Cytokinin and Inhibitor Activities in the Avocado Fruit Mesocarp[1,2]

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ABSTRACT

No cytokinin activity was found in methanolic mesocarp extracts after purification with petroleum ether and ethyl acetate. Cytokinin activity did appear, however, in aqueous fraction after acid hydrolysis or passage through Dowex 50 (H+) ion exchange columns. The level of this activity, the fruit growth rate, and the cell division rate were found to be positively correlated (with each other).

An inhibitor to cytokinin activity in soybean callus bioassay was found in the petroleum ether fraction; this inhibitor is not abscisic acid. The activity of the inhibitor could be considerably reduced by raising the level of kinetin in the nutrient medium. The intensity of inhibition was found to be positively correlated with reduction in the rate of mesocarp growth.

The reduction in fruit growth rate may be considered to be due to the diminution of bound cytokinin activity and (or) increased activity of an endogenous inhibitor.

Fruit growth occurs both by cell division and by cell expansion. In most fruits, cells divide for only a short period after fruit set (10), the fruit growth after this period being due to cell expansion. The avocado is exceptional in that cell division continues as long as the fruit is attached to the tree, and even after the fruit has reached horticultural maturity (11). Since cytokinins are known to be regulators of cell division (6), it was of interest to test for their activity in avocado fruits.

MATERIALS AND METHODS

Avocado fruits of the Fuerte variety were picked once a month, starting 2 months after fruit set (June to November), from productive trees growing under good conditions. After weighing the fruit, the mesocarp was cut into 5-mm cubes which were immediately frozen in liquid air. Ten-gram (fresh weight) portions of the frozen material were freeze-dried, sealed under vacuum in small glass containers, and were stored for 1 to 6 months before use. The freeze-dried material was extracted by maceration with 80% methanol. The homogenate was placed in a refrigerator for 1 hr and then was filtered through a Buchner funnel. The filtrate was treated as follows. (Scheme 1).

Scheme 1. *Solvent Partition*
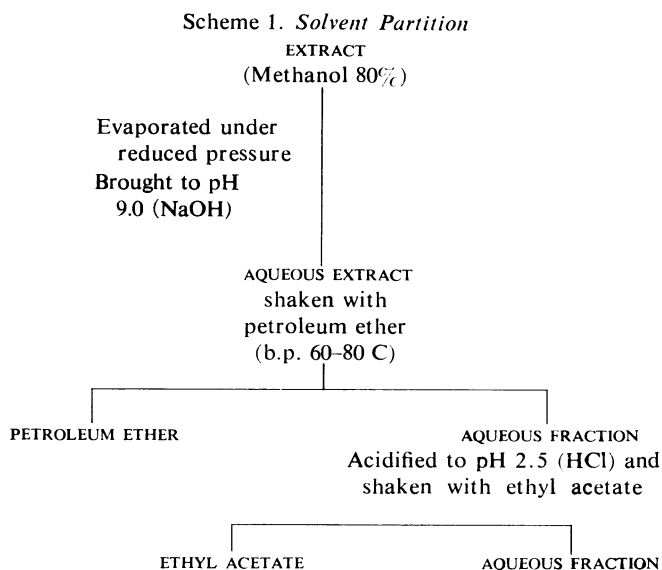
Partition Fractionation. The filtrate was concentrated under low pressure at 50 C until all of the methanol had evaporated. The aqueous residue was shaken four times with equal quantities of petroleum ether (bp 60–80 C). Immediately thereafter it was acidified to pH 2.5 with 1 N HCl, and extracted four times with equal volumes of ethyl acetate. The purified aqueous residue was tested before and after passage through an ion exchange column in the acid form, or by acid hydrolysis; all fractions were bioassayed after paper chromatography.

Column Fractionation. The column used was a Dowex 50 (H+) 200- to 400-mesh ion exchange column, 150 mm long and 20 mm wide. The aqueous concentrate loaded on the column was equivalent to 5 or 10 g fresh weight of the mesocarp material. The column was eluted with 120 ml of water, 120 ml of 70% methanol, 120 ml of water, 120 ml of 1.5 N ammonia, and 120 ml of water, in that order.

Hydrolysis. The aqueous concentrate was hydrolyzed in 1 N HCl at 100 C for 1 hr, after which the material was fractionated by shaking four times with equal volumes of ethyl acetate.

Paper Chromatography. Aqueous fractions were further separated by ascending paper chromatography by using isopropanol-ammonia-water (10:1:1) as solvent. Occasionally, butanol-acetic acid-water (4:1:1) was used for comparison. The front was allowed to migrate 20 cm.

Bioassay of Cytokinins and Inhibitors. The biological activity of the extracts was tested by the soybean cotyledon callus assay, a specific test for cytokinins. Miller's nutrient medium (8) with 0.8% agar (20 ml) was placed in 100-ml volume Erlenmeyer flasks.

