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Levels of indole-3-acetic acid in intact and decapitated coleoptiles as determined by a specific and highly sensitive solid-phase enzyme immunoassay *

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Abstract. A specific solid-phase enzyme immunoassay for the detection of as little as 3–4 pg of indole-3-acetic acid (IAA) is described. The assay involves minimal procedural efforts and requires only standard laboratory equipment. Up to 50 samples in triplicate, processed simultaneously, can be assayed and evaluated in 2.5 h. As little as 1 mg oat coleoptile tissue is sufficient for a quantitative IAA analysis and little or no extract purification is necessary. Using this assay, levels of IAA have been determined in coleoptiles of maize and oat. The distribution of IAA within single coleoptiles was quantitated and the production of IAA during the regeneration of the physiological tip in *Avena* coleoptiles was investigated. The changes in levels of IAA and other major phytohormones were quantitated during the growth of oat coleoptiles.

Key words: Auxin (immunoassay) – *Avena* – Coleoptiles (physiological tip) – Enzyme immunoassay – *Zea*.

Introduction

Basipetal gradients of growth-promoting substances (auxin) have been detected with bioassays in coleoptiles of dark-grown *Avena sativa* L. (Thimann 1934). Auxin produced in the coleoptile tip either by synthesis or release from bound forms (see e.g., Sheldrake 1973) is apparently translocated toward the base of the coleoptile. Growth is assumed to occur at that position in the gradient where the auxin concentration in the coleoptile reaches an optimal level (Went and Thimann 1937). When the tip of a coleoptile is removed, growth ceases for 1–2 h and is then resumed for several hours, even in excised coleoptile segments (Went and Thimann 1937; Anker 1973). This phenomenon is known as the "regeneration of the physiological tip" and is thought to be due to an induction of auxin production in the uppermost parts of the decapitated coleoptile whereby the auxin source of the tissue is regenerated (Anker 1973), although no data are available to support this idea.

Indole-3-acetic acid (IAA) is considered the main, if not the only auxin in coleoptiles of maize and oat (Bandurski and Schulze 1974). However, while the compound has been thoroughly characterized by gas chromatography-mass spectrometry (GC-MS) in diffusates from maize coleoptile tips (Greenwood et al. 1972), unequivocal identification of IAA in coleoptiles of *Avena sativa* L. has not been achieved. IAA has been found along with related compounds in whole, young shoots of dark-grown *Avena sativa* L. by Bandurski and Schulze (1974, 1977) with a GC-MS technique. Is was also detected in coleoptiles of etiolated *Avena* seedlings by the pyrone fluorometric assay (Mousdale et al. 1978).

The assumption that endogenous IAA is involved in the growth regulation of the *Avena* coleoptile is based on a wealth of indirect experimental evidence demonstrating, e.g., that exogenously applied IAA can substitute for the tip (see Went and Thimann 1937) and that it is transported in a strictly polar manner. However, endogenous levels of IAA have only rarely been determined directly, due to difficult analytical procedures which require processing of several grams of tissue (Bandurski and Schulze 1974, 1977; Mousdale et al. 1978; Zimmermann et al. 1976).

The present investigation was carried out in order to obtain more quantitative information on the occurrence and distribution of IAA in coleoptiles and its relation to coleoptile growth. To detect the minute quantities of IAA expected in the tissue, we have, based on a sensitive radioimmunoassay procedure for

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Abbreviations: ABA = abscisic acid; BHT = butylated hydroxytoluene; BSA = bovine serum albumin; IAA = indole-3-acetic acid; TBS = Trishydroxymethylaminomethane buffered saline

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IAA (Weiler 1981), developed a new, still more sensitive and specific enzyme-immunoassay (EIA) for IAA. The assay involves only minimal procedural efforts and equipment and yields quantitative and precise results at picogram levels in less than two hours for large batch assays. The assay principle used here is applicable to any other plant hormone as well.

Materials and methods

Chemicals. Indole acetic acid and p-nitrophenylphosphate (Sigma 104) were purchased from Sigma, Munich, FRG; bovine serum albumin from Serva. Alkaline phosphatase from calf intestine (Grade I, sp. act. > 2,500 U mg^{-1}) was from Boehringer, Mannheim, FRG.

Materials and equipment. The polystyrene solid supports used were Dynatech ELISA-grade "Removawell" cups (No. M 79 A) or Gilford EIA cuvette-packs (No. 1,413 × 79). Absorbance readings were carried out with a Gilford manual EIA reader at 405 nm. Removawell cups were manipulated in styrofoam racks (M76, Dynatech) holding 96 cups each. A compatible shaker for 4 plates (=384 samples) from Dynatech (AM 69) was used to mix reagents. Reagents were added with the Eppendorf Multipette 4780 System.

Plant material. Hybrid maize kernels (*Zea mays* L., cv. 'Anjou 21' from Nordsaat, Münster, FRG) were soaked in running tap water for ca. 24 h and then sown on moist vermiculite. They were kept in darkness for 48 h and subsequently treated with red light (1.6 W m^{-2} from 3 Phillips TL 20 W/15 Red fluorescent bulbs filtered through a Red filter No. 501/3 mm (Röhm and Haas, Darmstadt, FRG) for 1 h. After growth in darkness for another 20–30 h, the coleoptiles achieved a height of ca. 2.0–3.0 cm (average: 2.5 cm) and were thus used in the experiments.

Avena sativa L. cv. 'Victory' (1979 harvest; Svälof, Sweden) caryopses were soaked in running tap water for 10 h and then incubated on wet filter paper in darkness for another 12 h. The germinated caryopses were exposed to red light (as for maize) for 3 h and subsequently transferred to moist vermiculite. The seedlings were grown in total darkness for 70–80 h and harvested when 2.5–3.0 cm long (Went and Thimann 1937).

Processing of plant material. All manipulations were carried out under red light (1.6 W m^{-2}). For the determination of the gradient of diffusable IAA in maize or *Avena*, coleoptiles of 2.5–3.0 cm in length were used. These were cut at the node, placed on a cooled glass plate, and nicked 1 cm from the tip. The upper region of the coleoptile was removed without damaging the primary leaf and the latter was then pulled out. The entire coleoptile was then sectioned with an extra-thin razor blade. Six sections were normally cut, the two uppermost of 0.25 cm and the rest of 0.5 cm each. The coleoptile sections were then transferred to pre-weighed vials containing 2 ml water and kept on ice until 10–20 segments had been collected. The vials were weighed again to allow for fresh-weight determination of the segments and kept in darkness at room temperature for 3 h. Subsequently, the water was removed with a pipet and transferred to glass tubes containing 20 µl of a 1 mg ml^{-1} ethanolic solution of butylated hydroxytoluene (BHT). The aqueous solution was dried down under air, redissolved in 200 µl methanol and treated with an excess of ethereal diazomethane. The methylated residue was dried down and taken up in 1 ml 0.1 M Tris, pH 7.5. When single coleoptiles were used for diffusable IAA determination the cut segments were transferred to pre-weighed tubes containing 0.2 ml H_2O. The coleoptiles were removed after 3 h at room temperature and the water residue treated as above.

