J Plant Growth Regul (1996) 15:39–49

Journal of
Plant
Growth
Regulation
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Occurrence of Aromatic Cytokinins in Oil Palm (*Elaeis guineensis* Jacq.)

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Received March 20, 1996; accepted March 21, 1996

Abstract. The natural occurrence of 6-benzylaminopurine, 6-(2-hydroxybenzylamino)purine (*ortho*-topolin), 6-(3-hydroxybenzylamino)purine (*meta*-topolin), their ribosides and 9-glucosides is reported using specific antibodies to these groups of compounds in high performance liquid chromatography/enzyme-linked immunosorbent assay (HPLC/ELISA). Compounds were identified by their retention times and differential cross-reactivities with six antisera in analyses carried out in two laboratories using different HPLC gradient systems. Identities were confirmed by immunoaffinity purification followed by HPLC with on-line UV spectrum analysis. Further confirmation of the occurrence of *ortho*-topolin riboside and isopentenyladenine-9-glucoside was obtained from gas chromatography-mass spectrometry analysis of permethylated HPLC fractions of an extract of oil palm tissues. The aromatic cytokinins, and in particular, *ortho*-topolin riboside, were found in a variety of oil palm tissues at concentrations exceeding those of the isoprenoid cytokinins, zeatin, isopentenyladenine, dihydrozeatin, and their ribosides. The 9-glucosides of isopentenyladenine and zeatin were more abundant than those of the aromatic types. The cross-reactivity of benzyladenine compounds with antibodies to isopentenyladosine is discussed in relation to the interpretation of ELISA data.

Key Words. Oil palm—Cytokinin—Topolin

Abbreviations: Except where otherwise specified abbreviations for names of isoprenoid cytokinins used in this paper follow the convention proposed by Crouch et al. (1993). Aromatic cytokinins follow the terminology of Strnad et al. (1994). BA, N^6-benzyladenine; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; GC-MS, gas-chromatography-mass spectrometry; *m*T, *meta*-topolin; *o*T, *ortho*-topolin; TEAA, triethylammonium acetate; IAC, immunoaffinity chromatography; SPD, spectral photodiode.

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The presence of N^6-benzyladenine (BA) and its *meta*- and *ortho*-hydroxy-substituted derivatives (*meta*-topolin, *m*T and *ortho*-topolin, *o*T) in plant tissues has been reported in a variety of species (Ernst et al. 1983, Horgan et al. 1975, Nandi et al. 1989, Strnad et al. 1992b, 1994, 1996).

With the availability of antibodies to these families of compounds and the use of combined HPLC/ELISA it is becoming clear that they are of widespread occurrence in plants (Strnad 1996, Strnad et al. 1992b, 1994). We now report the presence in various of tissues of oil palm (*Elaeis guineensis* Jacq.) of compounds with specific cross-reactivity to antibodies to BA, *m*T, and *o*T, with HPLC retention times corresponding to those of known synthetic standards and at concentrations equal to or exceeding those of the isoprenoid cytokinins. These results have been confirmed using affinity purification followed by HPLC with on-line UV spectrum monitoring by the methods of Nicander et al. (1993), and using gas chromatography-mass spectrometry (GC-MS) on selected HPLC-purified fractions. This paper also contains previously unreported data on the isoprenoid cytokinins in these tissues.

Materials and Methods

Antibodies

Antibodies to benzyladenine, *o*-hydroxybenzyladenosine, and *m*-hydroxybenzyladenosine were produced by Strnad et al. (1990, 1992a, 1992b), Strnad (1996) and made available by them for collaborative studies.

Synthetic Standards

Standard compounds of BA, *m*T, *o*T, their ribosides and 9-glucosides, synthesized by the methods described in Kuhnle et al. (1977), were kindly supplied by Dr. J. Hanus. Tritiated standards (selectively labeled

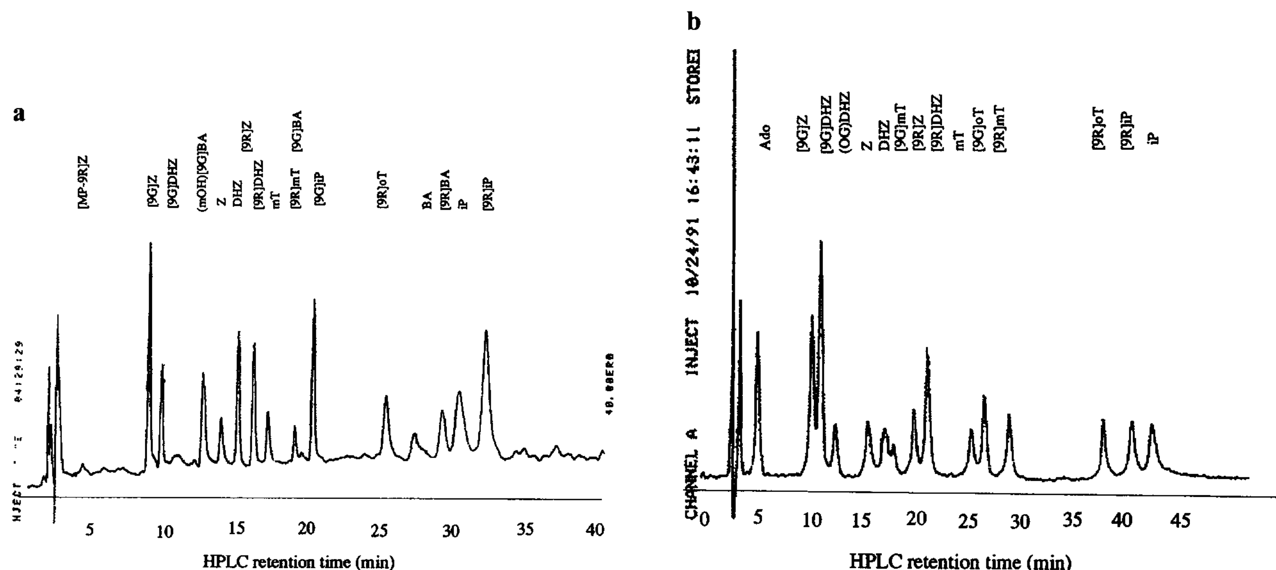


Fig. 1. Reverse phase HPLC separation of standard cytokinins. **a**, methanol gradient in use in Cambridge (see Table 2). Column: Anachem 150 mm × 4.6 mm, 5 μm C_{18} ODS; flow rate 1.5 mL · min^{-1}. Solvent : aqueous methanol + 0.2 mM TEAA, pH 7.0. Gradient: 0 min, 15% methanol; 15 min, 40%, 40 min, 40%; 40.1 min, 100%; 50 min, 100%. **b,** acetonitrile gradient in use in Olomouc (see Table 1). Column: Separon SGX 250 mm × 4 mm, 7 μm C_{18} ODS; flow rate 1.2 mL · min^{-1}. Solvent: aqueous acetonitrile + 40 mM TEAA, pH 3.5. Gradient: 0 min, 7% acetonitrile; 10 min, 8%; 30 min, 15%; 40 min, 23%; 50 min, 25%; 52 min, 25%.

at C2) of high specific activity (1.1 TBq · mmol^{-1}) of BA, [9R]BA, *m*T, [9R]*m*T, *o*T, and [9R]*o*T were also made available from this source.

