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Relationship between Indole-3-Acetic Acid Levels in Apple (*Malus pumila* Mill) Rootstocks Cultured *in Vitro* and Adventitious Root Formation in the Presence of Indole-3-Butyric Acid[1]

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ABSTRACT

***In vitro* rooting response and indole-3-acetic acid (IAA) levels were examined in two genetically related dwarfing apple (*Malus pumila* Mill) rootstocks. M.26 and M.9 were cultured *in vitro* using Linsmaier-Skoog medium supplemented with benzyladenine (BA), indole-3-butyric acid (IBA), and 1,3,5-trihydroxybenzoic acid (PG). Rooting response was tested in Lepoivre medium supplemented with IBA and PG. IBA concentrations of 12.0 and 4.0 micromolar induced the maximum rooting percentages for M.9 and M.26, respectively. At these concentrations rooting response was 100% for M.26 and 80% for M.9. Free and conjugated IAA levels were determined in M.26 and M.9 shoots prior to root inducing treatment by high performance liquid chromatography with fluorescence detection and validated by gas chromatography-mass spectrometry using $^{13}[C_6]$IAA as internal standard. Basal sections of M.26 shoots contained 2.8 times more free IAA than similar tissue in M.9 (477.1 ± 6.5 *versus* 166.6 ± 6.7 nanograms per gram fresh weight), while free IAA levels in apical sections of M.26 and M.9 shoots were comparable (298.0 ± 4.4 *versus* 263.7 ± 9.3 nanograms per gram fresh weight). Conjugated IAA levels were significantly higher in M.9 than in M.26 indicating that a greater proportion of total IAA was present as a conjugate in M.9. These data suggest that differences between M.26 and M.9 rooting responses may be related to differences in free IAA levels in the shoot base.**

In vitro clonal propagation of woody species has often been difficult because many species do not readily produce adventitious roots. The most successful method for adventitious root induction in woody species has been the exogenous applications of synthetic auxins (NAA, IBA)[3] to stem cuttings and shoots cultured *in vitro*. Many lines of evidence suggest that auxins play an important role in adventitious root formation (3, 5, 6, 9–12, 19, 26). Young leaves and buds generally promote rooting and may be replaced by exogenous auxin (19). Several investigators have reported positive correlations between endogenous IAA levels in cuttings and number of roots produced per cutting (24).

In this study, adventitious root formation was examined in two dwarfing apple (*Malus pumila* Mill) rootstocks. M.26 and M.9 apple rootstocks were selected because they are genetically related, but differ in rooting ability. M.26 is considered easy to root because a high percentage of stem cuttings produce roots under greenhouse conditions. M.9 is considered difficult to root because shoots require an etiolation treatment after budbreak in order to induce stem cuttings to root (3, 8). The difference in adventitious root formation between M.26 and M.9 is also apparent *in vitro*. Previous research has shown that M.9 requires higher exogenous auxin concentrations than M.26 for root induction (13, 14).

The differential response of M.26 and M.9 to exogenous auxin applications has led investigators to suggest that differences in rooting ability between M.9 and M.26 result from differences in endogenous auxin levels (16), auxin metabolism (11, 13, 14), or sensitivity of target cells to auxin (10). The objective of this research was to examine the relationship between endogenous IAA levels of M.26 and M.9 shoots cultured *in vitro* and the rooting response of shoots to exogenous auxins.

MATERIALS AND METHODS

Plant Material and Shoot Proliferation Conditions

Techniques and conditions for M.26 and M.9 establishment and proliferation have been previously described (12, 15). M.26 and M.9 apple rootstock cultures (*Malus pumila* Mill) were supplied by Dr. D. James (East Malling Research Station, England). Shoots were transferred to multiplication medium consisting of Linsmaier-Skoog salts (17) to which 4.4 μM BA, 0.5 μM IBA, 30.0 g/L sucrose, and 7.0 g/L Difco Bacto agar were added. The medium was adjusted to pH 5.2 and autoclaved. PG (1.3 mM) was added by filter sterilization. Cultures were grown in GA7 vessels (Magenta Corp., Chicago, IL) containing 50 mL of media and incubated in growth chambers at 25 ± 2°C with a 16 h photoperiod at an irradiance of 73 $\mu mol\ m^{-1}\ s^{-2}$ photon flux provided by cool-white fluorescent lamps. Shoots were subcultured every 30 d.