To determine extractable IAA, the coleoptile segments were kept cold during harvest in pre-weighed glass vials and their weights were immediately determined. They were then frozen in liquid N_2, ground, and extracted in the organic solvents (80% methanol or 70% acetone) for varying periods of time (see Results). After removal of the extracted tissue by centrifugation, the extracts were processed exactly as above. All extracts were stored at −18° C and assayed within 48 h.

For chromatographic resolution of the various coleoptile diffusates and extracts, 100 coleoptile tips of 0.5 cm length were placed in 5 ml water (for diffusates) or frozen in liquid N_2 (for extracts), the former were kept at room temperature for 3 h and the latter were extracted 3·1 h with methanol. The solutions were concentrated in vacuo after the addition of BHT, methylated, and applied to a Silica gel plate. Two IAA methyl ester reference spots were applied at either side of the streaked extracts after a 2-mm section of the silica surface had been scraped-off along the length of the plate to prevent contamination of the extract with the reference during development. The plate was developed either in $CHCl_3$:methanol (9:1) or $CHCl_3$:ethyl acetate (99:1) with chamber saturation. After development, 0.5 cm strips of the plate were cut, placed in 2 ml methanol and incubated overnight at 4° C. The strips were then removed, the absorbance of the eluates monitored at 254 nm and the solvent evaporated under air. The dried residue was re-dissolved in 0.5 ml 0.1 M TRIS, pH 7.5.

For studies on the growth of decapitated coleoptiles, oat seedlings were germinated as described above and grown until they had reached a height of ca. 2.5 cm. The uppermost 1 mm of each coleoptile was removed with a thin razor blade, and the seedlings were left in darkness. After varying periods of time, the upper 5 mm of the coleoptiles were harvested and either placed in water to allow for IAA diffusion or frozen in liquid N_2 for extraction with 70% acetone.

Growth measurements. Growth rates were determined from photographic recordings. During exposure, plants were illuminated with red light (1.6 W m^{-2}).

Determination of total IAA in maize and oat. The procedure of Bandurski and Schulze (1974) was followed in the extraction of 5-d-old, dark-grown seedlings of maize and oat, with the exception that the tissue was frozen in liquid nitrogen and then ground in a mortar prior to addition of 70% acetone. Two grams of oat and 5 g of maize tissue were used in the experiments.

Hormone spectrum of oat coleoptiles. Caryopses were sown on vermiculite as described above. The point of germination was reached when the radicules were ca. 1 mm long. Starting at 28 h after germination and every 9 h thereafter, 50 coleoptiles were separated from the primary leaf and frozen in liquid N_2, extracted 3·1 h in 80% methanol, and then processed as all other extracts. Hormones in the extracts were immunoassayed as described previously (Weiler 1980a, b; Weiler and Wieczorek 1981; Weiler and Spanier 1981).

Preparation of IAA-specific antibodies. Anti-IAA antibodies were obtained by immunization of rabbits with an IAA-bovine serum albumin conjugate as described earlier (Weiler 1981). From the fraction of the antiserum previously characterized by radioimmunoassay the antibodies were isolated by the standard method of precipitation with ammonium sulfate (Hurn and Chantler 1980). Briefly, the immunoglobulins were precipitated in 45% sat. $(NH_4)_2SO_4$ and the pellet collected by centrifugation, washed with half-saturated ammonium sulfate, dialyzed against 10 l H_2O for 24 h, and finally lyophilized. From 10 ml antiserum, approximately 200 mg lyophilized material were recovered. The antibodies were stored at −18° C.

Coupling of IAA to alkaline phosphatase. The enzyme tracer for the EIA was prepared by coupling IAA through its carboxyl group to amino residues on the enzyme with the carbodiimide method. IAA (10 µmol) was dissolved in 0.1 ml dimethylformamide and diluted with 0.1 ml H_2O. Fifteen µmol of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) in 0.1 ml water were then added and the solution adjusted to pH 6.4 with 0.01 M NaOH. After stirring continuously at room temperature for 1 h this solution was added, in 20-µl aliquots over a period of 90 min, to a solution of the alkaline phosphatase consisting of 0.1 ml enzyme (1 mg protein) and 0.2 ml 50% dimethyl-formamide (DMF) in H_2O, pH 6.4. The reaction was continued at room temperature, in the dark, for 15 h. The slightly turbid solution was then dialyzed first against 1 l 10% DMF in H_2O for 8 h and then against 3·5 l TRIS buffered saline (TBS: 50 mM Tris, 1 mM $MgCl_2$, 0.01 M NaCl, pH 7.5) for 3 d at 4° C. There was no loss of enzyme activity under these conditions.

Adsorption of antibodies to polystyrene. The property of protein to adsorb to polystyrene surfaces was used as the basis for the solid-phase EIA. The standard procedure for coating was as follows: The lyophilized immunoglobulins were dissolved in 50 mM $NaHCO_3$ pH 9.6 to a concentration of 10 µg ml^{-1}. This solution was incubated with the solid supports for 15 h at 4° C; 0.2 ml of the coating solution were used in every cup (Dynatech) and 0.3 ml in every cuvette (Gilford). After incubation, the solution could be saved for re-use. The coated surfaces were then incubated at room temperature for 15 min with 0.01% BSA in TBS to block any remaining protein adsorption sites. The surfaces were then washed with TBS and immediately used in the immunoassay.

Preparation of standards. A stock solution of IAA methyl ester was prepared by methylating 1.75 mg IAA with ethereal diazomethane and dissolving the product in 1 ml MeOH. The solution was stored at −18° C and was stable for at least 3 months. A 1:10 dilution of the stock was made in MeOH and subsequent dilutions were done in water to give a final range of 0.01–50 pmol/50 µl. The standard solutions were stored at 4° C and were prepared fresh every week.

