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Author: Urszula Nowak, Fortunat Młodzianowski

Citation style: Nowak Urszula, Młodzianowski Fortunat. (1980). The influence of kinetin on regeneration of the protonema from isolated protoplasts of the moss Funaria hygrometrica L. Sibth. "Acta Societatis Botanicorum Poloniae" (Vol. 49, no. 3 (1980) s. 195-203), doi 10.5586/asbp.1980.017

ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 49, nr 3: 195—203
1980

The influence of kinetin on regeneration of the protonema from isolated protoplasts of the moss *Funaria hygrometrica* L. Sibth.

URSZULA NOWAK, FORTUNAT MŁODZIANOWSKI

Department of General Botany, Institute of Biology,
Adam Mickiewicz University, Al. Stalingradzka 14, 61-713 Poznań, Poland

(Received: December 3, 1979)

Abstract

No stimulating effect of kinetin was noted on formation of the cell wall surrounding isolated protoplasts of the moss *Funaria hygrometrica*, although kinetin at a concentration of 1μM distinctly accelerated division of cells obtained from protoplasts and stimulated formation of the protonema and gametophores. At higher kinetin concentrations (5 μM) protoplasts, and later cells, increased their volume, whereas regeneration processes took place considerably more slowly than in the control.

INTRODUCTION

Protoplasts isolated from the moss *Funaria hygrometrica* by means of mechanical and enzymatic methods are capable of growth and regeneration of the cell wall (Binding, 1966; Gwóźdź, Waliszewska, 1979). Successive stages of this process are similar to those observed in protoplasts of other mosses such as *Physcomitrella patens* or *Polytrichum juniperinum* (Stumm et. al., 1975; Gay, 1976). Regeneration of the cell wall and first division, as also changes in the shape of regenerated moss cells, take place after a relatively long period from the moment of isolation, as compared to higer plants.

The stimulating effect of cytokinin on cell division in moss protonema leading to formation of gametophore buds is known (Szweykowska et al., 1968; Szweykowska et al., 1969). These phenomena must be accompanied by acceleration of processes connected with formation of the cell wall. It thus appeared of interest to observe the effect of cytokinin on formation of the cell wall in isolated protoplasts of moss protonema and on the behaviour of regenerated cells.

MATERIAL AND METHODS

Protonema cultures were obtained from *Funaria hygrometrica* spores grown on Kofler (1959) nutrient medium with microelements according to Heller (1953). The protonema grew in Erlenmayer flasks at a temperature of 24°C under continuous illumination of around 100 lux.

Six day old protonema were plasmolized in 0.3 M mannitol with an addition of 0.02% Tween 80 for 30 minutes, then washed with 0.3 M mannitol. 4% cellulase "Onozuka" (Yakult Biochemicals Co. Ltd. Nishi-Japan), 3% Macerozyme R-10 (Yakult Biochemicals Co. Ltd. Nishi-Japan), 3% Rohzyme H-150 (Rohm and Hass Co. Philadelphia, Penn., USA) in a water solution of 0.3 mannitol, pH 5.7, were used for isolation. Liberation of protoplasts took place at room temperature for 4 hours. The protoplasts were separated by filtration through a ϕ 50 μm mesh silk gauze, centrifuged for 3 minutes at 100 × g and washed twice with 0.25 M mannitol. The sediment was mixed with the nutrient medium at a ratio of 1:2 so that the number of protoplasts was around 2×10^5/ml. The nutrient medium contained the basic components according to Kofler (1959) and microelements according to Heller (1953), with an addition of 0.15 M mannitol, 0.1 M glucose, 100 mg/l mesoinositol and kinetin at concentrations of 1 and 5 μM. Initial experiments in which 0.1, 1, 5, and 10 μM concentrations of kinetin were applied showed that 1 μM constituted the optimal concentration and 5 μM the maximal for growth in the nutrient medium.

The protoplasts suspension was cultured as hanging drops in Petri dishes sealed carefully with Parafilm and placed in a humidified glass container followed by maintaining at a temperature of 20°C under daylight.

All of the manipulations were conducted under sterile conditions and the experiments were repeated four times. Percental vitality of protoplasts was estimated on the basis of the ratio of the total number of protoplasts to vital ones. Cell wall regeneration was detected by means of Calcoflour White st. staining, and examined under a fluorescence microscope.

