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Cytokinins of Dry *Zea mays* Seed: Quantification by Radioimmunoassay and Gas Chromatography–Mass Spectrometry

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Abstract. The endogenous cytokinins present in dry *Zea mays* seed were determined using both radioimmunoassay and gas chromatography—mass spectrometry. Similar values for bases and ribosides were obtained by the two methods. The cytokinins present in embryo and endosperm were estimated separately using radioimmunoassay; similar levels of cytokinins were found in these two tissues. The major cytokinins detected on a whole-seed basis were dihydrozeatin riboside, O-glucosyldihydrozeatin riboside, zeatin 9-glucoside, zeatin, and the nucleotides of zeatin, dihydrozeatin, and isopentenyladenine. Cytokinin levels in the mature dry seed were considerably lower than cytokinin levels published in the literature for immature seed. Unexpected activity in the radioimmunoassays was detected in the wash from the DEAE cellulose column chromatography step. The compound(s) responsible for this activity did not have the solvent partitioning characteristics of a cytokinin base or riboside. They eluted as a single fraction following high-performance liquid chromatography on a Zorbax C8 column; this fraction showed no activity in the *Amaranthus* bioassay for cytokinins, but inhibited the activity of authentic zeatin riboside present at an optimal concentration.

Germination refers to a precisely regulated pattern of physical, biochemical, and physiological events that is initiated with imbibition of the seed and is complete when the radicle of the developing seedling protrudes through the

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covering structures of the seed. Germination is regulated by internal (plant) and external (environmental) factors. Cytokinins have been implicated in the process of germination. It is considered that different plant hormones have specific functions in controlling the various processes necessary for germination (Khan 1971, 1975), and cytokinins have been assigned a permissive role (i.e., a role in countering the effects of inhibitors) because of their ability to alleviate inhibition of germination, in diverse species, caused by abscisic acid (ABA). It is widely accepted that cytokinins play a role in cell division and cell enlargement (Letham and Bollard 1961, Miller 1961, Fosket et al. 1977, Nishinari and Syono 1980), processes that may be important in germination.

Changes, most notably increases, in cytokinin activity as detected by bioassay have been observed upon stimulation of seeds to germinate (breaking of dormancy) or during the processes of germination and radicle elongation (Van Staden and Wareing 1972, Van Staden et al. 1972, 1973, Webb et al. 1973, Van Staden 1973, Van Staden and Brown 1973, Brown and Van Staden 1973, 1975, Dimalla and Van Staden 1977, Taylor and Wareing 1979, Smith and Van Staden 1978, Julin-Tegelman and Pinfield 1982). There is also a large volume of literature that indicates that applied cytokinins affect seed germination (see Khan and Tao 1978), but these results do not necessarily reflect the role the endogenous cytokinins play, particularly as the most commonly applied cytokinins (kinetin and benzylaminopurine (BAP)) are not naturally occurring.

Cytokinins have also been implicated in playing a role in early postgermination events, in particular in regulating reserve mobilization. Seeds store large amounts of reserves that are hydrolyzed after germination to sustain growth of the embryo. In monocots, the starch reserves of the endosperm are hydrolysed by α-amylase, which is secreted by the aleurone layer, and β-amylase, which is already present in the endosperm (for recent review, see Halmer 1985). In wheat seed, it has been suggested (Eastwood et al. 1969, Laidman et al. 1974) that the synthesis of α-amylase in aleurone tissues is induced by the sequential action of a cytokinin from the endosperm and a gibberellin from the embryo. Also, in barley grains, Khan and Downing (1968) found that ABA inhibition of α-amylase formation could be abolished by treatment with cytokinin (kinetin or BAP). In dicots, less is known about the mode of starch breakdown, but cytokinins may also play a role in the regulation of reserve breakdown in these species. The evidence for this is based on the ability of cytokinins to replace the embryonic axis in stimulating the synthesis and secretion of α-amylase (Gepstein and Ilan 1970, 1979, Locker and Ilan 1975, Metivier and Paulilo 1980, De Klerk 1986), an ability not usually shown by other phytohormones.

A possible role of endogenous cytokinins in radicle growth has been suggested. Smith and Van Staden (1979) observed an increase in radicle growth rate when excised maize embryos were cultured on medium containing zeatin (Z) compared with cytokinin-free medium. An even greater increase was observed when the medium contained cytokinin glucosides, but other compounds may have been responsible, because the cytokinin glucosides were extracted from *Ginko biloba* L. leaves and only partially purified. Similarly, the elongation of the radicle in isolated sycamore seeds could be induced by exogenous kinetin but not by gibberellic acid (GA) (Pinfield and Stobart 1972).

Although cytokinins have been implicated in the process of germination and

in early postgermination events, their exact function remains unknown. To elucidate this role of cytokinins, we require much more extensive information on changes in the levels and types of cytokinins within the seed during germination. However, there have been no definitive studies to identify the endogenous cytokinins present in dry seed that may be involved in the control of germination and subsequent seedling development for any species. In the present study we have therefore examined, by both gas chromatography–mass spectrometry (GC-MS) and radioimmunoassay (RIA), the types and levels of a wide variety of cytokinins in *Zea mays* seeds in order to provide information on which to base relevant metabolic studies.

Materials and Methods

Plant Material and Extraction Procedures

Dry seed of *Zea mays* L. (F1 hybrid, Iochief) was used. For the GC-MS quantification, whole dry seed (150 g) was extracted. For the study employing RIA, the same batch of dry seed (21.59 g; i.e., 100 dry seeds) was dissected into embryo (3.13 g) and endosperm (8.41 g). The difference in texture between the soft embryo and the hard endosperm was used to ensure that no residual endosperm was included with the embryo fraction. The portion (9.61 g) of the seed remaining after dissecting out pure embryo and endosperm, which was composed of scutellum and some endosperm and which we designated "remainder," was also collected and analyzed. Cytokinins were extracted by the method of Bieleski (1964) to minimize the action of nonspecific phosphatases.

