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Development of stomata in *Hordeum vulgare* L. under the influence of oleander glycosides and colchicine

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Abstract

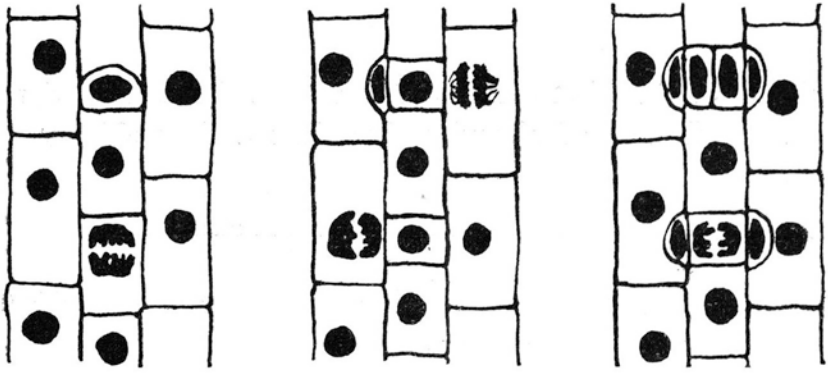
The effect of aqueous solutions of a 0.1 per cent mixture of oleander glycosides and of 0.1 and 0.5 per cent colchicine on the growth of seedlings, and particularly on the development of stomata was investigated in *Hordeum vulgare* L. The compounds were found not to penetrate with the same readiness through the coleoptile: glycosides passed very slowly, while colchicine rapidly. Growth inhibition of seedlings increased with the concentrations of the solutions applied, the time of incubation and the degree of damage to the coleoptile. Colchicine and glycosides cause a similar type of disturbances in all cell divisions leading to the formation of stomata. Most numerous disturbances were noted in phase II. The cause of these disorders lies in damage to the karyokinetic spindle and abnormal cytokinesis. As a result are formed the stomata with a changed number of guard cells or subsidiary cells, their shape is changed and sometimes also their orientation and the dimensions are reduced.

INTRODUCTION

Stomata in *Gramineae* are distributed in rows along the long axis of the leaf. They arise in three phases, occurring very regularly one after the other according to the schema shown below.

In phase I the asymmetrical division of some of the epidermal cells leads to differentiation of two cells: a smaller — guard mother cell and second large one which remains an epidermal cell. In phase II as the result of two asymmetrical divisions two subsidiary (or accessory) cells are formed by abscission on both sides of the guard mother cell. In phase III symmetrical division of the guard mather cell gives two guard cells. The mature stoma consists, therefore of four cells, two of which, the guard

cells are of a tibia-like shape, the remaining ones, known as subsidiary or accessory cells are ellipsoid in shape.



Phase I Phase II Phase III

A detailed analysis of the development of stomata in wheat, supported by electron-microscope studies is reported by Pickett-Heaps and Northcote (1966). In general it is an exceptionally stabilized process, there occur however deviations from the basic schema. These are spontaneous disturbances, the source of which is a change in the environmental conditions or in genetic factors (Stebbins and Shah 1960; Zeiger and Stebbins 1972).

Disturbances may also be experimentally evoked. This has been done by Stebbins et al. (1967) in investigations on young barley seedlings. They injured the plants mechanically and acted on them with various chemical agents from among which only 2-mercaptoethanol alone or in combination with gibberellic acid induced a certain protraction of the period of mitosis and disturbed the formation of subsidiary cells. Similar investigations with reference to the development and structure of stomata in *Lagenaria* after treatment of the plants with some of growth regulators (gibberellic and ascorbic acids, kinetin, coumarin, colchicine) were performed by Inamdar and Gangadhara (1975). In all cases the authors noted numerous anomalies in the structure, size and number of stomata formed. Disturbances in the structure of stomata caused by the action of colchicine were studied among other authors by Gavaudan (1938) in field-bean and by Weber (1943) in *Tradescantia*.

Another only recently known antimitotic agent are oleander glycosides. Their influence on the course of mitosis in meristematic root cells of *Allium cepa* and of the endosperm of *Haemanthus katharinae* were investigated and demonstrated by Tarkowska (1971a, b).

