Further Studies on the Double-leaf Formation in
Sesamum indicum L.

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Received June 9, 1961

It is known that double-leaves sometimes appear in some plants when the phyllotaxis changes from one system to another, for example, from the whorled system to the alternate one or from the decussate system to the alternate one (Fujita, 1949\(^1\)). Double-leaves were also induced experimentally in the course of study on phyllotaxis by Snow and Snow\(^2\)\(^-\)\(^5\). The present author obtained some double-leaves in Sesamum indicum L. by splitting the shoot apex of the dormant embryo\(^6\). Causes and processes of the double-leaf formation may not be always the same in those different cases. But it may be said that the double-leaf formation takes place commonly as a result of certain disturbances in morphogenetic functions of the shoot apex. In the present paper an analysis of the double-leaf formation in the incised apex will be made from such a viewpoint.

It was previously found in Sesamum indicum\(^7\) that few double-leaves were formed when the splitting of the shoot apex was made 24 hours after sowing, while they were often formed by the operation on the dormant shoot apex. In the former case, normal opposite leaves were formed instead of double-leaves. Therefore the double-leaf formation in this plant seems to be a characteristic morphogenesis of the shoot apex of a dormant embryo, resulting from its certain characteristic response to the operation. It seems therefore that the response of the 24-hour shoot apex to the operation may be different from that of the dormant apex, and the operation is no longer effective for the double-leaf formation on the 24-hour apex. This should be due to the difference of the stages of growth of the shoot apex. It is necessary to determine, first, at what stage of growth of the shoot apex within 24 hours after sowing the responsibility to the operation is lost, and secondly, how the response of the dormant shoot apex to the operation differs from that of the 24-hour shoot apex. It is appropriate in practice to put the latter question in another way, as follows: what difference can be seen between the regeneration processes of those shoots?

The present study deals with the two questions mentioned above. Information about those points may make it possible to present further explanation than that given in a previous paper\(^8\) on the cause and process of the double-leaf formation.

Material and Methods

The shoot apex of the embryo of Sesamum indicum L. was split longitudinally into halves, with a microscalpel made of a piece of thin razor blade, in a plane running through the two opposite first leaf primordia. The operation was made at intervals of 3 hours during 24 hours after the seeds were soaked in water. Embryos and seedlings were allowed to grow at 28-30\(^\circ\) under uninterrupted illumination at

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6,000 lux with fluorescent lamps. The observation was done about the external morphology of the first leaves 10 to 11 days after the operation, and their various forms were recorded. On the other hand, histological preparations of the shoot apex were made at various stages during 24 hours after sowing in order to check developmental changes in it. And one more set of histological preparations was made with the regenerating apices after the operation. Materials were fixed with formalin-acetic acid-alcohol, sectioned at 8 microns in thickness, and stained with Delafield's hematoxylin.

Results

1. Relationship between the operation stages and the rate of double-leaf formation.

After the operation, the halved apical meristems regenerated new shoots, and twin-seedlings were formed. The first leaf developing on each shoot took various forms, as shown in Fig. 1. The occurrence rate of each of the forms differed with operation stages. Although there were, as will be described later, found all the intermediate forms, they were classified into three types for convenience of recording. Those are the opposite-leaf, double-leaf and single-leaf types. The opposite-leaf type includes not only normal opposite leaves, but also leaves that approached to each other but remained separate (Figs. 1A and B). The double-leaf type includes the leaves in which two leaves are fused to various extent but two laminae are discernible (Figs. 1C–F). The single-leaf type includes the leaves in which the lamina looks quite single (Fig. 1G). These types are symbolized as Op-, D- and S-type, respectively.

![Fig. 1](image_url)

Fig. 1. Various forms of the first leaves that developed after the operation of the shoot apex of embryo. A shows a twin-plant, and B–G one of two shoots of a twin-plant. Cot: cotyledon. II: the second leaf. A, ca. x3. B–G, ca. x1.5.
The three leaf types occurred in various combinations on two shoots of the twin-seedling. Kinds of the combination are shown in the left column of Table 1. The combination D-D signifies that two shoots of the twin-plant both bear a double-leaf; D-S means that one shoot of the twins bears a double-leaf and the other a single-leaf, etc. The symbol Nu in the table stands for null and signifies that no leaf was formed, that is, shoot regeneration did not occur. In this way, the occurrence numbers of type combinations at each operation stage are given in Table 1.


