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# An Improved Disk Bioassay for Determining Activities of Plant Growth Regulators

Z. R. Zhao, Z. L. Wu, G. Q. Huang, and G. R. Li

Research Institute of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, China

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**Abstract.** An improved filter paper disk bioassay for determining activities of plant growth regulators was developed and evaluated. Indole-3-acetic acid (IAA), (±)-abscisic acid (ABA), and 6-furfurylamino-purine (kinetin) were dissolved in 95% ethanol at fixed dilutions. Specific concentrations of each growth regulator were then evenly dropped onto individual 6-cm paper disks and the solvent evaporated. The activities of the above three water-insoluble plant growth regulators in both solution and the disk assay were compared using the excised cucumber (*Cucumis sativus* L.) cotyledon root formation bioassay (IAA), wheat (*Triticum aestivum* L.) coleoptile straight growth bioassay (ABA), and cucumber cotyledon expansion bioassay (kinetin), and similar results were obtained at each concentration. The possible principle of this method has been studied using the cucumber cotyledon expansion bioassay. The results suggested that the disks, as carriers, had highly dispersed kinetin molecules on them and greatly accelerated the dissolution and diffusion of kinetin from disks to water.

The filter paper disk bioassay was originally devised to test microbial susceptibility to antibiotics (Barry et al. 1970, Bauer et al. 1966). Meudt and Bennett (1978) developed a disk bioassay for indole-3-acetic acid (IAA) and gibberellin ($GA_3$) using isolated first internode sections of etiolated bean plants (*Phaseolus vulgaris* L.), but did not calculate the actual concentrations of the chemicals. Anderson et al. (1987) also developed a disk bioassay for screening several plant growth regulators and suggested that it was rapid and qualitative. However, the actual concentrations of the growth regulators tested could not be determined by the method.

We often encounter water-insoluble compounds when screening synthetic and natural plant growth regulators. These sample are often formulated with emulsifiers. However, solvents and emulsifiers themselves may have definite promotion or inhibition properties, giving false results (Rao et al. 1976, Xu and Gu 1987). Furthermore, formulations are always complex and time-consuming. To simplify testing, we have improved the paper disk bioassay (Zhao et al. 1988) and used it to examine synthetic and natural plant growth regulators (Li et al. 1984, Zhao 1990, Zhao and Huang 1990, Zhao et al. 1991). In using it, we have detected a compound (chrysanthemic acid) which is water-insoluble and which significantly promotes rooting in excised cucumber (*Cucumis sativus* L.) cotyledons, mung bean (*Phaseolus radiatus* L.), and kidney bean hypocotyls (Zhao et al. 1991). This improved filter paper disk method has the following advantages over the previously described filter paper disk method (Anderson et al. 1987, Meudt and Bennett 1978). In our method, the actual concentration of each sample tested can be exactly calculated from an equation that we describe. Additionally, the method may be applied to assorted test samples, solvents, and excised plant materials.

The object of this study was to evaluate the improved filter paper disk bioassay for determining activities of plant growth regulators and to describe the possible principle of this improved method.

## Materials and Methods

### *Chemicals*

All reagents were analytical grade obtained from commercial suppliers.

### *Excised Cucumber Cotyledon Root Formation Bioassay Response to IAA*

The cucumber cotyledon root formation bioassay was carried out as previously described (Zhao et al. 1988, Zhao and Huang 1990). Cucumber (*Cucumis sativus* L. cv. Jinyan No. 4) seed was

sown on 0.7% agar and grown in the dark at 26°C. Cotyledons were excised from 3-day-old seedlings.

In the improved disk method, IAA was dissolved in 95% ethanol to give the following concentrations: 3, 10, 30, 100, 300, and 1000 ppm. The above solutions (0.3 ml) were evenly dropped onto individual 6-cm Xinhua qualitative filter paper disks and the solvent evaporated. To each treated disk, 3 ml of distilled water, as the incubation medium, were added to the bottom of each 6-cm petri dish; the IAA concentrations of these treatments were considered to be equal to 0.3, 1, 3, 10, 30, and 100 ppm. Disks treated only with 95% ethanol (evaporated) were used as controls. Ten cotyledons were placed on each disk.

