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An Improved Cytokinin Bioassay Using Cultured Soybean Hypocotyl Sections[1]

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ABSTRACT

This paper describes a modified soybean (*Glycine max*) tissue culture bioassay for cytokinins. Soybean hypocotyls were grown under sterile conditions and sliced into 1-mm sections. Sections were cultured for 5, 9, 13, or 22 days on a callus medium with zeatin or other cytokinins. The fresh weight of sections increased with the cytokinin concentration from 0.0005 to 1 μM zeatin; 2-fold concentration differences were readily distinguishable at 9 days. The assay should prove to have several advantages over the conventional soybean callus bioassay including convenience, lower variability between tissue samples, and improved resolution. Its specificity is comparable to that of the soybean callus bioassay.

A variety of bioassays for cytokinins have been developed (3) and the preferability of those involving a cell-division response has been explained by Miller (5) and Letham (4). However, cytokinin bioassays using callus have the disadvantage that callus cultures must be maintained to provide test tissue. The carrot root tissue culture assay eliminates the need for callus maintenance but the shallow slope of the dose-response curve makes the resolution of small cytokinin differences difficult (4).

In this paper a new tissue culture bioassay for cytokinins is described which has the advantages of the tissue culture assays described above and lacks some of their disadvantages. Seeds are easily stored and quickly grown to the size needed for the assay, and the low variability among tissue explants enables one to distinguish easily 2-fold concentration of differences even at relatively low cytokinin concentrations (about 1 nM zeatin). This improved resolution should make the assay especially useful in studies of zeatin metabolism and physiology. The assay is complete in 9 to 13 days as compared to 21 days for soybean or tobacco callus assays.

MATERIALS AND METHODS

Vermiculite was added to test tubes (25 × 150 mm) to a height of 40 mm. Eleven ml of 1/5× Hoagland's solution were pipetted into each tube. Tubes were covered with plastic caps, autoclaved for 20 min, and allowed to cool before addition of soybeans.

Soybeans (*Glycine max*, var. Kanrich or var. Kim in Table III only) were surface-sterilized in a 1.3% sodium hypochlorite solution for 4 min with occasional stirring, then rinsed 5 times with sterile distilled H_2O. One soybean was embedded in each tube, and the tubes were placed in a dark incubator at 30 C for about 7 days, when most hypocotyls had elongated to about 100 mm (giving about 100 tissue sections per seedling).

At the time soybean hypocotyl sections were to be cut, the tubes were inspected for signs of fungal or bacterial contamination. The few apparently contaminated seedlings were discarded. It was of use to sniff tubes of doubtful sterility, for even visually undetectable fungus produced a foul odor. Brown streaks on the hypocotyls were also considered signs of contamination.

A plastic mm ruler sterilized with 80% ethanol was placed within a sterile glass Petri dish (150 × 20 mm). A hypocotyl from one seedling was excised with a sterile razor blade above the primary root and just below the cotyledons. It was placed on the ruler in the Petri dish and 1-mm sections were cut from the entire hypocotyl with a sterile razor blade. Six such hypocotyls were sectioned, and one section from each was placed in small sterile plastic Petri dishes containing the media and the hormone to be tested. Duplicate dishes of six sections were used for the experiment shown in Figure 3 and Table II.

Hormone or other solutions to be tested were made up to the appropriate concentrations (10 or 4×, see below), filter-sterilized, and pipetted into sterile plastic Petri dishes. The soybean callus medium of Fosket and Torrey (1) was made up at 10/9 or 4/3 times the standard concentration, autoclaved, and dispensed with a sterile automatic pipetter into the dishes containing the hormone solution. In some experiments (Figs. 1, 2 and 4), 9 ml of the soybean callus medium were added to 1 ml of the hormone solutions in sterile plastic Petri dishes (60 × 15 mm). In other experiments (as in Fig. 3 and Table III), 3 ml of medium were added to 1 ml of hormone solution in smaller plastic dishes (35 × 10 mm). All hormone concentration given refer to final concentration. The soybean callus medium is always at the standard concentration (1). Plastic dishes were obtained from Falcon Plastics, Oxnard, Calif.

