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Endogenous isoprenoid and aromatic cytokinins in different plant parts of *Cocos nucifera* (L.)

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Abstract

The present study reports the analyses of both isoprenoid and aromatic cytokinins in the coconut palm by combined high performance liquid chromatography and group specific enzyme immunoassays (HPLC-ELISA). The results showed that the isoprenoid cytokinins were several fold more abundant than the aromatic cytokinins in each of the plant parts analysed: immature inflorescence, shoot apical meristem (SAM), spear leaf and embryo. Within the isoprenoid cytokinins, the most abundant ones by type were the zeatin- (Z-), the isopentenyladenine- (iP-) and the dihydrozeatin- (DHZ-) type in decreasing order for most plant parts studied, and individually, zeatin riboside (ZR) or zeatin riboside-5′-monophosphate (ZR5′P) depending on the part. In the case of the iP-type cytokinins, the results showed that its 9-glucoside was the most abundant one in most parts. The isoprenoid cytokinin profiles in coconut showed a predominant pattern of 9-conjugation as a major metabolism route for these cytokinins. Analyses also showed the occurrence of the aromatic cytokinin 6-benzylaminopurine (BAP) and its riboside (BAPR), 9-glucoside (BAP9G), and nucleotide (BAPR5′P). Their presence in coconut palm was unequivocally identified after permethylation by gas chromatography-mass spectrometry. They were more concentrated in the embryo and in the immature inflorescence than in the other two parts studied, however their concentration in each part was several times lower than that of isoprenoid cytokinins. All four were detected in each of the parts studied. The most abundant ones were BAPR and BAP9G in immature inflorescence; and BAPR in all of the other parts. When all cytokinins analysed are considered, differences between the plant parts studied were found. The zygotic embryos showed the highest content, double that in immature inflorescence, and five times more that in spear leaf and SAM. These differences are even greater when individual cytokinins are compared.

Abbreviations: BAP – 6-benzylaminopurine, BAPR – 6-benzylaminopurine riboside, BAP9G – 6-benzylaminopurine-9-glucoside, BAPR5′P – 6-benzylaminopurine ribotide, DHZ – dihydrozeatin, DHZR – dihydrozeatin riboside, DHZ9G – dihydrozeatin-9-glucoside, DHZR5′P – dihydrozeatin ribotide, ELISA – enzyme-linked immunosorbent assay, FW – fresh weight, GC-MS – gas chromatography-mass spectrometry, HPLC – high performace liquid chromatography, iP – isopentenyladenine, iPR – isopentenyladenosine, iP9G – isopentenyladenine-9-glucoside, iPR5′P – isopentenyladenosine-5′-monophosphate, monoclonal antibody immunoaffinity chromatography – mIAC, RT – retention time, SAM – shoot apical meristem, mT – *meta*-topolin, mTR – *meta*-topolin riboside, mT9G – *meta*-topolin-9-glucoside, oT – *ortho*-topolin, oTR – *ortho*-topolin riboside, oT9G – *ortho*-topolin-9-glucoside, Z – zeatin, ZR – zeatin riboside, Z9G – zeatin-9-glucoside, ZR5′P – zeatin ribotide, ZOG – zeatin-O-glucoside, ZROG – zeatin riboside-O-glucoside

Introduction

The coconut palm (*Cocos nucifera* L.) is a source of oil that is an important commercial product worldwide since, along with the palm kernel oil, it is the only source of short-chain fatty acids (Berger and Ong 1985). In coconut, vegetative propagation would allow significant yield increases by propagating high-yielding individuals. At present the coconut is only sexually propagated because of some morphological and physiological particularities; it has only one stem and does not produce any side shoots. Axillary buds only give inflorescences and the only vegetative bud is the shoot apical meristem (SAM), which builds the whole tree (Pannetier and Buffard-Morel 1986).

Cytokinins are plant growth regulators that control developmental processes in the different plant parts, such as cell division (Skoog et al. 1965), promote growth of lateral buds (Pillary and Railton 1983), delay senescence of intact plants (Nooden et al. 1990) and excised plant parts (Singh et al. 1992) and affect source sink/relationships (Kuiper et al. 1988). They are also widely used (in combination with auxins) to promote morphogenic responses in plant tissue culture *in vitro* (Krikorian 1995). However, nothing is known about the endogenous cytokinins in coconut palm and their role in the development of the crop.

In nature we can find cytokinins with an isoprenoid or with an aromatic side chain. Three groups of isoprenoid cytokinins can be found: the zeatin- (Z-), the dihydrozeatin- (DHZ-) and the isopentenyladenine- (iP-) type cytokinins. When analysis of cytokinins has been performed for several plant species, a great diversity of them has been found (see Kamínek (1992)) both for the isoprenoid types (Whenham 1989; Ulskov et al. 1992; Hansen et al. 1988) and the aromatic ones (see below).