Both the above named compounds cause significant disturbances in mitosis, owing to the inhibition and disorganization of the karyokinetic spindle and phragmoplasts, which lead to:
— the formation of restitution nuclei from the whole chromosome set undivided or divided in the kinetochore;
— separation of whole chromosomes or chromatides into several groups giving daughter nuclei of various sizes;
— disturbances in cytokinesis leading to the formation of polynucleate cells or whole groups of cells with incomplete cell plates and in the case of oleander glycosides even to cells without nuclei.

In view of the properties of both these compounds and the highly stabilized mechanism leading to the formation of stomata in grasses, studies were undertaken on the influence of oleander glycosides and colchicine on young barley seedlings with particular reference to their action on the forming stomata.

MATERIAL AND METHODS

Hordeum vulgare L., var. *nutans* was used for the experiments. The epidermis from the lower side (abaxial) was investigated in the first leaf of young seedlings. Preliminary experiments demonstrated that oleander glycosides are retained by the coleoptile and permeate very slowly into the leaf tissues. Therefore, during experiments with glycosides the coleoptile was longitudinally incised once, twice or completely removed. The same procedure was applied to the control seedlings. Colchicine, on the contrary, penetrates readily through the coleoptile, and after the end of incubation has to be thoroughly washed out since it accumulates between the caleoptile and the leaf base. When the coleoptile was incised or removed, colchicine produced such violent disturbances that in the postincubation period no stomata were formed. Therefore experiments with the use of colchicine were carried out on seedlings with intact coleoptile and compared with controls treated in the same way.

For the experiments seedlings 1 cm high were taken, immersed to half height for 12, 24, 48 and 72 hrs (incubation in aqueous solutions — tap water) of 0.1 per cent oleander glycosides (Oleander Gesamtglycoside, Laborchemikalien Carl Roth CHG, Karlsruhe-West) and in 0.1 and 0.05 per cent colchicine. After each of the above listed incubation times the seedlings were once more transferred to tap water (postincubation) for 24, 48 and 72 hrs. The culture was kept in a chamber under 16-hrs daylight of 5000 lux intensity. The temperature of the chamber was maintained at 24—25° C in the daytime and at 22—23° C at night, moisture was kept constant.

The seedlings were measured before and after incubation and their length increment was compared with that of the control seedlings injur-

ed in the same way. The whole plants were fixed in acetoalcohol (1 : 3 by volume). Analyses were performed on preparations of epidermis from the lower leaf surface stained with acetocarmine.

Advantage was taken of the red lumiscence of chlorophyll in ultraviolet light to check fresh material (unfixed) for the presence of chloroplasts in mature but abnormally developed stomata.

RESULTS

The influence of oleander glycosides and colchicine on seedlings growth is visualized in Table 1.

It results from these data that injury to the coleoptile affects the degree of growth inhibition in the seedlings. Glycosides and colchicine enhance this effect (elongation growth diminishes with increasing concentration of the solution applied and the time of incubation). Moreover, at the base of the seedlings exposed to colchicine, thickening due to colchicine accumulation was obserevd, moving upwards with growth of the seedlings in postincubation.

Both the compounds used induce disturbances in all cell divisions leading to the development of mature stomata. These disturbances are, as already mentioned, characteristic for both the compounds tested.

One of the most essential disturbances is the failure of formation of the metaphase plate as the consequence of which:

a) whole metaphase chromosomes (Plate I, fig. 3) or chromatides by way of restitution transformation, form polyploid nuclei. This happens most frequently under the action of colchicine.

b) meta- or anaphase chromosomes aggregate into separate groups giving subsequently micronuclei (Plate I, figs. 1 and 2. This type of disturbances appears usually after treatment with glycosides.

Both antimitotics produce also disturbances in phragmoplast and cell plate formation. Beside polynucleate cells without cell plate, there form cells with incomplete cell plate of varying length as seen in Figs. 9—11 (Plate II, arrows).

