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Levels of endogenous indole-3-acetic acid and indole-3-acetyl-aspartic acid during adventitious rooting in avocado microcuttings

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Abstract

Quantification of endogenous IAA and IAAsp was carried out during adventitious root formation in avocado microcuttings. Both auxin and conjugate were monitored in control cuttings (rooted in the absence of auxin) as well as in cuttings treated with a rooting promotor (IBA) or an auxin transport inhibitor (TIBA). Additionally, a histological study to follow root differentiation was carried out. In control cuttings IAA levels remained constant throughout the rooting process, however, in IBA-treated cuttings IAA levels increased 2-fold during the first 6 d. Addition of 200 μM TIBA induced a slight decrease of IAA levels and inhibited root formation.

As for IAAsp levels, both control and IBA-treated cuttings showed a big increase before root differentiation occurred and as the process went on, a progressive decrease took place. However, in TIBA-treated cuttings IAAsp levels not only did not increase but diminished progressively during the process. The role of auxin conjugates during the rooting process of avocado is discussed.

Key words: Avocado, IAA, IAAsp, rooting.

Introduction

In most woody plants adventitious root formation is a crucial step for *in vitro* clonal propagation. In avocado (*Persea americana* M.), juvenile microcuttings can be rooted up to 100% under appropriate culture conditions (Pliego-Alfaro, 1988), however, shoots obtained from *in vitro* cultured adult buds are extremely difficult to root (Barceló-Muñoz and Pliego-Alfaro, 1986; Pliego-Alfaro and Murashige, 1987).

The critical role of auxins on root formation is well established (Blakesley *et al.*, 1991). There are, however, different hypotheses to explain the neccessity for auxin during the process of adventitious root formation. Gaspar (1981) indicated that a decrease in auxin levels occurs at the initiation phase of the rooting process, while Jarvis (1986) sustained the opposite; more recently, Nordström and Eliasson (1991) have reported no variation in levels of free auxin throughout the rooting process. This controversy could be due to the fact that adventitious rooting can be divided into very different phases (Moncousin, 1988). It is also certain that endogenous auxin levels have been measured in different plants during the rooting process but a histological study has not always been carried out (Lovell and White, 1986); furthermore, we must take into account that sufficiently selective and sensitive methods for auxin quantification are relatively new (Sandberg *et al.*, 1987).

In this investigation we have tried to clarify the role of auxin during adventitious root formation in juvenile avocado microcuttings. Quantification of endogenous indole-3-acetic acid (IAA) and indole-3-acetyl-aspartic acid (IAAsp) has been made during the rooting process

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and under treatments known to increase or inhibit root formation. Additionally, a histological study to follow up root differentiation was carried out.

Materials and methods

Plant material and culture conditions

All the experiments were performed with microcuttings from 4–6-week-old avocado seedlings of the cv. Topa-Topa. Seeds were sterilized and germinated *in vitro* according to Pliego-Alfaro (1988). When shoots were 4–6 cm long, apical sections (2 cm) were removed and successively cultured into two rooting media as proposed by Pliego-Alfaro (1988). The first rooting medium in which cuttings were placed for 3 d was supplemented with indole-3-butyric acid (IBA) 5–100 μM; the second medium lacked IBA but contained activated charcoal (Sigma, neutralized powder), 1 g l^{-1}. Both rooting media contained the Murashige and Skoog salts (Murashige and Skoog, 1962) at 0.3 × standard strength and (in mg l^{-1}) sucrose, 30000; thiamine hydrochloride, 0.4; i-inositol, 100; and TC agar, 8000. After approximately 2 weeks the first roots were visible and virtually all shoots had rooted after 4 weeks.

When 2,3,5-triiodobenzoic acid (TIBA) was added, a double phase medium was used in the two rooting stages. The lower phase was a solid medium with the same composition as that described above for each stage. The liquid upper phase had the same composition as the solid phase, although lacking agar, and was supplemented with different concentrations of TIBA. Two experiments were carried out with TIBA. In the first one, the effect on rooting of 10–200 μM TIBA was evaluated in the absence of auxin, e.g. IBA was omitted in the first stage of the rooting process. In the second experiment, TIBA was used at a level of 200 μM and IBA (5–100 μM) was used in the first stage of the rooting process.

Extraction and quantitation of auxins

IAA and IAAsp acid were extracted and quantified using HPLC following the procedure described by Nordström and Eliasson (1991) during days 0, 3, 6, 9, and 12 of the rooting process. Three types of microcuttings were used, e.g. control, treated with 100 μM IBA during the first stage of the rooting process, and treated with 200 μM TIBA during the two stages of the rooting process.

