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Cytokinins of the Developing Mango Fruit[1]

ISOLATION, IDENTIFICATION, AND CHANGES IN LEVELS DURING MATURATION

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ABSTRACT

The cytokinin activity has been isolated and identified from extracts of immature mango (*Mangifera indica* L.) seeds. The structures of zeatin, zeatin riboside, and N^6-(Δ^2-isopentenyl)adenine riboside were confirmed on the basis of their chromatographic behavior and mass spectra of trimethylsilyl derivatives. Both trans and cis isomers of zeatin and zeatin riboside were also identified by the retention times of high performance liquid chromatography. In addition, an unidentified compound appeared to be a cytokinin glucoside.

The concentration of cytokinins in the panicle and pulp of mango reached a maximum 5 to 10 days after full bloom and decreased rapidly thereafter. The cytokinin level in the seed remained high until the 28th day after full bloom. The quantity of cytokinins in pulp per fruit increased from the 10th day after full bloom, the maximum being attained around the 50th day after full bloom. Similarly, the amount of cytokinins per seed increased from the 10th day after full bloom, reaching a peak on the 40th day and decreasing gradually thereafter.

A high percentage of fruit set in mango was persistently maintained by supplying 6-benzylaminopurine (1.5×10^3 micromolar) onto the panicle at the anthesis stage and by supplying gibberellic acid (7.2×10^2 micromolar) and naphthalene acetamide (3.1×10 micromolar) at the young fruit stage.

It is well known that cytokinin is an essential factor for the inflorescence differentiation and floret growth of grape (11, 24). Furthermore, it has become clear that cytokinin may play an important role in inducing fruit set of several plants (6, 9, 19). Until quite recently, BA,[2] in addition to GA_3 and β-naphthoxyacetic acid, have been used successfully for inducing parthenocarpic fruits in mango (4). But no information is available on the endogenous cytokinins of mango. This study was undertaken to examine the naturally occurring cytokinins in mango fruits, and to explore their role in growth and development.

In recent years, a number of researchers have described the utility of HPLC for the separation and determination of cytokinins in plant extracts (2, 15, 16). In this paper, I indicate the conditions for HPLC separation of cytokinins in mango fruit extracts using a reversed-phase micro-octadecyl (ODS) column in order to obtain the information for the subsequent routine analysis.

In a previous paper (5), I have shown the effectiveness of spraying with a mixture of GA_3 and NAAm onto the young fruit of mango, at the stage of about 0.4 cm diameter, in controlling fruit drop. In mango, however, there is a heavy drop of hermaphrodite flowers and young fruits amounting to 99% or more (14). Therefore, I have investigated the possibility of using chemical reagents as a more effective means of checking fruit drop. In the present study, emphasis has been on relationships between exogenous plant growth regulators, BA + GA_3 + NAAm, and fruit set in mango, especially on the minimal number of applications of these reagents needed to obtain positive fruiting responses.

MATERIALS AND METHODS

Analysis of Cytokinin Activities in Mango Fruits.

Plant Materials. Mangos (*Mangifera indica* L. cv Irwin), from 7-year-old trees growing in the suburb of Chia-yi City, Taiwan, were used for the experiments. Seeds taken from 1.5-cm-diameter fruits derived from open pollinated flowers were used for the isolation and identification of the endogenous cytokinins. For quantitative analysis, separate samples of fruit were harvested at weekly intervals from flowering to the enlarged fruit stage. The fruit tissues were weighed, freeze-dried, and stored in darkness at −20°C until required for extraction.

