Phytochemistry, Vol 23, No 1, pp 99–102, 1984
Printed in Great Britain

0031–9422/84 $3 00 + 0 00

ANALYSIS OF 3-INDOLE CARBOXYLIC ACID IN *PINUS SYLVESTRIS* NEEDLES

GORAN SANDBERG, EINAR JENSEN* and ALAN CROZIER†

Department of Forest Genetics and Plant Physiology, The Swedish University of Agricultural Sciences, S-901 83 Umeå, Sweden, *Institute of Biology and Geology, University of Tromsø, N-9001 Tromsø, Norway

(*Received* 13 *May* 1983)

Key Word Index—*Pinus sylvestris*, Pinaceae, Scots pine, analysis, indoles, 3-indole carboxylic acid, 3-indole acetic acid, needles

Abstract—3-Indole carboxylic acid (ICA) has been characterized as an endogenous constituent of *Pinus sylvestris* needles Quantitative estimates of 3-indole acetic acid (IAA) and ICA, corrected for both sample losses and the conversion of IAA to ICA occurring during purification, indicate that *Pinus* needles contain 24 5 ± 6 5 ng IAA/g and 2 3 ± 0 4 ng ICA/g

INTRODUCTION

There are many reports in the literature on the catabolism of 3-indole acetic acid (IAA) in both *in vivo* and *in vitro* test systems In particular, the products and pathways of IAA catabolism in bacterial cultures and horseradish peroxidase preparations have been extensively investigated [1] Although several indoles have been implicated as products of IAA catabolism in higher plants [2], to date only 3-indole carboxaldehyde [3–8], 3-indole carboxylic acid (ICA) [3, 4] and 3-oxindole acetic acid [9] appear to have been identified in higher plant extracts on the basis of strict physicochemical evidence [10] It has, however, been suggested that at least in the Brassicaceae, rather than being natural constituents, indole carboxaldehyde and ICA may be artefacts generated during sample preparation [11, 12] The basis for this proposal is that members of that Brassicaceae contain the enzyme myrosinase along with glucosinolates such as glucobrassicin and neoglucobrassicin Myrosinase catalyses the conversion of glucosinolates to 3-indole acetonitrile which, under the acidic conditions that prevail at certain points during sample purification, can breakdown to produce a range of indoles including IAA which can be further degraded to 3-indole carboxaldehyde and ICA

The purpose of the present study was to investgate the occurrence of ICA in *Pinus sylvestris* and to determine if it is a true endogenous component or merely a breakdown product arising from IAA during sample preparation

RESULTS AND DISCUSSION

Qualitative analysis of ICA in an extract from 1 kg of *Pinus sylvestris* needles was attempted using the procedures outlined in Fig 1 Reverse-phase HPLC of the partially purified sample showed a major peak which co-chromatographed with ICA (Fig 2) This peak was collected and further analysed by normal-phase HPLC

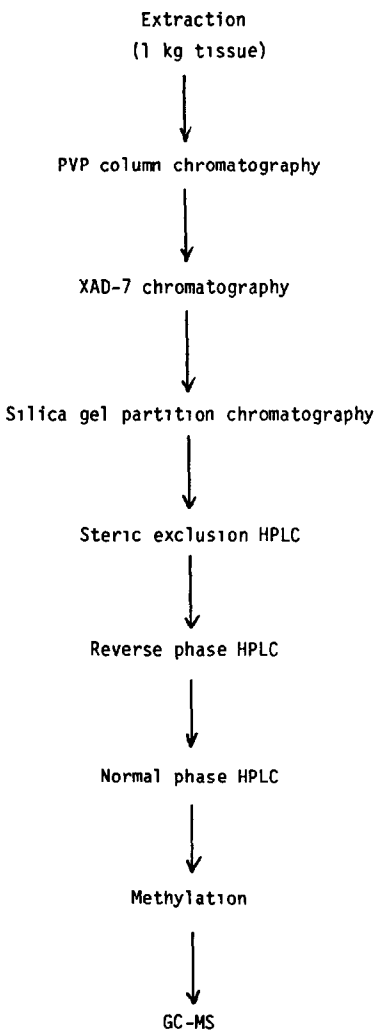

Fig 1 Procedures used for qualitative analyses of ICA in needles from *Pinus sylvestris*