Plant Samples

Oil palm tissue cultures were supplied by Unifield TC Ltd., Cambridge. Seeds supplied by Unilever Plantations and Plant Science Group were germinated following standard heat treatment to break dormancy (Hartley 1977) and sampled at an early stage of germination. At the stage selected the tigellum containing the root/shoot axis had come through the emergence pore and was separated from the haustorium (cotyledonary) tissue remaining embedded in the endosperm within the nut. Young immature inflorescences from clonal palms growing in a Malaysian plantation field trial (Pamol Plantations Sdn. Bhd.) were harvested by felling the palms, which were dissected in the field. The inflorescences were immediately frozen in liquid nitrogen and returned to UK in dry ice. [Data from analysis of some of these inflorescences has been reported by Jones et al. (1995).] They were stored at −70°C until used. Oil palm crown tissue was obtained from a palm (approximately 5 years old) grown in a glasshouse by Unilever Plantations and Plant Science Group, Cambridge. The palm was dissected, and the central crown tissue containing the meristem, leaf primorida, and actively growing rachis tissue at the base of young leaves was frozen in liquid nitrogen and stored at −70°C.

Extraction of Cytokinins

In Cambridge plant samples in liquid nitrogen were extracted in ice-cold aqueous ethanol (80% v/v) by the method of Kraigher et al. (1991). Samples for analysis in Olomouc were reduced to low volume under vacuum, taken up in ethanol, and sent to the Czech Republic. Samples analyzed in Cambridge were also reduced to low volume and purified by passage through a C_{18} Sep-Pak (Waters, Harrow, UK), which had been primed previously by passage of 10 mL of methanol, followed by washing with 10 mL of aqueous triethylammonium acetate (TEAA, 10 mM, pH 7.0). Cytokinins were eluted into a silanized flask with 10 ml of 40% (v/v aqueous methanol and the methanol removed in a rotary vacuum evaporator at 35°C. The residue was taken up in a small volume of water before HPLC separation.

The samples analyzed in Olomouc were purified as described in Strnad et al. (1992a). This included hydrolysis of ribosides using alkaline phosphatase followed by purification on DEAE-cellulose and C_{18} columns. Cytokinin extracted as riboside is therefore combined with the riboside fraction in these analyses. The extraction method used in Cambridge retains the ribosides with relatively little loss during processing.

HPLC

Samples in Cambridge were analyzed by reverse phase HPLC on a 150 × 4.6-mm ODS (C_{18}) 5-μm microsphere column (Anachem, UK) using a methanol gradient at pH 7 in the presence of TEAA (20 μM) with a flow rate of 1.5 mL · min^{-1} as described by Jones (1990). Those analyzed in Olomouc were chromatographed in either an acetonitrile gradient or a methanol gradient at pH 3.4 as described by Strnad et al. (1992b). Standard compounds were run before and after the experimental samples to establish retention times. Radiolabeled aromatic cytokinins were used as internal standards in some separations to establish recoveries. Otherwise in samples analyzed in Cambridge each plant sample was "spiked" with tritiated [9R]iP diol (50 μL, 17 mM, approximately 10,000 dpm) as described by Jones et al. (1995). This compound elutes at the same retention time as *o*T (normally about 22 min) but shows no detectable cross-reactivity with the *o*T antiserum.

In Cambridge the column eluate was collected into 1.5-mL Eppendorf tubes at 1-min intervals throughout the 40-min separation. The

