FURTHER EVIDENCE OF PERSISTENCE OF THE 2,4-D STIMULUS IN COTTON[1, 2]

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Although several reports in the literature indicate that 2,4-dichlorophenoxyacetic acid (2,4-D) or the stimulus therefrom does not normally persist in plants (**3, 4, 5, 6, 15**), it has been demonstrated by the authors that cotton is an exception to this generalization (**8, 11, 13**). This fact became apparent upon demonstration of a transmission of the stimulus into seed embryos formed subsequent to 2,4-D application. In studies (**7, 11, 12**) dealing with the effects of 2,4-D on the vegetative characters of the cotton plant, it appears that persistence is also shown. This paper deals with further experiments designed to show the continuing activity of 2,4-D in vegetative organs of the cotton plant as evidenced by (a) malformation and (b) recovery of the growth substance.

Experimentation and results

Plants in experiments 1 to 3 of this investigation were grown in 2- or 3-gallon jars of fertile Houston Black clay at College Station, Texas. Aqueous solutions of the sodium salt of 2,4-D, expressed in acid equivalent amounts, were utilized in all instances. Grasselli spreader was added at the rate of 2 ml. per liter of solution. The plants utilized in experiment 4 were grown in flats of fertile loam at Chicago, Illinois. The free acid of 2,4-D was utilized in this latter experiment. In all experiments the plants were grown under greenhouse conditions.

EXPERIMENT 1.—This experiment was performed to observe the malformation of leaves, both as to degree and number, on the main stem of cotton plants treated with known amounts of 2,4-D. Seeds of Stoneville 2B cotton, *Gossypium hirsutum* L., were planted February 27, 1950, and a series of five uniform plants was treated at the developmental stages of anthesis (May 5) and fruiting (May 20) with 0.04 mg. of 2,4-D per plant. This amount of 2,4-D was applied in 5 ml. of solution by spraying the entire plant, care being taken to prevent run-off. These two developmental stages were as characterized in previous publications (**12, 13**). At the time of treatment,

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plants sprayed at anthesis had produced 13 main stem leaves while those sprayed at the fruiting stage had 17. By July 7, plants of both series had produced a mean of 23 main stem leaves and a majority of the bolls on the plants had matured. Following boll maturity the plants again characteristi-

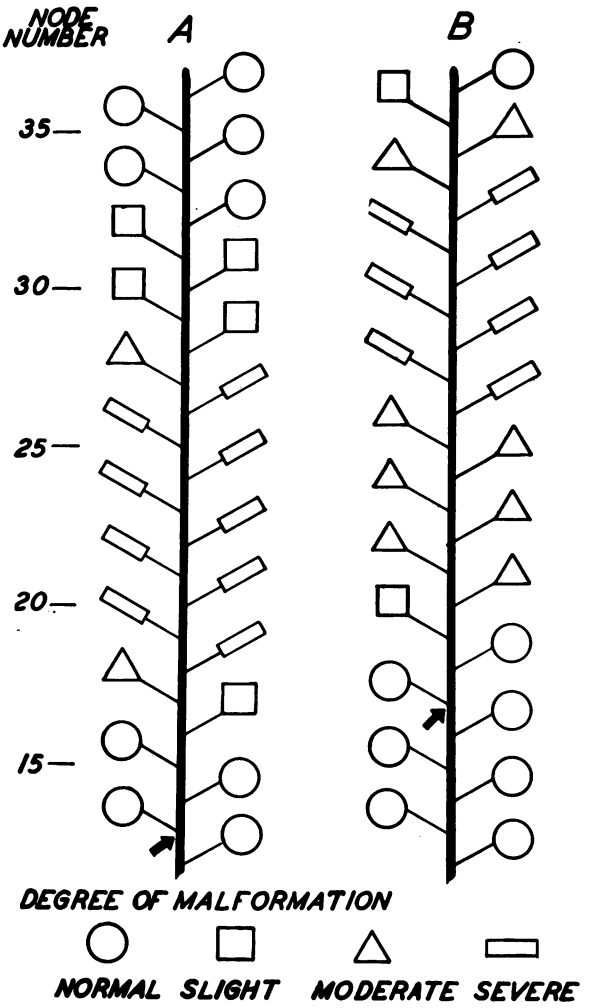

FIG. 1. Diagrammatic representation of malformation of leaves on main stem of Stoneville 2B cotton plants treated at anthesis (A) and fruiting (B) with 0.04 mg. of 2,4-D. Arrows indicate terminal macroscopic node at time of treatment.

