Fine structure of kinetin-treated protonema and kinetin-induced gametophore buds in *Funaria hygrometrica* 

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#### Abstract:

Besides occasional hypertrophy of grana and disintegration of stroma thylakoids occurring in some chloroplasts, no significant changes were found in ultrastructure of typical protonema cells treated for six days with kinetin. On the other hand, the fine structure of cells in kinetin-induced gametophore buds differed much from that of the protonema cells and showed characteristics of cells of with high metabolic activity and high division rates. The results indicate that cytokinins enhance development and differentiation in the protonema by activating only some of its cells, whereas the others remain unchanged or show symptoms of destruction and ageing. This is supported by the fact that in the presence of chloramphenicol, which prevents bud induction, kinetin acts synergistically with the inhibitor in producing degeneration and destruction of chloroplasts.

#### INTRODUCTION

There are relatively few studies concerning the ultrastructure of cytokinin-treated plant cells, and their results are very different depending on the plant material used. Neumann, Cireli and Cireli (1969) found that in carrot explants the high cell division rate induced by kinetin was not accompanied by any significant changes in the ultrastructure of cell organelles, with only a slight decrease in the number of mitochondria and ribosomes per cell. In onion roots, the stimulation of cell divisions occurring after a 1/2 hour treatment with kinetin was accompanied by an increase in number and size of vacuoles and by striking changes in the endoplasmic reticulum which was broken up into vesicles with abundant ribosomes associated with their memebranes (Hayat and Salama 1966). Several investigations showed an important role of cytokinins in the development of chloroplasts. Stetler and Laetsch (1965) found that kinetin is a specific requirement of cultured tobacco tissue for differentiation of proplastids into mature chloroplasts under light. In ageing leaves, cytokinins

prevent disintegration of various organelles (Shaw and Manocha, 1965; Butler, 1967; Dennis, Stubbs and Coultate, 1967); in particular they inhibit the degradation of the membrane structures of chloroplasts and mitochondria. Some authors (Kursanov et al. 1964; Sveshnikova, Kulayeva and Bolyakina, 1966) observed that in old yellow leaves, in which only chloroplasts with disintegrated grana were present, cytokinins induced a restoration of the thylakoid system and formation of new grana, together with new formation of chlorophyll and an enhancement of photosynthesis; similarly a restoration of the structure of mitochondria was observed.

One of the most striking biological effects of cytokinins is the induction of gametophore buds in the protonema of mosses (Bopp, 1965). The purpose of this study was to investigate this effect at the level of cell ultrastructure in the protonema and in the induced buds. The effect of chloramphenicol on the ultrastructure of kinetin-treated cells was also studied, as it has been shown previously that chloramphenicol inhibits the bud-inducing effect of kinetin (Szweykowska, Ratajczak and Schneider 1968), and brings about destructive changes in chloroplasts and mitochondria (Młodzianowski, Szweykowska and Schneider, 1970).

# MATERIAL AND METHODS

The protonema of Funaria hygrometrica was grown from spores on a nutrient medium consisting of mineral salts and glucose at 0.25% (Szweykowska and Handszu 1965). After ten days of growth it was transferred to a mineral medium with the addition of: 1) kinetin —  $5 \, \mu \text{M/l}$ , 2) kinetin —  $5 \, \mu \text{M/l}$ +chloramphenicol — 0.5 mM/l; the control material was transferred to a mineral medium with no additions. After six days, the material was fixed in KMnO<sub>4</sub> (Luft 1956) or in 1% OsO<sub>4</sub> solution in 0.1 M phosphate buffer of pH 7. At this time, numerous gametophore buds were present in the kinetin medium; no buds appeared in the control medium and in medium containing chloramphenicol.

In another series, kinetin (5  $\mu$ M/l) was added to a 10-day-old protonema culture in a mineral solution, and after three days (no buds visible yet), the material was fixed in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer of pH 7.