The plastic Petri dishes containing hypocotyl sections were placed in plastic trays, humidified with open dishes of H_2O, and covered with aluminum foil. Trays were incubated at 30 C in darkness unless otherwise indicated. At the end of the incubation period, the individual hypocotyl sections were weighed on an analytical balance sensitive to 0.1 mg.

Cell counts were obtained by macerating individual hypocotyl sections for a week in 1 ml of 5% chromic acid and counting five aliquots of each macerate in a hemocytometer or a Petroff-Hauser counting chamber.

RESULTS

Zeatin Dose-response and Time Course of Assay. Dose-response curves over the zeatin concentration range 0.0005 to 1 μM and 0.0005 to 0.05 μM obtained from soybean hypocotyl sections incubated for varying periods of time are illustrated in Figures 1 and 3. Already at 5 days, growth is evident, but the first usable dose-response curve occurs at about 9 days (Fig. 1). A threshold response is obtained at around 0.0005 μM and saturation has not been reached at 1 μM. The increase in fresh

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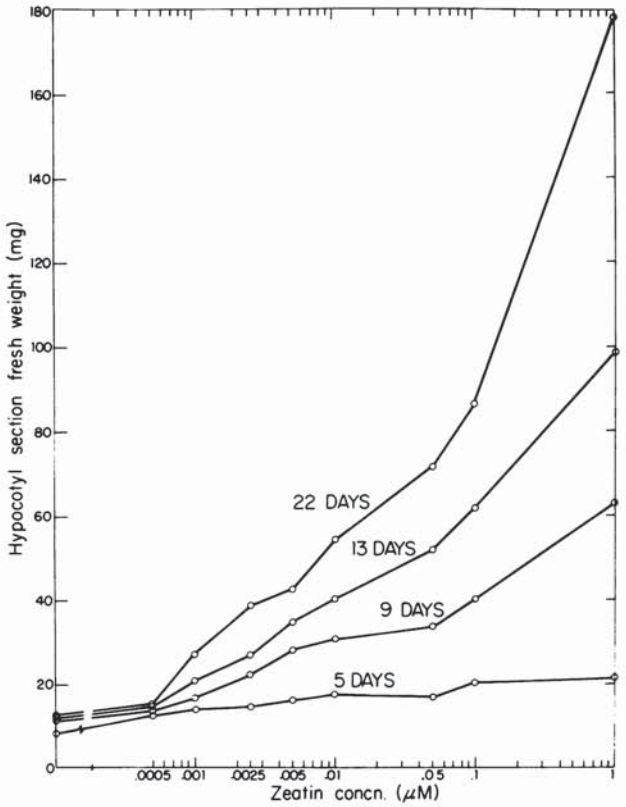

FIG. 1. Dose-response curve for soybean hypocotyl sections incubated on zeatin and soybean callus medium. Incubation times are as indicated. Each point represents the average fresh weight of six hypocotyl sections.

weight of zeatin-treated hypocotyl sections with time is shown in Figure 2. From 5 to 13 days, all four concentrations of zeatin cause an approximately linear increase with time in the fresh weight of sections. A decrease is evident in the rate of growth for all zeatin concentrations after 13 days, except for 1 μM zeatin on which sections continued growing at the same rate from 5 to 22 days.

Effect of Temperature and Light. The effect of temperature on the growth of hypocotyl sections was observed and it was found that 30 C was about optimal. Sections grown at 33 C on 0.005 μM zeatin did not significantly differ in weight from sections grown at 30 C at 9 days. Sections incubated at 25 C grew more slowly.

Under 100 ft-c of continuous cool-white fluorescent light sections grew faster than sections in darkness; however, hypocotyl sections without added zeatin grew markedly and weighed twice as much after 13 days as the control sections kept in darkness. Therefore, incubation in darkness was preferred because it gives a larger response to added hormone relative to control.