Extraction. Freeze-dried tissues, 4 g of flower or pulp or 2 g of seed, were extracted in cold (<0°C) ethanol (40 ml 80% v/v ethanol/g tissue) for 24 h three times. The ethanol extracts were filtered, combined, and reduced to the aqueous phase in vacuum. The solution was added to a standard volume (about 50 ml), adjusted to pH 2.5 with 1 N HCl, and partitioned four times with 50 ml ethyl acetate. The residual aqueous phase was then adjusted to pH 8.3 with 1.4 N NH_4OH, and partitioned four times with water-saturated *n*-butanol. Both the *n*-butanol and aqueous phases were taken to dryness in vacuum. The dried residue was dissolved in a small volume of phosphate buffer (10 mM KH_2PO_4, pH 6.4) and loaded onto a column (10 × 1.25 cm) of PVP with the same buffer to remove phenolics and pigments (8). The eluate was evaporated to dryness.

Qualitative Analysis. The dried residues of *n*-butanol and aqueous phases were individually dissolved in a minimum volume of 35% aqueous ethanol, and passed through a 40 × 1.8 cm (i.d.) Sephadex LH-20 column (3). The column was eluted with 35% aqueous ethanol as the mobile phase at a flow rate of 0.2 to 0.3 ml/min. Each fraction (6 ml) was monitored for UV absorption at 254 nm and then dried in a freeze-drying chamber. The cytokinin activities were tested by the soybean (*Glycine max* cv Acme) cotyledonary callus assay (12).

The above purification procedure was repeated, and the active fractions were collected. Further purification was accomplished by HPLC on a 29 × 0.4 cm (i.d.) column packed with μBondapak C_{18} (10-μm particle size; Waters Assoc.). The column was eluted at 0.5 ml/min with a solvent gradient linearly changed over 45 min from 30 to 70% aqueous methanol. The pH of the solvent was approximately 5. The HPLC chromatograms of the samples were compared to those of authentic cytokinins.

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[2] Abbreviations: BA, 6-benzylaminopurine; NAAm, naphthalene acetamide; 2iPA, N^6-(Δ^2-isopentenyl)adenine riboside; t-ZR, trans-zeatin riboside; c-ZR, cis-zeatin riboside; t-Z, trans-zeatin; c-Z, cis-zeatin; KE, kinetin equivalent; 2iP, N^6-(Δ-isopentenyl)adenine.

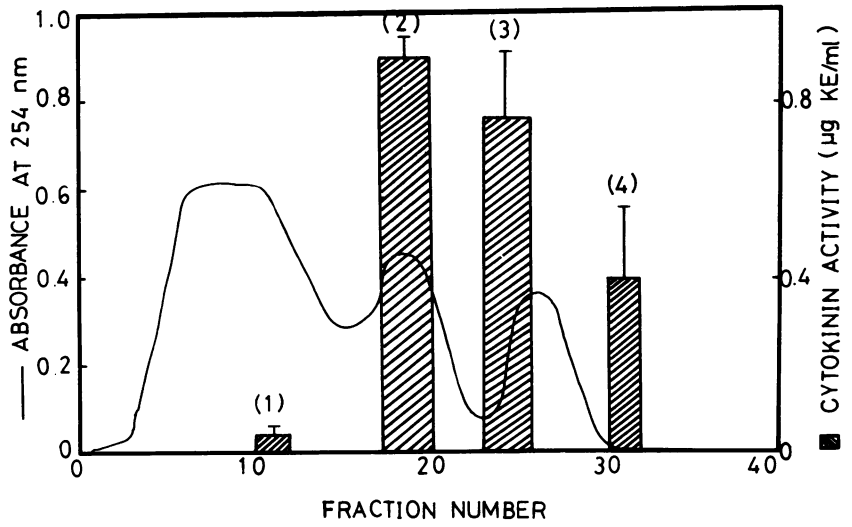

FIG. 1. Sephadex LH-20 gel filtration chromatography of *n*-butanol soluble cytokinins from mango seed (numerals in parentheses refer to the peak number). The cytokinin activities represented by the shaded areas of the column profile were obtained by the soybean cotyledonary callus assay. Vertical bars indicate SE.

