J Plant Growth Regul (1989) 8:263–272

Journal of
Plant
Growth
Regulation



# Relationship of Indole-3-Butyric Acid and Adventitious Rooting in M.26 Apple (*Malus Pumila* Mill.) Shoots Cultured In Vitro

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Received September 23, 1988; accepted March 28, 1989

**Abstract.** The role of indole-3-butyric acid (IBA) in adventitious root formation was studied by analyzing the uptake and subsequent metabolism of IBA in shoots of M.26 apple (*Malus pumila* Mill.) rootstock grown in vitro. Roots were induced by exposing shoots to 4 μM IBA and [$^3$H]IBA for 5 days in the dark and then transferring them to plant growth regulator (PGR)-free medium in the light until roots formed. Approximately 50% of the total radioactivity applied was taken up from the agar medium by the shoots during the 5-day incubation period in IBA. Indole-3-butyric acid metabolism was studied by extraction and high-performance liquid chromatographic (HPLC) separation of [$^3$H]IBA and metabolites from the basal sections of treated shoots. The major [$^3$H]IBA metabolite co-eluted with authentic [$^{14}$C]indole-3-acetic acid (IAA) suggesting that IBA was converted to IAA in the shoots. The proportion of newly synthesized IAA present as conjugates was higher at the end of the 5-day IBA treatment period than after 13 days in PGR-free medium. There appeared to be no conjugation of IBA at any time.

The role of auxins in adventitious root formation has been studied primarily by determining the uptake of radiolabeled auxins in stem cuttings and shoots cultured in vitro (Epstein and Lavee 1984; James 1983). These studies have suggested that uptake leads to the auxin concentrations required for root primordium initiation (James 1983). Synthetic auxins have replaced indole-3-acetic acid (IAA) for induction of roots from cuttings during commercial propagation because synthetic auxins are more effective than IAA. It has been suggested that the increased efficiency of the synthetic auxins indole-3-butyric acid

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(IBA) and naphthaleneacetic acid (NAA) over IAA for root induction, may be related to differences in the rates at which these auxins are metabolized in plant tissues (Jarvis 1986). Indole-3-butyric acid metabolism has been studied in *Vitis vinifera* and *Olea europaea* stem cuttings (Epstein and Lavee 1984); however, no information is available on the metabolism of IBA in shoots cultured in vitro.

The objective of this study was to examine the role of IBA in adventitious root formation in M.26 apple shoots cultured in vitro. Uptake of [$^3$H]IBA from the agar culture medium, and its subsequent distribution and metabolism were studied. Some conversion of IBA to IAA, and conjugation of newly synthesized IAA, was suggested. In light of these findings, the relationship between IBA uptake and metabolism and rooting is discussed.

## Materials and Methods

### *Plant Material and Shoot Proliferation*

Shoot cultures of M.26 apple rootstocks (*Malus pumila* Mill.), were obtained from Dr. D. James (East Malling Research Station, England). The apple shoots were originally proliferated in medium consisting of Linsmaier-Skoog basal salts (Linsmaier and Skoog 1965), 4.4 μM benzyladenine (BA), 0.5 μM IBA, 1.28 mM phloroglucinol (PG), and 3% sucrose (wt/vol) (Jones et al. 1977). The pH was adjusted to 5.2, 0.7% Difco Bacto agar (wt/vol) was added, and the medium was autoclaved at 100 kPa for 20 min. Phloroglucinol was added by filter sterilization. The shoots were incubated in growth chambers (Percival, Boone, IA, USA) at 25 ± 2°C under a photon-flux density of 73 μmol $m^{-1}$ $s^{-2}$, provided by cool white fluorescent lamps, for a 16-h photoperiod. Shoots were subcultured monthly. After 3 months, shoots were transferred to a multiplication medium similar to that described above except that IBA and PG were omitted. Shoots were then subcultured for an additional 18 months at which time it was assumed that no residual IBA or PG was present in the shoots.

### *Rooting Conditions*

The medium used for root induction consisted of Lepoivre salts (Quorin et al. 1977) and 3% sucrose (wt/vol) with no plant growth regulator (PGR) or with 4 μM IBA (IBAM). The pH was adjusted to pH 5.2, 0.7% Difco Bacto agar (wt/vol) was added, and the medium was autoclaved as described above.

