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Determination of Indole-3-acetic Acid in Douglas Fir Using a Deuterated Analog and Selected Ion Monitoring

COMPARISON OF MICROQUANTITIES IN SEEDLING AND ADULT TREE[1]

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ABSTRACT

Indole-3-acetic acid (IAA) content in shoot tips of Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) trees and seedlings was determined by combined gas chromatography-mass spectrometry using a deuterated analog (d_2-IAA) as an internal standard and the technique of selected ion monitoring. Ratios of the peak heights of the deuterated analog internal standard to endogenous IAA revealed a slightly higher content of IAA in seedlings compared with the shoot tips collected in June. The relatively high level of IAA (2.9 micrograms per gram fresh weight) in seedlings is discussed in relation to *in vitro* propagation of this species.

The induction of organogenesis in explants of Douglas fir (4) and pine (12) seedlings is in marked contrast with the low frequency of reported successes in obtaining similar results with organs excised from adult trees. The rooting response of cuttings of the juvenile phase of many coniferous species is also frequently different from that of cuttings of mature trees (6, 16). These discrepancies in performance of excised organs pose the question of what differences, if any, occur between the hormone concentrations in the mature and young phases of growth in trees. Unfortunately, plant organs used in studies of regeneration are seldom investigated for their content of growth regulators. The lack of information is particularly acute regarding the chemical background of tree species which are the source of explants used in tissue culture.

A recent analysis which used gas chromatography and mass spectrometry unequivocally demonstrated the presence of IAA in 15-year-old Douglas fir trees (5). The present study incorporates the s.i.m.[5] technique of combined GC-MS to determine the amounts of IAA in two different growth forms of Douglas fir. The specificity and accuracy of this sensitive method are enhanced by the use of a deuterated analog of IAA as an internal standard. This isotope dilution method for quantitative analysis has been used in our laboratory to determine the concentrations of polyamines in biological samples (11). A similar method was used by other workers who quantified IAA in human urine (7). This appears to be the first reported use of this method in the quantitative analysis of a plant hormone extracted from plant tissue.

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[5] Abbreviations: d_2-IAA: methylene-deuterated indole-3-acetic acid; GC-MS: gas chromatography-mass spectrometry; IAN: indole-3-acetonitrile; s.i.m.: selected ion monitoring; TFAA: trifluoroacetic anhydride.

MATERIALS AND METHODS

Synthesis of Internal Standard, Methylene d_2-IAA. A mixture of 75 mg of IAN in 0.2 ml of 40% NaOH was heated under reflux for 3 hr, or until no ammonia was detected with moist litmus paper (14). The solution was then acidified to pH 3.0 with 0.1 N HCl, and the IAA product was extracted using three portions of ethyl ether. The ether extracts were combined, dried over anhydrous magnesium sulfate, and evaporated to dryness *in vacuo*. TLC analysis of the synthesized product on a plate precoated with F-254 silica gel (EM Laboratories, Inc.) developed with methyl alcohol-ethyl acetate (2:8) with UV detection revealed both authentic IAA (Sigma Chemical Co.) and the synthetic product as fluorescence quenched spots of R_F 0.53. A trace of IAN was detected near the solvent front.

Following these preliminary experiments, synthesis of d_2-IAA was accomplished by substituting 40% NaOD in D_2O (Stohler Isotope Chemical Co.) in the hydrolysis procedure. A ^2H nuclear magnetic resonance spectrum confirmed the substitution of two deuteriums into the methylene group of the IAA product. The spectrum was obtained with a Varian HA-100 spectrometer using d_6-acetone as solvent with tetramethylsilane as the internal standard and exhibited the following resonances: δ (ppm) 9.9 (broad singlet, 1 H, N—H), 7.6 to 6.8 (complex multiplet, 5 H, aromatic Hs), 3.9 to 3.3 (multiplet, low intensity 2 H, —CH_2—). For reference, the spectrum of IAA exhibited δ (ppm) 9.9 (broad singlet, 1 H, N—H), 7.6 to 6.8 (complex multiplet, 5 H, aromatic Hs), 3.7 (singlet, 2 H, —CH_2—), and IAN exhibited δ (ppm) 8.15 (broad singlet, 1 H, N—H), 7.5-6.8 complex multiplet, 5 H, aromatic Hs), 3.5 (singlet, 2 H, —CH_2—).