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[3] Abbreviations: IBA, indole-3-butyric acid; FW, fresh weight; BHT, butylated hydroxytoluene; SPE, solid phase extraction; TBDMS, tert-butyldimethyl-silyl; PG, phloroglucinol; HFM, hormone-free medium; ACN, acetonitrile; NAA, naphthaleneacetic acid; GC-MS-SIM, gas chromatography-mass spectrometry-selected ion monitoring.

Rooting Response to Exogenous Auxins

Axillary shoot cuttings, 1.5 to 2.0 cm in length, were excised from 30-d-old cultures growing on multiplication medium. Individual shoots were transferred to 25 × 150 mm culture tubes containing 10 mL rooting medium so that the bottom 0.7 cm were inserted in the medium. The rooting medium consisted of Lepoivre salts and vitamins (21) supplemented with 30.0 g/L sucrose, 7.0 g/L Difco Bacto agar and IBA concentrations of 0.0, 0.1, 1.0, 4.0, 12.0, or 15.0 μM. The medium was adjusted to pH 5.2 and autoclaved. PG (1.3 mM) was added by filter sterilization. Shoots in rooting medium were kept in the dark for 5 d at 25°C for root induction. Shoots were then transferred to Lepoivre medium without IBA (HFM) to allow root development (12, 25). The average number of roots per rooted shoot and the rooting percentage (expressed as percentage of shoots producing at least one root), were recorded after 30 d in HFM. Duplicate treatments consisting of 10 explants each were used. The experiment was conducted twice.

IAA Determination

Free and conjugated IAA levels were determined in 1.5 to 2.0 cm axillary shoots excised from 30-d-old cultures. Sampling at this time provided an estimate of IAA levels prior to root induction treatment. Shoots were divided in half. Basal sections were in close proximity to the multiplication medium, while apical sections contained several leaves plus the apical meristem. Sample size ranged from 150 to 250 mg FW. Analyses were performed in triplicate. Determinations were repeated with similar results.

IAA Extraction and Purification

Extraction and purification of free and conjugated IAA were performed according to Nissen and Foley (20), with some modifications. Freshly harvested tissue was frozen in liquid nitrogen, and ground to a fine powder. Samples were extracted with 10 mL of 80% methanol/water (v/v) containing 1 μM BHT and 10 μL of methylene [^{14}C]IAA (10,000 dpm, 2.18 GBq/mmol, Amersham Corp.) as an internal standard. Samples were extracted overnight at 4°C. Extracts were centrifuged at 900*g* for 5 min. The supernatants were filtered, pellets washed with 80% methyl alcohol/water (v/v) and centrifuged. The supernatants were pooled, and then reduced to the aqueous phase under nitrogen at 40°C. The aqueous phase was then split into two equal parts. One aliquot was hydrolyzed according to Bandurski and Schulze (1) for total IAA determination. The aliquot hydrolyzed with 7.0 N NaOH was brought to pH 9.0 by addition of concentrated H_3PO_4. The aliquot for free IAA determination was adjusted to pH 9.0 with 1.0 N NaOH. Both aliquots were then partitioned with 2 mL ethyl acetate (2 times). The aqueous phase was adjusted to pH 2.7, extracted with 2 mL anhydrous diethyl ether (2 times) containing 1 mM BHT. The ether fraction was extracted twice with 2 mL 0.1 M phosphate buffer [pH 9]. The alkaline buffered extract was then partitioned, using dialysis, according to Liu and Tillberg (18). This procedure was modified by volume reduction of the three phases. Phase I extraction buffer (0.075 M K_2HPO_4, pH 2.7) was reduced to 75 mL, phase II (diethyl ether plus 1 mM BHT) was reduced to 75 mL, and phase III dialysis tubing buffer (0.1 M K_2HPO_4, pH 9.2) was reduced to 10 mL. Ascorbic acid (100 mg/L) was added to buffers. Samples were dialyzed in the dark for 20 h at room temperature.

After partitioning, IAA was extracted from phase III buffer using high capacity C_{18} SPE columns (J. T. Baker, Phillisburg, NJ) according to Nissen and Foley (20). IAA was eluted from C_{18} columns using 2.0 mL of 80% ACN/water (v/v). The aqueous ACN eluent was dried under nitrogen, resuspended in 100 μL ACN and held at −20°C until HPLC analysis.