†Permanent address Department of Botany, The University, Glasgow G12 8QQ, U K

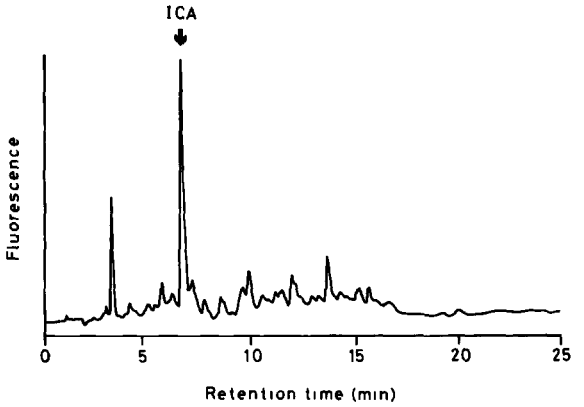

Fig 2 Reverse-phase HPLC of a semi-purified extract from *Pinus sylvestris* needles Arrow indicates peak co-chromatographing with ICA

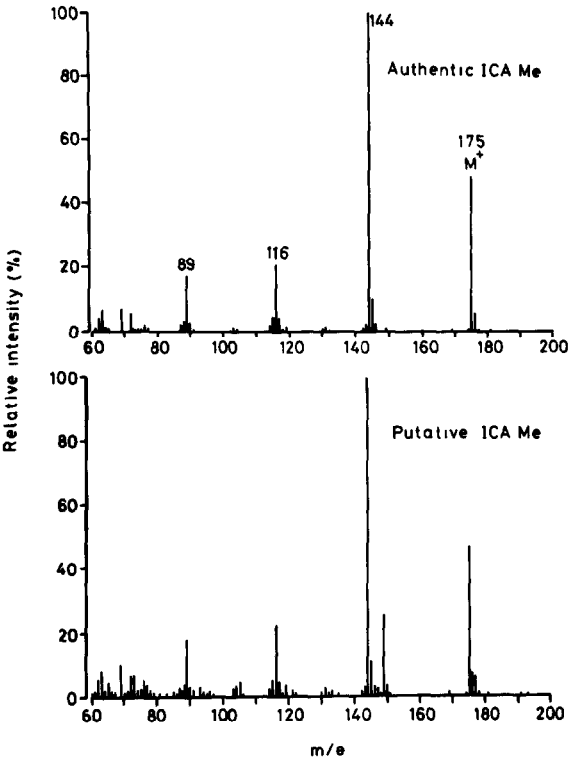

Fig 3 Electron-impact mass spectra of authentic ICA Me and putative ICA Me from a purified methylated extract of *Pinus sylvestris* needles

which showed a single fluorescent component which co-chromatographed with ICA The ICA-like material was methylated and analysed by GC/MS The predominant constituent in the total ion current trace yielded a mass spectrum very similar to that of authentic ICA methyl ester (ICA Me) (Fig 3) It can therefore be concluded that the purified *Pinus* extract contained ICA However, since at least a portion of the ICA pool could have originated from degradation of IAA during sample preparation, it is not possible to conclude further at this juncture that ICA is an endogenous constituent of *Pinus* needles