Immunoassay procedure. For the enzyme immunoassay, all solutions were maintained at 4° C. To each of the antibody-coated surfaces was added first 100 µl TBS (cups) or 200 µl TBS (cuvettes). Then, in a carefully timed sequence, 50 µl of the standards or 50 µl sample were added. The B_0 value (=maximum enzyme binding to coated surface) was determined in the absence of a standard by using 50 µl H_2O, and for the determination of unspecific binding (UB) an excess of IAA standard (200 pmol) was added. After mixing for 1 min the incubation vessels were kept at 4° C for 30 min. The appropriately diluted enzyme tracer (50 µl/cup) was subsequently added to all vessels and these were again mixed for 1 min and then incubated at 4° C for another 30 min. The solutions were then removed by aspiration and the vessels were rinsed twice with 0.25 ml TBS. For the assay of surface-bound alkaline phosphatase activity, 0.2 ml of a 1 mg ml^{-1} solution of p-nitrophenyl-phosphate in 50 mM $NaHCO_3$, pH 9.6, freshly prepared and equilibrated at 30° C, was added to each cup (0.3 ml to each cuvette). The enzyme reaction proceeded at room temperature for 60 min and was stopped with 50 µl of 5 M KOH per cup. The reaction solutions from cups were then transferred to Gilford EIA cuvettes containing 0.25 ml water and readings were then taken at 405 nm with the EIA manual reader (Gilford). Alternatively, when coated cuvettes were used, they were read directly. However, it is also equally possible to use any standard colorimeter or spectrophotometer. This procedure results in practically the same assay sensitivity when either cups or cuvettes are used.

The quantitative determination of IAA using the enzyme-immunoassay is based on the relationship between the enzyme activity in incubations with antibody but without added IAA and that of similar incubations with increasing concentrations of a standard IAA solution. This relationship can be represented by the following formula:

$$\frac{A_B - A_{UB}}{A_{B_0} - A_{UB}} \times 100 = \%(B/B_0).$$

Where A_B is the absorbance at 405 nm in the presence of a known amount of IAA (or in a given unknown sample); A_{UB} is the absorbance in the presence of a large excess (200 pmol) of IAA; and A_{B_0} is the absorbance in the absence of IAA. Linear standard curves which are to be preferred for manual evaluation of assay results are obtained by the logit-transformation of the relative binding parameter:

$$\text{logit}\,(\%\ B/B_0) = \ln \frac{B/B_0}{100 - B/B_0}.$$

In this study, a computerized result calculation was used and standard curves were generated by the spline-approximation method (Marschner et al. 1974; see Fig. 3) using the sigmoidal plot.

Results

Principle of method. One of the aims of the present study was to introduce immunological procedures even more sensitive and convenient than the radioimmunoassay to the analysis of plant hormones. The design of the assay should permit maximum reliability with minimal procedural effort and require inexpensive, standard laboratory equipment. We have, therefore, chosen a solid-phase enzyme immunoassay technique. Whereas, in the radioimmunoassay, a single radiolabeled antigen molecule gives rise only to very few decay events by which it can be detected, an enzyme label introduces a considerable intrinsic amplification factor. The use of enzyme rather than radioactive labels permits the application of the assay without complying with the regulations associated with the use of radioisotope. For the assay, polystyrene surfaces are uniformly coated with anti-IAA immunoglobulin (see Fig. 1). The subsequent steps are all carried out in a single reaction vessel, and all

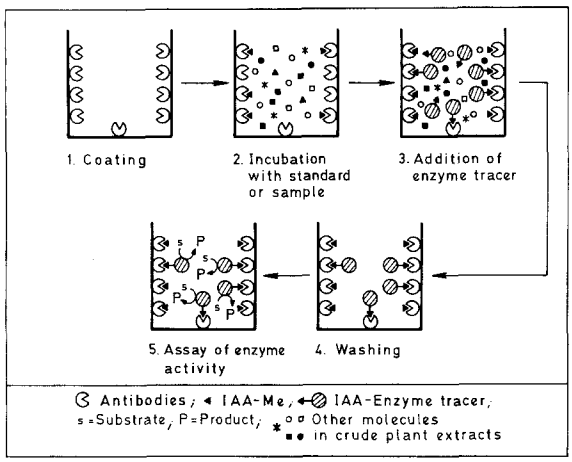

Fig. 1. Performance of the IAA solid-phase enzyme immunoassay

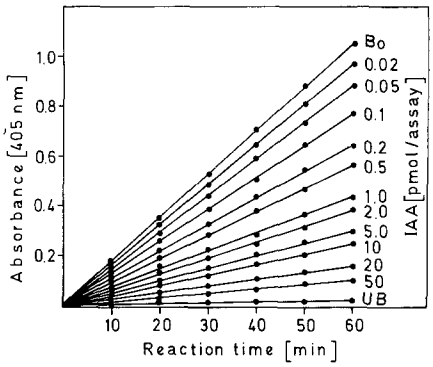

Fig. 2. Time course of p-nitrophenyl phosphate hydrolysis by IAA-alkaline phosphatase bound to IAA-antibodies on solid phase. Different amounts of enzyme had bound to the antibodies depending on the concentration of IAA included in the immunoassay

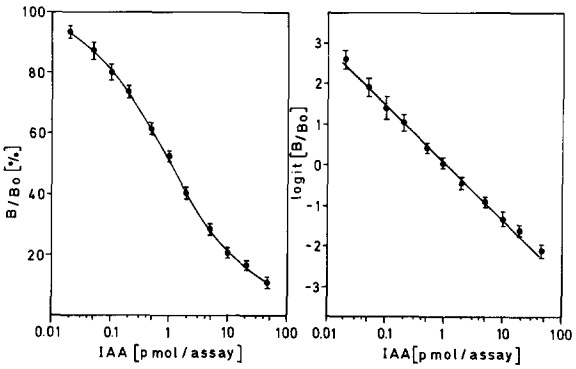

Fig. 3. Standard curves for the IAA solid phase enzyme immunoassay in the sigmoidal and linearized form. The standard was IAA-methyl ester. B = binding of tracer to antibody in the presence of IAA; B_0 = binding in the absence of IAA. Logit $B/B_0 = \ln [(B/B_0)/(100 - B/B_0)]$

separation steps are performed simply by aspiration, thus, the assay requires only a pipet and a simple, inexpensive colorimeter. The polystyrene cups or cuvettes used in our study as solid supports are both compatible with elaborate systems of assay mechanization which are commercially available. Large scale assays can be designed with both systems in which special spectrophotometers, interfaced with desk-top computers allow instantaneous calculation and a printout of results.

Basic parameters of assay. A range of immunoglobulin concentrations for the optimal coating of the polystyrene surfaces was tested and 10 μg ml^{-1} protein gave optimal results. The solution could be reused once and was then exhausted. The amount of tracer used per cup or cuvette was chosen arbitrarily to give a maximum antibody-bound activity under standard assay conditions of 2.7 pkat ($A_{405} = 1.0$ in 1 h, corresponding to a bound activity equivalent to 11% of the total activity added). Under these conditions, unspecific binding was 8% of the bound activity ($A_{405} = 0.08$ in 1 h). Thus, the useful absorbance range for a standard curve was ca. 0.9 when the enzyme reaction was terminated after 60 min (Fig. 2). Prolonged incubation times allow the use of even less tracer, with a concomittant rise in assay sensitivity. The indole-acetic acid-alkaline phosphatase conjugate (IAA-AP) had an immunoreactivity of 65% as checked by the total enzyme activity bound specifically to an excess of antibody. The IAA-AP when stored at −18° C in 50% glycerol did not lose any enzyme activity or immunoreactivity within 6 months. Stored in lyophilized form, it does not deteriorate for at least 2 years. Thus, a large batch of enzyme labeled IAA, once prepared, lasts for a much longer period of time than any radiolabeled IAA-derivative of high specific activity.

