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Nature and Amount of Auxin in Algae[1]

IAA FROM EXTRACTS OF *CAULERPA PASPALOIDES* (SIPHONALES)

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ABSTRACT

Evidence for the occurrence of indole 3-acetic acid in *Caulerpa paspaloides* extracts was obtained by bioassay, by high-performance liquid chromatography with an electrochemical detector, and by capillary gas chromatography combined with mass spectrometry. The amount of indole 3-acetic acid present was estimated to be about 1 milligram per kilogram fresh weight, with an error limit of one order of magnitude. This is in the range reported from angiosperms.

The giant coenocyte *Caulerpa prolifera* responds to inversion with respect to gravity by changing the location at which rhizoids form on the rhizome: the next rhizoids to differentiate after the rhizome is rotated 180° are on the new underside of the rhizome (20). On the basis of earlier experiments on *Bryopsis*, a related member of the *Siphonales*, it is reasonable to guess that auxin mediates this gravity effect. Not only did externally applied IAA induce rhizoids in *Bryopsis* (18), but extracts of untreated *Bryopsis* showed IAA-like activity in a bioassay, with more such activity from the basal half, which normally forms rhizoids (8). Added IAA stimulated rhizoid formation in *Chara* also (30).

Although Augier's review (3) lists many reports in which IAA-like activity was found in algal extracts by bioassays, the amounts calculated as IAA were usually very low compared to amounts reported for higher plants. Chemical identifications of the compounds responsible for the bioassay activity in algae have been unusually few. Only one group has identified IAA in algal extracts by MS (1) (in extracts of the brown alga *Undaria*). Phenylacetic acid (a compound with weak auxin activity in such higher plant bioassays as the *Avena* curvature or *Pisum* split-stem tests) was also identified in the extracts (2). Fries and Aberg (14) also used MS to identify phenylacetic acid in extracts of the green alga *Enteromorpha*. Fries showed that exogenous phenylacetic acid stimulated the development of *Fucus* grown in aseptic culture (13). Several other recent papers have cast doubt on the presence of IAA as an endogenous auxin of algae. Buggeln and Craigie (7) looked for 'bound IAA' in 10 species of algae, but found no evidence for it in their extracts on the basis of TLC and the Ehrlich color test. Dawes (9) used both the *Avena* coleoptile section bioassay and the indole-α-pyrone fluorescence test to look for IAA in *C. prolifera*, but also obtained negative results. Buggeln's suspicion that auxins are not endogenous regulators of algae was strengthened by his finding (6) that synthetic auxins did not induce any clear extra elongation compared with non-auxin analogues when tested on growth of *Alaria*.

These publications left us unconvinced about the absence of IAA in algae. Most of these earlier studies did not calculate loss of IAA during the extraction process—a loss that our earlier experience had shown us could be discouragingly large even with generally used procedures (15, 29). Furthermore, by discarding the initial boiling methanol extracts, Buggeln and Craigie probably threw away any free IAA in their algae.

Accordingly, we decided it would be worthwhile to reinvestigate *Caulerpa* for the presence of IAA, using improved procedures (17), including use of ^{14}C-labeled IAA as an internal standard to calculate losses of IAA in the steps of fractionation. In addition, we planned to follow the ^{14}C in the various fractions on the chance that IAA was being modified to a compound that would not appear as IAA in the tests used by earlier researchers or in the fractionations that we planned. Because so many investigators of auxin in algae have reported estimated 'IAA' concentrations much lower than those reported in angiosperms, we used a higher weight of starting material than they usually did, on the chance that the low estimates had described a real difference from angiosperms.

MATERIALS AND METHODS

Plant Material. *Caulerpa paspaloides* (Bory) Greville and *Caulerpa prolifera* (Forsskal) Lamouroux were collected off the Florida Keys and air-mailed in seawater to Princeton. There the algae were immediately placed in seawater, cleaned of macroscopic epiphytes, blotted dry, and frozen on dry ice for subsequent lyophilization. Different batches of the lyophilized material were extracted and analyzed differently by each of three coauthors, as indicated by their initials below.