For the samples to be analyzed by RIA, about 420 Bq of the following recovery markers [^{3}H]zeatin riboside ([9R]Z) (140.6 GBq mmol^{-1}), [^{3}H]dihydrozeatin riboside ((diH) [9R]Z) (189 GBq mmol^{-1}), [^{3}H]dihydrozeatin ((diH)Z) (1110 GBq mmol^{-1}), and [^{14}C]adenosine 5′-monophosphate (AMP) (2.15 GBq mmol^{-1}) were added to the extracting solvent to account for losses during purification and to validate the chromatographic procedures. Sources of the recovery markers have been given previously (Badenoch-Jones et al. 1987), except for the [^{14}C]AMP, which was purchased from Amersham International Ltd, Amersham, U.K. For the samples to be analyzed by GC-MS, deuterated analogues of the cytokinins to be assayed were added to the extracting solvent. As no deuterated analog of isopentenyladenosine 5′-monophosphate ([9R-5′P]iP) was available, deuterium-labeled isopentenyladenosine ([9R]iP) was added to the extract following nucleotide hydrolysis with alkaline phosphatase. The deuterium-labeled cytokinins were synthesized by procedures described by Summons et al. (1979).

Sample Purification Prior to Analysis by RIA

The scheme used to purify and fractionate crude samples prior to analysis by RIA is outlined in Fig. 1. This scheme was based on that described by Badenoch-Jones et al. (1987), and most of the purification procedures employed

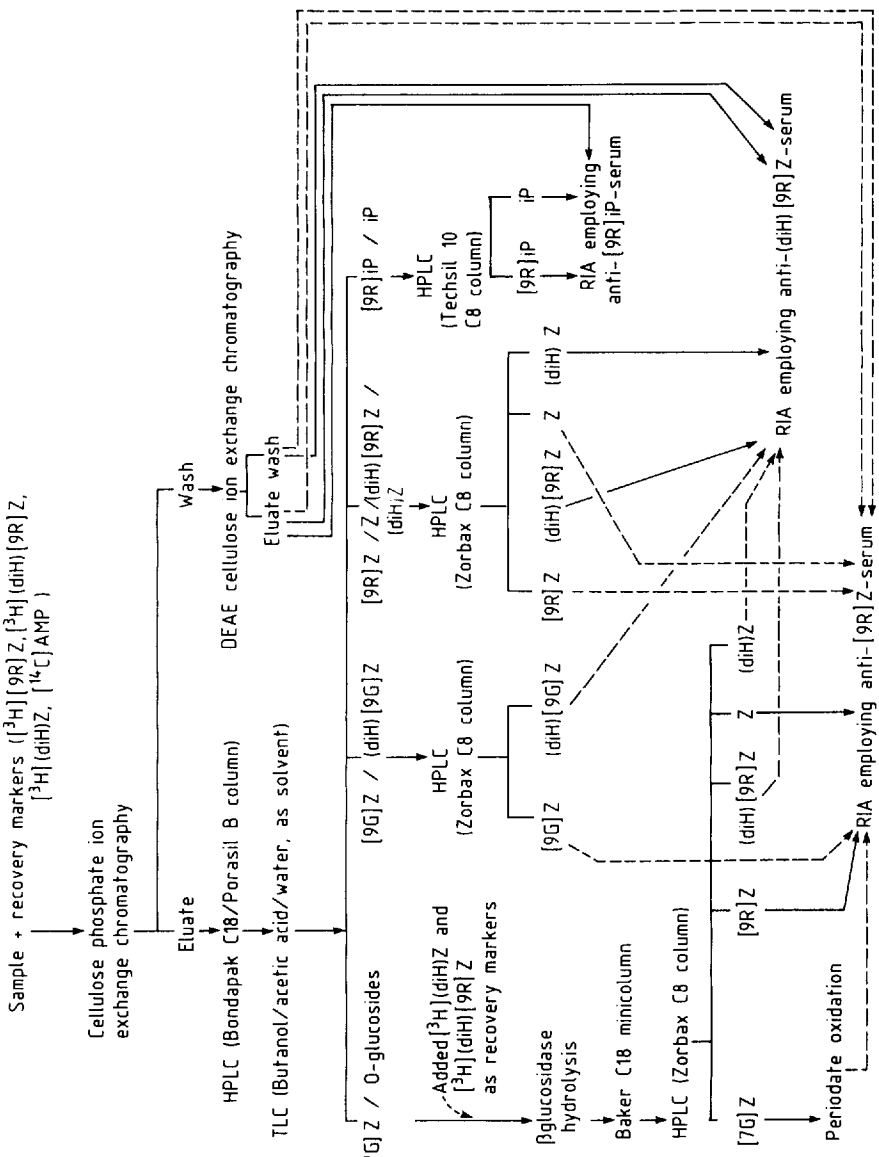

Fig. 1. Scheme for the separation of cytokinins in tissues of dry *Zea mays* seed prior to analysis by RIA.

in the present study have been described (Badenoch-Jones et al. 1987). An additional bulk high-performance liquid chromatography (HPLC) step on a Bondapak C18/Porasil B column (7.8 × 610 mm) (Waters Associates), as described by Badenoch-Jones et al. (1984b), was carried out following the ion exchange chromatography step on cellulose phosphate in order to ensure quality chromatography in the subsequent thin-layer chromatography (TLC) step and to reduce the number of TLC plates required. In the present study, [^{3}H](diH)Z and [^{3}H](diH)[9R]Z were added to the O-glucoside zone from TLC, prior to the β-glucosidase hydrolysis step, in order to estimate the recoveries of the O-glucosides in subsequent purification steps.