Statistical Optimization of Incubation Time of Assay. Table I presents Student's *t* values and *P* values for the differences in growth response between six sections incubated on low zeatin concentrations differing by only a factor of 2 or 2.5 for increasing periods of time. The weight differences at 9 days between the different hormone concentrations are all significant with $P = 0.025$ or lower. The sets of hormone differences shown have different incubation periods at which the lowest *P* value occurs; however, 9 to 13 days incubation is generally best for distinguishing small concentration differences. The resolution of such closely spaced hormone concentrations may become less clear (*i.e.* *P* value increases) if incubation is continued too long as is seen at 22 days for 2.5 *versus* 5 nM zeatin. In this case, the variability of the tissue section weights (standard errors) increased substantially from day 13 to 22.

Effects of Tissue Sample Size. The relatively low variability of this assay over the concentration range 0.5 to 50 nanomolar illustrated in Figure 3. The duplicates are similar and furthermore there is, in most cases, no difficulty in resolving 2-fold zeatin concentration differences using only six tissue pieces per treatment. This is illustrated in Table II where Student's *t* and *P* values are shown for a 2-fold and a 2.5-fold concentration difference. When all 12 tissue sections are used to calculate *t* values, the confidence level for the 2.5-fold concentration difference is improved from 0.025 to below 0.005. For the 2-fold concentration difference, it is improved from below 0.2 to below 0.05. This improvement in confidence level is due largely to the increased size of the *t* values which is directly due to the larger sample size. The use of 22 degrees of freedom instead of 10 makes a lesser contribution. Clearly it is of advantage to use 12 sections instead of 6 in doing bioassays in which small cytokinin differences are to be distinguished.

Hormone Specificity of Assay. The relative activity of some naturally occurring cytokinins, adenine derivatives, and other plant hormones are compared in Figure 4. The concentration of hormone or adenine derivative necessary to give a 14 mg fresh weight response was compared with the concentration of zeatin

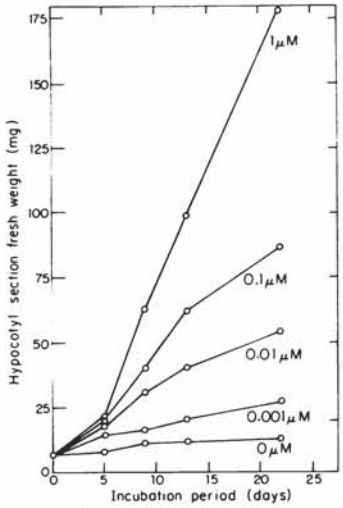

FIG. 2. Time course of growth of soybean hypocotyl sections incubated on different concentrations of zeatin. Each point represents the average of six hypocotyl sections.

Table I. *Effect of Incubation Time on Significance of Fresh Weight Differences between Treatments*

Six hypocotyl sections per treatment were incubated on different concentrations of zeatin for the indicated times and then weighed. The *P* values were found using Student's *t* test.

Incubation Time (days)	Zeatin Concentration (nM)					
	.5 vs 1		1 vs 2.5		2.5 vs 5	
	t	P	t	P	t	P
5	1.23	.25	0.27	.8	0.73	.5
9	2.55	.025	3.10	.01	2.88	.02
13	3.41	<.01	2.88	.02	3.96	<.005
22	3.95	<.005	2.92	<.02	1.17	<.3

necessary to give this response. Using this criterion of relative activity, zeatin is approximately 5 times more active than isopentenyl adenine; 10 times more active than isopentenyl adenosine; 30,000 times more active than adenine; and 300,000 times more active than adenosine. Adenosine monophosphate was inactive as were GA_3 and IAA over the concentration ranges tested (see Fig. 4).

