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Effect of water extract from leaves of *Nerium oleander* L. on mitosis

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Abstract:

The effect of water extract from leaves of *Nerium oleander* L. on the mitosis in meristematic cells of *Allium cepa* L. root tips has been studied. Observations were made on the changes during incubation and postincubation.
Significant disturbances were observed in the development of the mitotic spindle leading to the formation of polyploid and hypoploid nuclei capable of further division. Substances contained in the water extract, and causing the disturbances, are water soluble glycosides. Introductory observations under an electron microscope indicate that the glycosides desorganize the continuous fibres of the spindle which can be considered as the direct cause of the observed disturbances.

INTRODUCTION

Having studied for a long time the antimiotic properties of water extracts from plants which have anti-cancer attributes I became interested in oleander (*Nerium oleander* L.) which is mentioned by Taylor, McKenna and Burlage (1956) as being characterized by anti-cancer activity. Water extract of oleander has been known for a long time as a medicine for heart malfunctions, but its very interesting antimitotic activity has not been signalized as yet.

The term "antimitotic substances" is used to cover generally all chemical substances, which cause various types of disturbances in the course of nucleus division, and thus reduce the number of mitoses (regardless of the mechanism ich which these substances act). Classification of the antimitotic substances is very difficult since it should be based on the biochemical mechanism of their action. Various attempts at classifying them have been made among others by Kihlman (1955, 1956, 1966), Biesele (1958) and Deysson (1968).

Water extracts from various plants constitute, however, a separate group, that does not fit into any of the classifications mentioned above, since they comprise a mixture of various compounds, frequently little known or not known at all. Besides inhibiting mitoses they may also cause other disturbances in the life cycle of a cell.

It is the purpose of this paper to report the disturbances in mitoses caused by

water extracts from leaves of oleander. During the studies it was possible to establish that the substances causing the disturbances are the water soluble glycosides contained in the extract. Further stages of work concerning the action of a mixture of these glycosides are the subject of a separate publication (Tarkowska 1971).

Also introductory studies were performed *in vitro* and under electron microscope to investigate the effect of a mixture of glycosides on the cells of *Heamanthus katharinae* Bak. endosperm. They permit the determination of the direct cause of the disturbances observed under optical microscope. After completion of this phase of the work detailed results will be published, while now only the introductory information is presented in the discussion at the end of this paper.

MATERIALS AND METHODS

As test material meristematic cells of onion (*Allium cepa* L.) root tips have been used.

The bulbs were grown at room temperature (around 20°C) in tap water in dark 350 ml bottles, both aerated and not-aerated. No differences were found in the intensity and type of disturbances between these two variants. Roots for the experiments, on transfer from water to the extract were about 2 cm long. Both the water and the extract have been changed every 24 hours.

Extract from leaves of oleander (*Nerium oleander* L.) has been prepared in the following way. 10 g of dry, crushed leaves have been covered with 125 ml of tap water at room temperature and left for 4 hours. After that time the extract was filtered off and the same leaves have been covered with 125 ml of hot water and boiled slowly for 2 minutes, then left until cold and filtered again. Both the extracts were pooled together, shaken to aerate them, and the so prepared extract (extractum crudum) has been considered for further purposes as being 100%. Its pH was 6.1.

The time of action of the extract on the root tips (incubation), after which its effect on the dividing cells was checked, was: 2, 6, 8, 12, 24 and 48 hours. Other onions after 12, 18, 24 and 48 hours of action of the extract have been transfered back to tap water (postincubation) and during the following 72 hours the further fate of the incubated meristematic cells was investigated. Simultaneously control bulbs were grown in tap water for each of the different periods.

As basis for the observations squash preparations have been used, fixed in aceto-alcohol (1:3 by volume) for 4 hours and then stained with acetoorcein. The microtome method was used only accessorily.

RESULTS OF THE OBSERVATIONS

The water extract of oleander leaves used for the experiments was not toxic in action but did induce significant disturbances in the course of mitosis of the studied meristematic cells.

The first significant deviations from the normal state of the cells are observable after 4–6 hours of incubation in the extract and are manifest in a considerable reduction in length of the chromosome arms (figs. 1–3) the process progressing during the further incubation of the roots in the extract.

