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Determination of Endogenous Indole-3-Acetic Acid in *Plagiochila arctica* (Hepaticae)[1]

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ABSTRACT

Endogenous indole-3-acetic acid (IAA) was found in axenically cultured gametophytes of the leafy liverwort, *Plagiochila arctica* Bryhn and Kaal., by high-performance liquid chromatography with electrochemical detection. Identification of the methylated auxin was confirmed by gas chromatography-mass spectrometry. Addition of 57 micromolar IAA to cultures increased relative production of ethylene. This is the first definitive (gas chromatography-mass spectrometry) demonstration of the natural occurrence of IAA in a bryophyte.

Knowledge of endogenous growth regulators in bryophytes is very limited. There have been a few reports of the natural occurrence of cytokinins (8, 20) and ethylene (5, 15, 19), and evidence for gibberellins has been reviewed recently (11). Early reports (12, 17) of auxin in liverworts were based upon bioassay of chromatographed extracts, and the conclusions were sharply criticized (18). However, Thomas *et al.* (19) tentatively identified IAA in setae of *Pellia epiphylla* sporophytes using a double-standard isotope dilution assay with nitrogen-specific detection GLC.

We now report the natural occurrence of IAA in axenically cultured gametophytes of *P. arctica*. Evidence that IAA can promote ethylene production by these plants is also included.

MATERIALS AND METHODS

Plant Material. Initiation and maintenance of axenic cultures of *Plagiochila arctica* Bryhn and Kaal. have been described (2). Newly inoculated cultures consisted of gametophyte fragments suspended in 9 ml of liquid nutrient medium (modified Knudson's macronutrients, Hutners 'Metals 49,' 40 mM Mes [pH 4.8], and 1% D-glucose; [*cf.* 1]) in 30 ml serum-stoppered Wheaton bottles vented with cotton-plugged 19-gauge needles. For determinations of endogenous IAA, 14-week-old cultures were filtered, washed with distilled H_2O, and rapidly frozen on dry ice before lyophilization on a VirTis 10-010 Automatic Freeze-Dryer. Dried gametophytes were stored *in vacuo* at room temperature until analysis. In the two experiments to determine the effects of exogenous IAA on ethylene production cultures of 6 or 10 weeks' age were used.

Determination of Endogenous IAA. Vials of dried gametophytic tissue were divided into three replicate samples and each sample was weighed. Samples were extracted by plunging into 30 ml boiling 70% aqueous methyl alcohol (containing 0.4 mg ml^{-1} BHT[2]) for 3 to 5 min, then allowed to stand for 20 min after being ground in a mortar. The extract was filtered and the residue was washed with another 30 ml of 70% methyl alcohol. 1-[^{14}C]IAA (approximately 20 nCi; 59 mCi $mmol^{-1}$; Amersham Corporation, Arlington Heights, IL) was added to correct for losses. One sample was reduced to the aqueous phase on a rotary film evaporator at 50°C and partitioned against diethyl ether (three times, equal volumes) at pH 7 and then pH 3. The acidic ether fraction was dried over Na_2SO_4 at 2°C reduced to dryness, and taken up in 3 ml of 10 mM ammonium acetate. The other two samples were reduced to 1 ml, and 3 ml of 10 mM ammonium acetate was added. From this point, all samples were handled as in Law and Hamilton (13). Briefly, partial purification was achieved by two column chromatography steps. The first column consisted of 15 ml Sephadex G-10 over-layered with 7.5 ml carboxymethyl cellulose, and with 15 ml PVP over that. The column was equilibrated and the sample eluted with 10 mM ammonium acetate. Labeled fractions (20–65 ml) were pooled and applied to a DEAE-cellulose column (20 ml; H^+ form). After washing the loaded column with 50 ml of ammonium acetate, IAA was eluted with 15 ml of 1 N acetic acid. The eluant was then applied to a C_{18} Sep-Pak cartridge, washed with 5 ml of water to remove acid, and recovered in 2 ml HPLC-grade methyl alcohol. Sources of all reagents and materials used were the same as in Law and Hamilton (13). Samples were stored at −20°C until analysis by HPLC with electrochemical detection as previously described (13). A 15 cm × 4.6 mm 5 μm spherical C_{18} column (Alltech, Deerfield, IL) was used, and the system was operated isocratically at 0.90 v applied potential with a flow rate of 1 ml min^{-1}. Two solvents were employed in analyses; one consisted of 20% methyl alcohol:80% PAE buffer (50 mM $NaClO_4$, 5 mM sodium acetate, 1 mM EDTA, pH 5) and the second was composed of 39% methyl alcohol:61% PAE buffer pH 4.5. Retention times for IAA were 13.2 and 6.8 min, respectively. Twenty-five to 75-μl samples were injected, and amounts of IAA were estimated by comparisons of sample peak areas with those from known amounts of authentic IAA. Sample IAA was collected upon elution for liquid scintillation counting and calculation of endogenous IAA levels by the fractional recovery formula (13).

