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Auxins in Bean (*Phaseolus vulgaris* L. Cv. Zorin) Seedlings

By

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K e y w o r d s : Bean seedlings, auxin determination.

S u m m a r y

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The amount and composition of auxins were investigated in bean seedlings (*Phaseolus vulgaris* L.) cv. Zorin. Extracts of cotyledon, leaves, stems with shoots and roots were purified with Sep-Pak C_{18} cartridges and analysed by HPLC with fluorescent and photodiode array detectors. Biological activity was determined with an *Avena* bioassay.

The highest amount of indole-3-acetic acid (IAA) was detected in roots. In all analysed tissues bound IAA predominated. HPLC and bioassay confirmed IAA conjugates after hydrolysis with 7 N NaOH. In been seedlings, in addition to free IAA, some conjugates also showed biological activity.

Introduction

Plants contain minute amounts of the phytohormone, indole-3-acetic acid (IAA), as the free acid; this requires a sensitive detection method. IAA is labile and undergoes non-enzymatic oxidation in the presence of light, oxygen, and peroxides. Breakdown of endogenous IAA readily occurs during extraction and purification prior to analysis and it is a common practice to use an isotopically labelled internal standard to assess the extent of such losses (ERNSTSEN & al. 1986).

Techniques for the identification and quantitative analysis of IAA have recently been improved by the utilisation of high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS),

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and by the use of appropriate internal standards. For determination and separation of the indole derivates from biological material HPLC with UV- and fluorometric detectors is very useful (WURST & al. 1984). We combined this method with the *Avena* bioassay (LARSEN 1961) to determine the biological activity of auxins in bean seedlings, and with hydrolysis to confirm the presence of IAA conjugates.

Material and Methods

Seeds of *Phaseolus vulgaris* L. cv. Zorin were germinated in vermiculite at 25°C with a16 h light period. After 14 days, the seedlings were divided into leaves, stems with the apical shoots, apical shoots only and roots, homogenised in liquid nitrogen, freeze-dried at -80°C, and stored at -20°C until further analysis. For the purification and detection of auxins from plant tissues we used the modified method of NORDSTRÖM & ELIASSON 1991. All tests were performed in 2 series in triplicate for each sample. We homogenised tissues from at least 20 plants.

Extraction

100 mg of lyophilised powder with the antioxidant butylhydroxytoluol (BHT) were extracted in 18 ml of 5 mM K-phosphate buffer, pH 6.5, and kept for 1 h at 4°C. The extract was filtered, adjusted to 30 ml and divided into three 10 ml portions.

Purification by solid-liquid extraction

The samples were purified on Waters 500 mg SEP-PAK C_{18} columns that were activated with 10 ml of ethanol (EtOH) for approximately 20 minutes and conditioned with 5 ml of 5 mM K-phosphate buffer in parallel to a pH of 6.5 or 2.5. The 10 ml samples were first run through the columns conditioned to pH 6.5 and washed with 6 ml of 5 mM K-phosphate buffer pH 6.5. The eluate was acidified to pH 2.5 and applied to the columns conditioned at pH 2.5. The columns were rinsed with 2 ml of double-distilled water and eluted with 2 ml of 80% methanol (MeOH).

Determination, separation and quantitation by HPLC

The eluate was concentrated to 400 µl and analysed by a reversed phase HPLC (Nova-Pak C_{18}, 3.9 x 150 mm, Waters). Solvent A was 1% acetic acid; solvent B was 100% MeOH. The flow rate was 1 ml min^{-1}. The column was eluted using a linear gradient of 10-55% of solvent B over 30 min. A fluorescence detector (excitation at 254 nm, emission at 360 nm) and a photodiode array detector (absorption 280 nm) were used to measure the auxins. The retention times of IAA and indole-3-acetylaspartic acid (IAAsp) had been previously measured by the injection of 20 ng of the standard. The amounts were then calculated by comparing the peak areas of the samples to those of the standards.

Recovery

Recoveries were determined by adding 50.000 dpm [^{3}H]IAA (15-30 Ci/mmol, Amersham) before extraction. Losses were also evaluated by comparing the peak areas of standards immediately injected into the HPLC with equal amounts of the standards that had gone through the whole process of extraction and prepurification before injection into the HPLC. The IAA guantities reported were corrected for losses during work-up.

Hydrolysis of samples

IAA conjugates were hydrolysed under nitrogen with 7N NaOH, at 100°C for 3 hours (BIALEK & COHEN 1986, 1989).

The bioassay

The biologically active auxins were determined by the oat coleoptiles bioassay (LARSEN 1961).

Results and Discussion

Auxins in bean seedlings were identified by HPLC, on a column calibrated with standards, by light absorbency spectra and bioassay. The retention time of IAAsp was between the 14th and 15th minute and that of IAA about the 21st minute. The auxin spectrum, however, could change during the analysis and could thus not be used as the only auxin identification method. The bioassay was a useful additional method for the detection of biologically active auxins. The presence of IAA conjugates was confirmed by measuring IAA liberated by alkaline hydrolysis.

