An Improved Bioassay for Cytokinins Using Cucumber Cotyledons

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ABSTRACT

The cucumber cotyledon greening bioassay is frequently used for detecting cytokinins. Beneficial modifications of the original technique included using 5-day-old cucumber (Cucumis sativus L., cv. National Pickling) cotyledons treated with combinations of 40 millimolar KCl and various concentrations of cytokinins. A dark incubation period of 20 hours was followed by an exposure to light for 3.5 hours. Under these conditions, extremely low (0.0001 milligram per liter) concentrations of N6-benzyladenine, zeatin, kinetin, or zeatin riboside can be detected. Of the four cytokinins tested, kinetin appeared to be the least active. With these improvements, the assay is 10 times more sensitive than is the previously described cucumber cotyledon greening bioassay for cytokinins.

MATERIALS AND METHODS

Cucumber (Cucumis sativus L., cv. National Pickling) seeds were planted in vermiculite, in peat flats (20 x 15 x 7 cm) and germinated in the dark at 28°C. The cotyledons from 4- to 7-d-old plants were excised in dim green light, making certain that the hypocotyl hook was removed. The cotyledons were placed in 5-cm Petri dishes containing 3 ml of test solution which consisted of distilled H2O, cytokinins (various concentrations), 40 mM KCl, 10 mM CaCl2, or a combination of the cytokinin with either KCl or CaCl2. After preliminary studies, CaCl2 was eliminated. The dishes were returned to the dark at 28°C for 12, 16, 20, 24, and 28 h. After various incubation periods, they were exposed to fluorescent light with an intensity of 12.9 w/m2. After 3.5 h, the cotyledons were homogenized, and the Chl was extracted in 8 ml 80% acetone. The volume was brought up to 10 ml with acetone and then centrifuged at 2,500g for 10 min. The A of the supernatant were read at 663 and 645 nm. Chl content was expressed as A at 663 nm, or the total Chl levels were determined with a nomogram (12).

Table I. Effect of BA, KCl, or CaCl2 Alone and in Combinations on the Fresh Weight and Total Chl Level of Cucumber Cotyledons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh Weight</th>
<th>Total Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>mg/L</td>
</tr>
<tr>
<td>H2O</td>
<td>0.40</td>
<td>0.77</td>
</tr>
<tr>
<td>BA, 1 mg/L</td>
<td>0.43</td>
<td>1.66</td>
</tr>
<tr>
<td>KCl, 40 mM</td>
<td>0.41</td>
<td>3.29</td>
</tr>
<tr>
<td>CaCl2, 10 mM</td>
<td>0.38</td>
<td>0.95</td>
</tr>
<tr>
<td>BA, 1 mg/L + KCl, 40 mM</td>
<td>0.62</td>
<td>5.31</td>
</tr>
<tr>
<td>BA, 1 mg/L + CaCl2, 10 mM</td>
<td>0.44</td>
<td>1.12</td>
</tr>
<tr>
<td>KCl, 40 mM + CaCl2, 10 mM</td>
<td>0.41</td>
<td>2.82</td>
</tr>
<tr>
<td>BA, 1 mg/L + KCl, 40 mM + CaCl2, 10 mM</td>
<td>0.61</td>
<td>5.23</td>
</tr>
</tbody>
</table>

Table II. Total Chl Levels of 4- to 7-Day-Old Cucumber Cotyledons

The cotyledons were incubated in solutions for 20 h at 28°C in the dark and then exposed to light for 3.5 h. Values are the means of four replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4 Days</th>
<th>5 Days</th>
<th>6 Days</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/L Chl content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>0.6 g</td>
<td>1.0 fg</td>
<td>0.6 g</td>
<td>1.3 fg</td>
</tr>
<tr>
<td>BA, 1 mg/L</td>
<td>1.4 efg</td>
<td>2.4 cd</td>
<td>1.3 efg</td>
<td>1.7 def</td>
</tr>
<tr>
<td>KCl, 40 mM</td>
<td>0.8 g</td>
<td>1.9 de</td>
<td>1.4 efg</td>
<td>1.9 de</td>
</tr>
<tr>
<td>BA, 1 mg/L + KCl, 40 mM</td>
<td>2.8 bc</td>
<td>4.6 a</td>
<td>3.2 bc</td>
<td>3.6 b</td>
</tr>
</tbody>
</table>

* Means within rows and columns with similar letters are not significantly different at the 5% level of Duncan's multiple range test.

Previous studies have established that cytokinins accelerate chloroplast differentiation (11) as well as regulate and stimulate Chl production in etiolated cucumber cotyledons (7, 9). The increase in Chl production was found to be proportional to the concentration of cytokinin, and this response provides a sensitive, yet rapid, bioassay for cytokinins (7).