From the relative abundance of ions at m/e 285, 286, and 287 in the mass spectrum of derivatized d_2-IAA (description follows) the per cent deuterium incorporation into the methylene group was found to be 95%. The ratio d_2/d_0 (m/e 287/285), was approximately 125. By base-catalyzed exchange using IAA, d_2-IAA was formed in approximately the same isotopic purity (7).

Derivatization. Standard solutions of IAA (1.0 mM) in methanol and of d_2-IAA (0.8 mM) in methanol were prepared and stored at −20 C. Aliquots of the standard solutions were placed in 1-dram glass vials and were reduced to dryness under a stream of N_2. The dried samples were methylated using ethereal diazomethane (9).

Approximately 0.5 g of Na_2CO_3 was placed in a vial containing IAA methyl ester and enough TFAA (10) was added to cover the surface of the Na_2CO_3. The Na_2CO_3 neutralized the acid generated

from TFAA, thus preventing hydrolysis of the methyl ester. The vial was sealed with Teflon-lined screw caps and was heated on a steam bath for 5 min. Chloroform (200 μl) was added to the vial followed by slow addition of 2.5 ml of saturated aqueous Na_2CO_3, while the vial was cooled in ice to slow the evolution of CO_2. The vial was again sealed and centrifuged for 5 min. The chloroform layer at the bottom of the vial was transferred by pipette to a 0.3-ml conical vial. The solvent was evaporated under a stream of N_2 and the sample was ready for analysis by GC-MS.

GC-MS. Mass spectral experiments were carried out using a Du Pont 21-491B mass spectrometer interfaced with a Varian 2700 gas chromatograph. This system is equipped with a four-channel Du Pont MSID accessory for s.i.m. analyses. Outputs from the gas chromatograph flame ionization detector and the specific ion monitors were recorded on a Gould Brush 260 six-channel recorder. The gas chromatograph was equipped with a glass column (1.83 m × 2 mm) packed with 1.5% OV-101 on 100/120 Gaschrom Q. The column was temperature-programed from 170 to 240 C at 10°/min. Using this program, both derivatized IAA and derivatized d_2-IAA had a retention time of 5.1 min. For s.i.m. experiments, ions at m/e 285 (IAA) and m/e 287 (d_2-IAA) were monitored.

Standard Curve. A standard curve (Fig. 1) prepared by varying ratios of the derivatives of d_2-IAA and IAA was constructed to correlate peak height ratios (m/e 287/285) with actual molar ratios. This is necessitated by deviations of the ratio of peak height from molar ratios, due to deuterium isotope effects in molecular fragmentation (15). Aliquots of the two standard solutions (1 mM IAA and 0.8 mM d_2-IAA) were combined to approximate solutions with molar ratios (d_2-IAA/IAA) of 1:10, 1:3, 1:1, 3:1, and 10:1. The methanol solvent was evaporated under a stream of N_2 and the dry mixture of IAA and d_2-IAA was derivatized. Aliquots of the derivatized samples were injected into the GC-MS and the peak height ratios were determined by s.i.m. Individual points on the resulting standard curve (Fig. 1) are the averages of three injections. As little as 1.3 picomol were detected with a 4.5 ratio of signal to base line noise, but samples were typically 5-fold more concentrated.

Extraction and Partial Purification. Plant pieces were weighed within 15 min from the time of collection. The weighed plant samples were stored at −20 C until the time of their extraction. Although quantity and size of the plant pieces varied slightly from one collection to another, the following data can be taken as representative for the shoots and seedlings which were used in the study.

