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Endogeneous levels of indole-3-acetic acid and abscisic acid during the rooting of *Cotinus coggygria* cuttings taken at different times of the year

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Abstract. Cuttings of *Cotinus coggyria* cv Royal Purple rooted well in the spring but not at all later in the season. Levels of free and conjugated IAA and ABA were measured in cuttings taken at different times of the year. Hormones were measured in the leaf, the upper stem and the lower stem (rooting zone). In cuttings taken in early June the level of IAA was much higher than that of conjugated IAA. In late July the opposite was found. No significant differences in ABA levels were found although the ABA/IAA ratio changed dramatically.

1. Introduction

Woody species can conveniently be divided into three groups according to their ease of rooting. The first group includes those species with preformed root primordia in the annual shoots, such as *Salix*, *Ribes* and *Populus* [9]. The second group do not possess preformed roots but have a 'latent' rooting ability, and can be easily propagated, for example *Alnus* and *Tilia*. The third group have no preformed roots and possess no latent rooting ability. Typical examples of this group are *Abies* and *Pinus* species [13] which fail to root alone, or in response to standard rooting treatments. *Cotinus coggygria* cv Royal Purple is a deciduous woody shrub. It roots well in the spring [15], but exhibits a gradual decline in rooting ability during the growing season [15]. Seasonal rooting patterns are found in many other genera, for example *Syringa* [21], *Picea* [25] and *Populus* [22]. In the latter example, Smith and Wareing [22] found, using bioassay that the seasonal decline in rooting correlated well with a decline in endogenous auxin levels in the rooting zone of the stem.

In 1934, Thimann and Went [24] demonstrated that 'heteroauxin' stimulated adventitious rooting in the pea rooting test [26]. Since this early

work, many studies have been carried out on the involvement of auxin in the initiation of adventitious roots, using applied auxins and the analysis of the endogenous IAA. Measurement of endogenous plant growth regulators in the rooting zone of cuttings will begin to improve our understanding of adventitious root formation. Although most reports concern IAA, a number of workers have investigated the role of ABA in adventitious root initiation [10, 20]. ABA has been implicated in both the stimulation and inhibition of root initiation [2]. It is considered that the optimum concentration of IAA for the initiation of rooting is markedly supraoptimal for root extension. Thus IAA can be stimulatory at an early stage but inhibitory at a later one [14, 6]. Maldiney et al, [17] have suggested a similar interpretation of the stimulatory and inhibitory effects of ABA.

There have been relatively few reports investigating the endogenous levels of IAA and ABA in cuttings of woody plants, particularly using physico-chemical techniques. The aim of this study was to consider the variation in the levels of free and conjugated IAA and ABA in the rooting zone of *Cotinus coggygria* cv Royal Purple cuttings harvested in spring and summer. Variation in the levels of the plant hormones during the rooting period was also addressed, both on immediate excision of the cuttings and during the subsequent rooting period.

2. Material and methods

2.1 Plant material and sampling

Two clones of *Cotinus coggygria* cv Royal Purple were used, which differed in age and source. Clone SB had been planted and maintained as a hedge system for 6 years whilst clone LP had been planted out for just 1 year. The shrubs were pruned in late March to stimulate vigorous new growth later in the spring.

Clone SB: Cuttings of the whole of the young shoot (8–10 cm in length) were taken in early June. Leaves from the basal 3–4 nodes were removed prior to insertion into the propagating medium. Four replicate batches of twelve cuttings were taken for the rooting trial, and for the analysis of IAA and ABA a further four replicate batches of fifteen cuttings were harvested. Further batches of cuttings were taken in late July and late August. In late July, the mean length of the shoot was 53.2 $\pm$ 1.9 cm. The apical part of the shoot, including the shoot tip (10–12 cm in length) was taken as a cutting. Four replicate batches of ten cuttings were taken for the rooting trial. For the analysis of IAA and ABA, four replicate batches of fifteen cuttings were

harvested. In late August a similar rooting trial was carried out, but no material was analysed for endogenous hormones.

Clone LP: Cuttings of the whole of the young shoot (8–10 cm in length) were taken in early June. Four replicate batches of twelve cuttings were taken for assessment of rooting only. For the analysis of IAA and ABA, four replicate batches of fifteen cuttings were harvested at the time of excision. A similar batch of cuttings was harvested from the propagation bench after 7 and 14 days. Further batches of cuttings were taken from the shrubs in late July and late August, but for an assessment of rooting only with this clone.