The consequences of the above described disturbances in nuclear division and phragmoplast formation become particularly manifest in mature stomata, that is after a prolonged postincubation period. These disturbances appear in cell division in the III phase of stomatal development. They involve, therefore, nontypical guard cell and the subsidiary cells structure. In Figs. 1 and 2 (Plate I) polynucleate mother guard cells are visible which usually do not reach the third division, thus they do not produce guard cells. Disturbances in chromatin distribution may also occur during phase III and lead to the formation of binucleate and sometimes polynucleate guard cells (Plate I, fig. 4; Plate II, fig. 12).

Table 1

Length of seedlings incubated in colchicine and oleander glicosides as compared with that of control seedlings (mean values from 5 measurements)

Incubation time hours	Length of incubated seedlings, cm					Length of control seedlings, cm			
	colchicine intact coleoptile		0.1% oleander glicosides damage coleoptile			damage coleoptile			intact coleoptile
	concentration		incised once	inscised twice	removed	incised once	incised twice	removed	
	0.05%	0.1%							
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
12	1.6	1.6	1.5	1.5	1.5	1.5	1.5	1.5	1.6
24	2.8	2.0	2.0	1.8	1.7	2.0	2.0	2.0	3.6
48	3.1	2.2	3.0	2.0	1.8	3.3	3.0	3.0	6.4
72	3.5	2.5	3.0	2.0	1.9	6.5	5.0	4.8	9.8

Plate I

Fragments of lower (abaxial) epidermis taken from first leaf, Acetoalcohol (1 : 3), acetocarmine. Figs. 1—8 and 10—13 ca 1000 ×, Fig. 9 ca 600 ×

Fig. 1. Polynucleate guard mother cell. Oleander glicosides, 24 hrs of incubation.

Fig. 2. Polynucleate mother cell of subsidiary cells: Oleander glycosides, 24 hrs of incubation.

Fig. 3. Dispersed metaphase chromosomes in mother cells of subsidiary cells. 0.1% colchicine, 24 hrs of incubation.

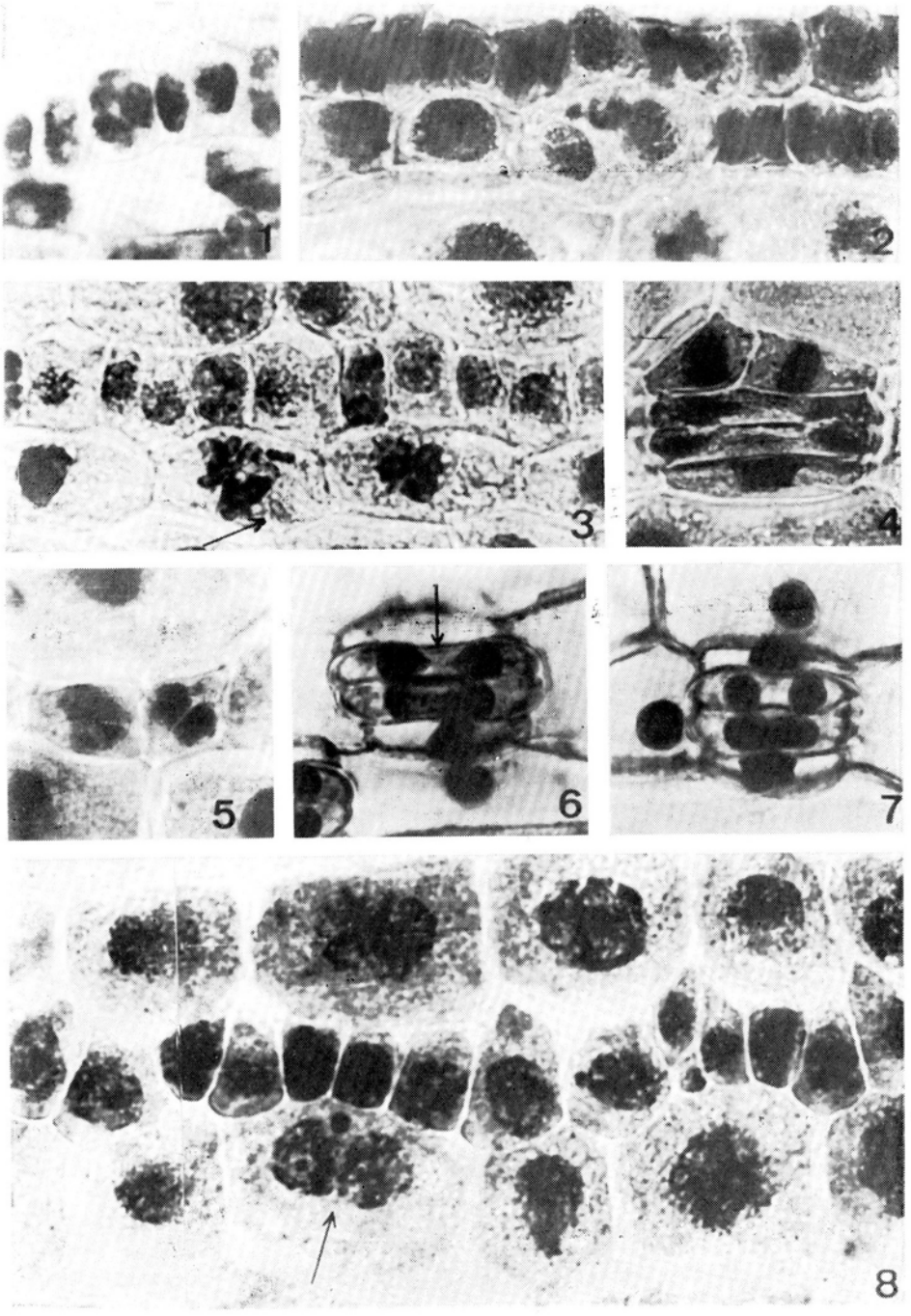
Fig. 4. Stoma with three subsidiary cells. 0.1% colchicine, 12 hrs of incubation and 48 hrs of postincubation.

