

pH Affects 1*H*-indole-3-butyric Acid Uptake but not Metabolism during the Initiation Phase of Adventitious Root Induction in Apple Microcuttings

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ABSTRACT. The influence of root initiation medium pH on root formation was investigated in relation to uptake and metabolism of applied IBA in microcuttings of *Malus ×domestica* Borkh. ‘Gala’ and ‘Triple Red Delicious’. Root formation and uptake of *H*³-IBA were related inversely to root initiation medium pH. Maximum root count (10.3 roots) and IBA uptake were observed at pH 4.0. Regardless of pH, overall root count of ‘Gala’ was higher (13.5 roots) than ‘Triple Red Delicious’ (4 roots). Uptake of IBA was highest at pH 4.0 for ‘Gala’ (1.7% uptake) and at pH 4 and 5 for ‘Triple Red Delicious’ (0.75% uptake). Metabolism of IBA was the same regardless of root initiation medium pH or cultivar examined. One-half of the IBA taken up was converted to a compound that coeluted with IBAsp during high-performance liquid chromatography. Apparently, pH regulates root formation by affecting IBA uptake but not metabolism. The level of auxin in tissue appeared unrelated to root formation between genotypes. Chemical names used: 1*H*-indole-3-butyric acid (IBA); 5-*H*³-indole-3-butyric acid (*H*³-IBA); indole-3-butyrylaspatic acid (IBAsp).

Involvement of auxin in adventitious root formation of macro- and micro-cuttings is documented extensively (Blakesley et al., 1991; Blazich, 1988). Propagule response to exogenous auxin frequently depends on applied concentration, net uptake and subsequent metabolism in tissue. Information regarding external factors controlling uptake and metabolism of applied auxin in cuttings and how tissue levels of auxin are related to adventitious root formation is limited (Blakesley, et al., 1991; Gaspar and Hofinger, 1988). Uptake of auxin into tissues and isolated cells can be affected by pH due to the weak acid nature of auxin molecules used to stimulate root formation (Rubery and Sheldrake, 1973).

Adventitious root initiation of *Malus ×domestica* microcuttings depended on pH of root initiation medium (Harbage and Stimart, 1996). Root count of these microcuttings was affected by IBA in a concentration-dependent manner modified by pH. Colorimetric assay of auxin remaining in root initiation medium after incubation of microcuttings suggested IBA uptake was related inversely to pH. However, the effects of pH on endogenous tissue levels or metabolism of IBA were unknown.

The purpose of this research was to examine further the role of root initiation medium pH on uptake and metabolism of IBA during initiation phase of adventitious root formation on two cultivars of *Malus domestica* microcuttings.

Materials and Methods

Shoot culture

Malus ×domestica ‘Gala’ and ‘Triple Red Delicious’ were obtained from Richard Zimmerman, U.S. Dept. of Agriculture, Beltsville, Md. Previous studies identified ‘Gala’ as an easy-to-root cultivar and ‘Triple Red Delicious’ as a difficult-to-root cultivar in terms of rooting percentage and root counts (Zimmer-

man and Fordham, 1985). Shoots were subcultured every 28 d onto 100 mL Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) modified to contain 0.56 mM myo-inositol, 1.2 μM thiamine HCl, 1.3 μM gibberellic acid, 0.49 μM IBA, 4.44 μM benzyladenine, 88 mM sucrose, and 7 g·L⁻¹ Difco Bacto agar. The pH was adjusted to 5.2 before addition of agar. All ingredients were added before autoclaving at 1.3 kg·cm⁻² at 121 °C for 15 min. Culture vessels were 475-mL (9.0-cm-diameter × 9.5-cm-high) clear glass jars covered with glass petri plate bottoms (10 cm in diameter) and a layer of plastic film. Ten to twelve shoots were placed in each jar. Cultures were maintained under continuous cool-white fluorescent lamps (40 μmol·m⁻²·s⁻¹) at 29 °C.

Shoots were harvested 21 d after subculturing for IBA uptake and metabolism evaluation. Shoot clusters were covered with sterile water in culture jars to prevent drying. Shoots were detached from the cluster at their bases with scissors and cut to shoot lengths with five nodes from the apex down. Cuts were made just above a node. The resulting cuttings are hereafter referred to as microcuttings or by cultivar names. Shoots were kept in sterile water for up to 5 min until placed on treatment medium.