Metaphase chromosomes initially become grouped relatively irregularily in the centre of the cell, but do not show a tendency to form a typical metaphase plate. In fig. 1 an example of "pseudospindle mitosis" (Deysson 1968) is shown. The chromosomes are scattered over the cell at the surface corresponding to the zone normally taken by the mitotic spindle. Two extremally located chromosomes are at the poles of the pseudospindle. There is no doubt that the extract causes significant disturbances in the formation and function of the mechanism responsible for chromosome movement. From the pictures obtained on squash preparations and an analysis of the microtome preparations it could be established that the bipolar mitotic spindle does not exist at all. However on the microtome sections, in the phase corresponding to normal metaphase it is possible to observe near chromosomes short tufts of kinetochore fibres (fig. 2 arrows), which would tend to indicate that in the first place damage comes to the continuous fibres of the mitotic spindle. These significant disturbances in the structure of the spindle influence the later behaviour of the chromosomes. The normal movement of chromatids to opposite poles of the cells does not take place.

Movements of chromosomes or chromatids within a cell are accidental and multidirectional. It is chracteristic that both whole chromosomes can be displaced, observable as x-figures (fig. 3) characteristic for c-mitoses, as well as single chromatids (fig. 4).

The disturbances in the dividing cells lead to two main effects:

— formation of polyploid restitution nuclei

— formation of micronuclei.

In the first case, whole chromosomes, with unsplit kinetochore (also the x — figures), or all the chromatids originally present in the central part of the cell (fig. 1, 3) aglomerate and form single polyploid restitution nucleus (fig. 5)

In the second case, that is on the formation of micronuclei either whole chromosomes aggregate into larger or smaller groups (fig. 6) or the chromatids form multipolar anaphases (fig. 4). The end effect is always the formation of hypoploid nuclei of various size with very variable chromosomal and genetic composition.

The number of chromosomes or chromatids aggregating into the groups mentioned above and later forming nuclei of various sizes can be very variable, usually around 4–7 chromosomes but it can also be 1 or 2 chromosomes (fig. 6).

The number of groups of chromosomes in a cell forming from the full chromosome complement of the given cell can also be variable. From 2 to 8 have been observed (fig. 4, 6, 7). The formed groups can be connected by chromosomal or chromatid bridges (fig. 7), and the groups can coalesce before the nuclei are formed. Thus the final number of nuclei formed in a cell can be lower than the number of groups of chromosomes observed initially. A special case of uneven chromosome

EXPLANATIONS OF FIGURES

Plate I

Figs. 1 and 3–8 cells from squash preparations, fixed in acetoalcohol (1:3) and stained in acetoorcein. Fig. 2. Microtome section, CrAF, 0.5–1–20, toluidine blue. Magnification ca 1500x

Fig. 1. Chromosomes strongly shortened. Their arrangement in the cell demarcates the region of the pseudospindle. After 6 hours of incubation.

Fig. 2. "Tufts" of kinetochore fibres seen near the metaphase chromosomes (arrows). After 12 hrs of incubation.

Fig. 3. X-figures before the formation of a restitution nucleus. After 8 hrs of incubation.

Fig. 4. Multipolar anaphase. After 6 hrs of incubation.

Fig. 5. Formation of a restitution nucleus from chromosomes unsplit in kinetochores (arrows). After 12 hrs of incubation.

Fig. 6. Formation of micronuclei. After 10 hrs of incubation.

Fig. 7. Uneven separation of chromosomes. Chromatid bridges visible. After 8 hrs of incubation.

Fig. 8. Promatephase immediately after the breaking of the nuclear envelope in micronucleus containing 2 chromosomes. After 24 hrs of incubation and 48 hrs of postincubation.

Plate II

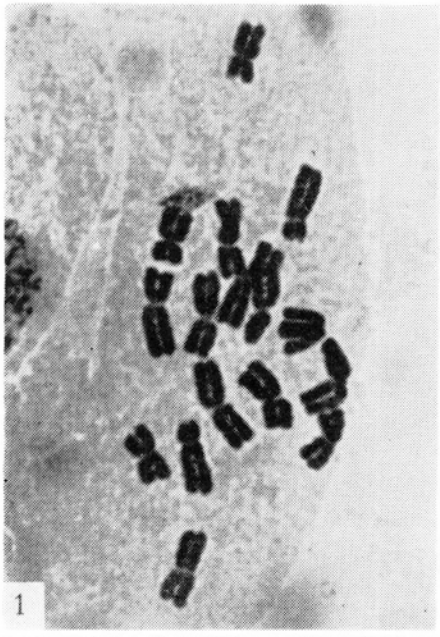
All cells from squash preparations following acetoalcohol and acetoorcein. 36–48 hours of postincubation following 24 hrs of incubation. Magnification ca 1000×

Figs. 9–11. Various stages of mitosis. Divisions synchronized: Fig. 9 in a 3 nucleate cell, Fig. 10 — in a cell with incomplete cell plates, Fig. 11 — an example of incomplete synchronization of divisions in one cell complex.

