle’ cuttings. Data were obtained from the average of two to four replicates ± SE. Each replicate consist of 4 pooled stem base cuttings.

For stems, the IBA increase in ‘Coastal Dawn’ was more significant than in ‘Royal Mantle’ with the peak occurring later, i.e. at day 3 in ‘Coastal Dawn’ and at day 1 in ‘Royal Mantle’ **(Figure 6).** Stem IBA levels of both cultivars declined after reaching their peaks, but for ‘Royal Mantle’ it slightly increased again from day 3 to day 7 **(Figure 6).**

In contrast to stem IAA **(Figure 5),** stem IBA levels of both cultivars still remained relatively high (190-200 ng) compared to the control levels (8-30 ng/g FW; **Figure 4**) at day 7.

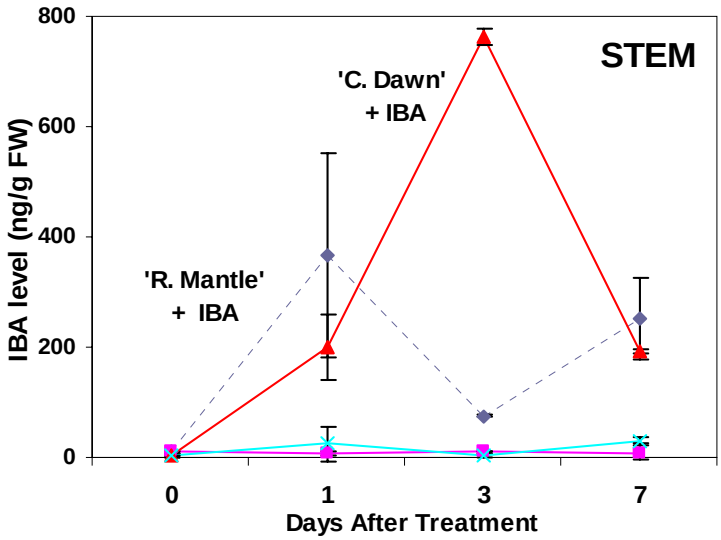

Figure 6. Time course changes in endogenous IBA levels (ng/g FW) in stem bases of 'Coastal Dawn' and 'Royal Mantle' cuttings. Data were obtained from an average of two to four replicates ± SE. Each replicate consist of 4 pooled stem base cuttings.

3.5. IBA transport pattern in Grevillea

Transport of [^{3}H]-IBA was measured in plants with 6-8 fully expanded leaves (plant height between 115-140 mm). The distance that the radioactivity was transported was not different between the plants of both cultivars during the 6 hours after application. At 6 h, the applied [^{3}H]-IBA had not been transported more than 20-30 mm from the point of application of both cultivars **(Figure 7).**

In terms of proportion radioactivity transported **(Figure 7),** 'Royal Mantle' transported a higher proportion of radiolabel than 'Coastal Dawn' after 6 h. In 'Royal Mantle' only a proportion of 0.66 ± 0.02 of the applied [^{3}H]-IBA remained in the application point, meaning a proportion of 0.34 has been transported down. In 'Coastal Dawn' the proportion of radioactivity transported after 6 hour was only 0.23 (the proportion of the remaining [^{3}H]-IBA was 0.77 ± 0.02).

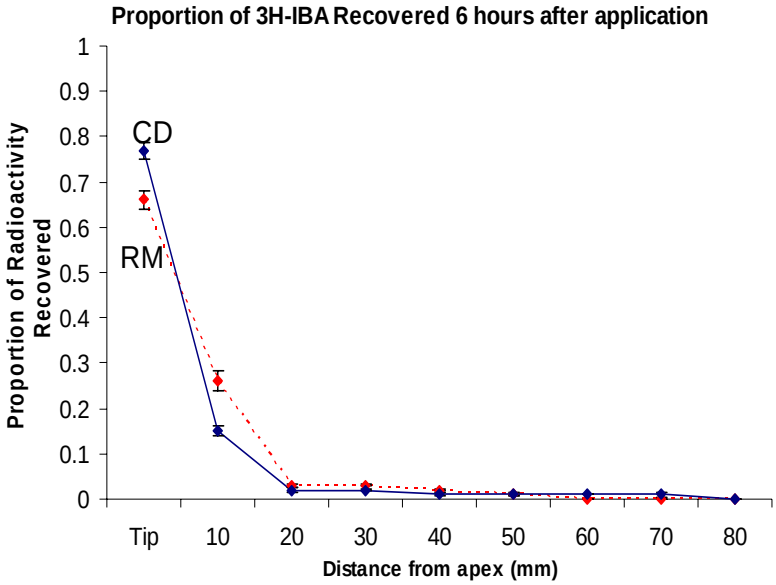

Figure 7. Distribution of radioactivity in *Grevillea* 'Coastal Dawn' and 'Royal Mantle' cuttings 6 hours after supplying [3H]-IBA (14.8 kBq per plant) to the apical bud. Each point is the value for 3 segments from 3 different plants. Data are expressed as a proportion of the total radioactivity recovered.