Aqueous Acetone Extraction for Bioassay (K.F.). The improved procedures of extraction and fractionation worked out by Iino *et al.* (17, Fig. 4) were followed, with changes mostly resulting from the larger weights of algal tissue extracted. All organic solvents were glass-distilled (from Burdick and Jackson), and all glassware was acid-washed before use. A preliminary experiment used 111 g lyophilized *C. paspaloides* (equivalent to 1.05 kg fresh weight), and the main extraction used 392 g lyophilized *C. paspaloides* (initially 3.34 kg fresh weight). For the latter, extraction was with 3 L of cold 80% acetone, containing 300 mg butylated hydroxytoluene as antioxidant. After blending small batches for 4 min or less each, we added to the combined batches 1.02×10^6 dpm of [1-^{14}C]IAA in 12.5 μl of 80% acetone as an internal standard. (The location and amount of the radioactivity was monitored at each stage of further treatment by taking 10- to 50-μl aliquots for counting in a liquid

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scintillation counter.) The material was shaken for 4 h in a dark coldroom. The tissue was then filtered by suction through Whatman No. 542 filter paper, washed with 80% acetone, then re-extracted overnight, and filtered as before. After reduction in volume on a rotary evaporator at 35°C, the extract was again filtered under suction and the pH was adjusted to 6.8 with 2 M K_2HPO_4 before applying it to a PVP column (37 g in a 13.5-cm high column). The column was eluted with 0.1 M K_2HPO_4 (pH 8.0), the fractions with most radioactivity being saved. The rest of the procedure was the same as that described in the flow chart of Iino *et al.* (*e.g.* adjusting to pH 3.0, partitioning with cold water-washed ether), except that more Na_2SO_4 (42 g) was needed in the first column to accommodate the larger sample. After developing the TLC, successive zones A to E (see Table I) were separated on the basis of their differing colors under UV. Fraction B, which largely overlapped the IAA marker spots, was scraped off the TLC and run through the Na_2SO_4 column, as Iino *et al.* (17) describe, and dried. All fractions were then dissolved in ethanol before evaporating to dryness to measure auxin activity with the standard coleoptile-section test (22) (instead of dissolving in methanol for the fluorimetric test used by Iino *et al.*).

Avena sativa (cv Victory) was the bioassay plant. Ten 6-mm coleoptile sections in 3 ml of 2% sucrose in phosphate buffer were used in each Petri dish. Dilution series of both calibration IAA and the unknowns were prepared. Length was measured after 22 h in the dark at 25°C. Estimates of IAA present, as read from the standard curves, were corrected for [^{14}C]IAA present, as measured by liquid scintillation counting of aliquots.

The medium, after use in the bioassay and after removal of the coleoptile sections, was frozen; one of the aliquots which had shown high auxin activity was then analyzed by HPLC (as described below) to see if any residual IAA was still present.

Radioisotope counting was done by adding 10- to 50-μl aliquots to glass vials containing 10 ml of scintillation fluid composed of 2,5-diphenyloxazole; 1,4-bis(5-phenyloxazol-2-yl)benzine; Triton X-100; and toluene. Channel ratios with an external standard were used for quench corrections.

Aqueous Methanol Extraction of Small Weights of Tissue for HPLC with an Electrochemical Detector (R.H.H.). Conditions for the HPLC were similar to those used by Law and Hamilton (23). An Alltech C18 reverse-phase column (25 cm × 4.6 mm) was used, with a mobile phase of 20% methanol and buffer (0.1 M sodium perchlorate, 0.01 M acetic acid, and 0.001 M EDTA) at pH 5, and a flow rate of 1.1 ml/min. The electrochemical detector (LC-2A with glassy carbon electrode TL-5, from Bioanalytical Systems, West Lafayette, IN) on the Glenco isocratic unit was operated at 0.95 v, which gave a clear response to 1 ng IAA.

One-half g of lyophilized *C. paspaloides* or *C. prolifera* was boiled 3 min in 70% methanol containing 20 mg butylated hydroxytoluene. The tissue was ground in a mortar with sand in additional 70% methanol. After filtration and washing, [1-^{14}C] IAA was added as an internal standard. The combined methanol extract (120 ml) was concentrated to about 5 to 10 ml on a rotary evaporator at 50°C, then adjusted to pH 8.0 with 0.1 M K_2HPO_4. After two extractions with ethyl ether, the aqueous fraction was adjusted to pH 3.0 (H_3PO_4, 3 N), then extracted four times with ethyl ether. The ether was dried at 4°C over anhydrous sodium sulfate and then evaporated in the rotary evaporator. The residue was dissolved in 4 ml of 20% methanol (in 0.01 M ammonium acetate) and then subjected to the small column clean-up method of Law and Hamilton (23). The sample eluted from the DEAE-cellulose column was divided in half, acidified with a few drops of 6 N HCl, and then eluted from a C18 SEP-PAK cartridge with one ml methanol for each half sample.