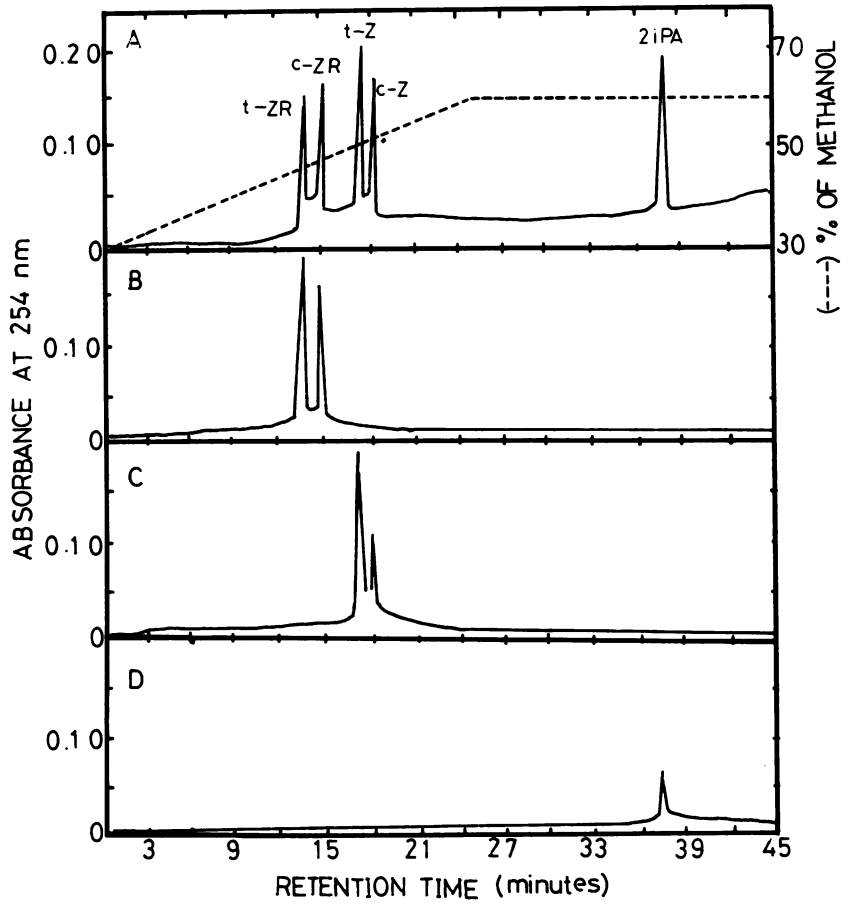

FIG. 2. HPLC analysis of the cytokinin-active fractions obtained from the Sephadex LH-20 elution profile in figure 1. A, Cytokinin standard; B, peak 2; C, peak 3; D, peak 4.

The HPLC instrumentation used for the separation of cytokinins was a Hewlett-Packard chromatograph 1084B equipped with dual 5800 p.s.i. pumps, a solvent programmer, a variable injector, an optional wavelength UV detector, and data system interface.

GC-MS analysis was performed with a Varian CH7 spectrometer equipped with a System 150 data system connected with a Varian 1200 gas chromatograph. A jet-type separator was used. The cytokinin-active materials recovered from the Sephadex LH-20 column fractions were trimethysilylated and analyzed by GC-MS. The samples were run on a 100 × 0.3 cm (i.d.) glass column packed with 3% OV-1 on silanized chromosorb-W with temperature programmed from 200 to 325°C at 6°C/min with a helium flow rate of 15 ml/min. The mass spectra of suspected cytokinins were compared with those of authentic cytokinins obtained in the

Table I. *GC-MS Obtained with Samples from Mango and with Authentic Cytokinins*

Peak numbers refer to peaks eluted from the analytical Sephadex LH-20 column.

Sample	Peaks in Mass Spectrum																
TMS_4-zeatin riboside	639	$(M^+)^a$	624	551	550	536	520	406	348	321	320	276	259	246	231	217	201
Peak 2	639	(M^+)	624	551	550	536	520	406	348	321	320	276	259	246	231	217	201
TMS_3-zeatin	435	(M^+)	346	332	304	292	280	272	264	207	191	156					
Peak 3	435	(M^+)	346	332	304	292	280	272	264	207	191	156					
TMS_4-2iPA	551	(M^+)	508	481	348	333	318	259	245	232	230	217	202	188	160	147	
Peak 4	551	(M^+)	508	481	348	333	318	259	245	232	230	217	202	188	160	147	

[a] Molecular ion.