Patterns and concentrations of cytokinin metabolites in different plant organs vary as reported for *Phaseolus vulgaris* (Hammerton et al. 1996) and *Pistacia vera* (Ahmadi and Baker 2000). Differences in composition and concentration have also been reported between xylem sap and phloem sap (Komor et al. 1993; Kamboj et al. 1998). Regarding abundance, Z-type cytokinins are the major ones found in most studies (Koshimizu and Iwamura 1991; Wagner and Beck 1993; Hammerton et al. 1996; Dieleman et al. 1997; Ahmadi and Baker 2000). Although, when care has been taken to preserve iP nucleotides, these were found to comprise the majority of the cytokinins in *Phaseolus vulgaris* (Hammerton et al. 1996). In the above mentioned studies aromatic cytokinin analysis was not performed, however, in recent years there has been increasing awareness that aromatic cytokinins occur naturally in many plant tissues, and methods developed by Strnad et al. (1992, 1994) has made it possible to use by combined high performance liquid chromatography and enzyme-linked immunosorbent assay (HLPC/ELISA) methods for their routine analysis. They include 6-benzylaminopurine (BAP), the *meta*-topolin (mT) and *ortho*-topolin (oT) families. The first natural compound of this type has been isolated by Horgan and co-workers from mature poplar leaves (Horgan et al. 1973) and identified as 6-(2-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (Horgan et al. 1975). It was later isolated from fruits of *Zantedeschia aethiopica* (Chaves das Neves and Paiss 1980a) together with 6-(2-hydroxybenzylamino)-2-methylthio-9-β-D-glucofuranosylpurine (Chaves das Neves and Paiss 1980b). The occurrence of this type of cytokinin has been also reported for a *Pimpinella anisum* L. cell culture (Ernst et al. 1983), *Populus* x *canadiensis* Moench (Strnad et al. 1992, 1994, 1997) and oil palm (Jones et al. 1995, 1996).

The latter are the only studies reported so far for a palm species. The present paper describes the analysis of the different types of cytokinins, including both the isoprenoid and aromatic ones, in aerial parts of another palm species, the coconut (*Cocos nucifera* L.).

Materials and methods

Chemicals and reagents

Cytokinins: iP, iPR, Z, ZR, DHZ, DHZR, BAP, BAPR were purchased from Sigma. oT, oTR, oT9G, mT, mTR and mT9G were synthesised as described by Holub et al. (1998). Before use all cytokinins were purified by HPLC as described by Strnad (1996). Bovine serum albumin, ovoalbumin and 4-nitrophenylphosphate were purchased from Fluka; DEAE-cellulose and acid phosphatase from Sigma, Sep-Pak C_{18} cartridges from Waters Assoc., methanol and acetonitrile for chromatography from Merck. Tritium labelled cytokinins, immunogens and alkaline phosphatase tracer syntheses as well as immunisation schedule and isolation of immunoglobulins were performed as described by Strnad (1996).

Plant material

Aerial parts from three different 15 year old coconut palms (var. Green Malayan Dwarf) were sampled at San Crisanto Yucatán, Mexico. The parts sampled were the immature inflorescence-5 (taking the first open inflorescence as zero)–stem apical meristem (SAM), the spear leaf and zygotic embryos (collected from nuts of 12 months after pollination). The samples were weighed, deep frozen in liquid nitrogen and stored in a refrigerator at −70 °C for two weeks until they were lyophilised and sent to Olomouc, Czech Republic for analysis. The samples were analysed in triplicate.

Cytokinin Analyses

Cytokinins from each plant part studied were, extracted and purified by the methods of Kraigher et al. (1991) and Faiss et al. (1997). The extracts were purified using combined DEAE Sephadex-Sep Pack C18 columns. Cytokinin bases, ribosides and glucosides retaining on a reverse-phase cartridge were eluted in 5 ml 80% methanol (v/v) and after drying B fraction (basic cytokinins) was obtained. After washing with 10 ml distilled water the DEAE-Sephadex column was coupled to another Sep Pack and cytokinin nucleotides were eluted with 10 ml 6M HCOOH. The nucleotides retained on the C18 cartridge were eluted in 5 ml 80% methanol, dried and dephosphorylated (fraction NT). The conversion of cytokinin nucleotides to their dephosphorylated forms was carried out using acid phosphatase for 30 min in the dark (25 °C, 0.05 U ml^{-1}, Sigma P-3627, EC .3.1.3.2) in 40 mM amonnium acetate buffer (pH 6.5), as described by MacDonald et al. (1981). Both fractions were immunopurified on a cytokinin monoclonal antibody column (Faiss et al. 1997). Cytokinin-O-glucosides (fraction OG) occurring in PBS eluates from the immunoaffinity columns were treated with β-glucosidase and re-purified on the same monoclonal column. All three immunopurified fractions were separated by reversed-phase HPLC and assayed by ELISA following the methods described in Jones et al. (1995). The aromatic cytokinins were analysed by the method of Strnad (1996). The following modifications of the methods have been used for cytokinin analyses:

1. HPLC separation was realised on Microsorb C18 (Rainin, 150 × 4.6 mm, 3 μm particle size);
2. the HPLC elution was performed with a methanolic gradient in (A): 10% methanol in 40 mM acetic acid (AcA) adjusted to pH 3.4 with triethylamine, and (B): 80% methanol in 40 mM AcA. The following gradient sequences were used: 0 min, 90% A + 10% B, 10 min, 60% A + 40% B, 14 min, 65%A + 35% B, 18 min, 50% A + 50% B, 24 min, 50% A + 50% B, 26 min, 100% B, 30 min, 100% B, 31 min, 90% A + 10% B, the flow rate was 0.6 ml/min;
3. aliquots of 50 μL were used in different group specific ELISAs;
4. the resulting immunohistograms were quantified on the basis of cross-reactivity and losses of tritiated cytokinin recovery markers;
5. the endogenous content was also calculated from the integration of HPLC peaks when the cytokinin levels were higher than 5 pmol.g^{-1} FW. Recoveries were usually higher than 60%. Total cytokinin content was calculated from the sum of the individual cytokinins analysed.

Identification of cytokinins

Cytokinin identity was confirmed by photodiode-array HPLC detection and by gas chromatography-mass spectrometry (Jones et al. 1996; Strnad et al. 1994). HPLC fractions containing appropriate cytokinin were evaporated in 1 ml hypovials (Pierce, Chester, UK) and permethylated using methyl iodide in dimethylsulphonyl carbanion. The samples were dried in a stream of N_2. The permethylated cytokinins were dissolved in 10 μl of methanol and 3 μl injected on to a 30 m × 0.25 mm PTE-5, (Supelco, INC, Bellefonte, PA, USA) 0.25 μm i.d. capillary column: He carrier gas, flow rate 2 ml/min, 2 min at 40 °C, from 40 °C at 10 °C min^{-1} to 300 °C and 300 °C for 10 min. The mass spectrometer was an electron impact HP 6890/HP5073 operating at electron energy of 70 eV with an autosampler HP 7673 (Hewlett Packard) in MSD regime: TIC, 45–550 amu, and 2.91 scan/s.

Statistics

The data shown in this paper represent the means ± SD of the analysis of tissues coming from three different plants. The data were submitted to analysis of variance (ANOVA). The significance differences were determined by test of Newman-Keuls.

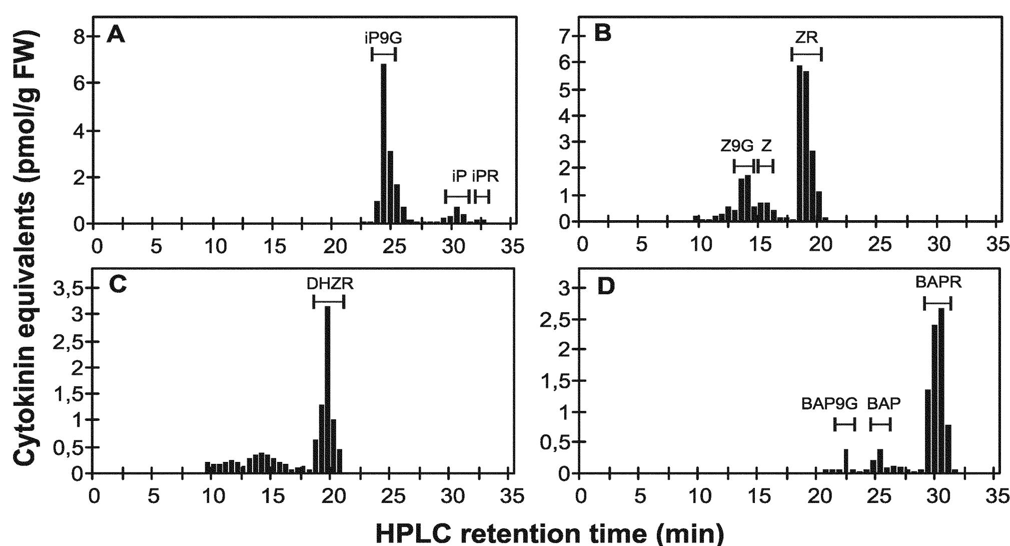

Figure 1. Immunodetection of isoprenoid and aromatic cytokinins in HPLC fractionated extracts from zygotic embryos of *Cocos nucifera* L. by ELISAs for isopentenyladenosine (A), zeatin riboside (B), dihydrozeatin riboside (C), and 6-benzylaminopurine riboside (D). Retention times of appropriate [2-^{3}H] or [^{3}H] cytokinin standards are indicated by horizontal bars.

Results

Cytokinin immunodetection and identification

The aerial parts of coconut palm were screened by direct ELISAs specific for individual cytokinin groups. The extracts containing considerable cytokinin immunoactivity (expressed as pmoles of riboside equivalents per gram of fresh weight) were further analysed by the ELISAs of individual HPLC fractions. The molecular recognition properties of the antibodies used enabled the ELISAs to be used to detect and quantify free bases, ribosides and 9-glucosides (see Figure 1) in basic (B) as well as in the nucleotide and the O-glucoside fractions after dephosphorylation and deglucosylation, respectively. The presence of cytokinins in the coconut extracts was determined on the bases of their co-elution with an authentic and radioactive standard upon HPLC and their relative activities in different ELISAs (see Figure 1B–D). The assays showed highly specific regions of cytokinin immunoactivity in HPLC fractions corresponding to standards with almost zero interfering cross-reactivity between separate assays. The quality of assays was further improved by introduction of monoclonal antibody immunoaffinity chromatography (mIAC, Figure 2). An example of the mIAC purified zygotic embryo extract is shown in Figure 2 (see for comparison with Figure 1 - same sample in ELISAs).

