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PLANT GROWTH SUBSTANCES IN ROOT CULTURES OF *LYCOPERSICON ESCULENTUM*

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Abstract—Cultured roots of *Lycopersicon esculentum* were found by GC-MS to contain gibberellins A_1 and A_3, zeatin riboside, indole-3-acetic acid and abscisic acid, the last two in both free and conjugated forms. These growth substances were detected also in the used culture medium, which in addition contained zeatin and dihydrozeatin riboside.

INTRODUCTION

Although it is widely accepted that representatives of the major classes of plant growth substances are involved in root growth and development there are relatively few reports where their presence in roots has been established rigorously [1, 2]. In many cases identifications were based solely on chromatographic separations and bioassays. However, mass spectrometry has been used to identify gibberellins (GAs) in intact roots of *Spinacea oleracea* [3] and *Triticum aestivum* [4], and cytokinins in roots of *Raphanus sativus* [5], *Chichorum intybus* [6], *Oryza sativa*[7] and *Phaseolus vulgaris* [8]. This technique was also used to establish unequivocally the presence of abscisic acid (ABA) in roots of *Zea mays* [9], *Persea gratissma* and *Helianthus annus* [10] and of indole-3-acetic acid (IAA) in roots of *Z. mays* [11–13].

The detection of growth substances in intact roots gives no information on the origin of these compounds, and it is possible that they are imported from the shoots. Excised roots which can be grown in culture on nutrient media lacking growth substances provide a suitable experimental system for examining the inherent capacity of roots to synthesise growth substances. While there are several reports [14–16] that cultured roots contain substances with hormone-like activity, there are very few definitive identifications. IAA and ABA were detected by GC-MS in root cultures of *Pisum sativum* [17] and *Phaseolus coccineus* [18]. There is also strong evidence that root cultures of *Z. mays* are able to synthesize small amounts of IAA [19].

The aim of this work was to establish unequivocally whether growth substances are present in cultures of excised tomato roots.

RESULTS AND DISCUSSION

A clone of excised tomato roots was initiated and maintained in culture for three months by weekly subcultures before growing up sufficient material for extraction. Growth substances were extracted from the roots and medium separately. After separating into acidic and basic fractions purified extracts were derivatized and analysed by GC-MS. The following compounds were identified by comparison of their mass spectra with those of authentic compounds. GA_1, GA_3, ABA and *trans*-zeatin riboside were present in the roots and medium; *trans*-zeatin was found only in the medium.

Since GA_3 is found commonly as a contaminant in laboratories working with growth substances a second extract was made from cultured roots and medium. In this second experiment, additional precautions were taken to avoid contamination by working-up the spent medium immediately. In addition to the compounds identified previously, the presence of IAA and cellulase-hydrolysable conjugates of the acidic growth substances was also investigated. The mass spectral evidence for the identification of growth substances in the roots and medium is presented in Table 1, and this information is summarized in Table 2. The presence of GA_3 was confirmed in roots and in medium, but only trace amounts of GA_1 were detected in both extracts. Other GAs or GA conjugates, if present, were below the level of detection of the mass spectrometer. These results confirmed the earlier finding that cultured tomato roots contained substances with GA-like activity in the dwarf pea and dwarf maize bioassays [16] and our own observations of compounds active in the *Rumex* and barley endosperm assays at the retention volume of GA_3 on HPLC.

IAA was detected in the free acid and cellulase-hydrolysed acid fractions from both the roots and medium, although the amount of free IAA in the roots was very small. It is interesting to note that several earlier attempts to show the presence of IAA in this tissue have failed [10, 20, 21] although the methods employed were presumably not as sensitive as the present one. The conclusive identification of IAA in root cultures of maize [19], pea [17] and runner bean [18] has been reported previously. ABA was detected in free and conjugated forms in both the roots and medium of tomato cultures. This is in agreement with reports that ABA is present in

Table 1. Comparison of the relative intensities of significant ions in the mass spectra of putative growth substances detected by GC/MS in extracts of cultured tomato roots and growth medium with those of standards

Compound	Relative intensity (%)									
GA_3 MeTMSi	*m/z* 504	489	477	445	388	370	347	208		
Standard	100	9	5	9	10	11	17	34		
Roots acids	100	10	5	9	5	9	10	59		
Medium acids*	100	13	—†	23	—	27	17	37		
IAA MeTMSi	*m/z* 261	202	186	130						
Standard	38	100	4	10						
Root conjugates	40	100	6	31						
Medium acids*	43	100	3	20						
Medium conjugates*	40	100	3	7						
ABA Me	*m/z* 278	260	246	205	190	175	162	147	134	125
Standard	1	6	5	6	100	4	44	10	54	35
Root acids	1	4	4	5	100	5	38	8	37	36
Root conjugates*	—	9	—	16	100	7	38	25	53	86
Medium acids	2	1	4	9	100	5	49	12	44	31
Medium conjugates*	0‡	1	3	0	100	4	21	13	32	11
Zeatin tBDMS	*m/z* 447	432	390	315	302	274				
Standard	10	13	37	93	100	34				
Medium	19	34	40	100	88	33				
Zeatin riboside TMSi	*m/z* 639	624	549	536	320	245	230	217	201	147
Standard	43	43	55	78	51	57	57	78	70	100
Roots	—	6	13	15	9	37	16	29	32	100
Medium	9	20	28	45	34	63	46	73	65	100
Dihydrozeatin riboside TMSi	*m/z* 641	626	366	322	259	245	230	217	147	
Standard	12	21	11	100	22	38	82	49	52	
Medium	4	8	4	36	27	69	52	52	100	

*Mass spectra contained some contaminating ions;
†Ion absent at this *m/z* value.
‡Relative intensity <0.5%.