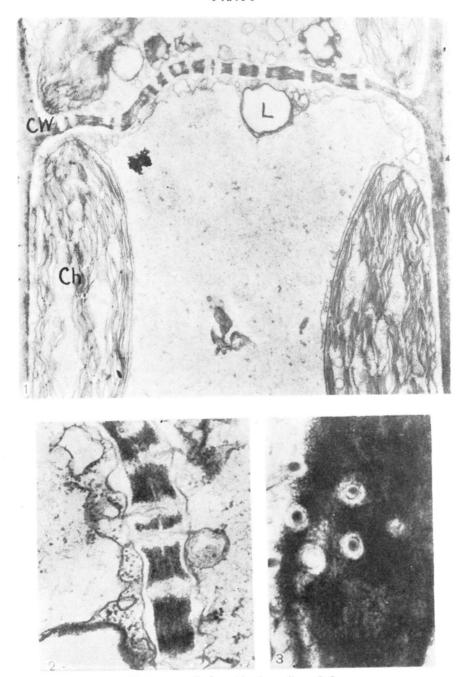
The material was dehydrated with ethanol and propylene oxide, and embedded in Epon 812. Sections were contrasted with uranyl acetate and lead citrate according to Reynolds (1963). The micrographs were taken with a JEM-7A electron microscope.

## RESULTS

The fine structure of the protonema of Funaria hygrometrica has been described in a previous paper (Młodzianowski, 1970). No significant changes in structure of the protonemal cells were found after the addition of kinetin to the culture medium, particularly during the first 3 days of kinetin treatment. This is illustrated by the photographs in plates I—III. The neighbouring protoplasts in rows of

cylindrical cells of the protonema filaments were connected by numerous plasmodesmata (figs. 1-3). Endoplasmic reticulum (ER) in the form of continuous cisterns was observed only occasionally. The ribosomes occurring freely in the ground cytoplasm were often seen in polyribosome configurations (fig. 5). Numerous lipid bodies and less numerous microbodies were observed similarly as in the control material. The nucleoplasm showed a fibrillar as well as granular structure, with the granules forming occasionally small agglomerations (fig. 4). The structure of nucleoli was variable. This variability, in relation to the age of cells, has been ealier pointed out by Bopp (1955). In fig. 4 a nucleolus after osmium fixation is shown, with an electron-dense peripheral zone separated from the central part by a narrow light. space. The mitochondria had a well developped system of tubules (fig. 6), and oval or rod-like profiles. Special attention has been devoted to the structure of chloroplasts as it is known that cytokinins are particularly involved in processes of their development. The fixation of the material in glutaraldehyde proved to be particularly suitable for preservation of the chloroplast structure. In this fixative the grana were much less swelled than in KMnO<sub>4</sub> or OsO<sub>4</sub> (Młodzianowski 1970). Besides, after glutaraldehyde fixation, ribosome-like particles and nucleoid-like regions could be seen in the chloroplasts (figs. 7 and 8). The structure of the majority of chloroplasts was much the same in control and kinetin-treated protonema. However, after six days of kinetin treatment, the grana of some chloroplasts were very large and lense-shaped, with densely packed lamellae (figs. 9 and 11). The outer compartments of these grana were often swollen (fig. 9). Chloroplasts of this kind were observed after glutaraldehyde as well as osmium fixation. The excessive development of grana as well as the enlargement of their outer compartments may be a symptom of cytokinin overstimulation which was also observed by Dennis et al. (1967) in young leaves of Brussels sprouts and by Farineau and Roussaux (1970) in pea cotyledons. It also may be considered as a manifestation of degeneration (Ashton 1963), as generally in the kinetin-treated protonema chloroplasts with such destruction symptoms as swelling, disintegration of the stroma thylakoids and disruption of the surrounding membrane (figs. 9-10) were much more frequent than in the control material. This might be connected with the intensification by kinetin of differentiation and ageing of the protonema, and the induction of large numbers of gametophore buds which form competition centres leading e.g. also to a decrease of chlorophyll content in the protonema (Brandes 1967). The destructive changes in chloroplasts may constitute a considerable part of these ageing processes occurring in the protonema under the influence of cytokinins.

