

Endogenous Auxin and Ethylene in the Lichen *Ramalina duriaei*¹

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ABSTRACT

Indole-3-acetic acid (IAA) levels and ethylene evolution rates were measured in a fruticose lichen *Ramalina duriaei* collected from carob trees growing in northeast Israel. IAA levels were estimated by gas liquid chromatography with electron capture detection of the pentafluorobenzyl ester and also by enzyme-linked immunosorbent assay following methylation. The identity of the isolated IAA was confirmed by gas chromatography-mass spectrometry of both the methyl and the pentafluorobenzyl ester. IAA levels in lichens 1 year after transplanting to an air-polluted urban site were found to be lower than in the control thalli left at a nonpolluted, rural site. The material from the latter contained about 2.5 micrograms per gram fresh weight free IAA and 0.5 microgram per gram fresh weight conjugated IAA, while the urban material contained 0.3 microgram per gram each of free and conjugated IAA. Ethylene production rate was 1.0 nanoliter per gram fresh weight per hour in the material from the rural site and 1.5 nanoliters per gram fresh weight per hour in material from the urban site.

MATERIALS AND METHODS

Biological Material. Thalli of the fruticose lichen *Ramalina duriaei* (De Not.) Bagl. were used. *Ramalina duriaei* is a symbiosis between an unknown ascomycetous fungus and *Treboxia*. The source of the material was a grove of old carob trees (*Ceratonia siliqua* L.) at HaZorea (Esdraelon Plain, northeast Israel). Leafless twigs (40–60 cm long) from trees that were covered by the lichen thalli were picked in July 1984 and divided into two groups. The twigs in group 1 were secured with a PVC cord and suspended from branches of trees at HaZorea. The twigs of group 2 were transferred to the Kefar HaYaroq Junction (near Tel Aviv) where they were suspended from olive trees, about 30 m from a very heavily traveled motorway. A detailed description both of the study sites and of the level of environmental pollution, as reflected in the accumulation of heavy metals, was published previously (9). In July 1985, all the lichens were removed from the branches, put into plastic bags, and stored at -20°C until analysis. Dry weight determination showed that lichens from both sites contained approximately 20% water.

Determination of Endogenous IAA. Samples of lichen (0.5–1.0 g) were extracted by homogenization in 70% acetone (5) containing 100 mg/L butylated hydroxytoluene with an Ultra Turrax² homogenizer. After blending, 200 nCi of [2-¹⁴C]IAA (59 mCi/mmol, Amersham Corporation, England) was added to each sample. After standing at 4°C overnight the homogenates were reduced to the aqueous phase on a rotary evaporator at 50°C, centrifuged 10 min at 12,000g, and the supernatant was divided into two equal parts. One part was kept for determination of free IAA and the other was hydrolyzed, at 100°C for 3 h under N₂, for the determination of conjugated IAA (5). Following hydrolysis the sample was brought to pH 2.5 with concentrated HCl (on ice) and then passed through a C18 Sep-Pak (Waters Associates) cartridge. The cartridge was washed with distilled H₂O to remove salt and the IAA was eluted with 2 ml of distilled methanol. Four ml of 10 mM ammonium acetate was added and further purification was obtained by ion-exchange chromatography, derivatization, and TLC prior to analysis by EC-GC.³

DEAE-Sephadex Column Chromatography. Columns for chromatography were prepared with a 3 ml bed volume of

A lichen is a symbiosis which consists of algal and fungal components that grow together to form a unit of consistently recognizable structure and appearance. In most lichens, the fungal component is ascomycetous and the algal components are either blue-green or green algae.

While there have been a fair number of reports on auxins in algae (1, 4, 13) and fungi (10), there is, to the best of our knowledge, no information on endogenous IAA or ethylene production by lichens. In this paper we report the estimation of IAA levels and ethylene production in the lichen *Ramalina duriaei* and the identification of IAA by GC-MS. The lichens for this study were taken from an area subjected to automobile pollution and from a nonpolluted area since prior studies (8, 9, 15) have shown that polluted environments alter a variety of physiological parameters of this slow growing association. We were interested in determining if the IAA levels in lichens were related to their physiological status.