Fig. 5. Tropokinesis in guard mother cell. 0.05% colchicine, 24 hrs of incubation and 48 hrs of postincubation.

Fig. 6 and 7. Constriction of nucleus in mature guard cell (arrow) leading to the formation of two nuclei. Oleander glicosides, 72 hrs of incubation and 72 hrs of postincubation.

Fig. 8. Inhibition of formation of stomata. Row of guard mother cells showing disturbances; a polynucleate cell (arrow) and polyploid mother cells of subsidiary cells are seen. 0.1% colchicine, 72 hrs of incubation and 72 hrs of postincubation.

Plate I



An interesting case is the constriction of the cell nucleus in the already formed stoma. In this way a two-nucleate guard cell arises (Plate I, figs. 6 and 7). Another, only sporadically noted, type of disturbances after application of the two tested compounds is the change in orientation of the guard cells (Plate I, fig. 5), sometimes by as much as 90° in relation to the leaf main axis (Plate II, fig. 13).

Most numerous disturbances were observed in phase II of development (formation of subsidiary cells) both after the action of colchicine and of glycosides. Frequently the mother cells of the subsidiary cells do not manage to divide, and, owing to this, the stomata are without subsidiary cells (Plate II, fig. 9, heavy arrows). Still more frequently, as the result of chromatin separation into several groups, together with inhibition of cytokinesis, the mother cells of the subsidiary cells remain polynucleate (Plate I, fig. 8, Plate II, fig. 9) or they have small incomplete cell plate (Fig. 9, thin arrow). Stomata have also been noted with 3 (Plate I, fig. 4), or sporadically with 4 subsidiary cells, as well as a complete change in the shape and size of the subsidiary cells (Plate II, fig. 12).

After 72 hrs of exposure to colchicine stomata may not form at all. Plate I, fig. 8 shows a fragment of the upper part of a leaf where stomata should be developed. As the results of disturbances, however, in the successive mitoses a row of undivided guard mother cells remained, and on both its sides ordinary polyploid mother cells of subsidiary cells are seen.