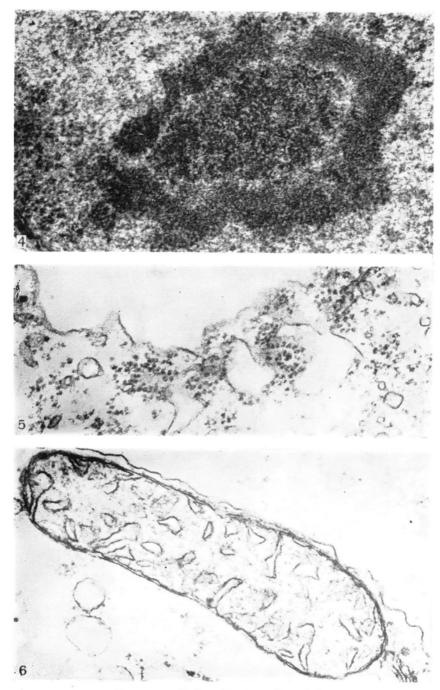
Gametophore buds appeared only in cultures treated with kinetin. They consisted of numerous isodiametric or slightly elongated cells forming three-dimensional, moruloid aggregations at the ends of short lateral branches of the protonema (fig. 12). The ultrastructure of the bud-cells differed much from that of protonema cells. The former contained a very dense cytoplasm with many free ribosomes, often in polyribosome configurations, and were also richer in endoplasmic reticulum. The granular ER was situated mostly along the cell walls and formed bead-like distensions



Protonema cells from kinetin medium. OsO4.

Fig. 1. Longitudinal section. 15 000 x. Fig. 2. Plasmodesmata on longitudinal section. 40 000 x. Fig. 3. Plasmodesmata on the cross section. 40 000 x.

Abbreviations for Plates I-VIII



Protonema cells from kinetin medium. OsO4.

Fig. 4. Nucleolus, 60 000 x. Fig. 5. Polyribosomes in the cytoplasm. 57 000 x. Fig. 6. Mitochondrion, 52 000 x.

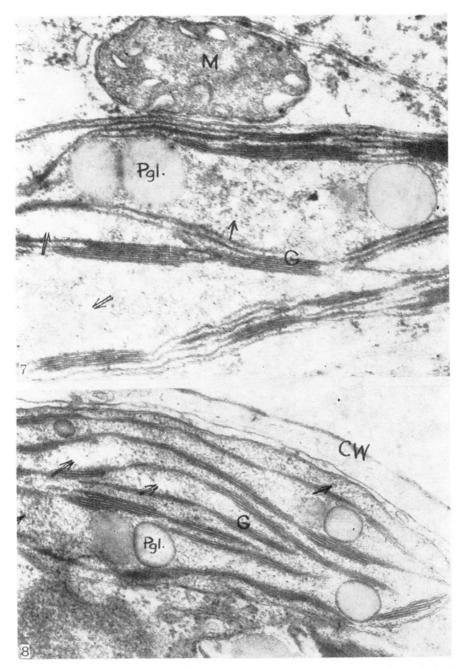
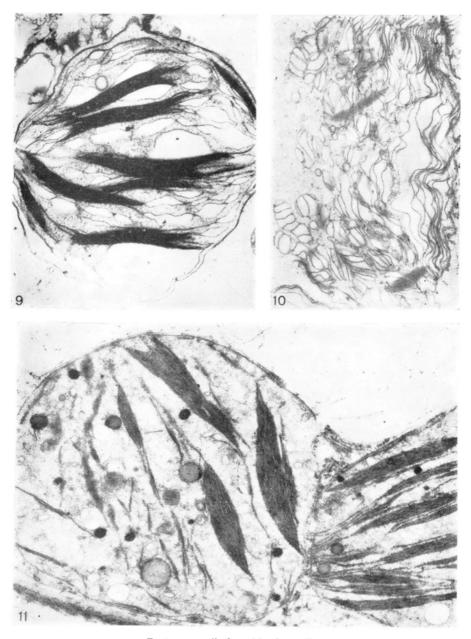
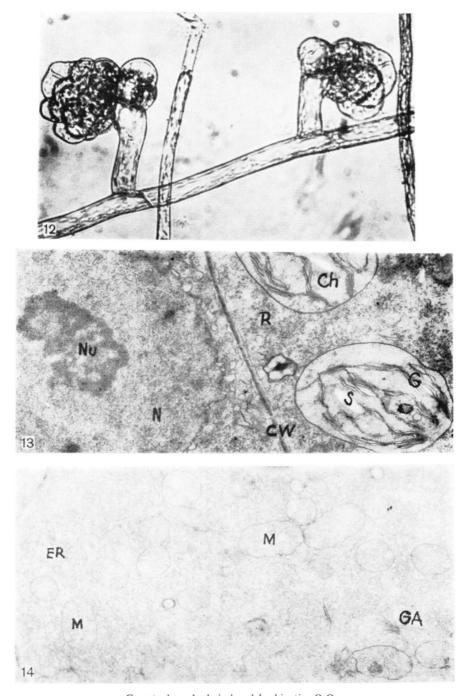


