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CYTOKININ PRODUCTION BY TOMATO ROOT: IDENTIFICATION OF A MAJOR CYTOKININ PRODUCED BY THE ROOT AND ENVIRONMENTAL FACTORS AFFECTING THE PRODUCTION

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Introduction

It has been well documented that the root tip is a major site of cytokinin production in higher plants^{5,6,10,16}, and the content of cytokinin in xylem sap suggests that environmental conditions around the root system affect the cytokinin production¹¹. The change in cytokinin production which induced by an environmental factor is responsible in part for the developmental aspects of plants. The amount of cytokinin transported from the roots to shoots markedly shows seasonal changes that are well correlated with the rate and characteristic of plant growth. Cytokinin level in xylem sap of *Populus* increased by low temperature treatment of the plant³. Flooding² and water stress⁴ caused a decrease in cytokinin content of the saps.

We have previously reported that tomato root tips cultured *in vitro* produced cytokinins and the cytokinins were released from the roots into the medium⁹. The major cytokinin found in the medium seemed to be zeatin riboside. After 7-day culture, the amount of cytokinin accumulated in the medium was approximately eight times more than that in the root tissues. Furthermore, various nutritional and hormonal factors affected the production⁷.

We report here the identification of a major cytokinin produced by tomato roots, and the effects of various environmental conditions on cytokinin production by tomato root using the root tip culture method.

Materials and Methods

Root tip culture

Tomato root tip cultures were carried out as reported previously⁹. Tomato seeds (*Lycopersicon esculentum* Mill. cv. Hagoromo) were sterilized with 0.4% sodium hypochlorite solution for 30 min, washed three times with sterile water and then germinated at 25°C in the dark in modified White's medium. After

seven days, root tips about 1 cm long (ca. 1.5 mg in fresh weight) were excised from the seedlings. Unless otherwise indicated, three root tips were transferred to a 100-ml Erlenmeyer flask containing 10 ml of the medium and cultured for seven days.

Cytokinin extraction and bioassay

At the end of each culture, the roots were removed from the medium by filtration. The medium (400–500 ml) was adjusted to pH 2.5 with 1 M HCl and then passed through a Dowex 50W-X4 (50–100 mesh, H⁺ form) cation exchange column of 50 ml volume. The column was washed with 500 ml distilled water and eluted with 500 ml 3 M NH₄OH. To avoid heat decomposition of cytokinins, the column was cooled with ice cold water until all the resin had converted to NH₄⁺ form. The eluate was evaporated to dryness below 40°C, and the resulting residue was chromatographed on papers with isopropanol : ammonia : water (10 : 1 : 1 v/v). The chromatograms were cut into 10 equal R_f strips, and each of them was placed directly into a bioassay medium for cytokinin. Cytokinin activity were determined using soybean callus bioassay⁹. The amount of cytokinin in each medium was calculated using as a reference of the growth curve for authentic zeatin. The threshold cytokinin concentration for detection by this assay system is ca. 20 ng/l.

Identification of a major cytokinin in the medium

Our previous report suggested that the major cytokinin produced by tomato roots is zeatin riboside. To confirm this, cytokinin fractions obtained from various root cultured media were combined (equivalent to ca. 87 l of medium) and used for identification of the cytokinin. The eluate from Dowex 50 W column was evaporated to remove ammonia and the resulting aqueous residue was passed through an Amberlite XAD-2 column of 200 ml volume. The column was washed with water thoroughly and eluted with one l of 40% ethanol. The eluate was evaporated to dryness and the resulting residue was subjected to silica gel thin layer chromatography (Analtech, Silicagel G) developed with chloroform : methanol (9 : 1 v/v). The zone identical to zeatin riboside was scraped off and eluted with methanol. The eluate was purified further by high performance liquid chromatography (HPLC) of Novapak C₁₈ column (Waters). The column was eluted with acetonitrile : water : acetic acid (15 : 85 : 0.1 v/v) at a flow rate of 1 ml/min, and the elution profile was monitored at 260 nm. Based on the retention time, a fraction corresponding to authentic zeatin riboside was collected and analyzed by field desorption (FD) mass spectrum.

