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Identification and Quantification of Indole-3-methanol in Etiolated Seedlings of Scots Pine (*Pinus sylvestris* L.)[1]

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ABSTRACT

Combined gas chromatography-mass spectrometry has been used to identify indole-3-methanol in a purified buffer extract from etiolated seedlings of *Pinus sylvestris* L. Quantitative estimates obtained by high performance liquid chromatography with fluorescence detection, corrected for losses occurring during purification, indicated that etiolated seedlings of *P. sylvestris* contained 19.7 ± 1.4 nanograms (± standard deviation) indole-3-methanol per gram fresh weight. The stability of indole-3-methanol at different pH levels was investigated. The rate of conversion, to a less polar unidentified substance, was enhanced with increasing acidity.

There are many reports in the literature on the catabolism of IAA. Both a decarboxylative and a nondecarboxylative pathway are suggested (*e.g.*, 3, 11, 17). Reliable identifications of some of the possible catabolic products of IAA have been obtained with both *in vitro* and *in vivo* test systems. The suggested major metabolites of the frequently studied decarboxylative pathway are HMO[2], 3-methyleneoxindole, IAld, and ICA (see 22). Surprisingly, as it could be either an intermediate or an end product, relatively little attention has been paid to the possible involvement of IM in IAA catabolism.

Formation of IM has, however, been shown by incubation of IAA with purified HRP (10, 19), as well as with an isolated wheat peroxidase isoenzyme (26). Grambow and Langenbeck-Schwich (10) demonstrated that the fate of IAA was dependent on the occurrence and concentration of H_2O_2 and phenols in the incubation medium. The presence of phenolic compounds resulted in formation of IM as the major catabolite, while in their absence, HMO was the dominant product. *In vivo* incubations of IAA with segments of *Pisum sativum* (7, 13), *Orobanche* sp. (14), and *Phaseolus vulgaris* (7) also yielded IM as one of the catabolic products. Indirect evidence for an IM pathway in plants comes from the identification of BIM, a proposed degradation product of IM, as a product of IAA catabolism in both a crude enzyme extract from *Zea mays* (3) and HRP (24). The possible production of IM in a HRP/IAA system is also pointed out by Nakajima and Yamazaki (15). Their suggestion is based on the low ratio between oxygen consumed and IAA added.

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[2] Abbreviations: HMO, 3-hydroxymethyloxindole; IAld, indole-3-aldehyde; ICA, indole-3-carboxylic acid; IM, indole-3-methanol; HRP, horseradish peroxidase; BIM, 3,3′-bisindolylmethane.

IAld (4, 6, 23) and ICA (20) are the only components in the decarboxylative pathway that have been identified as endogenous constituents in higher plants on the basis of strict physicochemical evidence (2). Many previous reports have pointed out IM as a possible catabolic metabolite of IAA (*e.g.* 10, 13). To our knowledge, however, the natural occurrence in plants has never been examined. The purpose of the present study was therefore to investigate the presence and quantity of IM in extracts of etiolated seedlings of *Pinus sylvestris* L., and to determine if IM is a genuine endogenous component or merely a breakdown product of IAA, formed during sample preparation.

MATERIALS AND METHODS

Plant Material. Seedlings of Scots pine (*Pinus sylvestris* L.) were grown in the dark at 20°C for 16 d in peat. Upon harvesting, the plant material was frozen in liquid N_2 and stored at −20°C prior to extraction and analyses.

Extraction and Purification. The cotyledons and hypocotyls of the etiolated seedlings were homogenized in 0.1 M phosphate buffer (pH 8.0) (0.5 g·ml^{-1}) containing 0.02% w/w of the antioxidant sodium diethyldithiocarbamate. The tissue homogenate was ultrasonicated for 15 min, filtered and saturated with $(NH_4)_2SO_4$ to precipitate proteins. All these steps were performed at 4°C. The extract was then centrifugated for 40 min at 46,000*g* in a refrigerated centrifuge. For IM analyses, the supernatant was adjusted to pH 8.0 with 1 M NaOH, slurried with insoluble PVP (1 g·25 g^{-1} tissue fresh weight) and passed through a column packed with 1 g of Amberlite XAD-7 (1). When IAA was analyzed, the pH was adjusted to 2.7 with 1 M HCl before the XAD column. The XAD-7 column was eluted with 10 ml of ethyl acetate and hexane (70:30, v/v). The aqueous phase of the eluate was discarded and 0.02% w/w of the antioxidant added to the organic phase prior to being reduced to dryness under a stream of N_2 in preparation for HPLC.