<table>
<thead>
<tr>
<th>Combinations of leaf types on twin-seedlings</th>
<th>Operation stages</th>
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<tbody>
<tr>
<td></td>
<td>0h.</td>
</tr>
<tr>
<td>D—D</td>
<td>1</td>
</tr>
<tr>
<td>D—S</td>
<td>13</td>
</tr>
<tr>
<td>D—Nu</td>
<td>10</td>
</tr>
<tr>
<td>S—S</td>
<td>2</td>
</tr>
<tr>
<td>S—Nu</td>
<td>2</td>
</tr>
<tr>
<td>Nu—Nu</td>
<td>3</td>
</tr>
<tr>
<td>S—Op</td>
<td>3</td>
</tr>
<tr>
<td>D—Op</td>
<td>0</td>
</tr>
<tr>
<td>Nu—Op</td>
<td>6</td>
</tr>
<tr>
<td>Op—Op</td>
<td>0</td>
</tr>
<tr>
<td>Total number</td>
<td>40</td>
</tr>
</tbody>
</table>

It seems necessary, next, to simplify the kinds of leaf type combinations in order to clarify the relationship between the operation stage and the effect of the operation.

Comparing various forms of the first leaves, it was found that they could be put in order according to the degree of fusion. As shown in Fig. 1, there are intermediate forms between the opposite leaf and the double-leaf, and the degree of fusion of the double-leaf gradually increases until a single-leaf is formed. Therefore, the single-leaf can be regarded as an extreme form of the double-leaf. That the single-leaf is not mere one of two leaves of the first pair is decided by the following facts. First, its insertion position is opposite to the cotyledon, not in the intercotyledonary plane as is the case with the normal first leaves. Secondly, its shape is wider than a usual leaf of the first pair. Moreover, the decision is supported by comparing the vascular system of the single-leaf with that of the double-leaf. A and B in Fig. 2 are the cross sections of the petiole and the insertion region of a double-leaf, respectively. In this petiole, two sets of normal vascular system are included, and in the insertion region...
there is a large leaf gap formed by fusion of two gaps. C and D in Fig. 2 are the sections of the petiole and the insertion region of a single-leaf, respectively. This petiole is wider than that of a normal leaf, and contains a wide vascular bundle that appears to be formed by union of two. The leaf gap is also wider than normal.

For the foregoing reason, the D-type and the S-type leaves can be regarded as those of the same category. Accordingly, the combinations D—D, D—S, S—S, D—Nu and S—Nu can be classified as one and the same category, D-group. On the other hand, Op—Op and Op—Nu are included in the Op-group. The latter group is the opposite category to the D-group, if the effect of the operation is judged only from the viewpoint of formation or non-formation of the double-leaf. Then, Op—D and Op—S form

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Fig. 2. A and B, transverse sections of the petiole and the insertion region, respectively, of a double-leaf. C and D, transverse sections of the petiole and the insertion region, respectively, of a single-leaf. ×33.

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Fig. 3. A, occurrence rate of type combinations on a twin seedling. B, occurrence rate of leaf types.
an intermediate group.

In this way, it is possible to give simple figures indicating a relationship between
the operation stage and the effect of the operation. In Fig. 3A, the occurrence
rates of above-stated three groups of leaf types, translated from Table 1, are plotted.
In Fig. 3B, on the contrary, are plotted the occurrence rates of the leaf types on
separate shoots apart from the combination on a twin-seedling.

From Fig. 3, it is evident that the leaves of D-group are formed mainly in 6
hours after sowing, but thereafter decrease rapidly in their formation rate, and are
scarcely formed later than 15 hours after sowing. On the contrary, it is clearly shown
that the opposite leaves are very few till 6 hours, then increase rapidly, and later
than 15 hours they are formed most frequently. Therefore it is concluded that the
operation between 6 and 15 hours after sowing had no effect to induce the double-
leaf formation.

2. Cytohistological changes in the shoot apex during 24 hours after sowing.

Cytohistological changes in the shoot apex during 24 hours after sowing were
studied in order to find some relations between the developmental changes in the
shoot apex during the experimentation period and the changes of the operation effect
just described above.

Dormant shoot apex (Fig. 4A): The shoot apex of a mature dormant embryo
is only a small meristematic region lying between the cotyledons. Two primordia of
the first foliage leaves are situated oppositely on both shoulders of the shoot apex,
and the narrow, rather concave shoot apex proper lies between them. Histological
construction of the shoot apex is not fully developed yet. Its complete zonation and
stratification are established several days after germination.