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² Mention of a 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.

³ Abbreviations: EC, electron capture; PFB, pentafluorobenzyl; IAA-PFB, indole-3-acetic acid pentafluorobenzyl ester; ELISA, enzyme-linked immunosorbent assay; FW, fresh weight.

DEAE-Sephadex (acetate form) equilibrated with 10 mM ammonium acetate. After application of the sample, the columns were washed with 10 mM ammonium acetate and IAA was eluted with 1 N acetic acid. Fractions containing radioactivity were pooled, applied to a C18 Sep-Pak cartridge which was then washed with distilled H₂O to remove the acid, and the IAA was eluted with 2 ml of distilled methanol. The methanol was evaporated under vacuum and the residue was taken up with 0.5 ml of a distilled methanol:acetonitrile (1:1) mixture, and transferred to reaction vials for derivatization. Following evaporation of the solvent and resuspension in acetone, PFB esterification was performed essentially as described by Epstein and Cohen (7).

Thin Layer Chromatography. TLC was done on Merck Kieselgel 60 F254 plates. The plates were washed with distilled methanol prior to chromatography and samples were developed with chloroform:ethyl acetate (90:10) solvent. In this solvent IAA remained in the origin, IAA-PFB had an R_f of 0.8, and excess PFB had an R_f 0.9. IAA-PFB was eluted from the silica gel with 1 ml of distilled toluene for GLC and radioactivity counting.

Gas-Liquid chromatography. GC was performed using a Varian 3300 gas chromatograph equipped with a ⁶³Ni EC detector (7). Nitrogen was used as a carrier gas at 1 ml/min with a 25 m SGE vitreous silica bonded phase SCOT capillary column (SGE 25QC2/BP 10/0.25). Injections were made in the splitless mode and toluene was used as the injection solvent. Under these conditions IAA-PFB had a retention time of 16.0 min.

For standardization of detector response, a sample of [2-¹⁴C] IAA (200 μ l) was derivatized as above and eluted from the TLC plate. The amount of IAA in 1 μ l was calculated from its radioactivity and was usually about 500 pg/ μ l. This was then used to calculate the amount of IAA per unit area in the GLC peaks. Following radioactivity counting the amount of IAA in the tissue was calculated using the isotope dilution equation (19).

Enzyme-Linked Immunosorbent Assay. Preparation of IAA-specific antibodies and the solid-phase enzyme immunoassay utilized were according to Sagee *et al.* (20). The assay was performed following methyl esterification of the IAA sample collected after the C18 Sep-Pak column step. Samples were methylated with ethereal diazomethane (6).

GC-MS. Samples for mass spectral analysis were prepared as described above for hydrolyzed samples. IAA-PFB purified as described was of sufficient purity for direct mass spectral analysis by GC-MS; however, the methylated sample of IAA was additionally purified by reverse phase HPLC (Fig. 1). The sample was dissolved in 100 μ l 50% methanol-water and injected onto a 4.6 \times 250 mm Whatman ODS-3 5 μ column and eluted with

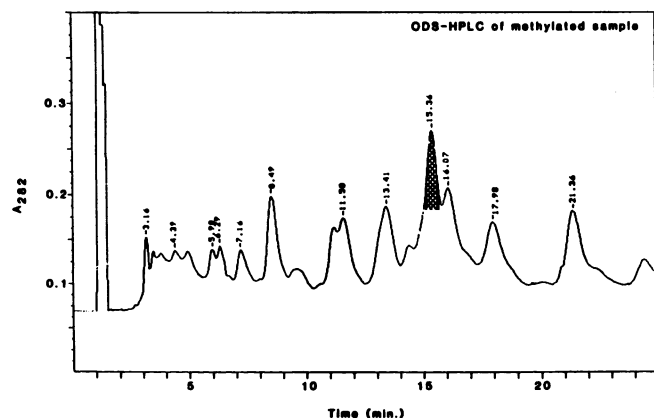


FIG. 1. Elution of the partially purified and methylated lichen sample from a 4.6 \times 250 mm Whatman ODS-5 μ C₁₈ HPLC column by 50% methanol-water at 1 ml/min. Shaded peak was collected for analysis by GC-MS.