Aqueous Methanol Extraction for GC-MS Analysis (W.P.J.). An amount of *C. paspaloides* weighing 1.73 kg fresh weight was lyophilized. The 209 g of material resulting were extracted with 2.74 L of 80% methanol, filtered under suction, re-extracted with 80% methanol, then filtered and extracted a third time. The extract was reduced in volume on a rotary evaporator under vacuum at 38°C and subsequently stored in a deep freeze. The aqueous extract was partitioned three times against petroleum ether (boiling point, 30–60°C) to remove pigment. The pH of the aqueous extract was adjusted to 8.00 with 0.1 N NaOH prior to three partitionings against ethyl acetate. After the aqueous extract was adjusted to pH 3.00 with 0.1 N HCl, it was partitioned three times against ethyl acetate. This acidic ethyl acetate fraction was reduced in volume under vacuum on a rotary evaporator, then lyophilized, taken up in 10 ml methanol, evaporated to dryness and, after being placed in a desiccator overnight, found to weigh 0.820 g.

This procedure was intended primarily to look for gibberellins in *Caulerpa*: before cleaning up the fraction further with HPLC, an aliquot was tested for identifiable substances (which might be lost during HPLC treatment). Accordingly, a small fraction (3.1 mg) was methylated twice with diazomethane, and 3.1 μg of the methylated fraction was silylated with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide for capillary GC-MS.

The gas chromatography was carried out on a Dani model 3800 with a flame ionization detector. The wall-coated, open tubular column was of fused silica, 25 m × 0.2 mm, using 0.25

Table I. *Bioassay Estimate of IAA in Caulerpa paspaloides*

The *Avena* coleoptile section assay was used to estimate IAA activity in TLC zones of a purified acetone extract of 392 g freeze-dried *Caulerpa* (fresh weight originally 3.34 kg). Radioactively labeled IAA was added to the original extract as an internal standard and marker.

TLC Zone	RF				
	A 0–0.28	B[a] 0.28–0.50	C 0.50–0.51	D 0.51–0.58	E 0.58–1.0
dpm from [1-^{14}C]IAA as internal standard	16,250	322,249	22,920	5,020	4,354
IAA activity from bioassay (μg)	*ca.* 50	3,312	*ca.* 50	Not assayed	Not assayed
dpm expressed as μg [^{14}C]IAA	0.0222	0.441	0.0314		
IAA equivalent in fraction minus the contribution of the [^{14}C]IAA (mg)		3.31			

[a] Unlabeled IAA, run on side channels of the TLC as markers, ran to RF 0.25 to 0.41.

μm OV-1 as the stationary phase. Helium was the carrier gas. Temperature was programmed to start at 150°C, rising to 300°C at the rate of 3°/min. The solvent was methylene chloride:silylating agent (10:1, v/v). Electron-impact MS was performed on a computerized VG model 7050 machine, using 24 electron volts, and 220°C as source temperature. A series of *n*-alkanes was added as retention standards (as Kováts recommended, 21). Scan rate was 0.7 s/decade. Approximately 1600 full scans were made during the 45 min of GC output. The distribution graph of m/z 202, an expected base ion for indoles, revealed a major peak in scan 416 at 10:22 retention time. The full scan, with preceding and following scans subtracted as background, was then plotted and compared with the MS of authentic IAA similarly methylated and silylated.

HPLC was used for further purification. A 208-mg aliquot of the original acid-ethyl acetate fraction was dissolved in 50% methanol and filtered through a Millipore syringe filter, and then 500 μl (equivalent to 51.9 mg) were injected into a Laboratory Data Control HPLC. The preparative, C18 reverse-phase column (Technicol), used only for plant extracts, had just been freshly regenerated and washed overnight with methanol. After 7 min of isocratic 30% methanol, the gradient started and rose to 100% methanol in 25 min with a straight-line setting on the gradient-controller. Flow-rate was 2.5 ml/min, and 1 min collections were used throughout. After removing 0.25 ml from each vial (for gibberellin bioassays, to be reported on later), the remaining contents of several pairs of vials were pooled, evaporated in the hood, then methylated and silylated for capillary GC-MS, which was run as described above.

