Plant Growth Regulation 8: 215–223, 1989.

Identification of indole-3-butyric acid as an endogenous constituent of maize kernels and leaves

EPHRAIM EPSTEIN[1] KAI-HSIEN CHEN[2] & JERRY D. COHEN[3]
[1]*ARO, The Volcani Center, Bet Dagan, Israel.* [2]*Department of Botany, University of Maryland, College Park, MD 20742, USA** and [3]*USDA ARS Plant Hormone Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA*

Key words: IAA, IBA, conjugated IBA, rooting, *Zea mays*

Abstract. Indole-3-butyric acid (IBA) was identified by thin layer chromatography, gas liquid chromatography and gas chromatography-mass spectrometry in kernels and leaves of corn (*Zea mays*) var. Hazera 224. Free and ester conjugated IBA were present in dry and germinating corn kernels and leaves. This is the first report of IBA in a monocotyledonous plant and, as far as we know, the first evidence for the presence of conjugated IBA.

1. Introduction

The auxin indole-3 butyric acid (IBA) is very effective in the promotion of rooting on a wide variety of plants and is being used commercially to root many plant species world-wide [16]. It is well accepted that IBA is a synthetic auxin [14, 16], however there are early reports of its identification by paper chromatography and bioassay as a natural product in potato peelings [5]. It has also been identified as an endogenous auxin by gas chromatography in *Nicotiana* [4]. We have shown that IBA is present in tobacco [7] and cypress leaves [11]. Recently IBA has been identified by GC/MS in pea root nodules [2] and pea root and epicotyl [15]. In this work we show evidence for the presence of free and ester conjugated IBA in kernels and leaves of maize (*Zea mays*), an important monocotyledonous crop plant.

2. Materials and methods

2.1 Plant material

The field corn var. Hazera 224 and Stowell's Evergreen sweet corn were used

* Current address: Department of Horticulture, National Taiwan University, Taipei 10764, Taiwan R.O.C.

in this study. Kernels were imbibed in water for 24 hrs and then planted in vermiculite in the dark at 24 °C, and 7 days after sowing, 15 g of kernels and 15 g of leaves were removed for homogenization in 100 ml of 70% acetone [3] using an Ultra-Turrax homogenizer. Non-germinating kernels of maize (15 g) were ground in a Weiley mill prior to extraction by Ultra Turrax. After homogenization, 1 µl (50 ng) of IBA-5-^{3}H (7.5 Ci/mmol, Nuclear Research Center, Dimona, Israel) was added to each sample and allowed to stand overnight at 4 °C. The homogenates were then reduced to the aqueous phase using a rotary evaporator at 50 °C, and were then centrifuged for 10 min at 12,000 g. The supernatant was removed and half was hydrolyzed with 1N NaOH for 1 h at room temperature to liberate ester conjugated IBA [2] while the other half was used for free IBA determination. Samples for free IBA and samples hydrolyzed with NaOH were brought to pH 3.0 with HCl and extracted three times with an equal volume of chloroform. The organic phases were pooled, evaporated to dryness and redissolved in 4 ml of 10 mM ammonium acetate for DEAE-Sephadex chromatography.

2.2 DEAE-Sephadex column chromatography

Columns were prepared with a 3 ml bed volume of DEAE Sephadex (acetate form) equilibrated with 10 mM ammonium acetate. After sample application, the columns were washed with 5 ml of 10 mM ammonium acetate followed by 5 ml of hexane, acetonitrile, ethyl acetate and methanol, and IBA was then eluted with 2% acetic acid in methanol [6]. The radioactive fractions were pooled, evaporated to dryness in a rotary evaporator, and the precipitate dissolved in 0.5 ml of methanol for TLC or HPLC analysis.

2.3 Thin layer chromatography (TLC)

Samples from the DEAE Sephadex column were methylated with ethereal diazomethane [8] and then applied as a band on Merck Kieselgel 60 F254 TLC plate and developed with hexane:ether (60:40 v/v). The R_fs of methylated IAA and IBA in this system were 0.7 and 0.8, respectively. They were identified on the plates under UV light and by cutting off a strip and treating with Ehrmann reagent [9] The indole bands were eluted from the silica gel with 1 ml distilled methanol for gas chromatography.