Fig. 7. Chloroplast of protonema cell from the kinetin medium 3 days of treatment. Glutaraldehyde. 55 000 x. Fig. 8. Chloropast of protonema cell from control medium. Ribosome-like particles (arrow), nucleoid-like region (double arrow). Glutaraldehyde. 50 000 x.



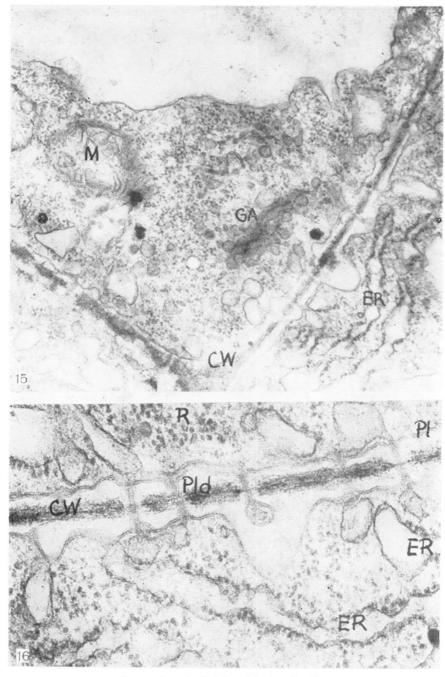
Protonema cells from kinetin medium

Fig. 9. Chloroplast with dense arrangement of grana thylakoids and swellings of marginal compartments. OsO<sub>4</sub>, 20 000 x. Fig. 10. Degenerating chloroplast. OsO<sub>4</sub>, 15,000 x. Fig. 11. Dense arrangement of numerous grana thylakoids. Glutaraldehyde. 24 000 x.



Gametophore buds induced by kinetin. OsO4.

- Fig. 12. Buds seen in light microscope.
- Fig. 13. Two cells of a bud. 12000 x.
- Fig. 14. Cytoplasm of a bud cell. 16 000 x.



Gametophore buds induced by kinetin. OsO4.

Fig. 15. Fragments of three cells of a bud. 50000 x.

Fig. 16. Enlarged fragment of fig. 15. 100 000 x.

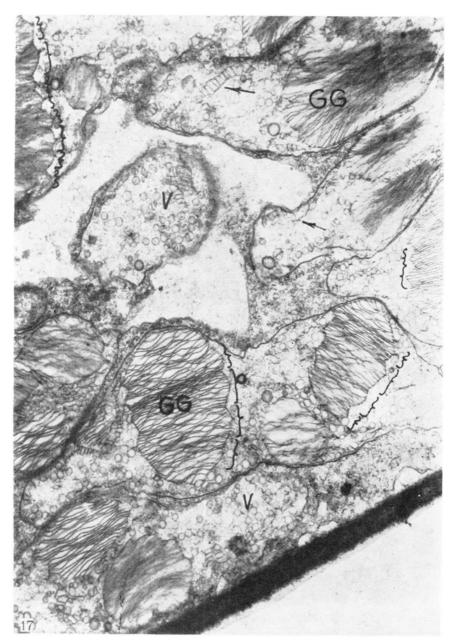
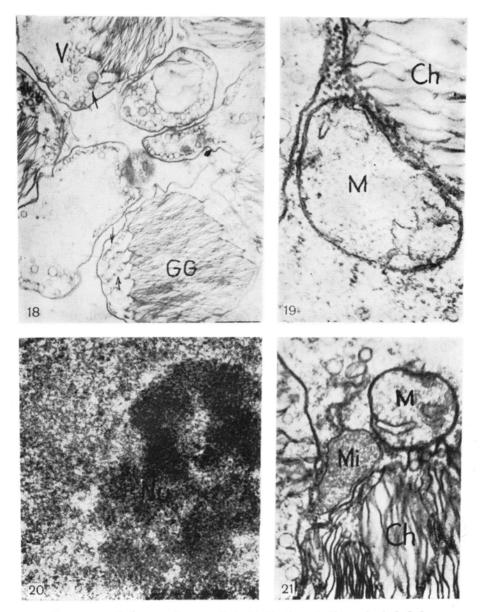