Several major cytokinin UV absorbing peaks with retention times, UV spectra, and immunoreactivity of appropriate standards were found. The peaks were zeatin riboside, dihydrozeatin riboside, isopentenyladenine-9-glucoside, and 6-benzylaminopurine riboside and are designated with abbreviations in Figure 1. The peak at position CK1 and CK2 (RT: 24.06 min + λ_{max} = 272 nm and RT: 27.38 min + λ_{max} = 275 nm, respectively) occurring in all samples analysed had also a cytokinin-like spectrum, but its structure remains still unclear.

The identity of different type of cytokinins tentatively identified by their HPLC retention times and antibody cross-reactivity was further confirmed by electron impact gas chromatography-mass spectrometry (GC-MS, Figure 3). This analytical technology was further improved by development of capillary GC separation of permethylated cytokinins (Figure 3A). The GC system separating 18 different isoprenoid and aromatic cytokinins was used to identify natural cytokinins present in the coconut extracts. The GC eluate was scanned continually between 45–550 amu with an on line electron impact mass spectrometer. The following compounds were identified in coconut palm extracts by this method: Z9G, Z, ZR, iP9G, DHZR, BAP, BAPR, and BAP9G. The mass spectrometric data are, however, shown for BAP cy-

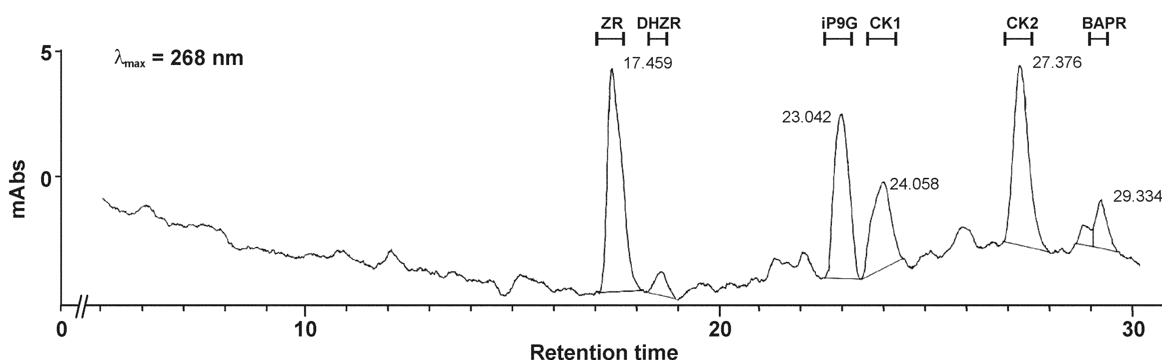

Figure 2. Diode-array HPLC analyses of immunoaffinity purified sample from zygotic embryos of *Cocos nucifera* L. Abbreviations above the traces indicate peaks with retention times. ZR - zeatin riboside; DHZR - dihydrozeatin riboside; iP9G - isopentenyladenine-9-glucoside; BAPR - 6-benzylaminopurine riboside.

tokinins only as these occur quite unusually in plant tissues and have been identified in two different plants only (Ernst et al. 1983; Nandi et al. 1989b). To further confirm the presence of the BAP-like substances in the coconut samples, the BAP immunoactive tissue extracts were combined, immunopurified by mIAC, fractionated by HPLC and corresponding UV/ immunoreactive fractions collected, combined and permethylated. Almost pure cytokinins obtained in this way were separated by capillary GC and structure elucidated under electron impact (70 eV) MS conditions.

The natural BAP, BAPR and BAP9G eluted after permethylation from the GC column at 24.94 min, 31.61 min, and 33.41 min (Figure 3A) and exhibited $[M^+]$ at 253 (di-methyl BAP, Figure 3B), 413 (tetra-methyl BAPR, Figure 3C), and 457 (penta-methyl BAP9G, Figure 3D), respectively. The most indicative ions were at 120 (methyl-benzylamine), 135 (adenine), 148 (N^6-substituted adenine), 224 (BAP), 238 (methyl BAP). Thus, GC-MS analysis clearly showed that the natural compounds coincided with the corresponding parameters of the BAP cytokinins.

Total cytokinins

Analyses of different types of cytokinins in the coconut palm showed that the plant part with the highest concentrations of total cytokinins (isoprenoid + aromatic) was the zygotic embryo with 96 pmoles/g FW and the immature inflorescence with 45 pmoles/g FW, whereas the SAM and the spear leaf had less than 25 pmoles/g FW each (Figure 4).