cally exhibited rapid main stem elongation and leaf initiation. On September 22, plants of both series had produced a mean of 33 main stem leaves and were in their second flowering period. By December 1 only three additional main stem leaves per plant had been produced of which only the latest formed was free of malformation. During the time that this experiment was

in progress, plants treated at anthesis produced a mean of 16 malformed main stem leaves and those sprayed at fruiting had produced 17. The degree of malformation of these leaves is illustrated in figure 1. The various degrees of severity are as characterized in a previous publication (**12**).

This experiment was repeated in the period between November 27, 1950, and June 1, 1951. The plants grown during this time produced a mean of 15 malformed main stem leaves. The pattern of leaf malformation was similar to that observed in the earlier experiment (fig. 1).

EXPERIMENT 2.—Since it has been pointed out by numerous workers that the major portion of 2,4-D applied to leaves is taken up within a short time after application (**2, 14, 17**), it would appear that the response observed in experiment 1 was sufficient to demonstrate the persistence of the 2,4-D stimulus. The possibility existed, however, that the response was not due to persistence but to continued 2,4-D absorption from the initial leaf surface application. An additional factor which might account for the above noted response would be the presence of sufficient leaf primordia within the terminal bud to delay the appearance of an effect that occurred at the time of 2,4-D application. Although this latter possibility seemed remote on the basis of published data concerning vegetative buds (**9**), it too could not be eliminated without checking. Consequently this experiment was conducted to determine whether or not these two factors had an influence on the apparent persistence. Stoneville 2B cotton plants were treated at the floral primordia stage (**12, 13**) on December 19, 1950, with 0.01 mg. of 2,4-D per plant. Application in this case was made within lanolin rings on the third and fourth true leaves, 0.05 ml. of a 100 p.p.m. solution being placed on each leaf. After intervals of 1, 2, 3, 5, and 12 days the treated leaves were removed from a series of plants by cutting the petioles at their junction with the leaf blade. The leaves on an additional group were allowed to remain on the plants for the duration of the experiment (82 days). Nine plants were included in each series. Plants of all series had ceased to produce malformed main stem leaves by March 11, 1951, at which time final observations were made. By this time, also, the plants had passed through one reproductive cycle and many bolls had matured.

Under the conditions of this experiment, it is apparent from the data in table I that 2,4-D absorption by the plants continued over a period of about five days. It was also observed that the number of malformed leaves formed was greater with application of 0.04 mg. of 2,4-D per plant than when 0.01 mg. was applied. With the 0.01 mg. application the maximum number of main stem leaves malformed was nine.

At the time of the 2,4-D application, dissection of terminal main stem buds of a series of plants indicated the presence of five to six leaves or leaf primordia. The largest two or three of these (maximum length approximately 11 mm.) possessed macroscopic petioles. By observing treated plants, it was determined that one or two of the larger leaves still within the bud at the time of treatment did not show any malformation upon expan-

TABLE I

INFLUENCE OF TIME ALLOWED FOR 2,4-D ABSORPTION ON NUMBER OF MALFORMED MAIN STEM LEAVES PRODUCED BY STONEVILLE 2B COTTON PLANTS.

Days	Number of malformed leaves	Total number of nodes	Leaves produced after treatment
1	1.1	19.2	10.8
2	2.0	20.8	12.4
3	4.7	19.4	11.1
5	7.9	18.9	10.3
12	8.0	19.7	10.9
82	9.0	19.8	10.9

sion. Assuming that the plants which were treated with 2,4-D had terminal buds equivalent to those dissected, the conclusion is indicated that the largest young leaf to be malformed was approximately 4 to 5 mm. in length, and the maximum number of leaves or leaf primordia present within the bud at the time of treatment that could be malformed was four or five.

EXPERIMENT 3.—Since it had been previously shown that the 2,4-D stimulus is translocated into the developing seed embryos during fruit development, it appeared desirable to determine the extent to which this stimulus would continue to make its presence evident in main stem leaf malformation if flowering was prevented. Thus a short-day variety of *Gossypium hirsutum* L., Marie Galante, was selected and grown in a 24-hour day by extending natural day length with incandescent lights. The seeds were planted on May 25, 1951, and the resultant plants treated on June 27. Two treated series of 12 plants each, receiving approximately 0.02 and 0.04 mg. of 2,4-D per plant, were utilized. The leaf blade dip method of 2,4-D application as described by ERGLE and DUNLAP (7) was used. After five days the treated leaves were cut off midway along the petioles. Vegetative laterals produced by the plants during the course of the experiment were pruned off as they developed.