Fig. 17. Chloroplasts of protonema treated with kinetin and chloramphenicol. OsO<sub>4</sub>. Giant grana composed of packets of numerous lamellae (brackets). Numerous vesicles are present in the stroma, so me of them in the form of linearly arranged bead-like structures (arrows) 20 000 x.



Protonema cells from medium supplied with kinetin and chloramphenicol. OsO4.

Fig. 18. Chloroplasts with giant grana and stroma thylakoids disintegrated into chains of vesicles (arrow). In some chloroplasts only numerous vesicles in chains of bead-like structures along the plastid periphery are present.  $10\,000~\rm x$ .

- Fig. 19. Mitochondrion with reduced tubules. 50 000 x.
- Fig. 20. Fragment of nucleus with a nucleolus, 60 000 x.
- Fig. 21. Microbody and mitochondrion with reduced tubules. 30 000 x.

leading to ER fragmentation (figs. 15 and 16). The Golgi structures consisted of many cisterns and numerous accompanying vesicles with electron-dense contents which indicated their high physiological activity. Many of them were located near the cell walls. The mitochondria were very numerous, in sections usually round or oval, with numerous and well developed tubules (fig. 14). The chloroplasts were less numerous than in the protonema cells, and their lamellar system was less developed. Starch grains were often observed in their stroma. The protoplasts of the bud-cells were connected by numerous plasmodesmata. The plasmalemma showed a wavy outline and in some parts a distinct layering. The nuclei were spherical or elipsoid and in nucleoli a nucleolonema and pars amorpha could be distinguished (fig. 13).

The kinetin-induced bud formation can be prevented by chloramphenicol (Szweykowska, Ratajczak and Schneider 1968), an inhibitor of protein synthesis dependent on S-70 ribosomes occurring in plant cells in mitochondria and chloroplasts (Sissakian et al. 1965; Wilson et. al. 1968). The effect of chloramphenicol on the ultrastructure of protonema cells in Funaria has been described in a previous paper (Młodzianowski, Szweykowska and Schneider 1970). The inhibitor caused disintegration and reduction of the internal membrane systems in chloroplasts and mitochondria. In this study, the stimultaneous effect of both factors, kinetin and chloramphenicol, on the cell ultrastructure was investigated. In the presence of both substances similar changes were observed in cells as those described for chloramphenicol alone, but more pronounced. In chloroplasts, giant grana were formed composed of a large number of lamellae in which distinct packets composed of 14-16 lamellae could be distinguished. In some grana, eight or more of such packets occurred (fig. 17 and 18). The stroma thylakoids disintegrated, frequently forming bead-like rows of vesicles, often arranged in stacks which indicated their divisions (fig. 17). In some chloroplasts, no lamellar structures were present, and only vesicles arranged often in chains of bead-like structures along the plastid periphery were observed (fig. 18). Ribosome-like granulations in chloramphenicol-treated chloroplasts had less distinct outlines than the control ones treated only with kinetin (fig. 17). This might indicate a partial loss of ribosome building material, a conclusion supported by the finding of Young and Nakada (1970) that in Escherichia coli chloramphenical caused a loss of about 14% of ribosomal proteins, in a fraction known to contain the initiation factors for messenger translation. No changes were observed in cytoplasmic ribosomes. In mitochondria, the number of tubules was much reduced (figs. 19 and 21). No changes were found in the nucleus structure. A fragment of nucleolus with nucleolonema is shown in fig. 20. The shape of the microbodies and the density of their matrix also showed no alterations (fig. 21).

