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SOME EFFECTS OF PLANT GROWTH-REGULATING SUBSTANCES ON MICROORGANISMS

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During recent years, much work has been done on the effect of various synthetic plant hormones on higher plants. This suggested that the same compounds may also have some effects on lower forms of living organisms—the bacteria, yeasts, molds, and actinomycetes.

Little work has been reported on the effects of these compounds on microorganisms. Indole acetic acid was found by Leonian and Lilly (1937) to be inhibitory to several fungi and algae. Brannon and Bartsch (1939), however, tested the effect of several of the growth regulators on *Chlorella vulgaris* and found marked stimulation when the chemicals were used in low concentrations.

Stevenson and Mitchell (1945) reported that 0.02 per cent of 2-4-dichlorophenoxy acetic acid or its sodium salt inhibited, and 0.1 per cent prevented, the growth of several species of bacteria; molds, however, were much more tolerant to the chemical. Culler (1946) in general confirmed these results and further noted greater susceptibility of gram-positive than of gram-negative bacteria.

The investigation reported in this paper was undertaken to increase our knowledge of the effects of a few plant growth-regulating substances on several bacteria and on a few molds and actinomycetes.

METHODS AND MATERIALS

Chemicals used.—The sodium salts of 2-4-dichlorophenoxy acetic acid, β-naphthoxy acetic acid, 4-chlorophenoxy acetic acid, and β-indole butyric acid were used in the experiments.

These were prepared by suspending the weighed acid in water, and titrating with N/1.NaOH using phenolphthalein as the indicator.

Serial dilutions were prepared in 0.05 molar phosphate buffer with pH 6.2. Resulting concentrations ranged from 0.00001 to 1.0 per cent.

TEST ORGANISMS

BACTERIA

Aerobacter aerogenes
Aerobacter cloacae
Bacillus subtilis
Brucella abortus
Corynebacterium diphtheriae
Escherichia coli
Sarcina lutea
Serratia indica
Serratia marcescens
Staphylococcus aureus

MOLDS

Aspergillus niger
Mucor sp.
Penicillium notatum

ACTINOMYCETES

Streptomyces griseus
Streptomyces lavendulae

Tests of growth.—Growth on plates was tested using the ratio of 90 ml. agar basal medium to 10 ml. of salt solution. Difco nutrient agar was used for *B. subtilis*, *A. cloacae*, *S. lutea*, *S. aurens*, *S. indica*, and *S. marcescens*. Tryptose-glucose agar was used to test the growth of *B. abortus*, *C. diphtheriae*, *S. aureus*, and *E. coli*. Difco malt agar was prepared for testing *P. notatum*, *A. niger*, and *Mucor* sp. Glucose-asparagine agar was used for *S. griseus* and *S. lavendulae*.

Tests for turbidity were set up as follows. Nine ml. amounts of broth were pipetted into standard 7″ x 7/8″ test tubes. To these were added 1 ml. amounts of the various dilutions of the plant growth-regulating substances. Difco nutrient broth was used as the basal medium in testing *S. lutea*, *B. subtilis*, *S. aureus*, and *E. coli*. Tryptose-glucose broth was employed in testing growth on *B. abortus*, *C. diphtheriae*, *S. aureus*, and *E. coli*.

The tubes were inoculated with 0.2 ml. of a broth suspension of the organisms and incubated at 37° C. for 90 hours. The amount of growth as shown by turbidity was determined by obtaining galvanometer readings, using an Evelyn Photoelectric colorimeter, at various time intervals throughout the incubation period. Light filter #540 was used. The photometric densities were then calculated from the galvanometer readings.

Biochemical tests.—Biochemical tests were made using nutrient gelatin, and glucose, sucrose, lactose, tryptone, and M.R.V.P. broths. These media were prepared to have final concentrations of 0.1, 0.01, and 0.001 per cent 4-chloro-phenoxy acetate, 2-4- dichlotophenoxy acetate, and β-naphthoxy acetate.