Axillary shoots, 1.5 to 2.0 cm in length, were excised from 30-day-old cultures growing on multiplication medium and were transferred individually to 25 × 150-mm culture tubes containing 10-ml PGR-free medium or IBAM. Shoots were incubated in the dark for 5 days at 25°C for root induction and were then transferred to culture tubes containing 10-ml fresh PGR-free medium. Shoots were then maintained in the light under growth chamber conditions as previously described (James and Thurnbon 1979; Welander 1983). The average number of roots per rooted shoot and the percentage of rooted shoots (ex-

pressed as percentage of shoots producing at least one root) were recorded after 7, 10, 13, 19, 25, and 31 days in PGR-free medium. Ten explants were used per treatment and the treatments were run in duplicate. The experiment was conducted twice.

### *Uptake and Distribution of [$^3$H]IBA in the Shoots*

The uptake and translocation of IBA during root induction were examined by inducing roots in M.26 shoots in IBAM-treated medium to which [$^3$H]IBA was added (sp act 268.25 GBq/mmol, gift from Dr. Jerry Cohen, USDA, Beltsville, MD, USA). A 300 µl aliquot of IBAM containing 80,400 dpm of [$^3$H]IBA was dispensed into 1.5-ml polypropylene microcentrifuge tubes which had been cut in half transversely. The resulting volume of the microcentrifuge tube was 0.5 ml. Microcentrifuge tubes were then placed in 13 × 65-mm culture tubes. Single shoots, 1.5- to 2-cm long, were excised from the multiplication medium and placed in the microcentrifuge tubes. Shoots were incubated in the dark for 5 days. The shoots were then transferred to PGR-free medium and maintained for 13 days in the light as described above.

The uptake and translocation of radiolabeled IBA were determined by harvesting shoots at the end of the 5-day dark treatment period and after 13 days on PGR-free medium. The shoot bases in contact with the medium were rinsed with 100 mM phosphate buffer (pH 9.0) and then blotted dry. Shoots were divided into a 1-cm basal section, upper stem section, and leaves. Plant parts were frozen in liquid nitrogen, lyophilized, weighed, and stored at −20°C. Tissue samples were oxidized for 1 min in a Packard Tri-Carb Sample Oxidizer Model 306. Tritiated water resulting from sample oxidation was dissolved automatically in a 17-ml mixture of Monophase-S and Permafluor V (15/2) (vol/vol) (Packard Instruments Co., Downers Grove, IL, USA). Radioactivity was determined using a Beckman model LS 8000 liquid scintillation counter. Four plants were harvested for each sample, with four replicate samples per sampling time. The experiment was conducted three times.

Indole-3-butyric (4 µM) agar samples were oxidized to determine initial radioactivity in the medium and at the end of the 5-day dark incubation period. Samples of PGR-free medium were oxidized at the end of the 13-day period to determine if any radiolabel had been extruded while the shoots had been in PGR-free medium. The phosphate buffer used to rinse the bases of the shoots was also sampled for radioactivity. Combustaid (0.2 ml) (Packard Instruments Co.) was added to agar samples to enhance oxidation, and samples were counted as previously described. Corrections were made for combustion recovery, quenching, and scintillation counting efficiency.

### *[$^3$H]IBA Metabolism*

Shoots were placed in [$^3$H]IBA-containing IBAM for 5 days in the dark and then transferred to PGR-free medium as previously described. Shoots were harvested 5 days after being placed on IBAM and 13 days after transfer to