Dissection of terminal main stem buds at the time of treatment revealed six to seven leaves or leaf primordia present, the largest being 5 mm. in length. This was small compared with the young leaves of the Stoneville variety at time of treatment (11 mm.). The largest leaves present in the terminal bud of the Marie Galante variety were often observed to be malformed upon expansion, particularly with the 0.04 mg. application of 2,4-D. The sequence up the main stem of increasing severity of leaf malformation was similar to that previously indicated for the Stoneville variety (12). One significant difference in this instance was noted, however. With Marie Galante cotton, terminal stem growth did not cease once the leaves became very severely malformed, but rather leaf initiation continued, with as many as 16 or 17 very severely malformed leaves being produced before the start of a regressive series in severity of leaf malformation.

This experiment was terminated on September 14 at which time none of the treated plants were yet producing normal main stem leaves. In the

interval between treatment with 2,4-D and termination of the experiment (80 days) plants receiving 0.02 mg. or 0.04 mg. of the growth-regulator produced a mean of 26 malformed leaves (fig. 2). Of this number, 24 were produced after the 2,4-D treated leaves were removed from the plants. Of the malformed leaves produced, 13 and 16 for 0.02 and 0.04 mg., respectively, were of the very severe type. These latter figures do not represent the maximum number of very severely malformed leaves that would have

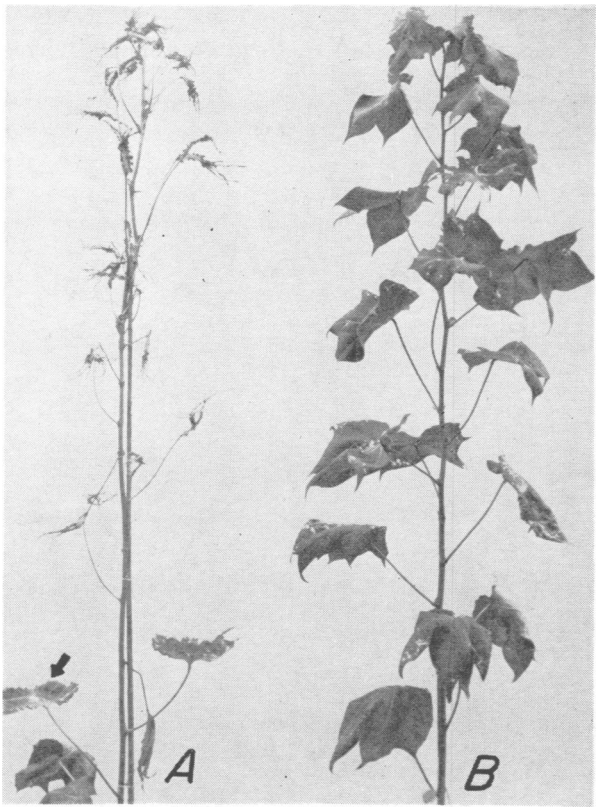

FIG. 2. Top portion of Marie Galante cotton plants, (A) treated with 0.04 mg. of 2,4-D and (B) untreated. Arrow indicates smallest expanding leaf at time 2,4-D treated leaf was removed from plant.

been produced had the plants been allowed to continue growth, for 18% of the plants of the 0.02 mg. series and 42% of those of the 0.04 mg. group were still initiating leaves with this degree of malformation at the termination of the experiment.

EXPERIMENT 4.—At the termination of the preceding experiment, the top two macroscopic nodes plus the terminal buds of plants of the 0.04 mg. series were preserved in anhydrous ether. On December 18, 1951, this material was ground in a mortar with 50 ml. of peroxide-free ether acidified with 2 ml. of 1 *N* HCl. After grinding, this material was placed in a stoppered

flask and allowed to stand at room temperature until January 28, 1952, at which time the ether was filtered off and made to volume. A known amount of lanolin was dissolved in an aliquot of this ether extract and then the ether was evaporated. Weighed amounts of the preparation were applied to the cotyledons of a series of 16-day-old Stoneville 2B cotton seedlings. The first true leaves of these seedlings were still within the apical bud at the time of the lanolin applications. The second day following treatment, the cotyledons showed moderate epinasty and in a week the expanding true leaves showed malformation. The leaf and stem malformations exhibited by these seedlings after a month were characteristic of those induced by 2,4-D (fig. 3).

