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CHANGES IN THE EPIDERMAL PATTERN OF THE SEPALS OF HYDRANGEA MACROPHYLLA (THUNB.) D.C., "OTAKSA" IN THEIR SUCCESSIVE GROWTH PHASES. FORMATION OF NEW STOMATA, DISINTEGRATION OF OLD STOMATA

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ABSTRACT

The sepals of *Hydrangea* flowers which are pink during anthesis, afterwards turn green through the formation of chloroplasts. During this process the sepals grow to a considerable extent. From about 200 mm² they expand to around 900 mm². As a result of the greening process, the character of an insect-attracting flower organ changes into that of a foliage leaf. Parallel to this runs a change in anatomical pattern. The pattern changes of the lower epidermis were the object of special interest in the present investigation. Apart from the formation of new stomata during certain stages, disintegration of old stomata actually takes place. As far as the present authors are aware, no mention of this phenomenon is to be found in the literature.

An excess of newly-formed stomata causes a considerable increase in the stomatal frequency and in the stomata index. A certain succession of processes involved in the change in anatomical pattern is discussed.

1. INTRODUCTION

The immediate reason for the present investigation was the publication of HELIGE and WEBER (1950) on the stomatal frequency of sepals of *Hydrangea* flowers (*Hydrangea opuloides* C. Koch var. *regula*). From their publication it appears that during the greening process that takes place at the end of the flowering period, the number of stomata of the lower epidermis of the sepals increases considerable; the upper epidermis does not show any stomata at all.

Helige and Weber did not make use of the Stomata Index, a quantity introduced by SALISBURY (1928). The latter author pointed out that stomatal frequency is highly variable. As a matter of fact, a high stomatal frequency can equally well be due to the formation of a large proportion of stomata mother cells as to the lack of expansion, the epidermal cells remaining very small in size. Salisbury therefore considered it convenient to have a means to express the frequency of stomata independent of their spacing through growth of the intervening unspecialized epidermal cells. The following expression, called the Stomata Index, was proposed:

$$I = \frac{S}{E + S} \times 100,$$

in which S is the number of stomata per unit area and E the number of epidermal cells in the same area.

As we are interested in anatomical patterns of leaves (NILLESEN and KARSTENS (1955); GOEDBLOED-GIEL, QUENÉ-BOTERENBROOD and KARSTENS (1958); KANIS and KARSTENS (1963)), it was considered worth-while to investigate the changes in the stomatal apparatus of sepals of ageing *Hydrangea* flowers by making use of quantitative methods to determine, among other things, the stomata index in flowers in anthesis and in different phases of greening.

2. MATERIAL AND METHODS

Flowers of *Hydrangea macrophylla* (Thunb.) D.C. var. "*Otaksa*" grown in the Botanic Garden were used. *Hydrangea macrophylla* (Thunb.) D.C. is a synonym of *Hydrangea opuloides* C. Koch. Unfortunately, we had no material of the var. *regula* investigated by Helige and Weber at our disposal.

Fresh flowers cut during the period from August to November were used. Each flower was given a Roman numeral, and the component sepals were numbered 1–4 (see Fig. 1 A). From each sepal, surface sections were prepared from five selected areas (see Fig. 1 B). Counting of the stomata and the epidermal cells was facilitated by making use of a cross-hatched ocular micrometer and a camera lucida. Care was taken to avoid the areas above the veins, as these exhibit a deviating pattern. The surface of the sepals to be investigated was determined by means of graph paper. For expressing the extent of greening, an arbitrary scale of five degrees (0–4) was used.