Histological procedures

Stem sections (5 mm long) from the basal part of the three types of microcuttings used for extraction and quantification of auxins, were sampled on the same days described above and fixed in a formalin–acetic acid–alcohol solution. The fixed pieces were dehydrated in an ethanol–butanol series and embedded in paraffin. Transverse sections (10 μm thick) were hydrated and stained according to Gerlach (1969).

Results

Effect of exogenous auxins and TIBA on rooting

Juvenile avocado microcuttings generally root up to 100% when cultured in a medium lacking auxin (Pliego-Alfaro, 1988). Addition of IBA in the range 5–100 μM during the first rooting stage (3 d) did not alter the rooting percentage obtained in control cuttings; however, the highest level of IBA (100 μM) significantly increased the number of roots/culture (Fig. 1). At this concentration 80% of the shoots developed a large basal callus. Higher concentrations of this auxin do not increase the number of roots/culture (Pliego-Alfaro, 1988).

The presence of TIBA (an inhibitor of polar auxin transport), 10–200 μM, in the liquid medium (upper phase) during both stages and in the absence of auxin, diminished the rooting percentage. The highest concentration used (200 μM TIBA) completely inhibited root formation (Table 1).

Addition of IBA (5–100 μM) to the solid phase during the first 3 d of the rooting process, in the presence of 200 μM TIBA in the liquid phase, partially overcame the inhibition caused by this compound (Table 2).

Histological studies

Microcuttings rooted in basal medium: At day 0 the stems of microcuttings showed typical collateral vascular bundles. The cells of the cambium did not show any type of activity and the xylem contained highly lignified vessel elements (Plate 1A). After 3 d in the rooting medium, the cambium became active and some cell nuclei appeared densely stained. From days 3 to 6, cambial derivatives divided and formed radial rows of cells around the xylem.

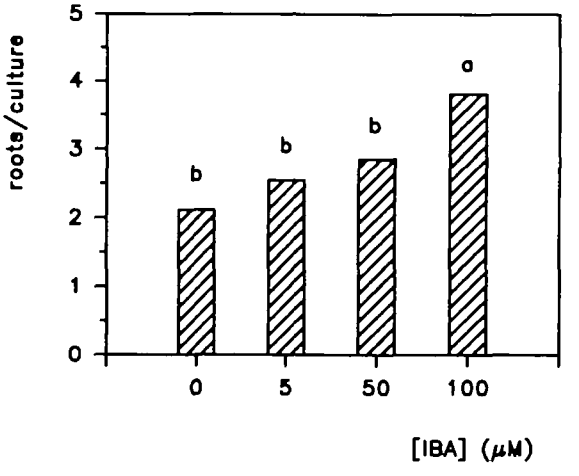

Fig. 1. Mean number of roots/culture after addition of different IBA concentrations to the first rooting medium. Treatments with the same letter are not significantly different ($P<0.05$).

Table 1. *Influence of TIBA in the absence of auxin on the rooting percentage of avocado cuttings*

Brackets: 95% confidence intervals. Data taken after 4 weeks.

TIBA (μM)	% Rooting
0	85 (62–97)
10	90 (69–97)
50	40 (19–64)
100	20 (6–44)
200	0 (0–17)