Table 2. Growth substances identified in cultures of excised tomato roots

	Roots	Culture medium
GA_1	t	t
GA_3	+	+
Zeatin*	—	+
Zeatin riboside*	+	+ +
Dihydrozeatin riboside*	—	+
ABA	+ +	+ +
ABA conjugate*	+	+
IAA	t	+ +
IAA conjugate*	+	+ +

*Growth substance identified after treatment with cellulase.
+ + Strong mass spectrum obtained; + Weak spectrum obtained; t Trace amount tentatively identified on the basis of the presence of several ions at the expected GC R_t.

excised roots of pea [17] and runner bean [18]. In the latter work 20–30% of the total ABA was found in the agar medium.

trans-Zeatin riboside was found in the roots and medium of the tomato cultures whereas *trans*-zeatin and *trans*-dihydrozeatin riboside were detected only in the medium. Since these cytokinins were found after cellulase treatment (see Experimental) it is possible that *in vivo* they are present as conjugates. Nevertheless, these results confirm previous reports which suggested that excised roots of tomato produce considerable amounts of cytokinins, which are released into the medium [14, 15]. In these reports PC separations and bioassay indicated that the cytokinins were zeatin riboside and, in small amounts, zeatin.

Thus, there is strong evidence that detached roots of tomato are able to synthesise IAA, ABA, GAs and cytokinins. At present no precise quantitative data are available, but, as indicated in Table 2, a relatively high proportion of IAA and cytokinins is released into the culture medium. The sustained growth of the roots in the absence of exogenous growth substances confirms that they are autonomous for these compounds. However, it is possible that root growth *in vivo* is influenced also by externally produced growth substances.

EXPERIMENTAL

Root material. Tomato seeds (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) were sterilized with 1.6% v/v NaOCl soln and allowed to germinate in the dark at 25° in 9 cm sterile Petri dishes. After 4 days, root tips (1 cm) were excised and transferred to 100 ml Erlenmeyer flasks containing 50 ml modified White's

medium [22, 23]. The medium contained 1.5% sucrose, and iron was added as the Fe^{3+}, Na^+ salt of EDTA (36.8 mg/l). A clone was established from a selected culture as previously described [24, 25]. After 3 and 12 months in culture (12 and 48 subcultures, respectively) root material for extn was grown in penicillin flasks containing 500 ml medium. Each flask was inoculated with 15 root tips (1 cm) and incubated at 25° for 21 days. At harvest the roots were sepd from the medium by filtration, washed with *ca* 50 ml H_2O and surface dried. The bulked root material and medium were stored at −20°. Each flask yielded *ca* 2g fr. wt of roots.

Extraction of growth substances from medium. Medium (14.4 l) was pumped in 1.5 l portions at 4 ml/min through a preparative Bondapak Porasil B (37–75 μm) HPLC column (15 cm × 0.9 i.d.). After each portion the column was washed with 2 mM HOAc (150 ml) and the growth substances eluted with 80% MeOH in 2 mM HOAc (150 ml). After removing the MeOH *in vacuo* from the combined eluates, growth substances were purified as described below.

Extraction and purification of growth substances from roots and medium. Root material (*ca* 70g fr.wt) was homogenized in 80% aq. MeOH containing 10 mg/l butylated hydroxytoluene (750 ml) and extd in this solvent at 4° for 4 hr. After filtration the residue was re-extracted with MeOH (500 ml) at 2° overnight. The residue was removed by filtration and the combined filtrates concd to an aq. soln *in vacuo*, adjusted to pH 3 with 1 N HCl and partitioned against equal vol. of EtOAc (× 4). The organic phase was then partitioned against 1/5 its vol. of 5% aq. $NaHCO_3$ (× 3), which was subsequently adjusted to pH 3 with 1 N HCl and partitioned against equal vol. of EtOAc (× 4). This final EtOAc phase, which contained the acids, was washed once with 1/3 its vol. of H_2O at pH 3, cooled to −20° to remove residual H_2O, and reduced to dryness *in vacuo* at 40°. The extract was then dissolved in 50 mM K-Pi buffer at pH 8.5 (50 ml) and poured into a column of PVP (5 ml) that had been pre-washed with 50 mM K-Pi buffer at pH 3 (50 ml). The PVP was washed (× 4) with 5 ml portions of 50 mM K-Pi buffer at pH 8.5 and the combined eluate was adjusted to pH 3.5 (1N HCl) and applied to a C_{18} silica SepPak cartridge that had been pre-washed with MeOH (5 ml) followed by 2mM HOAc (10 ml). The cartridge was washed twice with 2 mM HOAc (5 ml) and the acids eluted with 80% MeOH in 2 mM HOAc (5 ml), which was then reduced to dryness *in vacuo*.