Additionally, in order to obtain quantitative data on the intensity of cell wall regeneration, measurements were carried out in a Spekol with a medium pressure HQE 40 fluorescence lamp supplied with a KP fluorescence attachment and an additional ZV type amplifier. The suspension of cells was stained for 10 minutes with 0.01% Calcofluor and 0.25 M sorbitol. This was followed by centrifuging and washing the sediment with 0.25 M sorbitol. Ten drops were taken from the final

PLATE I

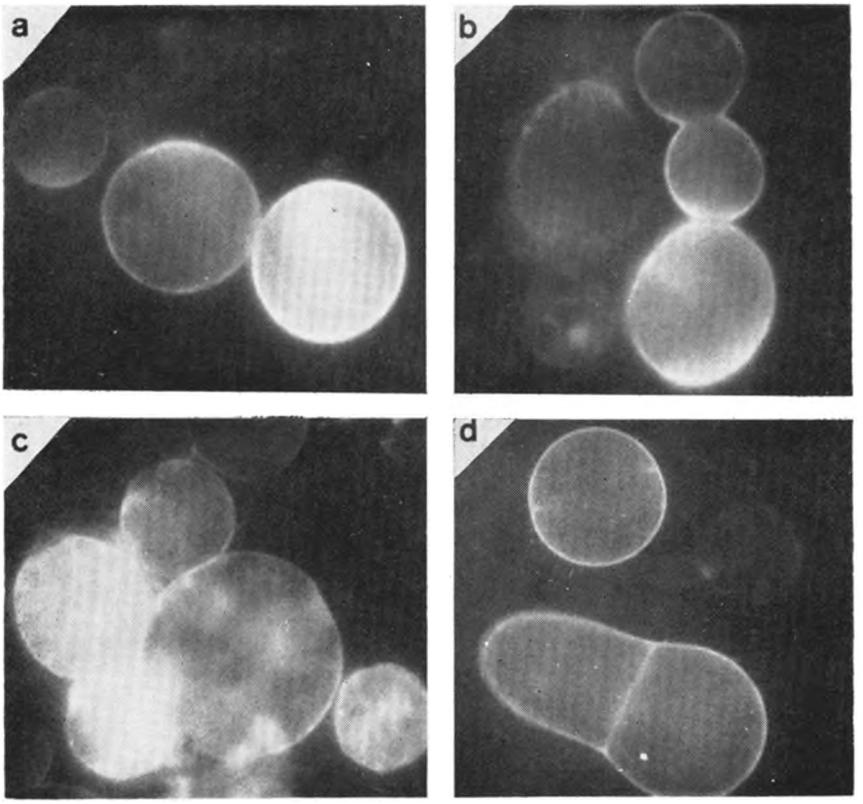

Fig. 1. Protoplasts of *Funaria hygrometrica* after 3 days of culture, stained with Calcofluor: a, b — control medium; c, d — medium with 1 μM kinetin. 1200 ×.