Results

Identification of a major cytokinin produced by tomato roots

HPLC profile of cytokinin fraction obtained from the medium showed the

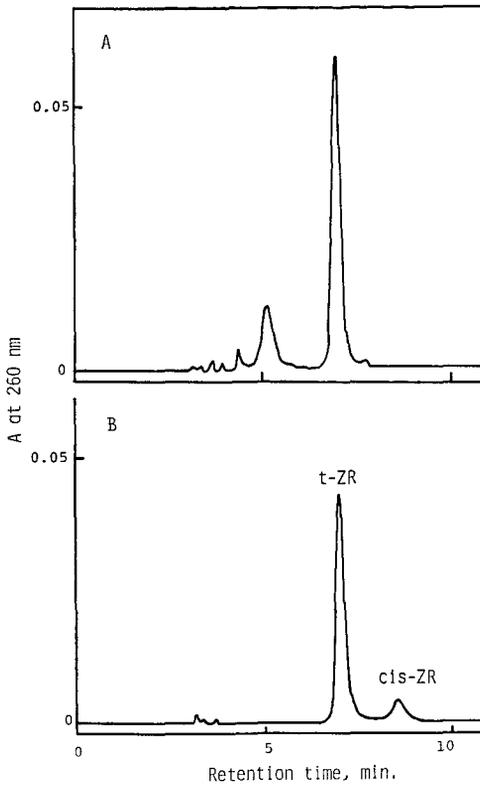


Fig. 1. Separation of root-produced cytokinin by high performance liquid chromatography on a column of Novapak C_{18} with acetonitrile: water:acetic acid (15:85:0.1, v/v). A, zeatin riboside fraction obtained from medium of tomato root culture; B, authentic zeatin riboside.

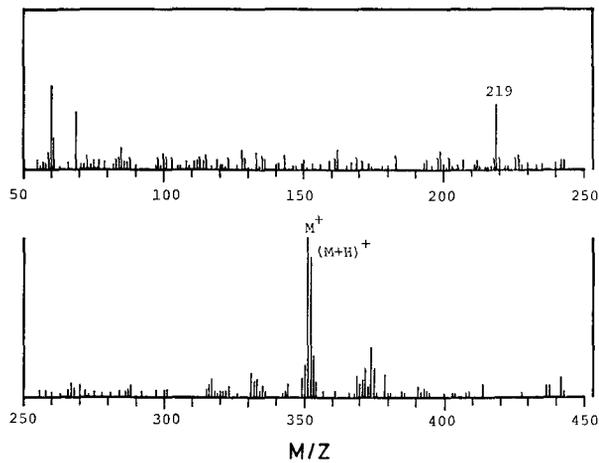


Fig. 2. FD-mass spectrum of the isolated substance corresponding to trans-zeatin riboside.

presence of a substance corresponding to *trans*-zeatin riboside (Fig. 1). The FD mass spectrum of the substance indicated that the substance has a molecular weight of 351 (Fig. 2). Furthermore, the substance stimulated strongly soybean callus growth just as authentic zeatin riboside. Thus the substance was identified as *trans*-zeatin riboside.

Temperature

Since temperature is a primary determinant of growth rate of plants, effect of temperature on the cytokinin production by tomato root was firstly examined at 20°C, 25°C and 30°C. After 7-day culture, cytokinin fraction was extracted from each medium and chromatographed on papers and then assayed for cytokinin activity. As shown in Fig. 3, two major peaks of cytokinin activity were always found at Rf 0-0.1 and 0.5-0.7 on the chromatograms. The fast-moving activity corresponds to zeatin riboside and the slow-moving one corresponds to its metabolite⁵. Although a maximum root growth was found at 30°C (mean fresh weight per root at 20°C, 25°C and 30°C was 11.4 mg, 19.0 mg and 20.7 mg respectively), the maximum cytokinin production was attained at 25°C. Total amount of cytokinin produced by one root at each temperature was 383 pg, 437 pg and 220 pg. Another difference observed was a slight decrease in the level of metabolite of zeatin riboside (Rf 0-0.1) with increasing the temperature.

