A COMPARATIVE STUDY OF DIFFERENT METHODS OF DETERMINING ACTIVITIES OF GROWTH-PROMOTING SUBSTANCES [1]

Felix G. Gustafson

PLANTS RESPOND to growth-promoting substances in a number of ways, but most of these responses are of such a nature that they are not readily measurable. However, during the last decade attempts have been made to utilize many of these responses to determine growth-promoting activities; while some of them are only qualitative, others are at least semi-quantitative.

There is no unanimity among investigators as to the accuracy or appropriateness of the various methods in general use; in fact, the situation is exactly the opposite. The reason for this disagreement, as the writer sees the situation, is partly due to prejudice and partly to the difference in viewpoint. One group has been mainly interested in discovering new chemical compounds which would elicit one or more of the many responses of plants to growth-promoting substances. These investigators have been for the most part interested in qualitative result, though they have also attempted to obtain some information as to relative activity of the different compounds. This group has used various plants grown under normal greenhouse conditions. Then there are those investigators who are interested in discovering the mechanism of the action of growth hormones on plants. The etiolated oat coleoptile grown under rigidly controlled conditions has been the main test object of this group, though etiolated pea seedlings have also been used to some extent. A third and more recent group of growth hormone investigators has in the main been attempting to determine the amount of hormone present in plants, and to associate this with growth habit, age, environment, etc. In other words this group has been interested in the application of the results of growth hormone studies to an understanding of the growth of plants.

Several investigators (among them Zimmerman and Hitchcock, 1937; Thimann and Schneider, 1939; and Avery, Berger and Shalucha, 1942) have recognized the fact that different test methods give different results. To emphasize this fact further the writer decided to try out, in his laboratory, tests representative of all schools so that the results, all of which would be obtained by the same investigator under comparable conditions, could be more accurately compared than if done by several different investigators in different laboratories. The writer has had access to a number of new compounds which fact has made the investigation doubly interesting and worth while.

METHODS AND MATERIAL.—After some preliminary experimentation it was decided to study stem curvature, epinasty, and gall formation in the tomato, the parthenocarpic development of fruits in the tomato, bud inhibition and enlargement (gall formation) of the cut stem of the sunflower, and curvature of the *Avena* coleoptile. The John Baer tomato was used throughout for the experiments on stem curvature, epinasty, and gall formation (enlargement of the stem at the point of application of the lanolin paste); the plants were grown in the greenhouse and were usually about 12 to 15 inches in height; careful choice as to size was made in each experiment. The parthenocarpic experiments were for the most part conducted in the field. The Russian mammoth sunflower grown in light was used throughout in the experiments on bud inhibition and gall formation, (Laibach, Mai, and Müller, 1934). These plants were used when the first internode above the cotyledons was 3 to 4 centimeters long, except in some experiments where the influence of distance of application of the chemical was studied. Only plants with both cotyledons present and in good condition were chosen. The Victory oats, obtained from Batavia, New York, were used for the *Avena* experiments.

The following chemicals were used: 2–fluoreneacetic (2–FlA), 4–fluoreneacetic (4–FlA), 9–fluoreneacetic (9–FlA), 7–acenaphthaleneacetic (7–ANA), β–7–acenaphthalenepropionic (β–7–ANP), diphenylacetic (Diϕ A), 3, 4–dihydronaphthaleneacetic (3, 4–diHNA), 2–ethyl–1–naphthaleneacetic (2–ethNA), β–naphthoxyacetic (NOA),[2] naphthaleneacetic (NA), naphthalenepropionic (NP), naphthalenebutyric (NB), indoleacetic (IA), indolepropionic (IP), indolebutyric (IB), phenylacetic (ϕA) acids, and naphthaleneacetamide (NAA). When applied to tomatoes or sunflowers, the chemicals were mixed with lanolin; in the experiments with *Avena* the chemicals were applied in the usual way in an agar block.