HPLC. Solvents were delivered via a LDC model 396 pump when isocratic elution was used, and via Waters model 45 and 510 pumps when gradient elution was utilized. Samples were introduced off-column via a Valco injector with a 100 μl loop. Column effluent was monitored with a Spectra-Physics SP-970 spectrofluorometric detector (excitation, 285 ± 5 nm; emission, 360 ± 10 nm) with a 5 μl flow cell. When radioactive components were analyzed, effluent leaving the fluorometer was directed to a Reeve Analytical radioactivity monitor operating in the heterogenous mode, with a 170 μl flow cell packed with a cerium-activated lithium glass scintilator. When necessary, radioactivity was quantified using an instagel scintillant and a LKB-Wallac Ultrabeta 1210 scintillation counter.

Normal phase HPLC was performed using a 240 × 4.6 mm i.d., 5 μm Nucleosil CN column eluted at a flow rate of 0.9 ml

min^{-1} with a mobile phase of hexane-ethyl acetate (75:25 v/v). One % of acetic acid was included in the mobile phase when IAA was analyzed. A 200 × 4.6 mm i.d., 5 μm Nucleosil C-18 column was used for reverse phase analyses. Samples were eluted at a flow rate of 0.6 ml min^{-1} with 10 mM Na-phosphate buffer (pH 6.5)-methanol (70:30, v/v) when IM was analyzed and with water-methanol-acetic acid (59:40:1, v/v) when IAA was analyzed. Ion pair chromatography was performed with a mobile phase of 0.05 M phosphate buffer (pH 6.5) with 20 mM tetrabutylammonium hydrogen sulfate-methanol (70:30, v/v) at a flow rate of 0.9 ml min^{-1}. Gradient elution comprised a 20-min program of 50 to 100% methanol in 10 mM Na-phosphate buffer (pH 6.5) at a flow rate of 1 ml min^{-1}.

GC. GC was carried out on a Hewlett-Packard 5880 equipped with a flameless NP detector. The column was a 15 m × 0.25 mm i.d. fused silica column. The cross-linked stationary phase was DB-1, with a film thickness of 0.25 μm. The injector temperature was 250°C, and the splitless injection technique was used. The oven temperature profile was held at 60°C for 1 min, then programmed at 30°C min^{-1} to 140°C, held for 3.2 min, and then programmed at 10°C min^{-1} to 160°C. The detector temperature was 200°C. Before analysis, samples were silylated with 15 μl acetonitrile and bis(trimethylsilyl)trifluoro acetamide at 60°C for 10 min.

Combined GC-MS. The instrument used was a Hewlett-Packard 5710A gas chromatograph linked via a jet separator to a VG micromass 7070H mass spectrometer equipped with a DEC PDP 8A computer system. Samples were silylated as above and introduced via an injector at 200°C onto a 15 m × 0.25 mm i.d. fused silica column. The column temperature was initially 40°C, and after injection increased with 30°C min^{-1} to 130°C, thereafter up to 170°C over 16 min. The carrier gas flow rate was 1 ml min^{-1}. The interface temperature was 220°C. Positive ion electron impact spectra were recorded at 70 ev with a 0.7 $decade^{-1}$ scan rate and a 0.5 interscan delay.

Chemicals. IM and IAA were purchased from Sigma Chemicals Co. and [2-^{14}C]IAA (2.04 GBq $mmol^{-1}$) from Amersham International pc. [^{14}C]IM (2.04 GBq $mmol^{-1}$) was prepared from [2-^{14}C]IAA by enzymic conversion as described by Grambow and Langenbeck-Schwich (10) and purified by reverse and normal phase HPLC. The radiochemical purity was confirmed by HPLC-on line radiocounting. The identity of both IM and [^{14}C] IM was confirmed by combined GC-MS. BIM was synthesized from indole and formaldehyde as described by Grambow *et al.* (9). The identity of the product was determined by melting point (169°C), IR spectroscopy, and NMR, and it proved to be pure on gradient reverse phase HPLC with fluorescence detection.