Confirmation of IAA Identity. In addition to co-chromatography with authentic IAA in two solvents, two procedures were employed to confirm electrochemical peak homogeneity and to

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[2] Abbreviations: BHT, butylated hydroxytoluene; MeIAA, methyl ester of IAA; Hyp-protein, hydroxyproline-rich protein.

tentatively confirm identity. One sample was methylated with diazomethane (16), and co-chromatography with MeIAA standard was confirmed (R_t = 18.8 min in the 39% methyl alcohol solvent). Progress of the methylation reaction was monitored by collecting IAA and MeIAA for counting as they eluted from the HPLC detector and calculating amounts of free and derivatized IAA. In a second procedure, the potential applied across the electrochemical cell was altered, and ratios of sample peak areas at several potentials were compared with area ratios of standard IAA.

To obtain a mass spectrum of the putative IAA, 920 mg of dried gametophytic tissue was extracted, partitioned against diethyl ether, and purified as above. The sample (in 2 ml methyl alcohol) was methylated and then evaporated in a small glass vial under N_2. The sample was taken up in a small volume of acetonitrile and injected into a LKB 9000A GC-MS with a 2.75 m × 2 mm column of 3% SP-2250 on 80/100 Supelcoport (Supelco, Bellefonte, PA), and electron impact ionization at 70 ev, ion source of 250°C, He carrier gas flow of 16 ml min^{-1}, and a temperature program of 5 min at 150°C, increasing 10°C min^{-1} thereafter to 240°C. MeIAA eluted at 16 min.

Ethylene Determinations. Since serum-stoppered culture vessels were vented with cotton-plugged needles, gas samples were easily removed from the air space over the culture medium after flaming the needles of sampling syringes. In each experiment, 1 ml of filter-sterilized IAA solution was added to each of five replicate bottles so as to give a working concentration of 57 μM. One ml of nutrient medium was injected into control cultures. Immediately after addition of IAA or medium, the gases in the air space (about 20 ml) were exchanged with room air by withdrawing the plunger of an inserted 50-ml syringe twice.

Gas samples (1 ml) were withdrawn just after treatment and 24 h later. Ethylene analyses were performed on a Varian 3700 GLC equipped with a flame ionization detector, using a Carbosieve B 60/80 mesh stainless steel column (Supelco). Conditions employed were: temperature settings of 160°C for the injector and 220°C for the detector; gas flow rates (ml min^{-1}) of 30 (N_2, H_2) and 300 (air). Ethylene produced by the tissue was identified on the basis of retention time and co-chromatography with authentic ethylene. Relative production was calculated on the basis of peak heights recorded on a Varian 9176 strip chart recorder.

RESULTS

IAA in the gametophytic tissue was identified by electrochemical detection, and identity was confirmed by GC-MS (Fig. 1).

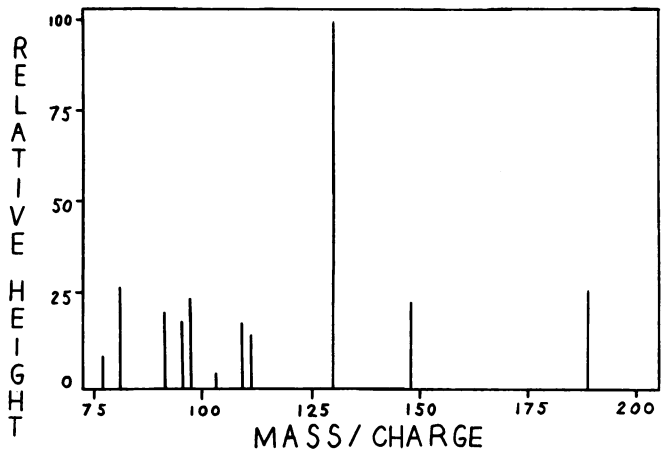

FIG. 1. Mass spectrum of methylated IAA isolated from *P. arctica* gametophyte cultures. Conditions are described in text. Spectral lines typical of methylated IAA are (mass/charge) 189, 130, 103, and 77.