Assay sensitivity. As compared to the RIA procedure for IAA (Weiler 1981) this enzyme-immunoassay exhibits a ca. tenfold higher sensitivity. For maximum sensitivity, both methods use IAA methyl ester as standard and methylated samples. This has the additional advantage of higher stability of IAA methyl ester as compared to the free acid. As Fig. 2 demonstrates, as little as 20 fmol (3.5 pg) IAA sufficiently reduce the surface-bound enzyme activity in the cuvettes to be readily detectable. The standard curve extends from 20 fmol to 50 pmol (3.5 pg–9 ng) of IAA (Fig. 3). Thus, the sensitivity of this assay matches the most sensitive physico-chemical procedure for IAA presently available (Crozier et al. 1980).

Assay capacity and accuracy. In contrast to physico-chemical methods, which are 'one-channel' methods, immunoassays uniquely provide the possibility of batch-processing of samples. The assay described here is routinely used in our laboratory with manual pipetting and sample reading to process and evaluate 50 samples (plus standards) in triplicate within 3 h time. Automated equipment for the entire assay procedure has been used in test trials to process and evaluate as much as 200 samples in triplicate within 5 h. Recovery of IAA added to extracts before processing averaged 90%. The coefficient of variation for sample triplicates throughout the measuring range is 5.3%. Between-assay coefficients of variations ($n = 6$ assays) averaged 9.2% throughout the measuring range. The EIA correlates closely with the RIA procedure ($r = 0.932$, slope $= 0.96$, $n = 39$).

Assay specificity. The results of a detailed cross-reactivity study are presented in Table 1. As compared to the RIA procedure (Weiler 1981) this assay is slightly more specific. Structurally related indoles and IAA-derivatives, when present in roughly compa-

Table 1. Cross-reactivities of anti-IAA antiserum adsorbed in solid phase, on a molar basis (all compounds diazomethane-treated)

Compound	Cross reactivity (%)
Indole-3-acetic acid	100
Indole-3-acetone	35
Indole-3-butyric acid	10.2
Indole-3-acetamide	8.6
1-Naphthylacetic acid	6.7
Indole-3-acetyl-L-aspartic acid	6.5
Indole-3-acetaldehyde	6.3
Indole-3-acetonitrile	2.8
Indole-3-propionic acid	2.5
Indole-3-pyruvic acid	1.5
Indole-3-lactic acid	0.5
Indole-3-acrylic acid	0.5
1-Naphthylacetic acid-β-D-glucosyl ester	0.5
Indole-3-acetyl-β-D-glucosyl ester	0.5
Indole-3-aldehyde	0.3
5-Hydroxyindole-3-acetic acid	0.3
Indole-3-glyoxylic acid	0.2
Tryptamine	0.2
Indole-2-carboxylic acid	0
Indoxyl-β-D-glucoside	0
2-Naphthylacetic acid	0
2,3-Dichlorophenoxyacetic acid	0
2,4-Dichlorophenoxyacetic acid	0
3,5-Dichlorophenoxyacetic acid	0
Phenylacetic acid	0
Imidazoleacetic acid	0
Urocanic acid	0
L-Tryptophan	0
D-Tryptophan	0

rable amounts, will not or only slightly affect the IAA value obtained in a crude extract. However, excessive amounts of those compounds will have to be removed prior to the IAA determination. It has been found sufficient in these cases to prepare acidic ether fractions of extracts. These can be assayed directly.

Levels of IAA in shoots and coleoptiles of maize and oat

a) Levels of extractable IAA in shoots of maize and oat. For comparison of the results of our assay with published data, 5-d-old, dark-grown shoots of maize (*Zea mays* L. c.v. 'Anjou 21') and oat (*Avena sativa* L. c.v. 'Victory') were extracted according to the method of Bandurski and Schulze (1974). From the acetone extract, an acidic ether fraction was prepared, methylated, and assayed directly thereafter for free IAA. This fraction was separated by TLC and it was found that besides the main immunoreactive peak corresponding to IAA-methyl ester, two minor peaks of immunoreactive material were also present. The

Table 2. Concentrations of free and base-labile IAA in shoots of maize and oat (FW = fresh weight)

Material	Free IAA pmol g^{-1} FW	"Bound" IAA pmol g^{-1} FW	Reference
Avena sativa L.			
5 d old etiolated shoots	92	29	Bandurski and Schulze 1974
4 d old etiolated shoots	465	4,747	Zimmermann et al. 1976
3.5 d old coleoptiles	115–126	–	Mousdale et al. 1978
5 d old etiolated shoots	98	126	this paper
Zea mays L.			
5 d old etiolated shoots	138	1,885	Bandurski and Schulze 1974
5 d old etiolated shoots	1,034	1,839	this paper

value for free IAA in Table 2 is accordingly corrected for such interference. The residual extract was hydrolyzed in 1 M NaOH for 1 h at room temperature, re-extracted, and methylated to determine the alkali-labile IAA. Table 2 compares the EIA data obtained in the crude fractions with the literature values for which highly purified material had to be used. The values are in general agreement except that we have found a higher value for free IAA in our maize variety.

b) Distribution of extractable and diffusable IAA within the coleoptile of maize and oat. Tips of 2.5 and 5 mm length of maize and oat coleoptiles (2.5–3.0 cm long) were kept in water in the dark for 3 h at room temperature. Repeated control experiments revealed that under these conditions, a linear increase in IAA concentration in the incubation medium over a period of 8 h for maize and 4 h for oat occurred. From the experiments (see Table 3) a maximum rate of IAA release, in the shorter tip preparations, of 7.3 pmol h^{-1} (maize) and 0.84 pmol h^{-1} (oat) was calculated. The values compare favorably with those estimated from bioassay data (5 pmol h^{-1}) for maize coleoptiles (Gillespie and Thimann 1963) and with the data of Bandurski (1979) who calculated an IAA flow per hour and per shoot in the order of 5–10 pmol. Thin layer chromatography (TLC) analysis of the crude diffusates which were assayed after methylation, revealed that the majority of the immunoreactive material in both maize and oat cochromatographed with authentic IAA methyl ester thus proving the validity of the results (c.f. Fig. 4 and 5).

