Note

Cytokinin variation in the sap of male and female *Gymnocladus dioica*

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Cytokinins act as plant growth hormones and have been associated with the development of sexually differentiated tissues[1]. Different forms of cytokinin have been extracted from tree sap and various plant tissues[2–4]. In cherry trees increased cytokinin levels have been related to increased fruit set. Specific endogenous cytokinins in *Mercurialis annua* have been associated with sexual differentiation[5]. Female plants were found to contain zeatin, while zeatin ribotide was found only in male plants. Both male and female plants contained isopentenyladenosine and ribosyl-*trans*-zeatin. Analysis showed that males contain more of the cytokinins common to both genders. The evidence suggests that the sex of other dioecious plants may be determined by the cytokinin concentrations found in their sap.

The analysis of cytokinins on octadecylsilica (ODS) has been reported previously[6–9]. Morris *et al.*[10] and Brenner[11] used ODS columns to concentrate cytokinins from aqueous plant media. Stevens and Westwood[12] used disposable ODS columns to concentrate cytokinins from xylem sap. This was followed by additional solvent extractions. Stafford[13] and Kim *et al.*[14] used neutral polystyrene columns to concentrate cytokinins derived from plant sources. Definitive identification and quantitation of cytokinin residues is often accomplished by gas chromatography–mass spectrometry (GC–MS)[15–18].

In these experiments we used disposable ODS columns to concentrate sap cytokinins from the Kentucky coffee tree, *Gymnocladus dioica*, a legume native to the eastern United States. Trees grown in California as part of a botanical diversification project have prospered. These deciduous trees provide summer shade and offer little obstruction to winter sunlight. While males are prized as lawn trees, females produce many 5 × 15 cm flattened pods containing large (1 × 1.5 cm) seeds. When saturated by rain, the fallen pods release a hazardously slippery gum, creating problems for homeowners.

This work was undertaken to determine the cytokinin concentrations of Kentucky coffee tree spring sap as a possible indicator of sex. This information could be used to cull females from nursery grown saplings.

EXPERIMENTAL

Chemicals

Reagent grade chemicals, HPLC grade solvents, and glass distilled water were used throughout the study. All solvents were passed through appropriate 0.5-μm filters before use. Reference compounds were synthesized in this laboratory as previously reported[19]. All glassware was silanized prior to use by coating the surface with a 10% dimethyldichlorosilane solution in dichloromethane, drying the exposed surfaces, and rinsing with methanol.

Plant samples

Leafless trees were tapped by boring 9 × 25 mm holes through the bark and cambium layers. Sap samples were aspirated with a hand operated vacuum pump. Selected samples were quick frozen within minutes of aspiration by spraying droplets of sap into liquid nitrogen through a 23-gauge needle. The frozen droplets were collected in a large extraction thimble immersed in the dewar. These samples were held at −72°C until analyzed. Other samples were transferred to polyethylene bottles and kept on ice for several hours before being frozen and held at −30°C.

Sample preparation

Samples were concentrated on disposable ODS columns (SPE-10, 3 ml, J. T. Baker) wetted with 3 ml of acetonitrile at an a aspirator produced vacuum of 1 p.s.i. or less. Once wetted, the column was not allowed to go dry until elution was completed. Wetted columns were conditioned with 2 ml of 0.1 M ammonium acetate at pH 7 before sample introduction. Sap samples of 20 ml were made to 0.1 M with respect to ammonium acetate before loading on the column. The column was washed with 2 ml of 0.1 M ammonium acetate. Adsorbed materials were eluted with 2 ml each of 30, 65, and 100% acetonitrile, diluted with 1% acetic acid in water (0.175 M) where applicable. Eluates were concentrated on a rotary evaporator at 30°C, transferred to vials with 50% methanol and made to volume for high-performance liquid chromatographic (HPLC) analysis.

Standard preparation

Standards were diluted to 20 ng/ml in 50% methanol. Aliquots of 20 μl were diluted with 20 ml of 0.1 M ammonium acetate, loaded on conditioned preparatory columns and treated as samples. Similar aliquots of standards were diluted with 6 ml of 65% acetonitrile in 1% acetic acid and treated as samples eluates to estimate mechanical and absorbtive losses.