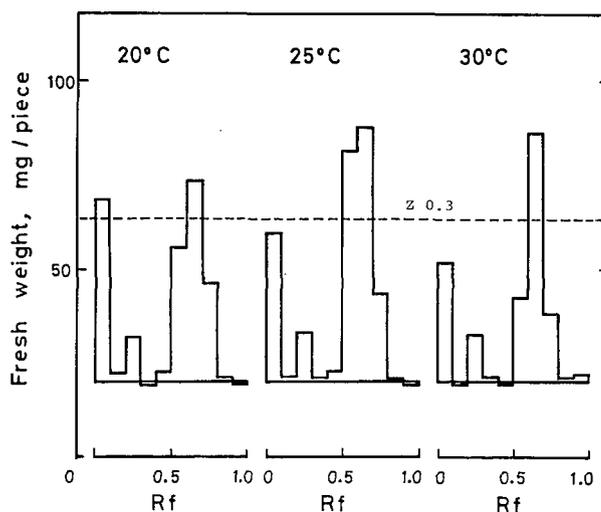


Fig. 3. Effect of culture temperature on cytokinin production by tomato roots. Cytokinin fractions equivalent to 150 ml medium were chromatographed on papers with isopropanol: ammonia: water (10:1:1, v/v) and assayed using soybean callus. The broken line represent callus yield obtained with 0.3 $\mu\text{g/l}$ zeatin.

pH of medium

In order to examine effect of pH of the medium, initial pH of each medium was adjusted to ranging from 3.5 to 8.0 with 1 M HCl or 1 M NaOH, and then the root tips were cultured in each medium. The roots failed to survive at pH 3.5. Since the amount of zeatin riboside and that of its metabolite showed a similar fluctuation, the total amounts were shown in Fig. 4. At pH 4.0, the highest cytokinin production was observed. Above pH 4.0, the amounts of cytokinin sharply decreased with increasing the pH, and reached a minimum at pH 5.6. The amounts turned to increase above pH 5.6. The root growth was not affected largely by the pH.

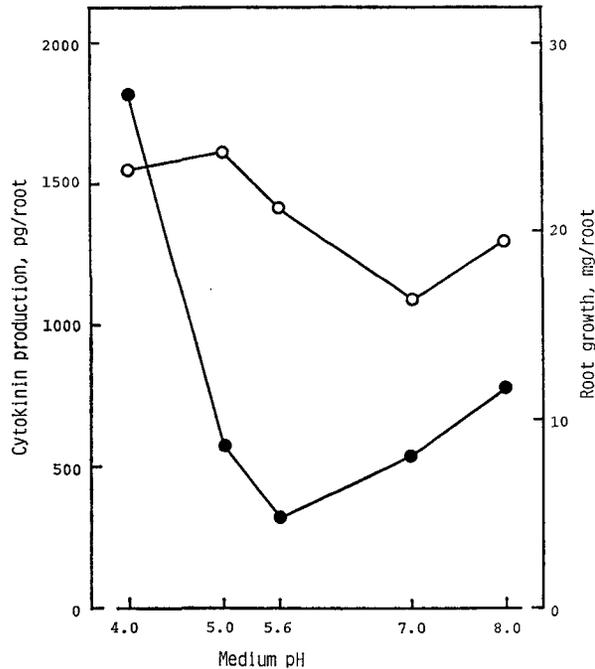


Fig. 4. Effect of pH of the medium on cytokinin production by tomato root (●) and the root growth (○). Cytokinin activity was detected by soybean callus assay and the amount of cytokinin was calculated using as a reference the growth curve for authentic zeatin.