Microcutting rooting

ROOT INITIATION. Microcuttings were treated with liquid root initiation medium containing 43.8 mM sucrose, 30 μM IBA and 10 mM 2[*N*-morpholino] ethanesulfonic acid (Harbage and Stimart, 1996). The pH was adjusted to 3.0 by adding H₃PO₄ followed by KOH to the desired pH before autoclaving. The pH was measured after autoclaving and remained unchanged. Under aseptic conditions, one microcutting was inserted into a plastic support fitted into the bottom of a 25×95-mm glass shell vial with the basal 4 mm submerged in 3 mL of root initiation medium. Vials were capped with plastic closures. Microcuttings were incubated in the dark at 30 °C for 11 h.

ROOT DEVELOPMENT. Microcuttings were transferred under aseptic conditions to glass vials as described previously containing one-half strength MS minerals, minus the Fe, and 22 mM sucrose. Microcuttings were maintained in the dark at 30 °C for an additional 85 h for 4 d (11 h root initiation plus 85 h root development). This period is when adventitious root primordia are initiated (Harbage et al., 1993). Microcuttings were placed under continu-

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Table 1. Analysis of variance for the influence of root initiation medium pH and cultivar on root count of *Malus × domestica* microcuttings after 11 h incubation in root initiation medium containing 30 μ M IBA followed by 14 d in root development medium.

Source of variation	df	Mean squares (root count)
pH	4	62.15***
Cultivar	1	2260.21**
pH \times cultivar	4	26.91 ^{NS}
Error	85	15.52

^{NS},***,** Nonsignificant and significant at $P \leq 0.001$ or 0.01, respectively.

ous cool-white fluorescent lamps ($30 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 23 °C for 14 d, at which time roots were counted based on the number of roots emerged through the surface of the stem from each microcutting distinguished with a hand lens (10 \times). We have shown previously that this number agrees with the number observed histologically with a light microscope (Harbage et al., 1993).

Rooting response to pH

Rooting response of both cultivars was evaluated on root initiation media buffered with 2[*N*-morpholino] ethanesulfonic acid at pH 3.0, 4.0, 5.0, 6.0, or 7.0. Microcuttings were handled as described previously for root initiation and development. A completely random design was used with nine or ten replications per treatment. A replication will hereafter be referred to as one microcutting placed in one glass shell vial.

IBA uptake from root initiation medium

RADIOISOTOPE ASSAY. Uptake of H^3 -IBA by microcuttings from root initiation medium adjusted to a range of pH levels was measured. Root initiation medium contained 30 μ M IBA plus 1478.3 Bq·mL⁻¹ H^3 -IBA at pH 3.0, 4.0, 5.0, 6.0, or 7.0. Specific activity of root initiation medium was 4.93 Bq \times 10⁴ Bq/ μ mol IBA. Microcuttings were treated with root initiation medium for 11 h. At the end of the 11-h incubation, microcuttings were removed, rinsed with glass distilled water, placed in scintillation vials with 10 mL scintillation fluid (Ready Solve II; Beckman Instruments, Fullerton, Calif.), extracted overnight, and counted on a liquid scintillation counter (model 9000; Beckman Instruments). A completely random design was used with nine replications per treatment.

IBA metabolism

H^3 -IBA DELIVERY. Metabolism of IBA taken up by microcuttings was evaluated in root initiation medium with 30 μ M IBA plus 2.8 \times 10⁵ Bq·mL⁻¹ H^3 -IBA at pH 4.5 and 7.0. The H^3 -IBA was provided by Jerry Cohen, U.S. Dept. of Agriculture, Beltsville, Md. Specific activity of root initiation medium was 9.2 \times 10⁶ Bq/ μ mol IBA. Microcutting stem bases were incubated in 1 mL of root initiation medium contained in 1.5-mL microcentrifuge tubes placed in shell vials as described above and capped with plastic closures. Cultures were incubated for 11 h in darkness at 30 °C.