2.4 High performance liquid chromatography (HPLC)

HPLC analysis was performed on a Varian 5000 chromatograph with a Rheodyne 7125 sample injector (50 µl loop), Varian UV-100 detector at

280 nm, and a Hewlett-Packard 3390A integrator. The column was a Whatmann ODS RP_{18}, (5 μ). HPLC was operated isocratically with a mobile phase of 20% acetonitrile containing 1% acetic acid at a flow rate of 1 ml/min. Fractions (1 ml) were collected using a Gilson TDC 80 fraction collector and those fractions containing radioactivity were pooled, dried *in vacuo*, and dissolved in methanol for subsequent analysis by GLC and GC/MS.

2.5 Gas-liquid chromatography (GLC)

Samples were methylated as described and 1 μl was injected onto 30 m × 0.25 mm i.d. fused silica capillary DB-5 column (J&W Scientific) using a flame ionization detector with a Varian 3300 gas chromatograph. Injections were made in the splitless mode, and nitrogen was used as a carrier gas at 1 ml/min. The GLC conditions were injector port at 250 °C, detector at 280 °C. Initial oven temperature was 100 °C for 2 min with a heating program of 5 °C/min up to 200 °C.

2.6 Gas chromatography-mass spectrometry (GC/MS)

GC/MS identification was performed with a Finnigan model 5100 GC/MS/DS combination using electron impact ionization. The GC was equipped with a fused silica capillary column SE-54 (J&W Scientific), 30 m × 0.25 mm i.d. Spectra were taken by both continuous and reconstructed ion chromatography (RIC) scans and by selected ion monitoring (GC-SIM-MS).

2.7 Confirmation of results

All of the initial isolations and identification were done at the Volcani Center laboratory in Israel. Additional confirmation was provided by repeating the isolation and identification in the Plant Hormone Laboratory in Beltsville, U.S.A. Conditions for the analysis in both Israel and the U.S. were similar, except that a Water's HPLC was used and GC/MS analysis was done with a J&W DB-17 fused silica WCOT column on a Hewlett-Packard 5992 quadrupole instrument in the laboratory in the U.S. Only dry seeds (kernels of Stowell's Evergreen and Hazera 224) were analyzed in the U.S. For Stowell's Evergreen the ^{3}H-IBA was recovered without measurable isotope dilution, indicating that this plant material contained no detectable IBA. IBA was isolated and identified by GC-SIM-MS (ions m/z 130 and 217) and by full scan GC/MS in the Hazera 224 seed material.

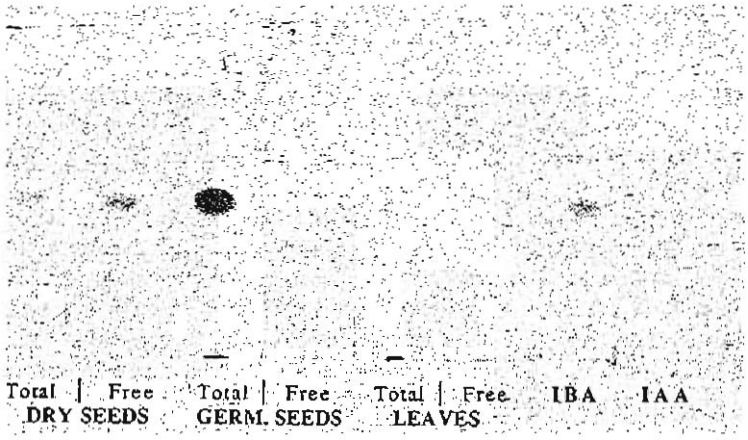

Fig. 1. Thin layer chromatography of methylated extracts of kernels and leaves of corn variety Hazera 224 and standard IBA. Chromatography Solvent: hexane:ether 60:40. Visualization done by Ehmann reagent [9].

