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Cupule Formation on Seedlings of *Galinsoga Ciliata* (RAF.) Blake and *Helianthus Annuus* L. Following Exposure to 2, 4-Dichlorophenoxyacetic Acid

By S. N. POSTLETHWAIT

INTRODUCTION

Early experiments involving the use of various growth-promoting substances were concerned generally with formative effects and morphological changes induced by these substances. Later experiments were of a more practical nature in which the hormones were tested to determine their numerous commercial applications. The results of morphological investigations disclose the most common reactions to be: (1) cell division, (2) cell enlargement, (3) differentiation of the newly formed tissues, especially into vascular structures, (4) changes in the thickness of cell membranes, and (5) organ building manifested in the development of root primordia, or rarely as bud primordia (Bausor et. al., 1940). Only a few studies have been concerned with the response of leaves to 2, 4-D (Burton, 1947, Felber, 1948). The major emphasis of other morphological reports has been placed on the response of stems and other plant organs to this hormone.

In the present paper the writer reports an unusual development of fused leaves of *Galinsoga ciliata* and *Helianthus annuus* in response to treatment with 2, 4-D. Fusion of leaves of a ten-inch treated tobacco plant was observed by Zimmerman and Hitchcock (Zimmerman and Hitchcock, 1942). The plant had been sprayed with an aqueous solution of P-chlorophenoxyacetic acid. Van Overbeek (Van Overbeek, 1947) reported "the writer was shown by Crafts at his experimental fields at Davis, California, a series of wild oat plants which had germinated in soil treated with high concentrations of 2, 4-D. Not only was the germination much reduced, but the plants that did germinate had tubular leaves with margins grown together in the same manner as found in coleoptiles and onion leaves." The brief discussion of fused leaves by Zimmerman and Hitchcock and by Van Overbeek makes it impossible to determine whether the fusion they described is the same phenomenon as the fusion described by the writer.

Because of the cup-like nature of these fused leaves the term "cupule" will be applied to the structure resulting when two or more leaves are fused along their margins to give the appearance of one organ (Fig. 1).

MATERIALS AND METHODS

Plant material for study was obtained from the following experimental series:

Series A. Four, five inch pots were filled with soil. Two hundred achenes were distributed evenly over the soil surface in each pot. The soil in three pots was treated with 200 cc. of an aqueous solution of 10, 25, and 50 ppm 2, 4-D respectively. The fourth pot, untreated, was retained as a control.

Series B. A flat 10" x 10" x 2" was filled with soil. Galinsoga achenes were sprinkled liberally on the soil and then one liter of a 25 ppm aqueous solution of 2, 4-D was applied with a fine sprinkler.

Series C. Normal Galinsoga seedlings bearing 6 to 8 expanded leaves were sprayed with an aqueous solution of 100 ppm 2, 4-D.

Series D. A flat 10" x 10" x 2" was filled with sand. Approximately 100 sunflower seeds were sown in the flat and then sprinkled with one liter of a 25 ppm solution of 2, 4-D.

Series E. Normal sunflower seedlings bearing 2 expanded epicotyledonary leaves were sprayed with an aqueous solution of 2, 4-D.

Aqueous solutions of 2, 4-D were prepared from a commercial product containing 14% 2, 4-Dichlorophenoxyacetic acid. Galinsoga achenes were obtained from plants growing naturally outdoors. All experiments were conducted under usual greenhouse conditions.

Photographs were taken and collections made at regular intervals. Histological material was killed and fixed in F. A. A., dehydrated in butyl-alcohol, sectioned at 10 μ to 15 μ and stained with safranin and fast green.

OBSERVATIONS AND RESULTS

External appearances

Series A and B. The preliminary experiments (series A) revealed that more cupules were produced in soil treated with 25 ppm than in either lot treated with 10 or 50 ppm 2, 4-D, consequently, further experiments (series B) were conducted using this concentration of 2, 4-D.

Cupules could be observed with the naked eye 10 to 15 days after germination. Approximately 50% of the seedlings produced cupules while the remainder showed little or no visible injury.