HPLC IAA Analysis

Samples were analyzed by reversed-phase HPLC with fluorescence detection. The instrument for HPLC purification and quantitation of IAA was an LDC Gradient Master with two Constametric II Pumps coupled to an Applied Biosystems (Foster City, CA) 980 fluorescence detector. Fluorescence detector excitation was at 220 nm and emitted light was monitored using a 350 nm band pass filter. HPLC grade solvents were ACN (American Burdick and Jackson, Muskegon, MI) and water (EM Science, Cherry Hill, NJ) containing 0.1% (v/v) glacial acetic acid (J. T. Baker, Phillipsburg, NJ) to produce a mobile phase with pH 2.7. The column was a 250 by 4.6 mm 5 μm Bakerbond C_{18}. The solvent program was a 15 min linear gradient from 10 to 50% ACN/water (v/v). At a flow rate of 1.5 mL/min IAA had a retention volume of 15.4 mL and K' of 4.4. Twenty μL loop injections were made. IAA quantitation was made by comparison of peak areas to standard curves of authentic IAA, and corrected for losses during extraction and purification by an isotope dilution technique (22).

GC-MS-SIM IAA Analysis

Internal standard (200 ng of $^{13}C_6$ [benzene ring]IAA, gift from Dr. J. Cohen, USDA, Beltsville, MD) was added to samples analyzed by GC-MS-SIM. Samples were processed as previously described. The entire sample was purified by HPLC and peaks which cochromatographed with authentic IAA were collected into 15 mL silanized, conical centrifuge tubes. Water was added to reduce ACN content to less than 5%. IAA was extracted twice with 2 mL diethyl ether, and the ether fraction dried under nitrogen. Samples were resuspended in 50 μL of ACN, and derivatized by incubation with 50 μL of *N*-methyl-*N*-(tert-butyl-dimethyl-silyl) trifluoroacetamide (Pierce Chemical Co.) for 45 min at 60°C. Samples were again dried under nitrogen at 40°C, and resuspended in 10 μL HPLC grade ethyl acetate.

Gas chromatography was carried out on a Hewlett Packard model 5790 Chromatograph equipped with a 30 m, DB-1 (J&W Scientific) capillary column (i.d. 0.25 mm; film thickness, 0.25 μm). The carrier gas was helium at a velocity of 40 cm/s. GC temperature increased from 125 to 250°C at a rate of 10°C/min. The mass spectrometer was a ZAB model HS-2F (VG Analytical, Wythenshave, UK). Source temperature was 180°C, ionizing voltage was 70 eV, and trap current was 100 μA. Resolution was 1000 and electron multiplier voltage was 2700 V. For GC-MS-SIM analysis, 1 μL on-column injections were made. Ions monitored included those

at m/z 232 and 238 (base peaks of putative IAA and internal standard), 289 and 295 (molecular ions of IAA- TBDMS and $^{13}[C]_6$-IAA-TBDMS), and 130 and 136, quinolinium ions of putative IAA and internal standard. Quantitative analysis of IAA levels was accomplished according to Nissen and Foley (20). The ratio 289/295 was used to verify the analysis as suggested by Cohen *et al.* (4).

RESULTS

Rooting Experiments

The presence of IBA in the rooting media significantly increased the number of roots/rooted shoot in M.26 and M.9; however, M.26 produced significantly more roots than M.9 at all IBA concentrations tested (Fig. 1). IBA concentrations for 0.1 to 15.0 μM did not significantly increase root production in M.26, while IBA concentrations from 0.1 to 4.0 μM significantly increased the number of roots/rooted shoot in M.9.

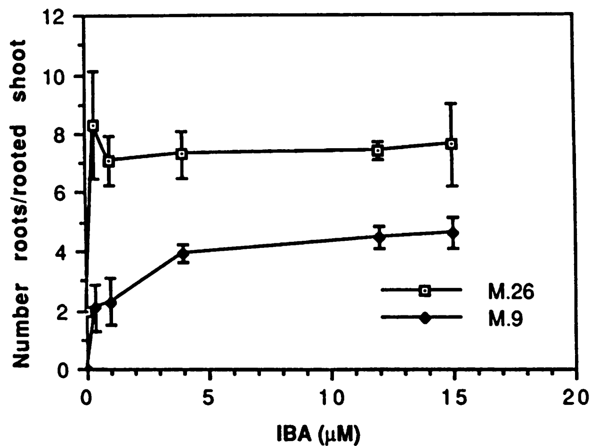

Figure 1. Effect of IBA on number of roots produced by M.26 and M.9 shoots. All shoots exposed to IBA for 5 d in the dark at 25 ± 2°C before transfer to hormone free medium in the light for 30 d. The experiment was performed twice, each time with 10 explants per treatment, and the results were pooled. Vertical bars denote SE of the mean.