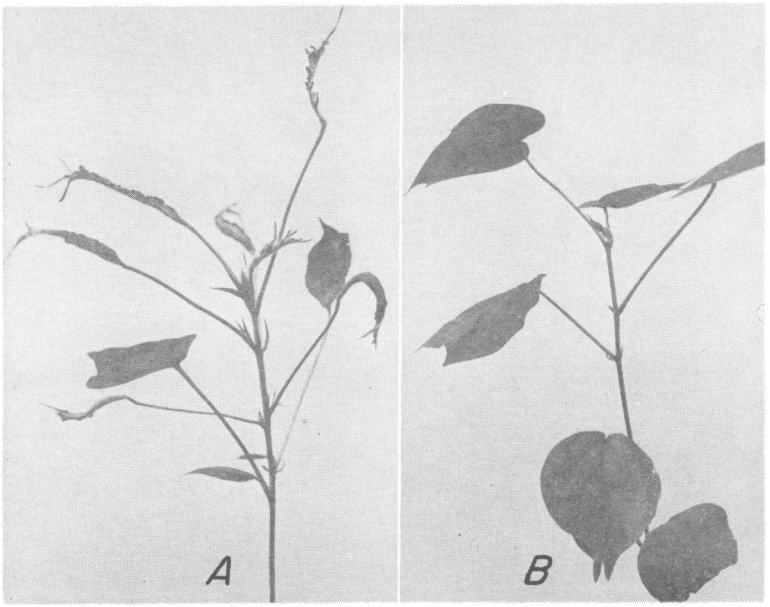

FIG. 3. Stoneville 2B cotton 30 days subsequent to treatment with (A) extract from 2,4-D treated Marie Galante plants and (B) extract from untreated Marie Galante plants.

Two and one-half months after the extract application, the top leaves of the treated plants were very severely malformed and the terminal meristems had ceased to initiate new leaves (**12**). In this interim the plants had produced a mean of seven leaves. After removal of the cotyledons to prevent contamination by any residual growth substance remaining in the lanolin smear, the shoots of these plants were extracted with acidified ether in the manner already described, an aliquot mixed with lanolin and smears were applied to a second series of seedlings. Again the seedlings exhibited characteristic 2,4-D malformation. One and one-half months later, the shoots of these seedlings (minus cotyledons) were harvested and extracted with acidified ether. These seedlings had also produced very severely malformed main stem leaves and terminal stem growth had ceased. A mean of seven

leaves per plant had been produced. Application of this extract to a third series of cotton seedlings likewise produced malformations characteristic of 2,4-D.

Extracts of normal plants were also made. When cotton seedlings were treated with these extracts, applied in lanolin, in no instance were any malformations observed.

Three months subsequent to the initial application of lanolin-extract mixture, another preparation was made from an aliquot of the Marie Galante extract to be checked against a series of lanolin applications containing known amounts of 2,4-D. Stoneville 2B cotton seedlings were again utilized in these bio-assays. After one month the plants treated with the extract were compared with those subjected to known amounts of 2,4-D. Using the degree of plant malformation as an index, it was calculated that the top two nodes plus the terminal bud of each of the Marie Galante plants (from experiment 3) yielded growth substance equivalent to 0.006 mg. of 2,4-D. If this growth substance was actually 2,4-D, it represents approximately 15% recovery of the amount originally applied to the leaves of the Marie Galante plants after they had grown for a period of three months and had produced 26 leaves. To eliminate the possibility that the observed effects were due to an endogenous growth regulator which had been greatly increased in concentration by 2,4-D application, determinations of the alkali and acid lability of the extract were made. After standing in 13 *N* sulfuric acid or after boiling in 1 *N* sodium hydroxide, no diminution of activity of the extracted substance was noted, indicating that this material was not any of the known naturally occurring growth substances (**17**).