The cotyledons in no case fused to form the cupule. The cupule was formed by the fusion of the first two leaves above the cotyledons. Leaf margins at the tip of the cupule did not fuse, resulting in the formation of a small opening or "ostiole" (Fig. 2). Frequently one edge only of 2 leaves was fused (Fig. 1). Less fre-

quently, fusion occurred some distance inward from the margins of the leaves (Fig. 3).

Seedlings in which the cupules were forming could be distinguished readily from those not developing cupules by observing the color and shape of the cotyledons. The cotyledons of the cupule-forming seedlings were always a darker blue-green in color and more elongated than those of normal seedlings.

The total surface of the cupules was never as great as that of two fully expanded normal leaves of the same age. The petioles of the two fused leaves commonly unite to form a cylinder or tube, bearing the cupule on its summit and whose base surrounds the stem apex. As seedling growth continues the cupule no longer enlarges. This new growth usually bunches up within the fused petioles and finally forces its way out through one side, apparently by mechanical rupture of the petiole tissue (Fig. 2). The cupule is pushed aside by the new growth and soon dies. A second cupule was not formed on any seedling.

Series C. *Galinsoga* plants of this series occasionally produced fused leaves. The plants at the stage of growth used in this series apparently reacted to a solution of 100 ppm 2, 4-D in much the same manner as did the seedlings of series B to a 25 ppm solution. In series C the relative length of leaf margin fused, was less, and the entire cupule was frequently larger than those formed in series B. The petioles of the fused leaves were united, forming a hollow cylinder in which the apical meristem continued its growth. This later growth forced its way to the exterior of the cupule either by a mechanical break in the petiole tissue, shoving the cupule to one side, or by merely pushing upward through the cavity formed by the cylinder-like fusion of the petioles and leaf blades.

Series D. Seedlings emerged from the soil six days after planting. Treated seedlings grew much slower than the control seedlings and were a darker green in color. The first pair of epicotyledonary leaves seldom fused, were much reduced in size (Fig. 5) and bore very little chlorophyllous tissue. The second pair of cotyledonary leaves (Figs. 5 and 6) were fused in approximately 20% of the treated seedlings.

Further growth of the sunflower seedlings bearing cupules was similar to that of *Galinsoga* described in series B. The apical meristem continued to grow, emerging either from the cavity produced by the fused petioles and laminas (Fig. 5) or by a mechanical break through the fused petioles. In the latter case the cupule was

forced aside as the stem continued to elongate (Fig. 7) and did not enlarge further during the course of the experiment.

Series E. Cupule formation in this series appeared similar to cupule formation observed in Galinsoga, series C. The stem apex often continued to grow from the center of the cupule to form a perfoliate structure. In other cases the growing stem tip burst through the fused petioles, pushing the cupule aside. The cupules (Fig. 4) were formed by the fourth or fifth pair of leaves, on the treated plant, i.e. the third or fourth pair of leaves expanding after treatment. The progression of events following cupule formation was the same as that described for plants of the previous series.

HISTOLOGICAL RESPONSES

Series A and B. Serial sections were made transversely and longitudinally through cupules on Galinsoga stem tips and through stem tips of control plants. Sections were made at various stages of growth in order to follow the course of cupule development. The apical meristem of normal Galinsoga in longitudinal section is approximately 100 μ in width and 50 μ in height. Leaf primordia are produced terminally, the entire apex apparently being involved in the differentiation (Fig. 9). Primordia are oppositely arranged and expand very rapidly. Leaves are frequently half expanded by the time the next pair of primordia are differentiated. No portion of the leaves of the normal seedling were fused.

Cupules at a very early stage (Fig. 8) have the vascular bundles oriented opposite each other in 2 groups of 3 each (Fig. 10). An "upper" epidermis and a "lower" epidermis are differentiated but there is no sign of separation of the two petioles at the region where normal separation would occur. The vascular bundle groups remain opposite each other and continue as normal ramifications into the lamina. The bundles that would ordinarily occur in the midribs occupy positions opposite each other in the cupule. There is no histological evidence to indicate demarcation of the leaf margins in either the fused petiole or lamina tissue.