3.6. Distribution of [3H-IBA] after top and basal application to Grevillea *cuttings*

3.6.1. Basal Application

Following basal application of [^{3}H]-IBA, no significant differences were observed between the two cultivars in the distribution of transported [^{3}H]-IBA at 6 h (data not shown) and 24 h in the leaf and upper stem **(Figure 7).** The applied radiolabel in 'Coastal Dawn' tends to be accumulated in the leaf **(Figure 8).**

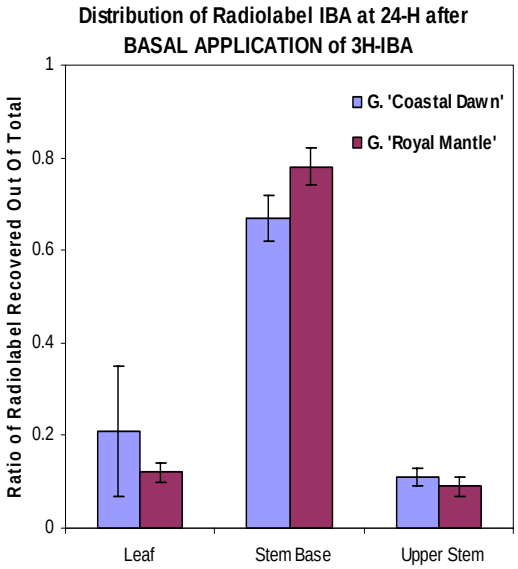

Figure 8. Distribution of radioactivity in *Grevillea* 'Coastal Dawn' and 'Royal Mantle' cuttings 24 hours after supplying [^{3}H]-IBA (14.8 kBq per plant) to the stem bases. Each point is the value for 3 segments from 3 different plants. Data are expressed as a proportion of the total radioactivity recovered.

3.6.2. Top Application

Following top application, most radioactivity (> 50 %) in the two cultivars was still remained at the point of application (upper stem) at 24-h after application of [^{3}H]-IBA **(Figure 9 A and B).** However, there were significant differences between the two cultivars in the distribution of in the leaf (P=0.016) and stem base (P= 0.007). In 'Coastal Dawn', most of the transported [^{3}H]-IBA moved to the leaf rather than the stem base. This is shown by a significant increase of the proportion of [^{3}H]-IBA recovered in the leaf from 6 to 24 h **(Figure 9 A).** In contrast, the transport in 'Royal Mantle' seemed to lead to an increasing accumulation in the stem base rather than the leaf **(Figure 9 B).** This indicated that 'Coastal Dawn' had different distribution pattern from 'Royal Mantle' of the applied [^{3}H]-IBA.