Table 3. Levels of diffusable IAA in coleoptile tips of maize and oat

Species	Length of tips (cm)		Diffusable IAA (pmol/tip h)	Duration of IAA release (h)	Total release of IAA (pmol/tip)
Zea mays L. (Coleoptiles of 2.5–3.0 cm in length)	0.25		5.3 9.6 5.1 8.2 8.3		
		average:	7.3 ±2.0	8	58.4
	0.50		2.5 1.9 2.6 3.1		
		average:	2.5 ±0.50	8	20.0
Avena sativa L. (Coleoptiles of 2.5–3.0 cm in length)	0.25		0.61 0.72 1.20		
		average:	0.84±0.30	4	3.4
	0.50		0.58 0.58 0.70		
		average:	0.62±0.07	4	2.5

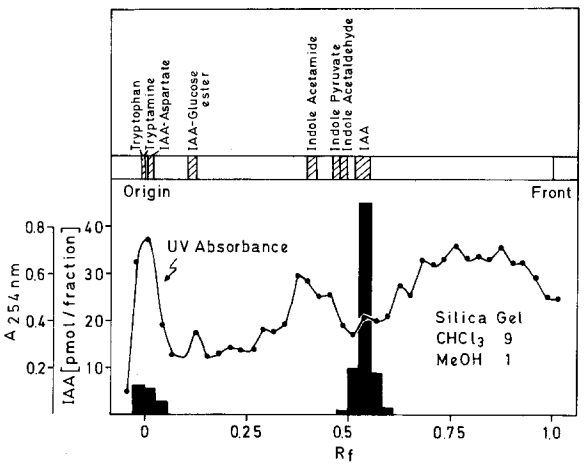

Fig. 4. Distribution of immunoreactivity on chromatograms of water diffusates from *Zea mays* L. coleoptile tips. The crude solution was methylated prior to chromatography (References treated the same)

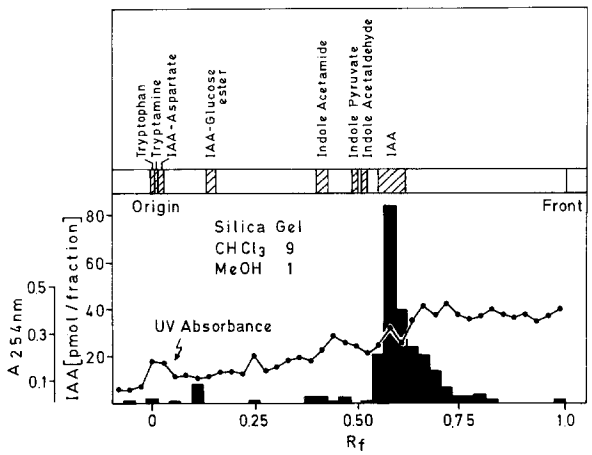

Fig. 5. As Fig. 4, but for *Avena sativa* L.

It is known that bacterial production of IAA from tryptophan occurs under non-sterile conditions and that this can account for a considerable fraction of the IAA extracted from non-sterile tissue (Libbert et al. 1968). To determine if such bacterial activity would lead to any additional production of IAA during the collection of diffusates, the following control experiments were carried out. Coleoptile tips of maize and oat were incubated in the presence of 25 and 50 μM tryptophan and tryptamine for 3 and 6 h, respectively; in addition, intact coleoptiles were placed with their tips down in tryptophan and tryptamine solutions. Up to 3 h, no additional release of IAA in the presence of either tryptophan or tryptamine was observed. After 6 h, significantly increased values were observed for tips and inverted coleoptiles placed in tryptophan (total conversion: $1.2 \cdot 10^{-3}$–$2.2 \cdot 10^{-3}$% of the tryptophan present for inverted coleoptiles and $3.8 \cdot 10^{-3}$–$3.0 \cdot 10^{-3}$% for the tips). In tryptamine, no effect was observed after 6 h. Similar results were obtained for oat. Up to 3 h (=standard incubation time) in both inverted coleoptiles and tip incubations, no detectable production of additional IAA occurred in the presence of 50 μM tryptophan.

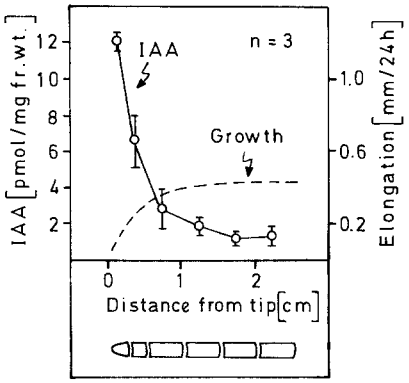

Fig. 6. Distribution of endogenous, diffusable IAA in single coleoptiles of *Zea mays* L. cv. 'Anjou 21'. Bars represent ± s.d. for three different coleoptiles. The segments were placed in 200 µl water and the IAA allowed to diffuse for a period of ca. 6 h at room temperature in the dark. The growth pattern was determined from photographic measurements of marked coleoptiles

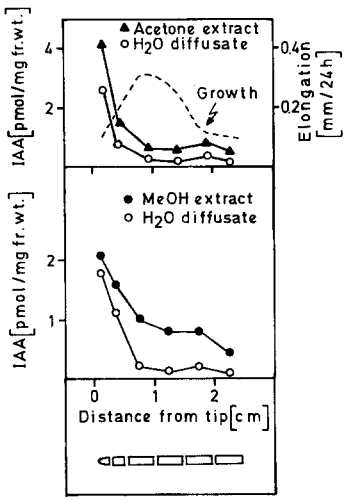

Fig. 7. Extractable and diffusable IAA content from *Avena sativa* L. cv. 'Victory' coleoptiles. 10–20 coleoptiles were combined in an average experiment. 2.5 cm long coleoptiles were dissected as shown and either placed in water or directly extracted into acetone or methanol after freezing in liquid N_2 and grinding. The growth pattern was determined from photographic measurements

The total extractable IAA in the tips was compared with the maximum yield obtained by the diffusion method. For 2.5 mm tips of oat coleoptiles, acetone extracts gave, after base hydrolysis and ether extraction, a total IAA value of 3.7 pmol/tip. This compares with the maximum yield of 3.4 pmol/tip obtained by diffusion. Of the extractable IAA ca. 60–70% represents the free acid, the remainder is released after base hydrolysis in 1 M NaOH. Prolonged extraction (>2 h) with 80% methanol, but not with 70% acetone, yields additional and variable amounts of IAA in both tips and basal parts of coleoptiles, especially when the tissue is homogenized.

