

Studies on Growth Regulators. I. Improved *Avena* Coleoptile Elongation Test for Auxin¹

J. Claude Sirois

Plant Research Institute, Canada Department of Agriculture, Ottawa, Canada

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Summary. A modified procedure is presented for the bioassay of auxin using *Avena* coleoptile segments. The modifications introduced result in a substantial improvement of the commonly used coleoptile elongation tests.

The proposed test retains the simplicity of physical requirements and of manipulation characteristic of all elongation tests. The main feature of the proposed test is that it permits the measurement of the elongation of coleoptile segments as being directly proportional to the concentration rather than to the logarithm of the concentration of indoleacetic acid. With an accuracy and a range comparable to those of the standard *Avena* curvature test, the proposed bioassay overcomes the limitations for the quantitative assessment of auxin inherent in other coleoptile elongation tests. An additional advantage is provided by the present procedure: two 5 mm segments from each coleoptile can be utilized thus doubling the number of assays hitherto possible with a given number of coleoptiles.

The number of modifications (1, 5, 6, 7, 9) proposed for the original *Avena* coleoptile straight growth test for auxin (3) is a good indication of its usefulness, but it also reflects its inadequacy.

The general acceptance (2, 8, 11, 12, 15) of the coleoptile straight growth test method in its varied forms stems from the advantageous features which it offers over the classical standard *Avena* curvature test (16) and the deseeded *Avena* test (14) methods. These advantages are: simplicity of physical requirements, ease of manipulation, and width of range in which the growth of the coleoptile segments is proportional to the logarithm of the concentration of auxin assayed. Furthermore, the sensitivity of response to indoleacetic acid (IAA) of some straight growth tests is comparable to (7, 9, 10) or even greater than (8, 10) that of the standard *Avena* curvature test (16).

All the straight growth tests, however, have serious limitations for analytical work because they lack both accuracy and specificity. (A forthcoming report of the second phase of the present investigation will deal with the specificity of the straight growth test method). The lack of accuracy of the straight growth tests as compared to that of the curvature test must be ascribed to either the measurement of the coleoptile response or to the nature of the response itself, which suggests some physiological problems. A wide divergence of tissue tolerance to IAA application exists between the 2 assay

methods. In the straight growth test, the coleoptile withstands and responds positively to concentrations of IAA higher than 2.0 mg/liter (8, 10) whereas in the curvature test the same tissue is limited in its response to concentrations lower than 100 μ g/liter (17). Even if the differences in testing conditions between the 2 methods could possibly account for the difference in the tissue tolerance to IAA, it can hardly explain why cell elongation, when measured by 1 method, responds linearly to IAA concentration, whereas by the other it responds linearly to the logarithm of the concentration of IAA assayed. If the 2 methods of assay for IAA are measuring the same process it is logical to expect its measurement to be made on the same scale. The study of this problem over a period of years has led to the development of a straight growth test method where cell elongation is proportional to the concentration of IAA rather than to the logarithm of the concentration. The proportionality between cell elongation and IAA concentration necessarily restricts the range of the test to that of the standard *Avena* curvature test; this limitation however is of little consequence when compared to the increased accuracy which results from it.

Materials and Methods

Throughout this work, seeds of the hullless oat cv. Brighton were utilized. Seeds of uniform size were surface sterilized for 5 minutes in a 2% (w/v) Na hypochlorite solution, rinsed in running tap water for 1 hour, and soaked in distilled water

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for an additional 2 hours. They were then placed at a 45° angle to the vertical, with the embryo down, on a 2-ply tissue paper supported by glass rods fixed at half an inch from the bottom of a plastic tray containing distilled water. The trays were kept in the dark room at 24° for 20 hours at 30 cm from a red light source supplied by two 15 w Sylvania Grolux fluorescent tubes wrapped in 2 layers of No. 14 Cinemoid filter (Strand Electric Company, Toronto), to eliminate radiation of wavelengths shorter than 580 m μ . The seeds were then transferred to an incubator at 26° in the dark for 48 hours. Under those conditions the first internode (mesocotyl) growth was completely inhibited and the coleoptile and the enclosed primary leaf grew to the desired length of about 20 mm.

For the bioassay the coleoptiles were excised by means of razor blades mounted between brass blocks essentially in the fashion described by Bentley (1) and, as is customary, the apical 3 mm were discarded. Ten segments for each treatment were transferred into 5 cm Stender preparation dishes provided with ground glass covers, and containing, in addition to the solution to be assayed, a 2.5 cm² piece of Whatman No. 1 chromatography paper. The piece of paper was included after it was found in other phases of the work (12,13) that pieces cut from chromatograms could be tested directly, bypassing the elution step sometimes used in biochromatography. The lengths of the coleoptile segments were measured under 10-fold magnification after a 20 hour incubation period in the dark at 26°. As pointed out before (2), in the low range of IAA concentrations tested the floating aeration is adequate, and consequently there is no provision in the procedure for further aeration of the system.