High-performance liquid chromatography

Analyses were performed on an ODS column (30 cm × 3.9 mm I.D., C_{18} μBondapak, Waters Assoc.). Eluent flow was delivered by two M6000A Waters pumps. The gradient was produced by a Waters 660 solvent programmer. The output of a Tracor 970 variable wavelength detector (set at 264 nm) was recorded on a Hewlett-Packard (HP) 5880A gas chromatographic integrator equipped with an external input board. Samples were eluted with a 5–30% 1-h concave gradient of acetonitrile in 1% acetic acid–water, at a flow of 1.4 ml/min. HPLC fractions were collected to separate free bases from ribosides and glucosides.

Permethylated derivatives

The HPLC fractions were concentrated on a rotary evaporator at 30°C, transferred to conical reaction vials and dried under nitrogen. The dried fractions were permethylated as in the method by Stafford *et al.*[13]. The permethylated derivatives were diluted to 100 μl with ethyl acetate and analyzed in 2-μl aliquots.

Permethylated HPLC fractions were analyzed on an HP 5972A gas chromatograph connected to an HP 5970B mass selective detector by a capillary direct interface maintained at 280°C. The derivatives were separated on a 25 m × 0.20 mm I.D. crosslinked dimethylsilicone column with a 0.1-μm film thickness. Experimental runs were temperature programmed in two steps, first at 25°C/min from 60–180°C and then at 6°C/min from 180–280°C, for splitless mode injections with the injector at 260°C. The ionization voltage is fixed at 70 eV. A column head pressure of 16 p.s.i. was maintained.

Analysis of tree sap samples was accomplished using the mass selective detector in the selected ion monitoring (SIM) mode. During each GC–MS run, the system controller monitored several pre-set time windows for either diagnostic or base peak ions. The diagnostic ions were used for identification. Base peak ion integration values were used for quantitation. These pre-set time windows were based on Kováts retention index values of the cytokinins of interest.

Cytokinin standards were eluted from both preparatory and analytical columns. Aliquots of these standards were permethylated. SIM base peak integration values were used to generate a standard response curve which was linear over the range of cytokinin ribosides found in the tree sap samples. The quantities of cytokinins present in the samples were estimated from the standard curve.

RESULTS AND DISCUSSION

Mechanical handling and absorptive losses of standards at the levels used in recovery experiments varied with the individual cytokinin. *cis*-zeatin, ribosyl-*cis*-zeatin and isopentenyladenine showed consistent losses of about 15%. The other tested cytokinins showed losses of 10% or less. Aliquots of mixed standards, representing 400 ng of each cytokinin, were diluted with 6 ml of 65% acetonitrile to simulate elution from a preparatory column. The HPLC analysis of these standards showed losses similar to aliquots of standards actually eluted from preparatory columns.

The experimentally determined Kováts retention indices for permethylated cytokinins of interest in this study are shown in Table I. A C_{31} alkane standard was not available for chromatography. Values based on the interpolated C_{31} retention time are underlined. The values given result from co-chromatography of alkane and derivatized cytokinin standards.

Fig. 1 shows a total ion current plot of permethylated cytokinin ribosides from typical female *vs.* male Kentucky coffee trees. These plots were obtained by monitoring the base peak ion for the three cytokinin ribosides found in tree sap samples at the expected elution times. The three peaks were identified in order of elution as isopentyadenosine, ribosyldihydrozeatin and ribosyl-*trans*-zeatin from their retention indices and the SIM of their diagnostic ions.

Tree sap samples held at -72°C during storage were assayed for phosphatase activity with *p*-nitrophenyl phosphate as the substrate. These samples exhibited low