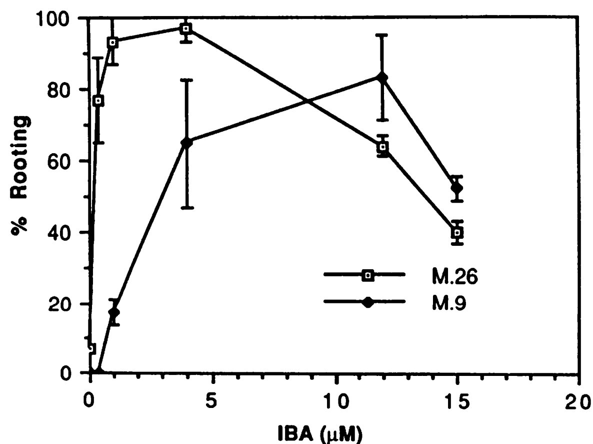

Figure 2. Effect of IBA on M.26 and M.9 rooting percentages. All shoots exposed to IBA for 5 d in the dark at 25 ± 2°C before transfer to hormone-free medium in the light for 30 d. The experiment was performed twice, each time with 10 explant per treatment, and the results were pooled. Vertical bars denote SE of the mean.

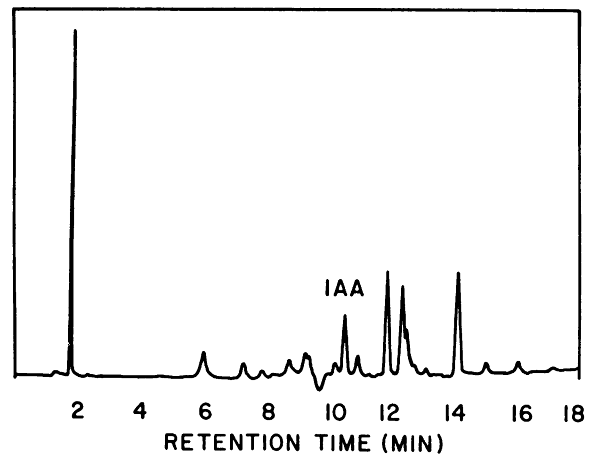

Figure 3. HPLC chromatogram of purified stem extracts from apple shoots cultured *in vitro*. Column: 5 μm Bakerbond C18 250 × 4.6 mm ID: gradient elution: 10 to 50% ACN/water + 0.1% acetic acid (v/v); flow rate: 1.5 mL/min; detector: applied biosystems 980 fluorometer, excitation 220 nm, emission using 350 nm band pass filter.

The maximum rooting percentages for M.9 (80%) and M.26 (100%) were obtained with IBA concentrations of 12.0 and 4.0 μM, respectively (Fig. 2). In the absence of IBA, rooting percentages were 0 for M.9 shoots and 7% for M.26 shoots.

IAA Determination

Free and conjugated IAA levels were determined in shoots prior to root inducing treatment. A typical HPLC/fluorescence chromatogram of purified extract from apical sections of M.26 shoots is shown in Figure 3. IAA eluted without interfering peaks, and recovery ranged from 40 to 60%. HPLC fluorescence determinations of IAA were validated by analyzing representative tissue samples by GC-MS-SIM, using $^{13}C_6$[benzene ring]IAA as internal standard. IAA levels determined by HPLC fluorescence showed good agreement with those samples analyzed by GC-MS-SIM (Table I). A typical mass spectrum of putative IAA from apical sections of M.26 shoots containing $^{13}[C_6]$IAA is shown in Figure 4. The base peaks of IAA-TBDMS ester result from the loss of the tert-butyl group (M-57) to form the ion at m/z 232. The quinolinum ion (m/z 130) is characteristic of 3-substituted indole compounds.