In addition to those cytokinins shown in the scheme of Badenoch-Jones et al. (1987), in the current study we also attempted to estimate dihydrozeatin 9-glucoside ((diH)[9G]Z) and the nucleotides of Z, (diH)Z, and isopentenyladenine (iP).

To separate (diH)[9G]Z from zeatin 9-glucoside ([9G]Z) with which it cochromatographs following the TLC step, the [9G]Z/(diH)[9G]Z mixture was subjected to HPLC (Zorbax C8 column (9.4 × 250 mm)). Using 25% (v/v) methanol in 0.2 M acetic acid as solvent, at a flow rate of 4.5 ml min^{-1}, retention times of [9G]Z and (diH)[9G]Z were 6.98 and 8.16 min, respectively.

The HPLC eluates from the Zorbax C8 and Techsil 10 C8 columns were collected, starting at the end of the void volume and continuing until well after the known elution volume of the last compound of interest. The eluates were collected as a series of equal volume fractions (~1.4–1.8 ml) using a Gilson 201 fraction collector. Aliquots (5%) of each fraction from the Zorbax C8 column were taken for scintillation counting to locate the fractions that contained the markers [^{3}H][9R]Z, [^{3}H](diH)[9R]Z, and [^{3}H](diH)Z (hydrolysates of O-glucoside fractions contained only [^{3}H](diH)Z and [^{3}H](diH)[9R]Z). These data enabled the precise pooling of fractions containing putative [9R]Z, (diH)[9R]Z, and (diH)Z prior to analysis by RIA. Fractions that would contain putative Z and [7G]Z (and also [9R]Z in the case of O-glucoside hydrolysates) were determined with reference to the elution times of the above tritiated markers and the elution times of authentic Z, [9R]Z, (diH)Z, (diH)[9R]Z, and [7G]Z standards. Again, appropriate fractions were pooled prior to analysis by RIA. To determine which fractions from the Techsil column might contain putative iP and [9R]iP, reference was made to the retention times of authentic iP and [9R]iP standards. The chosen fractions were not pooled, but assayed separately. A similar approach was adopted for the analysis of putative [9G]Z and (diH)[9G]Z fractions eluted from the Zorbax C8 column.

The nucleotides of Z, (diH)Z, and iP, which are present in the cellulose phosphate wash, were estimated following purification on DEAE cellulose as described by Badenoch-Jones et al. (1984a). RIAs, using each of the three antisera, were performed directly on the eluate (expected to contain the nucleotides) and on the wash from DEAE cellulose.

When estimating Z, (diH)Z, iP, and their nucleotides, corrections were made for the cross-reactivity of these bases and the 5′-monophosphates with their corresponding antisera (for cross-reactivity data see Badenoch-Jones et al. 1987). Authentic (diH)Z nucleotide standard has recently become available, and its percentage molar cross-reactivity values with various sera, as deter-

mined in the present study, are as follows: anti-(diH)[9R]Z serum, 48.5%; anti-[9R]Z serum, 1.32%; and anti-[9R]iP serum, 0.05%. In estimating nucleotide concentration, no attempt was made to correct for the cross-reactivities of nucleotides with each of the noncorresponding antisera (all these cross-reactivities were low (2% or less) (see Badenoch-Jones et al. 1987 and the above data)).

Sample Purification Prior to Analysis by GC-MS

The scheme used to purify and fractionate crude samples prior to analysis by GC-MS is outlined in Fig. 2. This scheme was originally designed to consist of as few chromatographic steps as possible in order to maximize the throughput of samples and to permit GC-MS analysis to be used on a routine basis. To enable many samples to be processed at one time, we attempted to develop TLC methods that adequately purify samples prior to GC-MS analysis. However, it was found that further sample purification (HPLC) was necessary before GC-MS analysis. Procedures shown in Fig. 2 and not covered by the descriptions or references given above include alkaline phosphatase hydrolysis, which was carried out as described by Parker et al. (1978), and TLC on cellulose, which was carried out using a 0.6-mm layer with isopropanol:acetic acid:water (5:1:1, v/v/v), as solvent.

Following chromatography on cellulose, the cytokinins were located in relation to dyes (Rf's in parentheses) as follows: zone 1 (iP and [9R]iP) between malachite green (0.93) and acridine yellow (0.83); zone 2 (Z, (diH)Z, [9R]Z, (diH)[9R]Z) between acridine yellow and phenol red (0.64); and zone 3 ([9G]Z, [7G]Z, (OG)Z, (diH OG)Z, (OG)[9R]Z, and (diH OG)[9R]Z) between phenol red and fissamine flavine FFS (0.29). Malachite green, acridine yellow, and fissamine flavine FFS were obtained from G. Gurr, and phenol red was obtained from Elliots and Australian Drug Pty. Ltd., Sydney, N.S.W., Australia. Cytokinins were eluted with methanol:acetic acid:water (10:1:10, v/v/v). iP and [9R]iP were separated by HPLC on the Zorbax C8 column using 70% (v/v) ethanol in 0.2 M acetic acid as solvent at a flow rate of 4.0 ml min^{-1}. The retention times of authentic [9R]iP and iP standards in this system were 4.3 and 9.0 min, respectively. RIA was used to locate the fractions containing cytokinins (endogenous plus deuterated analogues) following HPLC during sample purification prior to quantitative analysis by GC-MS.