The sensitivity and specificity of several cytokinin bioassays, including those based on leaf senescence and callus formation, have been reviewed by Letham (13). In a comparative study, Dumbroff and Brown (5) found that the cucumber cotyledon greening bioassay (7) was convenient and sensitive, and it provided a more linear response than did either the oat-leaf yellowing test (16) or the soybean callus test (14). Since then, the cucumber cotyledon assay has been used for studying the effects of herbicides, such as S-ethyl dipropylthiocarbamate, which stimulates (17), and fluoridone, which inhibits, Chl production (4).

Brenner et al. (1) found that the cucumber greening assay (7) provided a precise method for cytokinin quantification and identification. This bioassay, in conjunction with chemical assays, is used routinely for the confirmation of biological activity (2).

The amount of Chl produced by etiolated cucumber cotyledons after exposure to light is dependent on the age of the cotyledons (8) as well as on the dark incubation period (3). Green and Muir (10) found that the combination of KCl and CaCl2 in both the presence and the absence of kinetin, also increased the Chl content of cucumber cotyledons. They suggested that the relationship between potassium and the response to cytokinin provided for a superior bioassay.

The objective of this study is to present a superior assay for cytokinins through modification of our previous bioassay (7), taking into consideration such factors as age, incubation periods, and the addition of KCl and CaCl2 to the cucumber cotyledons.

1 Supported by a grant from the Natural Science and Engineering Research Council of Canada.
RESULTS AND DISCUSSION

In a preliminary experiment (Table I), it was found that the incubation of 7-d-old etiolated cotyledons for 24 h with N\textsuperscript{6}-BA, KCl or CaCl\textsubscript{2} alone had no effect on their fresh weights. However, a combination of BA + KCl increased the fresh weights, while CaCl\textsubscript{2} in combination did not alter this effect. BA or KCl alone stimulated Chl production, and an additive effect was achieved when they were combined. CaCl\textsubscript{2} with BA and KCl alone or in combination, did not alter their stimulatory effect on the cotyledons. The ability of KCl to enhance the stimulatory effects of BA on fresh weight and Chl production in cucumber cotyledons partially supports findings of Green and Muir (10). However, in our experiments, the addition of 10 mM CaCl\textsubscript{2} to the BA + KCl combination did not appear to have any beneficial effect on the cotyledons' response. In a study with CaCl\textsubscript{2}, Tanaki and Tsuji (15) demonstrated that the addition of 10 mM CaCl\textsubscript{2} did not significantly affect Chl content in cucumber cotyledons; in fact, higher concentrations were found to be inhibitory. For these reasons, the use of CaCl\textsubscript{2} was discontinued.

The cucumber greening assay for cytokinins is being used in several laboratories. The cultivar of cucumber does not seem to be an important factor. In our experience, the temperature at which the seeds are germinated, time of exposure to light, and the age of the plants (7) are important factors in detecting cytokinin concentrations below 0.01 mg/L.

An optimal age of plants for a maximum response to cytokinin was determined. In this study, 4- to 7-d-old cotyledons were used. The greatest effect of the BA + KCl treatment was found to be on 5-d-old cotyledons (Table II). In this treatment, the Chl production was 460\% higher than it was in the H\textsubscript{2}O control.

In a study of 6-d-old cotyledons, Dei (3) found that, the longer the dark incubation period, the lower the amount of Chl formed. Using 5-d-old cotyledons, we have tested incubation periods from 12 to 28 h to determine the optimal response to cytokinin. The best response was obtained after 20 to 24 h of dark incubation (Table III). Treatment of the cotyledons with BA or KCl and exposure to light for 3.5 h stimulated Chl production, and, with the BA + KCl combination, there was 467 to 550\% as much Chl as there was in the H\textsubscript{2}O control. The longer incubation period of 28 h did not promote as great a response, possibly because of the partially anaerobic conditions during incubation. The effect of anaerobiosis has been discussed in detail by Dei (3).

For purposes of a bioassay, it was decided to use 5-d-old cotyledons, an incubation period of 20 h, a combination of various concentrations of cytokinins with 40 mM KCl, along with light exposure time of 3.5 h. Under these conditions, the control cotyledons were still in the lag phase of growth and had a very low Chl A. The lowest concentration (0.0001 mg/L) of cytokinins + 40 mM KCl had detectably higher levels of Chl than did the control containing KCl. The increase in Chl was found to be proportional to the log of the concentration (Fig. 1). Of the four cytokinins tested, kinetin was found to be the least active.

The role of potassium in enhancing the response to cytokinin is not clear. Speculations as to its possible regulatory role as an osmoticum, an enzyme cofactor, or a nonspecific promoter of metabolism are discussed by Green and Muir (10) and Elliot (6).

The method described by Fletcher and McCullagh (7) could generally measure a cytokinin concentration of 0.001 mg/L. With minor modifications in this assay, it is now possible consistently to detect concentrations as low as 0.0001 mg/L of cytokinins. Sometimes, it is possible to detect responses to concentrations as low as 0.00001 mg/L. The new modified assay is relatively more sensitive than are several of the assays for cytokinins described previously. We are now presenting an improved, rapid, simple, and highly sensitive bioassay for cytokinins.

LITERATURE CITED

on Plant Growth Substances, Lausanne, pp 98–100