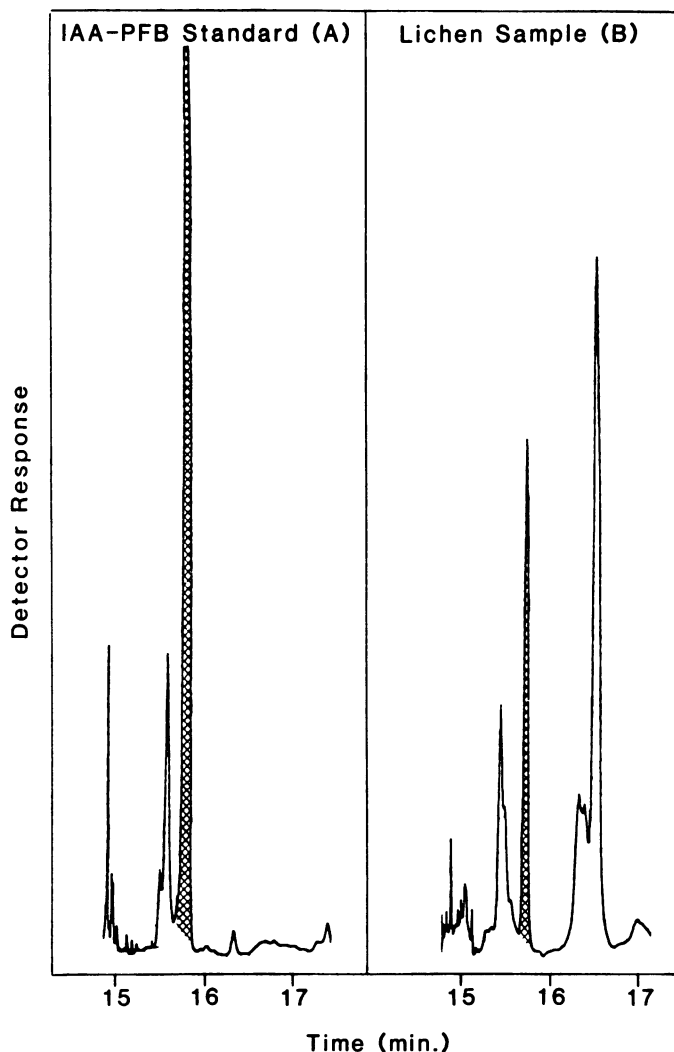


FIG. 2. Capillary gas chromatographic elution profile of a standard of the pentafluorobenzyl ester of IAA (A) and of the partially purified and derivatized lichen sample (B). Shaded peaks are at the retention time of IAA-PFB.

50% methanol-water at a flow rate of 1 ml/min HPLC was performed on a Waters Associates instrument equipped with a Rheodyne 7125 valve. The eluant was monitored using a Kratos 770 UV detector at 282 nm and 1 ml fractions were collected using a Gilson 201B fraction collector. The fraction containing methyl IAA was evaporated and resuspended in 30 μ l of ethyl acetate. Mass spectra were obtained on a Hewlett-Packard 5992a GC-MS. For analysis of the methyl ester of IAA, the GC conditions were as follows: injector at 300°C, He as carrier gas at 1 ml/min, and oven at 130°C for 2 min with the column vented followed by temperature programming at 16°C/min. For the PFB ester, similar conditions were used except the initial oven temperature was 200°C.