All cuttings harvested for hormone analysis were immediately frozen in liquid nitrogen and later freeze dried. For hormone analysis, cuttings were divided into leaf, upper stem and the lower stem which constituted the 'rooting zone'. Care was taken to avoid exposure of the cuttings to water stress.

2.2 Propagation environment

Cuttings were inserted to a depth of 2.5 cm directly into a mist propagation bench containing a 1:1 peat perlite medium. The entire bench was covered by a clear polythene frame to reduce fluctuation in humidity. The medium was heated to 18° C, the aerial environment 22–25° C, with a night minimum of 18° C.

2.3 The extraction and purification of IAA and ABA

The methods used for the analysis of IAA in *Cotinus* tissue have been described previously [7,8]. Essentially, plant material was homogenised and extracted into methanol. An internal standard of 100 Bq of [2-^{14}C] IAA (specific activity 11.2 MBq mg^{-1}) and 133 Bq of [2-^{14}C] ABA (specific activity 3.5 MBq mg^{-1}, Radio-chemical Centre, Amersham, UK) was added at this time. The methanolic extracts were reduced to near dryness under reduced pressure and taken up in 0.5 M phosphate buffer.

2.3.1 Purification of the free acids (IAA and ABA)

The aqueous extract (pH 8.0) was washed with diethyl ether, extracted into diethyl ether at pH 3.0 and concentrated under reduced pressure. The residue was resuspended in 0.5 cm^3 of 0.05 M phosphate buffer (pH 8.0) and applied to a column of insoluble polyvinylpyrrolidone (Polycar AT). The column was eluted by gravity flow with 0.05 M phosphate buffer (pH 8.0). The aqueous eluate, still containing both IAA and ABA was extracted into

diethyl ether at pH 3.0, reduced to near dryness and taken up in 10 cm^3 of water (pH 3.0) and reverse phase chromatographed on a Sep-pak cartridge (Waters Associates). The extract was loaded onto an equilibrated Cartridge and the free acids eluted in 40 % methanol. This was reduced to dryness and the sample was stored at − 20° C until required.

2.3.2 Hydrolysis of the conjugated acids

NaOH was added to the aqueous phase (pH 3.0) after extraction of the free acids, to convert it to a 1M solution of NaOH (pH 11.5). Internal standards of [2-^{14}C] IAA and [2-^{14}C] ABA were added and the solution was incubated for 1 hour at 30° C. The extract was adjusted to pH 3.0 and extracted in diethyl ether. This was then purified in the same way as the 'free' acids.

2.4 Preparative HPLC separation of IAA and ABA

Each sample was separated by HPLC (Applied Chromatography Systems) on a 5 μm Hypersil ODS column (150 × 22.5 mm). The mobile phase for ion pair reverse phase chromatography was 0.01 M tetraethylammonium chloride (TEA) in 0.001 M phosphate buffer at pH 6.6, running as a gradient with 15–35 % methanol, rising at 1 % min^{-1}. The flow rate was 2 cm^3 min^{-1}. The retention times of authentic IAA and ABA standards were determined by a spectrophotofluorimeter (Perkin Elmer MPF 43A) and a UV absorbance monitor (Perkin Elmer LC75) respectively. The fractions containing IAA and ABA were collected, reduced under vacuum and stored in methanol at − 20° C.

2.5 HPLC analysis of IAA

Each sample was completely dried and derivatised to 2-methyl indolo-α-pyrone (2-MIP) as described previously [7]. Essentially, 40 μl of a 1:1 mixture of acetic anhydride:trifluoroacetic acid was added to the dried sample. This was then separated by ion suppression reverse phase HPLC on an analytical 5 μm Hypersil ODS column (250 × 4.5 mm). 2-MIP was detected through a spectrophotofluorimeter with excitation wavelength 445 ± 5 nm and emission wavelength 480 ± 10 nm. The fraction containing 2-MIP was collected and the recovery of [^{14}C] 2-MIP was determined using a scintillation counter.

2.6 GC-ECD analysis of ABA

An aliquot of the purified extract was taken to determine the loss of [^{14}C] ABA up to that point. To the remainder 100 ng of 'mixed isomers' ABA

Table 1. Rooting of *Cotinus* cuttings (clones SB and LP) taken in early June, late July and late August. Assessment was made 6 weeks after excision

Month and clone	% of cuttings rooted	Number of roots > 10 mm per rooted cutting
June SB	39.6	6.0 ± 0.7
June LP	81.3	21.0 ± 2.0
July SB	0	-
July LP	0	-
August SB	0	-
August LP	0	-

standard of known composition was added, which constituted an internal standard for analytical GC-ECD. The extract was methylated using ethereal diazomethane and taken up in methanol prior to analysis by GC-ECD (Pye Unicam, GCV). Samples were injected in 1 μl of methanol onto a glass column containing 1.5% OV17 on Gas Chrom Q 80–100 (Phase Sep), running on a programme from 200–240° C. The injector and detector temperature were 250° C and 280° C respectively.