3. Results

IBA was identified in the kernels and leaves of Hazera 224 field corn by TLC (Fig. 1), GLC (Fig. 2) and by GC/MS (Fig. 3). We did not find detectable levels of IBA in Stowell's Evergreen corn kernels. TLC of extracts of dry corn kernels, and corn kernels and leaves seven days after sowing gave distinct blue spots at the R_f of IBA and IAA when viewed under UV light. Additional evidence was the characteristic blue color for IBA after dipping in Ehmann reagent which can detect nanograms of IBA (Fig. 1). The intensity of the color shows that the tissue had more IBA after hydrolysis with 1N NaOH. This indicates that IBA was released from conjugates by the hydrolysis. GLC chromatograms obtained from methylated samples extracted from corn kernels and leaves are shown in Figure 2. The methylated derivatives of putative IBA in the corn are indicated on the chromatogram by the peaks at $R_t = 25.9$ min. These peaks are at the identical retention time as the R_t value of authentic IBA. Figure 3 is the reconstructed ion chromatogram for masses 130 and 217 and the mass spectra of methylated samples of corn kernels and leaves. The mass spectrum corresponding to the

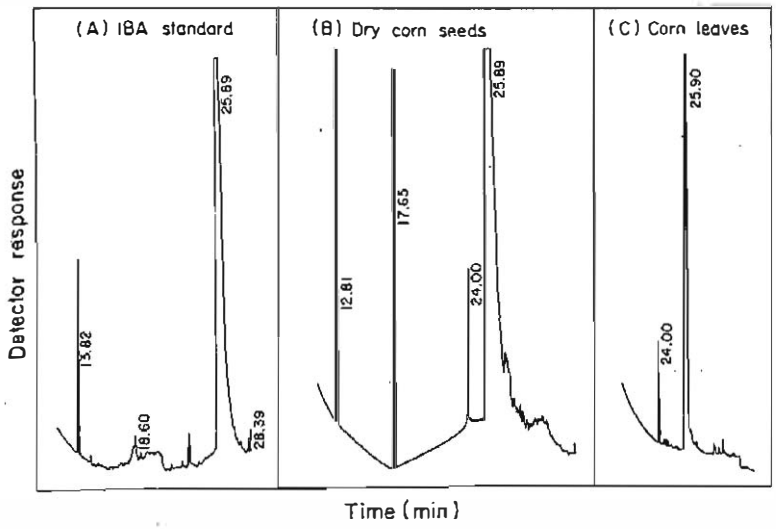

Fig. 2. Capillary gas chromatographic elution profile of authentic methyl IBA (A), partially purified and methylated samples of kernels (B) and leaves (C) of maize variety Hazera 224.

RIC peak at 25.5 min yields the fragmentation pattern typical of 3-substituted indoles. The molecular ion ($m/z = 217$) is quite stable and relatively little fragmentation occurs. The side chain cleavage with retention of the methylene carbon and ring expansion results in a base peak at $m/z = 130$, the quinolinium ion.

4. Discussion

The auxin IBA, which has largely been referred to as "a synthetic auxin", was found in large amounts in kernels and leaves of maize variety Hazera 224 but not Stowell's Evergreen. This is the first identification of IBA by GC/MS in a monocotyledonous plant and as far as we know, the first evidence for the occurrence of bound IBA. We found in our prior work that IBA was a better promoter of root initiation than IAA [19]. It increased root formation on the entire length of mung bean cuttings, compared to a small number of roots at the base of the cuttings after treatment with IAA. Previous reports suggested that IBA may be involved in physiological processes in some plant tissues. Blommaert [5] found more IBA than IAA