Clippers were applied near the tips of terminal branches, and small shoots were removed from a sexually mature specimen of Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco). The tree was located on the property of the Oregon Regional Primate Research Center in Beaverton, Oregon. The tree was estimated to be at least 40 years of age, as based on the number of whorls of branches. The shoots were collected in early June of 1977, a few weeks after the first sign of bud break. The subtending older shoot system, *i.e.* that produced during the growing season of 1976, was cut away just prior to weighing. Two of the shoots which were selected for uniformity had an average length of 2.6 cm and a combined fresh weight of 1.27 g.

Seeds provided by the Weyerhaeuser Corp. were sown in a soil-Vermiculite mixture. Seedlings received one application of Hoagland solution during their early period of growth. At the time of extraction, which was 3 weeks after sowing, the seedlings were without visible foliage leaves and had cotyledons which measured 1.5 to 2.0 cm in length. A horizontal cut was made with a scalpel blade immediately below the cotyledonary node, and the hypocotyl and root were discarded. The seedling tips used in the analysis were comprised of the distal portions of the central axes, the cotyledons and any unexpanded leaves. Twenty-four seedling tips had a combined fresh weight of 0.4 g.

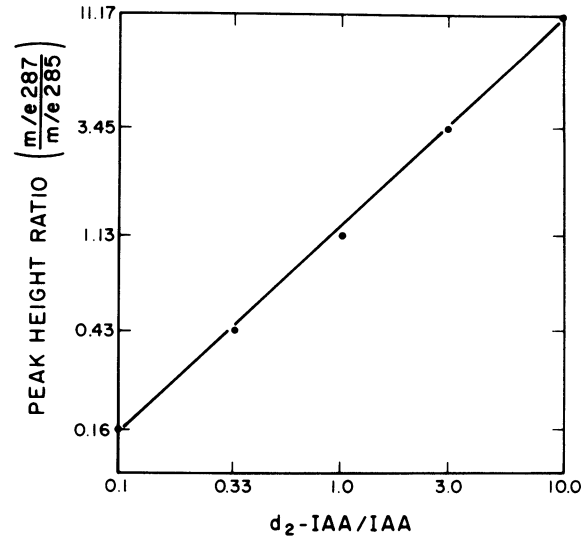

FIG. 1. Standard curve constructed to correlate varying molar ratios of d_2-IAA/IAA with heights of peaks due to ions at m/e 287 and 285 as determined by selected ion monitoring. Each point represents an average of three determinations. Sensitivity was 1.3 picomol, with 4.5 ratio of signal to base line noise.

Plant pieces were ground in cold acetone in a chilled mortar. An aliquot of the standard methanol solution of d_2-IAA was added to the homogenate. The quantity of this internal standard was comparable to the expected amount of endogenous IAA as determined from earlier experiments, some of which lacked the inclusion of the deuterated analog. In the case of the cotyledon extract, d_2-IAA was added at 5 μg/g fresh weight. The tree extract received d_2-IAA in the amount of 3.9 μg/g fresh weight. One-half of the homogenate was transferred to a centrifuge tube, sonicated in an ultrasonic glass cleaner (Cole Parmer model 8845-30) for 2 min, and subjected to 20,000*g* for 40 min at 0 C. The pellet was suspended in acetone and resonicated. Following another centrifugation, the two supernatants were combined and the acetone was removed *in vacuo*, at ambient temperature. The following method for partitioning and recovery of the extract was slightly modified from that described by DeYoe and Zaerr (5). The aqueous residue was adjusted to pH 8 with 40% NaOH, and the alkaline extract was partitioned against ethyl ether three times, discarding the ether phase. The aqueous phase was adjusted to pH 2.5 with 0.1 N HCl, and extracted four times with ether. The combined ether fractions were evaporated to dryness and the resulting residue redissolved in 3 to 4 ml of chloroform. The chloroform and any remaining water in the flask were collected by pipette and transferred to a conical centrifuge tube. Vigorous agitation of the tube was followed by slow speed centrifugation. The lower phase which contained the IAA and d_2-IAA was transferred to a conical vial. The chloroform was removed under a stream of N_2 and the sample was methylated with ethereal diazomethane. Methylated crude extract (containing the deuterated analog) and authentic methyl ester of IAA were spotted on F-254 TLC plates and developed in chloroform-ethyl acetate (2:1). The area of silica gel corresponding to the R_F (0.58) of methylated IAA in the extract was collected. Sonication of the silica gel in the presence of acetone followed by slow speed centrifugation were performed twice. The solvent was transferred to a vial and evaporated under a stream of N_2. The methylated endogenous IAA and its d_2 analog were derivatized with TFAA and analyzed by GC-MS. Steps of extraction and purification are given in Figure 2.

