Formation of Double Leaf by Amo-1618 and 2, 4-D in Sesamum indicum*

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Received June 3, 1968

Abstract

When the embryo of Sesamum indicum, peeled out of seed coat and deprived of one of the cotyledons, is treated with Amo-1618 at the concentration of 200 ppm for 48 hours, the first leaf primordia develop to a double leaf on the decotylated side of the shoot apex. The formation of double leaf is restricted to the case in which one cotyledon is removed and the treatment with Amo-1618 is made within 6 hours after sowing.

2, 4-D induces gamophylls, which partly resemble the double leaf, when it is applied to the embryo deprived of one cotyledon. It also induces gamophylls in the presence of both cotyledons and even when applied to the embryos grown for 24 hours after sowing.

Histological observations on the shoot apices of the treated embryos suggest that Amo-1618 induces double leaf by causing structural changes in the apical meristem and leaf primordia, whereas 2, 4-D induces gamophyll by stimulating the interprimordial region in the peripheral zone of the shoot apex.

All other chemicals examined, CCC, IAA, NAA, GA, and MH, have no effect of producing double leaf or gamophyll when used in the same manner as Amo-1618 or 2, 4-D was.

Since Mitchell et al. investigated the plant growth reducing properties of some nicotinium compounds, various chemicals having such properties have been listed up as growth retardants, and a vast amount of investigations on the physiology of those substances have been accumulated (cf. review by Cathey, 1964). In general, growth retardants reduce stem elongation but never cause malformation or other formative changes.

A growth retardant Amo-1618 was reported to inhibit particularly cell expansion and division of the subapical meristem without inhibiting leaf initiation in Chrysanthemum morifolium. It was also shown that Amo-1618 was highly effective to sesame plant in reducing stem growth. When it was applied to sesame seedlings through soil treatment, it induced in them dwarf characters such as short internode, thick stem and dark-green leaves. Recently, Courduroux reported that apical as well as subapical cells of the buds of Helianthus tuberosus cultured for long period on a medium containing Amo-1618 were affected and the buds lost the ability to sprout and continued to form tubers.

* Partly supported by the Grant in Aid for Scientific Researches from the Ministry of Education, No. 4007.

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Some authors have shown that 2,4-D induced gamophyllous cohesion of leaves when it was applied to the bud primordium\(^8\) or shoot tip\(^9\) in several plants. It is of interest above all that Haccius\(^11\) and Haccius and Trompeter\(^12\) induced syncotyly, i.e. cohesion of cotyledons by one margin only, in *Eranthis hiemalis* by treating immature seeds with 2,4-D. The induced syncotyly strikingly resembles in various respects the state of double leaf of sesame seedling, which is formed by cohesion of the first leaf primordia when the shoot apex of embryo is incised along the intercotyledonary plane\(^13\).

In the present investigation, it is attempted to get the double leaf by treating the embryo with Amo-1618 and 2,4-D. In addition, some other chemicals including growth retardant and auxins are likewise tested. The results of application of those substances to the sesame embryos in various conditions will be presented.

**Material and Methods**

Seeds of *Sesamum indicum* were stripped of seed coat with a dissecting needle after being immersed into water for about 30 minutes. Embryos thus peeled out were treated with chemicals.

*Treatment with Amo-1618*. The treatment with Amo-1618 was carried out immediately after the embryo was peeled out, or after varying periods from the time of sowing. In the former case, embryos were immediately deprived of one of the cotyledons (Fig. 2A), and then placed on the filter paper soaked with 5 ml of 200 ppm solution of Amo-1618 in a Petri dish. The treatment was continued for 48 hours. Embryos deprived of both cotyledons and those bearing both cotyledons were also treated in the same way. When the treatment was started at varying times after sowing, embryos were kept for varying periods after stripping of seed coat on a wet filter paper with water in a Petri dish, then they were deprived of one of the cotyledons and subjected to the treatment. Eight series of treatment were started at 3, 6, 9, 12, 15, 18, 21, and 24 hours after sowing, and continued for the subsequent 48-hour period respectively.

*Treatment with 2,4-D*. 2,4-D was applied at the concentrations of 2 and 0.5 ppm for 24 hours to the embryos at 0- and 24-hour stages in the same way as Amo-1618 was done.

Following the treatment, embryos were transferred on to the wet filter paper with water in Petri dishes, allowed to grow for several days, and then transplanted on vermiculite in glass pots poured with 0.1% solution of "Hyponex"**. Whole course of culture of the embryos and seedlings, including the treatment period, was carried out at 28-30\(^\circ\) under continuous illumination at 4000 lux with day-light fluorescent tubes.