In the cells of the leaf primordia as well as of the shoot apex proper, cytoplasm
stains intensely, but reserve materials (aleurone grains) are far less than in the cells
of the rib-meristem below. Difference in histological differentiation between the apex
proper and the leaf primordium seems very minute at this stage.

The cells of the apical meristem of the dormant embryo show strong shrinkage
of protoplasm under the technical procedures employed in this study.

6-hour shoot apex (Fig. 4B): The 6-hour shoot apex is nearly the same as that
of the dormant embryo, except that leaf primordia have swollen up a little by
hydration. The cells contain many aleurone grains, and show intense shrinkage of
protoplasm. The cells still appear to remain inactive at this stage.

9-hour shoot apex: The cells of the apical meristem do no longer show shrink-
age of protoplasm. In the cells of the leaf primordium, the cytoplasm stains deeply,
and small-sized aleurone grains appear in place of larger ones. On the other hand,
the cells of the shoot apex have digested most of aleurone grains.

The cells of the apical meristem seem to have become active physiologically by
this time.

12-hour shoot apex (Fig. 4C): Aleurone grains have almost disappeared from the
cells of the leaf primordium and the shoot apex, while the cells of the rib-meristem
still contain plenty of them. All the cells stain intensely and appear to be fully
activated.

15-hour shoot apex (Fig. 4D): Rising of the leaf primordium gets more con-
spicuous, and its cells stain deeper than those of the shoot apex. Cell divisions occur
for the first time at this stage in the leaf primordium, but not yet in the shoot apex
proper. The leaf primordium now begins to grow.
Fig. 4. Longitudinal sections of the shoot apices of germinating embryos. A, B, C and D, apices at the stages of 0, 6, 12 and 15 hours after sowing, respectively. A, ×210; B and C, ×245; D, ×225.

Fig. 5. Sections of regenerated shoot apices after the operation of the dormant shoot apex. A and B, 3 and 4 days after the operation, respectively. Note the epidermis, formed on the wounded side of the apex, extending down to the level of the arrow. cot: cotyledon. ×230.
Fig. 6. A, B and C, three sections from a regenerated apex, 4 days after the operation of the dormant shoot apex. A and C, the side sections, and B, the median section of the apex. ×170. D, E and F, three sections from a regenerated apex, 2 days after the operation of the 1-day-grown shoot apex. D and F, the side sections, and E, the median section of the apex. ×210. Arrows indicate the lowermost end of the epidermis formed on the wounded side. cot: cotyledon.
24-hour shoot apex: Cell divisions take place in the shoot apex proper as well as in the leaf primordium, but more frequently in the latter. Staining of the cells of the leaf primordium is deeper than those of the shoot apex proper.

Within 24 hours after sowing, whole dimension of the shoot apex slightly increases mainly by cell enlargement rather than by the increment of cell number. But the relatively large rising of the leaf primordium is due not only to cell enlargement but also to cell increment.

From above observations, it may be said that the cells of the shoot apex of the embryo remain dormant till 6 hours after sowing, then become gradually active, but real growth occurs first in the leaf primordium 16 hours after sowing and then in the shoot apex proper.

By comparing these changes in the shoot apex with the changes in occurrence rate of double-leaf, it seems possible to draw a conclusion that the operation on the shoot apex can cause the double-leaf formation when the shoot apex stays dormant but the effect by the operation gradually decreases as the shoot apex gets metabolically active even before the beginning of growth, and finally it becomes lost after beginning of growth of the shoot apex.

3. Regeneration of the shoot apex.

Whether the first-leaf primordia develop as a double-leaf or as opposite leaves after the operation must be determined in the course of shoot regeneration. Therefore, some difference is expected to be found between the regeneration process of the dormant shoot apex and that of the growing shoot. In fact, a significant difference was found between these two processes.

It has been observed that the new shoot apex regenerated only from the uninjured surface of the incised apex, but never from the wounded surface, and that the wounded surface of the apical meristem showed mere wound responses, such as cell divisions parallel to the wounded surface or callus formation on the wound. These results also apply to the present case, except that callus was not formed on the wound.

But a remarkable fact observed in the present study is the formation of new epidermis on the wounded side of the incised apex. As will be described below, it is significant that the new epidermis could be observed in the shoot apex bearing a double-leaf or a single-leaf, but not in the shoot apex bearing opposite leaves.