Attention must be drawn to the fact that the flowers of *Hydrangea* — at least this applies to our material — are not actinomorphic but zygomorphic or even do not exhibit a distinct symmetry at all. It often occurs that the situation is as represented in Figure 1 A. Sepal No. 1 is the smallest and No. 3 is the largest of the sepals, while Nrs. 2

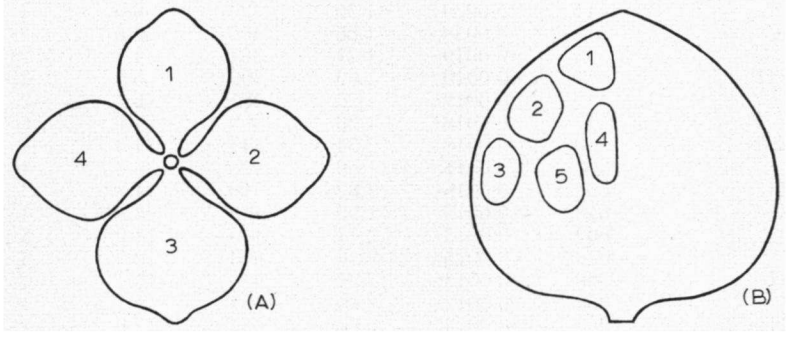

Fig. 1. *Hydrangea macrophylla* (Thunb.) D.C. "*Otaksa*". A. Outline of a flower. Sepals numbered 1–4. B. The five areas investigated in each sepal.

TABLE 1

Flower no.	Sepal no.	Sepal surface in mm²	Reciprocal surface value	Stomata index	E + S per unit area	S per unit area	Degree of greening
VII	1	69	0.0145	1.07	2246	24.1	0
VII	2	78	0.0128	1.07	1998	21.4	0
VII	3	79	0.0127	1.38	1540	21.2	0
VI	2	97	0.0103	1.66	964	16.0	0
IV		109	0.0092	1.51	1228	18.5	0
XVI	2	111	0.0090	1.96	1000	19.6	0
VIII	3	122	0.0082	1.37	815	11.0	0
II		147	0.0068	1.67	1068	18.0	0
VIII	4	152	0.0066	1.87	857	16.0	0
XVI	3	162	0.0062	1.63	820	13.4	0
XVI	1	173	0.0058	1.66	791	13.1	0
VIII	2	174	0.0057	1.84	813	15.0	0
VIII	1	210	0.0048	1.54	804	12.4	0
XVI	4	245	0.0041	1.83	744	13.6	0
XI	2	253	0.0040	1.78	521	9.3	0
XIII	1	273	0.0037	1.85	545	10.1	0
III		286	0.0035	2.09	622	13.0	0
XIV	1	304	0.0033	1.61	516	8.3	2
X	4	312	0.0032	2.12	452	10.0	1
XIII	2	320	0.0031	1.46	472	6.9	0
V	2	330	0.0030	2.20	484	10.5	0
XI	1	363	0.0028	1.32	476	6.3	0
XI	3	366	0.0027	1.68	449	8.2	0
X	3	377	0.0027	1.67	472	8.0	1
XIV	4	398	0.0025	1.70	499	8.5	2
XV	2	401	0.0025	1.53	431	6.6	4
V	1	403	0.0025	1.91	471	9.0	0
XII	3	403	0.0025	1.48	454	6.7	2
XIV	2	413	0.0024	1.31	528	6.9	2
X	2	416	0.0024	2.05	443	9.1	1
XI	4	419	0.0024	1.94	511	9.9	0
V	3	452	0.0022	1.35	430	5.8	0
V	4	459	0.0022	1.04	424	4.4	0
XVIII	3	472	0.0021	2.07	421	8.7	3
XV	4	473	0.0021	1.23	487	6.0	4
XII	4	494	0.0020	1.56	384	6.0	2
XVIII	1	505	0.0020	2.19	448	9.8	3
XV	1	511	0.0020	1.56	423	8.6	4
XII	2	512	0.0020	1.86	409	7.6	2
XII	1	533	0.0019	1.21	387	4.7	2
XVIII	2	534	0.0019	1.63	399	6.5	3
XIV	3	540	0.0019	1.77	465	8.2	2
X	1	547	0.0018	1.20	399	4.8	1
XIX	3	560	0.0018	2.54	449	11.4	4
XV	3	608	0.0016	1.50	428	6.4	4
XVII	2	638	0.0016	2.82	510	14.4	3
XIX	2	648	0.0015	1.99	448	8.9	4
IX	2	662	0.0015	2.71	480	13.0	3
IX	1	673	0.0015	2.10	508	10.7	3
XVII	3	798	0.0013	2.91	528	15.4	4
XIX	1	836	0.0012	2.39	397	9.5	4
XVII	1	852	0.0012	2.80	457	12.8	4
XX	1	872	0.0011	1.75	400	7.0	4
XVII	4	924	0.0011	2.98	480	14.3	4

and 4 are intermediate and about equal in size. Flowers XVI, VIII, XI, XIV, V, XII, and XVII in Table 1 exhibit this type of symmetry very clearly. However, in Table 1 (column 2) the position of the sepals in the flower is not taken into account. The numbering is therefore only given for information as to the number of sepals per flower investigated.