In order to investigate the possible natural occurrence of ICA in *Pinus* needles, quantitative analyses of ICA and IAA were carried out using the analytical procedures and internal standards shown in Fig 4 Initially, 100 g samples of tissue were extracted along with 25 kBq [2-^{14}C]IAA (2 6 μg) and 10 μg ICA The amounts of unlabelled ICA and IAA remaining at the end of the analytical sequence were estimated from the size of the appropriate fluorescent HPLC peaks while radiolabelled species were quantified from the response of an on-line HPLC radioactivity monitor These data provided a measure of the percentage breakdown of IAA to ICA (Y) calculated from equation (1)

$$Y = \frac{(\text{ICA added})\,([^{14}\text{C}]\text{ICA detected}) \times 100}{(\text{ICA detected})\,([^{14}\text{C}]\text{IAA added})}\ \% \quad (1)$$

In a second series of experiments, 20 g of *Pinus* needles were analysed using 1 5kBq [2-^{14}C]IAA (156ng) as an internal standard As the amounts of IAA and ICA detected were substantially lower than those encountered in the previous experiments, the homogeneity of the reverse-phase HPLC peaks used for quantification was confirmed by normal-phase HPLC Quantitative estimates of endogenous IAA (X) and endogenous ICA (Z) were calculated according to equations (2) and (3)

While processing the data in this manner provides a means of estimating both sample losses and the amount of ICA originating from IAA, the relatively low specific activity of the [2-^{14}C]IAA and the lack of an ICA internal standard labelled with a different radioisotope necessitate a number of compromises in experimental design As a consequence, ICA losses had to be calculated indirectly from [2-^{14}C]IAA recoveries Furthermore, as values of Y could not be determined for individual extracts, allowance for this factor had to be based on a mean value obtained in preliminary experiments in which 25 rather than 1 5 kBq of [2-^{14}C]IAA was used as an internal standard

Application of equation (1) to the data obtained from three analyses of 100 g tissue samples, to which 25 kBq [2-^{14}C]IAA and 10 μg ICA had been added as internal standards, indicated that 0 6 $\pm$ 0 1 % of the IAA pool was broken down to ICA during purification Subsequently, three 20 g samples of *Pinus* needles and 1 5 kBq [2-^{14}C]IAA were extracted and analysed When processed by equation (2), the data obtained indicated an endogenous pool of 24 5 $\pm$ 6 5 ng IAA/g ($\pm$ s d) Equation (3) provided an estimate of 2 5 $\pm$ 0 4 ng ICA/g of which 2 3 ng was calculated to represent endogenous ICA and 0 2 ng assessed as having originated from the breakdown of both

$$X = \frac{(\text{IAA detected})\,([^{14}\text{C}]\text{IAA added})}{([^{14}\text{C}]\text{IAA detected})} - [(\text{sp act } [^{14}\text{C}]\text{IAA})\,([^{14}\text{C}]\text{IAA added})] \quad (2)$$

$$Z = \frac{(\text{ICA detected})\,([^{14}\text{C}]\text{IAA added})}{([^{14}\text{C}]\text{IAA detected})} - (X + [(\text{sp act } [^{14}\text{C}]\text{IAA})\,([^{14}\text{C}]\text{IAA added})] \times Y) \quad (3)$$

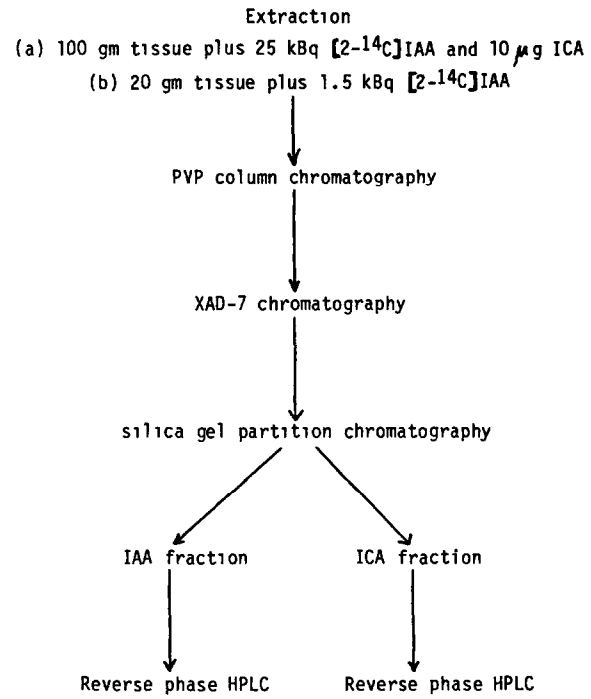

Fig 4 Procedures used for quantitative analysis of IAA and ICA in *Pinus sylvestris* needles