Developmental changes occurring in the shoot apices of the growing embryos and seedlings were observed every day during 10 days with a binocular microscope, and some of the apices were sampled and fixed with FAA (5 ml of formalin, 5 ml of glacial acetic acid, and 90 ml of 70% alcohol) and prepared for histological examination following the usual paraffin section procedure.

* Amo-1618: 4-hydroxy-5-isopropyl-2-methylphenyl trimethyl ammonium chloride, 1-piperidine carboxylate. 2,4-D: 2,4-dichlorophenoxyacetic acid.

** Manufactured by Hydroponic Chemical Co., Inc., Copley Ohio, U.S.A.
Other chemicals, CCC, NAA, IAA, GA, and MH*, were also tested as to the effect to produce double leaf. These were applied to the embryos deprived of one of the cotyledons in the same manner as that of Amo-application. Concentrations and duration of application are described in respective paragraphs later on.

Results

I. Effect of Amo-1618.
A. Treatment of 0-hour embryos.

Intact embryos. Embryos peeled out of seed coat were immediately subjected to the treatment with Amo-1618. In the seedlings developing from the treated embryos, growth of the stem was much reduced and the leaves became thicker than in untreated seedling. But histological examination of the shoot apex revealed that leaf initiation took place at a similar rate to the untreated seedling. The leaf arrangement in the treated plants was normally decussate, no double leaf being formed.

Embryos deprived of one cotyledon. When the embryos deprived of one of the cotyledons were allowed to grow without Amo-treatment, they grew nearly equally to intact embryos in growth rate, and the leaf arrangement did not undergo any aberration.

When the embryos were treated with Amo-1618 after one of the cotyledons was removed, a majority of developed seedlings bore a double leaf or a single leaf as the first leaf on the decotylated side of the shoot apex instead of a pair of opposite leaves (Fig. 1A-D; Table 1, 0 h). The remaining seedlings bore opposite leaves or "approached" leaves which were inserted at more or less smaller divergence angles than 180°. The smallest divergence angle observed was about 100°. As there are varied degrees of fusion and transitional forms from the double leaf to the single leaf, these two types may be reasonably regarded as belonging to the same category, the double-leaf class. There are likewise transitional forms from the opposite leaves to the most approached leaves. These leaves are classified into opposite-leaf class. Transitional forms from the approached leaf to the double leaf may be expected, but no such forms were found in practice. As described and discussed later on, the approached leaves are considered to have been affected only slightly by Amo-1618, whereas the double leaves are affected to much degree in their developmental

Fig. 1. Various grades of cohesion of the first leaves in Amo-treated, 21-day-grown seedlings. A, double leaf; D, single leaf; B and C, intermediate between double and single. II: leaf of the second node. ca. ×2.

process. Mere access of leaves does necessarily not lead to double leaf. It is therefore reasonable to distribute leaves into the two classes as above. Frequencies of the double-leaf class (D) and the opposite-leaf class (Op) are presented in Table 1. Three replications of experiments carried out on the 0-hour embryos gave similar high rates of double leaf formation (Table 1, column 0 h).

**Embryos deprived of both cotyledons.** The above observation suggests that the absence of one of the cotyledons is concerned with the double-leaf formation. Cohesion of leaves occurs on the side of the apex from which a cotyledon is removed. If both cotyledons are took off, what occurs?

When embryos deprived of both cotyledons were grown without treatment with Amo-1618, the development of the first pair of leaves was quite normal from morphological viewpoint, and nearly equal in growth rate to that in the intact seedlings during at least the first 3 days in spite of the absence of the cotyledons, the main source of nutrients. The growth of the first leaves seems to be supported by small amount of reserve material in the hypocotyl. However, subsequent growth and development of the leaves as well as the shoot apex slowed down and finally ceased.

When the decotylated embryos were treated with Amo-1618 for 48 hours, proper development of the leaves was never observed. Only slight outgrowths or centric organs were produced from the primordia of the first leaves. In about half the embryos the leaf primordia as well as the shoot apex degenerated. Accordingly the absence of a cotyledon on one side and the presence on the other are necessary conditions for the formation of double leaf.

**B. Changes in the apical meristem leading to the formation of double leaf.**

In the embryos treated with Amo-1618 after removal of one cotyledon, development of the first leaf primordia was almost completely suppressed during the treatment, i.e. during 2 days after sowing. The leaf primordia stayed in a buttress stage, whereas the shoot apex was enlarged in width (Fig. 2B). The enlargement of the apex seems to be based on the increase in cell number rater than cell volume. Under the wound surface made by the removal of the cotyledon, many periclinal walls are recognizable. The cell division activity under the would appears to elevate the shoulder of the apex at the decotylated side, resulting in the tilting of the apex toward the remaining cotyledon. In the enlarged apex, cells of the shoulder became vacuolated and the meristematic activity of the shoot apex came to be restricted to the proximity of the remaining cotyledon (Fig. 2B).