We could ascertain from the data of these seven flowers that each of the sepal types exhibits the general trend as to the relation between the quantities mentioned in Table 1 and the sepal surface. It seems certain that separate treatment of the sepal types would influence dispersion of the data favourably. Two points which exercise an unfavourable influence should be mentioned. In the first place, there is the fact that, with very few exceptions, all sepals of one flower exhibit identical degrees of greening simultaneously at all times. This means that petals with the same degree of greening can have very different surface areas and, therefore, the arrangement of the sepals in order of surface area (Table 1) cannot be considered completely satisfactory. Secondly, the present authors became aware of the fact that the phenomena to be described are subject to seasonal influence.

3. Discussion of the results

3.1. *Presentation of the data*

Table 1 gives a survey of the values of different quantities obtained from an investigation of 19 flowers and arranged according to the surface area of the sepals. For three flowers dating from the beginning of our study, no mention is made of which sepals were investigated. The first three columns of Table 1 require no special explanation; concerning the fourth column containing the reciprocal surface values it may be said that these values proved superior to the normal surface values in composing certain graphs.

The last four columns give the values for the stomata index, the total number of epidermal cells and stomata (E+S), the number of stomata (S), and the degree of greening of the petals. The values for E + S and for S are given per unit area of 1 mm^2.

From the values given in Table 1, some points are at once evident. It is obvious, first of all, that a certain relation exists between the size of the sepals on the one hand and the degree of greening on the other. It is, furthermore, evident that there is some trend as to the values of the stomata index, of E + S and of S.

To enable comparison of the quantities, some graphs are given in Figure 2. The surface areas of the sepals expressed in mm^2 are plotted along the abscissa on a reciprocal scale, in order to intensify the expression of certain features. This is only true for the more interesting parts of the graphs, i.e. those parts dealing with the greening period of the sepals. As a result, the data of the period preceding greening extend over a large part of the graphs, but this does not outweigh the advantages of the method.