In some guard mother cells interesting structures were found (after treatment with colchicine) — capsules of unknown chemical composition. These capsules usually fill a large part of the protoplast between the nucleus and the cell wall. They are of horse-shoe or irregular cylindrical shape. In polarized light they luminescence (particularly their outer edges), in ultraviolet light they emit their own pale green luminescence. The appearance of such structures in polyploid guard mother cells of *Faba vulgaris* also under the action of colchicine is mentioned by G a v a u d a n (1938). It is supposed that these capsules are the result of the specific action of colchicine on cells associated developmentally with guard cells, since in the subsidiary cells and in the epidermis this phenomenon has not been observed.

Spontaneous disturbances may occur in the development of stomata in control seedlings. They have been only seldom observed (1—1.5%) in *Hordeum vulgare*. They involve usually the absence, and sometimes an excess of subsidiary cells. After damage, and particularly, removal of the coleoptile the number of such disturbances increases.

Damage of the coleoptile in control seedlings influences also the length of the stomata. The mean length of a stoma in an intact seedlings is on the average 57 μ, und after removal of the coleoptile — 40 μ. Oleander glycosides and colchicine inhibit still more cell elongation. The shortest

Plate II

Fig. 9. Various disturbances in one row of stomata. Oleander glicosides, 48 hrs incubation and 48 hrs of postincubation. For details see text.

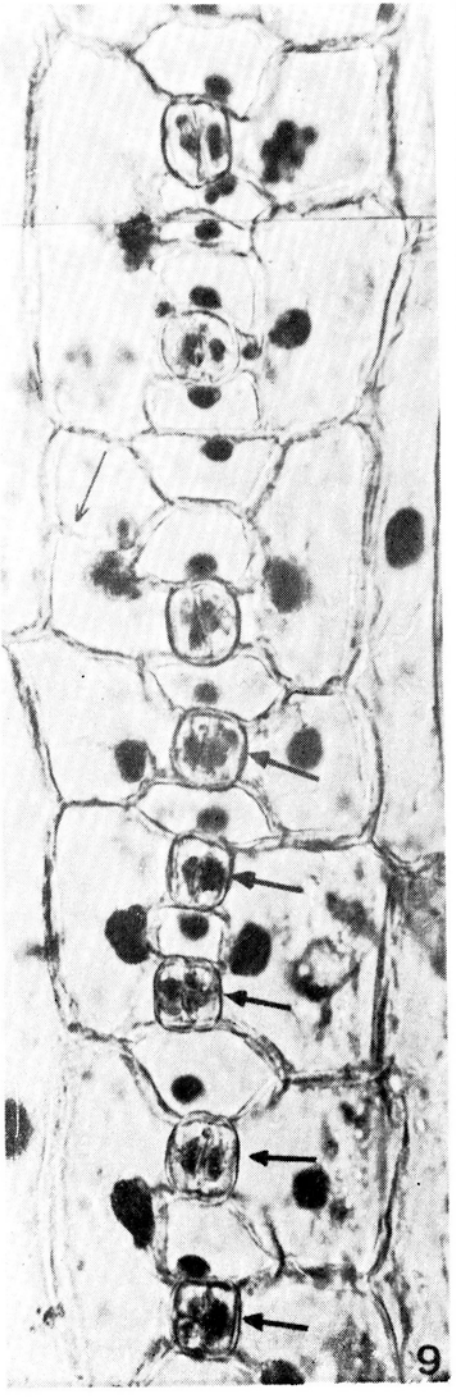
Fig. 10. Partial (short) cell wall in mother cell of subsidiary cell (arrow). Oleander glycosides, 72 hrs of incubation and 72 hrs of postincubation.

Fig. 11. Subsidiary cell changed in shape with patrial cell walls (arrow). Oleander glicosides, 48 hrs of incubation and 48 hrs of postincubation.

Fig. 12. Binucleate guard cells, lower subsidiary cell greatly elongated. Oleander glycosides, 72 hrs of incubation and 72 hrs of postincubation.

Fig. 13. Change of orientation of guard cells, by 90°. Oleander glicosides, 48 hrs of incubation and 72 hrs of postincubation.