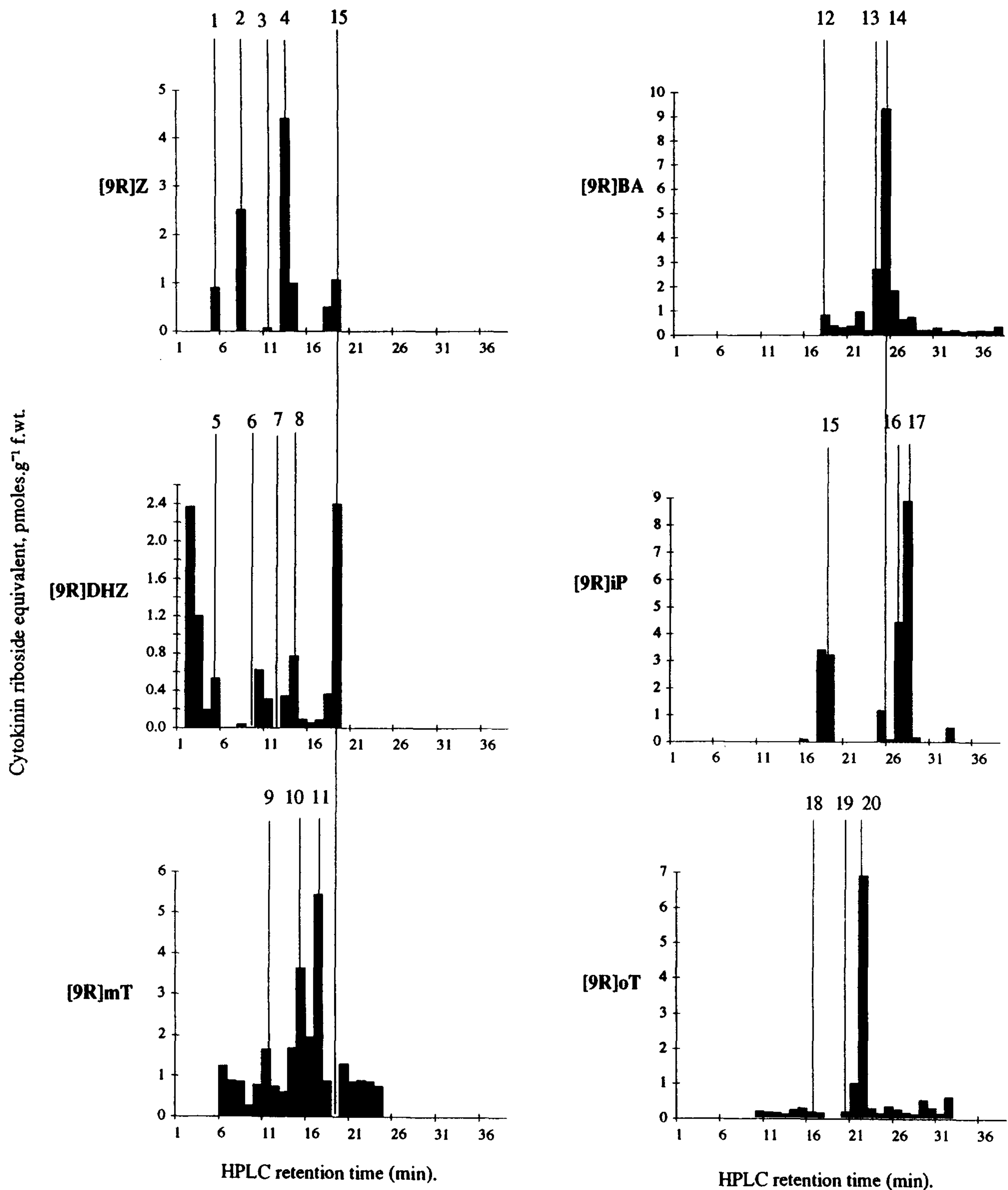


Fig. 2. HPLC/ELISA of oil palm seedling shoots using antibodies to isoprenoid and aromatic cytokinins. HPLC conditions followed Kraigher et al. (1991). ELISA was by the method of Strnad et al. (1992a). Standard retention times: *1*, [9R-MP]Z. *2*, [9G]Z. *3*, Z. *4*, [9R]Z. *5*, [9R-MP]DHZ. *6*, [9G]DHZ. *7*, DHZ. *8*, [9R]DHZ. *9*, [9G]*m*T. *10*, *m*T. *11*, [9R]*m*T. *12*, [9G]BA. *13*, BA. *14*, [9R]BA. *15*, [9G]iP. *16*, iP. *17*, [9R]iP. *18*, [9G]*o*T. *19*, *o*T. *20*,[9R]*o*T.

Table 1. Isoprenoid and aromatic cytokinins in oil palm tissues.[a]

	Cytokinin content (pmol riboside equivalent · g^{-1} fresh weight)									
						Inflorescence (Clone 90a)				
						Leaf axil 11		Leaf axil 12		
	Embryo		Embryoids	Leaf axil 6		Normal	Abnormal	Normal	Abnormal	Abnormal
Cytokinin	Haustorium	Tigellum	in vitro	Normal	Abnormal	female	female[b]	female	female	male
[9G]Z	0.3	1.5	0.6	2.5	1.4	15.0	10.0	19.0	20.7	3.0
Z	0.3	N.D.	0.2	0.1	1.0	N.D.	N.D.	N.D.	N.D.	N.D.
[9R]Z	0.7	3.9	0.3	N.D.	3.1	5.6	32.6	10.4	19.7	2.5
[9G]DHZ	6.5	0.4	5.6	0.8	1.2	0.9	1.7	1.8	1.8	N.D.
DHZ	N.D.	0.3	0.8	0.8	1.0	N.D.	N.D.	N.D.	N.D.	N.D.
[9R]DHZ	1.4	0.3	4.5	1.8	0.6	0.4	2.1	N.D.	1.5	N.D.
[9G]iP	2.5	1.7	2.9	0.2	10.0	N.D.	1.0	1.5	1.0	N.D.
iP	N.D.	N.D.	0.2	0.3	1.9	N.D.	N.D.	1.7	N.D.	N.D.
[9R]iP	0.6	0.1	0.1	0.1	0.8	3.5	N.D.	0.5	0.2	N.D.
[9G]*m*T	0.2	0.5	—	—	6.0	N.D.	N.D.	0.1	N.D.	N.D.
*m*T	0.4	0.5	1.4	2.2	5.0	N.D.	N.D.	0.1	N.D.	N.D.
[9R]*m*T	0.7	0.2	2.3	—	7.0	N.D.	3.2	N.D.	N.D.	116.0
[9G]*o*T	N.D.	N.D.	1.3	0.2	4.4	N.D.	N.D.	N.D.	N.D.	N.D.
*o*T	N.D.	N.D.	0.5	0.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
[9R]*o*T	41.0	46.0	0.6	N.D.	0.3	25.2	49.5	24.4	22.8	32.0
[9G]BA	N.D.	0.5	0.8	0.5	—	2.0	N.D.	N.D.	N.D.	N.D.
BA	1.7	1.8	0.4	0.6	0.3	1.6	4.3	1.5	2.2	2.2
[9R]BA	2.2	1.7	0.1	0.6	0.3	N.D.	2.1	2.4	2.2	1.7

[a] Samples were analyzed in Olomouc, CR, by HPLC/ELISA following the method of Strnad et al. (1992a) using antiboidies to [9R]Z, [9R]DHZ, [9R]iP, [9R}*m*T, [9R]*o*T, and [9R]BA. N.D., not detected. —, not determined. All results are means of duplicate assays.
[b] Mean of two inflorescence samples.

solvent was removed in a Univap centrifugal evaporator, and the fractions taken up in water before analysis by ELISA.

In Olomouc fractions were collected at 30-s intervals at a flow rate of 1 mL · min^{-1} over a separation period of 20 min.

ELISAs

The samples were analyzed by the method of Strnad et al. (1992a, 1992b). In Cambridge the detailed method (type of plates, layout of samples, and standards) was as described for the isoprenoid cytokinins in Jones et al. (1995), whereas the Olomouc assays were as reported by Strnad et al. (1992a, 1992b) for isoprenoid cytokinins and *o*T.

Immunoaffinity Chromatography (IAC) of Isoprenoid and Aromatic Cytokinins

The methods used followed the principles described by Nicander et al. (1993). Anticytokinin antibodies were affinity purified on an appropriate polylysine-agarose column (MacDonald and Morris 1985). The purified antibodies were bound to Affi-Gel (Bio-Rad, Richmond, CA, USA). Each IAC column contained 0.4 mL of the immunoaffinity gel of mixed, affinity-purified antibodies against isoprenoid and aromatic cytokinins in a 3-mL polypropylene syringe. A precolumn contained 0.4 mL of Affi-Gel with immobilized bovine gamma-globulin. Both immunocolumns in tandem were conditioned in PBS (20 mM Na_2HPO_4, 15 mM NaCl, pH 7.2). The prepurified extracts from DEAE-C_{18} columns were dissolved in 0.5 mL of PBS and passed through the immunocolumns five times. They were then washed with 5 mL of PBS, 10 mL of H_2O and eluted with 3 mL of ice-cold methanol. Immediately after elution, the gels were washed with water and equilibrated with PBS for the next series of samples. Methanol was removed from the eluates under a nitrogen stream.