In the solution method, IAA was made into the Na salt by dissolving 3 mg IAA in 0.3 ml of 0.1 N NaOH, then diluting with distilled water as required. Three milliliters of the above solutions were pipetted onto a 6-cm Xinhua filter paper disk and 10 cotyledons were placed in a 6-cm dish. Distilled water was used as control.

The cotyledons were incubated in a darkroom (26°C) for 5 days. The number of roots formed at the bases of the 10 cotyledons was then counted. Each treatment was replicated three times and the experiment was duplicated.

### *Wheat Coleoptile Straight Growth Bioassay Response to ABA*

The wheat coleoptile straight growth bioassay was performed as previously described (Nitsch and Nitsch 1956, Zhao et al. 1988). Wheat (*Triticum aestivum* L. cv. Fengkang No. 8) seeds were sown on 0.7% agar and grown in the dark at 26 ± 1°C. After 3 days, coleoptiles of the germinating seeds were 2–3 cm long. The apical 2–3 mm of each coleoptile was removed. From each coleoptile stump the uppermost 5-mm section was cut off. Sections of coleoptiles were floated in distilled water for 2 h.

In the improved disk method, ABA was dissolved in 95% ethanol to give 0.01, 0.1, 1, 10, and 100 ppm. Then, 0.05 ml of the above solutions were evenly dropped onto individual 2.2-cm Xinhua qualitative filter paper disks and the solvent evaporated. To each treated disk, 0.5 ml of 0.01 M phosphate-citrate buffer containing 2% sucrose (pH 5.0) was added in the bottom of a 2.2-cm dish. The ABA concentrations of these treatments were considered to be equal to 0.001, 0.01, 0.1, 1, and 10 ppm. Disks treated with 95% ethanol (evaporated) were used as controls.

In the solution method, ABA solution was made by dissolving 5 mg ABA in 2.5 ml 95% ethanol, then diluting with the above buffer. Then, 0.5 ml of each solution and a Xinhua filter paper disk were placed in each 2.2-cm dish. The control was buffer only.

Ten coleoptile segments were placed in each 2.2-cm dish. The tests were conducted at 546 nm. The coleoptile segments were then incubated in the dark (26 ± 1°C). After 20 h, the length of 10 coleoptile segments was measured. Each treatment was replicated three times and each experiment was duplicated.

### *Cucumber Cotyledon Expansion Bioassay Response to Kinetin*

The cucumber cotyledon expansion bioassay was done as previously described (Chen et al. 1979, Zhao et al. 1988).

In the improved disk method, the preparation of 6-furfurylamino-purine (kinetin) and the preparation of the bioassay were performed as described for the excised cucumber cotyledon root formation bioassay (vide supra).

In the solution method, kinetin solutions were made by dissolving 3 mg kinetin in 0.7 ml of 0.1 N HCl and then diluting with distilled water as required. Using a 6-cm Xinhua paper disk, 3 ml of the above kinetin solution and 10 cotyledons were placed in each 6-cm petri dish. Distilled water was used as the control.

The cotyledons were incubated in the dark (26 ± 1°C). Three days later, the fresh weight of 10 cotyledons was recorded. Each treatment was replicated four times and the experiments were triplicated.

### *Response of Cucumber Cotyledon to Kinetin Extracts from Disks*

The preparation of the bioassay and of kinetin were also made as described in the section for the excised cucumber cotyledon root formation bioassay (vide supra).

In the first experiment, the paper disks were treated with 0.3 ml of different concentrations of kinetin and were divided into three groups: to each treated paper disk, 3 ml distilled water were added to the bottom of a 6-cm petri dish for 0, 24, and 48 h before treatment. The actual kinetin concentrations of these treatments were considered to be equal to 0.3, 1, 3, 10, 30, and 100 ppm.