In longitudinal section (Fig. 12) the apical meristem is completely surrounded by the fused petioles. These fused petioles form a small elongated cavity directly above the meristem. This space is the result of partial differentiation of epidermis on what would ordinarily have been the adaxial side of the petiole. Numerous plant hairs develop inside the cavity thus formed.

No other abnormal meristematic activity is evident and elongation of cells appears to proceed in a normal fashion. Palisade and

spongy tissue differentiate in a properly oriented manner despite the variation in position of the leaf blade.

Series C. The formation of cupules in this foliage treatment series was infrequent. Complete studies have not been made but evidence indicates that these cupules are the result of the same phenomenon as those formed in series A and B and that the histology would be similar.

Series D and E. Serial sections were made transversely and longitudinally through cupules borne on the stem tip of treated sunflower seedlings and through stem tips of control plants. Sections were made at various stages of growth in order to follow the course of cupule development.

During the early stages of growth of sunflower seedlings the leaves are oppositely arranged but as the plant becomes older the phyllotaxy changes, becoming alternate (spiral). The apical meristem during the first stage of leaf arrangement is similar to that of *Galinsoga*, however, not all of the apical meristem is involved in the differentiation of leaf primordia and the leaves expand relatively slower than in *Galinsoga*.

Microscopic sections show that the course of cupule development is almost identical with that of cupule development in *Galinsoga*. The leaf midribs are opposite each other (Fig. 13) and there is no indication of a break in the lamina tissue between the two midribs. A cavity (Fig. 14) is formed by the fused laminae during the course of expansion of the cupule.

The fusion phenomenon was the only abnormality observed in the pair of leaves forming the cupule. Palisade, spongy, and vascular tissues were normally differentiated and oriented.