TABLE I

KOVÁTS RETENTION INDICES OF PERMETHYLATED CYTOKININ STANDARDS

| Alkane carbon number	t_R (S)	Cytokinin	t_R (S)	Kováts retention index
20	617.7			2000
		Isopentenyladenine	637.4	2027
21	688.8			2100
		Dihydrozeatin	733.2	2157
22	766.6			2200
		cis-Zeatin	776.9	2113
		trans-Zeatin	792.1	2231
23	847.8			2300
24	932.3			2400
25	1016.9			2500
26	1102.1			2600
27	1185.8			2700
28	1269.2			2800
		Isopentenyladenosine	1285.4	2820
29	1349.0			2900
		Ribosyldihydrozeatin	1377.2	2935
		Ribosyl-cis-zeatin	1426.8	2997
30	1428.2			3000
		Ribosyl-trans-zeatin	1441.0	3016
31	1503.8			3100
		Zeatin-O-glucoside	1566.9	3184
32	1579.3			3200
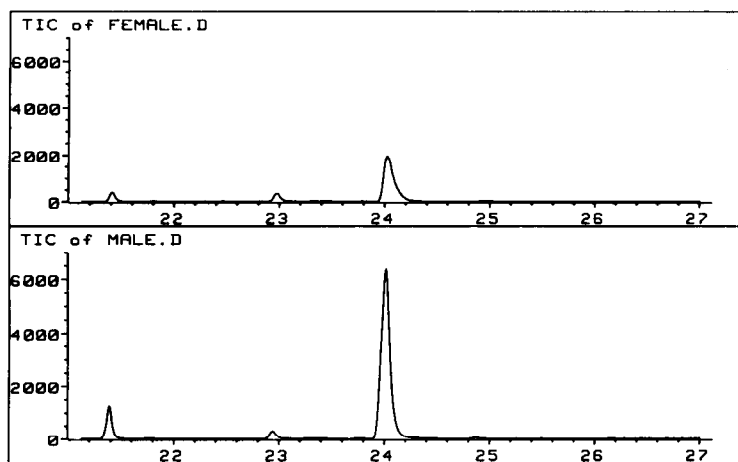
Fig. 1. Total ion chromatogram of the base peak ions of cytokinins found in female and male Kentucky coffee tree sap. Pre-set time windows: 21.0–22.5 min, m/z 391 (isopentyladenosine); 22.5–23.5, m/z 162 (ribosyldihydrozeatin); 23.5–24.5, m/z 390 (ribosyl-cis-zeatin, ribosyl-trans-zeatin); 24.5–27, m/z 230 (zeatin-O-glucoside).

levels of phosphatase activity. Hydrolysis of the samples with phosphatase did not increase identifiable cytokinin bases or ribosides indicating the absence of cytokinin phosphates in these samples. Traces of isopentyladenine and *trans*-zeatin in amounts too small to quantitate, were found only in the sap of male trees. *trans*-Zeatin ribosyldihydrozeatin and isopentyladenosine were found in the sap of both male and female trees.

TABLE II

CYTOKININS FOUND IN TREE SAP (μg/l)

tr = trace.

	Ribosyl-trans-zeatin	Ribosyldi-hydrozeatin	Isopentyl-adenosine	trans-Zeatin	Isopentyl-adenine
Male 1	100.5	12.5	29.5	tr	tr
1A	101	12	27.5	tr	—
2	211.5	31	10	tr	—
3	70.5	14	14.5	—	—
4	91.5	29	11	—	—
Female 1	37.5	15	8.5	—	—
1A	36.5	14	8.5	—	—
2	17	6	1.5	—	—
3	25.5	11	5	—	—

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The quantitative estimates of the sap cytokinins appear in Table II. The cytokinins of samples run in duplicate vary less than 10% based on external standards carried through the analytical procedure. The use of labeled cytokinins as internal standards would provide even better analytical control. Ribosyl-*trans*-zeatin was the major cytokinin found in every sap sample tested, but males contained significantly more of this cytokinin than did females ($a = 0.01$, 2-tailed t-test). In the samples tested the quantities of isopentyladenosine found in males was consistently higher than in females, but more samples would be needed to evaluate a relationship between isopentyladenosine and gender.

These analyses tend to confirm a relationship between endogenous cytokinins and sexual difference. If these trees exhibit similar differences in cytokinin concentration before maturity, assays could be used to identify nursery grown female saplings before permanent transplantation. The method could be extended to the sexual selection of dioecious crop plants.

These assays demonstrate the use of disposable ODS columns as a one-step concentration and partial clean up in the determination of cytokinins from plant sources. The columns are designed for replicate analyses offering a potential for savings in analytical time and equipment related expense.

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