Fig. 12. A poly-nucleate cell

Figs. 13–15. Cell complexes with uni and poly-nucleate cells. In figs 13 and 14 there are distinct regions without nuclei (an)

Plate I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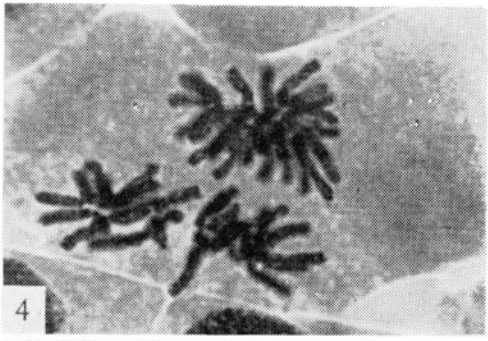
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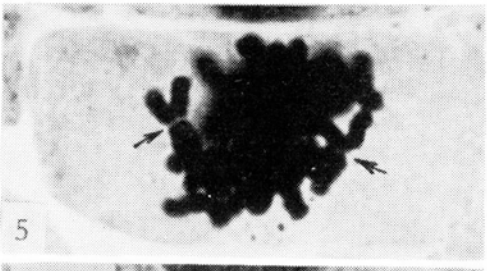
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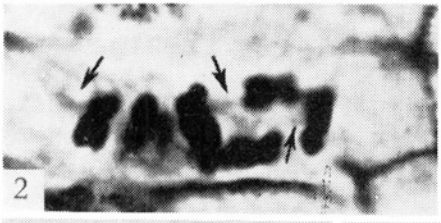
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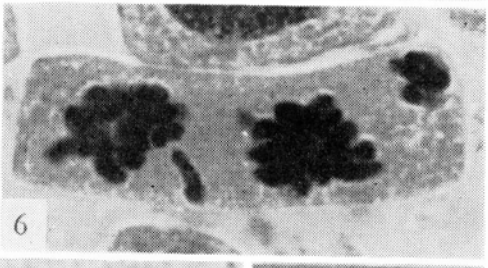
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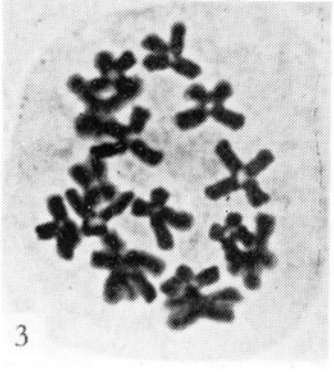
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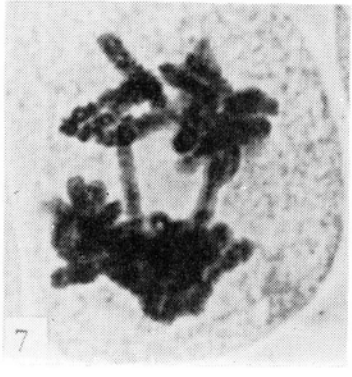
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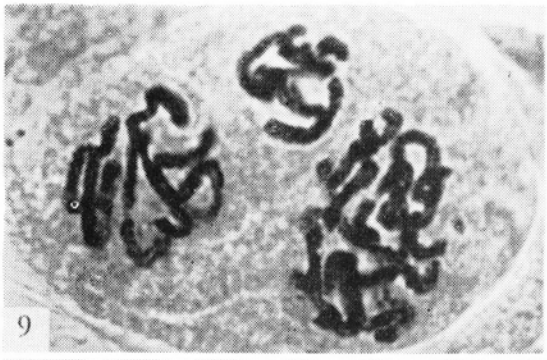
Plate II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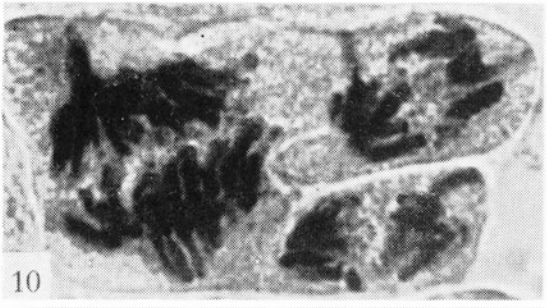
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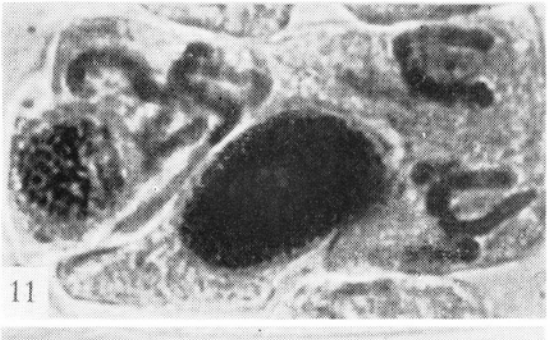
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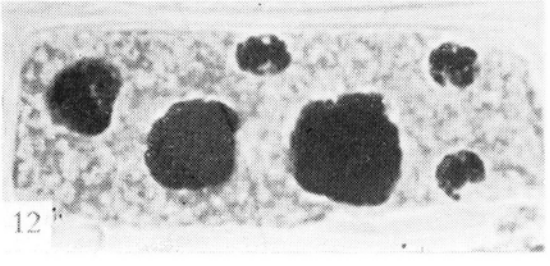
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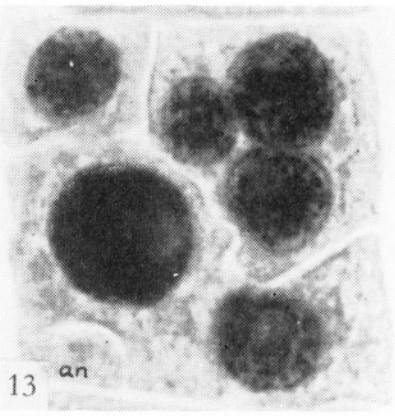