Isoprenoid cytokinins

The plant parts with the highest concentrations of total isoprenoid cytokinins were the zygotic embryos with 92 pmoles/g FW and the immature inflorescence with 40 pmoles/g FW, whereas the SAM and the spear leaves had less than 20 pmoles/g FW each (Figure 4). A similar pattern could be observed for the concentrations of the individual isoprenoid types of cytokinins (Table 1).

With respect to individual isoprenoid cytokinins, all fourteen CKs analysed were detected in each of the parts studied. The most abundant ones are: ZR (23 pmoles/g FW), iP9G (5 pmoles/g FW), ZROG (3 pmoles/g FW) and ZR5′P (2.5 pmoles/g FW) in immature inflorescence (Table 1); ZR (10 pmoles/g FW), Z9G (2 pmoles/g FW), ZR5′P (2 pmoles/g FW), and ZROG (2 pmoles/g FW) in spear leave (Table 1); ZR5′P (6.5 pmoles/g FW), ZR (3 pmoles/g FW), and Z9G (2.5 pmoles/g FW) in SAM (Table 1); and ZR5′P (23 pmoles/g FW), ZR (20 pmoles/g FW) and iP9G (20 pmoles/g FW) in zygotic embryo (Table 1).

In all the tissues analysed the concentrations of the DHZ-type cytokinins were very low (less than 3 pmoles/g FW) than those of the other types of isoprenoid cytokinins, with the exception of the zygotic embryo where relatively high levels of DHZR (5.73 pmoles/g FW) and DHZR5′P (6.03 pmoles/g FW) were detected (Table 1).

Aromatic cytokinins

The highest concentrations of total BAP cytokinins were present in the zygotic embryo with 4.2

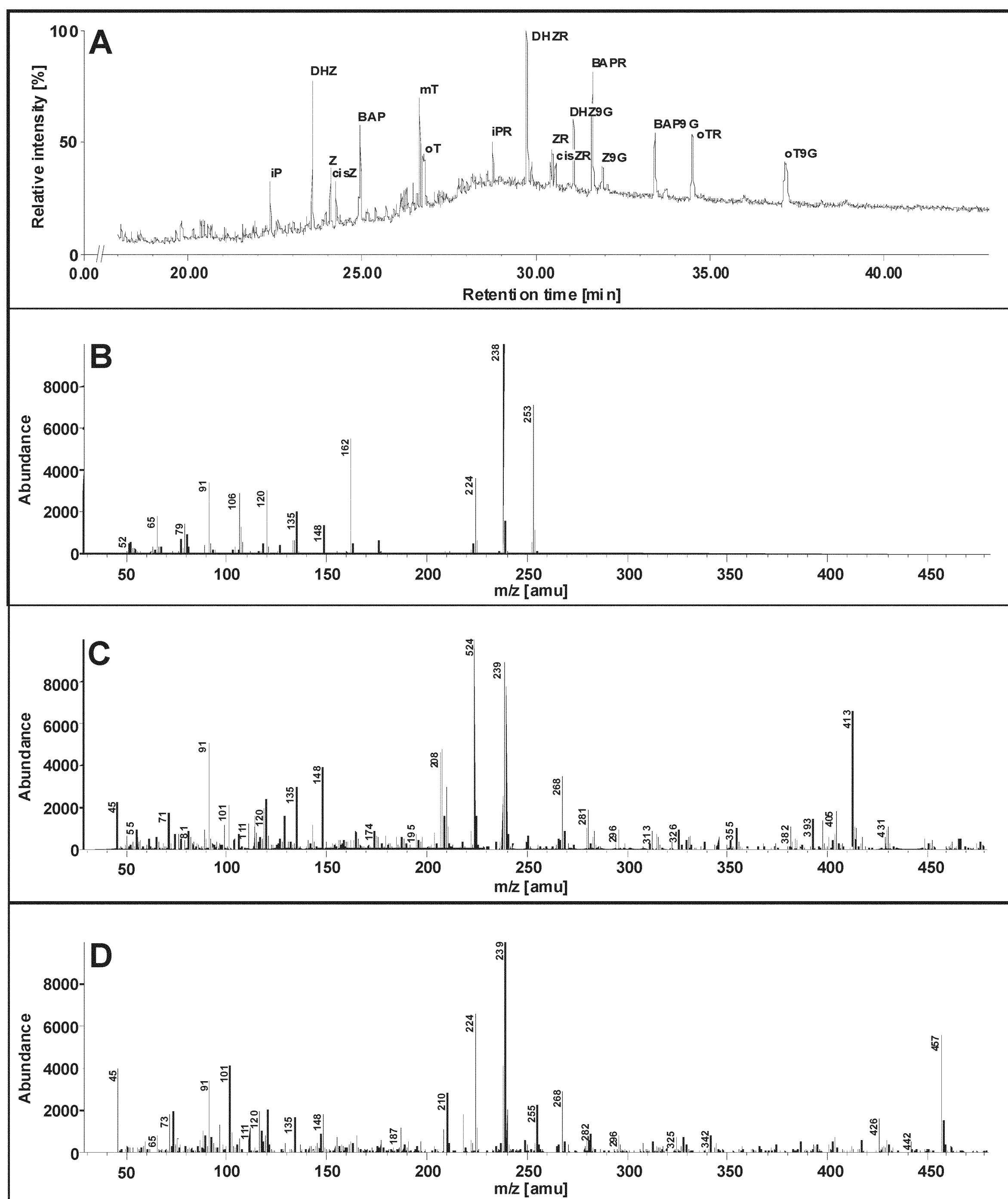

Figure 3. Separation of permethylated cytokinins by capillary gas-chromatography on Supelco μPTE column (A) and electron impact mass spectra (70 eV) of putative permethylated 6-benzylaminopurine (B), 6-benzylaminopurine riboside (C), and 6-benzylaminopurine-9-glucoside (D) from combined extracts of coconut palm tissues.