Number of root tips per flask

The effect of number of root tips was examined by changing the number ranging from one to six. Increasing the number decreased drastically cytokinin production (Fig. 5). It seems unlikely that this decrease is due to lack of nutritional factors, because the root growth was not affected largely. A possible explanation of this result is that increasing the number of root reduces oxygen concentration in the medium and the cytokinin production is strongly dependent

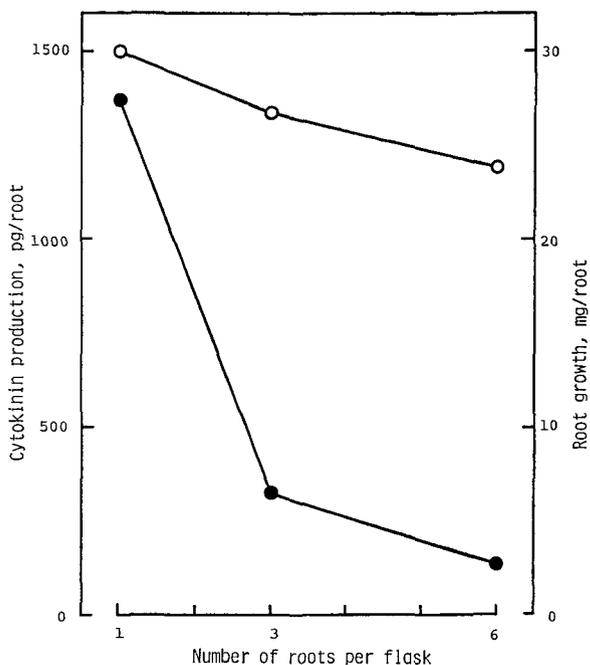


Fig. 5. Effect of number of roots per flask on cytokinin production by tomato root (●) and the root growth (○).

on the oxygen availability.

Shaking and aeration

It is therefore of interest to examine effects of shaking and aeration of cultures, both of which provide an aerobic condition, on the cytokinin production. Shaking culture at 100 rpm on a rotary shaker largely increased the mounts of zeatin riboside and its metabolite (Table 1). The total amount of cytokinin produced by one root was doubled by the shaking, while the root growth was not affected.

Aerated culture was carried out in a jar (7.5 × 15 cm) containing 500 ml medium and 150 root tips. The jar was aerated with moistened air (sterilized by two cotton filters) using glass sieve at a flow rate of ca. 150 ml/min for seven

TABLE 1. Effect of shaking culture on cytokinin production by tomato roots and the root growth

Culture method	Cytokinin production (pg*/root)		Total	Root growth (mg fresh weight/root)
	Zeatin riboside	Metabolite of zeatin riboside		
Stationary	243	50	293	19.6
Shaking	527	90	617	20.5

* Zeatin equivalent

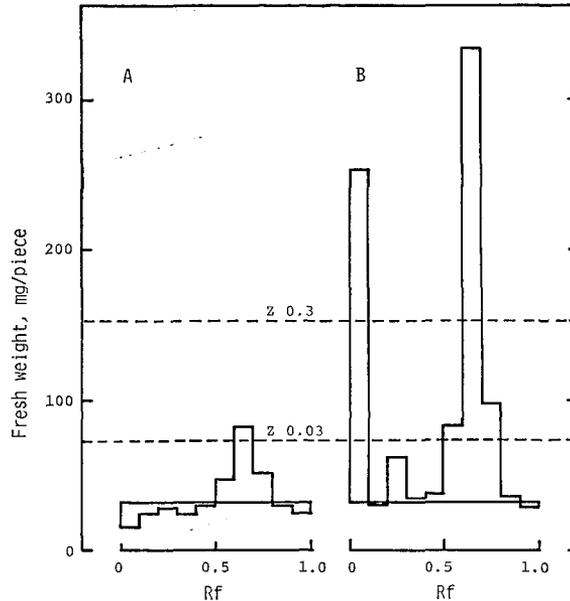


Fig. 6. Comparison of the cytokinin production under non-aerated culture (A) with that under aerated culture (B). Cytokinin fraction equivalent to 150 ml medium was chromatographed on papers with isopropanol: ammonia: water (10:1:1, v/v) and assayed using soybean callus. Broken lines represent callus yield obtained with 0.03 μg and 0.3 $\mu\text{g/l}$ zeatin.

days. A jar without aeration was used as a control. The amounts of zeatin riboside and its metabolite were extremely increased by the aeration (Fig. 6). Total amount of cytokinin produced by one root under non-aerated and aerated conditions was 27 μg and 1013 μg respectively. In this case, the root growth was also increased markedly by aeration, each fresh weight per root being 4.0 mg and 29.3 mg.