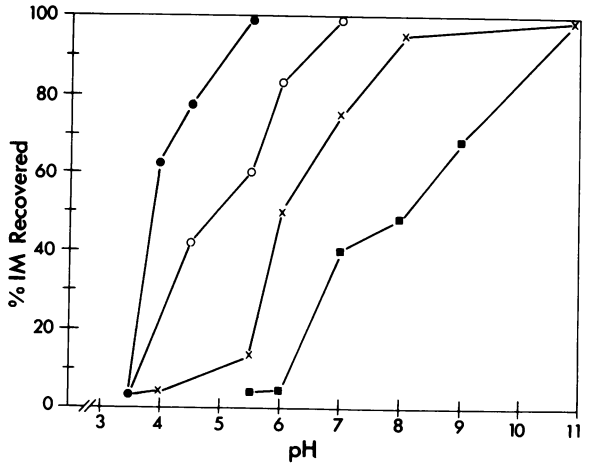

FIG. 1. Relative recovery of IM in incubations in 0.01 M phosphate buffer and methanol (60:40 v/v) under different pH, 21 d at −20°C (●), 1 d at room temperature (○), 6 d at room temperature (×), 21 d at room temperature (■). Samples were analyzed on isocratic reverse phase HPLC.

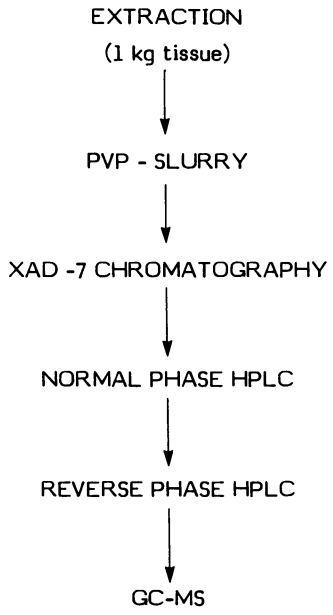

FIG. 2. Procedures used for the analyses of IM in etiolated seedlings of *P. sylvestris*.

RESULTS AND DISCUSSION

It is often claimed that IM is unstable, but documentation on the subject is limited (*e.g.* 3, 10). To examine suitable extraction and purification conditions, the stability of IM at different pH levels and temperatures was investigated. This showed that IM was spontaneously converted to an unidentified less polar compound. The rate of conversion increased with decreasing pH and was apparently instantaneous at pH 3.5 (Fig. 1). However, at neutral and basic pH values, IM was relatively stable, especially at −20°C. These observations are in agreement with the findings of Thesing (25) who noted a breakdown of IM under acidic conditions, but described IM as stable under neutral and basic conditions. The degradation of IM under acidic conditions has also been described by other investigators (3, 12). The proposed products obtained from IM degradation include BIM (25), 3-methyleneindolenine (3), and an unknown polymer (12). Magnus *et al.* (13) reported that 3-methoxymethylindole and BIM were products of IM formed during chromatographic analyses. The component formed from IM in the present investigation was more polar than BIM when analyzed on gradient reverse phase HPLC. The results above suggest that failure to detect IM as a catabolite of IAA could well be due to samples being exposed to acidic conditions (*e.g.* 8, 16).

Qualitative analyses of IM in an extract from 1 kg etiolated *P. sylvestris* seedlings was carried out using the procedures outlined in Figure 2. Normal phase HPLC of the partially purified sample showed a peak co-chromatographing with authentic IM (Fig. 3). Further purification of this fraction on reverse phase HPLC revealed a major peak with the same retention time as standard IM. The capacity factor for IM in these systems was 5.5 and 4.5, respectively. The peak from reverse phase HPLC was collected and silylated and a mass spectrum was obtained that closely matched the spectrum of di-trimethylsilyl-IM (Fig. 4). The derivatized HPLC reverse phase fraction was also analyzed by capillary GC with nitrogen-phosphorus detection. This chromatographic system also indicated the presence of IM in the extract (Fig. 5). Thus, on the basis of co-chromatography in three