Spectral Photodiode Array (SPD)-HPLC Detection of Cytokinins

Cytokinins for SPD-HPLC were separated on a Microsorb C_{18} column (150 mm long, 4.6 mm inner diameter, 5 μm pore size; Rainin, Woburn, MA, USA) at a flow rate of 0.5 mL · min^{-1} with a gradient of solvent A (10% methanol + 0.5% HAc, pH 3.35, adjusted with triethylamine) and solvent B (80% methanol + 0.6% HAc, pH 3.6) according to the following gradient profile; 0 min 90% A + 10% B; 20 min 50% A + 50% B; 39 min 45% A + 55% B, 40 min 100% B (washing), 50 min 90% A + 10% B (initial conditions). Samples of 100 μL were injected and 0.5-mL fractions collected, which were mixed with 2 mL

Table 2. Isoprenoid and aromatic cytokinins in oil palm tissues.[a]

Cytokinin	Seedlings (shoots)	Ramets (shoots)	Crown	Embryoids Clone 1	Embryoids Clone 2[b]	Embryoids Clone 3	Embryoid mean of three clones	S.D.±
	Cytokinin content (pmol riboside equivalent · g^{-1} fresh weight)							
[9R-MP]Z	N.D.	0.7	1.6	3.1	N.D.	2.2	2.7	0.6
[9G]Z	2.5	6.5	33.0	83.0	41.0	6.9	44.0	38.0
Z	0.1	0.4	0.5	N.D.	N.D.	1.2	1.2	—
[9R]Z	4.4	5.2	3.5	12.4	4.6	8.9	8.6	3.9
[9RMP]DHZ	N.D.	0.8	1.6	0.0	0.6	0.3	0.3	0.3
[9G]DHZ	0.6	0.8	1.4	0.2	2.7	0.2	0.2	0.0
DHZ	N.D.	0.2	N.D.	N.D.	N.D.	N.D.	—	—
[9R]DHZ	1.1	3.2	0.2	—	3.2	5.4	4.3	1.6
[9R-MP]iP	N.D.	N.D.	1.9	1.2	1.2	0.3	0.9	0.5
[9G]iP	6.6	12.3	31.0	129.0	78.0	70.0	92.0	32.0
iP	N.D.	2.3	0.1	2.0	2.5	0.3	1.6	1.2
[9R]iP	13.3	5.2	2.3	7.2	1.2	0.8	3.1	3.6
[9G]*m*T	2.1	1.4	N.D.	0.9	0.5	2.9	1.4	1.3
*m*T	4.0	2.5	N.D.	1.6	1.6	4.3	2.5	1.6
[9R]*m*T	5.5	4.2	N.D.	2.7	1.8	8.2	4.2	3.5
[9G]*o*T	N.D.	0.0	N.D.	N.D.	0.0	0.1	0.1	0.1
*o*T	0.3	0.4	N.D.	0.3	0.6	0.3	0.4	0.2
[9R]*o*T	7.8	8.6	N.D.	9.8	7.6	8.3	8.6	1.1
[9R-MP]BA	—	—	0.5	—	—	—	—	—
[9G]BA	0.8	1.0	0.4	1.4	0.3	1.3	1.0	0.6
[BA]	N.D.	2.0	0.6	1.0	1.3	3.6	2.0	1.4
[9R]BA	12.0	9.3	0.8	9.9	7.9	10.2	9.3	1.3

[a] Samples were analyzed in Cambridge UK by HPLC using the method of Kraigher et al. (1991) followed by ELISA by the method of Strnad et al. (1992a). All results are means of duplicate assays. N.D., not detected. —, not determined.
[b] Produces plants with abnormal flowers.

of UltimaGold scintillation cocktail (Packard-Canberra, IL USA). Handling losses were estimated by measuring isotope dilution of the available cytokinin standards.

The UV absorbance between 240 and 300 nm was monitored on-line with an SPD-M6A detector (Shimadzu, Japan) for 40 min. This wavelength interval was scanned approximately 10–15 times/s. Spectra were produced by integration of up to 50 peak scans, which were averaged to produce spectra of high accuracy. Peaks were integrated further at 265, 268, and 270 nm using CLASS-LC100 software (Shimadzu, Japan)

GC-MS

Specific cytokinin UV-absorbing fractions were combined and dried in a nitrogen stream in 1 mL Pierce Reacti-Vials (Pierce, Richmond, CA, USA) and in vacuo over P_2O_5. Dry samples were permethylated using dimethyl sulfinyl ions as reported previously for *m*T cytokinins (Strnad et al. 1996). The permethylated cytokinins were dissolved in 25 μL of methanol, and 10 μL was injected onto a 30-m × 0.25-mm μPTE-5 (Supelco, Bellefonte, PA, USA) capillary column. They were separated using He carrier gas, flow rate 2mL · min^{-1}, temperature gradient from 150 to 300°C at 10°C · min^{-1} and 300°C for 10 min. Electron impact mass spectra were obtained with an HP 5890A (Hewlett Packard) mass spectrometer at an electron energy of 70 eV.