Quantitation was performed by the fractional recovery formula (13). Estimates of endogenous IAA in each of three tissue samples are presented in Table I. Each estimate is based on a minimum of three injections.

Calculation of IAA content after methylation (Table II) shows that the putative IAA peak was homogenous before methylation, and that the reaction with diazomethane approached completion. The electrochemical detector has different sensitivities toward IAA and MeIAA and thus injections of standards of both are required.

Alteration of the potential applied across the electrochemical cell changes the relative oxidation of standards and peak components and hence electrochemical peak areas also change. Different compounds respond in various ways to each applied potential. Injections of authentic IAA were made at our usual operating potential of 0.90 v and at four other potentials. The ratios of mean peak areas at each of the latter potentials to the mean area at 0.90 v were calculated. Sample injections were made at these potentials, and area ratios calculated in the same manner for putative IAA and two unidentified components (Table III). If the only electrochemically detected component of the putative IAA peak is, in fact, IAA, then sample area ratios should be within experimental error of authentic IAA ratios. Electrochemical contaminants in the peak, which would cause an overestimation of IAA content, most probably would alter the area ratios from those observed with standard IAA. The oxidative behaviors of the two unidentified contaminants illustrate the different response.

Addition of IAA to gametophyte cultures substantially increased ethylene production by the tissue relative to cultures without exogenous auxin. In the first experiment, ethylene levels in the culture flasks were increased an average of 2.8-fold in the presence of IAA compared to controls. In the second experiment, IAA induced a 1.9-fold increase in ethylene production over 24 hr. Analysis of variance of the relative ethylene production indicated highly significant differences between treated and untreated cultures ($P < 0.002$ and $P < 0.02$ for the first and second experiments, respectively).

DISCUSSION

This paper presents the first determination of endogenous IAA in a bryophyte with definitive (GC-MS) identification. The estimated levels we report, assuming a 1:10 approximate conversion of dry weight to fresh weight, are well within the range of levels reported in higher plant studies (7), especially in consideration of the fact that many of these studies analyzed auxin-rich tissues or organs rather than whole plants. Work is is progress to verify the quantitation procedure employed in this paper by comparison with GC-MS determination of samples with tetradeuterated IAA. All these levels are far below those estimated for free IAA in rapidly elongating *Pellia* setae (19). Our study differs from that of Thomas *et al.* (19) in some important respects. We used axenic cultures of gametophytes, while the *Pellia* study focused on rapidly growing sporophytic tissue collected from the field under nonsterile conditions.

Several comments might be made about the method used for determination of endogenous IAA in this paper. The sensitivity (to 1 pmol IAA per injection) and selectivity of electrochemical detection, coupled with a relatively high recovery, allow determinations with very small amounts of tissue. Ether partitioning prior to column chromatography substantially decreases recovery, but reduces electrochemically detected contamination. Though not necessary for IAA determination in *P. arctica*, partitioning may be useful with other tissues. We have observed that interference from contaminants, occasionally encountered in analyses by this method, can be eliminated by slight alterations of HPLC solvent pH or methyl alcohol content, or by alteration

Table I. *Calculated Endogenous IAA Levels in P. arctica Gametophytes*

Three samples of cultured gametophytic tissue were extracted, purified, and analyzed as described in the text. Sample A was ether-partitioned during purification.

Sample	Dry Wt	[^{14}C]IAA Added	[^{14}C]IAA Recovered	Mean Sample[a]	Endogenous IAA
	mg	*pmol*	*%*	*pmol*	*pmol/g*
A	399.4	290.1	37.7	265.2	664
B	203.5	303.9	66.0	150.0	737
C	346.0	300.2	68.2	225.1	651

[a] Based on at least three injections.