TISSUE EXTRACTION. Microcuttings were removed, rinsed with glass distilled H₂O, placed in 1.5-mL microcentrifuge tubes, and frozen in liquid nitrogen (Chen et al., 1988). Tissue was macerated and extracted in 600 μ L of (in %) 65 isopropanol : 35 0.2 M imidazole buffer at pH 7.0 (extraction buffer) for 1 h, and centrifuged at 10,000 g_n for 5 min. Supernatant was placed in a 5-mL pear flask. The pellet was resuspended in 400 μ L extraction buffer and centrifuged, and the supernatant combined with the first. No measurable radioactivity remained in the final pellet. Combined supernatants were dried in a rotary evaporator to remove extraction

buffer and resuspended in (in %) 10 acetonitrile : 90 H₂O containing 2.5% formic acid (v/v), and extracts were filtered through 0.45- μ m filters. Extraction was on ice.

SEPARATION. The H^3 -IBA and metabolites were separated by high-performance liquid chromatography using a controller (model 680; Water's Associates, Milford, Mass.), 2 pumps (Water's 6000A), an injector with a 500- μ L injection loop (Rheodyne 7125), an absorbance detector (654 nm) (Water's 440), a scintillation fluid delivery module (Beckman 110B), a radioisotope flow detector (Beckman), and a fraction collector (Gilson 201). The system was connected to an IBM-compatible computer interfaced with Chromatographics software (Beckman). The column was a 4.6 \times 250-mm C-18 column (Resolvex; Fisher Scientific, Pittsburgh) with 10- μ m spherical particles. The mobile phase was achieved using acetonitrile (A) and 2.5% formic acid (B) in a linear gradient from 10% A : 90% B to 60% A : 40% B in 20 min and maintained at 60% A : 40% B for an additional 10 min. Flow rate was 1 mL·min⁻¹. IBA and its metabolites were identified in tissue extracts by comparison with authentic IAA (1*H*-indole-3-acetic acid), IAGlu (indole-3-acetylglucose), IBA, and IBAsp.

METABOLITE HYDROLYSIS. Metabolites coeluting with IBAsp were studied for release of free IBA (Bandurski and Schulze, 1977). Equivalent sized fractions corresponding to IBAsp were collected, adjusted to 10 mL with glass distilled H₂O, poured over 2.8 g NaOH in a plastic vial with a Teflon lined screw cap, and heated to 100 °C for 3 h under nitrogen gas. Aqueous mixtures were titrated to pH 2.5 with HCl, partitioned twice against ethyl acetate, and poured over one-third its volume of anhydrous sodium sulfate for 1 h to remove remaining H₂O. The ethyl acetate was filtered, dried on a rotary evaporator, redissolved in 500 μ L initial high-performance liquid chromatography solvent, and analyzed by high-performance liquid chromatography. A completely random design was used with four replications per treatment.

Results

ROOTING RESPONSE. Root count was affected significantly by pH and cultivar with no significant interaction between these main effects (Table 1). Maximum root count was on 'Gala', which was three times greater than the count of 'Triple Red Delicious' (Fig. 1A). The highest root count was at pH 4.0 (Fig. 1B). This count decreased as pH increased between pH 4.0 and 7.0 (Fig. 1). Root count at pH 3.0 was below that of pH 4.0 and 5.0, but above that of pH 6.0 and 7.0.

IBA UPTAKE. Uptake of H^3 -IBA was affected significantly by a

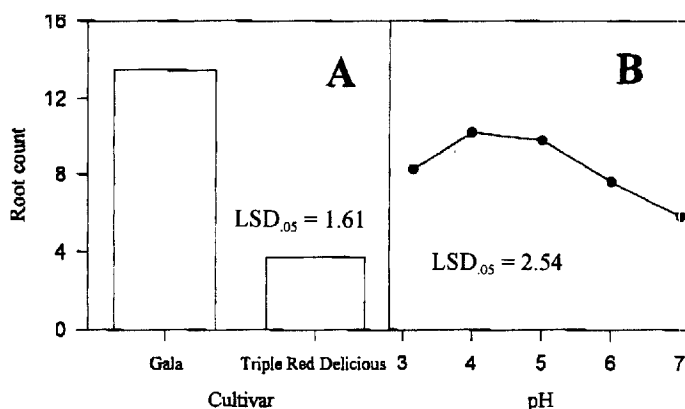


Fig. 1. Root count of *Malus × domestica* as influenced by cultivar (A) and pH of root initiation medium (B) after 11 h incubation in root initiation medium containing 30 μ M IBA followed by 14 d in root development medium.