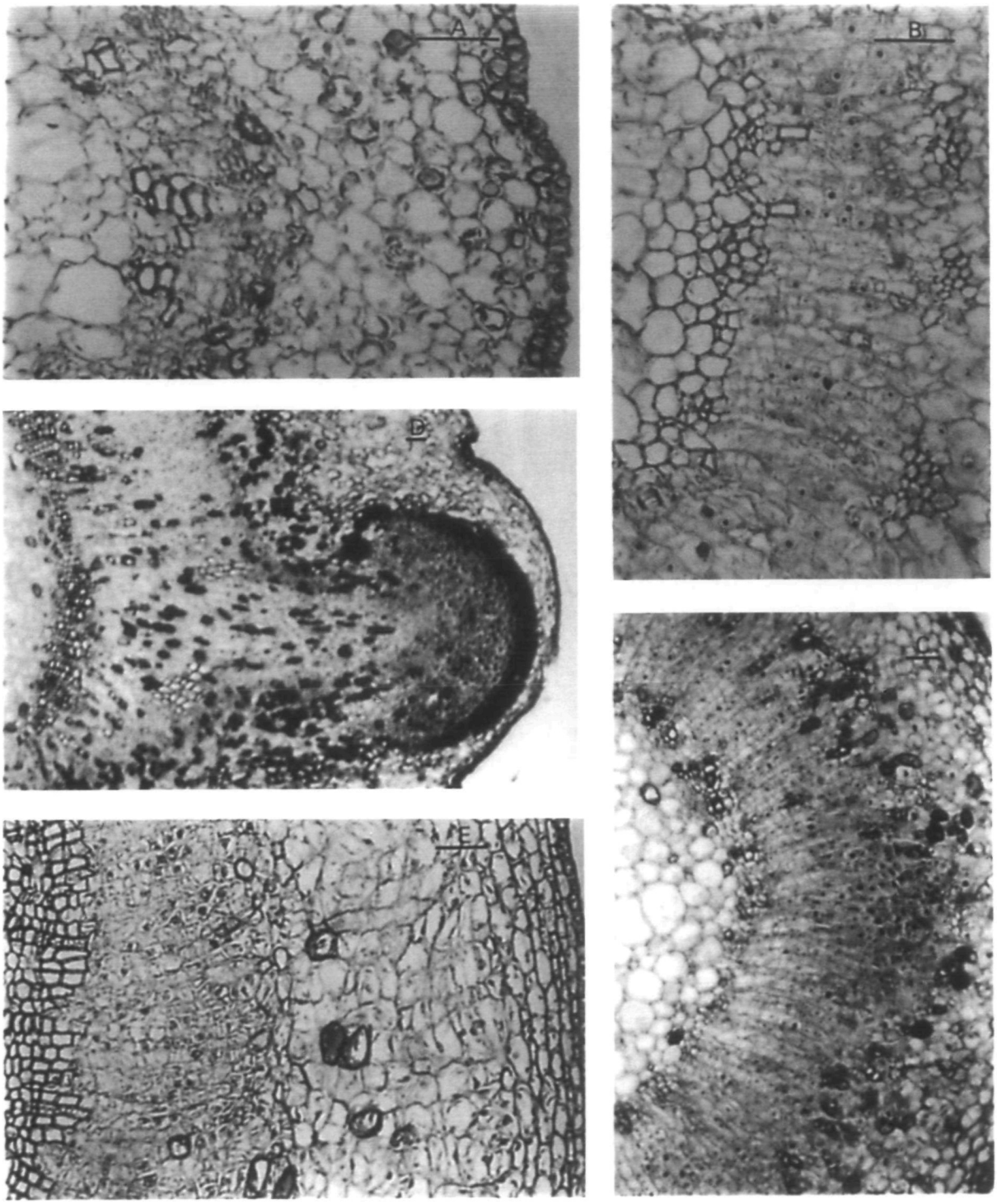

Plate 1. Transverse sections of avocado stems at different days of the rooting process. (A–D) Cuttings rooted in basal medium; (E) cutting treated with 200 μM TIBA. Scale bars = 50 μm.

Table 2. *Influence of IBA on the rooting percentage of avocado cuttings in the presence of 200 μM TIBA*

Brackets: 95% confidence intervals. Data taken after 4 weeks.

IBA (μM)	% Rooting
0	0 (0–17)
5	40 (19–64)
50	40 (19–64)
100	35 (16–62)

The development of secondary tissue was also observed; a ring of xylem and groups of phloem fibres could be observed (Plate 1B). During days 6–9, some of the cambial derivatives formed meristemoids, these meristemoids always appeared very close to the phloem showing the characteristic curved form produced by a change in the cell division planes (Plate 1C). From days 9 to 12 the primordia continued their development, the highly stained cells of the periphery were presumably those of the root cap. Differentiation of tracheary elements in the lower part of the root primordia were also visible at that time (Plate 1D). By day 15, the first roots had started to break the epidermis and became visible at the base of the cutting.

Microcuttings treated with 100 μM IBA: Addition of 100 μM IBA to the first rooting medium (3 d) did not alter the differentiation pattern observed in control cuttings. As expected, cambial development was more pronounced and the number of primordia per shoot was higher than in control cuttings.

Microcuttings treated with 200 μM TIBA: Addition of 200 μM TIBA in both stages of the rooting process did not prevent cambium division nor differentiation into xylem and phloem cells. However, in no case could signs of primordia differentiation be observed (Plate 1E).