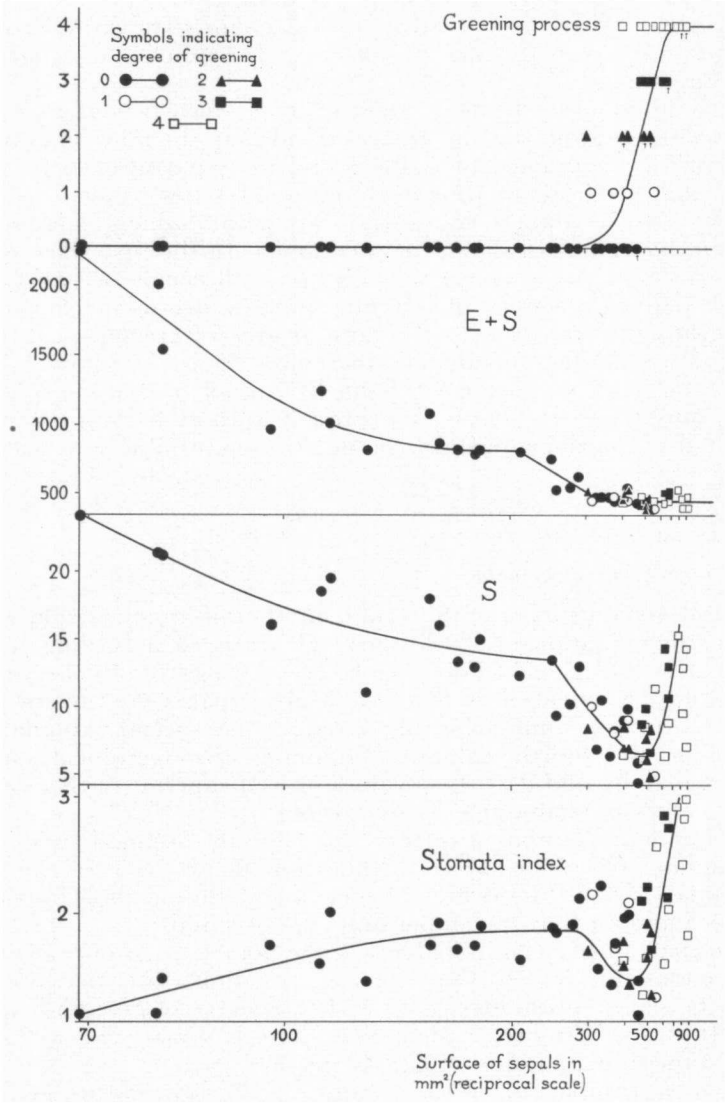

Fig. 2. *Hydrangea macrophylla* (Thunb.) D.C. "*Otaksa*". Graphical presentation of data in Table 1. From top to bottom: Greening process, E + S per unit area, S per unit area, Stomata index. The arrows in the top graph indicate coincidence of two values.

3.2. *Greening process*

The graphs bring out several interesting points in connection with the process of greening of the sepals. First of all, there is a distinct relationship between growth of sepals and the process of their turning green. This greening process is very interesting because of the fact that the sepals originally present as pink-coloured, insect-attracting flower organs change in character and become more or less the same as foliage leaves. From the graph it may be said that, in the main, for the plant under investigation sepals close to 300 mm² in size will start turning green.

The growth process itself was not investigated directly by measuring sepals in relation to time. The only point about which we are sure is that sepals do grow for a long time after anthesis is over. In the absence of data it is not possible, however, to co-ordinate our findings connected with growth with the process of growth itself (see the next paragraph).

3.3. *Number of epidermal cells* (E + S)

The total number of epidermal cells E + S, i.e. the sum total of ordinary epidermal cells (E) and of stomata (S) per unit area, exhibits the existence of two phases during the life-time of the sepals, each showing an initial decrease in cell number per unit area and gradually ending up with the cell number per unit area remaining constant. This means that in the growth periods represented by the descending parts of the graph, cell division is non-existent or its rate is too low to keep the cell number per unit area on the same level. Theoretically even disappearance of cells could take place during these periods.

On the other hand, the horizontal parts of the graph show that during the corresponding periods, cell division must be active in order to allow the number of cells to keep space with the expansion of the sepals. Especially the horizontal part of the second phase represents an important period in the life-history of the sepals, this being the period in which they expand from about 400 mm² to 900 mm².

Table 2 summarizes the data bearing on these phenomena,

TABLE 2

E + S per unit area	Surface of sepals in mm²	Part of the graph
2246 ⟶ 820	70 ⟶ 175	1st descending part
820	175 ⟶ 210	1st horizontal part
820 ⟶ 450	210 ⟶ 400	2nd descending part
450	400 ⟶ 900	2nd horizontal part

We have drawn attention to the fact that lack of quantitative data on the growth of the sepals has made it impossible to co-ordinate the phenomena connected with growth with the growth process itself. This is to be deplored because combined investigations of

growth and the internal structure of the plant on a quantitative basis might give results of considerable interest. It seems quite possible that the case of *Hydrangea*-sepals with two successive phases of development would be found to be comparable with well-known examples of plant organs in which simple measurements have established two growth periods, such as the sporangiophore of *Phycomyces* (ERRÉRA, 1884) and the inflorescence stalk of *Taraxacum* (MIYAKE, 1904).

3.4. *Number of stomata* (S)

A third item to be discussed is the number of stomata per unit area during the growth process of *Hydrangea* sepals. As can be seen from the graph, the number of stomata per unit area decreases gradually during the first phase of expansion described in paragraph 3.3. This result suggests that during this period of expansion no new stomata are formed or that the rate of formation is insufficient to keep the number at the same level.