Table 2. Analysis of variance for the influence of root initiation medium pH and cultivar on uptake of H^3 -IBA by *Malus × domestica* microcuttings during 11 h incubation in root initiation medium containing 30 μ M IBA with 1478.3 Bq·mL⁻¹ H^3 -IBA.

Source of variation	df	Mean squares (uptake)
pH	4	2.71***
Cultivar	1	1.42***
pH × cultivar	4	0.77***
Error	80	0.12

***Significant at $P \leq 0.001$.

pH × cultivar interaction (Table 2). Uptake of H^3 -IBA by 'Triple Red Delicious' from root initiation medium increased slightly between pH 3.1 and 4.0, was similar between pH 4.0 and 5.0, and decreased between pH 5.0 and 7.0 (Fig. 2). Uptake of H^3 -IBA by 'Gala' from root initiation medium increased sharply between pH 3.1 and 4.0 and decreased sharply between pH 4.0 and 7.0. Uptake of H^3 -IBA by 'Gala' was higher than 'Triple Red Delicious' from pH 3.0 through 5.0 and similar to 'Triple Red Delicious' at pH 6.0 and 7.0. Maximum uptake for 'Gala' was twice that for 'Triple Red Delicious'. A brown discoloration was observed on the basal 1 to 2 mm of microcuttings at pH 3.0. Samples of radioactivity remaining in medium following microcutting incubation plus radioactivity extracted from shoots equaled 100.1% (\pm standard error of 1.1%) of radioactivity delivered in root initiation medium.

IBA METABOLISM. Three radioactive peaks were identified in shoot extracts of both cultivars at pH 4.5 and 7.0 (Table 3). The first peak (peak 1) eluted at 3 min and was <10% of the total radioactivity of the combined three peaks at each pH level. The second radioactive peak (peak 2) coeluted with IBAsp and IAA with radioactivity ranging from 40.7% to 58.8% of each total. The third radioactive peak (peak 3) coeluted with the standard IBA with radioactivity ranging from 32.7% to 51.9%. Relative amounts of peaks 2 and 3 were unaffected significantly by cultivar, root initiation medium pH or their interactions (Table 3). Radioactivity counts in the tissue pellet after extraction were <1% of the total radioactivity (supernatant plus pellet). Radioactivity extracted from 'Gala' and 'Triple Red Delicious' at pH 4.5 and 7.0 averaged 1.69%, 0.65%, 1.14%, and 0.75%, respectively, of the total radioactivity in root initiation medium. Retention time by high-performance liquid chromatography of IBAsp and IAA were similar and unresolved in the gradient system used (Fig. 3). Basic hydrolysis of peak 2 from both cultivars at pH 4.5 and 7.0 resulted in release of a product that coeluted with standard IBA with recovery of 86.4%, 72.4%, 73.3%, and 90.0% for 'Gala' and 'Triple Red Delicious' at pH 4.5 and 7.0, respectively (Fig. 4).

Discussion

This study showed that pH regulated uptake of H^3 -IBA from root initiation medium into apple microcuttings but did not affect metabolism of IBA in the tissue. The pH was related inversely to root count and uptake between pH 4 and 7 in 'Gala'. Tissue levels of H^3 -IBA were almost six times higher at pH 4 than at pH 7 (Fig. 2). Similarly, tissue levels of H^3 -IBA were almost three times higher at pH 4 than at pH 7 for 'Triple Red Delicious'. About half of the H^3 -IBA taken up by shoots was metabolized into IBAsp during the 11-h exposure period and metabolism of H^3 -IBA in microcuttings of the easy-to-root 'Gala' was not different from the hard-to-root 'Triple Red Delicious' at pH 4.5 or 7.0 (Fig. 3).

Auxin has been implicated as a causal agent in adventitious root

formation (Jarvis, 1986). Reduced rooting ability associated with ageing of *Phaseolus aureus* Roxb. cuttings was related to lower auxin uptake in older cuttings (Jarvis and Shaheed, 1986). Auxin uptake failed to account for rooting differences between easy-to-root cultivars versus difficult-to-root cultivars of *Olea europaea* L. cuttings (Epstein and Lavee, 1984) or microcuttings of *Malus pumila* Mill. (James, 1983). Previously, we showed that rooting of *Malus domestica* microcuttings depended on auxin supply during the stage of primordia initiation (Harbage et al., 1993). We showed also that root counts and loss of IBA from root initiation medium were related inversely to pH (Harbage and Stimart, 1996). The present study showed that IBA taken up by microcuttings was related inversely to pH, which indicates that more roots formed at reduced pH as a result of increased IBA uptake.