3. Results

3.1 Rooting

The two clones of *Cotinus* (SB and LP) were treated separately because of the possibility of variation which can occur in ornamental nursery stock [23]. Also there was an age difference between the two clones. Rooting of the June cuttings was assessed after 7 weeks (Table 1). The rooting percentage of clone LP was significantly higher than clone SB ($P < 0.001$). Further, there was significantly ($P < 0.05$) more roots on LP cuttings at this time (Table 1). On cuttings which rooted, root emergence was first detected on most cuttings after 20 days. Cuttings from both clones were also taken in late July and again in late August. The rooting percentage was zero on each occasion.

3.2 Identification of IAA and ABA

The presence of IAA and ABA was confirmed unequivocally in the *Cotinus* tissue used in this work by Gas Chromatography-Mass Spectrometry (GC-MS) [5]. Further, a quantitative comparison between the HPLC technique

Table 2. The levels of free and conjugated IAA in leaves and stem of *Cotinus* cuttings (clone SB) at the time of excision in early June and late July. (a: not determined due to intense pigmentation)

	Free IAA		Conjugated IAA	
	June	July	June	July
	ng per g dry mass			
Leaf	130.9 ± 21.2	182.6 ± 19.4	37.9 ± 4.1	a
Stem-upper half	405.5 ± 67.0	40.4 ± 5.9	22.0 ± 3.6	457.8 ± 57.7
Stem-lower half	315.4 ± 41.2	15.9 ± 4.1	21.3 ± 2.5	672.0 ± 19.4
	ng per cutting			
Leaf	19.0 ± 3.1	166.7 ± 17.7	5.5 ± 0.6	a
Stem-upper half	15.8 ± 2.6	3.5 ± 0.5	0.9 ± 0.1	39.1 ± 4.9
Stem-lower half	13.2 ± 1.7	2.1 ± 0.6	0.9 ± 0.1	90.4 ± 2.6

used here, and a single ion monitoring GC-MS demonstrated that the HPLC technique is a reliable technique for the accurate quantification of IAA levels in *Cotinus* tissue [5].

3.3 Analysis of IAA and ABA in clone SB

The level of free IAA (per g dry mass) in leaf tissue of SB cuttings was very similar in June and July (Table 2). On the basis of ng per cutting, however, with the much higher leaf dry mass, the total amount of free IAA was very much higher in July cuttings. Inspite of this the level of IAA in the stem of July SB cuttings was considerably lower than that in June cuttings.

IAA and ABA bound as esters are released during mild hydrolysis treatment [18]. This treatment, as used in this work would not hydrolyse peptides. Conjugated IAA was present in all tissues extracted (Table 2). In contrast to free IAA, conjugated IAA was present in much higher levels in July. The ratio of free hormone to free plus conjugated hormone (F/F + C) reflects the ability of the tissue to conjugate plant hormones. Table 3 shows that the F/F + C ratio in July SB cuttings is considerably lower than that found in June. On a per cutting basis, in stem tissue, the total amount of free and conjugated IAA was very similar in June and July; 382.1 ng and 539.1 ng (IAA equivalents) respectively.

Very high levels of ABA were detected in the stems of both June and July cuttings (Table 4). Considerably less conjugated ABA was detected in the stems taken at both times (Table 4). The F/F + C ratio of ABA was almost indentical in both the upper and lower stem at both times (Table 3). However the ratio of free ABA to IAA in stem tissue was dramatically lower

Table 3. Ratio of free/free + conjugated IAA and ABA in leaves and stem of *Cotinus* cuttings (clone SB) at the time of excision in early June and late July. (a: not determined due to intense pigmentation)

	F/F + C ratio			
	IAA		ABA	
	June	July	June	July
Leaf	0.78	a	0.64	a
Stem-upper half	0.95	0.08	0.93	0.94
Stem-lower half	0.94	0.02	0.90	0.90

in July (Table 5). A negative correlation was found between the ABA/IAA ratio and rooting using Spearman's rank correlation test.