pmoles/g FW and in the immature inflorescence with 4.3 pmoles/g FW, whereas the SAM and the spear leaf had less than 3 pmoles/g FW each (Table 1). The aromatic cytokinins were, depending on the plant part, from 6 to 20 fold less abundant than the isoprenoid ones (Figure 4).

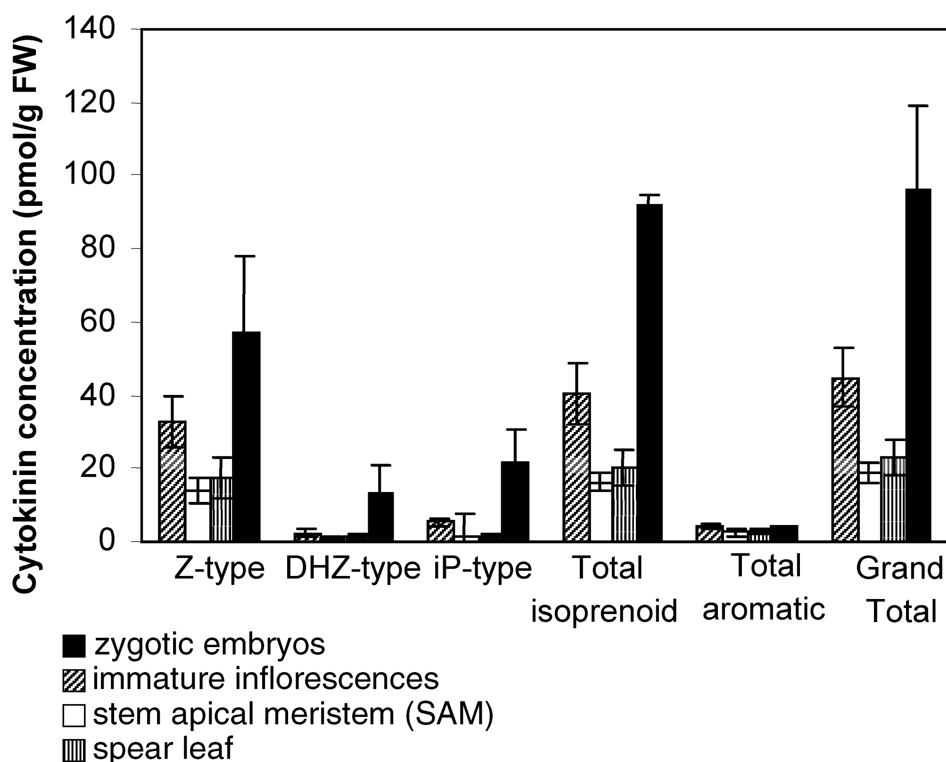

Figure 4. Endogenous concentrations of cytokinin metabolites in different aerial parts of *Cocos nucifera* L. Figures presented are means ± SD (n = 3). Different letters are significantly different at $p > 0.05$.

With respect to individual aromatic cytokinins, all four analysed were detected in each of the parts studied. The most abundant ones were: BAPR (2 pmoles/g FW) and BAP9G (1.6 pmoles/g FW) in immature inflorescences (Table 1); and BAPR in all of the other parts, zygotic embryo (2 pmoles/g FW), spear leaf (2 pmoles/g FW) and SAM (1.6 pmoles/g FW) (Table 1). Other members of the family of aromatic cytokinins were detected: *meta*- and *ortho*-topolin cytokinins, but were found at very low levels (below 0.5 pmol/g FW) in all samples analysed, therefore these results were not included for the calculation of the total cytokinins.

Discussion

Occurrence of both the isoprenoid and the aromatic cytokinins within a single plant species have been reported for only a few species, namely *Urtica dioica* (Wagner and Beck 1993), *Elaeis guineensis* (Jones et al. 1995, 1996) and *Medicago sativa* (Goicochea et al. 1996). These reports have shown that the isoprenoid cytokinins are the most abundant in different plant species. The present study reports the analyses of both isoprenoid and aromatic cytokinins in the coconut palm.