Chromatography was on a 12-m Hewlett-Packard cross-linked methyl silicone WCOT fused silica capillary column (Hewlett-Packard 19091-60312). Injections were made in the splitless mode and ethyl acetate was the injection solvent. Under these conditions methyl IAA had a retention time of 5.7 min and the PFB ester 4.2 min.

Ethylene Measurements. One g of freshly collected lichen (6 ml in volume) was placed in a glass tube with a septum. The samples were sprinkled with water before sealing. In one experiment, samples were sprinkled with a solution of 10 μ M IAA.

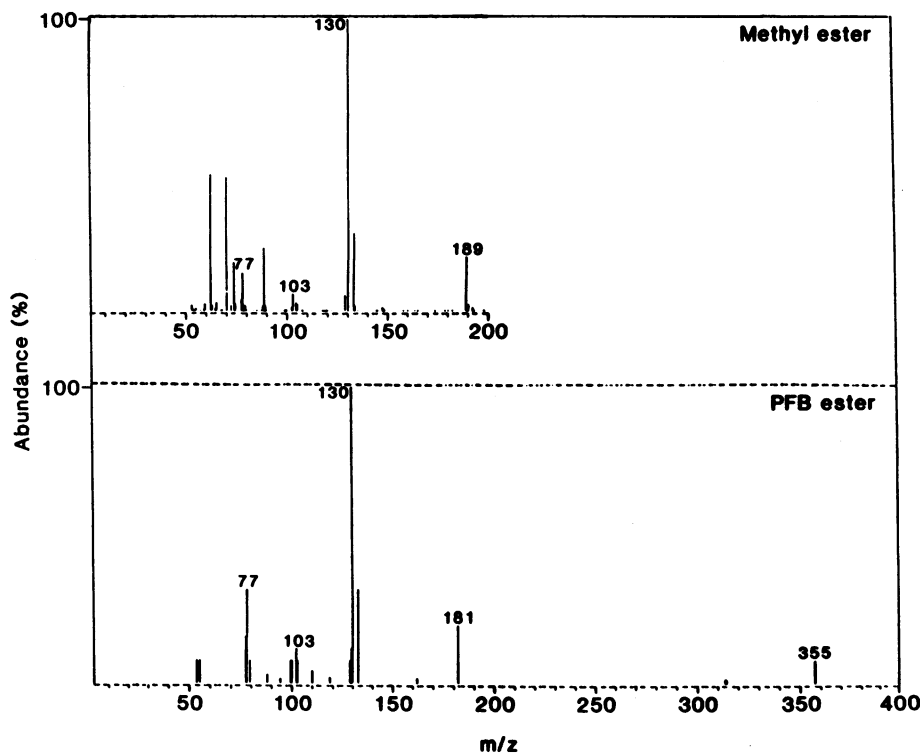


FIG. 3. Mass spectra of IAA isolated from lichen and derivatized to the methyl ester (upper spectrum) and to the PFB ester (lower spectrum). Characteristic ions for each compound (7, 22) are indicated.

Table I. Estimation of Free and Conjugated IAA in the Lichen *Ramalina duriaei* by EC/GLC and ELISA

Location	Free		Conjugated		Total	
	GLC	ELISA	GLC	ELISA	GLC	ELISA
	$\mu\text{g}\cdot\text{g FW}$					
HaZorea (low pollution)	2.40	2.50	0.45	0.55	2.85	2.95
Kefar haYaroq (highly polluted)	0.20	0.40	0.20	0.40	0.40	0.80

Three samples were analyzed from each location and values reported are the mean \pm SE. The lichens were kept in the vial for 18 h at room temperature. One ml of the air space was analyzed for ethylene by flame ionization GC.