endogenous IAA and IAA added to extracts as an internal standard This is the first report to establish that ICA is a natural constituent of higher plant tissues

EXPERIMENTAL

Plant material Current year needles of Scots pine (*Pinus sylvestris* L) were harvested in the last week of July 1982 from 2-year-old seedlings grown outdoors in mineral wool The plant material was immediately frozen in liquid N_2 and stored at −80° prior to extraction and analysis

Extraction and initial purification Needles were homogenized in −20° MeOH (0 5 g/ml) containing 0 02 mol/l of the antioxidant sodium diethyldithiocarbamate The antioxidant was similarly added to all solvents used in the preliminary purification steps as well as subsequent HPLC analyses The tissue homogenate was extracted for 2 hr at room temp filtered and the methanolic extract reduced to dryness *in vacuo* at 40° The extract was dissolved in 200 ml 0 1 M phosphate buffer (pH 8 0) and partitioned × 5 against half vols of petrol The buffer phase was then run through a 450 × 10 mm i d column of insoluble polyvinylpyrrolidone (PVP) which was further eluted with 300 ml 0 1 M phosphate buffer (pH 8 0) [13] The combined eluant from the PVP column was adjusted to pH 2 7 with phosphoric acid and run through a 150 × 10 mm i d column of Amberlite XAD-7 (300–1000 μm) [14] The column was eluted with EtOH and the 5–30 ml fraction, which contained both ICA and IAA, collected and reduced to dryness prior to HPLC

HPLC An Altex Model 110 pump was used for isocratic analyses while a Spectra-Physics Model 3500 liquid chromatograph was used for separations involving gradient elution Samples were introduced off-column via a Valco injector with a 100 μl loop Column effluent was monitored with a Spectra-Physics SF 970 spectrofluorimetric detector (excitation 285 ± 5 nm, emission 360 ± 10 nm) with a 5 μl flow cell When radioactive components were analysed, effluent leaving the fluorimeter was directed to a Reeve Analytical radioactivity monitor operating in the heterogeneous mode with a 170 μl flow cell packed with a cerium-activated lithium glass scintillator [15] A counting efficiency of *ca* 20% was obtained when ^{14}C-labelled solutes were analysed by reverse-phase HPLC An efficiency of *ca* 80% was obtained with normal-phase HPLC

Samples were initially purified by silica gel partition chromatography using a 500 × 10 mm i d column packed with a 10 m RoSIL silica gel support coated with a 0 5 M HCO_2H stationary phase (40% w/w) [16] The column was eluted at 2 ml/min with a mobile phase of hexane–EtOAc (4 1) saturated with 0 5 M HCO_2H The 14 0–16 5 ml and 16 5–18 0 ml fractions, which corresponded to the elution vols of IAA and ICA respectively, were collected for further analysis

Steric exclusion HPLC was carried out on an Ultrastyrogel support with an exclusion limit of 1500 daltons A 300 × 7 8 mm i d column was eluted with 0 5% HOAc in THF at a flow rate of 1 ml/min ICA had a retention vol of 7 1 ml

A 150 × 4 6 mm i d 5 μm LiChrosorb RP-18 column was used for reverse-phase HPLC analyses Samples were eluted at a flow rate of 1 ml/min with a 25 min gradient of 20–60% MeOH in 1% aq HOAc IAA and ICA had retention times of 6 3 and 6 7 min, respectively Fractions collected from reverse-phase HPLC for further analysis were diluted with 0 1% aq HOAc and absorbed onto a 200 × 4 mm i d Amberlite XAD-7 column, as described in the previous section, and eluted with EtOH prior to being reduced to dryness