3.4 Analysis of IAA and ABA in clone LP

Also with the LP shrubs, free IAA and ABA were measured at the time of excision of June cuttings, and again after 7 and 14 days from the mist bench. On day 0, the level of IAA (per g dry mass) was similar to that in clone SB in each of the tissues analysed (Figure 1). The levels of ABA in LP stem tissue were very much lower than those in SB (Figure 2 and Table 4). Consequently the ABA:IAA ratio in the rooting zone of LP cuttings (0.92) was much lower than that in SB cuttings in June (6.92) and July (99.9).

The level of IAA declined significantly over the first 7 days in the stem as a whole, more so in the rooting zone (Figure 1). Although the IAA in leaf tissue continued to decrease, by 14 days the level of IAA in stem tissue had started to increase, more so in the upper stem. The level of ABA after 7 days

Table 4. The levels of free and conjugated ABA in leaves and stem of *Cotinus* cuttings (clone SB) at the time of excision in early June and late July. (a: not determined due to intense pigmentation)

	Free ABA		Conjugated ABA	
	June	July	June	July
	ng per g dry mass			
Leaf	966.4 ± 25.3	438.3 ± 28.3	552.5 ± 71.5	a
Stem-upper half	2842.0 ± 44.2	2118.3 ± 426.2	226.9 ± 31.7	145.3 ± 9.2
Stem-lower half	2120.1 ± 135.2	1588.7 ± 145.9	224.5 ± 20.5	168.5 ± 21.1
	ng per cutting			
Leaf	140.1 ± 3.7	400.2 ± 25.8	80.1 ± 10.4	a
Stem-upper half	110.8 ± 1.7	181.1 ± 3.6	8.8 ± 3.6	12.4 ± 2.7
Stem-lower half	89.0 ± 5.7	213.7 ± 19.6	9.4 ± 0.9	22.7 ± 2.8

Table 5. Rooting percentage and the free ABA/IAA ratio in the lower half of the stem of *Cotinus* cuttings (clones SB and LP) harvested at the time of excision in early June and late July

	ABA/IAA ratio	Rooting percentage
June LP	0.92	81.3
June SB	6.72	39.6
July SB	99.92	0

was the same as that when the cuttings were excised (Figure 2), but much higher in each part of the cutting after 14 days.

4. Discussion

The primary objective of this work was to identify any relationship between the IAA and ABA status of the stock plant and subsequent rooting ability,

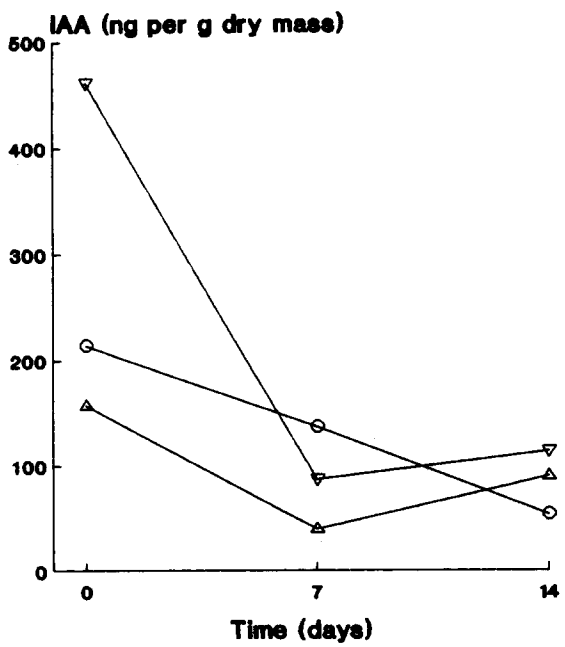

Fig. 1. Changes in the levels of IAA after excision of *Cotinus* cuttings (clone LP) taken in early June: (○) leaf, (△) upper stem, (▽) lower stem.