This slow decrease is followed by a dramatic one, followed in its turn by an appreciable recovery of the number of stomata per unit area. The very marked decrease takes place in the category of sepals with a surface area ranging from 253 to 500 mm^2, thus exhibiting an expansion by a factor of 2. The decrease in the number of stomata in this range, however, starts at 13 and drops to 5. The only possible explanation for this phenomenon, already alluded to in the foregoing paragraph, is that in this phase a certain number of stomata disappear. We will discuss this point separately (§ 3.6.).

During the increase in the surface of the sepals from 500 to 900 mm^2, there is, as already mentioned, a sharp increase in the number of stomata per unit area. This phenomenon can be only explained by the formation of new stomata. This formation must take place to a very considerable degree as compared with an expansion of the sepals from 500 to 900 mm^2, which means an expansion by a factor of 1.8, the number of stomata per unit area increasing by a factor of 3.

Helige an Weber mention an increase in the number of stomata per unit area of 1 mm^2 by an even higher factor of 4 by comparing pink sepals with green ones. If we do the same, a factor of only 1.5 results. A second difference as to the properties of the material used, lies in the fact that the absolute number of stomata per mm^2 is much higher in the material of Helige and Weber. While for pink and green petals these investigators found values of 14 and 53 respectively, these values lie considerably lower for our material, i.e. 10 and 15. It seems, therefore, that the material used by Helige and Weber cannot be directly compared with ours.

3.5. *Stomata index*

During the first phase of development the stomata index initially increases and then becomes linear. This means that in the beginning the proportion of ordinary epidermal cells to stomata gradually changes in favour of the latter. This is most probably due to the

formation of new stomata. In the flat part of the curve the stomata index remains the same, indicating that the proportion of epidermal cells to stomata also remains constant. In this period of sepal development growth is probably due only to expansion of already-present cells.

As to the second phase, the next part of the curve is the most interesting: the stomata index shows a sharp drop. We have suggested that the disappearance of stomata may explain this phenomenon. Without the conclusive evidence put forward in the foregoing paragraph, the decrease of the stomata index might be also explained by the formation of new ordinary epidermal cells. Lastly, the value of the stomata index rises again, even to about thrice its minimum value. The most acceptable explanation for this fact is, as was mentioned before, that there is considerable formation of new stomata.

3.6. *Formation and disintegration of stomata*

In connection with all that has been mentioned so far and what will be said presently, one must be well aware that in reality the situation is much more complicated. Apart from the complications discussed under "Material and methods", there are others deriving from the fact that different parts of one and the same sepal are not of the same age and, therefore, do not show the same developmental phase. Actually, the top part of the sepals is the first to develop, while the basal part follows later. This can be conveniently demonstrated in very young sepals. The apical part shows full-grown stomata and ordinary epidermal cells exhibiting the well-known undulated outline. The basal part, however, shows many stoma mother-cells and the outline of the ordinary epidermal cells is not yet typical. Consequently, the apical part of such very young sepals is the first to become coloured pink by the formation of the anthocyanin pigments which will later be present all over the whole sepal. During the greening process it is again the apical part that shows the phenomenon first. Therefore, everything so far put forward must be taken as a generalized picture.

In the foregoing paragraphs the possibility or the necessity for the formation of new stomata and the disappearance of old stomata has come up several times. If they prove to be real, these phenomena may have a direct bearing on the change in character of the sepals from a typical flower organ into a kind of foliage leaf. Teleologically speaking, it might be said that in connection with this change in function old inactive stomata have to disappear and new ones have to be formed. The disappearance of stomata would then typify the first phase of the change of the flower leaf into a foliage leaf, the formation of new stomata would then be characteristic of the second part, when actual greening takes place.

As to the formation of stomata, we observed the epidermal pattern around the later-formed stomata to be different from that of those whose differentiation takes place during the younger stages of the

sepals. The early formed stomata of *Hydrangea* sepals are surrounded by a circle of radiating epidermal cells, thus belonging to a type which, according to METCALFE and CHALK (1950), might be described as actinocytic. To the present authors this appears to be a regularly built variant of their anomocytic or irregular-celled type. Actually, the later-formed stomata belong to the typical anomocytic type (Plate I. A and B). The same difference in pattern can be observed in the illustration given by Helige and Weber. Furthermore, in the case of early-formed stomata the groups of surrounding epidermal cells exhibit a radiating striation of the cuticle. And lastly, the early stomata are situated on a slightly higher level than the ordinary epidermal cells, but the later ones lie on the same level.