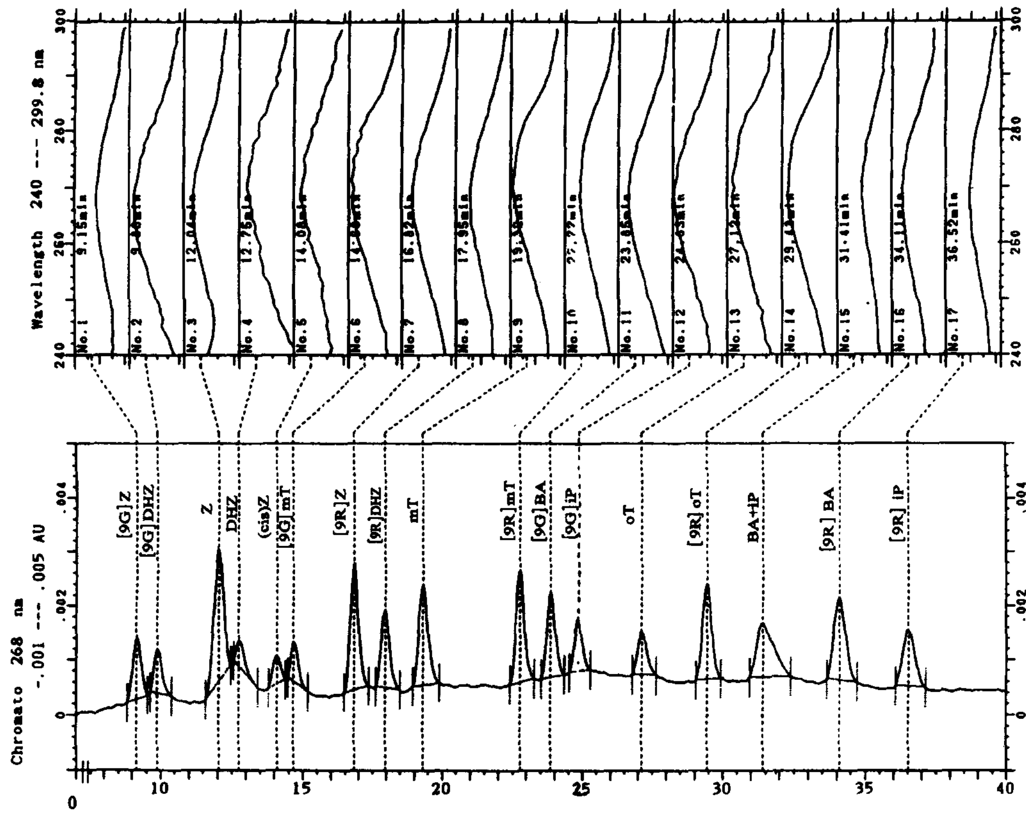
Results

HPLC Separation of Standards

Fig. 1, *a* and *b*, shows the separation of standard compounds routinely achieved using the neutral methanol gradient in use in Cambridge (Fig. 1*a*) and the acid acetonitrile gradient used in the Olomouc laboratory (Fig. 1*b*).

Both systems gave good resolution of the majority of cytokinins in mixtures containing free base, ribosides, ribotides, and 9-glucosides of the 3-isoprenoid types (Z, DZ, and iP) and the three aromatic types (BA, *m*T, and *o*T). This separation, combined with the high specificity of the different antibodies for their respective cytokinin

A



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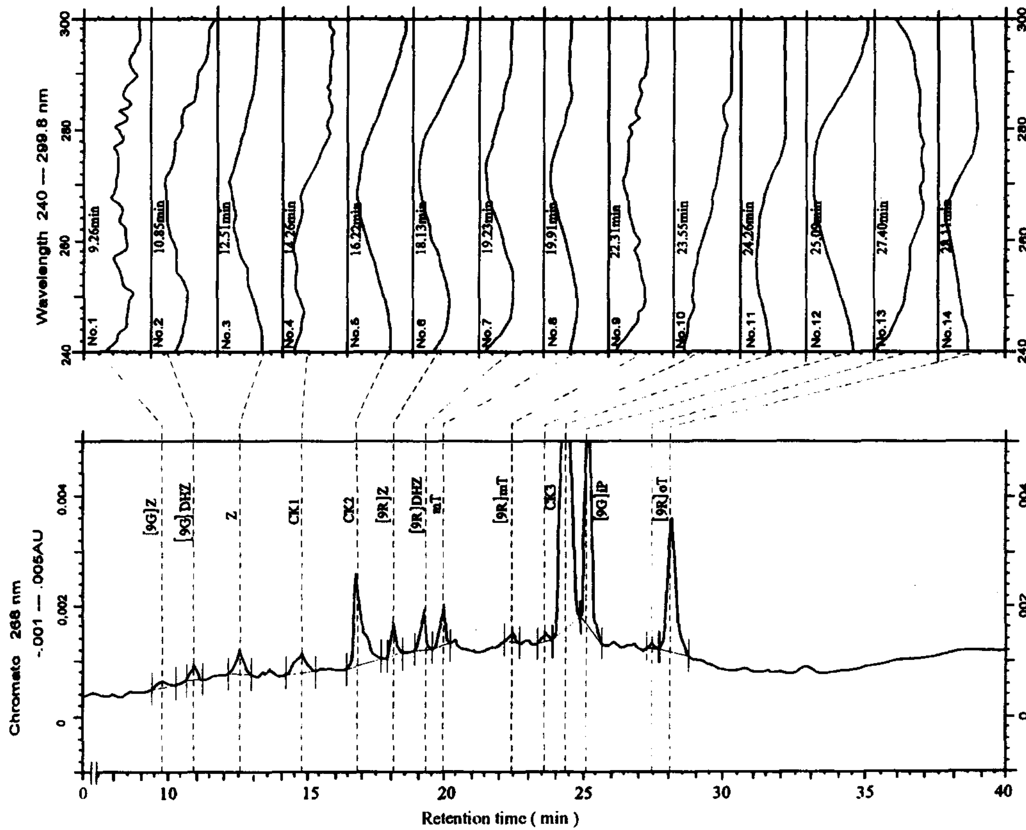

families, allows us to be reasonably confident of the identity of the compounds detected.

Cross-reactivity of HPLC Fractions to Specific Antibodies

The histograms presented in Fig. 2 show the cross-reactivities detected in HPLC fractions from an extract of oil palm seedling shoot tissue. It can be seen that there were highly specific reactions to cytokinins from all six groups in fractions corresponding to the retention times of known standards, with little interfering cross-reactivity between separate assays. This can be taken as good evidence of the presence of these compounds. In this series of experiments the HPLC runs were faster than usual, and the free bases and ribosides of iP and BA eluted in successive minutes, so these compounds were not well resolved. There was, however, clear evidence for the presence of ribosides of Z, *m*T, *o*T, and either ribosides or free bases of iP and BA. In other assays these compounds were resolved (see for example the analyses of embryoids in Table 1), and it is likely that the ribosides were the dominant components. Free base concentrations were consistently low. Activity in the ELISA for DHZ-type cytokinins was in this case not coincident with the retention times of standard compounds, although there was some signal corresponding to the riboside. Note the relative difference in peak heights at 13 and 14 min (peak 4, [9R]Z in Fig. 2 *a* and peak 8, [9R]DHZ, in Fig. 2 *b*) in relation to the response of the same fractions to antibodies to [9R]Z and [9R]DHZ. Otherwise the peaks found in assay with anti-DHZ were consistent with low cross-reactivity against other cytokinins present.

Cross-reactivities of various cytokinins in all of the isoprenoid and aromatic groups with the six antisera available have been reported by Huntley (1995). In general the free bases have about half the cross-reactivity of the corresponding ribosides, whereas the cross-reactivities of the 9-glucosides are variable depending on the antibody used.