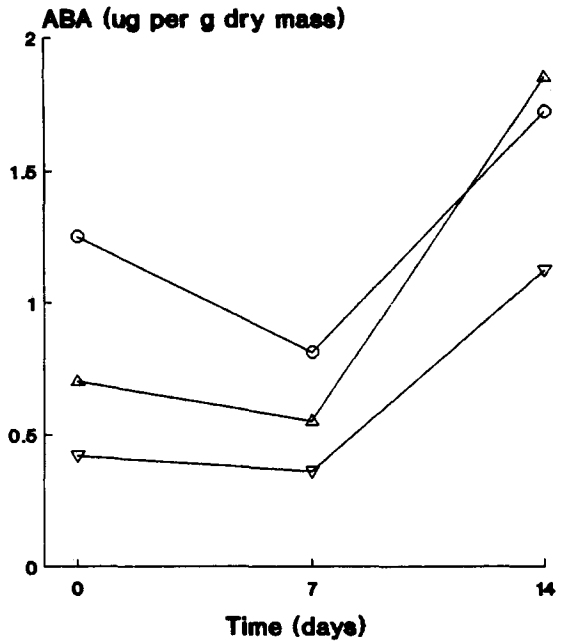

Fig. 2. Changes in the levels of ABA after excision of *Cotinus* cuttings (clone LP) taken in early June: (○) leaf, (△) upper stem, (▽) lower stem.

and to investigate how the levels of these hormones changed after cuttings were taken. The IAA status of the two clones of *Cotinus* used was very similar. In both clones rooting was significantly higher in June than in July and this correlated positively with the levels of free IAA. Smith and Wareing [22] reported a decline in auxin levels in the stem of *Populus* x *robusta* cuttings taken through the growing season. In the present study, early June cuttings were taken at the onset of rapid shoot growth. IAA has been implicated in the onset of cambial activity [16] and increased growth rate [1]. It might be predicted that in cuttings taken in late July at the end of the rapid growth phase, the levels of IAA would be much lower than those taken earlier in the season. The reduction in free IAA levels in July cuttings was accompanied by an increase in the concentration of conjugated IAA. Conjugated forms of IAA have been shown to act as a temporary storage form [19] and to afford protection against degradation by peroxidases [11]. Their role in *Cotinus* is unclear.

Both free and conjugated ABA was detected in *Cotinus*, the level of free ABA being similar to that found in cuttings of *Sequoiadendron* [3]. The levels

of ABA in *Cotinus* appeared to be relatively stable between June and July and no marked changes occurred in free or conjugated levels. This resulted in large changes in the ratio of free ABA to free IAA in the lower half of the stem. This ratio showed a significant negative correlation with rooting (Table 5). These data suggest that ABA is inhibitory to root initiation in *Cotinus* cuttings if the levels of IAA are low. High levels of ABA alone apparently do not inhibit rooting of *Cotinus* cuttings, a result also reported with *Rhododendron* [27] and *Populus* species [4].

Root emergence was noted on most cuttings within 20 days. Although anatomical studies were not carried out, it is almost certain that the primary event of adventitious root initiation took place during the first 14 days. The levels of IAA in LP stem tissue declined during this time. A similar pattern has been reported in cuttings of *Sequoiadendron* [3]. However in that, and the present study it is possible that a transient peak of IAA occurred between cutting excision and the later sample times. Such an increase has been reported in *in vitro* cuttings of grapevine [18] and in hypocotyl cuttings of *Phaseolus aureus* [5], and could be accounted for by the operation of the polar auxin transport process. These studies taken together suggest that an increase in IAA is necessary for the primary event of root initiation, but, as found with *Cotinus*, a subsequent decline allows the root initials to differentiate and develop into root primordia and emergent roots. Literature concerned with the role of endogeneous free and conjugated IAA and ABA is still scarce, and in some cases contradictory. We feel that a comment made by Doré in 1965 [12] is still relevant today; he noted that the large volume of literature pertaining to the control of rooting by applied plant growth regulators. However, relatively few of these reports have attempted to correlate endogeneous levels of plant growth regulators or receptors with rooting, either in the rooting zone or more precisely at the cell level.

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References

1. Bandurski RS, Schulz A and Cohen JD (1977) Photo-regulation of the ratio of ester to free indole-3-acetic acid. Biochem Biophys Res Commun 79: 1219–1223
2. Batten DJ and Goodwin PB (1978) Phytohormones and the induction of adventitious roots. In: DS Letham, PB Goodwin and TJV Higgins, ed. Phytohormones and Related