RIA

Antisera elicited in rabbits against [9R]Z, (diH)[9R]Z, and [9R]iP were used. These antisera have been characterized previously: anti-[9R]Z serum (Badenoch-Jones et al. 1984a), and anti-(diH)[9R]Z and anti-[9R]iP sera (Badenoch-Jones et al. 1987). The assay procedure was based on that developed by Weiler (1980), with minor modifications (Badenoch-Jones et al. 1987). Samples were dissolved in RIA buffer and assayed in triplicate. Where appropriate, an aliquot (up to 5%) of each fraction was taken in duplicate for the

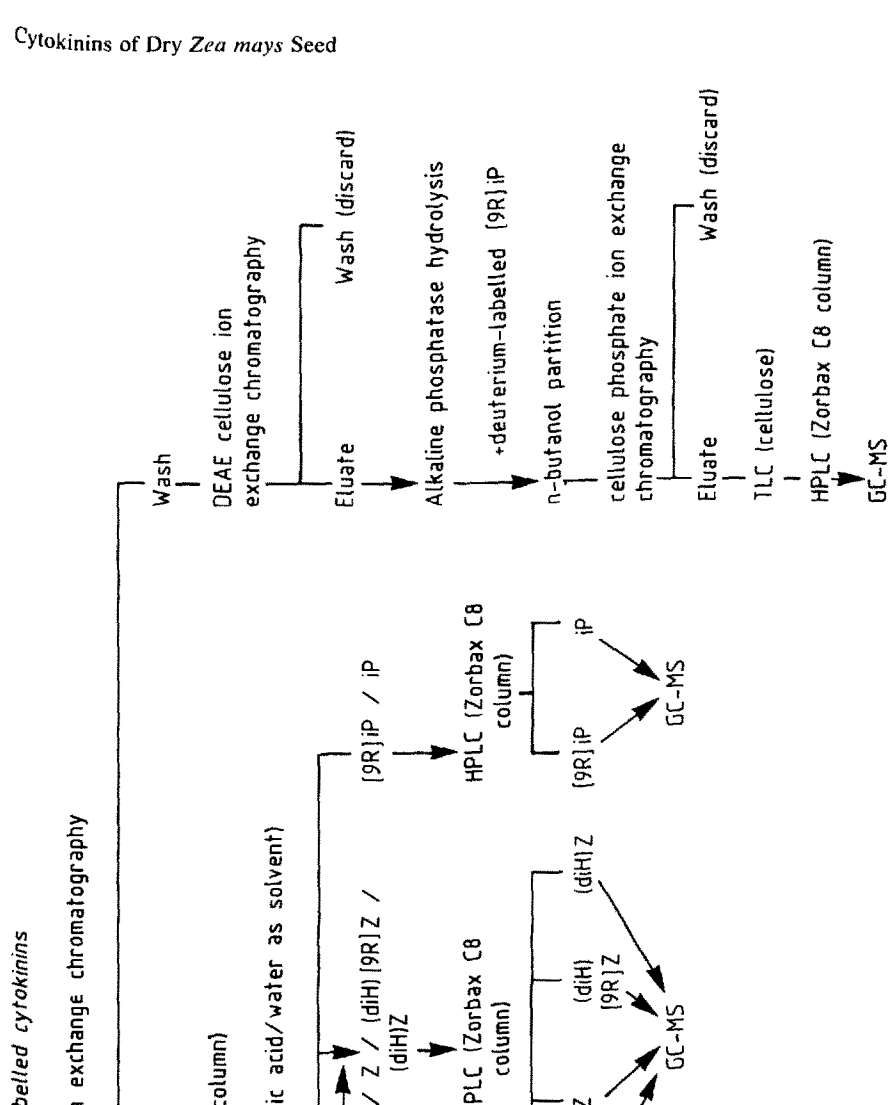

Fig. 2. Scheme for the separation of cytokinins in whole dry *Zea mays* seed prior to analysis by GC-MS.

Table 1. Ion pairs monitored for GC-MS analysis of the endogenous cytokinins in dry *Zea mays* seeds.

Cytokinin derivative	Ion pairs (labeled/nonlabeled ion)
TMS-[9R]iP	557/551 (M),* 542/536 (M-15)
TMS-[9R]Z	629/624 (M-15), 539/536 (b)*
TMS-(diH) [9R]Z	631/626 (M-15), 327/322 (B + CH_2O)*
tBuDMS-iP	319/317 (M), 304/302 (M-15)*
tBuDMS-Z	395/390 (M-57), 320/315 (a)*
tBuDMS-(diH)Z	436/434 (M-15), 394/392 (M-57)*

Key: M, molecular ion; M-15, loss of methyl radical from M; M-57, loss of *t*-butyl radical from M; a, loss of tBuDMS-OH from M; b, loss of TMS-OCH_2 from M; B, cleavage of ribosyl moiety with charge retention on the base.
* Most intense pair.

measurement of radioactivity in order to estimate recoveries. Cytokinin estimates were corrected for recovery according to the recovery of the corresponding radioactive markers.

For cytokinins for which recovery markers were not available, recoveries were assumed to be as follows: Z, as for [^{3}H](diH)Z; [9G]Z and zeatin 7-glucoside ([7G]Z), as for [^{3}H][9R]Z; (diH)[9G]Z, as for [^{3}H](diH)[9R]Z; the nucleotides, as for [^{14}C]AMP; and the O-glucosides (after the TLC step), as for [^{3}H][9R]Z, [^{3}H](diH)[9R]Z, and [^{3}H](diH)Z, combined (after the TLC step); in the steps subsequent to TLC, the recovery of the O-glucosides was corrected according to the recovery of [^{3}H](diH)Z and [^{3}H](diH)[9R]Z added after the TLC step (O-glucosylzeatin ((OG)Z) and O-glucosyldihydrozeatin ((diH OG)Z) according to the recovery of [^{3}H](diH)Z and O-glucosylzeatin riboside ((OG)[9R]Z) and O-glucosyldihydrozeatin riboside ((diH OG)[9R]Z), according to the recovery of [^{3}H](diH)[9R]Z. These assumptions as to recoveries might not be expected to result in large errors, except possibly in the case of [7G]Z (see Badenoch-Jones et al. 1987). A computer program based on that of Brooker et al. (1979) was used for assay evaluation and computation of results.