Fig. 5A shows a regenerating shoot apex 3 days after the operation of a dormant shoot apex. New shoot organization appears nearly completed. In the figure, the first leaf primordium is expected to arise on the right side of the new apex. What is worth mentioning here is the fact that a distinct epidermis covers the wounded side of the apex down to the level indicated by an arrow. This level of the lowermost end of the epidermis is considerably lower than that of the summit of the shoot, while at this stage the shoot apex itself shows no growth in height at all. Therefore, the epidermis on the wounded side must have been reorganized from the underlying cells of the wound, or formed from the tunica layer by its downward elongation along with the elongation of the subjacent tissue. Remains of dead cells attaching on the epidermis may support the former possibility. The lowermost part, at least, of the epidermis looks to be formed in this way. But the regeneration of organized tissue from the wounded surface of the apical meristem seems to be very difficult, and, in fact, it has never been reported. Although Ball stated that epidermis was formed from the sub-wound cells of the pith plug isolated from the sur-
rounding tissue of the shoot apex in *Lupinus*, the epidermis does not look to be typically organized one and the real epidermis seems to be formed as a result of growth of the regenerated apex. Thus, in the present study, the larger portion of the new epidermis was perhaps formed by downward extension of the tunica.

The downward extension of the tunica together with the elongation of the subjacent tissue causes a strong distortion in the outline of the shoot apex. The distortion of the external shape should be attended by the changes in the internal structure such as histological zonation or structural pattern in the shoot apex. Thus the fact of the epidermis formation on the wounded side of the incised apex suggests that profound disorganization and subsequent reorganization of the apical structure have occurred in the course of shoot regeneration.

Fig. 5B shows a regenerated shoot apex, 4 days after the operation, in which the epidermis has extended downward on the wounded side and a leaf primordium has started to grow. In comparison with the growth of normal first-leaf primordium, which attains a height of 600 to 700 microns and commences laminal development 4 days after sowing, that of the operated leaf primordium is very much retarded, because it takes about 3 days for reorganization of the new shoot apex. Examination of serial sections shows that this apex bears only one leaf primordium initiated opposite to the cotyledon.

A, B and C in Fig. 6 represent three sections taken from an apex bearing a double-leaf primordium, 4 days after the operation of a dormant embryo. A and C are both the side sections, and B the median section of the shoot apex. It can be seen in the B section that the basal parts of the primordia A and C are connected, that is, these two primordia are to grow into a double-leaf. In this apex the epidermis on the wounded side extends down to the level obviously lower than the summit of the shoot.

In all examples above mentioned, it is plain that the shoot apex itself has not yet grown in height at all, because it stays at the same level as in the dormant embryo, i.e., at the base of the cotyledons. Therefore it is doubtless that the epidermis extended downwards from the shoot summit.

In contrast with above examples, in the following example the operation was made 24 hours after sowing. D, E and F in Fig. 6 are the sections from the shoot apex 2 days after the operation. Both D and F represent the side sections, and E the median section of the shoot apex. On this apex two opposite leaf primordia developed. In this case, in contrast with above cases, the wounded side completely lacks epidermis up to the uppermost level. The wounded surface is covered only by irregular cell layer, not by an organized epidermis. This means that the tunica did not extend downwards, but extended only outwards enlarging the area of the apex. Accordingly it may be said that the apex did not undergo strong distortion in the course of regeneration. The development of this apex is much more advanced than that of the apex which formed new epidermis on the wounded side after the same growth period (cf. Fig. 5A). It is only one day retarded as compared with the intact apex. Therefore it is probable that the original organization of the apex was not so much disturbed by the operation, and the shoot apex regeneration took place readily by mere repairing or expansion, rather than by reorganization, of the halved apical meristem.

Thus the formation of organized epidermis on the wounded side of the incised apex is the characteristic of the shoot regeneration in the course of which leaves of D-group are formed. In other words, the disturbance of the apical organization, the epidermis formation on the wounded side of the apex during its reorganization, and
the formation of D-group leaves, are perhaps the closely connected and consecutive events.

4. Interpretation of the process of the double-leaf formation.

Previously the author9) presented an explanation of the process of the double-leaf formation. That, however, referred mainly to the approaching of two opposite leaves. Although the approaching of the leaf primordia must be one of the important conditions for the double-leaf formation, there still seems to remain some gap to be explained between a mere fact of spatial approaching of the leaf primordia and their fusion into one.

In relation to this point, interpretations of gamophyll formation induced by means of growth substance application give a good suggestion for the explanation of the double-leaf formation.