RESULTS

The mass spectra for derivatized IAA and d_2-IAA exhibit molecular ions at m/e 285, and 287, respectively. The most

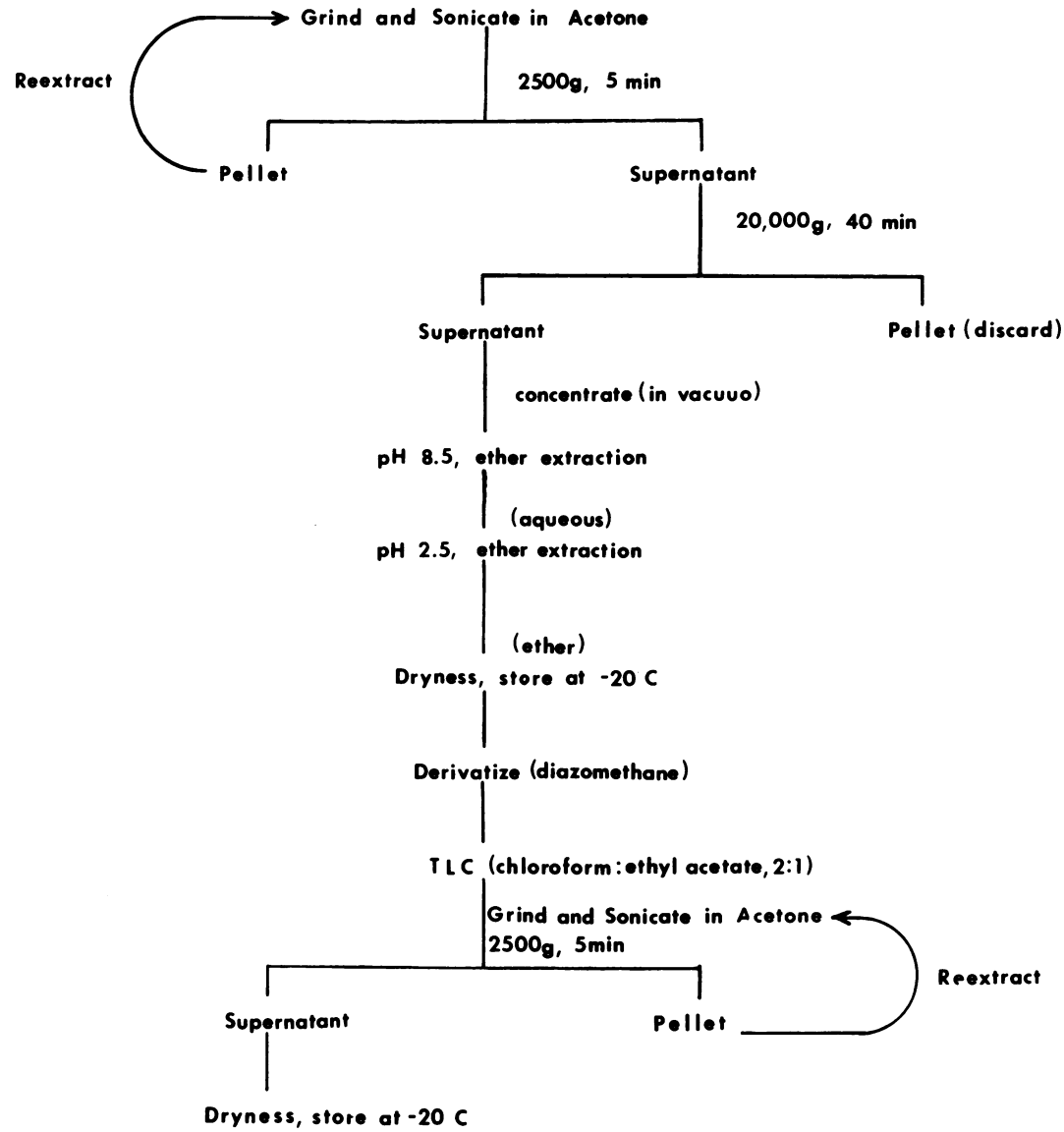

FIG. 2. Flow sheet for extraction and partial purification of endogenous IAA and deuterated analog.