GC-MS

GC-MS analyses were carried out using a VG 70E instrument interfaced to a VG 11/250 data system. The bases (Z, (diH)Z, and iP) were analyzed as the per-tBuDMS derivatives, and the ribosides ([9R]Z, (diH)[9R]Z, and [9R]iP) as the perTMS derivatives. The per-tBuDMS derivatives were made according to Hocart et al. (1986). The per-TMS derivatives were made as described previously (MacLeod et al. 1976). The ion pairs monitored are shown in Table 1, and the selected ion profiles obtained during the quantification of the tBuDMS derivatives of (diH)Z and Z are shown in Figs. 3a and 3b, respectively. The GC was fitted with a 25 × 0.3 mm ID bonded phase (BP-1)–fused silica capillary column (SGE), temperature programmed from 200 to 300°C at 8°C/min, and helium (0.6 kg/cm^2) was used as carrier gas. Ionization was by electron impact

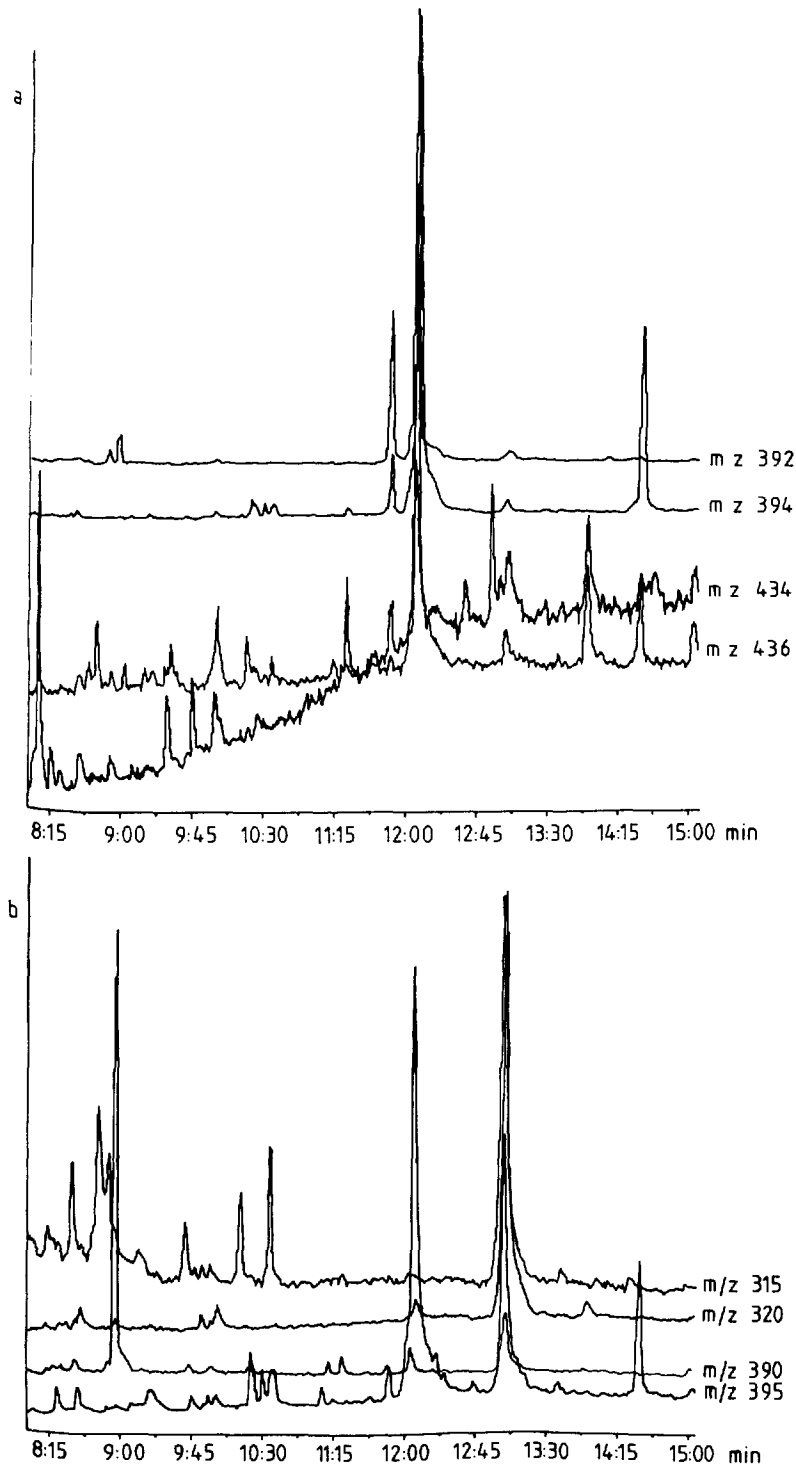

Fig. 3. Selected ion profiles obtained during the mass spectrometric quantification of the levels of endogenous (diH)Z (a) and Z (b) in dry *Zea mays* seed by the stable isotope dilution technique. The tBuDMS derivatives of (diH)Z and Z were eluted from the GC at 12:10 and 13:05 min, respectively.

(70 eV), and selective ion recording was carried out using magnet peak switching, with a sample time of 100 msec and a delay of 10 msec per peak.

For each sample, iP was analyzed individually, whereas Z and (diH)Z were analyzed as a mixture, and [9R]Z, (diH)[9R]Z, and [9R]iP (or [9R]Z and (diH)[9R]Z for the O-glucoside samples) were analyzed as a mixture. The ion currents for the two most intense high-mass ions for each compound and its labeled analog were monitored over a sample cycle time of 1.0–1.5 sec. Quantification was based on the relative intensities of these ions as observed at the elution time characteristic for each compound. Standard curves were constructed for each ion pair using the second and third injection of each sample to minimize memory effects. Results based on the most intense ion pair were confirmed by results calculated from the second pair of ions.

Bioassay

The *Amaranthus* bioassay was carried out as described by Biddington and Thomas (1973).