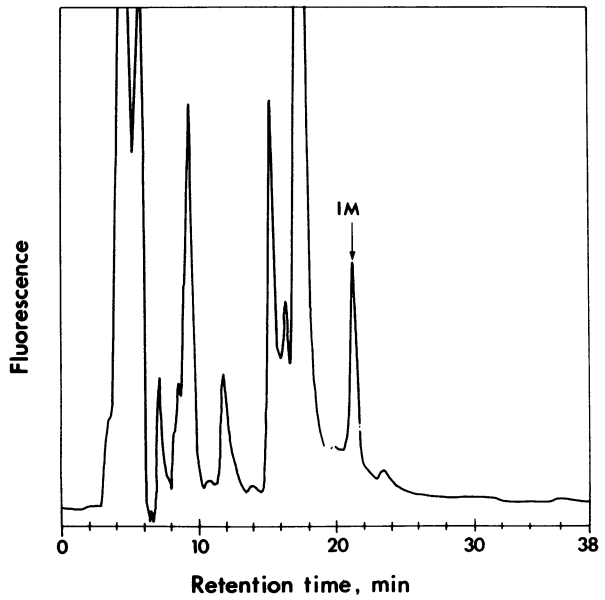

FIG. 3. Normal phase HPLC of a semi-purified extract from etiolated *P. sylvestris* seedlings. Arrow indicates peak co-chromatographing with IM.

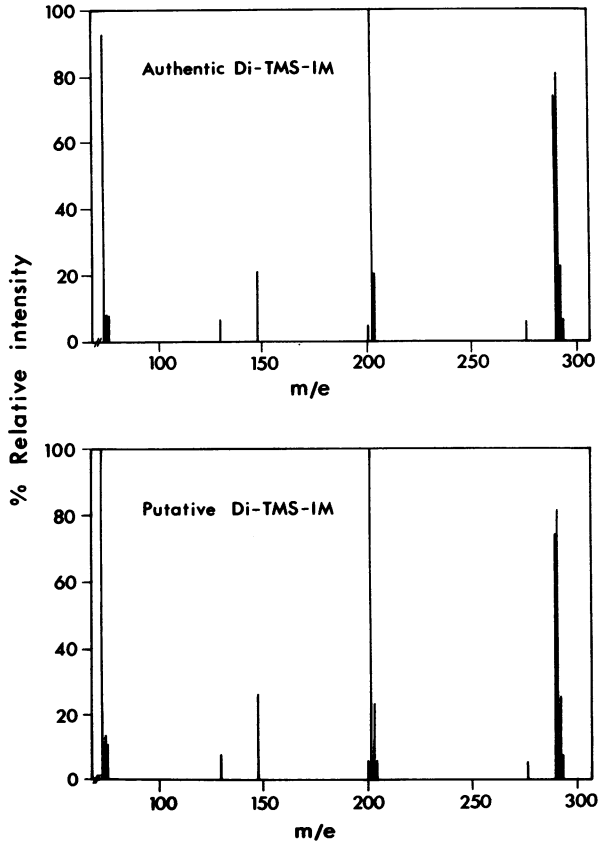

FIG. 4. Electron impact mass spectra of authentic IM and putative IM from etiolated *P. sylvestris* seedlings.

different separation systems and mass spectral data, it can be concluded that the purified *P. sylvestris* extract contained IM.

Because of the instability of IAA, at least a part of the IM pool detected could have originated from degradation of IAA during sample preparation. To investigate this possibility, 16.780 kBq (1440 ng) of [2-^{14}C]IAA was added to two 50-g samples of tissue