In the roots, at least four auxins were found: IAA, IAAsp and two unidentified peaks (Fig. 1). The peak corresponding to IAA, the peaks eluted at the 10th and 18th min, had a characteristic indole spectrum and were active in the bioassay. According to the results of base hydrolysis, the peaks eluted at the 10th min, at the retention time corresponding to IAAsp, and at the 18th min, were IAA conjugates. These peaks decreased or disappeared after hydrolysis of the crude extract, while the peak with the retention time of IAA increased, also showing biological activity. Although the 10 min peak contained impurities, IAA conjugates predominated in the roots. The calculated amount of IAA in roots was 70 ng/100 mg dry weight. As we know from the literature, the predominant forms of IAA in leguminous plants are amide conjugates (BANDURSKI & SCHULZE 1977). Our results confirmed that, in bean seedlings, the amide conjugates accounted for more than 80% of the total auxins (BIALEK & al. 1987, BIALEK & COHEN 1986). In cotyledons, leaves, and stems with apical shoots, the composition of auxins was similar to that in the roots, but their ratio changed (Fig. 2, 3, 4). As there was little analysed plant material (33.3 mg dry weight per replicate) the IAA level was below the detection limit of our method in these tissues.

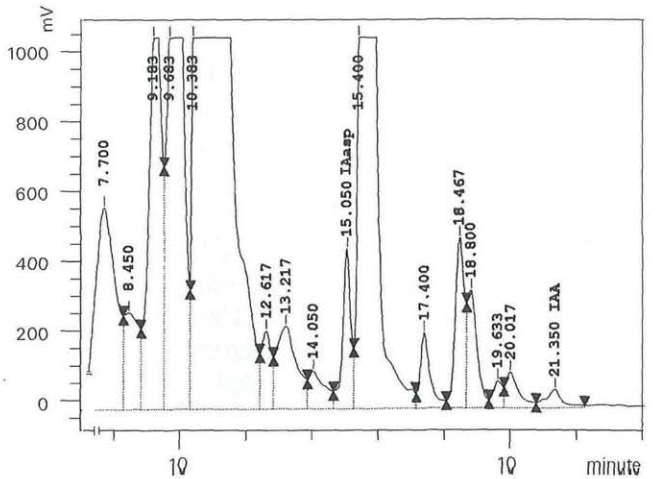

Fig. 1. A representative chromatogram of 33 mg dry weight seedlings roots. The shaded peak areas correspond to IAA and its conjugates.

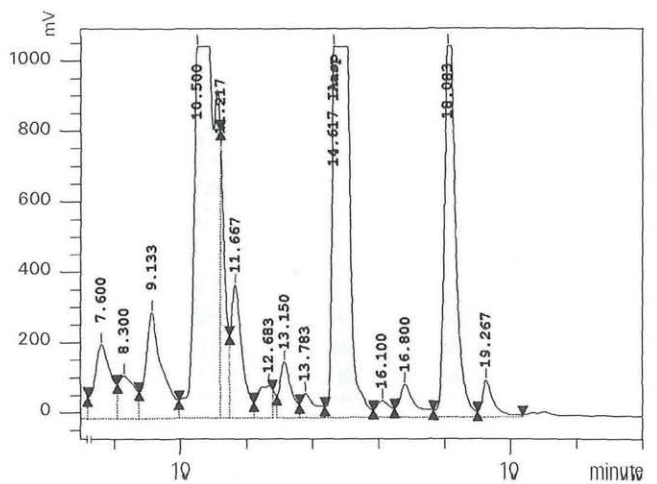

Fig. 2. A representative chromatogram of 33 mg dry weight cotyledons. The shaded peak areas correspond to IAA and its conjugates.

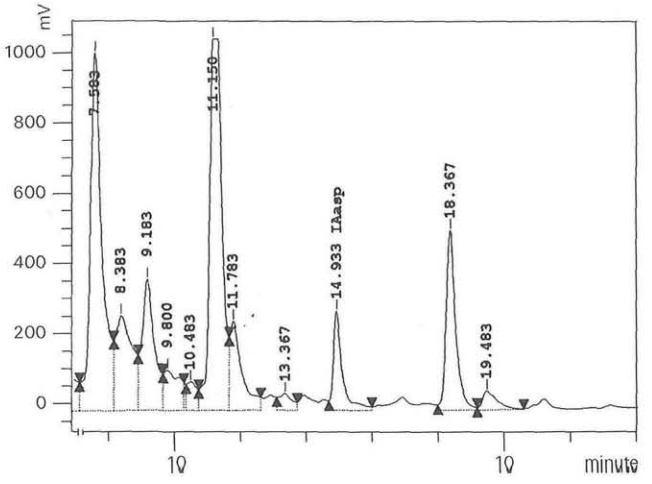

Fig. 3. A representative chromatogram of 33 mg dry weight seedlings leaves. The shaded peak areas correspond to IAA and its conjugates.

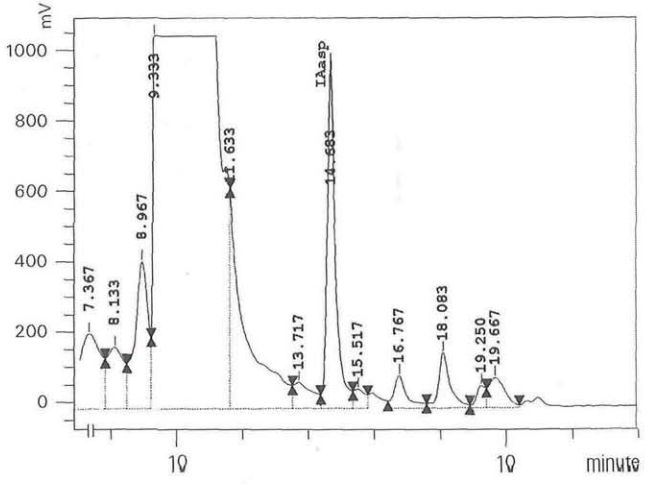

Fig. 4. A representative chromatogram of 33 mg dry weight seedlings stems with apical shoots. The shaded peak areas correspond to IAA and its conjugates.

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