RESULTS AND DISCUSSION

Three different extraction procedures and detection methods were used, and, taken together, they provide strong evidence that IAA was present in *Caulerpa*.

Aqueous Acetone and Bioassay. The preliminary experiment gave results similar to the main one, although our literature-inspired expectation of low levels of endogenous IAA prevented us from preparing a sufficient number of dilutions of the algal extracts to reach the linear part of the bioassay response curve. Dilutions for the main experiment were adjusted accordingly, and only those results are given in detail.

At the end of the extensive processing, we recovered 32% of the dpm in the original internal standard from fraction B, which included most of the zone typical of IAA (Table I). (No sizeable amount of radioactivity was found in other fractions.) Auxin activity, as measured by the *Avena* section assay of eluates from various zones of the TLC, and after correction for the miniscule contributions of ^{14}C in each zone (if the ^{14}C were assumed to be still with IAA), paralleled the activity indicated by radioisotope counting. The zones providing the 32% of the original ^{14}C standard also provided endogenous auxin activity equivalent to 3.3 mg IAA (Table I). Corrected for the 32% recovery, this would represent 3.1 mg IAA/kg fresh weight of *C. paspaloides*.

A control TLC, with [^{14}C]IAA run in the side-channels and dpm determined in the center, confirmed the interpretation that the IAA found in the center zone of the algal-extract TLC was not the result of transfer from the marker IAA run in the side channels.

HPLC. Medium that had showed IAA-like activity in the bioassay was tested for residual IAA by HPLC. A 44-μl aliquot of one of the active dilutions was injected. Only one peak appeared, and that was at the time expected for IAA (judging by calibrations made with IAA stock immediately before and after). Injection of 4 μl stock IAA (which was estimated to give the same response as did the 44 μl of unknown) added to 40 μl of the same dilution from the algal material gave a single peak at the same time as did the unknown alone and twice its height.

As an independent confirmation of the presence of IAA in *Caulerpa* extracts, the small-scale clean-up method (23) was used after ether partitioning, using 0.5 g duplicate samples. The results from HPLC with the electrochemical detector (Table II) again confirm the presence of IAA in *C. paspaloides*. After correction for the small contribution from the [^{14}C]IAA used as an internal standard and for the percent recovery (of approximately 40%), the average amount of endogenous IAA was calculated to be 6.25 μg/g dry weight. This is equivalent to 0.69 mg IAA/kg fresh weight. As additional evidence that the peak was due to IAA, half the sample in Experiment 2 of Table II was methylated with diazomethane and rerun with 60% methanol-buffer at pH 4.0. It was found to coelute with authentic IAA-methyl ester. Furthermore, when the presumptive IAA from *C. paspaloides* was run at two different voltages (0.954 and 0.894), the ratio of peak-areas was the same as that for authentic IAA (0.51).

A single determination of IAA in a *C. prolifera* extract, using 0.95 v on the detector, led to an estimate of 0.090 mg IAA/kg fresh weight.

GC-MS. Evidence that IAA was in an aqueous methanol extract of *C. paspaloides* came from the occurrence at the expected time (10 min) of a mass spectrum like that of methylated and silylated IAA when the acidic ethyl acetate fraction from the alga was similarly derivatized and run in the capillary GC-MS. The sample MS showed base ion of m/z 202. The molecular ion (M^+) 261 and the ion at m/z 73 were both at approximately 40% of the intensity of the base ion. Much smaller ions were at m/z 130 and 151. The retention time and the MS were confirmed from the MS library of the laboratory as those expected from authentic IAA after such methylation and silylation. Purification by HPLC of an aliquot of the acid-ethylacetate fraction, followed by methylation and silylation of the samples as before, and injection of aliquots along with the *n*-alkane series (as retention standards) into capillary GC-MS, provided even clearer evidence that IAA was in the extract. Figure 1 shows the total ion current from the MS of one such sample. (All peaks from scan 765 on are due to the *n*-alkane standards, as are those in scans 173, 256, 350, 450, 555, and 660.) The strong ion-current peak in scan 402 (arrow in Fig. 1) had the full mass spectrum shown in Figure 2. The spectrum, and retention time of authentic IAA, similarly tested, confirmed the identification of scan 402 as being that of methylated and silylated IAA (Fig. 3). (The ions at 86 and 151 in the spectrum of scan 402 are presumably from incomplete background subtraction.)