Three callus explants, each weighing approximately 8 mg, were planted in each Erlenmeyer flask. The explants were taken from

a stock culture growing on nutrient medium containing 1 mg/ liter of kinetin. Cultures were kept in the dark at 27 C and 80% relative humidity. Explants were weighed 25 to 30 days after planting. The intensity of inhibitory activity was determined in the soybean callus assay by adding chromatogram sections or inhibitory fractions to media containing various concentrations of kinetin.

RESULTS

The growth rate of Fuerte fruit and pericarp is shown in Figure 1, and relative growth rate of the Fuerte pericarp is shown in Figure 2. Fruit grows rapidly in the early months, the rate diminishing with fruit development. From September onward, the relative increase in weight is very small; the fruit weight increases continuously due to cell division and the expansion of cells that have recently divided. Cells were counted on transverse sections taken from the middle of the fruit; the average cell number along this radius of the pericarp was 160 in fruit picked in June, and this number increased during fruit growth to 295 in fruits picked in November.

The crude methanolic extract was fractionated as described in Scheme 1. This enabled us to differentiate between inhibitory and promotive substances. The results of the tests performed with the various fractions are given below.

Petroleum Ether Fraction. This fraction contained chlorophyll and the oily substances present in avocado fruit mesocarp. No cytokinin activity was detected; however, strong inhibitory activity was found at R_F 0.9 to 1.0. It was named "Inhibitor C."

This inhibitor was tested at different concentrations with various concentrations of kinetin. It was found that raising the kinetin level in the medium reduced the inhibitory action, and vice versa, i.e., raising the inhibitor level in the medium decreased the total growth induced by kinetin in proportion to the inhibitor concentration (Table I).

The intensity of inhibition was found to increase with fruit

Table I. *Response of Soybean Callus to Various Combinations of Kinetin and "Inhibitor C" in the Nutrient Medium*

Results are given as average callus growth (gram per flask). Standard error for the 5.0-mg kinetin per liter column: 0.10, 0.18, 0.18, 0.13, 0.09, and 0.05.

"Inhibitor C" Extracted from mg Fresh Tissue/Flask	Kinetin mg/liter in the nutrient medium			
	0.005	0.05	0.5	5.0
0	0.37	1.16	2.71	3.50
10	0.28	0.98	2.29	3.80
50	0	0.30	1.14	2.16
100	0	0.18	0.72	1.29
500	0	0	0.30	0.50
1000	0	0	0.16	0.38

FIG. 3. Activity of "Inhibitor C" during fruit growth. (R_F 0.9–1.0, eluted from chromatograms where petroleum ether fraction derived from avocado mesocarp, was run. Equivalent to 0.1 g fresh tissue per flask; nutrient medium contained 0.5 mg of kinetin per liter.
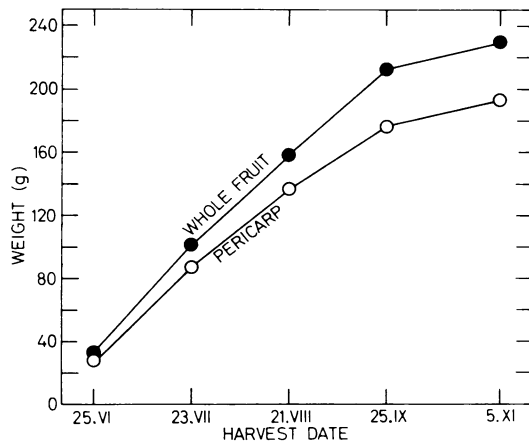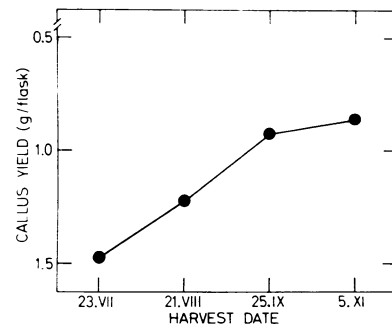
FIG. 1. Avocado fruit growth (var. Fuerte).
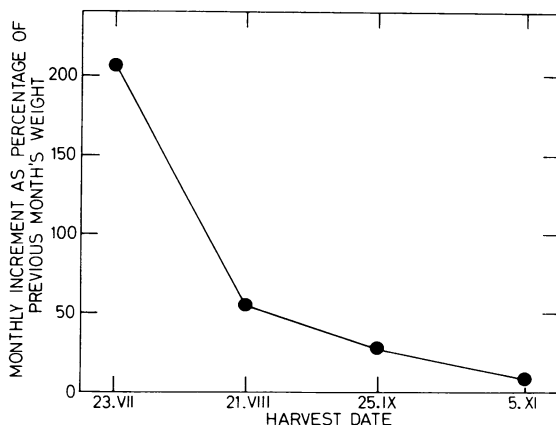
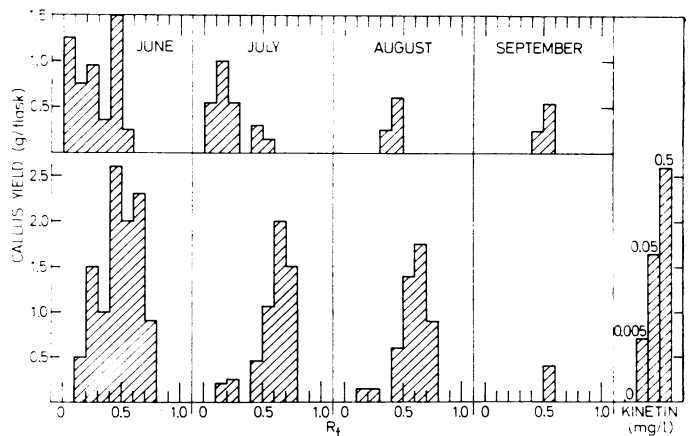FIG. 2. Relative rate of growth of Fuerte pericarp.