Accumulation of IAA into cells is determined by passive diffusion and influx and efflux carriers (Delbarre, 1996; Rubery and Sheldrake, 1974). Carrier effects are saturated at low concentrations above which uptake is determined solely by passive movement (Rubery and Sheldrake, 1974). Passive movement of auxins, which are weak acids, into cells depends on pH because at low pH (<4.7 pK for the acid) auxins exist predominantly in the undissociated form. The undissociated form is more soluble in lipid environments and passes readily across the plasma membrane (Rubery and Sheldrake, 1973). In cultured *Raphanus sativus* L., pH dependence of auxin-induced lateral root formation was overcome by addition of dimethyl sulfoxide (Blakely et al., 1986). These authors suggested that dimethyl sulfoxide acted by increasing plasma membrane permeability to auxin. Those results and our current findings indicate that applied auxin must cross a lipophilic barrier, perhaps the plasma membrane, to have a pronounced effect on root formation.

Auxin applied to promote adventitious root formation is subject to various metabolic pathways once inside plant tissue, with the degree of metabolism associated with differences in rooting. Applied IBA was converted into IAA in *O. europaea* cuttings

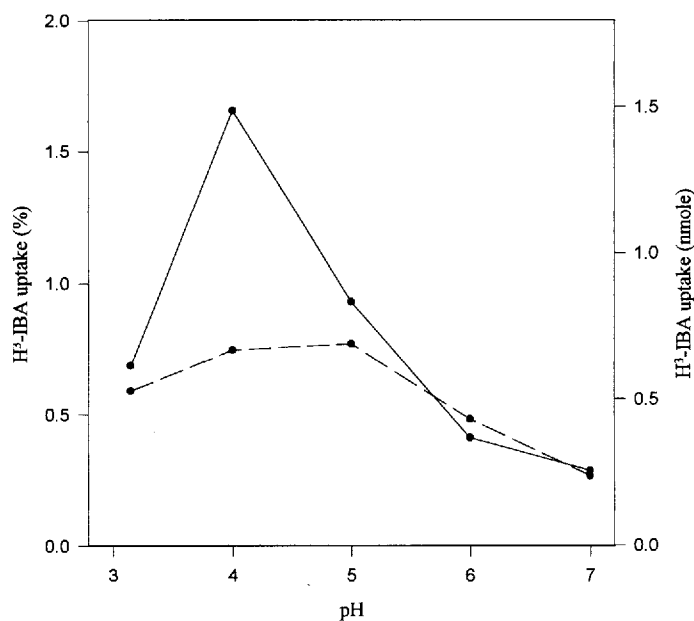


Fig. 2. Uptake of H^3 -IBA from root initiation medium by *Malus × domestica* 'Gala' (---) and 'Triple Red Delicious' (—) microcuttings in response to root initiation medium pH. Uptake is expressed as percentage of delivered H^3 -IBA and nm IBA per microcutting extracted from microcuttings after 11 h incubation in root initiation medium containing 30 μ M IBA with 1478.3 Bq·mL⁻¹ H^3 -IBA. LSD_{0.05} for percent uptake for 'Gala' and 'Triple Red Delicious' is 0.40 and 0.24, respectively.

Table 3. Influence of root initiation medium pH and cultivar on metabolism of H^3 -IBA taken up by *Malus × domestica* microcuttings during 11 h incubation in root initiation medium containing 30 μ M IBA with 2.8×10^5 Bq·mL⁻¹ H^3 -IBA per shoot.

Cultivar	pH	Percentage of total extracted H^3 -IBA ^z		
		Peak 1	Peak 2	Peak 3
Gala	4.5	6.2	49.6	44.2
	7.0	8.5	58.8	32.7
Triple Red Delicious	4.5	7.4	40.7	51.9
	7.0	8.3	45.3	46.4
ANOVA				
Effect	df	Mean squares		
pH	1	11.07 ^{NS}	191.60 ^{NS}	294.77 ^{NS}
Cultivar	1	1.00 ^{NS}	502.28 ^{NS}	458.40 ^{NS}
pH × cultivar	1	1.83 ^{NS}	21.33 ^{NS}	35.64 ^{NS}
Error	12	15.03	172.74	249.29

^zTotal uptake equaled 1.69 and 0.65 nM per microcutting for 'Gala' at pH 4.5 and 7.0, respectively and 1.14 and 0.75 nM per microcutting for 'Triple Red Delicious' at pH 4.5 and 7.0, respectively.