The curvature and epinasty experiments with tomato plants were set up according to the method of Zimmerman and Hitchcock (1937), i.e. the lanolin paste was smeared on one side of the stem either in the second or third internode from the top; the position varied with each experiment, but in later experiments the second internode from the top was used. The experiments on bud inhibition and gall formation were carried out with young sunflower plants. As previously pointed out by the writer (1941a), the Russian sunflower rarely produces branches when intact, but will do so when the apical

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[2] The three fluoreneacetic acids, 7–ANA, β–7–ANP, 3,4–diHNA, 2–ethNA and NOA were supplied by Dr. John Sheehan of the Chemistry Department of the University of Michigan. The NOA was also supplied by Dr. P. W. Zimmerman of the Boyce Thompson Institute, Yonkers, N. Y. NAA, NP, and NB were supplied by the American Chemical Paint Co., Ambler, Pa. To all of these the writer wishes to express his appreciation.

bud is removed. Some of the growth-promoting chemicals will, however, inhibit the buds as readily as the apical bud (Gustafson, 1941a). In these experiments the stem was cut about 1 centimeter above the cotyledons, and the lanolin paste applied to the cut surface. It had been shown earlier that stem length above the cotyledons could vary considerably without any influence. If only pure lanolin were applied to the cut surface, buds developed in the axils of the cotyledons within a few days, and the stem at the cut surface never enlarged or formed a gall. Experiments on the parthenocarpic development of fruits in tomatoes were conducted in the usual way (Gustafson, 1936) and so were the *Avena* experiments (Went and Thimann, 1937).

RESULTS.—*Curvature of the stem, epinasty and gall formation in tomato.*—Extensive experiments were conducted with tomatoes to determine the effectiveness of the different chemicals in inducing curvature and gall formation in the tomato stem. All of the above mentioned chemicals were used on nearly 1200 plants.

Of the seventeen chemicals used, only seven produced any response. As only qualitative observations could be made on stem curvature, epinasty and gall formation, comparisons were made between the responses of the plants to the different chemicals only when used in the same experiment. Thus the statement is usually made that the response was the same or less than naphthaleneacetic acid (NA), because as a rule this chemical was the most active in these experiments, or sometimes comparisons were made with indoleacetic acid (IA) instead. The results with each one will be noted briefly. The seven chemicals found to be active, with the exception of indolepropionic and 4–fluoreneacetic acids, were used in concentrations of .016, .062, .125, .25, .50, 1.0 and 2.0 per cent. The two lowest concentrations were not used with the above two chemicals.

Naphthaleneacetic acid (NA).—The three lowest concentrations gave strong curvatures within a day after application. In sixteen days a large gall had been formed at the point of application of the chemical. No epinasty was observed with concentrations lower than .25 per cent. With one per cent, large galls were formed in seven days. This concentration also suppressed the growth in length of the stem.

3, 4–Dihydro–1–naphthaleneacetic acid (3, 4–diHNA).—The three lowest concentrations caused less curvature than NA, but in sixteen days a good gall formation took place. A slight epinasty was produced with .25 per cent. Two per cent suppressed growth in length of the stem, and flower bud development was also retarded.

Naphthoxyacetic acid (NOA).—The three lowest concentrations gave curvatures comparable to .062 per cent 3, 4 diHNA, but much smaller galls were produced. Strong epinasty was produced with .25 per cent. The galls were larger than those produced by indoleacetic acid.

Indoleacetic acid (IA).—The three lowest concentrations produced curvatures comparable with NA. Some epinasty was also observed. The galls produced by these concentrations were much smaller than those produced by NA. Higher concentrations suppressed the growth of the stem in length. Sometimes light areas appeared some distance above and below the point of application of the chemical.

Indolebutyric acid (IB).—No curvature was produced by .016 per cent and only slight curvature with .062 and .125. Only a slight swelling of the stem was observed after sixteen days with .016 per cent; both .062 and .125 produced a swelling of the stem. Light spots like those produced with IA were also observed. Higher concentrations suppressed stem elongation.

Indolepropionic acid (IP).—Good curvatures were produced with .25 per cent, and the galls produced at the end of sixteen days were comparable in size to those produced by .125 per cent IA.