Plate II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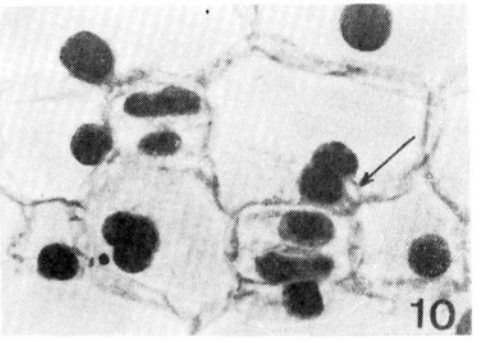
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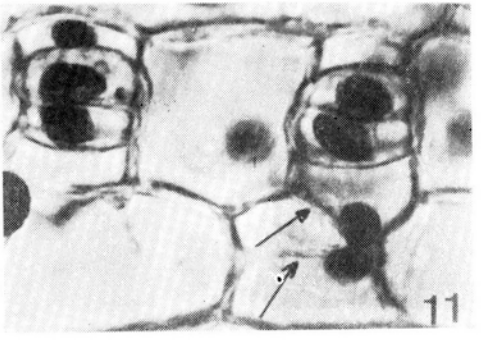
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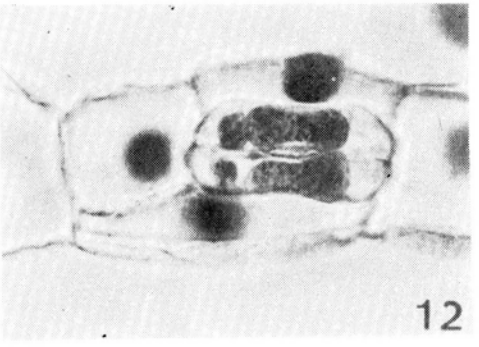
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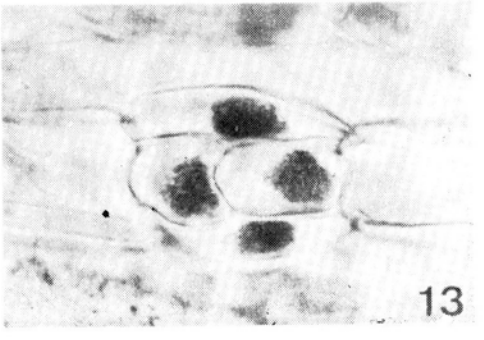
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stomata average 20-μ were observed after 72 hrs of incubation in glycosides after coleoptile removal and in 0.1 per cent colchicine (intact coleoptile).

The number of disturbances in the mitoses associated with development of stomata caused by injury to the coleoptile and the action of the tested agent were not counted accurately. During closer observation of the preparations, however, it could be noted that disturbances became more frequent with the increase of solution concentration, of the time of incubation and the degree of damage to the coleoptile. It was also found that during short incubation periods (12 hrs) most disturbances occur in phase I, and when this time is prolonged, the maximum of anomalies successively shifts to phase II and III. This is probably due to the cumulation of disturbances from the preceding phases and the occurrence of new disorders in the cells starting division.

An interesting problem is the presence of chloroplasts in the stomata with greatly changed structure. After treatment with glycosides chlorophyll luminescence in the guard cells was found to be reduced, whereas colchicine did not cause such changes — the number of chloroplasts and intensity of luminescence remained at the control level. Weber (1943) by acting with colchicine on *Tradescantia* leaves obtained sometimes even an increase in the number of chloroplasts in the guard cells and their appearance in the epidermal cells surrounding the stoma.

DISCUSSION

In young *Hordeum vulgare* seedlings the developing shoot is surrounded by a closely adherent coleoptile. Mechanical damage to it by incision, and particularly its removal, inhibit the growth of the seedlings. At the same time the incidence of disturbances in the development of stomata increases as compared with spontaneous disorders in intact control material. The mechanical factor is here the cause disturbing the normal development of stomata.