RESULTS

IAA was identified in the tissues of the lichen *Ramalina duriaei* by HPLC (Fig. 1), by GLC (Fig. 2), and by GC-MS (Fig. 3). Estimates of free and conjugated IAA in the lichen *R. duriaei* by both EC-GC and ELISA and from both a polluted and a non-polluted site are presented in Table I. In HaZorea (an area of relatively good air quality) the total amount of IAA was high (3.0 $\mu\text{g/g FW}$) and consisted mainly of free IAA (2.4 $\mu\text{g/g FW}$). Samples transferred to Kefar HaYaroq (an area of heavy automobile traffic) contained a much lower amount of total IAA (0.4 and 0.8 $\mu\text{g/g FW}$ by GC and ELISA, respectively) which consisted of equal amounts of free and conjugated IAA.

During incubation at room temperature (22°C), the amount of ethylene released by the lichen tissue from the rural site was 0.99 ± 0.05 nl/g FW·h and from the urban site 1.52 ± 0.04 nl/g FW·h. When samples from HaZorea were kept at 40°C for 2 h the rate of ethylene evolution increased to 2.8 nl/g FW·h. Samples from the rural site treated with 10 μM IAA showed no significant difference in ethylene evolution (1 nl/g FW·h) from untreated controls.

DISCUSSION

This report presents, to the best of our knowledge, the first definitive identification of IAA (by GC-MS) and the first determination of free IAA, conjugated IAA, and ethylene from lichens. Since lichens are very slow-growing associations, it was somewhat surprising to find high levels of IAA in the tissue. The level of IAA in the lichen from the nonpolluted air site was rather high, approximately 3 $\mu\text{g/g FW}$, and was mainly free IAA.

Jacobs *et al.* (13) reported 1 $\mu\text{g/g FW}$ of free IAA in the alga *Caulerpa* and Thomas *et al.* (21) found a very high level of IAA in the thalli of the liverwort *Pellia epiphylla*, almost 3 $\mu\text{g/g FW}$, all of it in the free form. However, when moss and liverwort were grown under sterile conditions, the levels of IAA were much lower: approximately 100 ng/g FW of IAA in the liverwort *Plagiochilla arctica* (16); 2.1 ng/g FW in the gametophytes of the moss *Physcomitrella patens* (3); and 5 ng/g FW in the protonema of the moss *Funaria hygrometrica* (14). Consequently, the high level in *Pellia* and lichens might come from bacteria associated with their tissue (17). Jacobs *et al.* (13) raised this possibility in their discussion on IAA in the algae *Caulerpa*. They conclude that "IAA is available to *Caulerpa*, whether it is synthesized by the alga itself, or by its associated bacteria, or by both." Until we can grow significant amounts of the lichen and the individual associated organisms in axenic culture, we will not be able to ascertain the source of the IAA.

The amount of ethylene that was produced by the slow-growing lichen thallus was in the same range as that found in active tissues such as meristematic plant tissues and nonsenescent leaves (2). Higher levels of ethylene evolution from samples from Kefar HaYaroq may indicate a stress-induced ethylene production as has been observed in higher plants (2). The rate of ethylene production was also affected by a short exposure to a temperature stress (40°C for 2 h). Auxin does not stimulate ethylene production in lichens and the pathway to ethylene production in fungi and other microorganisms that have been studied does not proceed through 1-aminocyclopropane-1-carboxylic acid (2, 18). Thus, one cannot relate ethylene evolution to the level of auxin in these tissues. The levels of IAA, both free and bound, were

lower in the lichens that were transferred to the air-polluted area. Previous studies showed that the lichens in the air-polluted site differed from those in the control site in other physiological parameters. They absorbed more heavy metals (Pb, Zn, Cu) (8), had significantly lower ratio of Chl *a* to phaeophytin *a* (9), and had significantly lower levels of ATP (15). It is most probable that the decrease in the IAA level in the lichens exposed to the urban site was a result of anthropogenic factors linked to automobile activity, and not because of climatic conditions, since the meteorological data are very similar at both sites of exposure (11, 12).

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