These tests were made on *E. coli*, *B. subtilis*, *A. aerogenes*, *S. aureus*, and *S. marcescens*.

Tests for development of resistance.—Nutrient broths were prepared containing concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, and 0.09 per cent of each of the chemicals. After inoculation and incubation, the tube with the highest concentration of chemical allowing growth was selected. Transfers were made from this tube to basal nutrient broth and to broth containing the next higher concentration of the chemical. Also, after incubation of the nutrient broth culture the next higher per cent solution was inoculated from it. The procedure was continued until no growth was obtained upon repeated transfer to media containing the higher concentration of the growth regulator. This was considered the limit of the resistance acquired by the test organism.

Morphologic studies.—Gram and methylene blue stains were prepared of all bacteria from both agar plates and broth cultures. Smears were made from the broth cultures at the time of each turbidity reading in an attempt to determine if any changes in morphology occurred during the incubation period.

The molds and actinomycetes were examined only for their gross morphologic characteristics.

RESULTS

Effects on growth.—*A. cloacae* when streaked on plates of nutrient agar containing serial concentrations of 0.1 to 0.000001 per cent of the sodium salts of β-indole butyric acid, 2-4-dichlorophenoxy acetic acid and β-naphthoxy acetic acid respectively and incubated, gave good growth in all concentrations except 0.1 per cent. After incubation for four days, the latter concentration permitted almost normal growth. Under the same conditions, *B. subtilis* appeared to be greatly inhibited for 24 hours, but on continued incubation good growth was obtained except at 0.1 per cent concentrations where there was complete inhibition.

Growth of *S. lutea* was greatly retarded for five days and when growth was obtained the pigment of the organisms on the test media was much deeper than for those grown on basal medium. *S. aureus* was completely inhibited for five days by 0.1 per cent β-naphthoxy acetate; with other chemicals there was decided inhibition at 0.1 and 0.01 per cent. The growth of *S. aureus* and *S. lutea* is shown

in Table I. *S. indica* and *S. marcescens* did not appear at all affected by 0.1 nor *E. coli* by 0.2 per cent of growth-regulators.

Growth of *C. diphtheriae* and *B. abortus* also appeared retarded for a period with growth equal to that of the control being obtained upon continued incubation. Complete inhibition for three days was obtained at 0.2 and 0.1 per cent. These results are shown in Table II.

Test plates of molds were observed over a 10-day period. *P. notatum* and *Mucor* sp. were inhibited somewhat at a concentration of 0.1 per cent. There

TABLE I

THE EFFECTS OF SODIUM SALTS OF GROWTH-REGULATORS ON THE GROWTH AND PIGMENT PRODUCTION OF *S. aureus* AND *S. lutea*

MEDIUM; SALT IN PER CENT	*S. aureus*			*S. lutea*		
	1 Day	2 Days	5 Days	1 Day	2 Days	5 Days
Control	3	4	4	4	4	4
β-indole butyrate						
0.1	1	2	4	0	0	2D
0.01	1	3	4	0	2	2D
0.001	1	3	4	0	2	2D
0.0001	2	4	4	0	2	3D
0.00001	2	4	4	0	2	3D
0.000001	2	4	4	–	1	3D
2-4-dichlorophenoxy acetate						
0.1	0	0	1	0	–	–D
0.01	1	1	1	0	1	3D
0.001	2	3	4	0	1	3D
0.0001	2	3	4	0	1	4D
0.00001	2	3	4	0	1	4D
0.000001	3	3	4	0	1	4
β-naphthoxy acetate						
0.1	0	0	0	0	1	3
0.01	–	2	3	–	2	3
0.001	1	2	3	0	2	3D
0.0001	2	3	4	0	2+D	3
0.00001	2	3	4	0	2+	3D
0.000001	2	4	4	1	2+	3

D = Deeper pigment than on control.
Growth: 4 = excellent; 3 = good; 2 = fair; 1 = poor; — = a few scattered colonies; 0 = no growth.

was marked inhibition of growth and sporulation of *A. niger* at 0.1 and 0.01 per cent of the acetates.