To the best of our knowledge, no concise data on the disintegration of stomata can be found in literature. HILLER (1884) describes for a number of flowers remarkable formations which, according to him, are "rudimentären oder in ihrer Bildung fehlgeschlagenen Spaltöffnungen". A copy of one of his illustrations is presented in Figure 3.

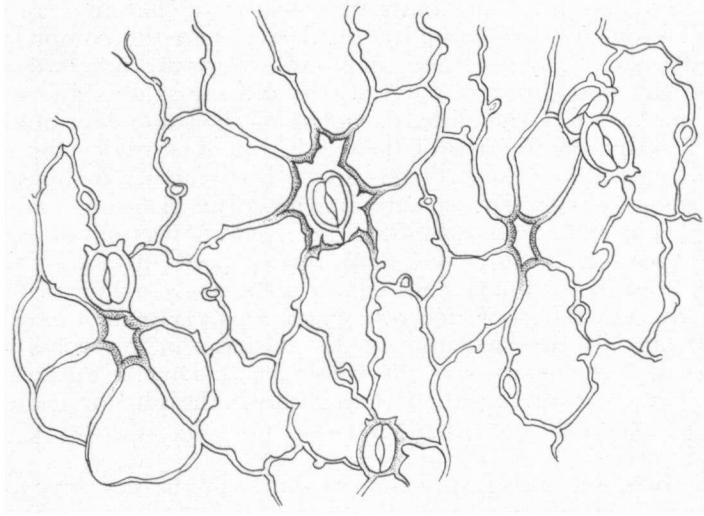

Fig. 3. *Lathyrus heterophyllus*. Flower epidermis. After Hiller (1884).

MÜLLER (1893) describes the same structures and appears to have the same opinion as Hiller. She mentions "das Vorhandensein normaler und sehr oft in Rückbildung begriffener und vollständig rückgebildeter Stomata" in the epidermis of flowers of a number of plants. By "Rückbildung" is meant not the disintegration of once normal stomata but a rudimentary state of these organs. This is apparent from the following passage: "an der unteren Epidermis genannter Arten .... sind sehr grosse und verschieden gestaltete Bildungen vorhanden, die als fehlgeschlagene Spaltöffnungen zu betrachten sind". PORSCH (1905) is of the opinion that "Jedenfalls die mehr oder weniger rück-

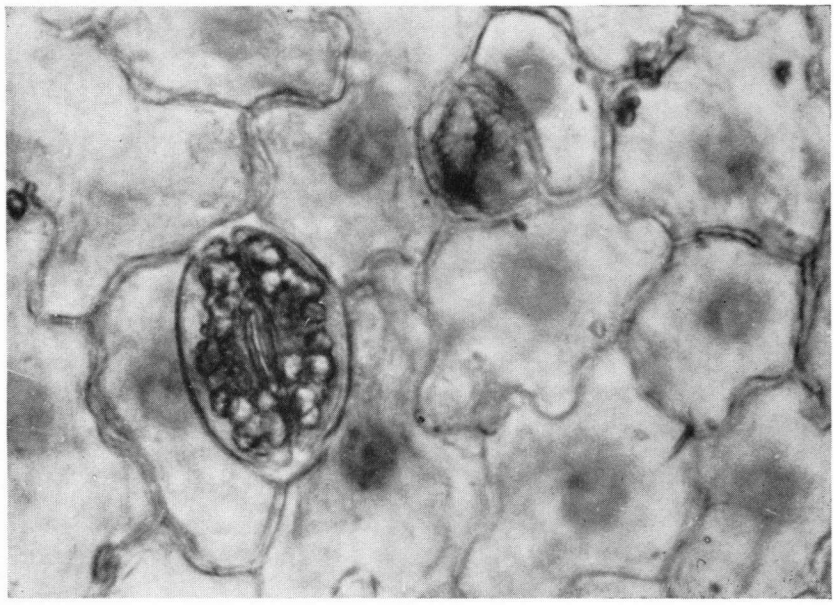

Plate Ia. *Hydrangea macrophylla* (Thunb.) D.C. "*Otaksa*". To the left, a fully-developed stoma of the later-formed, anomocytic type; to the right a stoma mother cell. 635 ×.