For maize it was found that of the total amount extractable in 70% acetone per tip (59 pmol), ca. 83% is present in alkalilabile conjugated form, 17% as free IAA. This material would account completely for the maximum yield of diffusable IAA (58 pmol per tip) obtained. However, as in *Avena*, long-term methanolic extraction released additional quantities of IAA from the tissue.

The high sensitivity and specificity of the enzyme immunoassay allowed a re-examination of the early bioassay data for the distribution of diffusable and, in addition, extractable IAA in coleoptiles (Went and Thimann 1937). Single tips or segments are sufficient for a quantitative analysis by our method. Figure 6 shows, on a fresh weight basis, the distribution of diffusible IAA within three single, 2.5-cm long maize coleoptiles. Figure 7 compares 2 typical experiments for diffusable and extractable IAA in 2.5-cm long *Avena* coleoptiles.

The hormone spectrum of developing Avena sativa L. coleoptiles. Coleoptiles, harvested at different growth stages were extracted exhaustively in 80% methanol

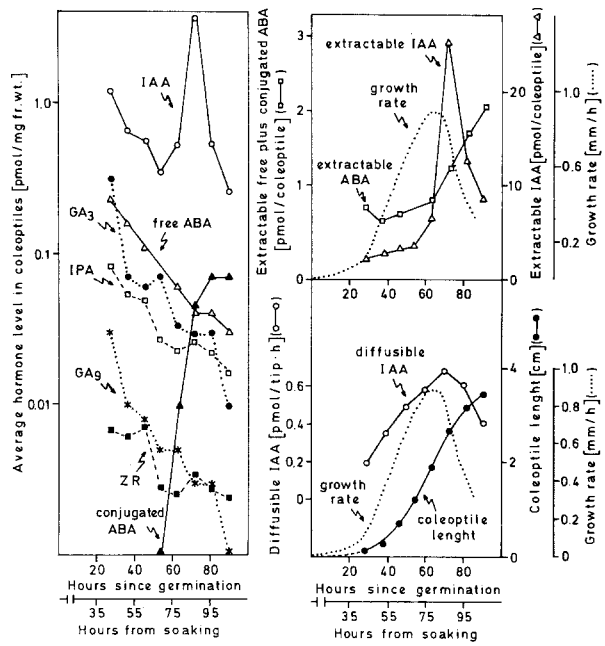

Fig. 8. Left: Levels of the principal growth regulators in growing oat coleoptiles. Right: correlation of coleoptile growth with diffusable IAA from tips (below) as well as with extractable IAA and ABA (free plus conjugated, above)

(3·1 h) and the extracts were assayed for a range of plant hormones by immunoassays (Fig. 8). In general, higher average concentrations of the hormones were found in young coleoptiles and their levels decreased during growth with two exceptions: a) the level of extractable IAA sharply rises 54 h after germination (coleoptile length: 1.2 cm), reaching a maximum at ca. 72 h (average coleoptile length of 2.7 cm),

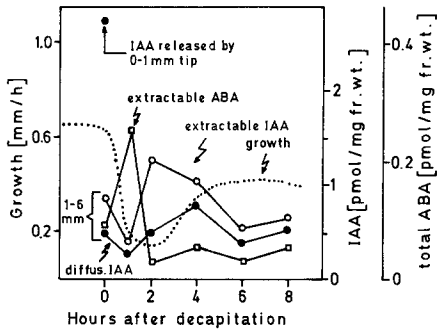

Fig. 9. Changes in the levels of diffusable and extractable IAA as well as extractable ABA (free plus conjugated) occurring during the 'regeneration of the physiological tip' in *Avena*. Coleoptiles were decapitated and the IAA and ABA contents of the upper 5 mm segments determined over an 8 h period (average from 2 typical experiments)

and then it decreases rapidly. b) Again, at a coleoptile length of 1.2 cm (maximum growth rate), the total amount of ABA extractable per coleoptile rises (Fig. 8). This is mainly due to an accumulation of conjugated ABA which became detectable in 1.2-cm-long coleoptiles. Thus, when the growth rate of the coleoptiles becomes maximum, a qualitative and quantitative change in ABA turnover occurs. In coleoptiles of standard length (2.5–3 cm), free and conjugated ABA is concentrated in the segment from 2.5–5 mm behind the tip and its level declines toward the tip and the base of the coleoptile (data not shown). Thus, ABA is clearly associated with the main growth area of the coleoptile.

The presence of free and conjugated ABA in coleoptiles was further confirmed by an immunoreactivity-distribution study (cf. Weiler 1980a) on thin-layer chromatograms of crude coleoptile extracts. Two immunoreactive fractions, cochromatographing with free ABA (R_f=0.51 in the solvent system toluene/ethyl acetate/acetic acid = 50+30+4) and with the polar ABA conjugates (R_f=0) were observed. Besides these two bands, no further immunoreactive material was detected on the plates. This strongly indicates the absence of any unspecific interference in the ABA values obtained for crude extracts (Fig. 8).

Furthermore, when coleoptile sections (0–12.5 mm) were incubated for 3 h at room temperature in the dark in 0.6 M mannitol, the level of free ABA increased significantly (60–100% in diverse experiments) as compared to controls. The levels of conjugated ABA did not change. A maximum of 10% of the total ABA found in the controls and in the mannitol-treated preparations was recovered from the incubation medium, ca. 90% were associated with the tissue. Thus, unlike IAA, ABA appears not to be readily exchangeable with the extracellular environment.

Levels of IAA and ABA in decapitated coleoptiles of Avena sativa L. Avena coleoptiles were decapitated as described in Materials and Methods and the changes in IAA and ABA concentration in the uppermost 5 mm segments were determined at various times thereafter. The average results from two typical experiments are shown in Fig. 9. It can be seen that after removal of the tip, both extractable and diffusable IAA decline (although not to zero) in the first hour following decapitation. Thereafter, both diffusable and extractable IAA rise significantly, followed by resumption of growth of the decapitated coleoptiles. Levels of extractable ABA (free plus conjugated) rise to a sharp peak one hour after decapitation and then decline to below the time-zero level (Fig. 9).