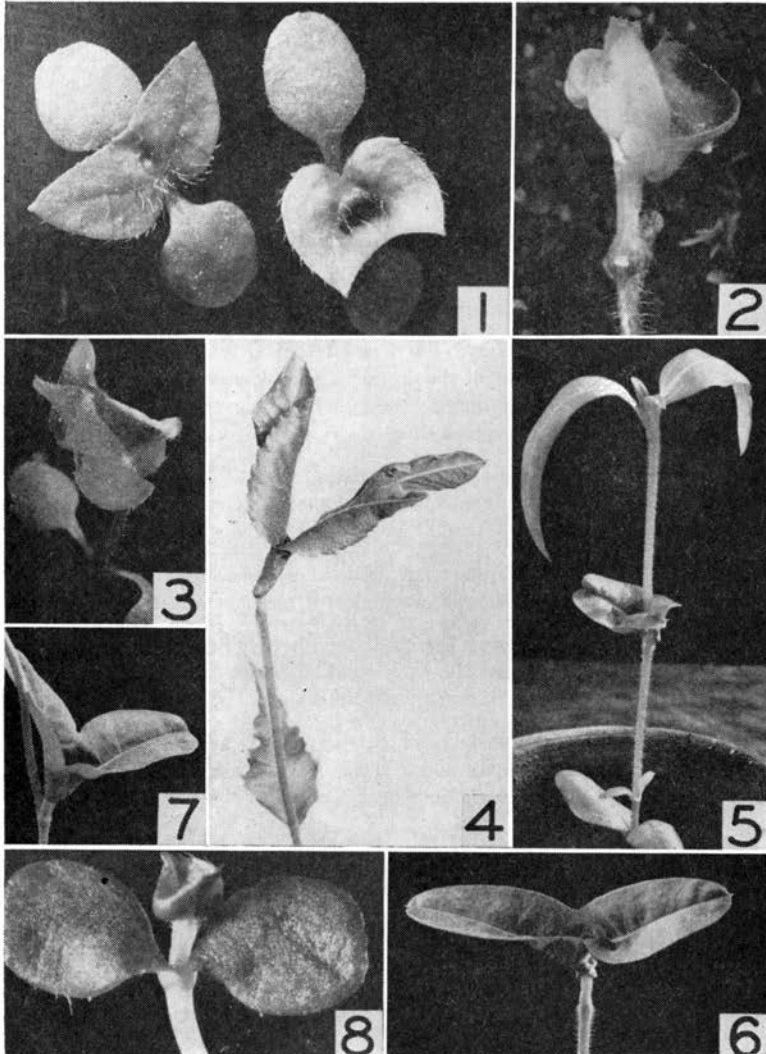
DISCUSSION AND CONCLUSIONS

The experimental production of fused leaves (cupules) has been reported infrequently in the literature (Zimmerman and Hitchcock, 1942, Van Overbeek, 1947). The occurrence of this phenomenon, as described by the writer, is closely correlated with exposure of plants to a sub-lethal concentration of 2, 4-D. The experimental plants must have oppositely arranged leaves. It is believed that the proximity of the margins of leaf primordia in opposite leaved plants makes possible the fusion of these margins by altering the destination of the intervening cells in such a way to form a circle of meristematic tissue. Growth of this circle of cells forms a cupule.

In Craft's experiment (Van Overbeek, 1944), high concentrations of 2,4-D were applied to the soil. "Tubular leaves" were