Auxin analysis

Microcuttings rooted in basal medium: No differences were found in the levels of endogenous IAA between the basal (first cm) and apical (rest of the cutting) parts of the shoots at the time the cutting was taken (day 0). Levels remained constant in both parts throughout the rooting process (Fig. 2A). IAAsp levels were also the same in apical and basal parts at day 0 when the ratio IAA : IAAsp was 1 : 4, however, in the apical part, IAAsp levels remained constant during the rooting process as with IAA levels, whilst in the basal part a 6-fold increase was found between day 0 and 6. This day coincided with cambium development and differentiation into vascular cells, but no meristemoids were visible yet. From day 6 to 12, IAAsp levels decreased and reached the levels found at day 0 (Fig. 2B).

Microcuttings treated with 100 μM IBA: Addition of 100 μM IBA to the first rooting medium induced a 2-fold

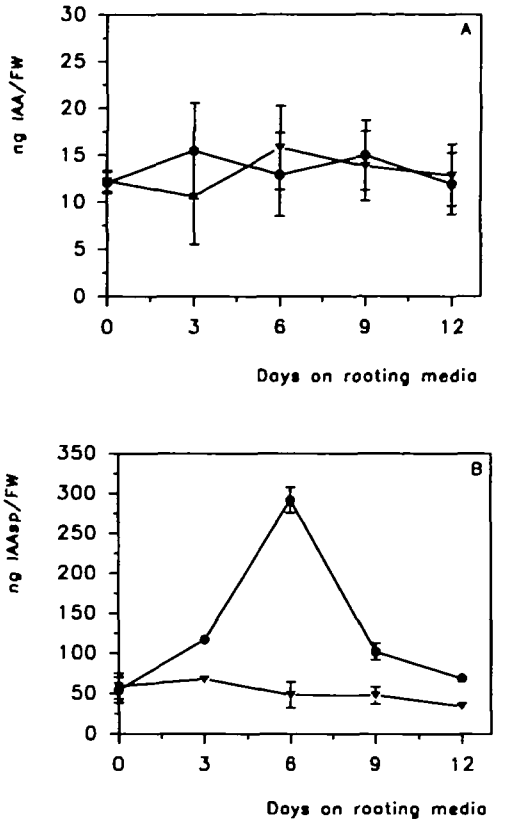

Fig. 2. Endogenous IAA (A) and IAAsp (B) levels in cuttings rooted in a basal medium at different days of the rooting process. Vertical bars represent mean ± SEM values. (●) Basal part; (▼) apical part.

increase in IAA levels from days 0 to 6 in the basal part of the cuttings, thereafter these levels diminished gradually and by day 12 reached the same values found at day 0 (Fig. 3A).

IAAsp levels showed a large increase between days 0 and 3 (Fig. 3B) and, as the process continued, the levels tended to decrease, although still remaining quite high (2 μg/gpf) by day 12.

Microcuttings treated with 200 μM TIBA: Addition of TIBA 200 μM to both rooting media completely inhibited primordia differentiation. IAA levels showed a slight decrease in the basal part of the cuttings (Fig. 4A). As for the IAAsp, the levels of this conjugate not only did not increase, as in all the other treatments, but diminished progressively during the rooting process (Fig. 4B).

Discussion

Juvenile avocado microcuttings show excellent rooting capacity when cultured in medium without auxin (Pliego-Alfaro, 1988). However, endogenous auxin levels must play a critical role in adventitious root formation since a decrease of polar auxin transport caused by 200 μM TIBA

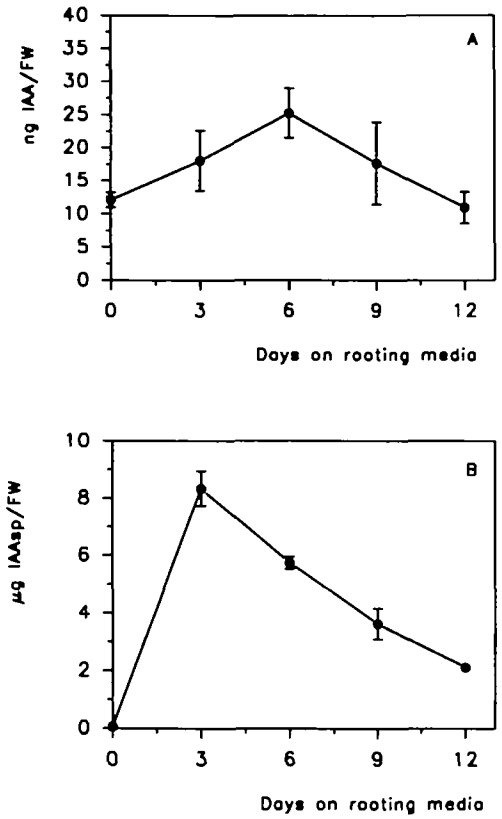

Fig. 3. Endogenous IAA (A) and IAAsp (B) levels in cuttings rooted in the presence 100 μM IBA at different days of the rooting process. Vertical bars represent mean $\pm$ SEM values.