Table 1 shows the results of analyses carried out in Olomouc of embryos, in vitro embryoids and three stages of inflorescence development from a normal flowering palm and one with abnormal (mantled) flowers. Anthesis occurs in the axil of leaf 20 ± 2. Leaf 0 is conventionally the unexpanded spear leaf in the center of the crown. The time course of inflorescence development was described by Corley (1976). The inflorescence in the axil at leaf 6 is very small (approximately 1.5 g including spathes). Rapid expansion growth takes place after leaf 8; by leaf 12 the spikelets are well differentiated, and abnormal flowers can be recognized. We think that the critical stage for the expression of floral abnormality occurs between leaf 5 and leaf 8 (Jones et al. 1995). Data for isoprenoid cytokinins in inflorescences in leaf axils 6, 7, and 8 were reported in Jones et al. (1995). Note that in this particular analysis ribotides were converted to ribosides by phosphatase hydrolysis before HPLC separation. All samples contained both isoprenoid and aromatic cytokinins. The most striking feature of the results is the generally high concentration of putative [9R]*o*T in all samples except embryoids and the very young inflorescence tissue. The isoprenoid cytokinins follow the pattern observed previously (Jones et al. 1995), with [9R]Z the most abundant active isoprenoid cytokinin and relatively high concentrations of [9G]Z and [9G]iP. Dihydrozeatin compounds were consistently present in zygotic and somatic embryos and most abundant in the zygotic embryo haustorium. This confirms our previous observations (Jones et al. 1995). Their presence in somatic embryos suggests that haustorial tissue is also present in these structures.

In Table 2 data are shown from analyses in Cambridge on embryoids and young shoot tissue from in vitro cultures compared with seedlings and the vegetative crown meristem with surrounding leaf base tissue. The figures are estimates of the concentration of each cytokinin species present in terms of riboside equivalents, corrected for recovery losses but not for differential cross-reactivities within each cytokinin group. Ribotides of *m*T and *o*T were not determined because we have no synthetic standards of these compounds and do not have their HPLC retention times. Tissues from three different clonal lines were analyzed. Again, all groups of cytokinins were represented, with high concentrations of [9R]*o*T in most samples. In this case the exception was in

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Figure 3. A, HPLC separation of immunoaffinity-purified cold and tritium-labeled cytokinin standards. The immunopurified mixture contained 100 pmol each of 18 cytokinins. Recovery of each tritium-labeled cytokinin was higher than 90%. Capacity of the 0.4-mL column was higher than 10 nmol. HPLC conditions were as described in the Materials and Methods section. **B,** analysis of immunopurified extract of oil palm inflorescence tissue (clone 90a, leaf axil 11, abnormal female) by photodiode array HPLC. Abbreviations above the trace indicate peaks with retention times, UV spectra, and immunoactivity corresponding to peaks with the same abbreviations as in 3 **panel A.** Z9G, zeatin 9-glucoside; dHZ9G, dihydrozeatin 9-glucoside; Z, zeatin; DHZ, dihydrozeatin; cisZ, *cis*-zeatin; [9G]*m*T, *meta*-topolin 9-glucoside; [9R]Z, zeatin riboside; [9R]DHZ, dihydrozeatin riboside; *m*T, *meta*-topolin; [9G]*o*T, *ortho*-topolin 9-glucoside; [9R]*m*T, *meta*-topolin riboside; [9G]BA, benzyladenine 9-glucoside; [9G]iP, isopentenyladenine 9-glucoside; *o*T, *ortho*-topolin; [9R]*o*T, *ortho*-topolin riboside; BA, benzyadenine; [9R]BA, benzyladenosine; [9R]ip, isopentenyladenosine.

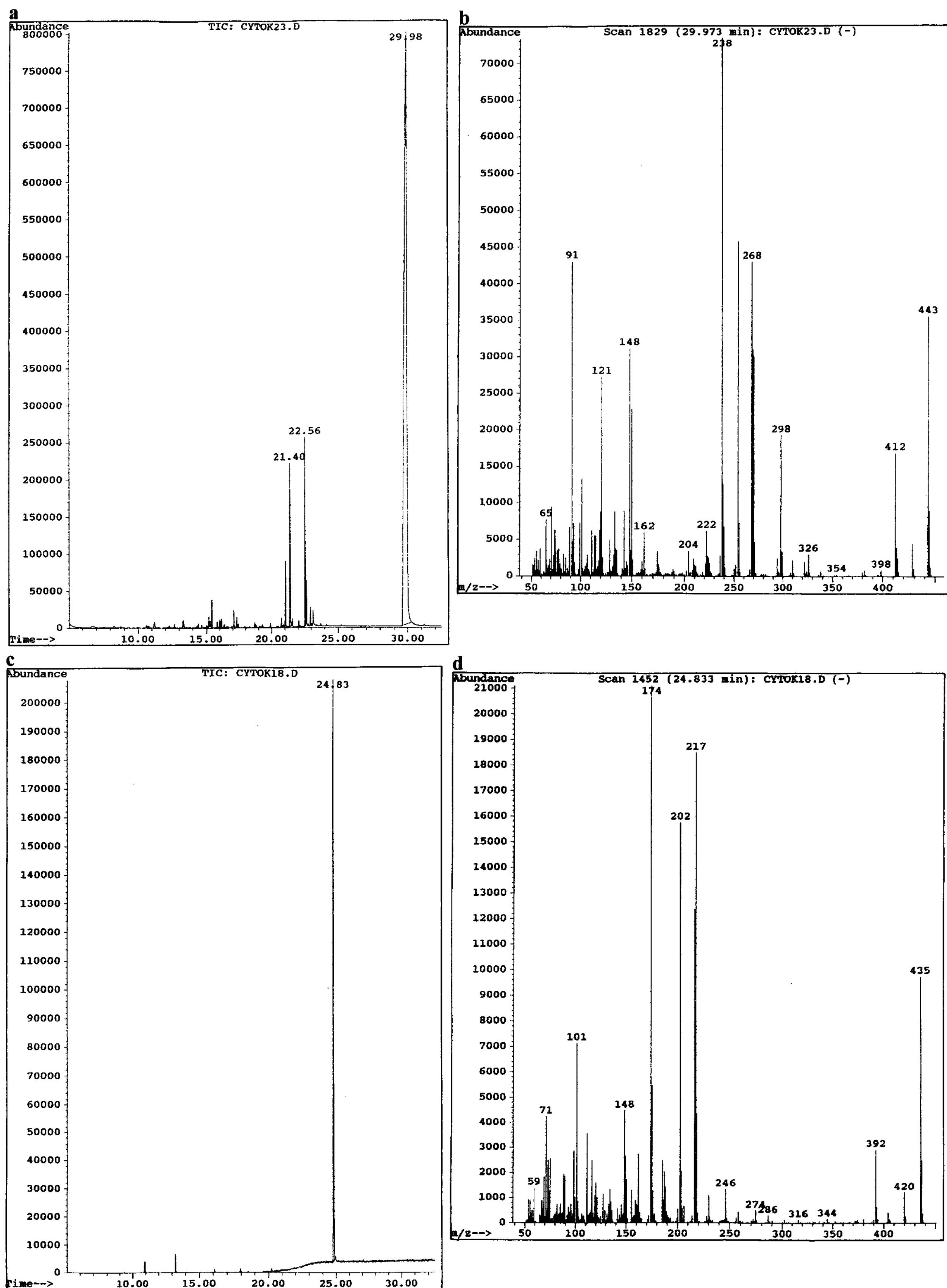

Fig. 4. Electron impact mass spectrum of putative permethylated *o*T riboside and isopentenyladenine 9-glucoside from combined extracts of immature inflorescences of oil palm tissues. *ortho*-topolin: **(a)** total ion concentration vs GC retention time. **(b)** ion mass spectrum of principal peak at 29.98 min. Isopentenyladenine 9-glucoside: **(c)** total ion concentration vs GC retention time. **(d)** ion mass spectrum of principal peak at 24.83 min.

