

NATURALLY OCCURRING GROWTH SUBSTANCES. II. AN IMPROVED STRAIGHT GROWTH TEST & ITS APPLICATIONS¹

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The sustained interest of many investigators in the chemistry and biology of plant growth stimulants has resulted in the development of a variety of bioassay methods for the qualitative detection and quantitative estimation of these substances, both synthetic and of natural occurrence. Among the most common techniques based on differential responses are the *Avena* curvature test (13) and the slit pea stem test (14). Common straight growth assays include the *Avena* section test (2), the pea section test (4), and several methods which employ root tissue (1,7,8).

Nitsch and Nitsch (9) examined a variety of other possible test objects and compared their suitability with that of previously reported methods. These workers found that sections cut from first internodes of Brighton oats were especially sensitive to growth promoters, reliable in response, and applicable over a wide concentration range. Unfortunately, this variety is in very limited supply, and fresh seed generally is quite difficult to obtain.

The present report describes a very satisfactory first internode test which has proved to be valuable for detecting the growth stimulants obtained from plant extracts as well as in searches for new types of synthetic plant growth regulators.

MATERIALS & METHODS

BIOASSAY: Most of the oat varieties employed in these experiments were obtained from local feed dealers. The Brighton seed was harvested on the Union Carbide Research Farm near Clayton, N. C., from stock originally supplied by the Canadian Department of Agriculture.

Seed was embedded to a depth of 3 to 5 mm in quartz sand contained in 9 × 14 inch pyrex trays, and the sand was then saturated with distilled water with precaution to avoid free-standing surface pools. The planted trays were stored in total darkness at a controlled temperature of 70 to 72° F for 5 days. All subsequent manipulations were conducted either in total darkness or in green light (principally 546 m μ) under conditions, described previously (9), which are without notable effect on internode growth.

At the appropriate stage of growth, 4.0 mm sections of the first internodes were cut with a coleoptile microtome (9,12) of Thimann's improved design.

Following immersion in distilled water for about one hour, eight to ten sections were transferred to a 16 × 125 mm test tube containing the desired concentration of test compound in citrate-phosphate buffer at pH 5.0 to which 2% by weight of sucrose had been added. The tubes were rotated at 1 rpm on a Wedco tissue culture roller for a period of time (generally 18 hr), the sections removed with a spatula, and their length measured with the aid of a photographic enlarger.

EXTRACTION OF GROWTH SUBSTANCES: Pea plants (*Pisum sativum* L., var. Alaska) were grown under the above conditions for 7 days after planting. The aerial parts were excised, washed with distilled water, immersed in purified methanol, and ground in a Waring blender. The suspension was stored in the dark at 4° C for about two hours, filtered by suction through paper, and the filter cake washed with cold methanol and oven-dried to constant weight.

Organic solvent was removed from the filtrate under reduced pressure on a rotating evaporator at temperatures below 40° C, the aqueous residue was extracted three times with peroxide-free ether, and the combined ether layers were washed several times with distilled water and dried over anhydrous Na₂SO₄ in the refrigerator. This colorless solution was evaporated to a small volume in vacuo and an aliquot chromatographed and assayed in the same manner as the aqueous fraction.

The combined aqueous layers were evaporated to small volume under reduced pressure and an aliquot (generally 1 ml) placed as a single streak near the bottom of a dry sheet of Whatman no. 17 paper which had been washed thoroughly in dilute hydrochloric acid, distilled water, and acetone in that order. Chromatography was carried out in a commercial Chromatocab at 25° C, employing a mobile phase of isopropyl alcohol—conc. NH₄OH—H₂O (80:5:15 by vol) in the ascending direction.

After about 20 hours, the chromatograms were air dried, cut into 10 to 15 horizontal segments, and an equal part of each segment added directly to the tubes containing buffer and sucrose. Corresponding parts were treated with appropriate standard color reagents.

Further concentration of the active components of the aqueous fraction was accomplished by extracting the entire chromatogram with purified acetonitrile containing between 5 and 10% methanol, followed by rechromatography on either Whatman no. 17 or no. 3 paper.

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RESULTS & DISCUSSION

Typical results obtained in growth experiments with first internode sections from several varieties of oats are compared in table I. The index was devised to express the relative value of each variety as a function of control growth, degree of elongation under chemical stimulation, and variability in both treated and untreated sections. A relatively high index indicates a large growth increment and a high degree of precision. From these data, it is apparent that the Forkeddeer variety provides internode sections of outstanding quality.

This variety of red oat (*Avena byzantina* C. Koch) was developed at the University of Tennessee Agricultural Experiment Station by Stanton in 1930 and 1931 (6) as a winter-hardy selection. Unlike Brighton, it is commonly available throughout the Southern and Middle Atlantic states. In our experience, seedlings have been notable for exceptionally uniform growth rate and habit, high incidence of germination, complete absence of fungal infection, and insensitivity to minor temperature fluctuations. The husk does not appear to influence the sensitivity toward exogenous growth stimulants.