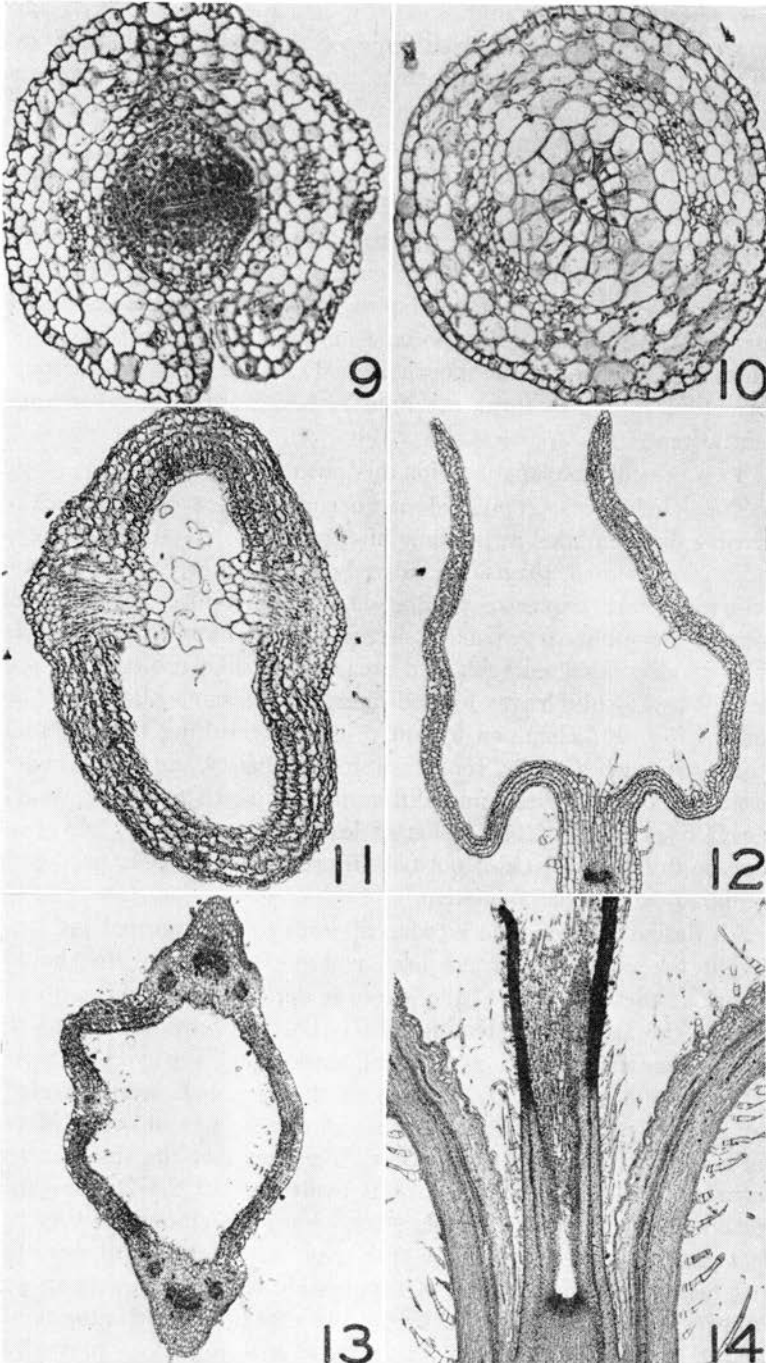
Explanation of Plate I

1. Fused leaves on *Galinsoga ciliata* seedlings from pre-emergence treatment series. Note that leaf margins were fused on one side only.
2. *G. ciliata* from pre-emergence treatment series bearing leaves fused to form a cupule. Note emergence of new growth through a break in the fused petioles.
3. *G. ciliata* from pre-emergence treatment series bearing a cupule with the leaves fused near the center of the leaf blades instead of at the leaf margins.
4. *Helianthus annuus* from foliage treatment series bearing a cupule. Note the peculiarly shaped leaf below the cupule. This leaf was a primordium at the time of treatment.
5. *H. annuus* from pre-emergence treatment series. Note the cupule about midway up the stem. The new growth has emerged from the center of the cupule giving it a perfoliate appearance. The large cotyledons may be observed at the base of the plant and immediately above them two small injured leaves. Topmost leaves also show injury, however, later leaves show a progressively lesser effect.
6. *H. annuus* from pre-emergence treatment series bearing a typical cupule.
7. *H. annuus* cupule that has been forced aside by elongating stem. New growth emerged from a break in the fused petioles.
8. Very young plant of *G. ciliata* bearing a cupule.



Explanation of Plate II

9. Transverse section of *G. ciliata* seedling immediately above the apical meristem. Note that the margins of the young leaves are separated.
10. Transverse section through fused petioles of *G. ciliata*. Note the hair filled area in the center of the section. An epidermis has been differentiated both inside and outside the other petiole tissue. The vascular bundles are in two groups of three each oriented opposite each other.
11. Transverse section through lamina tissue of a *G. ciliata* cupule. Midribs are evident opposite each other. Palisade cells have just begun to elongate.
12. Longitudinal section through cupule-bearing apex of *G. ciliata*. Apical meristem is surrounded by the cylinder of fused petiole tissues.
13. Transverse section through lamina tissue of a *Helianthus annuus* cupule. Midribs are oriented opposite each other. No break occurs in the tissue where normal leaf separation should take place.
14. Longitudinal section through a cupule-bearing *H. annuus* seedling. Note the cavity formed directly above the apical meristem. The leaf blades appear as two heavy black lines near the top of the picture.



formed on Wild Oat seedlings. It is to be expected that a high concentration of 2, 4-D would be required to produce tubular leaves on a monocotyledonous plant, since monocots appear relatively resistant to its effects. The "tubular leaves" apparently originated much in the same manner as the coleoptile.

The tobacco plant with fused leaves described by Zimmerman and Hitchcock (Zimmerman and Hitchcock, 1942) had apparently received somewhat less than a lethal dose of 2, 4-D. Applications of 2, 4-D in concentrations high enough to be lethal seem to kill or suppress the growth of the exposed plant before new leaves are formed. There also appears to be a minimum concentration which stimulates certain other morphological changes but not cupule formation. Cupules form within a rather limited range of concentrations.

Two possible explanations for this phenomenon are:

The fused leaves (cupule) do not occur when leaves have already become differentiated at the time of exposure to 2, 4-D. The cotyledons of treated plants are already well differentiated in the achenes before exposure to 2, 4-D, and therefore never fused. Leaves incompletely expanded at the time of exposure to 2, 4-D may develop fasciated veins and areas of gall-like growths but are never fused, while leaves formed later, on the same plant, may be fused (Fig. 4). Common formative effects resulting from 2, 4-D treatment, such as galls, root primordia, epinasty, etc., are usually produced by cells becoming differentiated, meristematic and abnormally elongated. The fusion of leaves reported here, however, may be due to the lack of normal differentiation of cells produced by the affected apical meristem.