Cell-division Response. Hypocotyl sections were incubated for 9 days on various zeatin concentrations, and their fresh weight and cell number were determined. The results presented in Table III show that zeatin at 0.0025, 0.025, and 0.25 μM strongly stimulates cell division in this assay in parallel with its stimulation of growth.

Calculation of the hormone-induced increase in fresh weight relative to the control divided by the increase in cell number

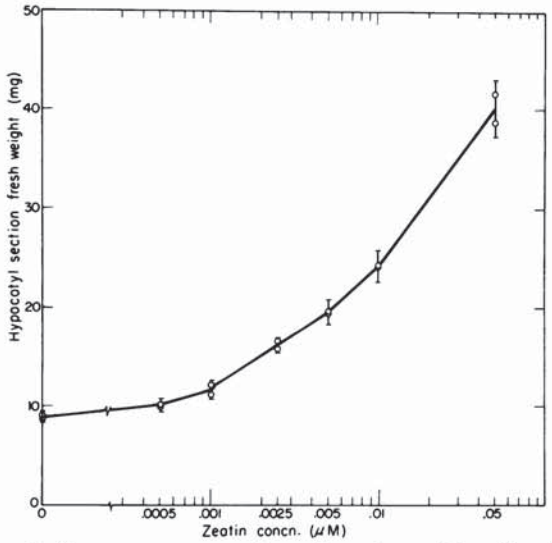

FIG. 3. Dose-response curve for soybean hypocotyl sections incubated for 9 days on zeatin. The curve is drawn through the average of the duplicate points. Each point represents the average of six hypocotyl sections. Vertical bars depict ± 1 SE of the mean of 12 sections.

Table II. *Effect of Sample Size on Significance of Fresh Weight Differences between Treatments*

Two dishes, each containing six hypocotyl sections, were used per treatment. Hypocotyl sections were incubated for 9 days. To calculate the Student's t value for the difference between treatments using six sections, the average fresh weight from each of two dishes at one zeatin concentration was compared with the average fresh weights of each of two dishes from the second concentration. The four t values were averaged, and a P value was read from a table. To calculate the t value for the differences between treatments using 12 sections, the average weight of the 12 sections from one treatment was compared with the average fresh weight of 12 sections from the second treatment.

Zeatin Concentration Differences (nM)	Number of Hypocotyl Sections			
	6 vs 6		12 vs 12	
	t	P	t	P
1 vs 2.5	2.54		3.6	<.005
	2.85	.025		
	2.42			
	2.17			
5 vs 10	1.68		2.2	<.05
	1.31			
	1.69	<.2		
	1.35			