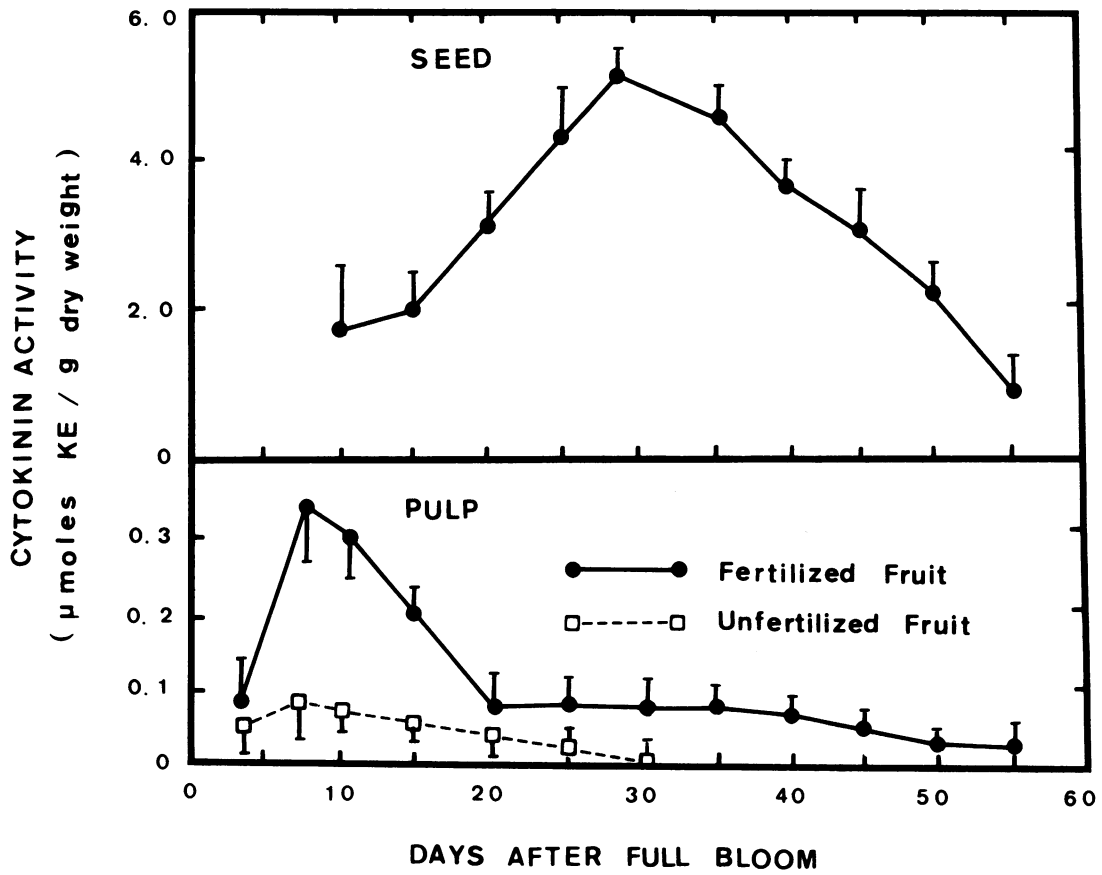

FIG. 3. Changes in cytokinin activities in the seed and pulp of mango at successive growth stages. The cytokinin activities are the sum total values of all cytokinin peaks on HPLC chromatogram. Vertical bars indicate SE.

same manner (7, 13, 17, 18, 21).