There was significant inhibition of *S. griseus* and *S. lavendulae* at concentrations of 0.1 and 0.01 per cent of the salts tested. It was observed that the pigment of *S. lavendulae* was much lighter on all media containing the salts; at a concentration of 0.1 per cent β-naphthoxy acetate and β-indole butyrate, and at 0.01 per cent 2-4-dichlorophenoxy acetate, no pigment was observed. This indicates a decrease in sporulation, since the pigment of *S. lavendulae* is due to spores. On the control plate good pigmentation was obtained.

It was decided to attempt to determine more accurately the effect on the growth of bacteria of the chemicals under test. For this purpose the turbidities of broth cultures during incubation were determined by the use of the Evelyn photoelectric colorimeter. Growths of *S. aureus*, *B. subtilis*, *C. diphtheriae*, *B. abortus* and

TABLE II

THE EFFECTS OF SODIUM SALTS OF GROWTH-REGULATORS ON *C. diphtheriae* AND *B. abortus*

MEDIUM; SALT IN PER CENT	*C. diptheriae*			*B. abortus*		
	1 Day	2 Days	3 Days	1 Day	2 Days	3 Days
Control	4	4	4	1	4	4
2-4-dichlorphenoxy acetate						
0.2	0	0	0	0	0	0
0.1	0	0	0	0	0	0
0.01	2	3-	4	1	3	3
0.001	3	3	4	1	4	4
0.0001	3	4	4	1	4	4
β-naththoxy acetate						
0.2	0	0	0	0	0	0
0.1	0	0	0	0	0	0
0.01	3	3	3	1	3	3
0.001	3	3	3	1	3	4
0.0001	3	3	3	1	4	4
4-chlorophenoxy acetate						
0.2	0	0	0	0	0	0
0.1	0	0	0	0	0	0
0.01	3	3	4	1	3	3
0.001	3	3	4	1	4	4
0.0001	3	3	4	1	4	4

Growth: 4=excellent; 3=good; 2=fair; 1=poor; — a few scattered colonies; 0=no growth.

TABLE III

THE EFFECT OF SODIUM SALTS OF GROWTH-REGULATORS ON *B. subtilis* IN LIQUID MEDIA

MEDIUM; SALT IN PER CENT	PHOTOMETRIC DENSITY				
	18 Hours	24 Hours	42 Hours	66 Hours	90 Hours
Control	.0580	.0605	.1249	.1675	.1871
4-chlorophenoxy acetate					
0.2	.0132	.0177	.0177	.0177	.0177
0.1	.0132	.0132	.0132	.0132	.0132
0.01	.0304	.0315	.0505	.0757	.1427
0.001	.0177	.0177	.0667	.1135	.1487
0.0001	.0223	.0315	.0706	.1249	.1739
0.00001	.0269	.0315	.0915	.1367	.1675
2-4-dichlorophenoxy acetate					
0.2	.0044	.0044	.0044	.0044	.0044
0.1	.0044	.0044	.0066	.0088	.0088
0.01	.0223	.0315	.0505	.1024	.1549
0.001	.0315	.0410	.1024	.1337	.1580
0.0001	.0362	.0410	.1024	.1302	.1659
0.00001	.0386	.0458	.1079	.1487	.1739
β-naphthoxy acetate					
0.2	.0044	.0066	.0088	.0177	.0177
0.1	.0088	.0132	.0132	.0132	.0132
0.01	.0339	.0362	.0655	.0969	.1308
0.001	.0269	.0362	.0862	.1024	.1739
0.0001	.0315	.0362	.1051	.1534	.1739
0.00001	.0458	.0505	.1367	.1612	.1739

S. lutea were markedly inhibited by 0.1 and 0.2 per cents of the salts and progressively less by lower concentrations. Typical data are presented in Table III for *B. subtilis*.