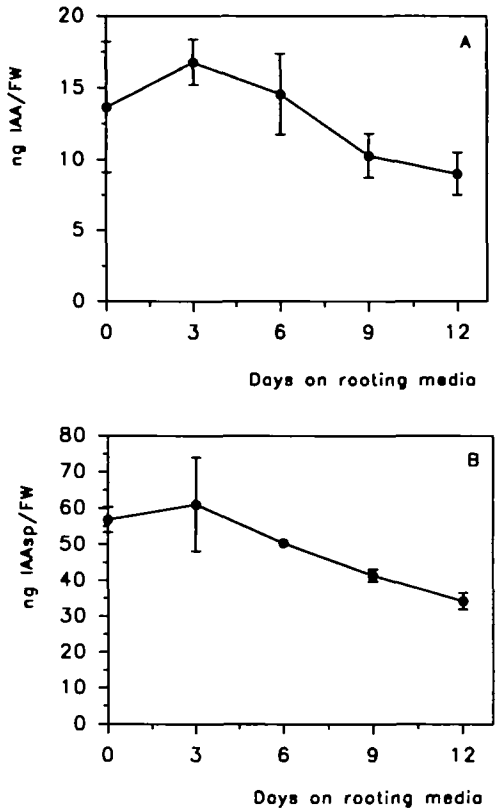

Fig. 4. Endogenous IAA (A) and IAAsp (B) levels in cuttings rooted in the presence of 200 μM TIBA at different days of the rooting process. Vertical bars represent mean $\pm$ SEM values.

completely inhibited root primordia differentiation, although not activation and development of cambial cells.

The increase obtained after using IBA on the number of roots per cutting is similar to that found in many other woody plants (Jarvis, 1986). IBA has been identified as an endogenous hormone in several plants (Badenoch-Jones *et al.*, 1984; Schneider *et al.*, 1985), however, its mode of action is not clear. It has been proposed that this hormone could exert its action through its conversion into IAA (Epstein and Lavee, 1984). In this investigation we have measured endogenous IAA and IAAsp levels after treatments with various concentrations of IBA.

In control microcuttings (rooted in the absence of auxin) endogenous levels of IAA remained constant throughout the rooting process; thus, no accumulation of IAA seemed to be necessary to initiate nor to sustain development of the root primordia. These results agree with those found in *Pisum sativum* (Nordström and Eliasson, 1991) but are in conflict with observations made by other authors, who proposed an accumulation of auxin at the beginning (Jarvis, 1986) or at the end (Gaspar, 1981) of the initiation phase during the adventitious root formation process. Nevertheless, in juvenile avocado cuttings an increase in the quantity of IAA reaching the base of the cutting must take place, as IAAsp levels increase during the first part of the rooting process. Thus, auxin would be required at the early stages of rooting, i.e. activation of cambial cells (days 0–3) and division of cambial cell derivatives (days 3–6). Formation of IAAsp could then be a mechanism of detoxifying excessive levels of free IAA as suggested by Nordström *et al.* (1991). The observed decrease in IAAsp levels which takes place in control cuttings during the initiation of root primordia (days 6–9), could be due to a release of free IAA through hydrolysis of the conjugate. This free IAA would be necessary during the early phases of root primordia formation as stated by Norcini and Heuser (1988). The conjugate could also be oxidized as has been shown in *Vicia faba* (Tsurumi and Wada, 1986) and *Populus tremula* (Plüss *et al.*, 1989). This oxidation, probably, must occur in IBA-treated plants, as the large increase and decrease suffered by IAAsp levels did not alter free IAA levels very much. Accumulation of IAAsp following auxin treatment has also been observed when rooting poplar shoots *in vitro* (Hausman, 1993). In this system and similar to the results obtained in avocado, decreasing IAAsp levels did not give rise to a paralell increase in free IAA. In any case, the results obtained with 200 μM TIBA indicate that conjugates, i.e. IAAsp, should serve as a source of free IAA necessary for root initiation.