Discussion

In this study it was found that the 2,4-D stimulus persisted in cotton plants for periods in excess of six months (experiment 1). As indicated by other workers (**5, 15**), this time interval is not too significant as an index to persistence if it represents a period in which the tissues being checked are in a relatively inactive or dormant state. In this instance, however, the cotton plants continued to exhibit growth. Demonstration of persistence of the stimulus in the vegetative growth of the Stoneville 2B variety was complicated by the reproductive phase, for fruiting results in reduced vegetative growth. With this curtailment of activity in the vegetative meristems and concurrent shift in loci of high metabolism to the reproductive structures, the 2,4-D stimulus is known to move into the developing bolls (**8, 11, 13**). In such cases, although the time interval over which the action of the growth regulator will show up is extended, the number of main stem leaves that will be malformed is not as great as if flowering had not occurred. This is illustrated in the results of experiment 3 in which a far greater number of main stem leaves were malformed in much less time.

It is quite obvious from this study that 2,4-D or its stimulus is long continuing in the cotton plant in that the number of malformed leaves initiated

greatly exceeded the number of leaves or leaf primordia present in the bud at the time of treatment with 2,4-D (experiments 2 and 3). With cotton, as was observed by WATSON (**16**) for beans, the larger leaves or leaf primordia present in the bud at time of treatment showed the least malformation. Because of the persistence of the 2,4-D stimulus in cotton, it is uncertain which size of leaves or leaf primordia are most vulnerable to the action of 2,4-D. It would seem, though, based on the sequence of leaf malformation, that the smaller the primordia the more severely it is affected. This malformation sequence, however, may be due to a greater amount of 2,4-D being present within the bud at the later periods of leaf initiation, thus the smallest primordia need not necessarily be the most vulnerable to 2,4-D action to obtain the results observed. The observation by BURTON (**1**) that more malformed leaves were initiated in cotton than observed in beans is undoubtedly accounted for, in part, by the longevity of the 2,4-D stimulus.

Whether a young leaf within the apical bud will be malformed by 2,4-D is determined to some extent by the amount applied (**12**). In experiment 3, for example, it was observed that 50% of the plants treated with 0.02 mg. of 2,4-D produced normal leaves after treatment, whereas only 8% of the plants subjected to 0.04 mg. did so.

The degree of persistence within the cotton plant (as measured by malformation of leaves) is to some extent determined by the amount of 2,4-D originally applied. For example, with Stoneville 2B cotton 16 to 17 main stem leaves were malformed if 0.04 mg. was applied whereas only 9 were distorted if the amount applied was 0.01 mg. (experiments 1 and 2). This is also shown in experiment 3 in which a greater percentage of the plants treated with 0.04 mg. of 2,4-D were showing very severely malformed terminal leaves at the close of the experiment.

In addition to the malformation of main stem leaves, persistence was demonstrated in this study by the extraction of a growth regulator from the meristems of treated plants. Even after long periods of growth, measured both in period of time and number of leaves initiated, a growth substance could be extracted which caused characteristic 2,4-D malformation when applied to other cotton plants. It was not definitely established that this regulator was 2,4-D, although the possibility of its being a naturally occurring auxin has been eliminated. The present observations, however, are very suggestive that the growth regulator is 2,4-D or some derivative thereof. On the other hand, since other compounds have been observed to induce 2,4-D-like leaf malformations in cotton (**10**), the possibility of its being some metabolite whose formation was 2,4-D-induced cannot be eliminated without further experimentation.

Summary

The persistence of the 2,4-D stimulus was demonstrated in the vegetative organs of two varieties of cotton, Stoneville 2B and Marie Galante. Evidence for this persistence was shown by (a) the period of time during which

the plants continued to produce malformed main stem leaves—up to six months, and (b) the number of malformed leaves produced—as many as 24. It was found that the number of malformed leaves formed was in excess of the number of leaves or leaf primordia in the terminal bud at the time of treatment, thus eliminating the possibility of an immediate effect not becoming evident for an extended time. That the persistence was not due to continued absorption of 2,4-D by the leaves was also established.

Persistence of the stimulus was further demonstrated by extraction of a growth-regulator from treated plants which induced cotton leaf malformations characteristic of 2,4-D. This regulator was extracted 80 days subsequent to the initial application of 2,4-D. Whether the growth-regulator was 2,4-D or a derivative therefrom was not clearly demonstrated. That this substance was not a naturally occurring auxin was established by its stability in alkali and acid solutions.

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