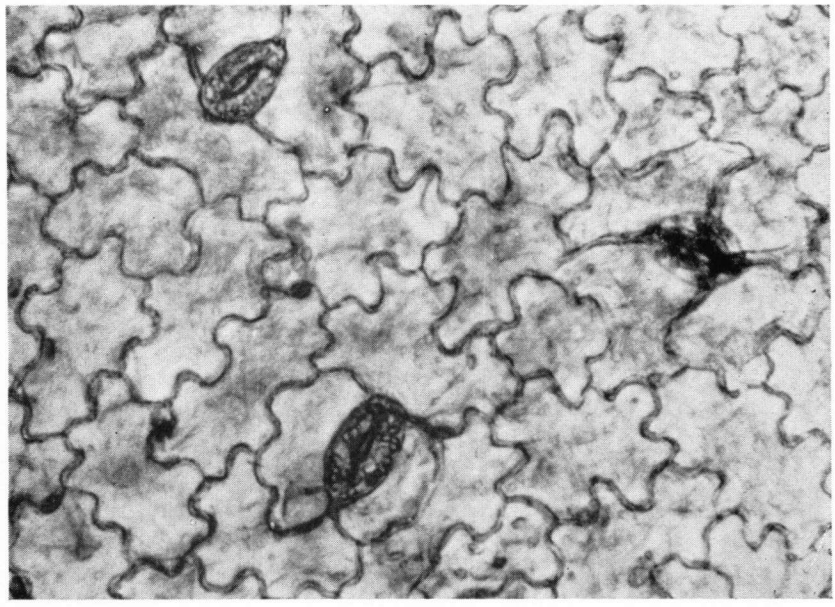

Plate Ib. *Hydrangea macrophylla* (Thunb.) D.C. "*Otaksa*". To the right, a disintegrating stoma of the early, actinocytic type. The radiating cell walls are already thickened. To the left, two stomata of the later type. 320 ×.

gebildeter Stomata auf Blumenblättern und Anthere physiologisch überflüssige Erbstücke darstellen, deren Auftreten in der Abstammung dieser Organe aus assimilierenden Blättern seine Erklärung findet". It is remarkable that none of these authors considers the possibility of stomatal disintegration even though the observed structures are only with difficulty to be interpreted as rudiments.

To the present authors the structures described seem to represent the cavity left after disintegration of the two guard cells, combined with certain changes in the surrounding cell walls. Besides the quantitative data discussed in § 3.4 and 3.5, we are able to present other arguments which make it acceptable that disintegration of guard cells of stomata actually takes place. It must be kept in mind that this process is linked with the whole complex of events occurring during the development of the successive pink and green stages of the sepals. As already mentioned, all processes start in the top of the sepals and proceed towards the base. This applies to the formation of stoma mother-cells, to the appearance of the waviness of the cell walls of the ordinary epidermal cells, the formation of chloroplasts accompanied by the disappearance of the anthocyanin pigment, the disintegration of the stomata, and finally to renewed synthesis of anthocyanin pigments.

As to the process of disintegration of the guard cells, this takes place as soon as the colouration of the sepals starts changing from pink to green. Furthermore, it always affects old stomata recognizable by the radial arrangements of the surrounding epidermal cells with a very wavy outline. The walls of the affected guard cells become thinner and thinner, the contents of the cells clot and lose their chlorophyll, and finally disappear. The cell walls of the surrounding epidermal cells radiating from the substomatal chamber become more apparent and acquire a greenish yellow colour. Gradually, the surrounding cells expand into the substomatal chamber and, finally, nothing is left but a discoloured cicatrice. The shape of these cicatrices always reflects the radial arrangement of the epidermal cells grouped around the affected stomata. There seems to be hardly any doubt that the radiating structures observed by Hiller (see Fig. 3) would not be identical with those observed by the present authors (see Fig. 4 and Plate I. B). It is interesting to mention that Hiller finds his radiating structures "meist an den oberen Theilen des Blattes", a finding that fits in very well with the acrofugal sequence of developmental processes of the foliar organs under discussion.