Under this treatment in which a slight decrease in endogenous IAA levels took place and the observed accumulation of IAAsp in control cuttings did not occur, differentiation of root primordia was not observed.

References

Badenoch-Jones J, Summons RE, Rolfe BG, Letham DS 1984. Phytohormones, rhizobium mutants and nodulation in legumes. IV. Auxin metabolites in pea root nodules. *Journal of Plant Growth Regulation* **3,** 23–39.

Blakesley D, Weston GD, Hall JF. 1991. The role of endogenous auxin in root initiation. I. Evidence from studies on auxin application and analysis of endogenous levels. *Plant Growth Regulation* **10,** 341–53.

Barceló-Muñoz A, Pliego-Alfaro F. 1986. *In vitro* propagation of the avocado rootstock G.A.-13. *I.A.P.T.C. Meeting, Minnesota,* Abstract **278.**

Epstein E, Lavee S. 1984. Conversion of indole-3-butyric acid to indole-3-acetic acid by cuttings of grapevine (*Vitis vinifera*) and olive (*Olea europaea*). *Plant and Cell Physiology* **25,** 697–703.

Gaspar Th. 1981. Rooting and flowering, two antagonistic phenomena from an hormonal point of view. In: Jeffcoat B, ed. *Aspects and prospects of plant growth regulators.* Monograph **6.** British Plant Growth Regulator Group, Wantage, 39–49.

Gerlach D. 1969. A rapid safranin-crystal violet-light green staining sequence for paraffin sections of plant materials. *Stain Technology* **44,** 210–11.

Hausman JF. 1993. Changes in peroxidase activity, auxin level and ethylene production during root formation by poplar shoots raised *in vitro*. *Plant Growth Regulation* **13,** 263–8.

Jarvis BC. 1986. Endogenous control of adventitious rooting in non-woody cuttings. In: Jackson MB, ed. *New root formation in plants and cuttings.* The Netherlands: Martinus Nijhoff Publishers, 191–222.

Lovell PH, White J. 1986. Anatomical changes during adventitious root formation. In: Jackson MB, ed. *New root formation in plants and cuttings.* The Netherlands: Martinus Nijhoff Publishers, 111–40.

Moncousin CH. 1988. Adventitious rhizogenesis control. New developments. *Acta Horticulturae* **230,** 97–104.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiologia Plantarum* **15,** 473–97.

Norcini JG, Heuser CW. 1988. Changes in the levels of [^{14}C]indole-3-acetic acid and [^{14}C]indole-acetyl-aspartic acid during root formation in mung bean cuttings. *Plant Physiology* **86,** 1236–9.

Nordström AC, Alvarado-Jacobs F, Eliasson L. 1991. Effect of exogenous indole-3-acetic acid and indole-3-butyric acid in internal levels of the respective auxins and their conjugation with aspartic acid during adventitious root formation in pea cuttings. *Plant Physiology* **96,** 856–61.

Nordström AC, Eliasson L. 1991. Levels of endogenous indole-3-acetic acid and indole-3-acetyl-aspartic acid during adventitious root formation in pea cuttings. *Physiologia Plantarum* **82,** 599–605.

Pliego-Alfaro F. 1988. Development of an *in vitro* rooting bioassay using juvenile-phase stem cuttings of *Persea americana* Mill. *Journal of Horticultural Science* **63,** 295–301.

Pliego-Alfaro F, Murashige T. 1987. Possible rejuvenation of adult avocado by graftage on to juvenile rootstocks *in vitro*. *HortScience* **22,** 1321–4.

Plüss R, Titus J, Meier H. 1989. IAA-induced adventitious root formation in greenwood cuttings of *Populus tremula* and formation of 2-indolone-3-acetyl-aspartic acid, a new metabolite of exogenously applied indole-3-acetic acid. *Physiologia Plantarum* **75,** 89–96.

Sandberg G, Crozier A, Ernsten A. 1987. Indole-3-acetic acid and related compounds. In: Rivier L, Crozier A, eds. *The principles and practice of plant hormone analysis.* London: Academic Press, 169–301.

Schneider EA, Kazakoff CW, Wightman F. 1985. Gas chromatography–mass spectrometry evidence for several endogenous auxins in pea seedlings organs. *Planta* **165,** 232–41.

Tsurumi S, Wada S. 1986. Identification of 3-hydroxy-2-indolenone-3-acetyl-aspartic acid as a new metabolite in *Vicia* roots. *Plant and Cell Physiology* **27,** 559–62.