# DISCUSSION

At a definite stage of development (10–16 days after inoculation of spores), the protonema of *Funaria hygrometrica* is highly sensitive to the presence of cytokinins in the medium, forming under their influence an excessive number of gameto-

phore buds. This process is followed by a decrease in dry weight and protein concentrations in the gametophyte cells, which indicates an enhancement of processes similar to ageing (Szweykowska, Ratajczak and Schneider 1968). Brandes (1967) found in protonema cells which were induced to develop into buds (probud cells) a striking increase of cytoplasmic RNA which occurred in spontaneous as well as kinetin-induced buds. It was characteristic that no increase occurred in other protonema cells, this indicating that only some specific protonema cells were responsive to the bud-inducing factors. Using autoradiography and labelled 6-benzyladenine, Brandes and Kende (1968) showed that radioactivity strikingly accumulated in the pro-bud cells and that the non-responding protonema cells contained only very little radioactivity.

Also in our experiments, no changes in ultrastructure were observed in typical protonema cells treated with kinetin; only occasionally symptoms of chloroplast ageing and degeneration could be seen in some of them. On the other hand, the cells of young buds had an ultrastructure characteristic for metabolically active meristematic cells, with a dense cytoplasm, numerous ribosomes and richly developed endoplasmic reticulum. These observations confirm, at the level of cell ultrastructure, that the response to exogenously applied cytokinins is probably different in various protonema cells. Ten days after spore inoculation the protonema differentiates (although in liquid media no morphological signs of such a differentiation into e.g. chloronema and caulonema occur at this time) into cytokinin-non-responsive cells and cells responding to cytokinins by bud initiation. Under the influence of cytokinins, these cells become RNA-rich centres giving rise to gametophore development. The buds constitute further centres of high physiological activity and active cell divisions, as shown by their ultrastructure characteristic for metabolically active meristematic cells. The non-responsive protonema cells which probably, as shown by Brandes and Kende (1968), do not even take up or at least do not accumulate cytokinins, do not change at first and eventually show symptoms of a decrease of vitality and of physiological ageing.

It is interesting that in the presence of chloramphenicol, which inhibits kinetininduced bud formation, kinetin not only does not diminish the disorganising effect of the inhibitor on the protonema cells, but even seems to act synergistically, increasing the extent of degeneration processes. In this case only the ageing-enhancing activity of kinetin seems to come into play.

The action of cytokinins on the protonema seems thus to be complex: they generally enhance processes of development and differentiation, in such a way that in some cells they induce meristematic activity leading to bud formation, and in the remaining cells they directly or indirectly promote processes of ageing.

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# Wpływ kinetyny na ultrastrukturę splątka i indukowanych przez nią pączków gametoforowych u Funaria hygrometrica

## Streszczenie

Dziesięciodniowy splątek Funaria hygrometrica przenoszono na 6 dni do pożywki zawierającej kinetynę lub kinetynę i chloramfenikol, a następnie utrwalano i badano ultrastrukturę splątka i wytworzonych w nim pączków gametoforowych. W komórkach splątka nie stwierdzono żadnych zmian, poza występującym w niektórych przypadkach przerostem gran w chloroplastach. Natomiast komórki pączków posiadały ultrastrukturę charakterystyczną dla komórek o aktywnym metaboliźmie i intensywnych podziałach: odznaczały się gęstą cytoplazmą, licznymi rybosomami, bogatym rozwojem retikulum endoplazmatycznego i struktur Golgiego i licznymi mitochondriami; natomiast chloroplasty były stosunkowo mniej liczne i słabiej zróżnicowane w porównaniu z komórkami splątka. Wyniki te wskazują, że pod wpływem cytokinin następuje przyspieszenie rozwoju i różnicowania się splątka, przy którym tylko pewne jego komórki ulegają aktywacji i dają początek pączkom gametoforowym, pozostałe zaś nie ulegają zmianie lub wykazują objawy rozkładu i starzenia. Wniosek o przyspieszaniu przeż cytokininy również procesów rozkładu i starzenia potwierdza fakt, że w obecności chloramfenikolu, który zapobiega indukcji pączków, kinetyna współdziała z inhibitorem zwiększając wywołane przez niego objawy degeneracji i rozkładu chloroplastów.