Normal-phase HPLC was carried out on a 250 × 4 6 mm i d 5 μm Nucleosil-CN column eluted at a flow rate of 1 ml/min with EtOAc–hexane–HOAc (73 25 2) The retention time of ICA was 5 8 min

Combined GC/MS The instrument used comprised a Hewlett Packard 5710A gas chromatograph linked via jet separator to a VG Micromass 7070H mass spectrometer equipped with a DEC PDP 8A computor system Samples methylated with ethereal CH_2N_2 in 10% MeOH were introduced via an injector at 240° into a 2 m × 2 mm i d glass column packed with 1% SP2100 on a 100–200 mesh Supelcoport support The column temp was held at 120° for 4 min after sample injection and then programmed from 120 to 200° at 16°/min The carrier gas flow rate was 30 ml/min The interface temp was 240° Positive ion electron-impact spectra were recorded at 70 eV with a 1 0 sec/decade scan rate and a 0 5 sec interscan delay

Internal standards When quantitative analyses were undertaken, 10 μg ICA and 10 μg IAA and/or [2-^{14}C]IAA (sp act 1 63 GBq/mmol, Amersham International) were added to methanolic tissue homogenates to facilitate the estimation of sample losses and the conversion of IAA to ICA encountered during purification

Acknowledgements—We wish to thank Karin Lundstrom and Karin Ljung for technical assistance Financial support to G S was provided by the Swedish Council for Forestry and Agricultural Research E J was supported by the Norwegian Research Council for Natural Sciences and Humanities A C received a travel grant from the British Council

REFERENCES

1 Sembdner, G, Gross, D, Liebisch, H-W and Schneider, G (1980) in *Encyclopedia of Plant Physiology*, N S, Vol 9 *Hormonal Regulation of Development I Molecular Aspects of Plant Hormones* (MacMillan, J, ed), p 281 Springer, Berlin
2 Schneider, E and Wightman, F (1978) in *Phytohormones and Related Compounds—A Comprehensive Treatise* (Letham,

D S, Goodwin, P B and Higgins, T J V, eds), Vol 1, p 29 Elsevier, Amsterdam
3 Jones, E R H and Taylor, W C (1957) *Nature (London)* **79**, 1138
4 Prochazha, Z and Sanda, V (1960) *Collect Czech Chem Commun* **25**, 270
5 Isogai, Y, Okamoto, T and Koizumi, T (1967) *Chem Pharm Bull* **15**, 151
6 Bourdoux, P, Vandervorst, D and Hootelé, C (1971) *Phytochemistry* **10**, 481
7 Chowdhury, B K and Chakraborty, D P. (1971) *Phytochemistry* **10**, 481
8 Shindy, W and Smith, O E (1975) *Plant Physiol* **55**, 550
9 Reinecke, D M and Bandurski, R S (1983) *Plant Physiol* **71**, 211
10 Bearder, J (1980) in *Encyclopedia of Plant Physiology*, N S, Vol 9 *Hormonal Regulation of Development I Molecular Aspects of Plant Hormones* (MacMillan, J, ed), p 9 Springer, Berlin
11 Gmelin, R and Virtanen, A I (1961) *Suomen Kem* **34**, 15
12 Gmelin, R (1964) *Collog Int C N R S* **123**, 159
13 Glenn, J L, Kuo, C C, Durley, R C and Pharis, R P (1972) *Phytochemistry* **11**, 345
14 Andersson, B and Andersson, K (1982) *J Chromatogr* **242**, 353
15 Reeve, D R and Crozier, A (1983) *Lab Pract* **32**, 59
16 Crozier, A and Reeve, D R (1977) in *Plant Growth Regulation* (Pilet, P E, ed), p 67 Springer, Berlin