PGR-free medium to determine the amounts of $^3H$ in the plants present as free and bound IBA and labeled metabolites. Freshly harvested tissue was frozen in liquid nitrogen and ground to a fine powder. Samples were extracted 15 h at 4°C with 10 ml of 80% methanol (MeOH)/water (vol/vol) containing 1 μM butylated hydroxytoluene (BHT) and 10,000 dpm methylene [$^{14}$C]IAA (2.18 GBq/mmol, Amersham Corp., Arlington Heights, IL, USA). The [$^{14}$C]IAA was added for detection of the IAA fraction. Extracts were centrifuged at 500 *g* for 5 min, the supernatants filtered, and pellets washed with an additional 3 ml of 80% MeOH/water (vol/vol). After centrifugation the supernatants were pooled and reduced to an aqueous phase under nitrogen at 40°C. The aqueous phase was adjusted to pH 9.0 with 1.0 N NaOH and then partitioned twice with 2 ml anhydrous diethyl ether containing 1 mM BHT. The aqueous phase was then adjusted to pH 2.7 with 1 N $H_3PO_4$ and extracted twice with 2 ml anhydrous diethyl ether containing 1 mM BHT. The ether fraction was extracted twice with 2 ml of 0.1 M phosphate buffer (pH 9.0). The alkaline buffer was then adjusted to pH 2.7 and further extracted using high-capacity $C_{18}$ SPE columns (J. T. Baker, Phillipsburg, NJ, USA) according to the procedure of Nissen and Foley (1987). Compounds of interest were eluted from the $C_{18}$ columns using 4.0 ml of high-performance liquid chromatographic (HPLC) grade acetonitrile (ACN) (American Burdick and Jackson, Muskegon, MI, USA). The ACN eluent was dried under nitrogen, resuspended in 25 μl ACN, and held at −20°C until HPLC analysis.

Samples were analyzed by reversed-phase HPLC and monitored with fluorescence detection using an LDC Gradient Master with two Constametric II Pumps coupled to an Applied Biosystems 980 fluorescence detector (Foster City, CA, USA). Fluorescence detector excitation was at 220 nm, and the emitted light was monitored using a 350-nm band pass filter. The column was a 250 × 4.6-mm 5 μM Bakerbond C18. High-performance liquid chromatographic grade ACN and water (EM Science, Cherry Hill, NJ, USA) containing 0.1% glacial acetic acid (vol/vol) (JT Baker) were used to create a 15-min linear gradient from 10–50% ACN/water (vol/vol) at a flow rate of 1.5 ml/min. Fractions of the HPLC eluent were collected at 1-min intervals from 0–17 min and assayed for radioactivity. Samples were counted by liquid scintillation spectrometry as dual-labeled to determine distribution of $^{14}$C and $^3$H radioactivity. The purification procedure was tested on [$^{14}$C]IAA and [$^3$H]IBA standards to check for losses during the different purification stages.

Samples used to determine the presence of bound IBA (ester + amide conjugates) and labeled metabolites were processed according to the protocol described above with the following modifications: after MeOH evaporation the aqueous phase was hydrolyzed according to Bandurski and Schulze (1977), cooled, and then brought to pH 9.0 by the addition of concentrated HCl.

Radioactivity was corrected for quenching and crossover (% spillover). Seven plants were used for each replicate, and two replicates were assayed per experiment. The experiment was conducted three times.

## Results

### *Effect of IBA on Adventitious Root Formation*

The presence of IBA had a significant effect on both the percentage of shoots

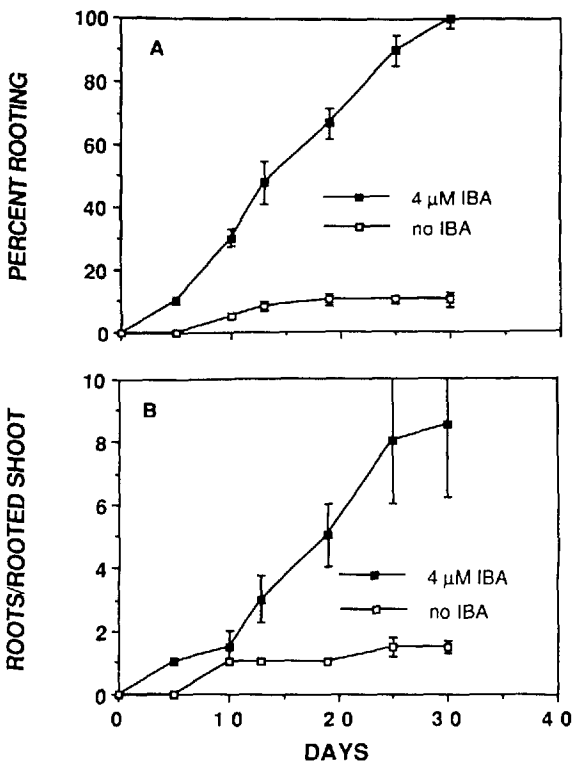


**Fig. 1.** Effect of IBA on percentage of rooting of M.26 shoots grown in vitro. Treated shoots were incubated in the dark for 5 days at 25°C on medium containing IBA. Control shoots were incubated on PGR-free medium in the dark. All shoots were transferred to fresh PGR-free medium at the end of the dark period, and then incubated for an additional 30 days in the light. Duplicate samples of 10 shoots each were used. Vertical bars denote SEM.