Discussion

Enzyme-immunoassay. Radioimmunoassay, recently introduced into the analysis of a variety of plant hormones (Pengelly and Meins 1977; Weiler 1979; Weiler 1980a, b; Walton et al. 1979; Weiler and Wieczorek 1981; Weiler 1981; Weiler and Spanier 1981) allows a rapid and reliable quantitative determination of several important hormones side by side (e.g., Weiler and Ziegler 1981). The technique for IAA has, however, been the least satisfactory of these assays because of the instability of the labeled IAA (Pengelly and Meins 1977) and the comparatively low sensitivity that was achieved (Pengelly and Meins 1977; Weiler 1981). The assay described here eliminates these drawbacks in that a) it employs enzyme-labeled IAA which has proven stable for more than one year either in lyophilized form or in solution, and b) it readily detects 20 fmol (3.5 pg) of IAA. This assay has a sensitivity comparable to the most sensitive fluorimetric procedure of Crozier et al. (1980), which allows the detection of ca. 1 pg of IAA but is time consuming and requires highly purified, high pressure liquid chromatography (HPLC) separated, IAA for a reliable analysis. The EIA can be performed in much cruder fractions, sometimes even in unpurified material (see Fig. 4 and 5). The problem of instability of IAA has been solved by using the IAA methyl ester as a standard and by methylation of extracts. The assay variability (5% average coefficient of variation for triplicate determinations) is comparable to the RIA procedure but it can be further improved by carrying out the sample incubation (step 2 in Fig. 1) as an equilibrium reaction instead of the short incubation used here which requires careful timing. Under equilibrium conditions, the background variability is reduced to allow the detection of 10 fmol (<2 pg) of IAA reproducibly. The main advantage of the solid-phase enzyme immunoassay, as described here,

over the radioimmunoassay is that it requires only minimal equipment and that its use is not restricted to special isotope laboratories. Its sample capacity is comparable to the RIA with the additional advantage that, whereas the RIA is limited by the time required to obtain a valid radioactivity determination (usually at least 2–4 min per sample), the manual absorbance readings of the EIA in the specially designed colorimeter we used takes a maximum of 16 min for 100 samples plus standards. This feature renders the assay ideally suited for on-line computation of results. Both types of solid support that we used to adsorb the antibody are comparable in sensitivity and reproducibility. They represent miniaturized systems for processing many samples, require minimum space and consume reagents economically. Extension of this approach to other phytohormones shows that the EIA is in principle applicable to the other classes of hormones as well (Jourdan and Weiler, unpublished).

IAA in coleoptiles of maize and oat. The amount of quantitative information on the occurrence of IAA in coleoptiles is very limited and is almost exclusively based on bioassay data. Despite this lack of information, coleoptiles are frequently used to study auxin, and in particular IAA, effects on growth. Most of this work was and is being carried out with coleoptile segments treated externally with auxin. The endogenous auxin status of the experimental tissue is not known, and the validity of conclusions derived from such experiments for the situation in vivo has been the subject of severe criticism (e.g., Mer 1968). The sensitive and specific enzyme immunoassay for IAA has allowed us the re-investigation of the distribution and levels of IAA in coleoptiles of all ages on a quantitative basis and to compare these results with those published in the literature. The values for free and base-labile IAA in shoots of oat and maize agreed well with the data by Bandurski and Schulze (1974) who used similar extraction and hydrolysis procedures. The higher values for free IAA found by us might reflect varietal differences which have been reported to be considerable (Sheldrake 1973). However, as Zimmermann et al. (1976) pointed out, changes in the extraction and hydrolysis procedures drastically affect the yields of free and esterified IAA. Hemberg and Tillberg (1980) have also reported extremely variable results for IAA from maize kernels depending on the extraction conditions. As Fig. 8 demonstrates, for *Avena*, levels of extractable IAA in developing seedlings may also vary considerably with time.

Recent work, (see Bandurski 1979) has shown that during the germination of maize kernels, IAA conjugates (mainly the myo-inositol ester) move from the endosperm to the shoot at a rate of ca. 5–10 pmol/h. From these conjugates, IAA is apparently liberated in the coleoptile tip and then translocated basipetally. Gillespie and Thimann (1963) have estimated by bioassay that maize coleoptile tips release ca. 4.5–5 pmol of IAA per tip and hour, independent of the length (3–6 mm) of the tip preparations used. The average value for our experiments (4.9 pmol released per tip and hour) closely agrees with this estimate. However, the sensitive immunological assay clearly showed that the yield of diffusible IAA was strongly dependent on the length of the tips used for the experiments. Shorter tips consistently yielded significantly more IAA than longer ones both in maize and oat (see Table 3). This supports the early observation of Went (1928). Thus, IAA release or production seems to occur only in the most apical part of the coleoptile tip. The data furthermore suggest that in the growing tissue adjacent to the tip, IAA is to a great extent either immobilized and/or metabolized.

The yield of diffusible IAA per maize coleoptile tip compares well with the total amount of IAA extractable per tip (see Table 3). Of this material, more than 80% is acetone-extractable as base-labile conjugates, the rest as free IAA. The estimated supply of acropetally moving esterified IAA (Bandurski 1979) would thus well account for the total IAA production of the maize coleoptile tip. For *Avena*, the IAA yield from tips by acetone extraction and by diffusion also agree well (see Table 3). A single tip yields 0.6–0.8 pmol h^{-1} of diffusible IAA. This is in the same range as the value calculated by Sheldrake (1973) from data of Went and Thimann (1937), 0.2–0.3 pmol h^{-1}. Two-thirds of the extractable IAA are present as free acid, the rest in the form of base-labile conjugates. Sheldrake (1973) has reported an acropetal movement of IAA as well as base-labile conjugates in the *Avena* coleoptile. However, it is not known at present whether this IAA supply from the endosperm would account for the hormone released by the tip or whether additional synthesis of IAA occurs in coleoptile tips of *Avena*. It was excluded that bacterial interference, which was reported to result in IAA production from tryptophan under non-sterile conditions in a number of tissues including coleoptiles (Libbert et al. 1968), contributed to IAA values during the collection of diffusates in our experiments.

Due to the sensitivity of the enzyme immunoassay, and its high selectivity for IAA, it was possible to obtain quantitative values for the distribution of diffusible and extractable IAA within coleoptiles of maize and oat. Single coleoptiles could be used for a quantitation. The auxin gradients found in early experiments by bioassay (Thimann 1934) have now been firmly traced back to the compound, IAA, for *Avena sativa* L. and *Zea mays* L. (Fig. 4–7). Extrac-

tion of coleoptile segments with organic solvent yields consistently more IAA than the diffusion method. This holds true especially when methanol is used as the solvent (cf. Fig. 7, lower part), indicating the presence of immobile forms of IAA in the tissue.

Our data suggest that consumption (immobilization and/or metabolism) of IAA is associated with the process of growth-initiation in coleoptiles: 1. The tissue level of IAA drops sharply in the first millimeters of the growth zone (see Fig. 6, 7). This cannot be due solely to IAA export, because longer tips (5 mm) yield less IAA than shorter (2.5 mm) tips by the diffusion method (Table 3). 2. From the data presented in Fig. 8, it can be calculated that there is a discrepancy between the amount of IAA produced by coleoptile tips over time and the amount of IAA extractable from these coleoptiles. During growth, an increasing amount of IAA released by the tip (as calculated by integration of the IAA production rate of tips over time) cannot be recovered from the coleoptiles by extraction. This apparently 'consumed' fraction of hormone is correlated ($r=0.99$) with coleoptile length, i.e., the growth achieved.