Quantitative Analysis. [8-^{14}C]Zeatin was used for estimating the efficiency of cytokinin recovery in the analytical procedure. Labeled zeatin (15,000 dpm) was added to the sample at the initial point of tissue extraction. This mass of zeatin was less than could be detected by HPLC. The recovery of [^{14}C]zeatin was estimated by measuring the radioactivity in an aliquot of the combined active fractions using normal scintillation technique.

The *n*-butanol phase described in the extraction procedure was used for the quantitative analysis of cytokinins throughout the experiment. After the combined *n*-butanol extracts were reduced to dryness and passed through the PVP column, the resulting residue dissolved in water (150 ml) was adjusted to pH 3.0 with acetic acid, and centrifuged at 18,000*g* for 20 min. Then, the suspension was percolated through a column of cellulose phosphate (NH_4^+ form equilibrated to pH 3.0). The column was washed with 0.05 N acetic acid followed by water and finally eluted with 0.3 N NH_4OH. An aqueous solution (10 ml) of the residue obtained by evaporation of the eluate to complete removal of NH_3, was adjusted to pH 3.5 and centrifuged at 18,000*g* for 20 min (20). The final cytokinin purification was achieved by separation on TLC and HPLC. TLC was performed on the silanized 20 × 20 cm PF_{254} (Merck) plates in 0.25 mm thickness, and developed with solvent methanol:H_2O (2:3, v/v). The quenching zones (R_F = 0.15–0.25, 0.30–0.35, and 0.40–0.50) under UV light at 254 nm were outlined with a soft pencil, scraped off, and extracted with 80% ethanol (1, 10). The ethanol extracts were taken to dryness. The solid residuals were chromatographed on a μBondapak C_{18}-column for HPLC analysis by the above methods. The UV-absorbing peaks on HPLC chromatogram were individually collected, freeze-dried, and bioassayed. Calibration curves of authentic cytokinins were constructed from the calculated peak heights of UV absorption at 254 nm. Simultaneously, estimates of the contents of each isolated cytokinin from plant extracts were measured by calculating the biological activities and UV peak heights (by comparison with cytokinin standards). In this step, I found that each UV-absorbing cytokinin-active component and its biological activity were approximately in agreement (data not