Based on three different extraction procedures and three different detection methods, our results provide strong evidence that IAA is present in *Caulerpa*. When corrections are made for the losses incurred in the processing of extracts, the concentrations of IAA in *Caulerpa* are estimated to be about 1 mg/kg fresh weight with an error limit of ± one order of magnitude. This is the range that others have reported for angiosperms. Some recent estimates of IAA amounts in angiosperms, based on improved methods, include 1.7 mg IAA/kg in rice seeds (4), 0.72 mg/kg fresh weight in Douglas Fir after correction for the 95% loss (11), and 0.19 mg IAA/kg fresh weight from young tobacco leaves (27). Although *Caulerpa* may be an exception among algae in containing such high levels, we consider it more likely that the negative results in the other publications on algae noted in "Introduction" were due to such factors as less thorough fractionation, lack of calibration of losses, smaller initial weight of algal material, and not having had the benefit of recent improvements in technique (17, 23).

Did the IAA found in the extracts come from *Caulerpa* or from bacteria? This is an obvious question, particularly in the light of research from Libbert's laboratory showing that the bacteria on angiosperms or on algae can greatly increase the amount of auxin extractable (24, 28). Thimann and Grochowska

Table II. *Estimates of IAA in Caulerpa paspaloides using HPLC with an Electrochemical Detector*

In each experiment, a 0.5-g freeze-dried sample was analyzed and the final sample volume was 2 ml. [1-^{14}C] IAA was added as an internal standard to the initial extract (22,680 dpm in the first experiment, and 55,638 dpm in experiment 2). The [^{14}C]IAA had a specific activity of 748 dpm/ng.

Injected Vol	IAA in Peak	Radioactivity in Peak	[^{14}C]IAA in Peak	[^{14}C]IAA Recovered	Sample	IAA Content
	ng	*dpm*	*ng*	%	*ng*[a]	*μg/g dry wt*
Experiment 1						
45 μl	24.9	201	0.27	39.3	62.7	5.57
45 μl	26.2	213	0.29	41.7	62.1	5.52
Experiment 2						
10 μl	6.8	116	0.16	41.6	16.0	6.40
25 μl	19.5	284	0.38	40.8	46.9	7.50
Av. of 4 determinations				40.8		6.25

[a] Sample ng $= \frac{\text{(ng in peak} - \text{ng [}^{14}\text{C]IAA in peak)}}{\text{\% recovery of [}^{14}\text{C]IAA} \times 10^{-2}}$

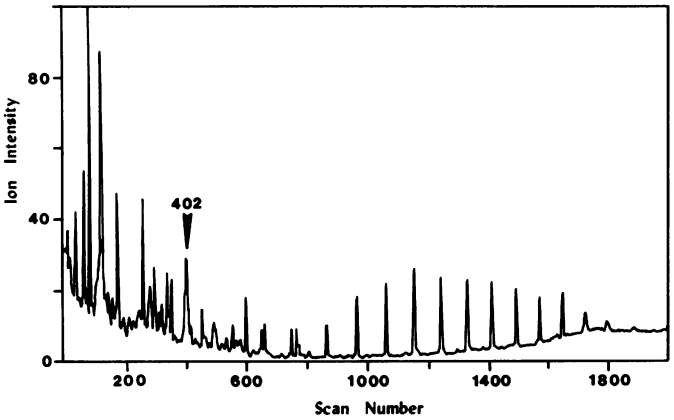

FIG. 1. Total ion current trace of all MS scans of a sample from the acid-ethyl acetate fraction of an aqueous methanol extract after it had been further purified by HPLC, then methylated and silylated for capillary GC-MS. A series of *n*-alkanes was added to the sample as retention standards. Scan 402 (arrow) had m/z 202, characteristic ion from indoles, as a major component.