As demonstrated by experiments, the permeability of the coleoptile to colchicine and glycosides is different: colchicine penetrates readily to the young leaf, while glycosides pass very slowly, hence it is necessary to incise the coleoptile in this case. This is an essential matter in the analysis of the influence of the chemical factor on the formation of stomata. It is possible that the selectivity of the coleoptile in respect to the chemical compounds applied by Stebbins et al. (1967) was the reason why they did not observe any disturbances. The chemical compounds they used may have permeated very slowly or been retained by the coleoptile (the authors do not mention any injury to this organ). Probably the kind of plant used should also be taken into account. Pickett-Heaps

(1967) stresses that colchicine penetration into very young wheat seedlings was so slow that the coleptile had to be incised. In *Hordeum* there was no such necessity, the colchicine accumulating between the coleoptile and leaf was even difficult to wash out. On the other hand, the experiments of Kanciruk (1973) showed that oleander glycosides penetrate through the coleoptile quicker and more readily in *Triticum* than they do in *Hordeum*. Probably various substances permeate in a different degree through the coleoptile of various genera or species of grasses.

As already mentioned at the beginning, the mechanisms of action of both the compounds tested are similar and consist in influencing the karyokinetic spindle and phragmoplast. Hence the occuring disorders in the development of stomata are analogous, colchicine acting, however, stronger. Incubation in 0.1 per cent colchicine produces as early as after 24 hrs disturbances almost in all the cells forming stomata, and after longer exposure inhibits completely development of the latter (Plate I, fig. 8).

Mitotic figures are characteristic for colchicine and for oleander glycosides. Mitosis disturbances occur in all three phases of development of stomata, but they are most frequent in phase II during the formation of subsidiary cells.

Damage or destruction of the karyokinetic spindle and disturbances in cytokinesis are manifested by the formation of polyploid or polynucleate cells as well as polynucleate cells with incomplete cell plates. The stomata formed as the result of these disorders have a different number of guard and subsidiary cells. These changes are associated with changes in shape and a considerable reduction of the dimension of the stomata.

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Rozwój aparatów szparkowych u Hordeum vulgare L. pod wpływem glikozydów oleandra i kolchicyny

Streszczenie

Badano wpływ wodnych roztworów 0,1% mieszaniny glikozydów oleandra oraz 0,1% i 0,05% kolchicyny na wzrost siewek, a szczególnie na rozwój aparatów szparkowych u jęczmienia (*Hordeum vulgare* L.). Obserwacje prowadzono w czasie 72 godz. inkubacji i 72 godz. postinkubacji. Ze względu na bardzo wolne przenikanie glikozydów przez koleoptyl, nacinano go lub usuwano. Kolchicyna natomiast przenika bardzo łatwo, a działanie jej jest tak silne, że eksperymenty z kolchicyną prowadzone były na siewkach z nieuszkodzonym koleoptylem.

Siewki utrwalano w acetoalkoholu w stos. objęt. 3 : 1. Obserwacje przeprowadzono na preparatach ze zdartej dolnej skórki 1-go liścia, barwionej acetokarminem.

Stwierdzono, że hamowanie wzrostu siewek wzrasta wraz ze stosowanym stężeniem roztworów, czasem inkubacji i stopniem uszkodzenia koleoptyla.

Kolchicyna i glikozydy oleandra wywołują podobny typ zaburzeń we wszystkich podziałach komórkowych (trzech fazach rozwojowych) prowadzących do wytworzenia aparatu szparkowego. Jednak największą ilość i najbardziej różnorodne zaburzenia obserwowano w II-ej fazie tj. przy powstawaniu komórek pomocniczych (dodatkowych). Ogólnie biorąc ilość anomalii wzrasta wraz z czasem inkubacji, stężeniem działających roztworów i stopniem uszkodzenia koleoptyla.

Przyczyną zaburzeń jest inhibicja lub uszkodzenie wrzeciona kariokinetycznego i nienormalny przebieg cytokinezy, przejawiające się powstawaniem komórek poliploidalnych lub wielojądrowych, oraz komórek wielojądrowych z niepełnymi przegrodami. Powstające w wyniku tych zaburzeń aparaty szparkowe mają zmienioną ilość komórek przyszparkowych i pomocniczych, zmienione kształty i niekiedy kąt ustawienia, oraz zmniejszone wymiary.