Three days after sowing, the leaf primordia showed some growth, attaining about 100 μ in height. At this stage of development, double leaves, single leaves and opposite leaves can be distinguished by the examination of the serial longitudinal sections (Fig. 2C, D). Double or single leaves developed on the decotylated side of the apex. Four days after sowing, these different leaf types were externally distinguished under a binocular microscope with ease (Fig. 3). If two primordia of the first leaves are separate from each other at this stage, they never form double leaf later on (Fig. 3F). So, structural changes leading to cohesion of the primordia must have occurred in the period of treatment, during which growth activities of the primordia were suppressed. The portion of the shoot apex between the two leaf primordia, which is, if normal, the site of one of the leaf primordia of the next pair, seems to be incorporated into the double leaf as its middle part. On the next node only one leaf develops on the opposite side to the double leaf in most cases (Fig. 1). A single leaf is formed as an extreme case of the double leaf from the area.
comprising the two primordia and the inter-primordial region (Fig. 3A). As in the case of formation of double leaf by longitudinal split of the shoot apex of the embryo, the center of the shoot apex shifts toward the remaining cotyledon, leaving leaf primordia toward the decotylated side (Fig. 2C, D). Then the divergence angle between these primordia may become smaller than 180°. If fusing does not occur between these primordia, they may develop as the approached leaves. The approach of the primordia is only a result of the positional shift of the apex center, not depending on the reorganization of the primordia, whereas double- or single-leaf formation is considered to depend on the suppression and reorganization of the primordia. Therefore, the opposite and approached leaves are put into one and the same category, and the double and single leaves into the other, as described above.

C. **Amo-treatment started at varying times after sowing.**

Since it was found previously\(^\text{13}\) that the rate of double-leaf formation by splitting the shoot apex of the embryo decreased steeply as the operation was carried out

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**Fig. 2.** Longitudinal sections of apical meristem in early steps of development in and after Amo-treatment. A, shoot apex in a 0-hour embryo; the broken line at the base of a cotyledon indicates the position from which the cotyledon is to be removed. B, shoot apex of an embryo after 2 days' treatment. The shoot apex has enlarged in width, tilted toward the remaining cotyledon, and the center of the apex has been shifted near to the cotyledon. C, shoot apex of a treated embryo, one day after the end of Amo-treatment, i.e. 3 days after sowing. A single-leaf primordium is growing on the shoulder of the decotylated side of the apex. D, shoot apex bearing a double-leaf primordium, 3 days after sowing. Ap: shoot apex; P: leaf primordium; Co: cotyledon; W: wound surface. ×120.
later than 9 hours after sowing, a series of experiments was undertaken to examine whether or not the same change in the rate of formation of double leaves occur when the time of Amo-treatment is delayed.

Amo-treatment was started at 3, 6, 9, 12, 15, 18, 21, and 24 hours after sowing. Observations were made 10 or 11 days after the treatment.

As shown in Table 1, double leaves were formed at high rates by the treatment started within 6 hours after sowing, but at 9-hour stage they abruptly decreased to a very low rate. Thereafter the Amo-treatment had no effect to produce double leaf, in other words, the shoot apex did not respond to Amo-1618 in producing double leaf.

Table 1. Relation between the starting time of Amo-treatment and the rate of formation of double leaves.

<table>
<thead>
<tr>
<th>Leaf type (%)</th>
<th>Starting time of Amo-treatment (hours from the time of sowing)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>70</td>
</tr>
<tr>
<td>Op</td>
<td>30</td>
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<td>Total number</td>
<td>20</td>
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D: double-leaf class, containing double and single leaves. Op: opposite-leaf class, containing opposite and approached leaves. In the columns of 0 hour and 9 hours, results of three and two replications of experiments are presented respectively.
II. **Effect of 2, 4-D.**

A. **Treatment of 0-hour embryos.**

*Embryos deprived of one cotyledon.* When 2, 4-D was applied at 2 ppm for 24 hours to embryos deprived of one cotyledon, growth of the embryos was heavily inhibited and the cells of the hypocotyl were strikingly hypertrophied. The leaf primordia were also markedly swollen and their development was arrested (Fig. 4A). Transections of these swollen primordia of 6-day-old embryos revealed lateral cohesion of them. The fusion occurred in all of 19 embryos treated. In one of them, leaf primordia developed to a tubular structure (Fig. 4B). This tube was open on the side facing the remaining cotyledon, thus resembling a double leaf. When 2, 4-D was applied for 18 hours at the same concentration as above, 20 out of 25 embryos treated formed such tubular leaves, 3 formed opposite leaves, and the remaining 2 degenerated.