^{NS}Nonsignificant.

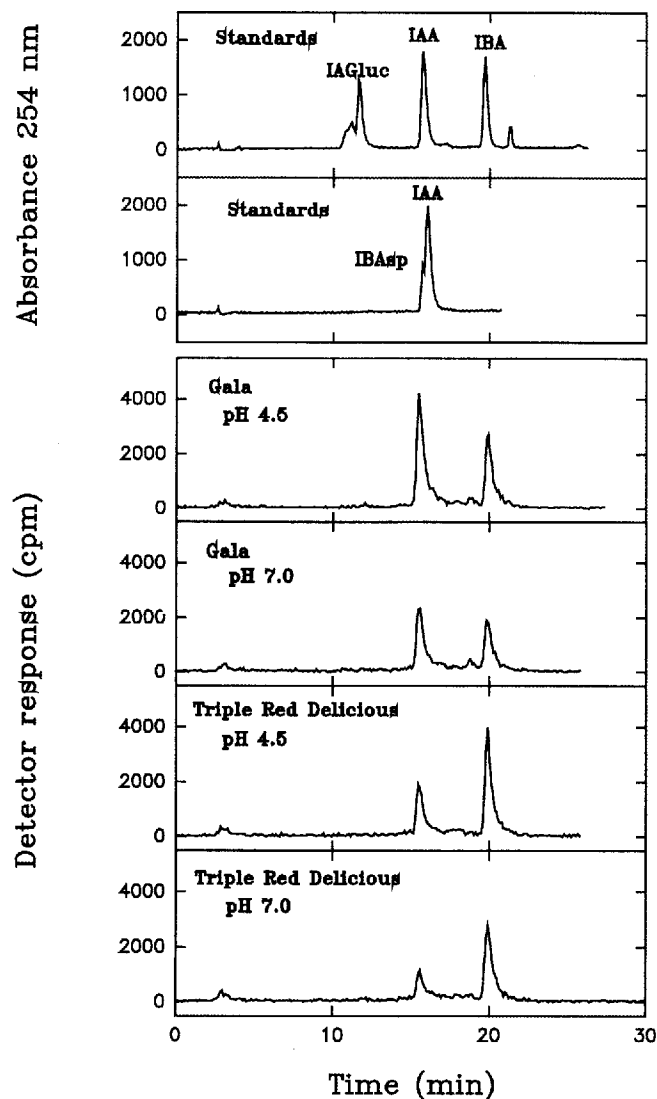


Fig. 3. Chromatograms of auxin standards analyzed with a UV absorbance detector and tissue extracts of *Malus × domestica* microcuttings after 11-h incubation in root initiation medium containing 2.8×10^5 Bq·mL⁻¹ H^3 -IBA, and analyzed with a radioisotope flow detector. IAGluc = indole-3-acetylglucose, IAA = 1*H*-indole-3-acetic acid, IBA = 1*H*-indole-3-butyric acid, and IBAsp = indole-3-butyrylaspartic acid. Tissue extracts from 'Gala' and 'Triple Red Delicious' at pH 4.5 and 7.0.