Results

In Table 2 are presented estimates of the cytokinin content of dry *Z. mays* seed as measured by GC-MS. Also given in Table 2 are the cytokinin contents of the embryo, endosperm, and "remainder" of dry *Z. mays* seed as estimated by RIA, and for each cytokinin, the ratio of its content in embryo to its content in endosperm. From the known weights and cytokinin contents of embryo, endosperm, and "remainder," the cytokinin content of the whole seed was calculated from the RIA data.

All the cytokinins detected by GC-MS were also detected by RIA, although iP and some of the O-glucosides were not detected in all parts of the seed. Some of the cytokinins we did not quantify by GC-MS were detected by RIA; [9G]Z, (diH)[9G]Z, zeatin riboside 5′-monophosphate ([9R-5′P]Z), and dihydrozeatin riboside 5′-monophosphate ([9R-5′P](diH)Z). Both RIA and GC-MS revealed that iP and [9R]iP were present in whole seed at lower concentrations than the Z and (diH)Z bases and ribosides. For each of the bases and ribosides, the estimates of whole-seed cytokinin concentration made by RIA and GC-MS were very similar. For the four O-glucosides, estimates of whole-seed cytokinin content made by GC-MS were higher than those made by RIA; however, both GC-MS and RIA revealed (diH OG)[9R]Z to be the major O-glucoside present, followed by (OG)Z, (diH OG)Z, and then (OG)[9R]Z. Data from RIA indicated the presence of considerable levels of [9G]Z in sweet corn seed and a lower, but still readily measurable, level of (diH)[9G]Z. RIA data indicated that quantitatively the major cytokinins in whole dry seed were [9R-5′P]iP, (diH)[9R]Z, [9R-5′P](diH)Z, [9G]Z, [9R-5′P]Z, (diH OG)[9R]Z, (diH)Z, and Z, respectively. Similarly, GC-MS data indicated that (diH OG)[9R]Z, (diH)[9R]Z, and Z, respectively, were quantitatively major cytokinins in whole

Table 2. Cytokinin concentrations in dry seed of Zea mays (ng cytokinin g^{-1} tissue).

	Embryo (RIA)	Endosperm (RIA)	"Remainder" (RIA)	Embryo/ endosperm (RIA)	Whole seed[a] (RIA)	Whole seed (GC-MS)
Bases						
Z	6.5	1.4	4.3	4.6	3.5	8.7
(diH)Z	4.1	2.9	5.4	1.4	4.2	5.5
iP	9.9	ND	1.8	—	2.3	3.7
Ribosides						
[9R]Z	1.3	1.9	3.1	0.7	2.3	1.5
(diH) [9R]Z	5.9	11.7	16.0	0.5	12.8	9.3
[9R]iP	0.4	0.7	0.4	0.6	0.5	0.9
Glucosides						
(OG)Z	ND	5.0	1.2	—	2.5	8.0
(OG) [9R]Z	ND	ND	0.2	—	0.1	2.8
(diH OG)Z	ND	2.1	3.1	—	2.2	6.4
(diH OG) [9R]Z	12.6	3.4	4.7	3.7	5.4	10.0
[7G]Z	[b]	[b]	[b]	[b]	[b]	NA
[9G]Z	3.6	8.3	15.1	0.4	8.3	NA
(diH) [9G]Z	ND	1.4	0.2	—	0.7	NA
Nucleotides						
[9R-5'P]Z[c]	6.4	6.6	6.8	1.0	6.7	NA
[9R-5'P] (diH)Z[c]	9.4	8.6	7.8	1.1	8.3	NA
[9R-5'P]iP[c]	9.5	27.6	6.9	0.3	15.5	0.70
Others						
DEAE wash (anti-[9R]Z serum)	4.9	1.2	3.2	—	2.6	—
DEAE wash (anti-(diH) [9R]Z serum)	2.3	0.5	1.3	—	1.1	—

Key: ND, not detected; NA, not assayed.

[a] Calculated from RIA data for embryo, endosperm and 'remainder'.

[b] If this compound occurs at all in these tissues, it will be at a concentration of less than 1.5 ng g^{-1} of tissue.

[c] Although designated here as the monophosphate, and calculated according to the cross-reactivity of the monophosphate, the antisera are likely to also cross-react with the di- and triphosphates (cross-reactivities unknown), and nucleotide estimates therefore may include a contribution from the di- and triphosphates.

seed, whereas [9G]Z and the nucleotides of Z and (diH)Z were not analyzed by GC-MS.

The estimate of iP nucleotide obtained by GC-MS was lower than that obtained by RIA. The GC-MS value will be an underestimate, because losses during sample purification prior to, and including, the alkaline phosphatase step were not accounted for.

RIA data indicated that for most cytokinins, differences between the levels in embryo and endosperm were not marked. However, iP appeared to be in the embryo and not the endosperm, whereas (OG)Z, (diH OG)Z, and (diH)[9G]Z appeared to be in the endosperm and not the embryo.

Surprisingly, when the DEAE wash fractions for each of the three tissues were assayed by RIA employing both the anti-[9R]Z and anti-(diH)[9R]Z sera, activity was detected in each instance (Table 2). This activity was further investigated by pooling the remaining DEAE wash fractions for each tissue and subjecting each of two aliquots of this pooled sample to HPLC on the Zorbax C8 column. The solvent was 0.2 M acetic acid for 5 min, followed by a gradient of 0–100% methanol in 0.2 M acetic acid (curve 8) for 25 min, at a flow rate of 4.5 ml min^{-1}. After 2 min, the collection of 17 equal fractions was begun. For both aliquots, strong activity was detected by RIA employing the anti-[9R]Z serum in only one fraction (fraction 14), although slight activity was also detected in the first fraction. Activity was detected in the same fraction when the anti-(diH)[9R]Z and anti[9R]iP sera were employed. Activity of this fraction was strongest in the [9R]Z assay and weakest in the [9R]iP assay.