All the results were analyzed statistically by the usual methods (4) and the least significance difference (LSD) at the 5% level is given in the legends of figure 1 to figure 5 for each experiment reported.

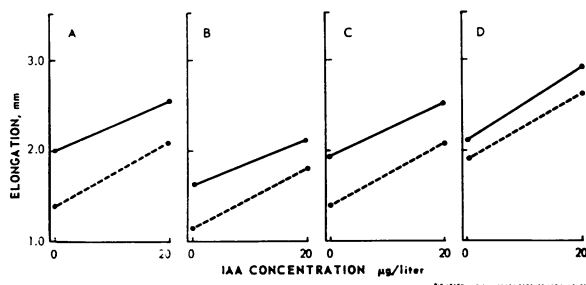


FIG. 1. Elongation of coleoptile segments treated or not with 0.02 µg of IAA in presence ●—● or absence ● - - - ● of: A) Mn²⁺ in the floating solution, or the following in the test solution, B) Mn²⁺, C) sucrose, D) sucrose and Mn²⁺. The respective LSD's in presence or absence of additives were A) 0.19 and 0.16, B) 0.19 and 0.18, C) 0.21 and 0.15, D) 0.20 and 0.19.

Results

The results obtained by Nitsch and Nitsch (8) in their detailed study of different factors affecting the response of coleoptiles to IAA were generally confirmed. Consequently, their procedure was followed without or with minor changes. A length of 5 mm for coleoptile segments, a 3-hour floating period as a pretreatment for the segments, and a buffer system at pH 4.8 at a final concentration of 0.005 M K₂HPO₄ and 0.0025 M citric acid for the test solution, were adopted as a routine for all tests. This buffer concentration was found adequate to maintain the pH below 5.0 when 2.5 cm² pieces of paper chromatograms developed in propanol-ammonia and air-dried for 1 hour were extracted in it. The presence of acetate in the chromatography solvent system makes it impossible to use the present assay. The exposure of chromatograms to a strong air current for 16 hours was insufficient to free the paper from traces of acetate which proved to be highly toxic to the coleoptile segments.

Study of the Factors Involved in the Modification of the Bioassay Method. The results of our study of some factors previously reported (8,11,15) to affect the response of coleoptile segments to IAA were at variance with these reports. They led to the introduction of modifications to the procedure common to straight growth test methods.

Effect of Mn²⁺ and Sucrose on the System. The addition of MnSO₄ at the rate of 1.0 mg/liter to the distilled water for the 3 hour period during which the coleoptile segments were floated had no effect on the differential growth between the control and IAA treated segments (fig 1A). Similarly, the typical results reported in figure 1, B, C, and D, show that the inclusion in the assay medium of 1.0 mg/liter of MnSO₄ and 2% (w/v) sucrose, either singly or in combination had practically no effect on the slope of the curves even if it promoted the overall elongation of the segments. The statistical analysis indicates that the addition of Mn²⁺ and of sucrose increased the variability of the response of the segments (fig 1). Since the sensitivity of the test, assessed by the slope of the curves depicting the differential growth due to IAA was not affected, and since its reliability, measured by the LSD was adversely affected by the presence of Mn²⁺ and sucrose, neither was included in the system.

Volume of Test Solution. Five quantities of IAA ranging from 0 to 0.08 µg were each tested in 0.5 and 1.0 ml of buffer. The results of these tests which are illustrated in figure 2 show that the response of the coleoptile segments to IAA was of the same magnitude in the 2 volumes. They clearly indicate that, within the limits stated, for any 1 quantity of IAA, coleoptile segment elongation is related to the actual amount of IAA in the solution independently of the concentration. The similarity of these activity-concentration curves (A, B, fig 2) with that of the standard *Avena* curvature test sug-

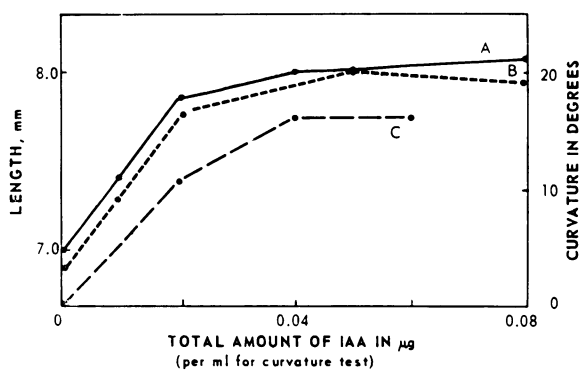


FIG. 2. IAA activity measured by (left ordinate) elongation of coleoptile segments assayed in A) 0.5 ml, or B) 1.0 ml of buffer, and by (right ordinate) the standard *Avena* curvature test C). LSD for A) 0.21, for B) 0.20, and for C) 2.2.