The type of stomatal disintegration described is fundamentally different from that investigated by FLAMM (1923). In the latter type, adjoining parenchyma cells fill up the substomatal chambers by thylloidal proliferation and the stomata themselves remain unchanged.

Lastly, by taking the disintegrated stomata into account we were able to eliminate most of the observed decrease in stomata index (§ 3.3). Indeed, only a slight decrease remained, probably due to the division of a number of epidermal cells in the course of forming stoma mother cells. We actually observed such a type of division,

since epidermal cells proved to divide a few times before giving rise to a stoma mother cell.

The present authors had the opportunity to investigate a small number of greening flowers of a second specimen of *Hydrangea macrophylla* "*Otaksa*". This plant had flowers with smaller and narrower petals and proved to differ in other respects as well. The stomata index showed no distinct rise during greening of the petals although dis-

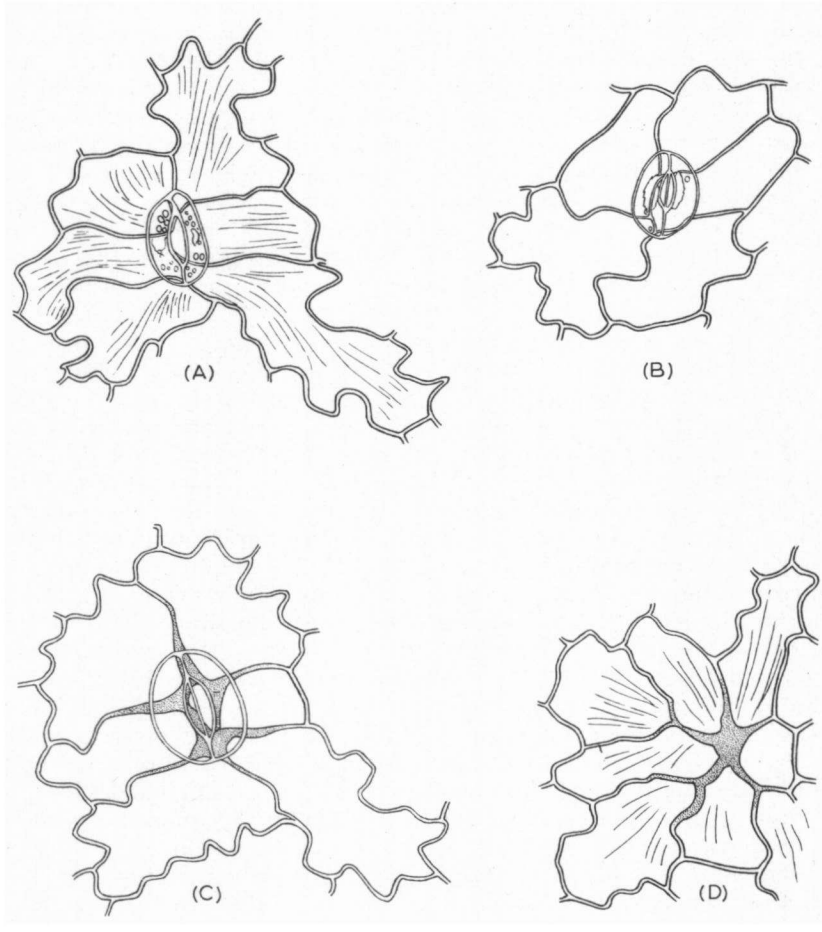

Fig. 4. *Hydrangea macrophylla* (Thunb.) D.C. "*Otaksa*". Successive stages of disintegration of stomata of the early, actinocytic type. A. The content of the guard cells is still present; cell walls already thin. B. The content of the guard cells has nearly disappeared. C. Guard cells empty. The cell walls have been drawn in but are in reality hardly visible. Radiating cell walls of the surrounding epidermal cells are thickened. D. Final stage of the process. Cicatrice closed. The striation of the cuticle is only shown in A and D. Flower XII, 4. 290 ×.

integration of old stomata and formation of new ones were observed, indicating that the processes under discussion may shift in relation to each other.

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