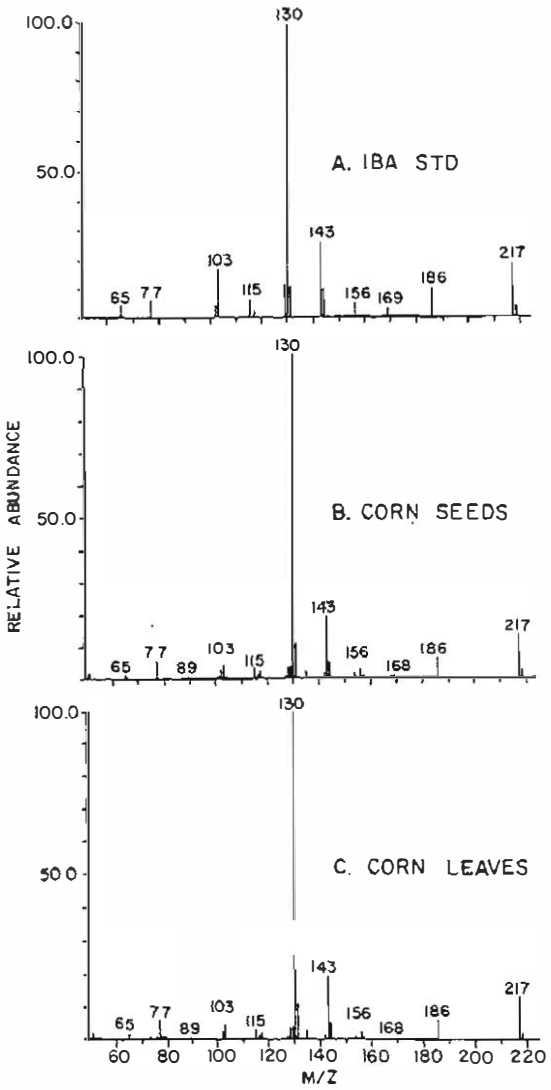

Fig. 3. Mass spectra of authentic methyl IBA (A) and the purified and methylated samples of kernels (B) and leaves (C) of maize variety Hazera 224.

in potato peelings at the beginning of sprouting. Bayer [4] found that tumor-prone *Nicotiana* had much more IBA than the normal plants. Chen [7] isolated IBA from tumor-prone *Nicotiana* hybrids and identified it by GC/MS, but was unable to find significant levels in the non-tumorous plants. The large amounts of IBA in maize kernels and its presence in the maize leaves suggest that this auxin is widely distributed in the plant. IBA may play a role in the germination and in the rooting of maize seedlings. We have shown that 1N NaOH hydrolysis increased the amount of IBA in the extract, thus suggesting that, like IAA [3], IBA is found in maize as ester conjugates.

Relatively few studies of IBA metabolism within the plant have been conducted. Andreae and Good [1] showed that pea tissues can conjugate exogenous IBA to form IBA aspartate, and recently Wiesman et al. [19] found that when cuttings of mung beans were incubated with IBA, more than 90% of the IBA was metabolized after 24 h. In mung bean and in olive cuttings [18] most of the IBA was converted to unidentified conjugates. Epstein and Wiesman [12] found that IBA-alanine increased the number of olive cuttings which rooted compared with IBA or IAA treatments. Epstein and Lavee [10] and Fawcett et al. [13] have demonstrated the conversion of IBA to IAA in plants. This evidence indicates that plants are able to synthesize, catabolize and conjugate this hormone. Our failure to find IBA in the Stowell's Evergreen cultivar of maize and Chen's finding that IBA was not present in non-tumorous tobacco plants suggest that it is not continuously present in plant tissues or that its accumulation is under genetic and possibly also developmental control. Went and Thimann [17] proposed that some unknown compounds (which they termed rhizocalines) might exist in the plant and act in conjunction with IAA in the formation of adventitious roots. Based on the plethora of publications relating application of IBA to adventitious rooting and the results of the present studies, as well as preliminary results from our laboratories indicating IBA is present in at least seven other species, we as a working hypothesis suggest that IBA could be such a natural rhizocaline.

The natural occurrence of IBA in plants has important implications related to indole biochemistry in higher plants. It is clear that plants are able to make indolealkonic acids with side chains longer than the three carbons present in tryptophan and are also able to reduce the chain length to form IAA [10, 13]. Knowledge of the biosynthesis of compounds such as IBA could provide new insight into the mechanism and regulation of auxin biosynthesis.