The effects observed through variation of several of the most important parameters affecting determination both of hormone profiles of plant extracts and the growth-promoting ability of new types of synthetic organic compounds are described in the following paragraphs.

GROWTH RATE: Figure 1 indicates the rate of growth of 4 mm Forkeddeer sections under standard conditions, both in the presence and absence of added indole-3-acetic acid (IAA). Unlike Brighton sections, both treated and untreated objects cease to grow after about 16 hours, resulting in a constant differ-

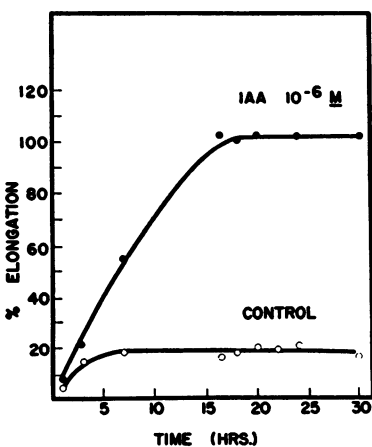


FIG. 1. Growth rate of Forkeddeer oat sections in buffer + sucrose.

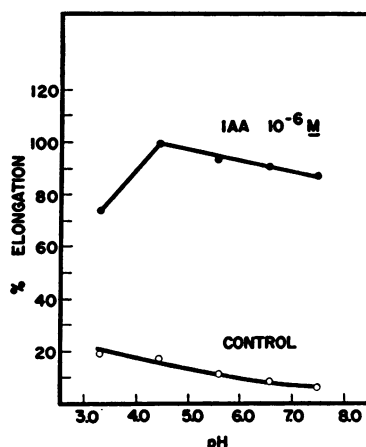


FIG. 2. Effect of pH on Forkeddeer oat sections in buffer + sucrose.

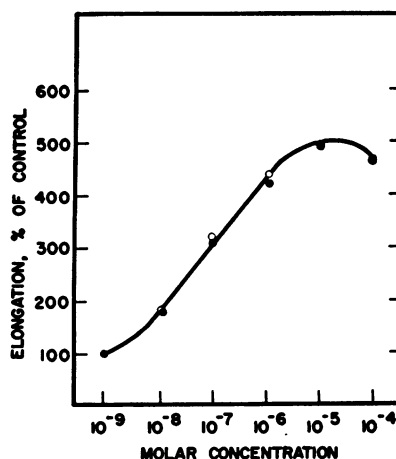


FIG. 3. Effect of auxin concentration on oat sections in buffer + sucrose + 10^{-6} M IAA. ○ Forkeddeer, ● Brighton.

TABLE I
GROWTH CHARACTERISTICS OF OAT SECTIONS

VARIETY	GROWTH*					
	CONTROL		IAA (10^{-6} M)		INDEX**	
	LENGTH, mm	STD. DEVIATION	LENGTH, mm	STD. DEVIATION	ELONGATION % OF CONTROL	F
	A	B	C	D	E	F
Forkeddeer	4.9	0.49	8.3	1.48	475	655
Clinton	4.6	0.44	6.3	1.73	410	540
Victory	4.5	0.48	6.4	2.44	520	445
Winter turf	4.5	0.48	7.7	3.98	695	365
Brighton	5.0	0.71	10.4	3.06	640	300
Tuxedo	5.2	0.87	9.3	1.79	450	290
Goats	5.0	1.05	8.3	2.91	440	140

* Initial section length 4.0 mm.

E
** F = $\frac{E}{BD}$

ence between the two. This flexibility in the duration of treatment has been found very advantageous where a variation in daily laboratory routine becomes necessary.

EFFECT OF pH: The variation of sensitivity and growth with changing pH is shown in figure 2. Like Brighton, Forkeddeer sections exhibit maximum response at a pH near four. However, the nearly parallel growth curves of the IAA-treated and untreated sections permit meaningful measurements of stimulation to be extended into the alkaline range. The significance of this observation may be demonstrated in investigations dealing with compounds such

as salts of organic acids and with hydroxy acids which form cyclic esters or lactones under acid conditions. Although most of our measurements have been made at pH 5.0, the shallow slopes of the pH-activity curves indicate that some latitude in buffering capacity is permissible.

EFFECT OF AUXIN CONCENTRATION: The ability of various concentrations of IAA to stimulate growth in sections of Brighton and Forkeddeer oats is shown in figure 3. Although Forkeddeer occasionally does not reach the maximum degree of stimulation observed in the other variety, the generally comparable response and range of sensitivity make it of exceptional value in the qualitative and semiquantitative evaluation of growth substance activity required in developing growth profiles of plant extracts by paper chromatography. Since, in most of these cases, the chemical nature of the growth promoters is not known with certainty, application of a truly quantitative measure becomes meaningless. In such instances, emphasis must be placed upon reliable detection of biological activity regardless of the chemical structure of the stimulant.

EFFECT OF SYNTHETIC GROWTH REGULATORS: Forkeddeer sections have been found to respond to a variety of synthetic growth regulators over wide concentration ranges. In general, concentration-growth curves are similar to those of figure 3. Compounds which have been examined include the halogenated phenoxyacetic acids, substituted cinnamic acids (manuscript in preparation), the indole-3-alkanoic acids and amides (3), and many other substituted indoles.