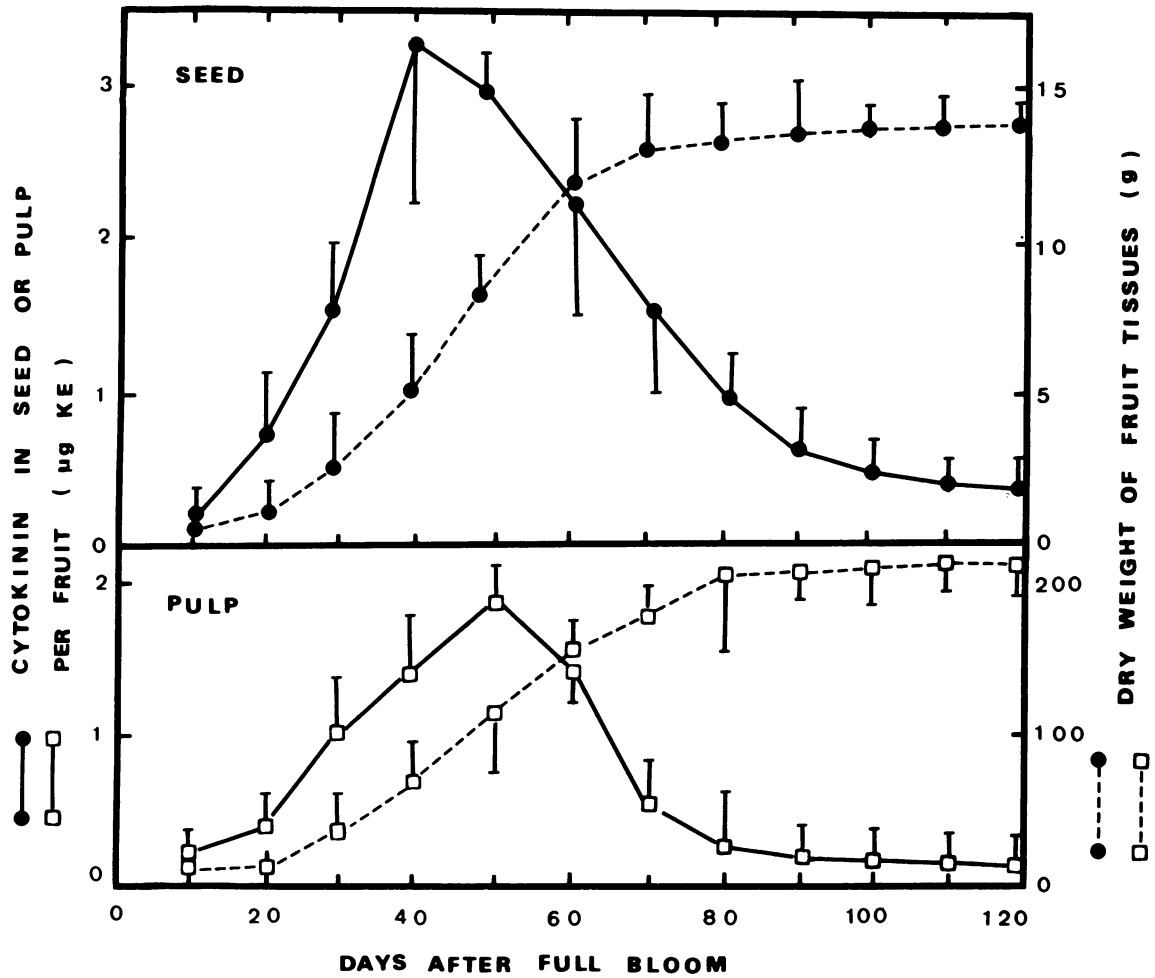

FIG. 4. Changes in cytokinin contents and dry weights in the seed and pulp per fruit at successive growth stages. Vertical bars indicate SE.

shown). These results clearly show that all cytokinin peaks on the HPLC chromatogram are homogeneous.

Reduction of Physiological Fruit Drop of Mango Treated with Plant Growth Regulators. At the anthesis stage, the following five treatments were made with authentic plant growth regulators sprayed on the panicle or young fruit: (a) GA_3 (7.2×10^2 µM) + BA (1.5×10^3 µM); (b) NAAm (3.1×10 µM) + BA (1.5×10^3 µM); (c) GA_3 (7.2×10^2 µM) + NAAm (3.1×10 µM); (d) GA_3 (7.2×10^2 µM) + NAAm (3.1×10 µM) + BA (1.5×10^3 µM); (e) control. The selected mango inflorescences were sprayed with BA containing a wetting agent, Tween 20, at the anthesis stage, and 20 d later the young fruits were sprayed once with GA_3 and/or NAAm as specified. Unsprayed trees were used as controls. The per cent fruit drop was counted at intervals of 10 d up to the stage of fruit maturity.

RESULTS AND DISCUSSION

CYTOKININ ACTIVITY IN YOUNG FRUIT OF MANGO

Qualitative Analysis.

Sephadex LH-20 Column Chromatography. The cytokinins in the *n*-butanol phase were separated by Sephadex LH-20 chromatography (Fig. 1). Four zones of biological activity were observed (fractions 10–11, 17–19, 23–25, and 31–32). Two cytokinin-active zones (fractions 10–11 and 17–19) were found also in the aqueous phase (data not presented). The use of Sephadex LH-20 columns is known to give a characteristic value for the ratio of V_e (elution volume) to V_t (bed volume) for each cytokinin: zeatin riboside, 1.0; zeatin, 1.2; 2iPA, 1.6; 2iP, 1.8 (23). V_e/V_t values in the active fractions 17–19, 23–25, and 31–32 were very close to those of zeatin riboside, zeatin, and 2iPA, respectively. However, one additional zone of biological activity, other than that of the employed authentic cytokinin standards, was found in the chromatogram of fraction 10–11. This compound is possibly a cytokinin-glucoside (3, 22).