Isoprenoid cytokinins

The results showed that in the coconut palm the isoprenoid cytokinins were several fold more abundant than the aromatic cytokinins in each of the plant parts analysed: immature inflorescence, SAM, spear leaf and embryo. Within the isoprenoid cytokinins, the most abundant ones were the Z-type, the iP-type and the DHZ-type in decreasing order for all plant parts studied, excepting spear leaf where the order was Z-type, the DHZ-type and the iP-type. Therefore these patterns of abundance agree with previous reports for other species. For instance the Z-type cytokinins comprised 90% of the total cytokinins in *U. dioica* (Wagner and Beck 1993), 80–90% in *R. hybrida* (Dieleman et al. 1997) and 43% in *Pistachio* seedlings (Ahmadi and Baker 2000).

Regarding individual isoprenoid cytokinins, the predominant ones were ZR or ZR5′P depending on the part: ZR in inflorescence and SAM; ZR5′P in

Table 1. Endogenous concentrations of individuals cytokinins in aerial parts of *Cocos nucifera* L. Values presented are means ± SD (n = 3). Different letters are significantly different (p > 0.05).

Cytokinins (pmol/g FW)	Tissue analysed			
	Immature inflorescence	SAM	Spear leaf	Zygotic embryo
Z9G	1.79 ± 0.38 [a]	2.68 ± 1.04 [a]	1.96 ± 0.36 [a]	2.59 ± 1.39 [a]
Z	0.84 ± 0.27 [b]	0.43 ± 0.04 [b]	1.13 ± 0.44 [b]	3.84 ± 2 [a]
ZR	23.05 ± 5.32 [a]	3.05 ± 0.93 [b]	10.05 ± 3.31 [b]	20.89 ± 7.07 [a]
ZR5′P	2.83 ± 0.50 [b]	6.53 ± 1.33 [b]	1.69 ± 0.60 [b]	23.93 ± 9.04 [a]
ZOG	0.84 ± 0.10 [a]	0.16 ± 0.07 [b]	0.15 ± 0.087 [b]	0.70 ± 0.19 [a]
ZROG	3.31 ± 0.38 [b]	1.03 ± 0.15 [d]	2.20 ± 0.65 [c]	5.43 ± 0.58 [a]
IP9G	4.92 ± 1 [b]	0.28 ± 0.18 [b]	0.69 ± 0.33 [b]	19.99 ± 8.52 [a]
IP	0.19 ± 0.03 [b]	0.08 ± 0.005 [b]	0.16 ± 0.07 [b]	0.39 ± 0.13 [a]
IPR	0.19 ± 0.03 [b]	0.086 ± 0.005 [b]	0.34 ± 0.034 [b]	0.83 ± 0.39 [a]
IPR5′P	0.1 ± 0.026 [b]	1.11 ± 0.12 [a]	0.17 ± 0.07 [b]	0.13 ± 0.075 [b]
DHZ9G	0.28 ± 0.07 [a]	0.11 ± 0.03 [a]	0.28 ± 0.10 [a]	0.64 ± 0.42 [a]
DHZ	0.28 ± 0.07 [a]	0.11 ± 0.03 [a]	0.28 ± 0.10 [a]	0.64 ± 0.42 [a]
DHZR	1.45 ± 0.86 [b]	0.35 ± 0.10 [b]	0.59 ± 0.27 [b]	5.73 ± 1.13 [a]
DHZR5′P	0.33 ± 0.085 [a]	0.25 ± 0.12 [a]	0.37 ± 0.27 [a]	6.03 ± 5.61 [a]
BAP9G	1.58 ± 0.54 [a]	0.52 ± 0.27 [b]	0.42 ± 0.29 [b]	0.90 ± 0.29 [ab]
BAP	0.44 ± 0.04 [a]	0.23 ± 0.16 [a]	0.32 ± 0.05 [a]	0.80 ± 0.47 [a]
BAPR	1.95 ± 0.52 [a]	1.63 ± 0.90 [a]	1.96 ± 0.60 [a]	2.03 ± 0.43 [a]
BAPR5′P	0.34 ± 0.07 [a]	0.26 ± 0.09 [a]	0.27 ± 0.14 [a]	0.48 ± 0.21 [a]

spear leaf; and in embryo ZR5′P and ZR, but also iP9G. In general, these results are consistent with previous studies showing ZR as the most abundant individual cytokinin in plants of *U. dioica* (Wagner and Beck 1993), *R. hybrida* (Dieleman et al. 1997) and *P. vera* (Ahmadi and Baker 2000). These studies showed that this cytokinin was also the most abundant one in most of the individual plant parts analysed. In the case of the iP-type cytokinins in coconut, the results showed that iP9G was the most abundant one in immature inflorescence, spear leaf and embryo; and iPR5′P in SAM. Similarly, in oil palm (regenerant plants) iP9G was the most abundant iP-type cytokinin (Jones 1990).

The isoprenoid cytokinin profiles in both coconut and oil palm show a predominant pattern of 9-conjugation as a major metabolism route for these cytokinins. Some conjugates are known to be active, however the ribosides, mainly ZR have been found as a form of transport of the cytokinins through the xylem to different parts of the plant (Dieleman et al. 1997), therefore the high concentrations of the 9-conjugates, might suggest that they play a role in the hormonal homeostasis in the coconut tissues. The 9-glucosides are regarded as inactive products of metabolism and are normally found predominantly in the vacuole (Wagner and Beck 1993). They can be regarded as evidence of high cytokinin turnover in coconut. It is, however, possible that they may be remobilised, and their relatively high concentration in the embryo suggests that they might have a storage role prior to germination.