4–Fluoreneacetic acid (4–FlA).—All concentrations used produced curvatures and galls of approximately the same magnitude as those produced by IB of corresponding concentrations. Two peculiarities characterize this compound. In concentrations as low as .25 per cent it produced a curling of the leaves, which has not been observed with any other chemical. The leaves rolled very much as if infested with aphids on the under side. Sections of the leaves showed that the cells on the upper side increased to an abnormal size. This chemical did not suppress stem growth as did all the others that had an effect on the plant. No studies were made on the chlorophyll content, but the leaves were definitely greener than the controls.

From the above it can be seen that such observations as have been set down are definitely not quantitative, although as great care as possible was exercised. This type of experimentation does not lend itself to exact measurements, and the only recourse for the investigator is to run large experiments with as uniform a lot of plants as can be obtained and by direct comparison to arrange the chemicals in some sort of a series. In the present experiments as far as the curvature of the stem and gall formation are concerned we find that naphthaleneacetic acid is definitely the most effective chemical and the others follow in the order indicated, NA>3,4–diHNA>NOA>IA>IP,>IB and 4–FlA. Controls with lanolin never produced any response.

Bud inhibition and gall formation in sunflower plants.—It has been known for many years that such chemicals as indoleacetic, indolebutyric and naphthaleneacetic acids inhibit lateral bud development (Thimann and Skoog, 1933). The writer has found that sunflower seedlings 8 to 12 inches tall are good experimental plants to show this. Plants of this size have a very small bud in the cotyledonary axil, but in intact plants this never develops any further. Uniform and vigorous plants with both cotyledons intact were selected and decapitated about 1 centimeter above the cotyledons. The desired chemical mixed with lanolin was then smeared on the cut surface; controls with pure lanolin were

TABLE 1. *Bud inhibition in sunflower plants with low concentrations of growth-promoting chemicals. The number of buds produced is shown in the columns under the proper concentrations during a period of time indicated for each experiment.*

	September 11, 1941 Run for 20 days, 16 plants used with each chemical			October 24, 1941 Run for 26 days, 18 plants used with each chemical				
Concentr.	.032%	.016%	.008%	.016%	.008%	.004%	.002%	Remarks
NOA	2	3	15	..	..	..	..	
3,4 diHNA	3	..	12	..	14	18	..	Gall formed even with .004%
IB	1	3	4	13	17	18	18	Slight gall formation even with .002%
IA	..	..	..	17	17	18	17	Gall as for IB
NA	0	0	..	0	0	0	0	Gall as for IB
Lanolin	..	..	..	..	.	..	..	All plants produced buds

run with each lot of plants. As none of these chemicals will indefinitely prevent lateral buds from developing unless renewed at frequent intervals, the number of plants producing buds was counted at frequent intervals and comparisons made with the control. Many of the chemicals used produced an enlargement (gall) of the stem near the point of application. However, not all chemicals, even when buds were inhibited, produced these galls. Both bud inhibition and gall formation were studied with all chemicals.

In preliminary experiments concentrations as high as one or two per cent were used to find out whether or not the chemical was active and later experiments had as their purpose determination of the degree of activity, and in these later experiments low concentrations were used. In the preliminary experiments it was found that seven compounds (2–ethNA, ϕA, DiϕA, 2–FlA, 9–FlA, 7–ANA and β–7–ANP,) did not inhibit lateral buds, although the first two produced sizeable galls.

TABLE 2. *Transportation as a factor in bud inhibition. The chemicals were used in a concentration of one per cent. The figures denote the number of plants out of 18 that produced buds after 26 days.*

	Stem cut 1 cm. above node	Stem cut at the node
Lanolin	18	..
Phenylacetic	10	3
Diphenylacetic	18	17
Ethylnaphthalene acetic	17	17
Naphthalene propionic	17	18

Of the remaining ten compounds, NP, NB, 4–FlA and NAA reduced the number of buds, but never completely inhibited their growth. Table 1 gives the results of two experiments with low concentration of the chemicals.

The table shows that NA is by far the most active, IB is next and NOA and 3,4 diHNA are least active. While this table does not show any appreciable difference between IA and IB, other experiments have indicated that the latter is considerably more active. Another fact the experiments bring out is that there is little or no relation between bud inhibition and gall formation; in the October experiment concentrations of 3,4 diHNA, IB, and IA which did not inhibit bud formation produced galls as large as those produced by NA, which did inhibit bud formation.