A detailed immunoassay analysis showed the presence in coleoptiles of several growth regulators other than IAA (Fig. 8), and their tissue levels in coleoptiles of different growth stages are given herein for the first time. Besides IAA, ABA is the most dominant one in terms of molar concentrations. The finding that ABA occurs in coleoptiles is important insofar as exogenously applied ABA strongly interferes in the IAA-induced process of elongation of excised coleoptile segments (e.g., Zenk 1970). The presence of free as well as conjugated ABA was demonstrated (Fig. 8) and several observations suggest a possible involvement of this hormone, together with IAA, in the endogenous regulation of coleoptile growth:

1. ABA is concentrated in the growth area of the coleoptile.

2. The increase in the amount of extractable ABA (see Fig. 8) correlates with the decrease in growth rate of the coleoptile. This decrease occurs in the presence of high amounts of both extractable and diffusible IAA (Fig. 8).

3. There exists an inverse relationship in the tissue levels of IAA and ABA during the regeneration of the physiological tip and the growth behavior of decapitated coleoptiles can best be explained on the basis of levels of both IAA and ABA (Fig. 9): The rapid reduction in growth rate after decapitation of coleoptiles is associated with increasing ABA- and decreasing IAA-levels, the resumption of growth 2–2.5 h after decapitation is preceeded by both a drop in ABA levels and a rise in IAA-levels. The endogenous presence of ABA in the *Avena* coleoptile has to be taken into account when auxin effects are studied in this tissue.

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References

Anker, L. (1973) The auxin production of the physiological tip of the *Avena* coleoptile and the repression of tip regeneration by indoleacetic acid (not by naphthylacetic acid and 2,4-dichlorophenoxyacetic acid). Acta Bot. Neerl. **22**, 221–227

Bandurski, R.S. (1979) Homeostatic concentrations of indole-3-acetic acid. In: Plant growth substances, pp. 37–49, Skoog, F., ed. Springer-Verlag, Berlin Heidelberg New York

Bandurski, R.S., Schulze, A. (1974) Concentrations of indole-3-acetic acid and its esters in *Avena* and *Zea*. Plant Physiol. **54**, 257–262

Bandurski, R.S., Schulze, A. (1977) Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiol. **60**, 211–213

Crozier, A., Loferski, K., Zaerr, J., Morris, O. (1980) Analysis of picogram quantities of indole-3-acetic acid by high performance liquid chromatography-fluorescence procedures. Planta **150**, 366–370

Gillespie, B., Thimann, K.V. (1976) Transport and distribution of auxin during tropistic response. I. The lateral migration of auxin in geotropism. Plant Physiol. **38**, 214–225

Greenwood, M.S., Shaw, S., Hillman, J.R., Ritchie, A., Wilkins, M.B. (1972) Identification of auxin from *Zea* coleoptile tips by mass spectrometry. Planta **108**, 179–183

Hemberg, T., Tillberg, E. (1980) The influence of the extraction procedure on yield of indole-3-acetic acid in plant extracts. Physiol. Plant. **50**, 176–182

Hurn, B.A.L., Chantler, S.M. (1980) Production of reagent antibodies. Meth. Enzymol. **70**, 104–141

Libbert, E., Wichner, S., Duerst, E., Kunert, R., Kaiser, W., Manicki, A., Manteuffel, R., Ricke, E., Schroder, R. (1968) Auxin content and auxin synthesis in sterile and non-sterile plants, with special regard to the influence of epiphytic bacteria. In: Biochemistry and physiology of plant growth substances, pp. 213–230, Wightman, F. and Setterfield, G., ed., Runge Press, Ottawa, Canada

Marschner, I., Dobry, H., Erhardt, F., Landersdorfer, T., Popp, B., Ringel, C., Scriba, P.C. (1974) Berechnung radioimmunologischer Meßwerte mittels Spline-Funktion. Ärztl. Lab. **20**, 184–191

Mer, C.L. (1968) Plant growth in relation to endogenous auxin with special reference to cereal seedlings. New Phytol. **68**, 275–294

Mousdale, D.M.A., Butcher, D.N., Powell, R.G. (1978) Spectrofluorimetric methods of determining indole-3-acetic acid. In: Isolation of plant growth substances, pp. 27–31, Hillman, J.R., ed. University Cambridge, Press

Pengelly, W., Meins, F., Jr., (1977) A specific radioimmunoassay for nanogram quantities of the auxin, indole-3-acetic acid. Planta **136**, 173–180

Sheldrake, A.R. (1973) Do coleoptile tips produce auxin? New Phytol. **72**, 433–447

Thimann, K.V. (1934) Studies on the growth hormones of plants. VI. The distribution of the growth substances in plant tissues. J. Gen. Physiol. **18**, 23–34

Walton, D., Dashek, W., Galson, E. (1979) A radioimmunoassay for abscisic acid. Planta **146**, 139–145

Weiler, E.W. (1979) Radioimmunoassay for the determination of free and conjugated abscisic acid. Planta **144**, 255–263

Weiler, E.W. (1980a) Radioimmunoassay for the differential and direct analysis of free and conjugated abscisic acid in plant extracts. Planta **148**, 262–272

Weiler, E.W. (1980b) Radioimmunoassay for trans-zeatin and related cytokinins. Planta **149**, 155–162

Weiler, E.W. (1981) Radioimmunoassay for pico-mol quantities of indole-3-acetic acid for use with highly stable [^{125}I]- and [^{3}H]IAA derivatives as radiotracers. Planta **153**, 319–325

Weiler, E.W., Spanier, K. (1981) Phytohormones in the formation of crown-gall tumors. Planta **153**, 326–337

Weiler, E.W., Wieczorek, U. (1981) Determination of femto-mol quantities of gibberellic acid by radioimmunoassay. Planta **152**, 159–167

Weiler, E.W., Ziegler, H. (1981) Determination of phytohormones in phloem exudate of tree species by radioimmunoassay. Planta **152**, 168–170

Went, F.W. (1928) Wuchsstoff und Wachstum. Rec. Trav. Bot. Neerl. **25**, 1–116

Went, F.W., Thimann, K.V. (1937) Phytohormones. Macmillan Co., New York

Zenk, M.H. (1970) Phytohormone and Genaktivität. Ber. Dtsch. Bot. Ges. **83**, 325–344

Zimmermann, H., Siegert, Chr., Karl, R. (1976) Veränderungen des Wuchsstoffes Indolyl-3-essigsäure bei der Keimung von *Avena sativa* L. Z. Pflanzenphysiol. **80**, 225–235

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