FIG. 4. Response of soybean callus to mesocarp extract, equivalent to 10 g fresh tissue per 100 ml of nutrient medium. Upper part: After column treatment; lower part: after acidic hydrolysis; on the extreme right: the activity of kinetin under the same bioassay conditions is presented for comparison.

development (as shown in Fig. 3), correlating well with the decrease in fruit growth rate.

Ethyl Acetate Fraction. Acid gibberellins, auxins, and abscisic acid are present in this fraction. Abscisic acid has been found in large quantities in avocado fruit mesocarp (7). We examined this fraction, with the wheat coleoptile elongation test for auxins and inhibitors and with barley endosperm test for gibberellins. In both tests, inhibition was found at R_F 0.6 to 0.8, the R_F value of synthetic abscisic acid. The substances from the inhibiting region were eluted and tested in the soybean bioassay. Amounts derived from 5, 50, and 500 mg fresh weight in 100 ml of nutrient medium did not inhibit growth of the callus. Tests conducted with higher concentrations revealed different activities (1).

Aqueous Fraction. In the procedure employed cytokinins should be found in the aqueous fraction; however, no such activity was observed in this fraction even after fractionation with petroleum and ethyl acetate, followed by paper chromatography. The possibility that endogenous inhibitors are masking cytokinin activity could be ruled out inasmuch as no inhibitory activity was observed in this purified aqueous fraction. When the aqueous fraction was passed through a strongly acidic Dowex 50 (H^+) column, the material eluted by NH_4OH contained cytokinins located on paper chromatograms at R_F values 0.1 to 0.2 and 0.6 to 0.7. The intensity of the activity varied with the stage of fruit development. It was high in the early samples, gradually diminishing with the reduction in the rate of fruit growth (Fig. 4). Also, after acid hydrolysis, cytokinin activity was present in the extract, located around the two R_F values at which cytokinins were detected after column treatment; it was generally stronger at R_F 0.7. The intensity of this activity varied with the stage of fruit development in a manner similar to that found after column treatment (Fig. 4).

DISCUSSION

It is generally accepted that cytokinins are substances which induce cell division in the presence of auxins. A positive correlation between cytokinin level and rate of cell division was found in plum and apple fruits (4), and in the opinion of Letham, cytokinins "may therefore play an important role in the regulation of cell division in both fruits" (6). It would seem reasonable, therefore, to assume that a tissue which is undergoing cell division over a long period of time, like that of the avocado mesocarp, would contain endogenous cytokinin. However, no cytokinin activity could be detected in chromatographed crude extract of avocado fruit mesocarp, even when no inhibitory activity interfered.

The appearance of cytokinin activity after further treatment of the inactive aqueous fraction can be explained by the presence of "bound" cytokinin, which can be liberated by acid hydrolysis

or by passage through an acid ion exchange column. The fact that the activity was found to be correlated with the avocado fruit growth rate and with cell division may indicate that the material(s) has a physiological role.

Kende (3) also found that acid hydrolysis transformed an inactive substance in a cell division assay into an active one, and he assumed that a bound form had been liberated. Since tRNA is precipitated in the extraction procedure, it cannot be considered as the source of cytokinin activity in the aqueous fraction.

Column fractionation has been used for purifying cytokinins in other studies (2, 5, 9); Heide and Skoog (2) attributed its effectiveness to removal of inhibitors. The present results indicate that this treatment may act in an entirely different way.

The neutral inhibitor(s) of cytokinin activity found in the petroleum ether fraction differs in its chemical properties from the inhibitor described by Letham (4). The pattern of change in the activity levels of these two endogenous inhibitors, as related to fruit development, also differs. The level of inhibition found by Letham was high when intensive cell division occurred in the fruit but was low when cell division stopped, whereas the level of inhibition in our studies followed the reverse pattern.

Our results show a direct correlation between the level of bound cytokinin and the rate of mesocarp growth, as well as an inverse correlation between the level of inhibitor and the rate of fruit growth. The reduction in growth rate may be due to change in the level of either the bound cytokinin or of the inhibitor, or both.

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