TABLE 3. *Transportation is not a factor in the difference in bud inhibition of different compounds. The figures denote the number of plants out of 16 that had produced visible buds 24 days after the application of the chemical at varying distances above the cotyledonary node. Concentrations used are given in the parenthesis next to the chemical.*

	7 cm.	5 cm.	3 cm.	1 cm.
NA (.008%)	0	0	0	0
3,4 diHNA (.008%)	7	8	7	5
IA (.016%)	9	10	11	8
IB (.016%)	6	3	4	6

To ascertain whether transportation might be a factor in the lack of inhibition shown by some compounds, several experiments were set up in which the chemical was applied at different distances from the cotyledons. First several experiments were run using four compounds which have little or no inhibiting effect. In these experiments the stem was cut 1 centimeter from the cotyledonary node and at the node. In the latter situation the chemical was applied within one or two millimeters of the bud.

It is evident from table 2 that transportation is not a factor except in the case of phenylacetic acid. This chemical produces a much greater inhibition when it is added at the node than when it is applied at a distance of a centimeter.

Experiments were also conducted to discover whether the difference in activity between those compounds that showed inhibition was due to transportation. In these experiments the stems were cut

TABLE 4. *Degrees of curvature induced in Avena coleoptiles by varying concentrations of a number of chemicals. In all Avena tests indoleacetic acid is the standard, and, as the curvature with this substance is not always the same, the curvature produced with it on the day each chemical was used is given for each experiment. In this way the activity of a particular chemical can be compared with IA and through it with others that were not used on the same day. The figures for each chemical under the different concentrations represent average curvatures for 12 or 24 seedlings.*

Chemical	IA 30 Microgm.	Concentrations in micrograms per liter 50	60	100	200	300	400	500	600	800	1000
IB	9.4	0	0	..	3.0	7.0	9.7	..	..	..	..
IP	14.6	..	..	..	0	..	0	..	0	0	0
φA	14.7	..	..	..	..	..	..	..	0	0	0
Diφ A	10.1	0	0	..	..	..	0	..	..	0	0
7-ANA	9.1	..	..	..	0	..	0	0	0	0	0
4-FlA	8.8	..	..	..	0	0	0	..	..	0	..
NA	14.7	0.7	..	1.2	4.6	..	8.0	..	..	..	..
NB	12.0	..	..	..	0	..	0	..	0	0	0
NP	13.3	..	..	..	0	..	0	0	0	0	0
NOA	9.8	..	..	0	0	0	00	..	0	0	0[a]
NAA	9.4	..	0	0	0	..	0	..	0	0	00
3,4-diHNA	17.1	..	..	0	0	..	0	..	3.3	4.8	6.0
2-EthNA	17.1	..	..	0	0	..	0	..	0	0	0

[a] A concentration of 2000 micrograms per liter was also without effect.

1, 3, 5, and 7 centimeters above the cotyledonary node and the chemical applied to the cut surface. Concentrations were used which had previously been found to cause partial or in the case of NA complete inhibition.

There is no evidence that the greater activity shown by NA in comparison with IA is due to the former being transported more readily through the stem.

One might summarize these experiments by saying that of the seventeen compounds studied, seven do not inhibit bud development, four inhibit only to a slight extent, and six completely inhibit development for a period of several weeks, if concentrations as high as one per cent are employed. The difference in ability to inhibit bud development is not due to transportation through the stem, except perhaps in the case of phenylacetic acid. There seems to be very little correlation between bud inhibition and the formation of galls, since at least two (2-eth-NA and φA) of the first group produce fair sized galls, and some of those which inhibit bud development, such as NAA, do not produce galls.

Parthenocarpy.—Since the writer (1942a) has previously made extensive studies of parthenocarpy, it was thought unnecessary, at this time, to investigate all of the compounds from this standpoint. Only nine of the seventeen compounds mentioned above are included in the present study, but at some time or another the writer has employed all of them except 7-acenaphthaleneacetic and β-7-acenaphthalenepropionic acids.