All tissues differentiate in what appears to be a normal fashion, except the epidermis of the area where the leaf margin should occur. If the epidermis of the leaves is derived from the marginal cells of the apical meristem (Fig. 9) it would appear that the 2, 4-D is demonstrating a greater influence on this particular zone of meristematic cells. If all areas of the meristem were affected equally one would expect the formation of a large gall of undifferentiated cells. This does not occur. The fact that the fusion may occur on one side only (Fig. 1) is evidence that 2,4-D does not always uniformly affect the meristem. A certain amount of torsion or twisting toward the fused side may be observed, suggesting that the 2, 4-D effect is that of suppression rather than activation. Suppression is also suggested by the fact that in no case is the total surface of fused leaves equal to the total surface of two normally expanded leaves of the same age.

Wrinkling of lamina tissue frequently occurs, forming secondary cups or cavities. This may be due to differentiation in a basipetal direction; the apex of the leaves becoming fixed in size first, then further expansion of the lamina could take place only by the bulging or wrinkling of the blade nearest the petiole. The twisting or torsion of the two leaves toward the fused side might also be partially explained in this manner. It is difficult to understand why fusion sometimes occurred nearer the center of the leaf lamina than the margin (Fig. 3). It is possible that the effect of 2, 4-D was not exerted until after a certain amount of physiological differentiation had already occurred in the plate meristem of the leaf primordium. If those cells towards the margin were physiologically differentiated beyond the point of 2, 4-D influence at this concentration, and, certain cells nearer the center were still susceptible, the margins would thus be differentiated while fusion (lack of differentiation) would occur in the areas of susceptible cells.

A second possible explanation of cupule formation may be that the activity of natural hormones already present is augmented by the addition of 2, 4-D. If this is true it would appear that there is a gradient of natural hormone distribution in the apical meristem with two points of high concentrations occurring on an axis perpendicular to the plane of leaf separation. Leaf primordia (Fig. 9) would have the greatest meristematic activity at these two points with a gradual decrease towards the margins or the two points at the edges of the plane of leaf separation. Here the natural hormone concentration is normally below the range of that inducing meristematic activity and leaf tissue is not formed at these positions. The opposite leaves thus become separated into two simple leaves.

If the natural hormone is supplemented with 2, 4-D, the additional stimulatory effect may be great enough to cause cell division at the edges of the plane of leaf separation. If this were true the cell division should occur in a complete circle about the apical meristem. All cells formed from this circle become differentiated into appropriate leaf tissue and thus a cupule is formed.

The concentration of 2, 4-D required to produce fused leaves in *Galinsoga* and *Helianthus* seedlings is very low (25 ppm) when applied to the soil prior to germination. It is not known whether the 2, 4-D is taken up by the roots or by the cotyledons and other embryo parts as they emerged from the seed coat. In the foliage treatment series of older plants as much as 100 ppm 2, 4-D is required. The higher concentration required by the older plants may be due to the total amount of 2, 4-D being apportioned among a number of apical meristems, thus decreasing its effect on each

individual one, or it may be that the apical meristems of the older plants are more resistant.

SUMMARY

1. 2, 4-D was applied to seedlings and to older plants of *Galinsoga* and *Helianthus* by treating the soil directly and by spray applications to the foliage. Concentrations used were slightly less than lethal.

2. The formation of fused leaves was studied in its gross aspects as well as microscopically. It is believed that the proximity of the margins of leaf primordia in opposite leaved plants makes possible the fusion of these margins by altering the destination of the intervening cells in such a way to form a circle of meristematic tissue. Growth of this circle of cells forms a cupule.

The conditions which give rise to cupules and the course of their formation are the same for both *Galinsoga* and *Helianthus*. Palisade, spongy and vascular tissues are differentiated in a normal fashion and are properly oriented. Later growth produced by the apical meristem emerges either directly from the center of the cupule or from a mechanical rupture in the fused petioles.

3. Two possible explanations for fusion of leaves are advanced:
- a. A suppression of differentiation by the 2, 4-D in the intervening cells of the apical meristem between margins of the leaf primordia, resulting in an uninterrupted circle of meristematic cells which produce the cupule.
 - b. The presence of 2, 4-D may augment the normal distribution of natural hormones in such a way as to produce the circle of meristematic tissue at the stem apex.

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