abundant fragment ions appear at m/e 226 and 228, as a result of loss of the carbomethoxy group from the respective molecular ions (Fig. 3). The molecular ions were used in selected ion-monitoring experiments, since the use of higher mass ions results in less background due to contaminants.

Heights of the molecular ion peaks obtained in three separate s.i.m. experiments (Fig. 4) were averaged and these average peak heights were used in calculating the ratio of d_2-IAA to IAA in the samples. The molar ratio, obtained from the standard curve (Fig. 1), corresponds to the ratio of the added analog d_2-IAA to endogenous IAA in the homogenate at the beginning of the extraction procedure. Using this ratio and the known amount of d_2-IAA added to the extract, the amount of endogenous IAA was calculated. Since the internal standard is essentially identical in chemical and physical properties with endogenous IAA, no correction for differential losses of the two species during sample handling is required.

The average corrected ratio of d_2-IAA/IAA in the shoots collected in June was 2.45, the amount of d_2-IAA added to the methanol extract was 3.9 μg/g fresh weight, and therefore, the concentration of endogenous IAA was 1.6 μg/g fresh weight. The seedling extract to which was added d_2-IAA in the amount of 5 μg/g fresh weight, gave a corrected ratio of 1.7, and a concentration of 2.9 μg IAA/g fresh weight.

DISCUSSION

The sensitivity of this isotope dilution method using a deuterated analog and selected ion monitoring permitted analysis of IAA using as little as 200 mg of tissue, fresh weight. The concentration of IAA as determined by this method was a few times higher than that reported by DeYoe and Zaerr (5) for Douglas fir shoots. However, our shoot samples included all of the newly produced leaves. This, as well as any genetic difference in the specimens, may account for the greater amount of auxin. Of greater importance is that the work of DeYoe and Zaerr (5) and our study show the concentration of IAA in growing shoots of Douglas fir trees to be well over an order of magnitude greater than in the vegetative tissues of herbaceous species such as *Pisum* (2) or *Phaseolus* (13). This relative abundance of IAA appears only for a short duration in conifer trees, and free IAA virtually disappears during dormancy (1, 5). It would at first appear that those means of propagation which depend on auxin, such as the rooting of cuttings, have their best chance of success sometime