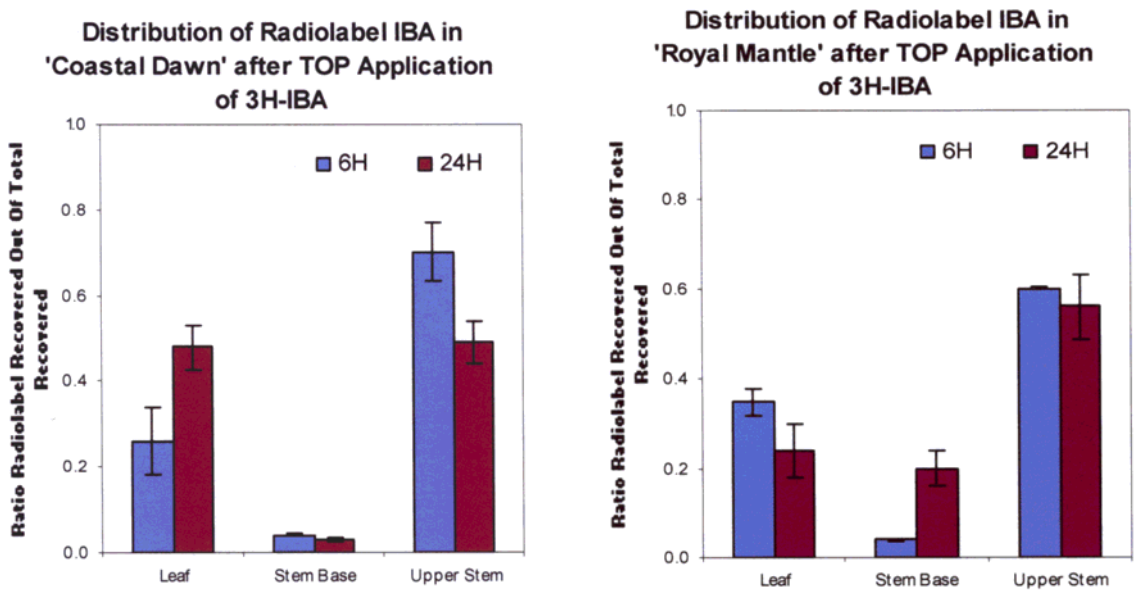

Figure 9. Distribution of radioactivity in *Grevillea* 'Coastal Dawn' (A) and 'Royal Mantle' cuttings (B) at 24 hours after supplying [^{3}H]-IBA to the stem base. Each point is the value for 3 segments from 3 different plants. Data are expressed as a proportion of the total radioactivity recovered.

4. Discussion

4.1. The Importance of endogenous auxin levels in adventitious root formation

The results of the IAA and IBA measurements in this study demonstrated that there were no significant differences in the endogenous stem IAA and IBA levels between the easy and difficult-to-root cultivars, either in summer or winter **(Figure 4).** Therefore no evidence was obtained to suggest that the difficult-to-root cultivar might have been deficient in the endogenous level of IAA or IBA.

However, differences in the auxin levels between seasons were noted. In summer when rooting percentage was higher, stem IAA levels were much higher, whereas stem IBA levels were lower than in winter **(Figure 5).**

Differences in endogenous IAA levels between seasons have been reported in *Pinus sylvestris* (Sandberg and Ericssson, 1987) and *Abies balsamea* (Sundberg *et al.*, 1987). Several others reported a decline in the IAA level in *Abies balsamea* trees along with the progression from active growth in July to quiescence in December, particularly reflected in the activity of the cambium (Sundberg *et al.*, 1987). Similar results were reported in *Pinus sylvestris* (Sandberg and Ericssson, 1987). These reports lead to a hypothesis that the level of IAA controls the rate and

seasonal growth, since IAA levels were elevated during the growing season compared to the dormant period.

It has been well documented that in the natural annual cycle of growth and development, woody plants go through periods characterised by shoot growth and other periods of non-growth or dormancy which are under both environmental and genetic control. This cycle of activity was hypothesised to correlate with rooting, i.e. the high rooting phase coincides with the resumption of growth and high cambial activity, whereas the low rooting phase occurs during winter dormancy and low cambial activity (Davies, 1984). However, the role of growth substances in the regulation of growth and dormancy, and the regulations of their endogenous levels in different seasons are still unclear. While the endogenous cambial IAA levels declined during the transition from activity to rest (Sandberg and Ericssson, 1987; Sundberg *et al.*, 1987), endogenous IBA levels of the plants were not measured in these studies

Measurements of endogenous IBA levels in plants have rarely been conducted and there are no published reports on endogenous IBA levels measured in different seasons. It is possible that the ratio of IAA to IBA, or the extent of metabolism of the applied IBA to IAA and/or to other metabolites is important for rooting. In this study, IBA metabolism – like the plant's metabolism in general – was more active in the warmer than in the cooler season, indicated by much lower level of IBA in summer than in winter.

4.2. Changes in endogenous auxin levels during adventitious root formation

4.2.1. IAA

In this study exogenous IBA application resulted in an increase in stem IAA and IBA levels in both easy and difficult-to-root *Grevillea* **(Figure 4 and 5).** An increase in the endogenous IAA level following IBA treatment has also been reported in avocado (Garcia-Gomez *et al.*, 1994) and *Sequoia sempervirens* (Blazkova *et al.*, 1997). The increase in the endogenous IAA level might have partly come from conversion of the applied IBA into IAA, as has been reported in apple (van der Krieken *et al.*, 1992), grapevine and olive (Epstein and Lavee, 1984).

