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 µmol·m⁻²·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 centrifuged 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 645 nm.

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:

\[
D = \frac{C \times 10}{W \times 1000} \text{ (mg/g)}
\]

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

\[
\bar{D} = \frac{D}{n} \pm \sqrt{\frac{\sum (D_i - \bar{D})^2}{n(n-1)}}
\]

\[
\frac{D}{D_0} = \frac{\bar{D}}{D_0} \pm \sqrt{\frac{\sum (D_i - \bar{D})^2}{n(n-1)}}
\]

D - total chlorophyll content of sample
D₀ - total chlorophyll content of control
\bar{D} - means of total chlorophyll contents of sample determined in parallel experiments
D₀ - means of total chlorophyll contents of control determined in parallel experiments
Dᵢ - 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
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**


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

<table>
<thead>
<tr>
<th>Concentration (mol/L)</th>
<th>D/D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA - Fletcher method</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>1.71±0.21</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>1.84±0.19</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>1.73±0.21</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>1.59±0.19</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>1.39±0.11</td>
</tr>
<tr>
<td>Control</td>
<td>1.00±0.11</td>
</tr>
</tbody>
</table>

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