When 2, 4-D was applied at 0.5 ppm for 24 hours, growth of the embryo took place to some extent. Three or four days after sowing, leaf primordia turned out to have formed gamophylls (Fig. 4C, D). Forty-five out of 47 embryos treated formed gamophylls, and in the remaining 2 the shoot apex degenerated.

These gamophylls are not perfectly symmetrical. On the side facing the remaining cotyledon the fusion is confined to the basal portion, whereas it reaches more higher level on the opposite side (Fig. 5A–C). Thus, they are cylindrical only at the basal part, and at more distal part they resemble a double leaf. These gamophylls...
ous double leaves differ characteristically from the double leaves induced by Amo-
treatment or splitting of the shoot apex of embryo, in that the positions of the
constituent leaves are always opposite across the shoot apex, there never occurring
mutual access of them on one side of the shoot apex.

**Intact embryos.**

When the embryos bearing both cotyledons were treated with 2,4-D at 0.5 ppm
at 0-hour stage for 24 hours, the first pair of leaves formed a cylindrical structure
in their basal region. They were separated higher in laminal region. Twelve out
of 21 embryos treated formed such gamophyllous leaves, and the others formed
separate opposite leaves.

**B. Treatment of 24-hour embryos.**

When embryos grown 24 hours were treated with 2,4-D at 0.5 ppm for 24 hours
after removal of one cotyledon, 11 out of 14 embryos treated formed gamophylls
and the remaining 3 formed separate leaves. In these gamophylls, there are only
slight differences in the extent of cohesion between two sides. In 8 out of 11 gamo-
phylls a little larger extent of fusion occurred on the side of the remaining cotyledon

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**Fig. 5.** Transverse sections of gamophylls induced by 2,4-D. A₁-A₃, three sections
representing the basal, middle, and upper portions of a gamophyll which was formed
by the 2,4-D treatment of 0-hour embryo deprived of one cotyledon. B, a gamophyll
formed by the treatment of 24-hour embryo deprived of one cotyledon. C, a gamophyll
formed by the treatment of 24-hour embryo bearing both cotyledons. Co: cotyledon
×55.
rather than on the decotylated side (Fig. 5B).

When the cotyledons were both left intact, in all 8 seedlings examined gamophyllous fusion was observed at least near the base of the leaf (Fig. 5C).

Thus, in contrast with Amo-1618, 2,4-D has the effect to induce cohesion of leaves in the 24-hour embryos, and even in the presence of both cotyledons.

III. Effect of other chemicals.

Following chemicals were applied to the 0-hour embryos deprived of one of the cotyledons.

CCC—This was applied for 48 hours at the concentrations ranging from 50 to 600 ppm. Growth of the embryos was inhibited during the treatment, but after removal of the chemical relatively good growth occurred in case of lower concentrations as compared with the growth after Amo-treatment. At higher concentrations (400 to 600 ppm) growth of the embryos and formation of chlorophyll in the cotyledons were inhibited for a long time after removal of the chemical. As to formation of double leaves, however, CCC was ineffective at any concentration tested.

IAA and NAA—These were applied at 1 ppm for 6 hours. Elongation of the hypocotyl was heavily inhibited, but hypertrophy was not brought about as was by 2,4-D. The first leaf primordia developed nearly normally, and neither double leaves nor gamophylls were formed.

GA—This was an unidentified mixture of different gibberellic acids. When embryos were treated with 10 ppm solution for 6 hours, the hypocotyl elongation was greatly accelerated and the leaf growth was also stimulated in elongation but weakened in laminal development. Leaves were quite normally opposite in position.

MH—This was applied at 10 ppm for 24 hours. Elongation of the hypocotyl was much the same as in the untreated seedlings, while the development of the first leaves was weakened. The first leaf primordia did not grow at all during 2 days after sowing. Four days after sowing, they became only slender rod-like outgrowths with very little or no lamina. As to the position they were all normally opposite.

Thus all these substances, whether growth retardant or auxin, or growth inhibitor, failed to produce double leaf or gamophyll when they were used in the same way as Amo-1618 or 2,4-D was done.