that rooted and the number of roots per rooted shoot. When IBA was present, 100% of the shoots rooted in 30 days (Fig. 1A), while only 10% of shoots without IBA rooted within the 30 days. The number of roots per rooted shoot increased steadily during the 30-day period reaching a maximum of 9 ± 3 (mean ± SE) roots per rooted shoot in treatments with IBA, but only 1.5 ± 0.4 roots per rooted shoot when IBA was not present (Fig. 1B).

### *IBA Uptake Experiments*

M.26 shoots absorbed ~50% of the $^3H$ radioactivity supplied during the 5-day treatment period (Table 1). Five days after exposure to [$^3H$]IBA, the major portion of radioactivity taken up by the shoots was located in the basal sections of the shoots. Thirteen days after transfer to PGR-free medium (18 days after initial exposure to IBA), 91% of the radioactivity remained in the 1-cm basal section of the shoot. After 13 days in PGR-free medium, roots were present in about 50% of shoots, and it was found that 4.4% of the radioactivity had accumulated in the roots at this time (Table 1). Although the percentage of

**Table 1.** Uptake and distribution of $^3H$ in M.26 apple rootstock grown in vitro[a]

| Plant structure | Days[b] | Distribution | | |
|---|---|---|---|---|
| | | %[c] | %[d] | dpm/g dry wt (dpm × $10^{-3}$) |
| Stem base | 0 | 49.8 | 96.6 | 10.7 ± 0.8 |
| | 13 | 48.3 | 91.0 | 10.1 ± 0.7 |
| Roots | 0 | NA[e] | NA[e] | NA[e] |
| | 13 | 2.1 | 4.4 | 2.1 ± 0.2 |
| Upper stem | 0 | 2.5 | 1.9 | 0.4 ± 0.1 |
| | 13 | 0.3 | 0.7 | 0.2 ± 0.0 |
| Leaves | 0 | 0.7 | 1.5 | 1.3 ± 0.3 |
| | 13 | 1.8 | 3.9 | 0.3 ± 0.1 |

[a] Shoots were exposed to [$^3H$]IBA for 5 days and then transferred to PGR-free medium for 13 days. Duplicate samples, consisting of seven shoots, were analyzed. Means are the average of three experiments.
[b] Days in PGR-free medium.
[c] Based on total amount of radioactivity applied.
[d] Based on total amount of radioactivity in shoots.
[e] No roots were present at 5 days.

radioactivity was similar to that in the leaves, the dpm/g dry weight was seven times greater in the roots than in the shoots (Table 1).

### *IBA Metabolism*

The extraction and purification procedure resulted in 70% recovery of [$^3H$]IBA and 80% recovery of methylene [$^{14}C$]IAA standards.

Extraction of [$^3H$]IBA-treated basal sections with 80% MeOH/water (vol/vol) showed that only 26% of the $^3H$ was extractable in MeOH. In comparison, when [$^3H$]IBA was added to shoots not previously treated with radiolabeled IBA, 98% of the radioactivity was extractable in MeOH. After the final partitioning against diethyl ether it was found that 18.6% of the radioactivity in the shoots initially extracted in the MeOH remained in the aqueous phase compared with 5.9% when [$^3H$]IBA standards were run. Distribution of radioactivity in the subsequent phases of the purification protocol was similar for both [$^3H$]IBA standards and plant samples. During solvent partitionings and solid phase extractions (SPE) using $C_{18}$ disposable columns, only 8.6% of total radioactivity taken up by the plants was lost. The total recovery after ACN elution from SPE columns was 17.4%.