Acknowledgements

This work was supported by a grant (U.S.-842-84) from the United States-Israel Binational Agricultural Research and Development Fund (BARD). Part of this work was carried out under cooperative agreement No. 56-32u4-7-001 (RSA-7-001-010) of the U.S. Department of Agriculture/Agricultural Research Service and the University of Maryland.

We would like to thank Dr. Lea Muszkat from the Department of Chemistry of Natural Products, ARO, Volcani Center, for her help with the GC/MS work.

Note

1. Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

References

1. Andreae WA and Good NE (1957) Studies on 3-indoleacetic acid metabolism. IV. Conjugation with aspartic acid and ammonia as process in the metabolism of carboxylic acids. Plant Physiol. 32: 566 571
2. Badenoch Jones J, Summons RE, Rolfe BG and Letham DS (1984) Phytohormones, *Rhizobium* mutants and nodulation in legumes. IV. Auxin metabolites in pea root nodules. J. Plant Growth Regulators 3: 23 29.
3. Bandurski RS and Schulze Aga (1977) Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiol. 60: 211–213
4. Bayer MH (1969) Gas chromatographic analysis of acidic indole auxins in *Nicotiana*. Plant Physiol. 44: 267–271
5. Blommaert KLJ (1954) Growth- and inhibiting-substances in relation to the rest period of the potato tuber. Nature 174: 970–972.
6. Chen K-H, Miller AN, Patterson GW and Cohen JD (1987) A rapid and simple procedure for purification of indole-3-acetic acid prior to GC-SIM-MS analysis. Plant Physiol 86: 822–825
7. Chen K-H (1987) Analysis of indole 3 acetic acid in tobacco genetic tumors and wheat GA_3 insensitive mutant "Tom Thumb" Ph.D. Thesis. University of Maryland
8. Cohen JD (1984) Convenient apparatus for the generation of small amounts of diazomethane. J. Chromatogr. 303: 193–196
9. Ehmann A (1977) The Van-Urk-Salkowski reagent a sensitive and specific chromogenic reagent for silica gel thin layer detection and identification of indole derivatives. J. Chromatogr. 132: 267–276
10. Epstein E and Lavee S (1984) Conversion of indole-3-butyric acid to indole-3-acetic acid by cuttings of grapevine (*Vitis vinifera*) and olive (*Olea europeae*). Plant & Cell Physiol. 25: 697 703
11. Epstein E, Muszkat Lea and Cohen JD (1988) Identification of indole 3-butyric acid

(IBA) in leaves of cypress and maize by gas chromatography-mass spectrometry (GC/MS). Alon Hanote 42: 917 919

12. Epstein E and Wiesman Z (1987) Improved vegetative propagation of olive cuttings with IBA-alanine. Olea 18: 35 38
13. Fawcett CH, Wain RL and Wightman F (1960) The metabolism of indolylalkenecarboxylic acids and their amides, nitriles and methyl esters in plant tissues. Proc. Royal Soc. London Ser. B. 152: 231 254
14. Nickell LG (1982) Plant Growth Regulators: Agricultural Uses. Berlin: Springer Verlag
15. Schneider Elnora A, Kazakoff CW and Wightman F (1985) Gas chromatography-mass spectrometry evidence for several endogenous auxins in pea seedlings organs. Planta 165: 232–241
16. Weaver RJ (1972) Plant Growth Substances in Agriculture. San Francisco: W.H. Freeman and Co.
17. Went FW and Thimann KV (1937) Phytohormones. NY: Macmillan
18. Wiesman Z and Epstein E (1987) Metabolism and transport of ^{3}H indole 3-butyric acid in cuttings of olive. Olea 18:29–33
19. Wiesman Z, Epstein E and Riov J (1988) Comparison of movement and rate of metabolism of indole-3-acetic acid and indole-3-butyric acid in mung bean cuttings Physiol Plant. 74: 556–560