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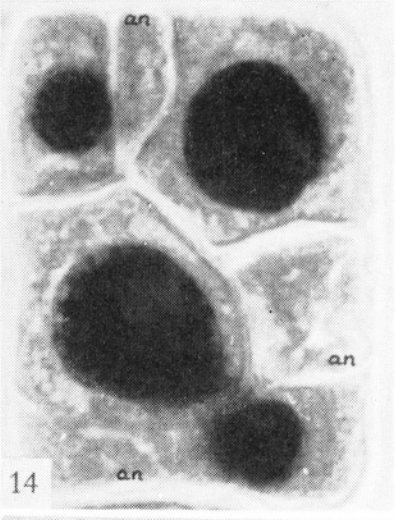

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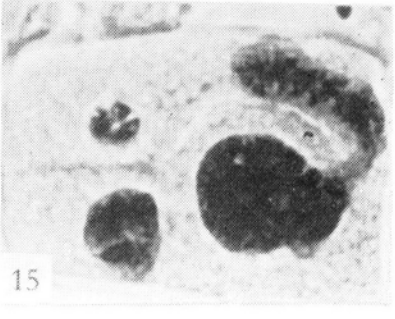
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division is the "somatic reduction", that is the separation of the metaphase chromosomes in somatic cells into two groups.

Very interesting and significant for this problem is the course of cytokinesis, which leads to the formation of cell plates between cells. In a few cases these cell plates do not form at all and the cells remain multi-nucleate (fig. 12). More frequently, however, cell plates form, either as complete, separating off a given region of a cell (fig. 13,14) or incomplete ones which cut off parts of the cell containing cytoplasm only (fig. 10, 15). Both types of cell plates can form during the incubation time, in various stages of the nuclear modifications, that is either during the formation of chromosome groups or after cell nuclei are completely formed. In the first period of postincubation the consequences of disturbances in the process of cytokinesis, caused under the direct influence of the extract during incubation, are the best observed (fig. 12–15). The complete cell plates formed between the sister nuclei usually divide the mother cell into several sister cells. The new formed cells have various sizes and shapes, and their nuclei, as was mentioned above, consist of a varied, accidentally aggregated numbers of chromosomes. Not every cell nucleus, together with the part of cytoplasm surrounding it will become separated out into a uninucleate cell. Frequently 2 nucleate cells form and more rarely cells with more nuclei (fig. 13, 15).

In both cases, that is following the formation of complete or incomplete cell plates, complexes of uninucleate and polynucleate cells form, the arrangement of which, size and shape indicate that they originate from the same mother cell. It also happens that parts of cytoplasm without nuclei become separated off (fig. 13, 14).