A transient increase in the IAA level has been reported as a necessary event prior to root formation (Moncousin *et al.*, 1989; Gaspar *et al.*, 1997; Kevers *et al.*, 1997), but this required level has not been quantified to predict how much increase is required for optimum rooting. Addition of 10 µM IBA to the rooting medium of avocado cultured *in vitro* induced a 2-fold increase in IAA levels at the basal part (Garcia-Gomez *et al.*, 1994). Similar level of increase

was reported in mung bean hypocotyl cuttings treated with 25 mg L^{-1} of IBA (Pan and Xingshan, 1999). The amount of IBA applied to these cuttings was well below that which was added in my experiment (40 000 ng per cutting). However, previous experiments have demonstrated that this dose induces rooting in *Grevillea*.

4.2.2. IBA

The increase in IBA levels following IBA treatment occurred in stems of both cultivars. Similar to the increase in endogenous IAA level after IBA treatment **(Figure 4),** the IBA increase in the stems of the difficult-to-root cultivar was more pronounced than in the easy-to-root cultivar with a later peak **(Figure 5).** These results agree with Blazkova *et al* (1997) who reported that IBA was accumulated more quickly and to a greater extent in unrooted mature than in rooted young clones. On the contrary, easy-to-root peach (Baraldi *et al*., 1993) and sweet cherry (Epstein *et al*., 1993) demonstrated a better ability to take up applied auxin than the difficult-to-root cultivar. The differences in the results obtained were probably due to difference in the species used and the experimental conditions employed - including the amount of IBA applied, since in Baraldi's and Epstein's report the plants were cultured *in-vitro.*

Following IBA treatment, stem IBA levels of both cultivars still remained relatively high (190-200 n/gFW) compared to the control levels (8-30 ng/g FW; **Figure 5)** at day 7. In contrast, the IAA levels rapidly decreased to near control levels at this time. This result confirmed that IBA is a more stable auxin than IAA, as has been also reported by De Klerk *et al* (1997) and Nordstrom and Elliasson (1991).

This study suggests that the dramatic increase in the stem IAA and IBA level following IBA application was not crucial for rooting. This conclusion was based on the fact that the increase in the IAA and IBA level in 'Royal Mantle', which is easier-to-root and more responsive to the applied auxin, was not as pronounced as that a in 'Coastal Dawn'.

4.3. Auxin transport

The two *Grevillea* cultivars demonstrated a similar rate of IBA transport **(Figure 7).** However, the distribution pattern of the applied IBA was different between the two cultivars **(Figure 9 A and B).** Distribution of plant hormones is one of the critical determinants in controlling plant growth (Brown, *et al*, 2001). After 24 h most of the applied [^{3}H]-IBA in 'Coastal Dawn' moved to the leaf, whereas in 'Royal Mantle' it moved to the stem base. The proportion of the radioactivity recovered in the 'Coastal Dawn' leaf increased significantly from 6 to 24 h after

application **(Figure 9 A),** whereas in the easy-to-root 'Royal Mantle' the proportion in the leaf decreased and the proportion in the stem base increased **(Figure 9 B).** Therefore 'Royal Mantle' cuttings seemed to have a higher ability to mobilise the apically applied [^{3}H]-IBA.

This finding was similar to those reported by Marks *et al* (2002) that apical application of auxin to the difficult to root species *Syringa* induced a higher percentage of rooting compared to basal application. Marks *et al* (2002) hypothesised that it is important that the applied auxin is transported through the polar auxin transport system in *Syringa,* and this mechanism was less critical for root induction in the easy-to-root *Forsythia*. However, in their system adventitious roots were formed at the base of the cuttings, whereas in my study application of IBA to the top of 'Coastal Dawn' resulted in some of the roots forming aerially. It is possible that this was due to a higher proportion of the applied [^{3}H]-IBA still being located in the upper stem and leaf at 24-h after application, resulting in an increase of IBA concentration in this area. This is consistent with the fact that in 'Royal Mantle' roots were formed at the stem base, where more of the applied IBA accumulated. This finding has highlighted a possible role of auxin transport and distribution in root induction in *Grevillea*.

4.4. Method of application

Apical auxin application to the top of cuttings resulted in a higher or comparable rooting success compared to basal application, and might potentially reduce the amount of auxin used to induce rooting **(Table 2).** It is important for the applied auxin to reach the competent cells to produce adventitious roots (Ford *et al.*, 2002). The effectiveness of top application as opposed to basal application might be due to more efficient delivery of auxin into these cells. The basally applied auxin might need to be redistributed within the cuttings, and retransported to the cutting base through the natural basipetal transport system (Jarvis and Shaheed, 1986; Ford *et al.*, 2002).