the crown meristem, in which it was not detected. As in Table 1, the isoprenoid cytokinins followed the familiar pattern for oil palm tissues, but a dominant feature was the relative abundance of the ribosides of BA, *o*T, and *m*T with generally lesser amounts of free base.

Cytokinin Analyses Using On-line Photodiode Array HPLC

An HPLC system to separate IAC-bound isoprenoid and aromatic cytokinins was developed to analyze immunopurified plant extracts. The HPLC eluate was scanned continually between 240 and 300 nm with an on-line UV spectrum detector. The HPLC retention times and UV spectra of 18 IAC-purified cytokinin standards collected from the chromatogram are shown in Fig. 3*A*. There was little effect of the different N^6-substituents on the spectra. Dihydrozeatin compounds have spectra shifted to wavelengths 1–2 nm longer than the other cytokinin groups.

The experiment presented in Fig. 3*A* also indicates that the IAC columns were able to bind all natural cytokinin bases including *trans*-zeatin, *cis*-zeatin, dihydrozeatin, isopentenyladenine, benzyladenine, *m*T, *o*T, as well as their nucleosides, nucleotides, and 9- and 3-glucosides. All 18 cold and labeled cytokinin standards of 100–500 pmol applied to an IAC column were recovered in high yield (90%, see Fig. 3*A*). These experiments indicate that the columns gave high recoveries and had maximum capacities for different cytokinins present in plant extracts. The IAC procedure tested on oil palm extracts is shown in Fig. 3*B*. Seven major peaks with retention times, UV spectra, and immunoreactivity of standards were found. The peaks were zeatin 9-glucoside, dihydrozeatin 9-glucoside, zeatin, zeatin riboside, dihydrozeatin riboside, *m*T riboside, *m*T, isopentenyladenine 9-glucoside and *o*T riboside and are designated with abbreviations in Fig. 3*B*. The peak at the position of CK2 had a cytokinin-like spectrum, but the structure remains unclear. The peaks CK1 and CK3 eluted at 14.72 and 23.55 min, but their spectra were unrelated to that of cytokinins.

GC-MS

The immunoaffinity-purified oil palm extracts of young inflorescence tissue were combined for subsequent identification of cytokinins by GC-MS (Fig. 4). The sample was fractionated by HPLC and UV-absorbing peaks corresponding with cytokinin retention times collected. Almost pure cytokinins collected in this way were permethylated, separated by GC, and the structure elucidated by MS working in electron impact mode (70 eV). According to the HPLC-UV analysis, [9R]*o*T (Fig. 4, *a* and *b*) and [9G]iP (Fig. 4, *c* and *d*) were present in the highest

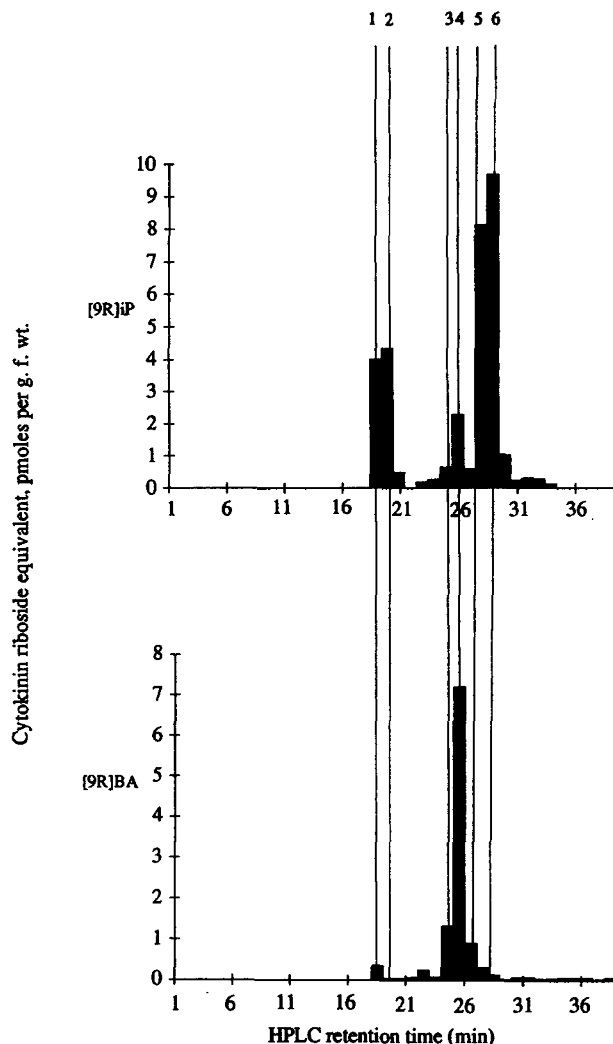

Fig. 5. HPLC/ELISA of oil palm ramets (shoots) showing cross-reactivity to antibodies to [9R]iP and [9R]BA. HPLC conditions were as in Kraigher et al. (1991). ELISA technique was as in Strnad et al. (1992b). Only fractions 18–38 were assayed. Standard retention times: *1,* [9G]BA; *2,* [9G]iP; *3,* BA; *4,* [9R]BA; *5,* iP, *6,* [9R]iP.

concentrations in the combined oil palm extract. The EI mass spectrum (70 eV) of the principal GC peak at 29.98 min exhibited an [M$^+$] at *m/z* 443, which matches that of a pentamethyl [9R]*o*T. The most indicative ions were at *m/z* 412 (loss of $-OCH_3$), 298 (di-Me *o*T with HCOH sugar fragment), 268 (di-Me *o*T), 254 (Me *o*T), 148 (N^6-substituted adenine), and 121 (Me-benzylamine). In the case of penta-Me [9G]iP ([M$^+$], 70 eV, *m/z* 435) the most intense ions in the spectrum arise by elimination of the side chain and loss of the tetramethylated ribose with proton transfer to the purine ring. The ions at *m/z* 420 and 392 arise by elimination of $-CH_3$ and $-C_2H_7$ from the side chain. The major fragment ions in the spectrum at *m/z* 217, 216, 202, and 174 are formed, respectively, by loss of glucose with proton transfer, loss of a proton by

this ion (presumably from the terminal methyl group), loss of methyl, and loss of $-C_3H_7$ from the side chain. The presence of an ion at *m/z* 148 in both spectra strongly indicates the existence of a side-chain at N^6 with at least one $-CH_2$ group (Shannon and Letham 1966). The evidence from MS therefore confirms the identification by retention times in HPLC and GC of *o*T riboside and isopentenyladenine 9-glucoside as the major cytokinin components of oil palm inflorescence tissue extracts. However, the levels of Z, *cis*-Z, [9R]Z, [9R]DHZ, and *m*T were too low to be identified by this method.