To obtain better indication of the relationship between oxygen availability and the cytokinin production, the amounts of cytokinin observed in these experiments were arranged in order of oxygen concentration which was measured by an oxygen electrode. Fig. 7 shows that both cytokinin production and root growth are strongly dependent on the oxygen concentration. However, at middle oxygen concentrations (8-18%), the dependency of cytokinin production was greater than that of the root growth.

Light

To examine effect of light, the cultures were maintained in growth cabinets under various light regimes. The illumination about 2,500 lux was provided by white fluorescent lamps. After 7-day culture, each medium was collected about three hours after lighting. As shown in Table 2, exposure to various light

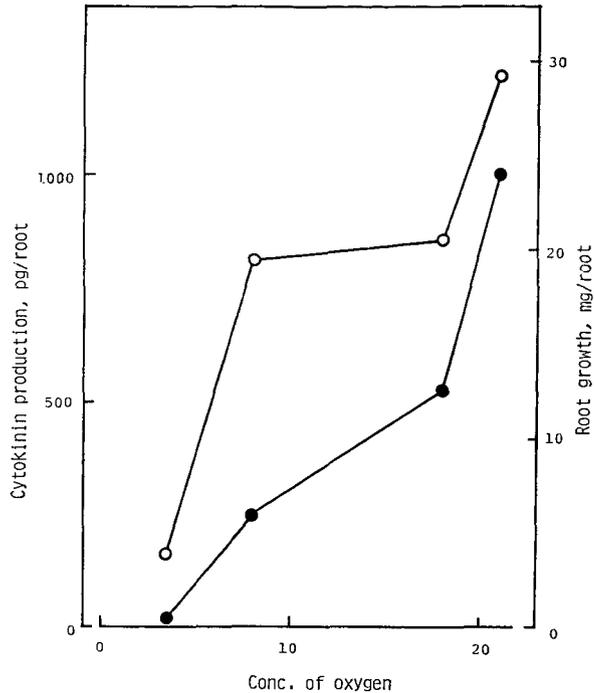


Fig. 7. Effect of oxygen concentration in the medium on the cytokinin production (●) and the root growth (○).

TABLE 2. Effects of various light regimes on cytokinin production by tomato roots and the root growth

Day length (hr)	Cytokinin production (pg*/root)			Root growth (mg fresh weight/root)
	Zeatin riboside	Metabolite of Zeatin riboside	Total	
0	293	30	323	21.3
6	390	160	550	31.1
12	250	160	410	35.0
18	253	187	440	32.2
24	240	83	323	35.8

* Zeatin equivalent

regimes resulted in increase in the amount of cytokinin. Short day cycle was most effective. Furthermore the amount of metabolite of zeatin riboside substantially increased under the photoperiod. The root growth was also stimulated by exposure to light.

Discussion

The data reported here indicate that various environmental factors induce

both qualitative and quantitative changes in cytokinins produced by cultured tomato roots. Concerning the effect of temperature, SKENE and KERRIDGE¹²⁾ found a qualitative differences in the cytokinins of xylem sap of grape vines grown at root temperatures of 20°C and 30°C, a slow-moving activity on a paper chromatogram which appears to be a metabolite of a cytokinin being absent from the 30°C samples. In the present study, the amount of metabolite of zeatin riboside decreased with increasing the culture temperature (Fig. 3). These results suggest that the high temperature inhibits metabolism of cytokinin.