Table II. *Methylation of P. arctica IAA Sample*

To test the tentative identity of a R_t IAA sample peak, the sample was methylated with diazomethane and analyzed by HPLC-electrochemical detection with 39% methyl alcohol:61% PAE buffer (pH 4.5). Analysis of peaks co-chromatographing with IAA and MeIAA (R_t = 6.4 and 18.8 min, respectively) is presented. The methylation reaction was 95.5% complete.

Peak, R_t=	Area	Equivalent	^{14}C Recovered		Specific Activity
	units^2	*pmol*[a]	*pCi*	*pmol*	*pCi/pmol*
IAA	3.9	0.7	11.7	0.2	16.7
MeIAA	19.8	14.9	248	4.2	16.6

[a] Based on detector responses of 5.5 units^2 pmol^{-1} IAA and 1.33 units^2 pmol^{-1} MeIAA at 0.84 v applied potential.

Table III. *Comparison of Relative Detector Responses with Alteration of Applied Potential*

Injections of standard IAA (24 pmol) and IAA from sample A (70 μl) were made with an applied potential of 0.90 v across the electrochemical detector and mean peak areas as (chart units)^2 calculated. Peak areas were also calculated for two unknown sample components. Then areas of standard and sample injection peaks at other applied potentials were determined, and the ratios (R) of areas at × v ÷ areas at 0.90 v were calculated for each component.

Applied Potential	Area			
	Standard IAA	Sample IAA	First Unknown	Second Unknown
v	*units*^{2} *(R)*			
0.90	152 (1.00)	55 (1.00)	10 (1.00)	41 (1.00)
0.95	182 (1.20)	68 (1.24)	21 (2.10)	18 (0.44)
0.80	112 (0.74)	41 (0.75)	15 (1.50)	12.5 (0.30)
0.75	84 (0.55)	30 (0.55)	15 (1.50)	13 (0.32)

of the applied potential. The method has been employed successfully for IAA determinations in *Pisum sativum* epicotyls (14), *Caulerpa paspaloides* (a coenocytic green alga) (W.P. Jacobs, K.F. Falkenstein, and R.H. Hamilton, personal communication, 1984), *Gossypium hirsutum* callus cultures (10), and *Avena sativa* coleoptiles, *Helianthus annuus* epicotyls, and *P. sativum* seeds (Law, unpublished results). Hence, this method has considerable potential for application to studies in which endogenous IAA levels are of interest.

No attempt was made to determine levels of conjugated IAA in *P. arctica* gametophytes. We have reported (13) an HPLC-electrochemical detection procedure to determine endogenous indoleacetylaspartic acid, an important conjugate found in some higher plants (9, 13). We did not detect this conjugate in *P. arctica* tissue. Zenk (21) applied large amounts of IAA to eleven bryophyte species, and none of these formed indoleacetylaspartic acid, though eight formed conjugates which he identified as IAA-glucose. No conjugated IAA was detected in elongated *Pellia* setae (19).

There have been other reports of ethylene production by bryophytes (5, 15, 19), but the findings that *P. arctica* produces auxin and ethylene, and that auxin induces ethylene production in this species, take on added significance in view of the Suppression Theory (2, 5). According to the Suppression Theory, auxin-induced ethylene may trigger deposition of Hyp-protein(s) in cell walls of specific merophytes and thus inhibit leaf development. Partial to complete suppression of development of leaf primordia from ventral merophytes seems to be a recurrent phenomenon among families and genera of leafy liverworts (3). Rather than an evolutionary loss or reduction of these primordia having occurred, a suppression of development is involved. Studies with inhibitors and antagonists (2, 4, 6) have implicated auxin, ethylene, and Hyp-protein in primordial suppression. Evidence that ethylene and Hyp-protein are endogenous to *P. arctica* has been reported (2, 5). Thus, while not proving the Suppression Theory, these results complete the demonstration of the natural occurrence of the postulated regulatory components in *P. arctica* gametophytes. Since these components are endogenous to sporophytes of higher plants, and can influence cell division and enlargement in these, a common mechanism for suppression of cell growth may be found in all divisions of land plants.

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