In the second experiment, the disks were divided into two groups: the first group consisted of three treatments. To each paper disk treated with 0.3 ml of 10, 30, and 100 ppm kinetin, respectively, 3 ml distilled water were added to the bottom of each 6-cm petri dish for 24 h before treatment; the second group also consisted of three treatments. To each disk treated with 0.3 ml of 100 ppm kinetin, 3, 10, and 30 ml distilled water were added, respectively, in each 6-cm petri dish for 24 h before treatment. Three milliliters of extracts from above treated disks were pipetted onto a 6-cm Xinhua paper disk without kinetin.

Ten cotyledons were placed in each dish. Disks with only 95% ethanol were used as controls. The cotyledons were incubated in the dark (26 ± 1°C). Three days later, the fresh weight of 10 cotyledons was taken. Each treatment was replicated three times and each experiment was duplicated.

## Results and Discussion

### *Effect of IAA on the Rooting of Excised Cucumber Cotyledons*

IAA at 0.3–100 ppm significantly promoted adventitious root formation of excised cucumber cotyledons. The optimal concentration for IAA was 30 ppm. The activities of IAA in both improved filter paper disk and solution methods were approximate at each concentration (Fig. 1).

### *Effect of ABA on the Elongation of Wheat Coleoptile Segment*

ABA at 0.001–10 ppm obviously inhibited the elongation of wheat coleoptile segments. The activities of each concentration of ABA in both the improved

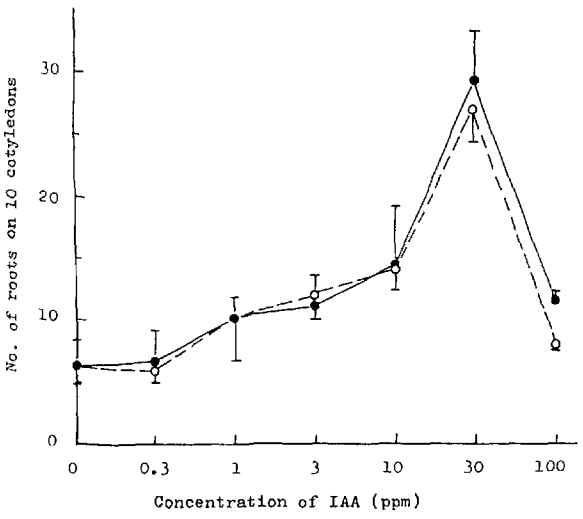


**Fig. 1.** A comparison of the activities of different concentrations of IAA in both paper disk (○—○) and solution (●—●) methods using the excised cucumber cotyledon root formation test. Vertical bars are ± SD.

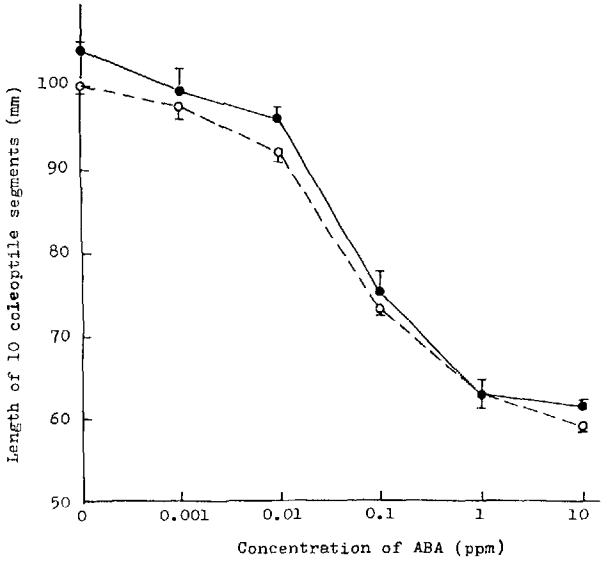


**Fig. 2.** A comparison of the activities of different concentrations of ABA in both paper disk (○—○) and solution (●—●) methods using the wheat coleoptile straight growth test. Vertical bars are ± SD.

filter paper disk and solution methods were also approximate (Fig. 2).