The first aq. phase (containing acidic and polar conjugates) and the first EtOAc phases (containing neutral conjugates) were combined and reduced to dryness *in vacuo*. After dissolving in H_2O (50 ml) and adjusting the pH to 4.5, the soln was treated with cellulase from *Aspergillus niger* (100 mg) at 37° overnight. The pH was then adjusted to 3 (1 N HCl) and the sample treated as for the free acid fraction to yield a cellulase-hydrolysed acid fraction.

Cytokinins were extd from the first aq. phase (after cellulase treatment and partitioning against EtOAc) by adjusting the pH to 8.5 and partitioning (× 4) against equal vol. of H_2O–satd *n*-BuOH, which was then reduced to dryness *in vacuo*. The extract was dissolved in H_2O (10 ml), adjusted to pH 3 (1N HCl) and applied to SP-Sephadex C-25 (H^+ form) [26], which was washed with H_2O at pH 3 (60 ml). Cytokinins were eluted with 0.2 M NH_4OH (40 ml), which was reduced to dryness, the residue dissolved in H_2O at pH 5.3 (5 ml) and applied to a C_{18} silica SepPak cartridge, which had been pre-treated with MeOH (5 ml) and H_2O at pH 5.3 (10 ml). After washing with H_2O at pH 5.3 (3 × 5 ml), cytokinins were eluted with 50% MeOH and the eluate reduced to dryness *in vacuo*.

HPLC. (i) *Acidic growth substances.* Free acids and acids released from conjugates by cellulase hydrolysis were applied in two aliquots in 20% MeOH in 2 mM HOAc (0.5 ml) to a Spherisorb 5 ODS column (25 cm × 0.4 cm i.d.) and eluted at 1 ml/min in a gradient of 28% MeOH in 2 mM HOAc to 100% MeOH over 40 min. 3 ml fractions were collected and taken to dryness *in vacuo*.

(ii) *Cytokinins.* Samples in 4% MeCN in dil. aq. triethylammonium bicarbonate (TEAB) (0.5 ml) were applied to a Hypersil 5 ODS column (25 cm × 0.4 cm i.d.) and eluted at 2 ml/min in a linear gradient of 4% to 20% MeCN in dil TEAB at pH 7 over 40 min. 27 1 ml fractions were collected.

Bioassay for cytokinin activity. From a work-up of 7 l of culture medium 10% of each HPLC fraction was dried down on filter paper discs in 50 ml conical flasks prior to bioassay using soybean hypocotyls [28]. Biological activity (90 ng zeatin equivalents) was detected in HPLC fractions 20 and 21 (same region as zeatin std) and in HPLC fractions 28 and 29 (120 ng zeatin equivalents, in a similar region to zeatin riboside std).

GC/MS. (i) *Acidic growth substances.* The HPLC fractions were methylated in MeOH (*ca* 50 μl) with ethereal CH_2N_2. HPLC fraction 5 (elution vol. 20–23 ml) was analysed for ABA directly after methylation. The other fractions and the remainder of fraction 5 were trimethylsilylated by the addition of *N*-methyl-*N*-trimethylsilyltrifuoracetamide (MSTFA) (10 μl) to the thoroughly dried samples in glass ampoules, which were then sealed and heated at 90° for 30 min. Samples were analysed using a Kratos MS80 GC-MS system. For analysis of GAs samples (1 μl) were injected into a fused silica WCOT bonded OV-1 column (25 m × 0.3 mm id) at an oven temp of 60° and a He inlet pressure of 0.4 kg/cm² with no split. After 0.5 min a split of 50:1 was opened and after 1 min the oven temp was increased to 240° at 15°/min and thereafter at 4°/min to 300°. The temp of the injector, interface and source were 200°, 270° and 200°, respectively. Data were acquired from 240°, the mass range being scanned from 700 to 50 mu at 1 sec/mass decade. An electron energy of 70 eV was used. The anylysis of ABA and IAA was conducted under similar conditions except that the oven temp was programmed from 60° to 200° at 15°/min and then at 4°/min to 250°. Data acquisition began at 200°.

(ii) *Cytokinins.* (a) N-9 ribosides. Fractions in which N-9 ribosides were suspected were treated with pyridine-MSTFA containing 1% trimethylchlorosilane (1:1) (5 μl) at 90° for 30 min to produce per TMSi derivatives. They were analysed by GC/MS as for the acids above except that samples were injd at an oven temp of 100°, which was maintained for 1 min and then increased to 295° at 20°/min and then maintained at this temp. Data acquisition began after 15 min.

(b) Free bases. Fractions in which free bases were suspected were treated with pyridine-*N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide containing *t*-butyldimethylsilylchlorosilane (1:1) (5 μl) at 90° for 30 min [29]. A GC temp prog of 100° for 1 min increasing to 240° at 20°/min and then to 295° at 4°/min was used. Data acquisition began at 240°.

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