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An improvement of cucumber cotyledon greening bioassay for cytokinins

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Abstract

The cucumber cotyledon greening bioassay for cytokinins was modified by using 95 % acetone-ethanol instead of 80 % acetone as extraction solvent. The cotyledons were extracted directly with a 2:1 (v/v) acetone-ethanol solution in dark for 24 hours, omitting the homogenization and centrifugation operations of the previous bioassay. The modified bioassay is more convenient and especially useful in screening cytokinin-active substance from a large number of samples.

Introduction

The cucumber cotyledon greening bioassay is frequently used for detecting cytokinins (Fletcher and McCullagh 1971, Klepper and Mcquirk 1995, Ricci *et al*. 2001, Wei-lun Chen *et al*. 1979). Fletcher *et al*. (1980) made some beneficial modifications of the bioassay by using 5-day-old cucumber cotyledon treated with combination of 40 mM KCl and various concentrations of cytokinins. As homogenization and centrifugation operations are still needed in the modified bioassay, it is not convenient enough to screen cytokinin-active substance from a large number of samples. Acetone-ethanol solution was recently reported to be a better extraction solvent of chlorophyll from vegetable leaves than aqueous acetone (Min-wen Yang 2002). It has the peculiarity of quick extraction speed, high extraction efficiency, good chlorophyll stability and determination accuracy. The objective of this study is to present an improved chlorophyll extraction method by using 95 % acetone-ethanol instead of 80 % acetone as extraction solvent and modify the operation of the previous bioassay. As most of the cytokinin-active compounds so far found fall into two main structure groups: purine and urea derivatives, two structurally typical cytokinins, 6-benzyladenine (BA) and N,N'-diphenylurea (DP), were selected as representatives of cytokinins.

Materials and methods

Cucumber seeds (Luming yan 4#) were planted in vermiculite and germinated in dark at 28 °C. The cotyledons from 5-day-old plants were exercised in dim green light, weighed and uniformly floated in 7-cm Petri dishes containing 5 mL of test solution containing 10^{-4} to 10^{-8} M of BA or 10^{-5} to 10^{-8} M DP and 40 mM KCl. BA or DP was dissolved in

minute quantity of DMSO and diluted with distilled water to given concentration. The DMSO content in the test solution was controlled to below 1 % (v/v), which was demonstrated having little or no effect on chlorophyll synthesis in the bioassay. Cotyledons placed in a solution of 5 mL of 40 mM KCl containing less than 1 % (v/v) DMSO was used as control. Each plate of sample and control were placed 10 pieces of cotyledons with the adaxial face down. Their weight was on average 0.1700±0.0050 mg. All the dishes were returned to the dark at 28 °C for an incubation of 24 hours and then exposed to fluorescent light with an intensity of 11 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 3 hours at 28 °C. The dishes of each sample and control were randomly divided into two groups. The cotyledons of the first group were homogenized, extracted with 10 mL 80 % (v/v) aqueous acetone, and centrifugated to get supernatant liquor with Fletcher method (Fletcher *et al*. 1980). The cotyledons of the second group was extracted directly with 10 mL 95 % acetone--ethanol 2:1 (v/v) solution in dark for 24 hours (new method). UV spectra of the extraction solutions of control within 600-700 nm were taken on a Shimadzu UV-2100 spectrometer. The total chlorophyll concentration was measured according to the method of Arnon (1949). The absorbance of the extraction solutions were measured using UV755B spectrophotometer at 663 and 645nm.

Results and discussion

In a preliminary experiment, cotyledons incubated in 40 mM KCl and minor DMSO (control) were treated with the Fletcher method and the new method. The absorbance spectra of the extraction solutions in the range of 600-700nm were similar (Figure), indicating that the total chlorophyll concentration of the extractions prepared with the new methods can also be determined according to Arnon formula. The total chlorophyll content (D) of cotyledons of each sample or control was therefore calculated according to the following formula:

$$D = \frac{Cx10}{Wx1000} (mg / g)$$

C - Total chlorophyll concentration of the cotyledon extraction, (mg/L)
W - Fresh weight of the cotyledons, (g)

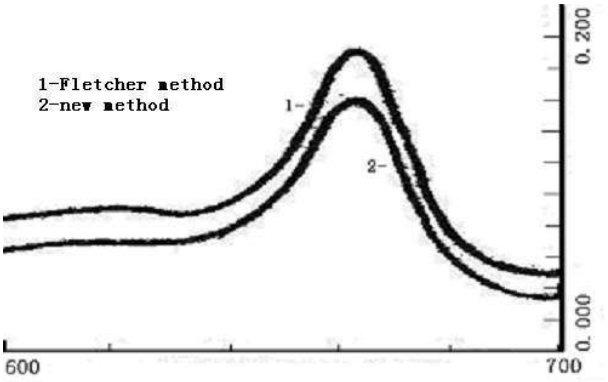

Figure. Absorbance spectra of the control cotyledon extractions obtained with the Fletcher method and the new method, Values are the means of three replicates.