The active fractions obtained from the Sephadex LH-20 fractionation of the *n*-butanol phase were then subjected to HPLC and GC-MS analyses for further identification.

HPLC. In the past (2), the Sephadex LH-20 column has often been used for cleanup of cytokinins from plant extracts. However, due to impurities, no attempt was made to obtain distinct UV peaks for cytokinins by this chromatographic technique. The resolving power of HPLC for cytokinins is now well documented. In this study, the cytokinin-active fractions obtained from Sephadex LH-20 column chromatography were dissolved in a minimum volume of 30% methanol, filtered through 0.45-µm-type Millipore filters, and charged onto the µBondapak C_{18}-column as described in "Materials and Methods." The first peak obtained in column fraction 10–11 was not investigated further. The other cytokinin-active fractions were resolved into five peaks of UV absorption by chromatography on the µBondapak C_{18} and comparison with cytokinin standards (Fig. 2). An early sharp peak of UV absorption (retention time, 13.3 min) in plant extracts corresponding to t-ZR of the activity of cytokinin standards. The four later peaks were at the retention times of authentic c-ZR, t-Z, c-Z, and 2iPA, respectively. The corresponding µM concentrations of the above cytokinins were: t-ZR, 3.6; c-ZR, 2.9; t-Z, 5.1; c-Z, 0.4; and 2iPA, 0.3, as determined by computer integration of HPLC

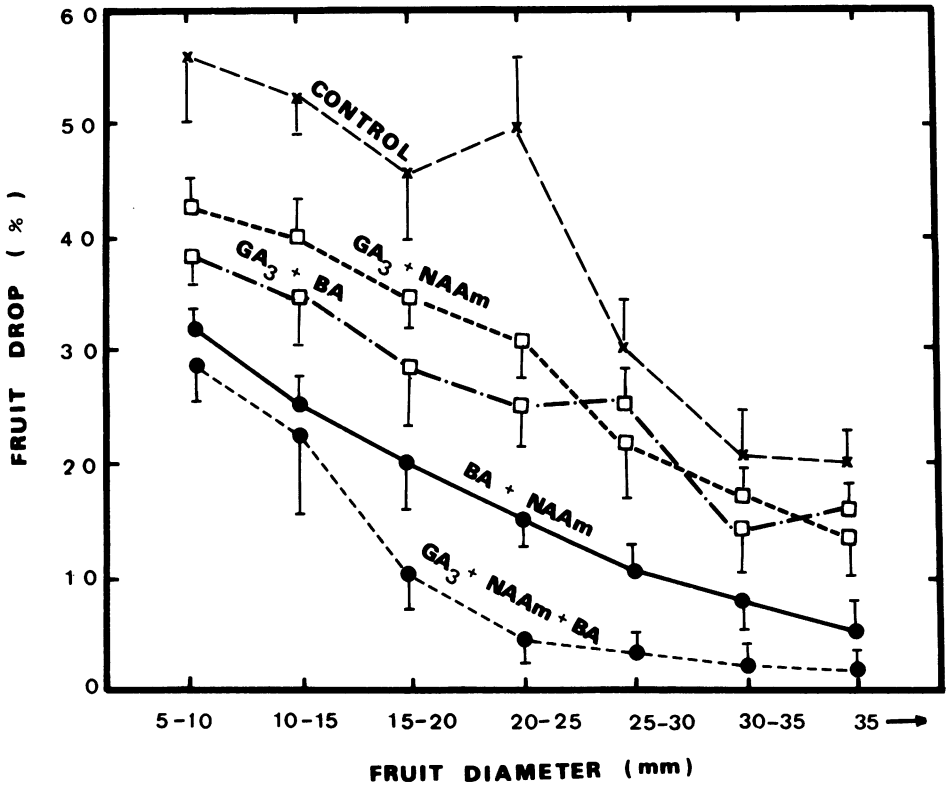

FIG. 5. Rate of fruit drop after treatment with plant growth regulators as related to fruit size. The mango panicles were sprayed with BA at the anthesis stage, and 20 d later the young fruits (about 4.0–4.5 mm diameter) were sprayed once with GA_3 and/or NAAm as specified. The per cent fruit drop was counted at intervals of 10 d up to the stage of fruit maturity. Arrow shows fruit size over 35 mm. Vertical bars indicate SE.