Fraction 14 was assayed for biological activity using the *Amaranthus* bioassay. For each original aliquot of pooled sample, two bioassay dishes were prepared, each containing approximately 1–5 ng [9R]Z equivalents, as estimated from activity in RIA. No biological activity was detected in any of these test dishes, whereas standard [9R]Z showed activity at concentrations of 1, 10, 50, and 100 ng per dish. However, it was observed that the appearance of the test cotyledons was different from that of control cotyledons (no added sample), the test cotyledons appearing bleached. Therefore, a further sample of whole dry seed was extracted and subjected to cellulose phosphate and DEAE cellulose chromatography. The DEAE wash fraction was then subjected to HPLC (gradient on Zorbax C8 column as described above). Fraction 14, which again showed activity in RIA, was divided into two dishes (about 4 ng [9R]Z equivalents per dish), one of which contained 50 ng [9R]Z standard, for bioassay. In contrast to the activity detected in the dish containing 50 ng standard [9R]Z alone, both test dishes showed no activity in the bioassay, with the cotyledons in both dishes appearing bleached.

When aliquots of the DEAE wash fraction from whole seed were partitioned against either n-butanol (pH 8) or ethyl acetate, no RIA activity was detected in the solvent phase.

Discussion

In a previous study (Badenoch-Jones et al. 1987), we developed techniques for quantifying by RIA, most of the known naturally occurring cytokinins. In the

present study, we have used these techniques to examine the cytokinins present in embryo and endosperm of dry *Z. mays* seed. This is one of the most extensive examinations to date of cytokinin levels in a tissue.

A comparison of cytokinin levels determined in the present study for dry *Z. mays* seed with those available for developing *Z. mays* seed reveals a reduction in cytokinin levels with maturity. For example, for the active cytokinins, Z and [9R]Z, concentrations in immature kernels are in the order of 100 ng g^{-1} tissue (Summons et al. 1979, Badenoch-Jones et al. 1984a), whereas the present study indicates that they are in the order of 5 ng g^{-1} tissue in dry seed. Our data are consistent with a decline in cytokinin activity in maturing seed that has been reported for a number of species: *Z. mays* (Miller 1967), *Pisum arvense* (Burrows and Carr 1970), cotton (fruit) (Sandstedt 1971), wheat (Wheeler 1972, Jameson et al. 1982), barley (Michael and Seiler-Kilbitsch 1972), *Lupinus albus* (Davey and Van Staden 1978, 1979), *Phaseolus* (Nesling and Morris 1979) and *L. luteus* (Summons et al. 1981). These previous data were largely obtained by bioassay, but for *L. luteus* seeds, they were obtained by GC-MS analysis. [9R-5'P]Z appears to be the major cytokinin in immature *Z. mays* seed (Letham 1973, Badenoch-Jones et al. 1984a), and although it remained a quantitatively important cytokinin in the mature tissue, its concentration declined considerably during maturation, from about 200 ng g^{-1} tissue to about 6 ng g^{-1} tissue. Unfortunately, there is not enough information on cytokinin types and levels in immature *Z. mays* seed to determine whether the levels of all cytokinins are lower in mature than immature seeds.

The present study has shown [9G]Z to be one of the major cytokinins in dry seed (8.3 ng g^{-1} tissue). Estimates of [9G]Z in immature *Z. mays* seed have varied from 15 (Summons et al. 1979) to 78 (Badenoch-Jones et al. 1984a) ng g^{-1} fresh weight tissue, so it would appear that the level of [9G]Z in *Z. mays* seed declined during maturity to a lesser extent than the levels of bases, ribosides, and nucleotides. (diH)[9G]Z was detected in small quantities in dry seed (0.7 ng g^{-1} tissue); this cytokinin has previously been identified, but not quantified, in immature *Z. mays* seed (Summons et al. 1980). The N-glucosides are probably more widely distributed than is evident at present, but they have been infrequently detected previously, largely because of their very low biological activity in conventional callus bioassays.

The substantial levels of the [9G]Z and the occurrence of (diH)[9G]Z in the *Z. mays* seed is an interesting feature, as these compounds are relatively stable metabolically (they undergo little cleavage of the glucose moiety) and are considerably less active than Z (see Letham and Palni 1983). The formation of these cytokinins may be a means of lowering cytokinin activity in maturing seed in order to create a quiescent state which may be essential for seed survival. The formation of these inactive cytokinins and of (diH OG)[9R]Z in mature seed may explain, in part, the reduction in cytokinin activity in mature seeds, especially as there is some evidence that, at least for *L. albus* fruits, the decrease in cytokinin levels in maturing seed is not due to cytokinin export (Davey and Van Staden 1981). (diH OG)[9R]Z, the major O-glucoside found in dry *Z. mays* seed, was also the major O-glucoside found in *L. luteus* seed at a late stage in development (Summons et al. 1981).

Previous studies have established that side-chain cleavage by cytokinin oxi-

dase (Whitty and Hall 1974) occurs actively throughout the sweet corn plant (roots and derooted seedlings—Parker and Letham 1974; leaves—Hocart 1985; seed at commercial maturity—Summons et al. 1980; germinating seed—Hocart 1985). Thus the substantial levels of (diH)[9R]Z and (diH OG)[9R]Z (putative storage form of (diH)[9R]Z—Letham and Palni 1983) occurring in the dry seed may be of importance in that these cytokinins are resistant to side-chain cleavage by cytokinin oxidase and therefore more likely to be involved in the germination process.

The presence of iP and [9R]iP in a monocot was demonstrated for the first time. Although these cytokinins have been identified in a few higher plant tissues (Dyson and Hall 1972, Watanabe et al. 1978, Dauphin et al. 1979, Dauphin-Guerin et al. 1980, Yokota and Takahashi 1980, Palni et al. 1983, Tsui et al. 1983), they may not be widely distributed (see Letham and Palni 1983).