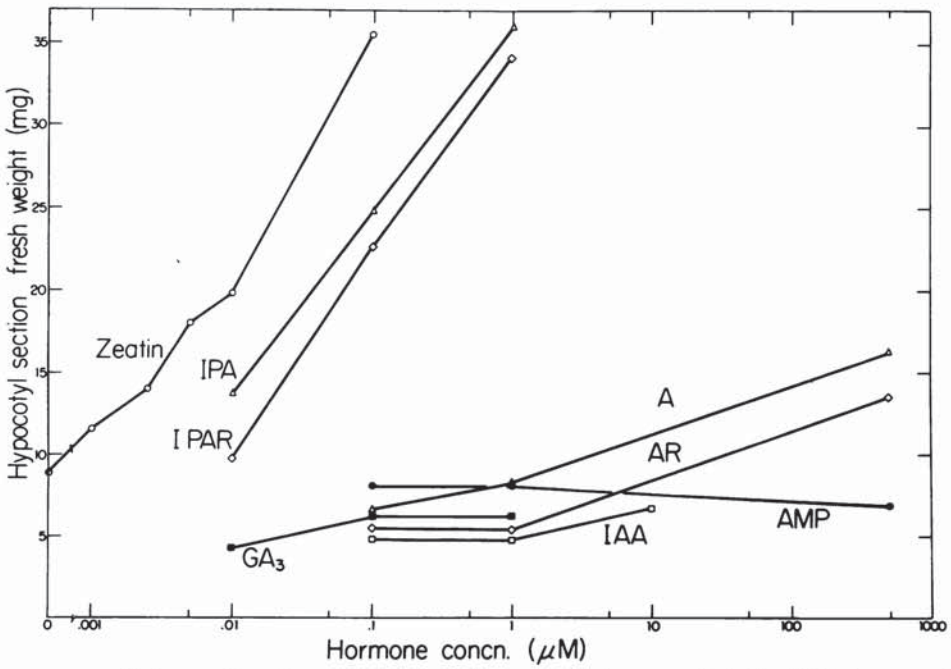

FIG. 4. Dose-response curves for hypocotyl sections incubated for 9 days on zeatin, isopentenyl adenine (IPA), isopentenyl adenosine (IPAR), gibberellic acid (GA_3), indole-3-acetic acid (IAA), adenine (A), adenosine (AR), and adenosine monophosphate (AMP). Points represent the average of six sections. The initial average weights of six sections were 4 to 6 mg.

Table III. *Effect of Zeatin on Growth and Cell Division in Soybean Hypocotyl Sections Cultured for 9 Days*

Values shown are for three individual sections and the average.

Zeatin Concentration (μM)	Hypocotyl Section Fresh Weight (mg)	Avg.	Hypocotyl Section Cell Number (cells x 10^{-4})	Avg.
0	10.1		53	
Control	11.7	11.4	37	45
	12.3		45	
	14.1		57	
0.0025	17.2	16.8	98	87
	18.2		107	
	23.6		167	
0.025	23.8	24.3	120	139
	25.5		129	
	31.0		298	
0.25	33.6	32.8	282	295
	33.8		306	

relative to the control gives a value of 0.012 μg/cell (range 0.009 to 0.014) over the hormone concentration range tested. This would correspond to an average cell volume of about 0.12 mm^3, equivalent to a cubic cell 23 μm on each side. Thus the growth of the soybean hypocotyl sections in this assay can be accounted for by the cytokinin-promoted production of new cells, although some expansion of pre-existing cells could also be involved.

DISCUSSION

The resolving power of the soybean hypocotyl bioassay described here is high. Two-fold zeatin concentration differences are easily distinguished at the lower range of the dose-response curve (0.5 to 5 nM). We expect that the resolution could be further improved by increasing the tissue sample size from two dishes to three or more dishes (Table II). In contrast, a comparison of the previously reported 5% confidence limits with reported values on the dose-response curves obtained with the carrot-root tissue culture assay suggests that 10-fold hormone concentration differences or greater might be necessary in order to be resolvable (4). Our experience shows that the soybean callus assay (5) often is quite variable, making the resolution of 2-fold concentration differences difficult in the concentration range 1 to 10 nM.

Sections cut from hypocotyls are much more uniform in volume than pieces of callus, and this can explain why duplicate averages of six sections are similar (Fig. 3). The standard error of the mean for 12 sections is usually about 7% as compared to 10% for 24 pieces of tobacco callus tissue as measured by Helgeson and Upper (2).

Although the soybean callus assay is reported to be able to detect zeatin at a concentration of 0.05 nM, our own results and those of our colleagues indicate that a workable lower limit for detection is about 0.5 nM. This is the same as the lower limit of the dose-response curves presented here and makes the assay presented here one of the more sensitive of the cytokinin bioassays.

The soybean hypocotyl section assay for cytokinins is as specific as the soybean callus assay (6). It gives no growth response to added GA_3 or IAA, or to adenosine monophosphate. Adenine and adenosine do cause some growth at 500 μM, and it is interesting to note that Miller (6) found 400 μM to be optimal concentration for adenine-promoted growth in soybean callus.

It is interesting that the addition of the ribose to isopentenyl adenine lowers the activity of this compound. The addition of the ribose group also lowers the cytokinin activity of adenine. Adenosine monophosphate shows no activity whatsoever; this may be ascribable to low uptake due to the phosphate group.

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