Discussion

The neutral methanol gradient system for HPLC separation of the isoprenoid cytokinins has been in routine use in the Cambridge laboratory for several years for analysis of isoprenoid cytokinins in oil palm tissues. In this work we have regularly observed specific cross-reactivity with antiserum to [9R]iP in fractions eluting at retention times appropriate to benzyladenine, its riboside, and 9-glucoside (usually eluting at about 25, 27, and 18 min, respectively) at which no known cross-reacting isopentenyladenine compounds would elute. Since BA is commonly used as a medium additive in tissue culture work we had already established that the isopentenyladenine antibody has a relatively high affinity for BA compounds, whereas the antibodies to zeatin riboside and dihydrozeatin riboside in use in our laboratory have very low cross-reactivities with them. These observations prompted the collaborative program between Cambridge University and the Czech Institute of Experimental Botany in Olomouc to use the antibodies to BA and its derivatives to check for the presence of aromatic cytokinins in oil palm tissues. Furthermore, in the Czech laboratory affinity purification, HPLC with on-line UV monitoring, and GC-MS have been used to confirm the identities of the cytokinins already tentatively identified by their HPLC retention times and antibody cross-reactivities. The results have confirmed those obtained by immunoassay and have given definitive mass spectra demonstrating that *o*T riboside and isopentenyladenine 9-glucoside are the major cytokinins present in many oil palm tissues.

It is clear from the data that representatives of all of these cytokinin families are present in a wide variety of oil palm tissues. The predominant forms of isoprenoid cytokinins are the ribosides and 9-glucosides, whereas the free bases are usually close to or below the detection limit. In the case of the aromatic cytokinins, ribosides of all three types were present, with lesser amounts of free base and rather low concentrations of 9-glucosides. The aromatic cytokinins were at very low concentrations in the vegetative crown tissues and in the youngest inflorescence analyzed (leaf 6) but were found in older inflorescences, embryos, embryoids, and shoot tissues of seedlings and ramets. In particular, the riboside of *o*T was consistently found to be the major cytokinin present, at concentrations up to 50 pmol $\cdot$ g^{-1} fresh weight. Without sample replication no conclusions can be reached on quantitative differences between tissue types.

The content of *m*T compounds was very variable among samples. It has been shown in poplar leaf tissue (Strnad et al. 1996, unpublished) that these compounds are regulated by light, and the concentration can vary over two or three orders of magnitude in a short time.

At present we do not know the functions of the different cytokinins, although observations of their use in tissue culture shows that plant tissues respond differently to different cytokinins. The *ortho*-topolins have low biological activity in conventional bioassays (Iwamura et al. 1980, Kamínek et al. 1987), but this may only imply that they affect physiological processes not previously associated with responses to cytokinins.

Data obtained in Olomouc (Table 1) confirm the presence of a high concentration of [9G]DHZ in the embryo haustorium, with much lower levels in the tigellum and other oil palm tissues, as reported by Jones et al. (1995).

The detection of BA and [9R]BA in the samples confirmed our suspicion that the previously unidentified cross-reactivity found at the same retention times in most of our ELISAs for [9R]iP was indeed due to the presence of these compounds. This observation emphasizes the difficulty inherent in the interpretation of ELISA results unless the compounds are separated by HPLC with accurately controlled retention times and checked with other antibodies, not only for nonspecific interference with binding but also for interference as a result of the presence of other cytokinins.

Fig. 5 shows an assay of shoots of oil palm ramets using antibodies to [9R]iP and [9R]BA. In this instance the HPLC run was again faster than average (part of the same experimental sequence shown in Fig. 2). The free bases and ribosides eluted in successive minutes and were not completely resolved as shown by the ELISA. Using the [9R]iP antibody a strong signal was obtained at the retention time of [9R]BA which could have been misinterpreted as iP free base. Using the antibody to [9R]BA it is clear that the correct interpretation is that the peak was due to the presence of about 8 pmol of [9R]BA per g fresh weight. The cross-reactivity of [9R]BA with the [9R]iP antibody, about 30%, would give a peak of the size observed. By contrast the cross-reactivity of isopentenyladenine compounds with the antibody to [9R]BA is very low, and little signal was obtained in this assay at the retention time of [9R]iP. A small peak was seen at the retention time of [9G]BA, overlapping that of the relatively strong [9G]iP signal. The absence of a response in the fraction at 18 min suggests that this was probably a genuine indication of

the presence of [9G]BA, but at this level it would not affect the quantification of [9G]iP.

We think that our experience of relatively high (30%) cross-reactivity of BA cytokinins with antibodies to isopentenyladenine in not confined to our own antibodies. Antibodies raised in Olomouc and Cambridge to [9R]iP share this property. Others using ELISA methods to estimate iP and its derivatives may get false results if their HPLC system does not fully resolve iP, [9R]iP, BA, and [9R]BA, which elute fairly close together in our (and most other) gradients. Fortunately the isopentenyladenine cytokinins have very low cross-reactivities with the anti-BA riboside antiserum, and this allows the compounds to be resolved when all are present together.

Acknowledgments. We thank Unilever Plantations and Plant Science Group for funding the work in Cambridge; Dr. C. J. Eeuwens of Unifield TC Ltd., Cambridge, for provision of oil palm tissue cultures and ramets; C. Donough of Pamol Plantations Sdn. Bhd, Kluang, Malaysia, and Dr. S. Mayes and Dr. B. G. Smith of Unilever Plantations and Plant Science Group, Cambridge, for oil palm inflorescences and crown tissue. We thank Dr. J. Hanus, Institute of Experimental Botany, Isotopic Laboratory, Prague, for the gift of both unlabeled and tritiated aromatic cytokinin standards; and Mgr. Jana Zalubilová for her help with figures. Research in the Czech Republic was supported by Academy of Sciences Grant A538402 and by Czech Grant Agency Grant 206/94/0641.

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