The presence of measurable levels of nucleotides in dry *Zea mays* seed is in agreement with the finding of detectable levels of "bound" cytokinin in dry *Z. mays* seed by Julin-Tegelman (1979). [9R-5'P]Z has previously been unequivocally identified in *Z. mays* seed (Letham 1973) and is probably widely distributed in higher plants (see Letham and Palni 1983). [9R-5'P](diH)Z and [9R-5'P]iP, identified in the present study, have not been previously identified in *Z. mays* seed, but they appear to occur in some plant tissues ([9R-5'P](diH)Z in *P. vulgaris* (Palmer et al. 1981) and *D. innoxia* crown-gall tumor tissue (Palni et al. 1983) and [9R-5'P]iP in male and female plants of *Mercurialis annua* (Dauphin-Guerin et al. 1980) and *D. innoxia* crown-gall tumor tissue (Palni et al. 1983). In all the previous studies, [9R-5'P](diH)Z and [9R-5'P]iP were identified as the corresponding ribosides in alkaline phosphatase–treated nucleotide fractions.

In the present study this was also the case for the GC-MS analysis of [9R-5'P]iP, but nucleotides were analyzed directly (not following alkaline phosphatase hydrolysis) by RIA. For quantifying the nucleotides by RIA, the same DEAE eluate was assayed using each of the three antisera, and no attempt was made to separate the individual nucleotides prior to their assay. This should not have caused any large error in quantifying the nucleotides, because they were all present in similar quantities, and for each antiserum only the corresponding nucleotide and not the other two nucleotides cross-reacted strongly (see Materials and Methods).

It was of interest to examine, separately, the cytokinins in endosperm and embryo of dry *Z. mays* seed, as these tissues have infrequently been isolated and analyzed individually. Smith and Van Staden (1978) have performed cytokinin analyses on isolated embryo and endosperm tissue of mature *Z. mays* seed (and of seed 3 days after imbibition). Their work suggested the presence of both O-glucosides and Z (or (diH)Z) bases and ribosides in both embryo and endosperm of mature seed; however, their cytokinin identifications were tentative, being based on bioassay following simple purification procedures, and quantitative data for individual cytokinins were not obtained.

Our results confirm the presence of Z, [9R]Z, (diH)[9R]Z, and O-glucosides in both embryo and endosperm (although not all the O-glucosides were detected in either of these tissues). They also reveal the presence of other types of cytokinins in both embryo and endosperm of dry *Z. mays* seed. Similar

levels of cytokinins were found in embryo and endosperm of dry seed. In mature *Z. mays* seed, Smith and Van Staden (1979) also found similar levels of cytokinins in embryo and endosperm, but the bioassays were done on tissue that had been cultured for 3 days. In contrast, the data of Thomas et al. (1978) for dry wheat seeds suggested that endosperm cytokinin levels were higher than those of embryo. The finding of similar levels of cytokinins in embryo and endosperm of mature *Z. mays* seed is also in contrast to the higher levels of cytokinin in endosperm than embryo found in developing seed—for example, *L. albus* seed (Davey and Van Staden 1979).

The detection of activity by RIA (with all three antisera) in the DEAE wash fraction, in which no cytokinins would be expected to elute, was surprising. It could not be explained by poor chromatography at the cellulose phosphate or DEAE steps, as recovery of radioactive markers after both these steps indicated adequate chromatography. It more likely reflected a lack of specificity of RIA for cytokinins, and we therefore attempted to further characterize the active compound(s). RIA activity in the DEAE wash chromatographed in a discrete fraction when subjected to HPLC on the Zorbax C8 column, suggesting that activity was due to either one or only a limited number of compounds. With respect to their extraction properties into ethyl acetate and neutral butanol, the compound(s) responsible for the RIA activity did not behave like cytokinin bases and ribosides. Initial *Amaranthus* bioassay studies suggested that the compound(s) had no cytokinin activity.

However, the ability of the HPLC fraction containing the active compound(s) to abolish the activity of standard [9R]Z in the bioassay complicates the interpretation of the results. This finding suggested the presence of an inhibitor of the bioassay response in this HPLC fraction, and therefore no conclusion can be made as to whether the compound(s) responsible for the RIA activity are biologically active. It is tempting to speculate that the compound(s) showing the activity in RIA may be the inhibitory compound(s) in the bioassay; i.e., they are natural "anticytokinins" with a close structural relationship to the cytokinins but with the ability to block the biological activity of naturally occurring cytokinins.

The detection of activity in RIA in the DEAE wash clearly indicates the importance of using rigorous and appropriate separative techniques prior to analysis of cytokinins by RIA. Such activity has also been found previously in the DEAE wash fraction of a lupin seed extract (Badenoch-Jones et al. 1984a). Data on cytokinin activity in embryo, endosperm, and "remainder" obtained by RIA, when combined to give activity on a whole-seed basis, generally correlated well with data available from GC-MS analysis indicating the reliability of RIA, when combined with appropriate separative techniques, for estimating cytokinins.

There was, however, some underestimation of the O-glucosides when analyzed by RIA. Underestimation of the O-glucosides might be expected (see Badenoch-Jones et al. 1987) because of a lack of O-glucoside recovery markers, even though an attempt was made in the present study to account for losses during the purification steps following the TLC step, by addition of recovery markers prior to β-glucosidase hydrolysis. RIA also proved to have a useful application in locating the HPLC fractions containing cytokinins prior to

quantitative analysis by GC-MS. The usual methods for doing this in the presence of a high UV background are based on either cochromatography with authentic radiolabeled cytokinins or the use of bioassay. Both of these alternatives suffer severe disadvantages—in the former case because high-specific activity radiolabeled standards are generally not available, and in the latter case because bioassay consumes a substantial part of the sample, and it is difficult to detect cytokinins that are very weakly active in bioassays.

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