A selective ion chromatogram from an extract of apical sections of M.26 shoots is shown in Figure 5. The ratio of ions at m/z 232/238 was 0.39 and the ratio of ions at m/z 289/295 was 0.38, verifying the analysis (4). Based on the amount and purity of internal standard added and the sample weight (250 mg), the IAA level was calculated to be 273.6 ng/g FW (20).

Free IAA levels were significantly higher (P = 0.01) in basal sections of M.26 shoots than in basal section of M.9 shoots, while free IAA levels in M.26 and M.9 apical shoot sections were comparable (Table I). M.26 and M.9 shoots contained significant amounts of conjugated IAA. The amount of conjugated IAA relative to free IAA (conjugated/free) was higher in M.9 than in M.26 (Table I).

Table I. *Free and Conjugated IAA levels in M.26 and M.9 Apple Rootstocks*

IAA was analyzed in shoots from 30-d-old cultures grown in Linsmaier-Skoog medium supplemented with 4.4 μM BA and 0.5 μM IBA.

Rootstock	Tissue	IAA[a]		Conjugated/free
		Free	Conjugated[b]	
		ng/g FW		
M.26	Apical section	298.0 ± 4.4 280.0 ± 9.1[c]	995.9 ± 22.0	3.3
	Basal section	477.1 ± 6.5 470.8 ± 8.1[c]	910.0 ± 10.6	1.9
M.9	Apical section	263.7 ± 9.3	1346.0 ± 91.4	5.1
	Basal section	166.6 ± 7.6 162.2 ± 8.7[c]	1023.4 ± 65.4	6.2
		hsd = 35.3[d]	hsd = 282[d]	

[a] Values are means ± SE (n = 6). [b] Values represent amide plus ester conjugates. [c] IAA levels determined by GC-MS-SIM; values are means ± SE (n = 3). [d] Honestly significant difference calculated using Tukey's w-procedure (P = 0.01).

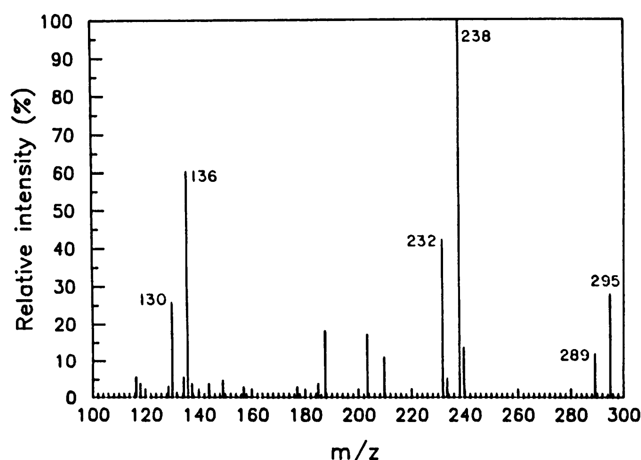

Figure 4. Mass spectra of putative IAA peak from extract of apical 1 cm of M.26 shoots grown *in vitro*. $^{13}[C_6]$IAA was added as internal standard.

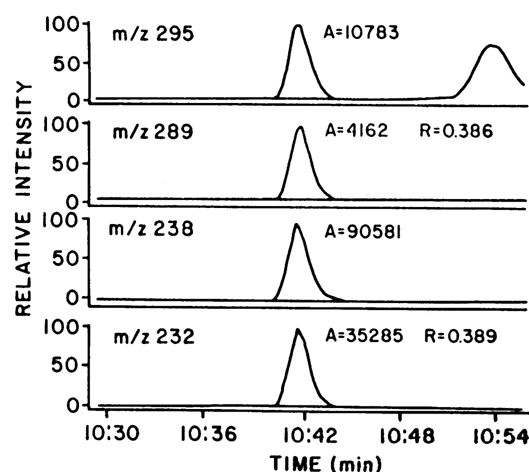

Figure 5. Selected ion chromatogram of extract from apical 1 cm of M.26 shoots grown *in vitro*. $^{13}[C_6]$IAA was added as internal standard. Ions at m/z 232 and 289 are from the base peaks, and molecular ions respectively, of the IAA-TBDMS. Ions at m/z 238 and 295 are the corresponding ions from $^{13}[C_6]$IAA-TBDMS. The chromatogram was obtained on a 30 m, DB-1 capillary column using helium as carrier gas. The mass spectrometer was a ZAB HS-2F (VG analytical).