The only instance of stimulation of growth by low concentrations of chemical was noted for *E. coli* by 4-chlorophenoxy acetate. These data are presented in Table IV.

In order to determine whether the increase in turbidity actually represented an increase in the numbers of viable cells in the media containing small amounts

TABLE IV

THE EFFECT OF 4-CHLOROPHENOXY ACETATE ON GROWTH OF *E. coli*

MEDIUM; SALT IN PER CENT	PHOTOMETRIC DENSITY			
	24 Hours	48 Hours	72 Hours	96 Hours
Control	.0757	.2076	.2182	.2255
4-chlorophenoxy acetate				
0.2	.0315	.0580	.1249	.1308
0.1	.0505	.0969	.1580	.1580
0.01	.0555	.1739	.2182	.2255
0.001	.0757	.2111	.2441	.2460
0.0001	.0706	.2147	.2403	.2480
0.00001	.0731	.2247	.2441	.2441
0.000001	.0783	.2218	.2441	.2480
0.0000001	.0783	.2218	.2441	.2499

TABLE V

CORRELATION BETWEEN PHOTOMETRIC DENSITY AND NUMBERS OF VIABLE CELLS OF *E. coli*

MEDIUM	NO. ORGANISMS PER ML.		DENSITY	
	*48 Hours	96 Hours	48 Hours	96 Hours
Control	200 M.**	370 M.	.2076	.2255
4-chlorophenoxy acetate 0.0001%	300 M.	840 M.	.2147	.2480
4-chlorophenoxy acetate 0.0000001%	500 M.	890 M.	.2218	.2499

* Incubation time of tube culture when sample was plated.
** M=million.

of 4-chlorophenoxy acetate, serial dilutions from representative tubes were made and plated in duplicate in nutrient agar. After 24 hours incubation, colony counts were made. Results showed a good correlation between numbers of organisms per ml. and photometric densities as is illustrated by Table V.

In general, turbidity determinations indicate that there is a period during which these chemicals in low concentrations inhibit growth, but that after prolonged incubation, growth equal to or nearing that in the control media is obtained. The results described above on the effect of 4-chlorophenoxy acetate on growth of *E. coli* are the exception. This was the only case in which a significant stimulation of growth was obtained.

Biochemical reactions.—The biochemical reactions of the microorganisms tested did not appear to be affected by growth in the presence of the test chemicals. There was one exception to this; *E. coli* in 0.1 per cent 4-chlorophenoxy acetate lactose broth produced acid but no gas. On transfer from the test solution to a tube of plain lactose broth, the organism formed both acid and gas as is customary for this bacterium. Reactions in the other media tested were not changed.

Development of resistance.—Three organisms were tested for ability to acquire resistance to these chemicals; they were *B. subtilis*, *S. aureus*, and *S. lutea*.

The parent strain of *B. subtilis* grew at a maximum concentration of 0.04 per cent β-naphthoxy, 4-chlorophenoxy, and 2-4-dichlorophenoxy acetates in 24 hours. Subsequent inoculations into higher percentages gave growth in 0.06 per cent but not in 0.07 per cent except with 4-chlorophenoxy acetate. It should be noted that although *B. subtilis* did not grow in 0.07 per cent solutions, growth was obtained in plain broth inoculated from tubes of this concentration which had been inoculated with the bacterium, indicating inhibitory rather than bactericidal effect of the acetates.

S. lutea grew in media containing 0.03 per cent solutions of the chemicals. Growth was obtained in 24 hours in 0.04 per cent when transferred from 0.03 per cent solutions. After four transfers through media containing 0.04 per cent acetates, growth was obtained in 0.05 per cent solutions. No growth was obtained in concentrations of 0.06 per cent.

S. aureus grew well in a concentration of 0.05 per cent of each of the three salts in 24 hours. Transfers into higher concentrations produced no growth above 0.07 per cent until the fifth day of incubation, when in tubes of 0.08 per cent acetates, growth was observed.