The radioactivity profile showed co-elution of [$^3H$] with peaks corresponding with authentic IAA and IBA (Fig. 2). High-performance liquid chromatographic retention volume of authentic IAA had a retention volume of 14.0 ml, and IBA had a retention volume of 19.6 ml.

High-performance liquid chromatographic determinations of extracts from basal sections of shoots incubated in [$^3H$]IBA for 5 days indicated that ~3% of the total $^3H$ incorporated in the shoots was obtained in the fraction which was

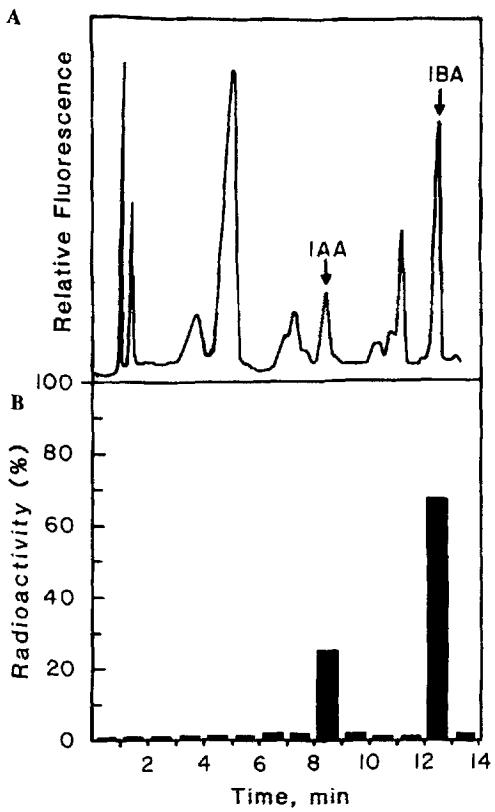


**Fig. 2.** Separation by reversed-phase HPLC of radioactive metabolites of [$^3$H]IBA extracted from M.26 shoots incubated for 5 days in [$^3$H]IBA. Duplicate samples, consisting of seven shoots, were extracted for determination of free IBA and IBA metabolites. The HPLC separation was monitored by fluorescences (**A**) and 1-min fractions were collected to determine the distribution of radioactivity (**B**). Conditions were as follows: column was a 5 μM Bakerbond C18, 250 × 4.6-mm ID; gradient elution 10–50% (ACN:water + 0.1% acetic acid, vol/vol); flow rate 1.5 ml/min; detector Applied Biosystems 980 fluorometer; excitation 220 nm; emission using 350-nm band pass filter.

co-eluted with authentic IAA, while 7.6% of the radiolabel incorporated in the shoots remained in the fraction co-eluted with IBA (Table 2). After alkaline hydrolysis there was an increase in radioactivity from 3.0–8.5% in the fraction co-eluted with authentic IAA. Alkaline hydrolysis did not cause an increase in the percentage or absolute amounts of radioactivity co-eluted with IBA, indicating a very low level of IBA conjugates at 5 days (Table 2).

After 13 days in the PGR-free medium, the percentages of radioactivity in the nonhydrolyzed samples co-eluted with IAA and IBA were 6.6 and 7.1%, respectively. Alkaline hydrolysis did not significantly increase the percentages of radioactivity in the fractions co-eluted with authentic IAA and IBA, indicating that the levels of IAA and IBA conjugates were negligible after the shoots had been in hormone-free medium for 13 days. No other major radioactive fractions were found.

**Table 2.** Radioactivity in IAA and IBA fractions extracted from M.26 shoots treated with [$^3$H]IBA

| HPLC fraction | Days[a] | Free (%)[b] | Bound (%)[b] | Total (%)[b] | Bound:total |
|---|---|---|---|---|---|
| IAA | 0 | 3.0 | 5.5 | 8.5 | 0.64 |
| | 13 | 6.6 | 0.8 | 7.1 | 0.07 |
| IBA | 0 | 7.6 | 0.5 | 8.4 | 0.09 |
| | 13 | 7.1 | 1.1 | 8.2 | 0.13 |

[a] Days in PGR-free medium. Shoots were exposed to [$^3$H]IBA for 5 days and then transferred to PGR-free medium for 13 days.

[b] Based on total radioactivity taken up by shoots. Duplicate samples, consisting of seven shoots, were analyzed. Means are the average of three experiments.