chromatograms and calculation of the peak areas. Clearly, zeatin riboside and zeatin are the major cytokinins in the seed of mango.

GC-MS. The active fractions eluting from the analytical Sephadex LH-20 column were evaporated to dryness and trimethylsilylated with 25 μl pyridine and mixed with 25 μl *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). The samples were silylated at room temperature for 30 min in a desiccator and individually examined by GC-MS. Characterization of the compound of peak 1, however, was not successful because of the limited amount available. In the other peaks, the molecular ions were always observed, and their elemental compositions were confirmed by exact mass measurement (Table I). Peak 2 had the same mass spectrum as zeatin riboside. And the other peaks were identified on the basis of MS and GC retention times as zeatin and 2iPA, respectively.

Quantitative Analysis.

Recovery of [^{14}C]Zeatin. Recovered radioactivity in the final extracts was 7,552 dpm. An average of 50 ± 1.3% of the [^{14}C]zeatin was recovered.

Changes in Endogenous Cytokinins during Growth of Mango Fruit. In panicle and pulp, a maximum amount of cytokinins was found 5 to 10 d after full bloom, when the fruit tissues reached 0.35 μM (KE/g dry weight) of cytokinins and gradually declined thereafter. The cytokinin concentration in the seed reached maximum values of up to 5.5 μM on the 28th d after full bloom (2.5 cm in fruit diameter), and then declined rapidly (Fig. 3). However, the total amounts of cytokinins both in the seed and pulp increased continuously, reaching maximum values, respectively, when the fruit was 40 and 50 d old (Fig. 4). At this stage, the fruit still continued to enlarge at a constant rate, even though the seed had reached the final size.

The cytokinin content of parthenocarpic fruits was extremely low as compared with that of fertilized fruits. Furthermore, the cytokinin activity decreased almost to zero at the marble stage (1.5–2.0 cm fruit diameter, red-green appearance) of parthenocarpic fruits (Fig. 3). The decrease coincided with the heavy fruit drop (100%) of parthenocarpic fruits of mango at this stage. However, cytokinins were also found in the parthenocarpic fruits prior to the marble stage, indicating that these substances are not necessarily products resulting from fertilization. Hence, the high cytokinin activity of fertilized fruits may be considered as resulting merely from strong sink activity in these fruits.

Effect of GA_3, NAAm, and BA in Different Combinations on Fruit Set of Mango. The panicles were treated with aqueous solution of BA at the anthesis stage and later with the other growth regulators in various combinations as previously mentioned (Fig. 5). The fruit setting (well-pollinated fruit) was promoted by treatment with growth regulators in this experiment. And the treatment with GA_3 + NAAm + BA was found to be the best for promotion of fruit set.

Chacko and Singh (4) demonstrated that, for parthenocarpic development of mango fruit, an initial application of cell division factors like cytokinin and later application of factors which promote cell enlargement such as auxin and gibberellin are necessary. However, the experiment showed that, even after spraying seven times, the per cent of successful fruit set up to maturity was only 60%. Consideration of the cost shows that the treatment lacks commercial value. The results of our study utilizing fewer applications of synthetic growth regulators for inducing fruit set and development give more positive indications. Our observations indicate that, in mango, gibberellins, auxins, and cytokinins are all involved in fruit development. Lack of any one growth regulator may lead to reduction in fruit set. It is assumed that external application of these plant growth regulators to the panicles or young fruits of mango will cause mobilization of metabolites to the sites of application. This problem is worthy of further investigation.

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