Parthefol16,17) investigated gamophyll induction by 2,4-D in Linum usitatissimum. After him, gamophyll formation depends on abnormal functioning due to 2,4-D of whole leaf-generating region ("initial ring"). That is, the simultaneous development of the whole initial ring results in the formation of a tubular union of leaves. Haccius and Schneider18) presented similar explanation in Galium aparine. They concluded that the morphogenetic pattern in the apical cone based upon some gradients of substances exuding from definite poles was flattened by excess amount of 2,4-D, and consequently separation of individual leaf primordia within the leaf generating region did not take place and the entire "Bildungsring" grew into a tubular structure (gamophyll).

Consulting these interpretations on gamophyll induction, the double-leaf formation may be explained as follows.

When the shoot apex of dormant embryo is split (Fig. 7A), the apex may undergo profound disorganization, as suggested in the preceding section. Bisected leaf primordia may also degenerate to various extent. Upon regeneration, the new apex center shifts toward outside (Fig. 7B), and the original central region (hatched area in Fig. 7B) comes to occupy the lateral region of the new apex. Then this region may become potential for leaf formation. In consequence, there occurs on one side of the apex a wide leaf forming area consisting of both the newly activated region and the degenerated leaf primordia. When the leaf primordia resume growth after some retardation period, the region between the primordia may grow with them. Thus two primordia may grow into a united structure (Fig. 7C). On that occasion, the narrow shoot apex of sesame embryo and accordingly small distance between two
leaf primordia may be one of the conditions which facilitate the process. Grade of disorganization of the shoot apex and the size of the regenerated apex may determine the degree of fusion. If the disorganization is strong and the regenerated apex is narrow, the leaf forming area may be small, and a single leaf may be formed.

On the other hand, the active shoot apex may undergo little disturbance in its structure when operated, and its original configuration may persist. Regeneration of the shoot apex may be performed by mere expansion of the halved apex. Therefore the leaf primordia may maintain their individualities and their growth may not be so much inhibited. They may only perform regeneration of their own structure, growing separately, although they may approach to each other more or less as a result of outward shift of the new apex center.

Summary

1) Double-leaves were formed in high frequency in Sesamum indicum by splitting the shoot apex of dormant embryo. But their occurrence rate decreased rapidly with delaying the operation stages from 6 to 15 hours after sowing, and later than 15 hours after sowing they were scarcely formed, opposite leaves being formed in place of them.

2) Histological observation revealed that the shoot apex of embryo stayed dormant till 6 hours after sowing, then became gradually activated metabolically, and that cell division occurred first 15 hours after sowing. Therefore plastochronic change hardly occurred by that time.

3) From above results, it may be concluded that the double-leaf formation is the characteristic response of the dormant shoot apex to the operation, and the response becomes different as the shoot apex gets active, even if its plastochronic stage is the same as in the dormant stage.

4) When the operation was made on the dormant shoot apex, an epidermis was formed on the wounded side of the apical meristem of the regenerated shoot, while it was never formed after the operation on the activated shoot apex. The epidermis was formed by downward extension of the tunica. This fact means that the apical meristem, when split at the dormant stage, undergoes strong distortion in the course of regeneration. The distortion in the external shape may be attended with the disturbance in the internal structure. This is a characteristic behavior of the dormant shoot apex.

5) Process of the double-leaf formation was interpreted from the viewpoint of disturbance in the structure and morphogenetic function of the shoot apex due to the operation.

References

摘 要

1. キマの胚の生長点を、対生する第1葉原基を通る面で二分すると、第1葉原基はしばしば双生葉となる。手術の時期をいわゆる変えてみると、双生葉は、手術が休眠状態の胚の生長点になされたときに形成されるが、胚が発芽活動にはいると、まだ生長は起こらなくても双生葉形成率は急に減り、細胞分裂が始まるころにはほとんどそれは形成されなくなることが知られた。

2. 休眠胚の生長点の手術ののちには、再生した生長点の、傷をうけた側にあらたに表皮が生じた。24時間胚の生長点ではこのことは起こらない。新しい表皮は tunica が下方に向かって引き伸ばされることによって形成される。したがって、この場合には生長点に強い歪曲が起こったことになる。生長点の内部構造にも強い変化が起こっているであろう。

3. 双生葉形成過程は、手術による生長点の構造および機能の乱れ、ならびにその後の再編成というところから説明された。（東京都立大学理学部生物学教室）