Discussion

The growth retardants generally reduce stem elongation without causing malformations of the plants. They can also cause increase in thickness of the stem and leaf of the treated plants5,14 or stimulate or retard flowering14,16. These resulting changes in plants are exclusively quantitative rather than qualitative or morphological. Recently Courduroux7 reported that buds of Helianthus tuberosus cultured for long period on the medium containing Amo-1618 (1 ppm) plus high concentration of sucrose (0.3 M) lost the ability to sprout and continued tuber formation. He considers that apical and subapical cells were irreversibly affected by Amo-1618. This may be the first example of something qualitative effect of Amo-1618 on the apical meristem. In the present investigation, Amo-1618 induced double leaves in sesame seedlings. The results presented in this paper may be the first observation on the morphological effect of Amo-1618, although it was manifested only under a particular condition that the chemical was applied within 6 hours after sowing to the embryos deprived of one of the cotyledons.
If embryos bearing both cotyledons are treated, the development of the first leaf primordia as well as the apical meristem are not altered, and the initiation of the succeeding leaf primordia goes on normally, as was observed in Chrysanthemum morifolium. If embryos from which both cotyledons are removed are treated, leaf primordia as well as the shoot apex degenerate. The removal of one cotyledon may make the shoot apex sensible to Amo-1618 and, on the other hand, the presence of the other cotyledon may protect the apex against total disorganization by the chemical. In the absence of one cotyledon, Amo-1618, if applied within 6 hours after sowing, suppresses the development of the first leaf primordia during the treatment, and also cause in the apical meristem considerable disturbance, resulting in the shift of the center toward the remaining cotyledon. Upon beginning development after the treatment, the leaf primordia develop into an united structure, a double-leaf primordium. During this period of suppression of development, the leaf primordia seem to be dedifferentiated to some extent and then reorganized so as to form the double leaf.

The suppression of the leaf primordia and the consequent formation of the double leaf are hardly or not effected by the treatment started later than 9 hours after sowing. This fact is comparable with the previous observation that few double leaves are formed by the longitudinal incision of the shoot apex of the embryo made later than 12 hours after sowing. As described previously, the sesame embryo becomes gradually activated from dormancy by 6 hours after sowing. The first cell divisions are recognized 15 hours after sowing in the leaf primordia. Even if growth is possible to occur before cell division begins, growth and development of the leaf primordia and shoot apex of the embryo in 9 hours after sowing seem to be very little, if any. Therefore the fact that the formation of double leaves is confined to the case in which Amo-treatment is made within 6 hours after sowing suggests that the formation of double leaf is dependent on the dormant state of the embryo, rather than on the early developmental stage of the leaf primordia. But the solution of this problem should await more studies, physiological and morphological, relating the early stages of germination.

In contrast with Amo-1618, 2,4-D induces gamophylls not only in the absence of one cotyledon, but also in the presence of both cotyledons. The gamophyll differs from a double leaf produced by Amo-1618 in the position of constituent leaves which are always opposite across the shoot apex. This suggests that leaf cohesion by 2,4-D is based on the stimulation of the interfoliar region on the peripheral zone of the shoot apex, not on the alteration in the configuration of the apical meristem.

References

14) Wittwer, S. H., and Tolbert, N. E., Amer.

摘 要

順：Amo-1618 および 2,4-D によるゴマの双生葉形成

生長抑制物質 Amo-1618 はゴマにおいて双生葉を形成させる効果がある。播種後 6 時間以内に、胚から子葉の 1 つをその基部から取り除いて、200 ppm の Amo 溶液を浸ませた濁紙上で 48 時間処理すると、その後生長してくれる第 1 葉は、子葉を除去した側で、双生葉となる。播種後 9 時間以上経った胚を同様に処理しても双生葉は生じない。また子葉が 2 側存在すると、処理時期の如何にかかわらず双生葉は生じない。

2,4-D (0.5 ppm) を子葉の 1 つを除去した胚に 24 時間作用させると、その第 1 葉は葉緑において合着して短状の構造となる。この合着は残存する子葉に面した側では葉の基部だけに限られるが、子葉を除去した側ではより上方向にまで及ぶ。従ってそれは Amo による双生葉に似た点も持っている。Amo と異なり、2,4-D は 2 子葉が存在しても、また播種後 24 時間を経過した胚に対しても葉の合着を起こさせる効果を持つ。Amo が、1 子葉の不在という条件下で、葉原基の発育を抑制しつつ茎頂分裂組織の体制に変化を起こさせることにより双生葉の形成をもたらすのに対し、2,4-D は茎頂分裂組織の葉原基間の部分を制限することによって葉緑の合着をひきおこすものと考えられる。

CCC, IAA, NAA, GA および MIH はすべて双生葉形成または葉緑合着をひきおこすのには無効であった。