The disturbances referred to above, concerning nucleus and cell division take place in dividing meristematic cells of root tips at the time when the root tips are immersed in the extract. This concerns both the cells which were dividing at the time when incubation was started and those which started dividing during incubation. The extract acts as a typical antimitotic substance, since it causes a constant reduction in the number of cells starting to divide. After 48 hours only few mitoses are observable or they are absent altogether. Thus even though both after 12 hours and after 48 hours similar figures can be found (groups of chromosomes, polynucleate cells, cell complexes), the length of incubation time affects the frequency with which the various figures occur. After longer incubation (24–48 hrs) polynucleate cells and cell complexes dominate.

After a definite time of action of the extract (incubation) (12, 18, 24 and 48 hrs) the recovery was studied by transferring the onions back into tap water (postincubation). In the first 24 hrs of postincubation it is usually possible to observe extension growth of the root tips amounting usually to a few millimeters. Microscopic observations have shown that this is caused primarily by cell elongation and not by increased cell division (that is increase in cell number), since the first normal mitoses after transferring to tap water appear only after a certain time. Thus when for example the extract acts for 24–48 hrs the first divisions during postincubation were observed only after 30 hrs. It was found that the longer was the time for which the root tips were cultured on the extract the more time elapses from the transfer of the onions back into water to the appearance of the first cell divisions.

Divisions also occur in the cell complexes, but this always happens later in comparison with the divisions in normal cells (with a 2n number of chromosomes). This delay varies between 12 and 24 hours. During postincubation all the phases can be observed of the division of micronuclei of various chromosome number and containing sometimes even only two chromosomes (fig. 8). Divisions in poly-nucleate cells and in cells with incomplete cell plates between the nuclei are usually synchronized (fig. 9, 10), though examples of incomplete synchronization can also be found (fig. 11). However mitoses do not occur in all cells that have nuclei with excessive or incomplete number of chromosomes. A considerable number of these cells become shifted in the root into the region of elongation and maturation, without prior divisions.

After having established the effect of the extract described above, an attempt was made to answer the question, which of its components is responsible for these disturbances. It is known that leaves of oleander contain, among other compounds, the glycosides which have strong cardial effects. Thus the suggestion aroses that they are responsible for the abnormal mitoses. In order to check this suggestion an experiment was conducted with a mixture of glycosides (Oleander Gesamtglykoside, Carl Roth Laborchemikalien, Karlsruhe-West). It was tested on root tips of *Allium cepa* using the same durations of exposure to the solution as was the case in the experiments with extracts. On squash preparations stained with acetoorcein almost identical mitotic disturbances have been found. It was also found that a 100% water extract from the leaves corresponds to a 0.05% water solution of the glycoside mixture. The results obtained and their detailed analyses are the subject of a separate publication (Tarkowska 1971).

DISCUSSION

There is no doubt that glycosides are the main acitve components of a water extract from leaves of oleander, responsible for the disturbances in the mitoses of meristematic cells of *Allium cepa* root tips. The main effect of their action is the disorganization of the structure and function of the mitotic spindle, which results in disturbances in the normal movements of chromosomes during division. In consequence restitution nuclei form from the whole complement of chromosomes or chromatids, or else they divide unevenly into several groups, which leads to the formation of micronuclei.

Studies on the effect of glycosides on the endosperm cells of *Haemanthus katharinae* Bak. *in vitro* and under electron microscope (Tarkowska — in preparation) permit the elucidation of the mechanism and direct cause of disturbances in chromosome movements. Results obtained under electron microscope indicate that the disorganization of the spindle in the primary phase of glycoside action is the result of the disorganization of the continuous fibres and later also of the kinetochore fibres. The centromeres themselves maintain for a long time the capacity to form microtubules. In an electrone microscope it was found that the "tufts" of fibres

observed under optical microscope near metaphase chromosomes (fig. 2) are bundles of kinetochore microtubules, which persist even during long periods of exposure of the dividing cells to glycosides.

Similar figures as those described in this paper have been observed under optical microscope by Molé-Bajer in the endosperm of *Haemanthus* after treatment with methanol (1965) and chloral hydrate (1967). The effect of chloral hydrate she has also studied under electron microscope (1969) and obtained very different pictures than have been obtained after treatment with oleander glycosides (Tarkowska 1971) Chloral hydrate originally destroys both the continuous fibres and the kinetochore fibres, and only after a longer exposure to chloral hydrate the kinetochore microtubules regenerate but first they have a converging arrangement near the centromeres after which they attain a dispersed arrangement, typical for normal mitoses.