The importance of basipetal auxin transport in rhizogenesis has been demonstrated by Marks and Simpson (2000) in *Syringa vulgaris,* a difficult-to-root species. Only apical application of auxin induced rooting in this species. In contrast, in an easy-to-root species *Forsythia,* auxin application was able to induce similar levels of rooting when applied to either end of the internode (Marks and Simpson, 2000). The results of their experiment indicate that accumulation of auxin for root induction in *Syringa* may only be possible if auxin transport is facilitated by the natural basipetal transport system. The general way of auxin application, i.e. basal treatment, may not lead to an increase in auxin concentration in the cells that would give rise to adventitious root formation.

In this study apical IBA application in *G.* 'Coastal Dawn' resulted in some roots formed aerially **(Figure 3).** Cuttings with roots formed aerially, particularly if formed near the shoot tip - will not usually survive because the roots may rot or dry out before reaching the media. To make these aerially rooted cuttings survive and grow into plants, the cutting bases **(Figure10 c**, the part with no roots) should be removed and the rooted shoots **(Figure 10 b)** replanted. This technique was successful in one experiment conducted in autumn. More studies should be conducted before this technique can be recommended in commercial propagation. In particular, previous experiments have indicated that *Grevillea* rooting is seasonal; therefore more experiments need to be conducted on a larger scale to determine the consistency of the results in different seasons.

Figure 10. 'Coastal Dawn'cutting (a) at the time of planting (b) the shoot formed aerial roots after treatment with top IBA at 4 g L^{-1} (c) stem base removed from the rooted part.

5. Conclusion

Rooting differences between the difficult and easy-to-root *Grevillea* cultivars were not likely to be related to their endogenous auxin levels or their ability to take up the applied auxin. This conclusion was based on the findings that (1) there was no significant difference in the endogenous IAA and IBA levels between the easy and difficult-to-root cultivars (2) both cultivars demonstrated an increase in endogenous IAA and IBA levels following IBA application, and the increase in the difficult-to-root 'Coastal Dawn' was more pronounced than in the easy-to-root 'Royal Mantle'.

A different distribution pattern of the top applied [^{3}H]-IBA was noted between the difficult-and easy-to-root cultivars. In contrast to the easy-to-root cultivar, the applied IBA in the difficult-to-root cultivar moved to the leaf rather than the stem base. These findings led to a conclusion that auxin distribution might explain the differing rooting ability of the two *Grevillea* lines.

6. Potential Application to the Domestication and Conservation of Australian Native Plants

These studies indicate that the auxin transport and distribution pattern may be an important factor in adventitious root formation. This knowledge could lead to technical improvements in the area of propagation applicable to difficult-to-root woody species used in floriculture, essential oils and forestry; (1) by providing information on the possible alternative method of auxin application to cuttings to ensure proper delivery of the applied auxin to the receptive tissues; (2) potentially reducing the amount of hormone used for propagation. Moreover, if the auxin pattern is shown to be critical to ease of rooting, it may also lead to a practical method of screening genotypes in future Australian plant selection programs.

The floriculture of Australian native plants is potentially a $ 250 M export industry. Overcoming limitations to vegetative propagation is crucial to the conservation and commercial development of Australian native flora.

7. Publication/Dissemination

Manuscripts are being prepared to publish the results of this project:

1. Santi Krisantini, Margaret Johnston, Richard Williams, Christine Beveridge. Adventititious Root Formation in *Grevillea*, an Australian Native Species. Submitted and accepted with corrections for Scientiae Horticulturae Journal.
2. Santi Krisantini, Margaret Johnston, Richard Williams, Christine Beveridge, John Ross. Identification and quantification of indole-3-acetic acid and indole-3-butyric acid, and Conversion of Indole-3-butyric Acid to Indole-3-acetic Acid by Cuttings of *Grevillea*, an Australian Native Species. To be submitted to Plant Growth Regulation Journal.
3. The results would also be presented at 7th Australian Native Flower Conference in May 2005.

A list of published papers related to this project is as follows:

- Santi Krisantini, Margaret Johnston, Richard Williams, Christine Beveridge. 2003. Propagation of *Grevillea*. The Combined Proceedings of the International Plant Progapators' Society (53):154-159

- Santi Krisantini, Margaret Johnston, Richard Williams. 2002. Rooting of two *Boronia* species as influenced by £-naphthalene acetic acid (NAA) and 2-chloroethyl phosphonic acid (CEPA). Propagation of Ornamental Plants (2): 16-21.
- Santi Krisantini, Margaret Johnston, Richard Williams. 2002. Can ethylene promote rooting in your cuttings? Sixth Australian Wildflower Conference Proceedings 2002.

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