Morphologic studies.—Smears from both solid and liquid media showed no change in morphology or staining reaction by Gram's method. In the stains made at each turbidity reading, there was apparently no change in cell shape or grouping at any time during the incubation period. Methylene blue stains of *C. diphtheriae* showed granules on smears from control cultures and on those of organisms grown in the presence of the growth-regulators.

No change in the gross morphology of the molds and actinomycetes was noted with the exception of the apparent decrease in sporulation of *A. niger* and *S. lavendulae*.

DISCUSSION

Both streak plates and broth cultures showed that the growth of *S. aureus*, *B. subtilis*, *C. diphtheriae*, *B. abortus*, and *S. lutea* was almost completely inhibited by 0.1 per cent 4-chlorophenoxy acetate, β-naphthoxy acetate, and 2-4-dichlorophenoxy acetate. On the other hand, the gram negative bacteria, *E. coli*, *S. indica*, *S. marcescens*, and *A. cloacae* grew well in a concentration of 0.1 per cent of the salts. These findings agree with the observation that, in general, bacteriostatic agents are more inhibitory to gram positive than they are to gram negative bacteria and specifically with Culler's (1946) data for 2-4-dichlorophenoxy acetate.

In broth cultures and on streak plates, *B. subtilis*, *S. lutea*, and *S. aureus* seemed to be greatly inhibited for a period of time, but on continued incubation growth nearly equal to that of the control was obtained. Apparently, these bacteria became adapted to the chemical. Tests to determine ability to acquire resistance to the salts were performed on these three organisms, and it was found that they did adapt themselves to growth in the presence of 0.2 to 0.3 per cent increase in the amounts of the acetates.

Many investigators have reported stimulation of growth of higher plants by the chemicals used in this investigation. The only case of apparent stimulation which we observed was that produced with *E. coli* by 4-chlorophenoxy acetate in

concentrations of 0.0001 per cent and lower. Here a significant increase in number of organisms was confirmed by plate counts and by turbidity determinations. Perhaps, extremely low concentrations of the other chemicals might stimulate growth of this and other organisms but no evidence was obtained to support this.

Changes in biochemical reactions were not observed except in one case. When *E. coli* was grown in lactose broth with 0.1 per cent 4-chlorophenoxy acetate added, acid but no gas was produced. Since hydrogenlyase is responsible for the production of gas from formic acid in the dissimilation of carbohydrate by *E. coli*, this enzyme may be inhibited in the presence of the growth-regulator. However, with this one exception, no evidence was obtained for the inhibition of specific enzyme activity.

It can be seen that the results obtained with 2-4-dichlorophenoxy acetate in this investigation correlate well with those found by other workers. Little has been published on the effects of the other growth-regulators on bacterial growth.

SUMMARY

Sodium salts of β-indole butyric acid, 4-chlorophenoxy acetic acid, 2-4-dichlorophenoxy acetic acid and β-naphthoxy acetic acid, when incorporated in media, were inhibitory to the growth of several gram positive bacteria in concentrations higher than 0.01 per cent. Gram negative bacteria were less readily inhibited.

Aspergillus niger and *Streptomyces lavendulae* were inhibited by 0.1 per cent of the salts and sporulation was markedly decreased by as little as 0.01 per cent.

A limited increase in resistance to the growth-regulating substances by *Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina lutea* was obtained by culturing the bacteria in increasing concentrations of the chemicals incorporated in media.

The only instance of stimulation of growth was noted for *Escherichia coli* when grown in the presence of 4-chlorophenoxy acetate in concentrations of 0.0001 per cent or lower.

No changes in morphologic characteristics or staining reactions, as a result of growth in the presence of the chemicals, were noted.

The only alteration in biochemical reaction observed was the failure of *Escherichia coli* to produce gas when grown in lactose broth containing 4-chlorophenoxy acetate.

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