### *Effect of Kinetin on the Expansion of Excised Cucumber Cotyledon*

Kinetin at 0.3–100 ppm clearly stimulated the expansion of cucumber cotyledons. The activities of each concentration of kinetin in the improved disk method were very similar to the level observed in the solution method. The fresh weight of cotyledons was linearly proportional to the concentrations of kinetin at 0.3–10 ppm (Fig. 3).

As shown in Figs. 1–3, the activities of three water-insoluble plant growth regulators (IAA, ABA, and kinetin) in both the improved paper disk and solution methods were very similar.

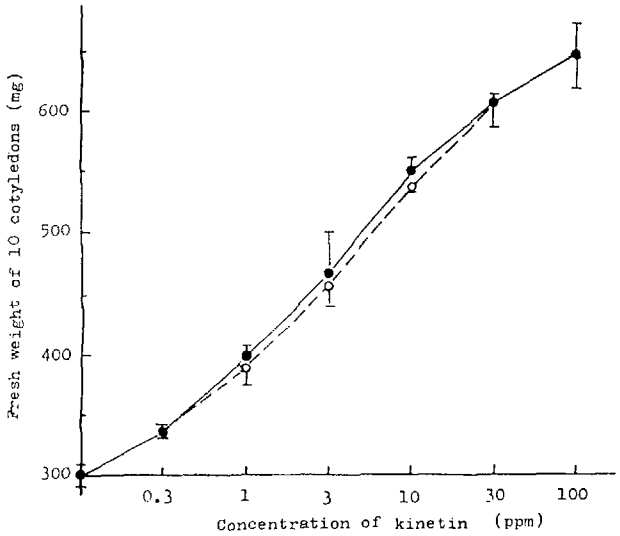


**Fig. 3.** A comparison of the activities of different concentrations of kinetin in both paper disk (○—○) and solution (●—●) methods using the cucumber cotyledon expansion test. Vertical bars are ± SD.

### *Response of Cucumber Cotyledon to Kinetin Extracts from Disks*

The results of the first experiment are shown in Table 1. The kinetin activities of extracts from disks which were treated with 0.3 ml of different concentrations of kinetin and then soaked in 3 ml distilled water for 0, 24, and 48 h, respectively, before treatment, were similar in the cucumber cotyledon expansion bioassay at each concentration. The actual kinetin concentrations of extracts from disks of the three groups were 0.3, 1, 3, 10, 30, and 100 ppm, that is, they were directly proportional to the quantity of samples contained on each of the disks. In general, each organic compound has its specific solubility in water. For example, triacontanol, which is very lipophilic also has a solubility of 10 mg/L (10 ppm)

**Table 1.** A comparison of kinetin activities of extracts from disks which were treated with 0.3 ml of different concentrations of kinetin and soaked in 3 ml of distilled water for varying times before treatment using the cucumber cotyledon expansion bioassay (26 ± 1°C, dark).

| Concentration of kinetin (ppm) | Fresh weight of 10 cotyledons (mg) Extracts from disks soaked for | | |
|---|---|---|---|
| | 0 h | 24 h | 48 h |
| 0 | 318 ± 0 (100.0) | 330 ± 14 (103.7) | 328 ± 11 (103.1) |
| 0.3 | 375 ± 10 (100.0) | 371 ± 11 (99.0) | 367 ± 12 (97.9) |
| 1 | 391 ± 4 (100.0) | 405 ± 15 (103.5) | 409 ± 10 (104.6) |
| 3 | 440 ± 4 (100.0) | 460 ± 27 (104.5) | 468 ± 13 (106.3) |
| 10 | 509 ± 18 (100.0) | 544 ± 31 (106.8) | 547 ± 24 (107.4) |
| 30 | 615 ± 10 (100.0) | 656 ± 69 (106.6) | 638 ± 5 (103.7) |
| 100 | 653 ± 35 (100.0) | 687 ± 10 (105.2) | 666 ± 42 (101.9) |