DISCUSSION

Previous researchers have investigated the rooting performance of M.26 and M.9 shoots *in vitro* (10, 11, 14). Welander and Snygg (26) reported 7 to 8 roots/rooted shoot for M.26 at IBA concentrations ranging from 1 to 10 μM. James and Thurnbon (13) reported 3 to 7 roots/rooted shoot for M.9, which they obtained by exposing shoots for 4 d to IBA concentrations ranging from 4 to 20 μM. In our study, exogenous applications of IBA induced an average of 8 and 4 roots/rooted shoot in M.26 and M.9, respectively. Rooting percentages we obtained were comparable to previous studies. James (10) reported 65% rooting for M.9, by exposing *in vitro* shoots to 15 μM IBA for 4 d. Welander (25) reported 100% rooting for M.26 at IBA concentrations ranging from 0.5 to 5 μM.

There is little available information on IAA levels in tissue cultured apple. IAA was only recently identified as an endogenous constituent in M.26 shoots cultured *in vitro* (23). To our knowledge there have been no previous reports of IAA levels in M.9, conjugated IAA levels in M.26 or M.9, or validation of IAA levels in apple shoots grown *in vitro* using $^{13}[C_6]$IAA as internal standard.

The levels of free IAA in M.26 shoots we measured were higher than those previously reported by Welander and Snygg (26). They reported values ranging from 42 to 56 ng/g FW free IAA for M.26 shoots cultured in auxin-free medium. Since we proliferated M.26 shoots in medium containing 0.5 μM IBA, the higher free IAA levels we determined in M.26 basal sections suggest the possibility of some IBA to IAA conversion during multiplication. The conversion of applied IBA to IAA has been demonstrated in olive (*Olea europaea*) and grapevine (*Vitis vinifera*) stem cuttings (5). Free IAA levels ranging from 250 to 950 ng/g FW have been reported for tissue cultured *Sequoia sempervirens* shoots (6). Conjugated (amide + ester) IAA levels of M.26 and M.9 shoots

were comparable to levels previously reported for 5-d-old maize shoots (1) and young cotton fruits (7).

Previous studies have suggested that the number of roots produced per cutting may be a function of the auxin level in the root generation zone (2, 9). Despite the development of refined techniques for IAA quantitation, little research has been published on endogenous IAA levels in stem cuttings and the possible relationship of those levels to adventitious root formation. Weigel *et al.* (24), using enzyme-linked immunosorbent assay (ELISA), reported a positive relationship between auxin levels in stem cutting of *Chrysanthemum morifolium* at the time the cuttings were taken and the number of roots formed 20 d later. We found similar results in that M.26 had higher free IAA levels in basal sections than M.9 immediately prior to root induction, and also produced more roots than M.9 did 30 d later. Our data support Le's (16) suggestion that differences in rooting ability between M.9 and M.26 might be due to differences in free IAA levels.

James (11) has suggested that the two rootstocks differ in their rates of auxin degradation. He based his hypothesis on research in which M.9 required 10 times more IAA to produce the same number of roots as M.26 (14) and M.9 absorbed twice as much [1-^{14}C]IAA as M.26, but produced only one-third as many roots (11). Our data does not fully support his hypothesis since free IAA levels in apical sections were similar in both rootstocks. If IAA degradation were higher in M.9 one would expect lower free IAA levels throughout the entire plant.

The IAA levels observed suggest the possibility that M.9 has a reduced ability to convert IBA to IAA. However, we cannot discount the possibility that IBA might be converted into IAA at a similar rate in both rootstocks, but M.9 might conjugate a greater percentage of newly converted IAA. A higher proportion of IAA in M.9 exists as either amide or ester conjugates compared with that in M.26 (Table I). James and Thurnbon (14) have previously shown that M.9 requires 10 times more IAA than M.26 for optimum rooting, which could be due to increased conjugation of exogenously supplied IAA.

In conclusion, results from the present study suggest that differences between M.26 and M.9 *in vitro* rooting response may be related to differences in free IAA levels in basal sections. These differences in free IAA levels between M.26 and M.9 basal sections may reflect differences in IBA metabolism and/or IAA conjugation.

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