The zygotic embryo showed a different profile for each of the types of cytokinins analysed. For example, high levels of the nucleotide of ZR were found and the levels of iP9G were similar to those of ZR. Higher concentration of iP9G was also detected in oil palm germinating somatic embryos (Jones 1990) and in oil palm regenerant plants (Jones et al. 1995). In coconut, the DHZ-type cytokinins DHZR and DHZR5′P were detected in relatively high concentration in embryos. Similarly, Jones et al. (1995) reported that the DHZ-type cytokinin DHZ9G accumulated in the haustorium of zygotic embryos of *E. guinenesis*. The first report that indicated that germinating zygotic embryos was a site of biosynthesis came from Nandi et al. (1988) utilising yellow lupin seed. This was corroborated with the utilisation of exogenous cytokinins that replaced the axis inducing expansion and chlorophyll formation in the cotyledons (Nandi et al. 1989a). The high levels detected in the zygotic embryos of coconut palm could be indi-

cating that is site of biosynthesis and the different cytokinins detected could be playing an important role during the germination of the seed. The resistance to degradation of the DHZ types by the cytokinin oxidase could explain the relatively high levels detected in the zygotic embryo of coconut palm. Therefore this compounds could be expected to elicit a prolonged hormonal response during the germination. In germinating oil palm embryos, Nair (1997) showed that although cytokinin concentrations decreased during germination due to increasing water content, the total amount per embryo increased, providing evidence of *de novo* synthesis. If cytokinins are hormones, then it is vital that the active signal molecules are metabolised rapidly and removed from the active site. Concentration is therefore not a measure of activity, it is transport and turnover that is important. Hence it is not surprising that the ribosides (transport) are high, as are glucosides (storage or dump), while the bases and possibly ribotides are kept at low concentrations.

Aromatic cytokinins

Aromatic cytokinins were first found to occur naturally in mature poplar leaves (Horgan et al. 1973, 1975) and *Zantedeschia aethiopica* fruits (Chaves das Neves and Paiss 1980a, 1980b). Since then, the presence of this type of cytokinin has been reported in other species: *P. anisum* L. (Ernst et al. 1983), *L. esculentum* (Nandi et al. 1989b), *P. canadensis* (Strnad et al. (1992, 1994, 1997)), *E. guineensis* (Jones et al. 1996) and M. *sativa* (Goicochea et al. 1996). The present study showed the occurrence of BAP and the metabolites BAP9G, BAPR and BAP5′P in coconut, therefore extending the list of species where these cytokinins have been detected. In addition to this discussion, 6-benzylaminopurine, its riboside and the corresponding nucleotide have been identified as major endogenous compounds in primary crown gall tumours of tomato (Nandi et al. 1989b). Unfortunately, it was impossible to distinguish if these cytokinins are synthesised within the primary tumour, or synthesised elsewhere in the plant and transported to the tumours as sink for these cytokinins. Furthermore, the identification of BAPR in an old anise cell culture (Ernst et al. 1983) was also done on cell level only. This would thus appear to be the first report on the identification of BAP cytokinins in natural plant material.

The BAP cytokinins were found to be more concentrated in the embryo and in the immature inflorescence than in the other two parts studied, however their concentration in each part was several times lower than that of isoprenoid cytokinins. All four were detected in each of the parts studied. The most abundant ones were BAPR and BAP9G in immature inflorescence; and BAPR in all of the other parts.

When all cytokinins analysed are considered, important differences between the plant parts studied are found. The zygotic embryos show the highest content, the double of that in immature inflorescence, and five times that in spear leaf and SAM. These differences are even greater when individual cytokinins are compared. For instance, the content of iP9G is more than twenty times greater in embryos than in spear leaf, and that of DHZR5′P is at least ten times greater in embryos than in any other of the parts analysed. Similarly, large differences for individual cytokinins between different plant parts have been reported in *Pistachio* seedlings, although the total cytokinin contents for the different plant part studied did not differ importantly (Ahmadi and Baker 2000).

The present work reports for the first time an analysis, qualitative and quantitative, of the endogenous cytokinins of coconut palm and their levels, including both the isoprenoid and the aromatic cytokinins. This type of studies could help us to explain the slow rate of development of the coconut palm, that could be due to the relatively low level of active cytokinins detected in SAM and spear leaves. Similar studies could be carried out to compare the contents of the different types of cytokinins in healthy vs diseased palms affected by lethal yellowing. This disease is characterised by the high rate of yellowing and senescence of the leaves (McCoy et al. 1983) that could be explained by a reduction of the endogenous cytokinins in the palms. These types of studies are currently in progress. In diseased palms stomata close permanently and this is believed to be a change central to the mode of action of the disease (León et al. 1996). The hypothesis of increased abscisic acid contents as a cause for this occurrence has been tested and no changes were observed (Martínez et al. 2000).

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