DETERMINATION OF HORMONE PROFILES: As an example of the utility of Forkeddeer sections for determining the array of growth promoters extractable from plants, the hormone profiles of both ethereal and aqueous extracts of the pea (*Pisum sativum* L., var. Alaska) are shown in figure 4. The quantitative variation in response may be due to the intrinsic difference in stimulatory ability among active compounds, the presence of growth inhibitors exhibiting the same Rf as the promoters, variation in the concentration of promoters, or a combination of these factors.

Chromatography of the ethereal extract reveals a zone of activity which corresponds in Rf with IAA (Rf 0.44). While no color was observed upon application of Ehrlich's reagent to these chromatograms, the quantity of IAA present might be too small to detect. On the other hand, several careful investigations of pea extracts (5, 11) have failed to demonstrate the presence of IAA, and the stimulation observed in the present case may be due to another compound. It should be noted that the profile of the ether extract is similar to that found by Phillips and coworkers (10) who employed Brighton sections in their biological tests. However, an important factor overlooked by these investigators was the

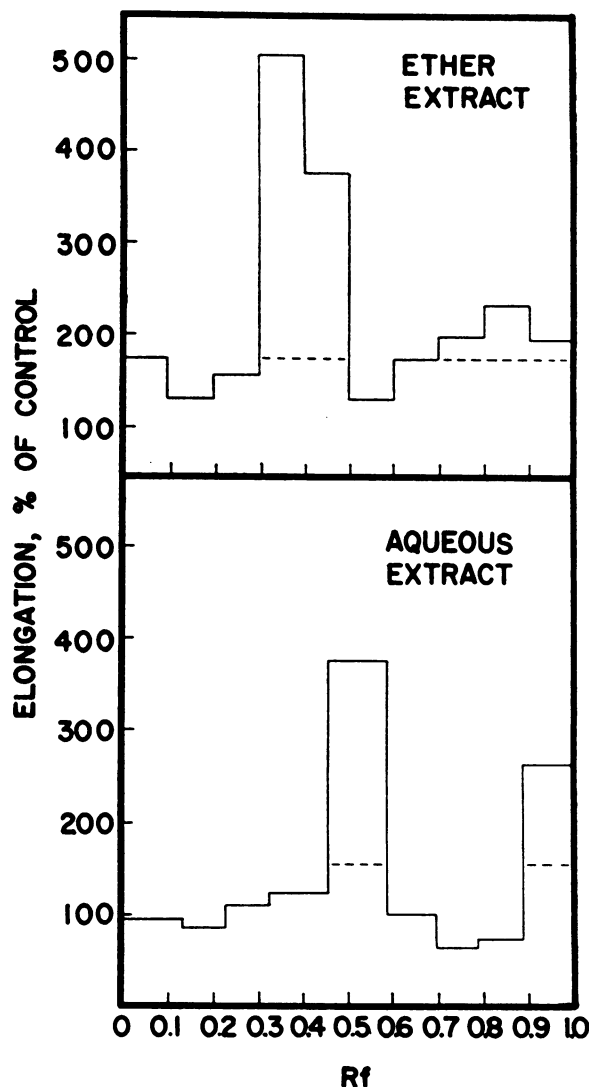


FIG. 4. Effect of Alaska pea extracts on growth of Forkeddeer oat sections in buffer + sucrose. Values above the broken line are statistically significant at the 1% level.

necessity for thorough removal of water from the ethereal layer prior to bioassay. We have found that even the small amount of water present in the organic layer (generally ca. 1.3% by weight at 20°C) will permit solution of sufficient quantities of growth stimulants normally insoluble in ether to result in misleading interpretations.

The aqueous residue remaining after extraction with ether provides several growth-promoting zones upon chromatographic examination. Although purple spots corresponding to several of these areas appear after treatment with Ehrlich's reagent (Rf. 0.34, 0.78, 0.89), Rf comparison with an extensive catalog of values for known indolic natural products still does

not provide firm evidence of their chemical nature.

Extraction of the thoroughly dried residue or its chromatogram with acetonitrile containing between 5 and 10% methanol, followed by paper chromatography, results in a hormone profile almost identical to that observed in the intact aqueous fraction. This leads us to believe that all of the growth-stimulatory substances of the Alaska pea may be accounted for in the organic extracts. The extended investigation resulting from these observations will be the subject of future communications.

SUMMARY

I. Examination and comparison of the characteristics of first internode sections from a number of oat varieties has led to the development of a convenient bioassay for chemical growth stimulants, both synthetic and of natural occurrence.

II. The Forkeddeer variety has been shown to provide sections of outstanding uniformity, sensitivity, range of effective concentration and pH, and availability.

III. Determination of hormone profiles of aqueous and ethereal extracts of the Alaska pea, based on the Forkeddeer bioassay, failed to provide conclusive evidence of the presence of indole-3-acetic acid in this variety. Several other unidentified growth stimulants, insoluble in ether, were detected.

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