The ratio of free-to-conjugated IAA at the end of the 5-day exposure period to IBA indicated that ~64% of the newly derived IAA was in the conjugated form. The conjugated IAA was reduced to 7% after 13 days in PGR-free medium. No conjugated IBA was detectable at either sampling time.

## Discussion

Exogenously supplied IBA significantly increased adventitious root formation in M.26 apple shoots grown in vitro. These results agree with those of James and Thurnbon (1979), Jones et al. (1977), and Welander (1983), who reported that IBA was required for root induction in several different apple rootstocks and cultivars. In our study, the observation that a 5-day treatment period with IBA was sufficient to produce 100% rooting supports the hypothesis that increased amounts of auxin are required during early stages of root formation (Haissig 1972; Kantharay et al. 1979; Welander 1983). Increased endogenous IAA concentrations have been reported during initiation of root primordia in tomato (Maldiney et al. 1987). Maintaining increased auxin concentrations by applying exogenous auxins, however, is known to inhibit the development of root primordia and subsequent root elongation, and may induce extensive callus formation in apple shoots grown in vitro (Hutchinson and Zimmerman 1987).

In this study, the percentage of [$^3$H]IBA taken up by M.26 shoots was similar to that reported for olive and grape stem cuttings (Epstein and Lavee 1984). Indole-3-butyric uptake was also comparable to the uptake of [$^{14}$C]IAA in M.9 and M.26 apple shoots grown in vitro (James 1983). The minimal upward translocation of the radiolabel, also reported by James (1983) and Epstein and Lavee (1984), was expected since auxin transport in stems is mainly basipetal (Goldsmith 1978). Accumulation of radiolabel in newly formed roots was consistent with the movement of auxin in roots, which is primarily acropetal (Goldsmith 1978). The radiolabel that accumulated in the roots could be a metabolite of IBA.

Our finding that a large amount of label was not extracted in 80% MeOH/water during the first extraction has also been reported by others for maize (Lee 1980) and apple leaves (Tapper and Davies 1968) exposed to 2-[$^{14}$C]IAA.

Lee (1980) has shown that, when [$^{14}$C]IAA is fed to plant tissues, this nonextractable fraction is associated with protein and carbohydrate fractions. These complexes might play a role as a reservoir of IBA and participate in homeostatic regulation as has been suggested for high-molecular weight IAA–amide complexes (Bialek and Cohen 1986; Cohen and Bandurski 1982).

We found that the amount of radioactivity which remained in the ether phase after extraction with the phosphate buffer at pH 9 was three times higher than expected as compared with results from partitioning the [$^{3}$H]IBA standards. These data suggest that the ether fraction may have contained some basic and/or neutral metabolites. In other purification steps, losses of radioactivity were small and similar for both standards and samples. This suggests that most labeled metabolites were removed during the first fractionation step.

Conversion of [$^{3}$H]IBA to [$^{3}$H]IAA by M.26 shoots in vitro was strongly suggested by co-elution of $^{3}$H with methylene [$^{14}$C]IAA during HPLC separation. Epstein and Lavee (1984) reported a similar conversion in olive stems. Conversion of IBA to IAA may lead to the necessary IAA concentration(s) for adventitious root formation and may explain the effectiveness of IBA in inducing adventitious roots. That the IBA was not conjugated would increase its effectiveness since it would be continually available as a pool of free IBA, able to be converted to IAA, during adventitious root formation. The conversion of IBA to IAA may result from enzymatic hydrolysis or cleavage of the side-chain by β-oxidation (Fawcett et al. 1958).

The increase in putative IAA after alkaline hydrolysis indicated that radiolabeled IAA was present as a conjugate. Conjugation of exogenously supplied auxins, which is known to occur rapidly in plants (Vijayaraghavan and Pengelly 1986), may serve as a mechanism to protect auxins from oxidation or provide for slow release of free auxins (Cohen and Bandurski 1982). In our study the decrease of $^{3}$H in the conjugated IAA fraction during the 13 days in PGR-free medium, during which roots were being formed, has also been reported in a similar study by Norcini and Heuser (1988). These results suggest that the conjugated IAA was being hydrolyzed to maintain free IAA levels necessary for root formation and development.

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