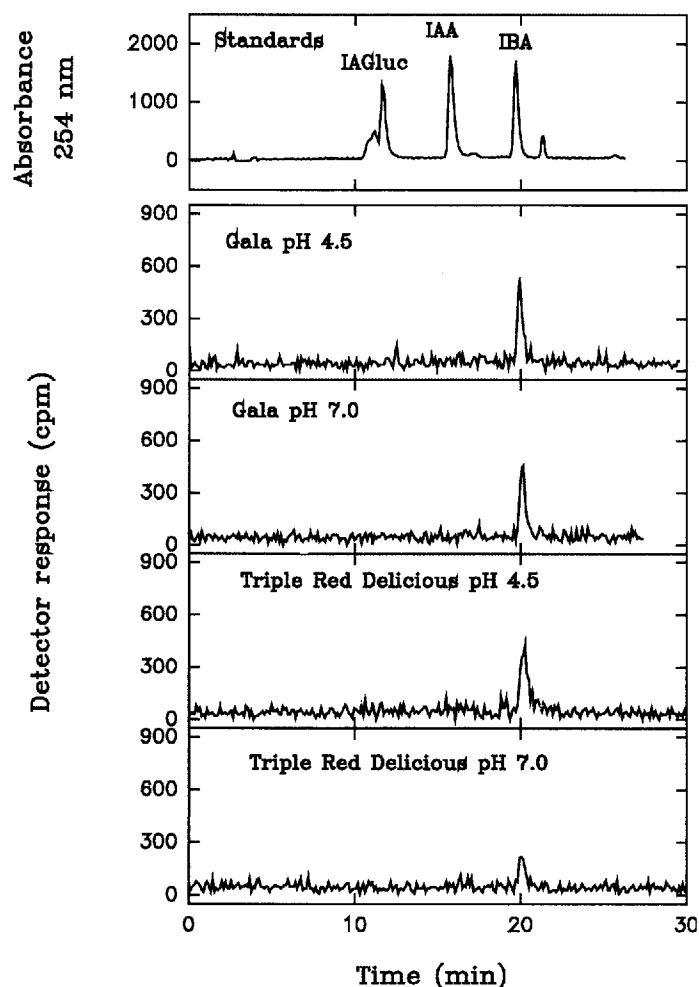


Fig. 4. High-performance liquid chromatography chromatograms of auxin standards analyzed with an ultraviolet absorbance detector and high-performance liquid chromatography microcutting extract fractions corresponding to the IAA/IBAsp retention time after alkaline hydrolysis analyzed with a radioisotope flow detector. Hydrolyzed fractions from 'Gala' and 'Triple Red Delicious' apples at pH 4.5 and 7.0 incubated in 2.8×10^5 Bq·mL⁻¹ H^3 -IBA for 11 h.

(Epstein and Lavee, 1984) and *M. pumila* (Alvarez et al., 1989b). The higher amount of free IAA produced in response to applied IBA was related to higher root counts in *M. pumila* 'M.26' compared to 'M.9' (Alvarez et al. 1989a). Therefore, increased root formation resulting from reduced pH of root initiation medium was due possibly to a pH affect on IBA metabolism. However, metabolism of IBA observed in this study was unaffected by cultivar or pH of root initiation medium. In all treatments, most applied IBA, extracted from tissue and separated by high-performance liquid chromatography, coeluted with free IBA or a metabolite with the same retention time as IBAsp (Fig. 3). The metabolite-released free IBA following harsh alkaline hydrolysis suggests that IBA was converted into IBAsp (Fig. 4). This result was consistent with studies on *Vigna radiata* (L.) R. Wilcz cuttings, which showed that most IBA taken up during root induction phase was converted into IBAsp (Weisman et al., 1988, 1989). In our study, IAA and IBAsp standards eluted with identical retention times, indicating similar chromatographic properties. Previous studies reporting metabolism of applied IBA into IAA may have mistaken IBAsp for IAA (Epstein and Lavee, 1984; Alvarez et al. 1989b). The difference in metabolites reported also may result from different IBA incubation times before tissue extraction. In this study, metabolism was evaluated after 11 h, whereas Alvarez et al. (1989b) evaluated metabolism after 5 d and Epstein and Lavee (1984) measured metabolism after 26 d. In our study, lack of differences in metabolism between cultivars or due to root initiation medium pH suggests that differences in metabolism failed to account for differences in rooting between these cultivars or differences in rooting due to pH as reported previously (Harbague et al., 1996).

Protocols involving application of auxin for root initiation would benefit from use of buffers to maintain a constant pH at an acidic level in the range of 4.5 to 5.0. This pH range might eliminate variability of auxin uptake and root formation due to variable root initiation medium pH, which can result from presence of plant material (Skirvin et al., 1986; Williams et al., 1990), medium nutrients and carbohydrate sources (Owen et al., 1991), and autoclaving (Owen et al., 1991; Skirvin et al., 1986). Reducing medium pH from near neutral to values of pH 4.5 to 5.0 would likely increase root initiation only if auxin supply was limiting. Protocols involving supraoptimal auxin levels would probably be unaffected by reduced pH, since this would only further increase auxin uptake.

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