gested that a comparison be made between the 2 types of assay. The results of a curvature test performed according to the standard *Avena* curvature test method (16) are reproduced in figure 2, C. They illustrate the almost perfect duplication of the results (A, B, fig 2) obtained by the present straight growth method.

But as shown in figure 3, curve A, when the elongation of the coleoptile segments is plotted against 0.01 μg increments of IAA, a break in the straight line between 0.02 and 0.04 μg of IAA was consistently observed. Many substances such as carbohydrates, organic acids, metallic ions, nitrate, and variations in the test conditions such as temperature, duration, buffer composition, pH, molarity, were tested in vain to overcome the drop in response depicted by the break in the curve. The

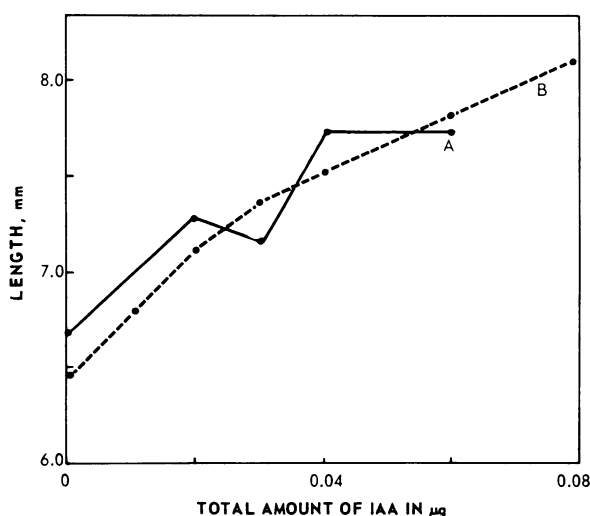


FIG. 3. Growth curves of coleoptile segments assayed in A) 1.0 ml, or B) 2.0 ml of buffer. LSD A) 0.20, B) 0.18.

failure to explain this drop on metabolic grounds led to the study of strictly physical factors which could cause it. A slight, but constant, upward curling of the coleoptile segments was observed when more than 0.02 μg of IAA was present in the assay dishes. The drop in response could be reflecting a reduction in the absorbing surfaces of the segments caused by the emergence of their ends from the solution. The return to the almost linear response of the segments to levels of IAA greater than 0.04 μg could be explained by the constancy of the segment curling. The lack of proportionality between the intensity of the curling and the concentrations of IAA greater than 0.03 μg would reduce its effect relatively to the overall elongation of the segments.

The obvious test for this hypothesis is to increase the volume of the test solution to prevent the emergence of the coleoptile segment ends. Since doubling the volume of the test solution from 0.5 to 1.0 ml had no effect on the response of the segments (fig 2), the volume was increased from 1.0 to 2.0 ml.

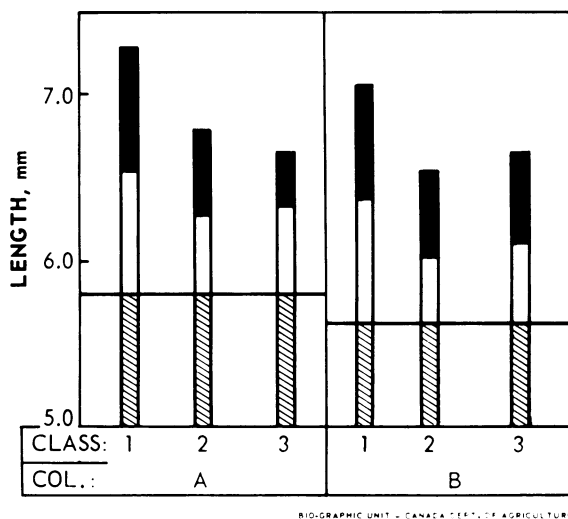


FIG. 4. Histograms showing lengths of coleoptile segments after 3-hour floating period (hatched sections), and added elongation during 20-hour test in buffer alone (blank sections) and in buffer plus 0.02 mg IAA (solid sections). LSD's for Col. A) 1, 2, 3 are 0.17, 0.17, 0.23, and for Col. B) 1, 2, 3, 0.19, 0.23, 0.29 respectively. See text for description.