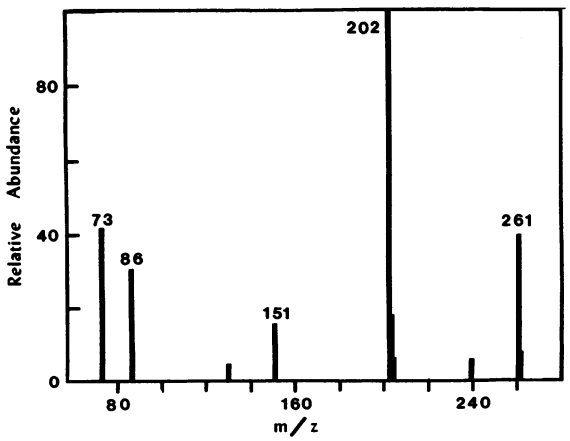

FIG. 2. Mass spectrum of scan 402 from the sample in Figure 1, after subtraction of scan 398 as background.

(31) have confirmed the angiosperm results. (The MS identifications of IAA and phenylacetic acid mentioned in "Introduction" were not from axenic cultures.) However, several authors have reported that bacteria are present inside *Caulerpa* (10, 12).

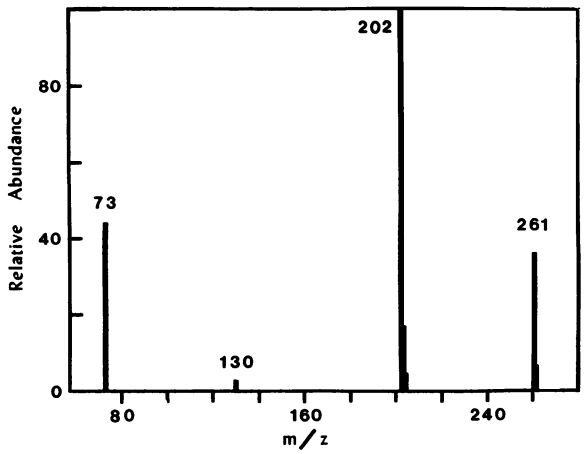

FIG. 3. Mass spectrum of authentic IAA-methyl-trimethylsilyl after capillary GC-MS as for the sample in Figures 1 and 2.

Under these circumstances, the most reasonable conclusion seems to be that IAA is available to *Caulerpa*, whether it is synthesized by the alga itself, or by its associated bacteria, or by both.

The doubts expressed by Buggeln (6) about IAA as an endogenous regulator of algal growth were based on tests of the effects of five synthetic auxins and their analogues on the elongation of blade-pieces from the brown alga *Alaria*. The auxins caused no more elongation than did the analogues, the latter known to show little auxin activity in angiosperms. However, Dawes (9) had already reported that indole-butyric and indole-propionic acids caused no growth stimulation in *C. prolifera* at concentrations which stimulated growth when IAA was added. Similar results were obtained with *Codium*, a genus also from the Siphonales, grown in unialgal culture and tested with IAA and four synthetic auxins (16). Such activity of IAA and absence of stimulatory activity by various synthetic auxins is in striking contrast with the situation in angiosperms, as Hanisak pointed out, and suggests that there are interesting differences in the metabolic control of IAA levels between algae and angiosperms. The activity of exogenous phenylacetic acid on *Caulerpa* has not been examined and its occurrence in *Caulerpa* remains to be documented.

The role of IAA in *Caulerpa* seems to be that of a growth regulator. IAA increased the production of new assimilators in both *C. sertularioides* (26) and *C. prolifera* (9) growing in plain seawater. The same IAA concentration that stimulated assimi-

lator production inhibited rhizome elongation in *C. sertularioides*. Such a differential effect of IAA on the various organs of *Caulerpa* provides an intriguing parallel with the complementary effects of GA_3, which had no effect on the production or elongation of blades but stimulated rhizome growth and rhizoid production (20).

Results so far suggest that IAA functions in *Caulerpa* to stimulate blade assimilators (and perhaps inhibit rhizome development) while GA_3-like substances stimulate rhizomes and are without effect on the blades. Present indications are that IAA is present throughout the plant, because Brennan and Jacobs (5) found that ^{14}C added to blade tips as [^{14}C]IAA was quickly distributed evenly throughout the giant coenocyte.

The presence of IAA in *Caulerpa* and the differential effects of GA_3 and IAA added to *Caulerpa* support the hypothesis that these hormones may be involved in mediating the gravity responses recently reported in *Caulerpa* by Jacobs and Olson (20) and Matilsky and Jacobs (25).

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