One of the special cases of uneven division of chromosomes induced by oleander glycosides is the so called "somatic reduction", that is the separation of metaphase chromosomes into two groups. This phenomenon has been described several times in the meristematic cells of root tips following treatment with various agents such as colchicine and sodium nucleate (Allen, Wilson and Powell 1950), various antibiotics (Wilson 1950) and low temperatures (Huskins and Cheng 1950).

As a result of the above mentioned disturbances in the movement of chromosomes, caused by extracts from leaves of oleander polyploid cells are formed as well as cells with a number of chromosomes greater or lower than 2n. The use of root tips for the experiment described above proved very convenient, since it permits the study of the reversibility of changes in cells with disturbances following a transfer of the root tips from the extract to water. It appears that the cell nuclei, both with the incrased and decreased chromosome numbers are capable of further divisions, which may have important genetic effects.

CONCLUSIONS

A study was made of the effect of a water extract from leaves of oleander (*Nerium oleander* L.) on mitosis in meristematic cells of onion (*Allium cepa* L.) root tips.

The following disturbances in the course of mitosis have been observed:

1. Strong shortening of the chromosomes,

2. Disturbances in the formation and development of the mitotic spindle, which results in:

a) formation of restitution nuclei from all chromosomes (unsplited at the kinetochore) or from chromatids,

b) separation of chromosomes or less frequently chromatids into several groups, from which nuclei of various sizes are formed (micronuclei).

3. Disturbances in cytokinesis leading to the formation of uni or poly-nucleate cells, as well as those comprising only cytoplasmic regions, without nuclei. The cell plates forming between nuclei can be complete or incomplete.

During postincubation normal mitoses were observed of both polyploid nuclei and hypoploid nuclei (containing even 2 chromosomes).

It was found that the disturbances referred to above have been induced by the glycosides contained in the water extracts from leaves of oleander (Tarkowska 1971). Introductory experiments with the use of an electron microscope have shown that the direct cause of the disturbances is the disorganization of the microtubules forming the continuous fibres of the mitotic spindle. The results obtained permit the suggestion that the correct, regular arrangement of continuous fibres is necessary for the normal bipolar division of the chromosomes at anaphase.

It is my pleasure to thank Prof. Dr J. Szuleta for suggestions and constructive criticism during the progress of experiments.

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Wpływ wyciągu wodnego z liści oleandra (Nerium oleander L.) na mitozę

Streszczenie

Badano wpływ wyciągu wodnego z liści oleandra (*Nerium oleander* L.) na mitozę w komórkach merystematycznych wierzchołków korzeni cebuli jadalnej (*Allium cepa* L.). Stosowano tylko jedno stężenie wyciągu, określone umownie jako 100%. Po działaniu wyciągu na korzenie w ciągu 6–48 godz. (inkubacja), cebule przenoszono do wody na okres 72 godz. (postinkubacja).

Stwierdzono następujące zaburzenia w przebiegu mitozy:

1. Silne skracanie chromosomów

2. Zaburzenia w tworzeniu się i rozwoju wrzeciona podziałowego, w wyniku czego następuje:
a) powstawanie jąder restytucyjnych z całego zespołu chromosomów (niepodzielonych w kinetochorach) lub chromatyd,
b) rozdzielanie chromosomów (częściej) lub chromatyd na kilka grup, z których powstają jądra różnej wielkości (mikrojądra).

3. Zaburzenia w przebiegu cytokinezy prowadzą do powstawania komórek jedno lub wielojądrowych, a także bezjądrowych terenów cytoplazmy. Powstające przegrody komórkowe mogą być całkowite lub niecałkowite.

W okresie postinkubacji obserwowano normalne mitozy zarówno w jądrach polyploidalnych, jak i hypoploidalnych (zawierających nawet 2 chromosomy).

Stwierdzono, że w/w zaburzenia wywołane są glikozydami zawartymi w wodnym wyciągu z liści (Tarkowska 1971). Wstępne badania w mikroskopie elektronowym wykazały, że bezpośrednią przyczyną występujących zaburzeń jest dezorganizacja mikrotubul tworzących włókna ciągłe wrzeciona podziałowego. Uzyskane wyniki pozwalają przypuszczać, że prawidłowy, regularny układ mikrotubul ciągłych jest konieczny dla normalnego 2-biegunowego rozdziału chromosomów w anafazie.