In Fletcher method, chlorophyll content or absorbance at 663 nm was used to show the cytokinin activity of the corresponding sample. In order to show cytokinin activity of the sample more clearly and quantitatively, we take the relative chlorophyll content of sample to control (D/D_0) to express its cytokinin activity. Three replicates of each sample or control were treated with the Fletcher method and the new method respectively. The results were statistically treated and summarized in Table.

$$\frac{D}{D_0} = \frac{\overline{D}}{\overline{D_0}} \pm \sqrt{\frac{\sum_{i=1}^{n} \left(\frac{D_i}{D_0} - \frac{\overline{D}}{\overline{D_0}}\right)^2}{n(n-1)}}$$

D - total chlorophyll content of sample
D_0 - total chlorophyll content of control
$\overline{D}$ - means of total chlorophyll contents of sample determined in parallel experiments
$\overline{D_0}$ - means of total chlorophyll contents of control determined in parallel experiments
D_i - total chlorophyll content of sample or control determined in parallel experiment No.i
n - total number of the parallel experiments for each sample or control

The data in Table showed that the new method, besides its convenience of omitting homogenization and centrifugation operations, is more accurate and

Table. Relative chlorophyll content of cotyledons treated with various concentrations of BA or DP

Concentration (mol/L)	D/D_0			
	BA - Fletcher method	BA - New method	DP - Fletcher method	DP - New method
10^{-4}	1.71±0.21	1.96±0.14	-	-
10^{-5}	1.84±0.19	2.40±0.08	1.09±0.12	1.42±0.13
10^{-6}	1.73±0.21	2.29±0.10	1.31±0.02	1.06±0.07
10^{-7}	1.59±0.19	1.41±0.06	0.99±0.07	1.07±0.05
10^{-8}	1.39±0.11	1.12±0.08	0.80±0.16	0.98±0.01
Control	1.00±0.11	1.00±0.09	1.00±0.11	1.00±0.09

reliable than the Fletcher method, especially for the samples with weak cytokinin activity. For instance, at the concentration of 10^{-8} mol/L of DP, the Fletcher method gave a relative chlorophyll content value (0.80) unreasonably much lower than the control while the new method gave a value (0.98) within the admissible determination error. We are therefore presenting an improved cytokinin bioassay that is especially useful in screening cytokinin-active substance from a large number of samples, where an identical dispose time is important to guarantee accurate evaluation.

References

Arnon D.I. 1949. Copper enzymes in isolated chloroplasts phenoloxidases in beta vulgaris. Plant Physiol., 24: 1-15.

Fletcher R.A., McCullagh D. 1971. Cytokinin-induced chlorophyll formation in cucumber cotyledons. Planta, 101: 88-90.

Fletcher R.A., Kalldumbil V., Steele P. 1980. An improved bioassay for cytokinins using cucumber cotyledons. Plant Physiol., 69: 675-677.

Klepper R.R., Mcquirk P.A.1995. Investigation of cytokinin activity in a commercial plant growth regulator by means of a cucumber cotyledon chlorophyll production bioassay. Proc.Plant Growth Regul. Soc. Am., 22: 264-267.

Min-wen Yang 2002. Study on rapid determination of chlorophyll content of leaves. Chinese Journal of Spectroscopy Laboratory 19: 478-481.

Ricci A., Carra A., Torelli A., Maggiali C.A., Vicini P, Zani F., Branca C. 2001. Cytokinin-like activity of N -substituted N-phenylureas. Plant Growth Regulation, 34: 167-172.

Wei-lun Chen, Yan-ru Song, Cheng Tsui 1979, Comparative study on the bioassay between N6, $O^{2'}$ -dibutyryl cyclic adenosine 3,5-monophosphate and cytokinin. Acta Botanica Sinic, 21: 263-268.

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