Naphthalenepropionic, naphthalenebutyric, 2-ethylnaphthaleneacetic, diphenylacetic, 2 and 9-fluoreneacetic acids and naphthaleneacetamide are not able to produce parthenocarpic fruits in the John Baer tomato. Previously (1942b) the writer has reported that NOA is more active than IB, and several investigators (Gustafson, 1941b); Howlett, 1941; and Strong, 1941) have found IB to be more active than IA. The new compounds found active are 3, 4-dihydronaphthaleneacetic and 4-fluoreneacetic acid. The former is definitely less active than its parent NA. Indolepropionic, and 4-fluoreneacetic acids are probably the least active and phenylacetic and indoleacetic are somewhat below naphthaleneacetic. Their effectiveness is not the same with all plants. Some years ago (1941b) the writer reported that for the crookneck summer squash and buttercup squash, naphthaleneacetic was much more effective than indolebutyric, yet the reverse holds true for the tomato. It must, however, be remembered that these statements are based on qualitative or, at the best, semiquantitative determinations.

Experiments with Avena.—The experiments so far discussed have been conducted with plants grown in the light and represent the material which is typical of that used by what may be termed one school of hormone thought. The other school has used as test material oats or peas grown in the dark and, therefore, etiolated. As this research has as its object the comparison of several commonly used methods, the influence of most of the chemicals on etiolated *Avena* has also been determined, by the usual *Avena* technic. In table 4 some of these determinations are given.

In no sense does the activity of any of these compounds compare with that of indoleacetic acid, when the curvature produced in the oat coleoptile is considered. In fact most of them are completely inactive. Of the fourteen chemicals used in the *Avena* experiments only four were active and the ones that had shown the greatest activity in other tests

TABLE 5. *Relative activities of the different chemicals are indicated by numbers, thus 1 is the most active; no activity is denoted by ..; when a chemical was not used in a test the space is left blank.*[a]

Name of chemical	Tests: Tomato: Curvature of stem	Tomato: Gall formation on stem	Tomato: Epinasty	Sunflower: Parthenocarpy	Sunflower: Bud inhibition	Sunflower: Gall formation	*Avena* test
Indoleacetic	4	4	3	4	3	1	1
Naphthaleneacetic	1	1	1	2	1	1	2
Indolebutyric	6	6	4	2	2	1	3
3,4–dihydronaphthaleneacetic	2	2	1	5	4	4	4
Indolepropionic	5	5	5	6	6	6	..
Naphthoxyacetic	3	3	4	1	4	4	..
4–fluoreneacetic	6	6	..	6	7	6	..
Phenylacetic	..	..	..	4	11	6	..
Naphthalenepropionic	..	..	..	..	7	6	..
Naphthalenebutyric	..	..	..	..	7	6	..
2–ethyl–1–naphthaleneacetic	..	..	..	..	..	6	..
Naphthaleneacetamide	..	..	..	..	7	..	..

[a] 2–fluoreneacetic, 9–fluoreneacetic, diphenylacetic, 7–acenaphthaleneacetic and β–7–acenaphthalenepropionic acids never produced any visible reactions in the plants treated with them.

were also found to produce curvature in *Avena*, though proportionately less when compared with indoleacetic acid. Three (IP, NOA and 4–FlA) that had generally been active in the other tests were conspicuously without effect on oats; it is true that with the exception of NOA in the production of parthenocarpy, they were never the most active in the other tests, but still they were fairly effective.

DISCUSSION.—Of the seventeen compounds used throughout this investigation, twelve were active in one or more of the seven tests which were made, but only four produced a response in all tests. In table 5 an attempt has been made to classify these compounds, but as pointed out earlier many of these tests are so rough that it is possible only to approximate the activity, and the table is, therefore, not very accurate.