The results reproduced in figure 3, curve B, confirmed that the response was independent of volume, and, as postulated, indicate that the failure of the segments to respond linearly at ca 0.03 μg of IAA was readily overcome by increasing the volume of the test solution.

Physiological Age of the Coleoptile, and Location of the Segment. Coleoptiles ranging in length at time of excision, from 16 to 28 mm were separated as follows: class 1, 18 mm \pm 2; class 2, 23 mm \pm 2; class 3, 26 mm \pm 2.

Two segments from each coleoptile were obtained in the following manner: the apical 3 mm

were discarded; the following 5 mm, (3rd to 8th from the apex) referred to as Col. A, and the next 5 mm (8th to 13th from the apex) referred to as Col. B, were then excised from the decapitated coleoptiles. The 6 groups of segments, consisting of Col. A and Col. B, obtained from each of the 3 classes, 1, 2, and 3, were floated for 3 hours in distilled water and assayed in presence or absence of 0.02 μg of IAA. The results obtained are reported in figure 4. They show the importance of a rigid selection of the coleoptiles, as to their length, to obtain reproducible results. It also appears that in order to obtain maximum response, the coleoptiles should not exceed 20 mm in length at the time of excision. Another interesting feature of these results is the response of the basal portion of the coleoptile, Col. B, to the IAA treatment. The response is approximately the same as the one obtained with the apical segment, Col. A. If the basal segment responds to IAA in a concentration range sufficiently broad to be useful in quantitative assays this feature would double the yield of useable segments.

Hundreds of tests have been performed by 4 different operators and the results of a typical assay reported in figure 5 show clearly that Col. B can be used for quantitative assays.

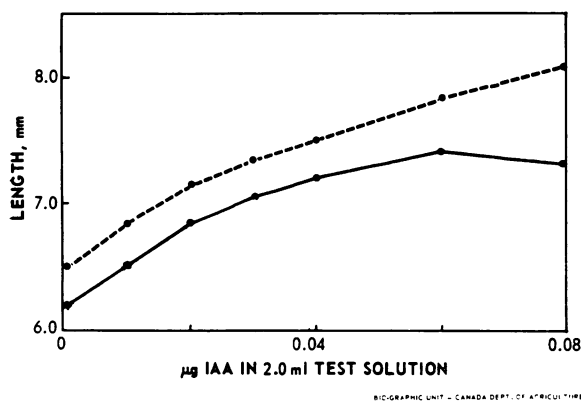


FIG. 5. Growth curves of coleoptile segments: Col. A) ● - - - ● (LSD, 0.19) Col. B) ● ——— ● (LSD, 0.20).

Conclusion

An attempt to improve the accuracy of the *Avena* coleoptile straight growth test for auxin has led to the development of the following procedure: Brighton oat seeds surface-sterilized with 2% (w/v) Na hypochlorite solution, rinsed in tap water for 1 hour, and soaked in distilled water for 2 hours. Seeds germinated on tissue paper in distilled water under red light for 20 hours at 24° and in the dark for 48 hours at 26°. Coleoptiles 20 mm \pm 2 in length decapitated at 3 mm from apex. Two 5 mm segments, Col. A, and Col. B excised and floated in separate Petri dishes, for 3 hours in distilled water.

Coleoptile segments transferred in units of 10 to each preparation dish containing a 2.5 cm² piece of chromatography paper, 2.0 ml of a 0.005 M K₂HPO₄-0.0025 M citric acid buffer at pH 4.8. Dishes incubated in the dark at 26° for 20 hours and coleoptile segment lengths measured under 10 \times magnification.

The present method retains advantageous features common to all straight growth tests over the standard *Avena* curvature test (17): simplicity of equipment, ease of manipulation, suitability for direct bioassay of paper chromatograms.

Furthermore, the use of the present procedure improves the usefulness of the commonly used straight growth tests (1, 2, 4, 5, 6, 8). It transforms the straight growth test from a qualitative or semi-quantitative test into a quantitative one having the high accuracy of the standard *Avena* curvature test. This was achieved by determining and making use of the relationship which exists between cell elongation and IAA. As shown by the standard *Avena* curvature test, cell elongation is directly proportional to the concentration of IAA. The presently proposed test demonstrates that an identical relationship between cell elongation and IAA concentration exists and can be assessed under the conditions imposed by the straight growth test method. This is at variance with the other straight growth tests which show that cell elongation is proportional to the logarithm of the concentration and not to the concentration of IAA.

The proposed procedure, by permitting the use of two 5.0 mm segments from the same coleoptile offers the additional advantage of doubling the number of assays hitherto possible with a given number of coleoptiles.

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