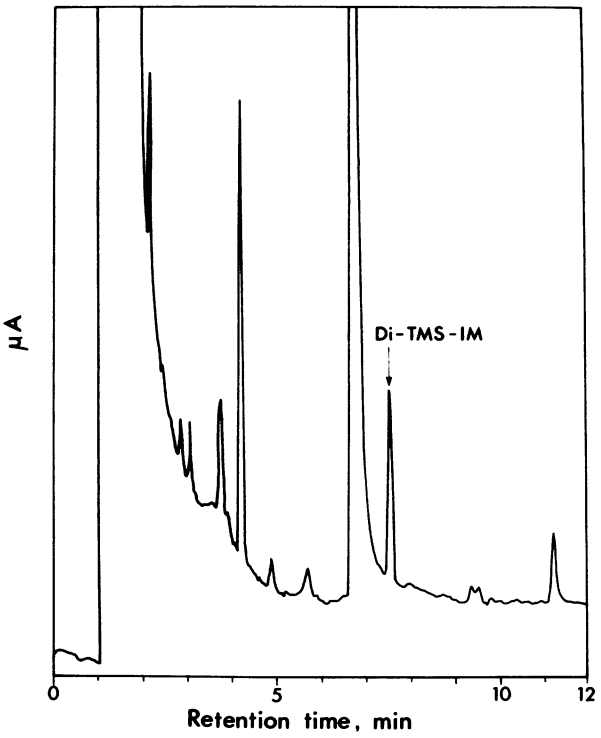

FIG. 5. GC-NP of a semipurified extract from etiolated *P. sylvestris* seedlings. Arrow indicates peak co-chromatographing with di-trimethylsilyl-IM.

immediately before homogenization. To minimize losses of any [^{14}C]IM formed, 1 μg of cold IM was added as carrier. The samples were treated as described in Figure 2, but were divided in a neutral and an acidic fraction on the XAD column. The fractions were then analyzed on normal phase HPLC connected to an on line radioactivity detector. The acidic fraction showed one single radioactive peak which co-chromatographed with IAA. The neutral fraction did not contain any detectable radioactive peak. The IAA and IM fractions were collected for more precise determination of the radioactivity by liquid scintillation counting. Assuming the same recovery for IAA as for IM, it was calculated that the breakdown of IAA to IM in the two samples was only 0.55% and 0.47%. However, this result does not exclude the possibility of degradation of other substances than IAA to IM during sample preparation.

The next step was to quantify the IM pool in the pine seedlings. A 150-g sample of tissue was homogenized and the homogenate divided into three equal parts. An internal standard of 1.482 kBq [^{14}C]IM (107 ng) was added to each aliquot. The amount of IM was estimated from the size of the appropriate fluorescent peak during reverse phase HPLC. The radioactivity in this fraction was quantified by liquid scintillation counting. The amount of IM was then calculated from the following isotope dilution equation (18):

$$X = \frac{([^{14}C]IM \text{ added})\ (IM \text{ detected})}{[^{14}C]IM \text{ detected}} - [(\text{specific activity } [^{14}C]IM)\ ([^{14}C]IM \text{ added})]$$

Application of this equation to the data from the three samples indicated that the *Pinus* seedlings contained 19.7 ± 1.4 ng IM g^{-1} fresh weight (± SD). The IAA pool was determined with the same procedures as described for IM. Thirty g of tissue was homogenized and divided into three equal parts. An internal standard of 558 Bq (48 ng) [2-^{14}C]IAA was added to each sample. The IAA pool was estimated to 175 ng ± 13.5 ng g^{-1} fresh weight

(± SD), which demonstrates that only a minor part of the obtained IM could originate from the IAA pool during sample preparation. The separation systems used, however, do not separate ICA and IAA. The IAA fraction was therefore collected and further analyzed on ion-pair reverse phase HPLC in order to investigate the possible contribution from ICA in the IAA peak. A trace of a possible ICA peak was detected in this system but it did not affect the amount of IAA estimated. The quantitative IM and IAA estimates compare with 24.5 ng IAA g^{-1} fresh weight, 2.3 ng ICA g^{-1} fresh weight, and 46 ng indole-3-ethanol g^{-1} fresh weight calculated to be present in light-grown *Pinus* needles (20, 21). These estimates (20, 21) are based on methanol extraction, and as a consequence not directly comparable with quantifications based on buffer extraction.

To date, relatively few studies have dealed with extraction methods for plant hormones (5). The advantage of buffer extraction is that it reduces the time-consuming purification work. The major disadvantage is the possibility of enzymic conversion during the extraction procedures. The precautions taken in this study to avoid enzymic activity, including ultrasonication, protein precipitation, and working at low temperatures showed an acceptable result according to the low conversion of IAA to IM. This indicates that buffer extraction is suitable for qualitative investigations. In the case of quantification, the estimated value should be considered as an approximative figure as is true for most quantitative hormone estimates.

With ICA and IM identified as endogenous substances in *P. sylvestris*, it may be suggested that a decarboxylate pathway of IAA metabolism exists. However, if IM is to be claimed as a product of IAA catabolism, more evidence is needed. Further studies dealing with enzymic conversion of IAA to IM in cell-free systems, protoplasts, and intact tissue of *P. sylvestris* are required.

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