Values are mean ± SD. Numbers in parentheses represent percent of activities of disks soaked in distilled water for 0 h.

at room temperature (Chen 1984). In this improved disk method, when kinetin is dissolved in ethanol and then placed onto paper disks, the molecules of kinetin are evenly dispersed on disks. The high dispersion of kinetin on disks greatly accelerated dissolution and diffusion, so when the solvent is evaporated and distilled water is added, the fast dissolution and diffusion of kinetin from disk to water should occur and the activities of kinetin may be detected.

The results of the first experiment also showed that kinetin activities of extracts in two latter groups (soaked for 24 and 48 h) were higher than the former group (soaked for 0 h) at each concentration except 0.3 ppm, but the difference between the two latter groups was slight (Table 1). It indicates that a period of time is required for the dissolution and diffusion of kinetin from disk to distilled water and that 24 h is sufficient.

The results of the second experiment are shown in Table 2. When disks containing 100 ppm kinetin were soaked in different volumes of distilled water (3, 10, and 30 ml) for 24 h before treatment, the kinetin activities of extracts from these disks were equaled to 10, 3, and 1 ppm, respectively, that is, they were inversely proportional to the volumes of water added.

According to the results obtained from Tables 1 and 2, each actual concentration of sample tested can be exactly calculated from the following equation

$$C = \frac{a}{b} \times 1000 \text{ (ppm) or } C = \frac{a}{b \times MW} \text{ (M)}$$

where C (ppm or M) is concentration, a (g) is the quantity of sample placed on each disk, b (L) is the volume of water added, and MW is the molecular weight of the sample tested. In our experiments, the activities of plant growth regulators tested were only related to the quantity of samples dropped onto disks and the volume of water added, and were not related to the shape, size, and thickness of paper disks, or whether the plant material contacted the paper disks or not, or the shape and the size of the container used.

**Table 2.** A comparison of kinetin activities of the disks treated with 0.3 ml of different concentrations of kinetin and kinetin activities of extracts from disks dropped with 0.3 ml of 100 ppm then soaked in different volumes of distilled water for 24 h before treatment using the cucumber cotyledon expansion bioassay (26 ± 1°C, dark).

| Concentration of kinetin (ppm) | Fresh weight of 10 cotyledons (mg) | | | |
|---|---|---|---|---|
| | | Extracts from disks soaked in different volumes of distilled water | | |
| | Disks | 3 ml | 10 ml | 30 ml |
| 1 | 403 ± 20 | | | |
| 3 | 449 ± 32 | | | |
| 10 | 517 ± 7 | 517 ± 14 | 441 ± 3 | 405 ± 7 |

Values are mean ± SD.

In summary, the improved disk method is a rapid, convenient and reliable bioassay. The actual concentration of samples tested can be exactly calculated from the above described equation and it may be applied to various water-insoluble samples, solvents, and excised plant materials. As this disk method has advantages over previously described filter paper disk methods (Anderson et al. 1987, Meudt and Bennett 1978), it may be widely applied to screening and studying plant growth regulators. This is especially true, when numerous samples are

screened. However, it may have its limiting factor. When the solubility of a compound in water is less than its effective concentration, this method is not applicable. In our previous experiments (Zhao and Huang 1990, Zhao et al. 1988), the solubility of plant growth regulators tested in water are generally larger than their effective concentrations. For example, the solubility of IAA, $GA_3$, and 2,4-dichlorophenoxyacetic acid (2,4-D) are 1500 mg/L (ppm), 5000 mg/L (ppm), and 600 mg/L (ppm), respectively (Hartly and Kidd 1983, Worthing 1983), whereas their effective concentrations are at 0.3–100 ppm, 0.01–1 ppm, and 0.01–1 ppm, respectively (Zhao and Huang 1990).

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