The amount of cytokinin liberated from the roots influenced greatly by pH of the medium (Fig. 4). WHITTY and HALL¹⁷⁾ reported that the optimal pH for cytokinin oxidase activity is 5.8 and the activity actually disappears at pH 4.0. The highest cytokinin production at pH 4.0 and the lowest at pH 5.6 found in the present study suggest that there is inverse relationship between the amount of cytokinin produced by roots and the activity of cytokinin oxidase.

The amount of cytokinin produced by roots appears to be strongly dependent of oxygen availability (Fig. 7). In sunflower plants, reduced amount of cytokinin in xylem sap was found when the root system was subjected to water flooding²⁾. It is generally recognized that the growth of a plant is stimulated by cultivation of soil. An aerobic condition around the root system may stimulate the growth of the plant by means of stimulation of cytokinin production.

In plants of *Xanthium strumarium*¹⁶⁾ and *Perilla flutence*¹⁾, short day treatment induced both qualitative and quantitative changes in cytokinins of xylem sap. In the present study, exposure to various light regimes induced the both changes in cytokinin produced by tomato roots (Table 2). Root tips of plants are known to possess phytochrome^{13,14)}. These results indicate that the root tip itself has an ability for perception of light, and thereby changes cytokinin production and metabolism.

In cultured tobacco cells, cytokinin synthesis is known to closely correlate to the cell cycle⁹⁾, suggesting the cytokinin synthesis is a normal accompaniment of cell division. In order to see correlation between the amount of cytokinin produced by tomato roots and the rate of root growth, the data obtained here were

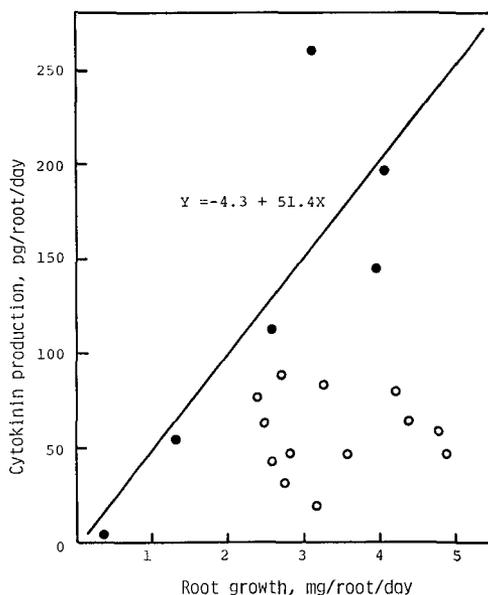


Fig. 8. Correlation between cytokinin production by tomato root and the root growth. As a whole, correlation coefficient is 0.25.

plotted in Fig. 8. As a whole, no correlation was found between them, correlation coefficient being 0.25. However, there are many cases where the root growth was high but the cytokinin production was poor (open circles). On the contrary, the opposite case could not be found. This may indicate that the amount of cytokinin produced by roots is primarily determined by the rate of cell division at their meristematic regions, and it is finally controlled by the activity of degradation enzyme. The regression line calculated without the open circles, $Y = -4.33 + 51.4X$, represents a maximum cytokinin producing ability of tomato root per unit growth, it being 51 pg/mg or 7.7 pg/mm.

Summary

Effects of various environmental conditions on cytokinin production by tomato roots (*Lycopersicon esculentum* Mill.) were examined using root tip culture *in vitro*. A major cytokinin produced by the roots was identified as *trans*-zeatin riboside by purification with HPLC and FD-mass spectrum. Culture temperature caused qualitative changes in the cytokinin. The amount of cytokinin produced by the roots was greatly affected by pH of the surrounding medium. Low and high pHs were promotive while neutral pH was inhibitory, suggesting the production is controlled by cytokinin oxidase. Increasing oxygen availability in the medium increased largely the amount of cytokinin and the root growth. This result suggests that both the cytokinin production and the root growth are fully dependent on the oxygen availability. Various light regimes induced a slight qualitative and quantitative change in cytokinin production. A maximum cytokinin producing ability of one tomato root per unit growth was estimated as 51 pg/mg or 7.7 pg/mm.

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