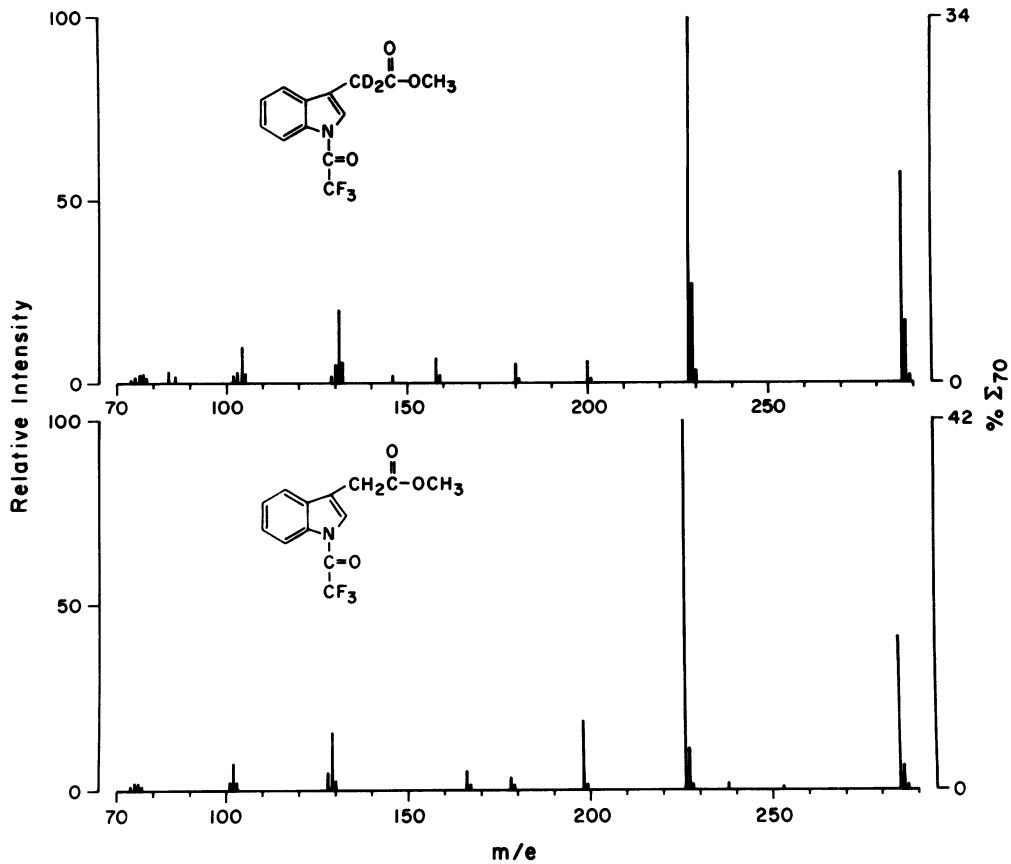

FIG. 3. Mass spectra of methyl esters of trifluoroacetylated d_2-IAA and IAA. Base ions (m/e 226 and m/e 228) result from loss of the carbomethoxy group from the molecular ions of IAA (m/e 285) and d_2-IAA (m/e 287), respectively.

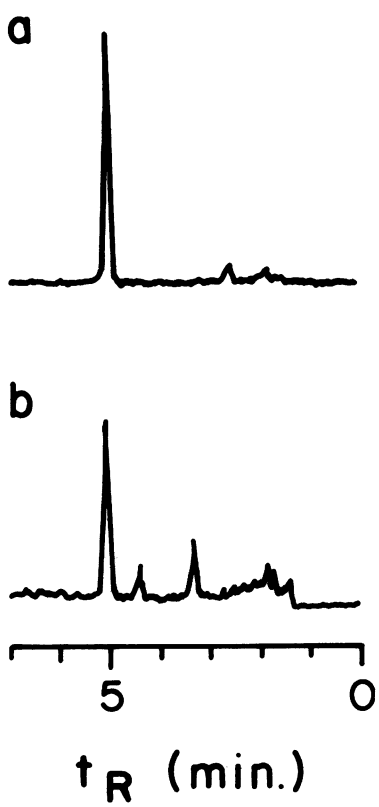

FIG. 4. Selected ion-monitoring traces of the molecular ions of *a*, added d_2-IAA (m/e 287) and *b*, endogenous IAA (m/e 285) in a sample of seedling extract.

during active growth of the shoot system. Studies of the rooting response in Douglas fir have shown instead that the application of auxin and removal of bud scales from cuttings gave no such increase in rooting ability at the time of bud break in late spring over that seen in cuttings taken in early December (8). Controlling factors in addition to auxin quite obviously influence the rooting of cuttings.

Results of the present study also showed that seedlings at about the same developmental stage as that which was found to be regenerative in culture (4) have a surprisingly high level of endogenous IAA. The necessity for adding IAA to nutrient media in order to stimulate shoot production in tissues which, when excised from the intact plant, contain a relatively high level of auxin, poses problems of interpretation for those workers who are concerned with the chemical control of morphogenesis. The spontaneous oxidation of IAA (3) and its enzymic degradation are worthy of consideration. The quantity of endogenous IAA and the fate of this important growth regulator have yet to be determined for an actively regenerating tissue system grown under conditions of sterile culture.

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