Compounds: A Comprehensive Treatise, Vol. 2, pp. 127–173. Amsterdam: Elsevier North-Holland Biomedical Press
3. Berthon JY, Maldiney R, Sotta B, Gaspar T and Boyer B (1989) Endogeneous levels of plant hormones during the course of adventitious rooting in cuttings of *Sequoiadendron giganteum* (Lindl.) *in vitro*. Biochem Physiol Pflanzen 184: 405–412
4. Blake TJ and Atkinson SM (1986) The physiological role of abscisic acid in rooting of poplar and aspen stump sprouts. Physiol Plant 67: 638–643
5. Blakesley D (1984) Rooting of Cuttings of Woody Plants. PhD Thesis Leicester Polytechnic
6. Blakesley D and Weston GD (in press) The role of endogeneous auxin in adventitious root initiation. Plant Growth Reg
7. Blakesley D, Allsopp AJA, Hall JF, Weston GD and Elliott MC (1984) Use of reverse phase ion pair HPLC for the removal of compounds inhibitory to the formation of the 2-methyl indolo-α-pyrone derivative of indole-3-acetic acid. J Chrom 294: 480–484
8. Blakesley D, Hall JF, Weston GD and Elliott MC (1983) Simultaneous analysis of indole-3-acetic acid and detection of 4-chlororindole-3-aceteic acid and 5-hydroxyindole-3-acetic acid in plant tissues by HPLC of their 2-methyl indolo-α-pyrone derivatives. J Chrom 258: 155–164
9. Carlson MC (1950) Nodal adventitious roots in willow stems of different ages. Amer J Bot 37: 555–561
10. Chin TY, Meyer MM and Beevers L (1969) Abscisic acid stimulated rooting of cuttings. Planta 88: 192–196
11. Cohen JD and Bandurski RS (1982) Chemistry and physiology of bound auxins Ann Rev Plant Physiol 33: 403–430
12. Doré J (1965) Physiology of regeneration in cormophytes. In: W Ruhland, ed. Encyclopedia of Plant Physiology, Vol. XV/2, pp 1–91. Berlin: Springer-Verlag
13. Hartmann HT and Kester DE (1975) Plant Propagation: Principles and Practices, 3rd Edition. New Jersey: Prentice-Hall
14. Jarvis BC (1986) Endogenous control of adventitious rooting in non-woody cuttings. In: MB Jackson, ed. New Root Formation in Plants and Cuttings, pp. 191–222. Dordrecht: Martinus Nijhoff
15. Kelley JD and Forret J (1977) Effect of timing and wood maturity on rooting of cuttings of *Cotinus coggyria* 'Royal Purple'. Proc Int Plant Prop Soc 27: 445–448
16. Little CHA and Wareing PF (1981) Control of cambial activity and dormancy in *Picea sitchensis* by indol-3-yl acetic and abscisic acids. Can J Bot 59: 1480–1493
17. Maldiney R, Pelese F, Pilate B, Sotta B, Sossountzov L and Miginiac E (1986) Endogenous levels of abscisic acid, indole-3-acetic acid, zeatin and zeatin-riboside during the course of adventitious root formation in cuttings of Craigella and Craigella lateral suppressor tomatoes. Physiol Plant 68: 426–430
18. Moncousin C, Favre JM and Gaspar T (1988) Changes in peroxidase activity and endogenous IAA levels during adventitious root formation in vine cuttings. In: MRS Kutacek, RS Bandurski and J Krekule, ed. Biochemistry of Auxins in Plants, pp. 331–337. The Hague: SPB Academic Publishing
19. Nowacki J and Bandurski RS (1980) *Myo*-inositol esters of indole-3-acetic acid as seed auxin precursors of *Zea mays* L. Plant Physiol 65: 422–427
20. Schmid VA (1972) Über die Steckholzbewurzelung bei einheimischen Laubholzarten mit besonderer Berücksichtigung der Verhältnisse bei *Populus tremula* L. Ber Schweiz Bot Ges 82: 14–38
21. Schmidt G (1978) Studies on some factors concerning the rooting of green cuttings of common lilac (*Syringa vulgaris*). Acta Hort 79: 79–87
22. Smith NG and Wareing PF (1972) The rooting of actively growing and dormant leafy

cuttings in relation to the endogenous hormone levels and photoperiod. New Phytol 71: 483–500
23. Sweet JB, Campbell AI and Goodall RA (1979) Improving the quality of hardy nursery stocks. ARC Res Rev 5: 1–3
24. Thimann KV and Went FW (1934) On the chemical nature of the root forming hormone. Proc Koln Ned Acad Wetensch 37: 456–459
25. Tognoni F, Kawase M and Alpi A (1977) Seasonal changes in rootability and rooting substances of *Picea glauca* cuttings. J Amer Soc Hort Sci 102: 718–720
26. Went FW (1934) A test for rhizocaline, the root forming substance. Proc Koln Acad Wetensch 37: 445–455
27. Wu FT and Barnes MF (1981) The hormone levels in the stems of difficult-to-root and easy-to-root rhododendrons. Biochem Physiol Pflanzen 176: 13–22