A study of the table will reveal many interesting facts. Perhaps the most striking and important one is that in no single instance does a compound occupy the same relative position in all tests; indoleacetic acid, for instance, occupies the supreme position in the *Avena* test, but in three others it holds fourth place; and naphthaleneacetic acid, which is accorded first place in five tests, is a very poor second in the *Avena* test; naphthaleneacetamide and 2–ethyl naphthaleneacetic acid are each active in one test, and naphthalenepropionic, and naphthalenebutyric each in two tests. It is obvious from this that no one test is sufficient to classify a compound. The curvature of the stem and epinasty of the tomato, used extensively by Zimmerman and Hitchcock, do not accord indoleacetic acid a very high position; yet it is the only one that would be detected in the low concentrations (30 micrograms/l) usually employed in the *Avena* test. On the other hand, using only the *Avena* test, one would come to the conclusion that naphthoxyacetic acid was not worth considering. Therefore, it seems to the writer that the purpose of an investigation should determine the method to be chosen. If the investigator is interested in determining the activity of a new chemical, then the methods employing green plants should be used; if, on the other hand, he wishes to determine the amount of growth hormone present in a plant, then the *Avena* method or perhaps some of its modifications is the one to use. It also seems inappropriate to formulate theories concerning the action of growth hormones, unless one takes into consideration all types of experiments.

The writer has had a second purpose in mind in running these tests, namely, a study of the relation between activity and structure of the compound, especially of the ring. As a by-product of some investigations in the Chemistry Department several new compounds or modifications were made available for growth studies. The dominant idea in this work was to learn to what extent the ring and also the position of the side chain could be modified without loss of activity.

Phenylacetic acid is active in inhibiting bud formation only when it is placed at the node near the bud, showing that slow transportation is the cause of low activity. This is in agreement with Went's idea (Went and White, 1939) that phenylacetic acid and other compounds which show no activity in the *Avena* test are inactive because of slow rate of transport in the plant. It was, therefore, decided to combine two benzene rings to form diphenylacetic acid to see whether this would be still less active. The new compound was completely inactive. Another group of compounds made available was that of the fluorenes, which might be thought of as compounds in which the two benzene rings of the diphenylacetic acid are further joined to one another through a CH_2 group. Of the three positions of attachment (2, 4, and 9) of the side chain, only the 4 position brought about activity. In the 2 and 4 positions the side chain is attached to a carbon atom next to a

double bond, whereas in the 9 position there is no double bond. For a discussion of structure and activity see Koepfli, Thimann and Went, 1938.

Compounds with the naphthalene nucleus have rivalled those with the indole nucleus in growth-promoting activities. Several modifications in the naphthalene nucleus were accordingly made. The smallest modification was made by introducing hydrogen into the 3 and 4 positions. As shown in table 5 this decreased the activity some, but not very much; it still produced responses in all tests. Another modification was to introduce the ethyl radical at the 2 position. This destroyed all activity of the parent compound, except the formation of the gall in the sunflower. By causing the acid side chain to form a five membered ring with the second benzene ring of the naphthalene nucleus, acenaphthalene is formed and two such compounds were synthesized, namely, 7–acenaphthaleneacetic and β–7–acenaphthalenepropionic acid. Both were completely inactive. By introducing an oxygen atom between the naphthalene ring and the acid side chain a series of new compounds have been formed. Of these only naphthoxyacetic acid was used. Lengthening of the side chain in this manner decreases the activity except perhaps the ability to form parthenocarpic fruits, which seems to have been increased. The effect on the oat coleoptile has been completely lost.

We thus see that changes, which seem only minor, modify or even destroy the growth-promoting activities of a compound.

SUMMARY

This series of experiments brings out the fact that no one of seventeen compounds used is equally effective in all of the seven tests employed to determine growth-promoting activities. Statements in regard to relative activities of growth-promoting chemicals should be based upon specifically named tests.

It seems also that some tests are better suited than others to measure specific activities and the test to be used should be chosen in accordance with the information desired. Thus the tests with green plants are more accurate in determining the effectiveness of a compound in producing roots or seedless fruit, for instance, than is the *Avena* test. On the other hand, the *Avena* test seems to be much more sensitive in determining the quantities of native hormone present in plants than are the other tests.

Modifications in the structure of the nucleus of a compound profoundly influence its activities.

Department of Botany,
University of Michigan,
Ann Arbor, Michigan

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