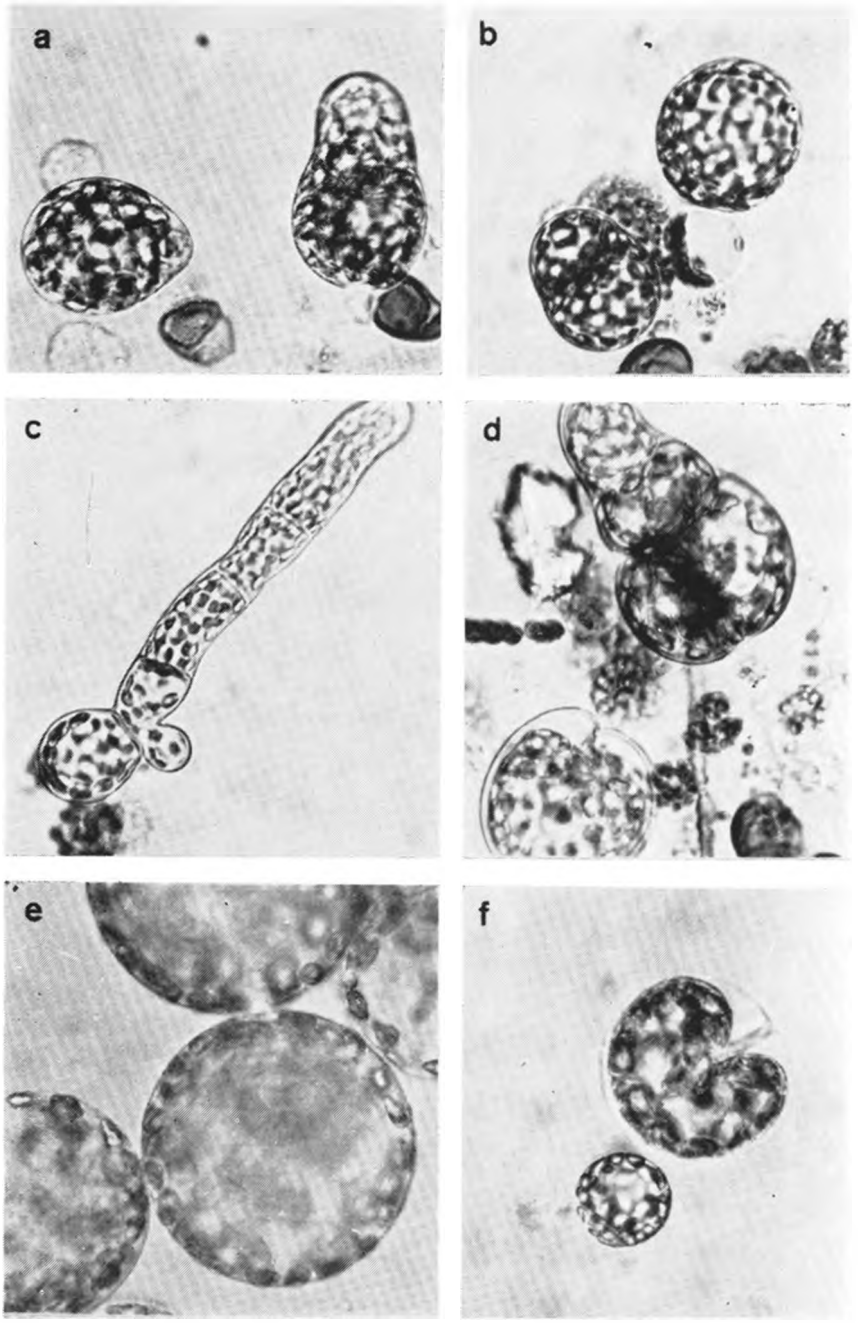

Fig. 6. Regenerated cells of *F. hygrometrica* growing on the control medium (a, b) with 1 μM kinetin (c, d) and 5 μM kinetin (e, f) after 7 days of culture. Photo c — 320 ×, all other — 800 ×.

PLATE III

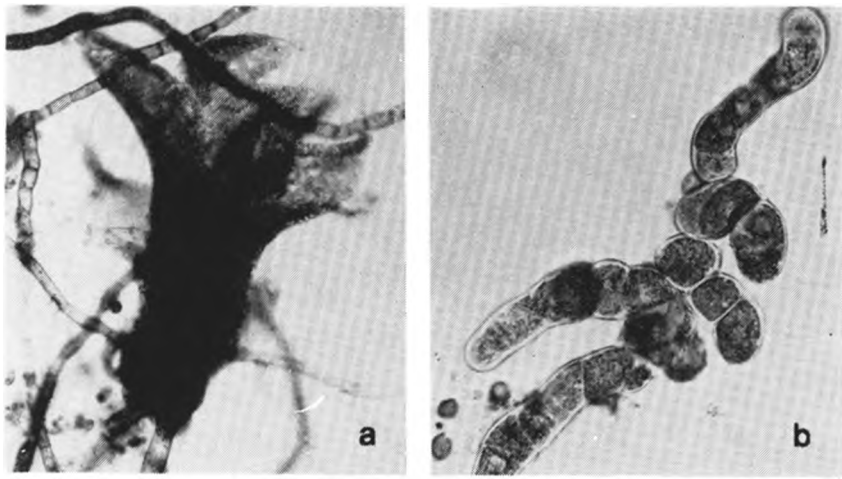

Fig. 7. *Funaria hygrometrica* after 3 months of culture of isolated protoplasts: a — gametophyte developed on 1 μM kinetin medium (150 ×), b — protonema growing on 5 μM kinetin medium (390 ×).

volume of protoplasts in 1 ml of the sorbitol solution, mixed with 3 ml of 0.25 M sorbitol, and fluorescence intensity measured immediately at a wave length of 405 nm.

RESULTS

1. Cell wall regeneration

Immediately after isolation and washing of protoplasts no characteristic fluorescence of the cell wall was noted. Begining of the formation of a new cell wall around protoplasts from the control and kinetin media (1 μM) was observed after 24 hours, the wall being regenerated with various intensity after 3 days of culture (Fig. 1). Apart from uniform fluorescence of the cellulose border, areas of various shape and distinct arcs more intensively fluorescing were visible (Fig. 1 a–c). After 7 days of culture around 95% of the protoplasts had walls totally regenerated. Fluorescence measurements after 1 to 3 days of culture showed no significant differences in the fluorescence intensity of cell walls between the kinetin and control material. Hence no stimulating influence was found of kinetin on the rapidity with which regeneration of the cell wall in *Funaria* protoplasts takes place.

2. Augmentation of protoplasts and cells

Funaria hygrometrica protoplasts freshly isolated from a 6 day old protonema had a diameter of 18.5 μm. Growth of protoplasts during the first three days of culture was intensive in all of the nutrient media. Fig. 2 presents the diameter of spherical protoplasts and later of cells from the control and from the 1 and 5 μM kinetin media after 3, 7 and 14 days of culture. Slowest growth was shown by protoplasts on the control medium. Only after one week, then already as cells, did they double their diameter, while on the 14th day cells with a diameter smaller than after 7 days of culture dominated in the population of spherical cells, this being due to the fact that all of the larger spherical cells divided or transformed into another form.

The diameter of protoplasts and cells on the 1 μM kinetin medium increased somewhat more rapidly. The diameter in cells almost doubled already after three days and even increased slightly during following days. Average diameters in the population of spherical cells were found to be smaller on the 14th day of culture than after 7 days, this being due also to the fact that the spherical cells divided.

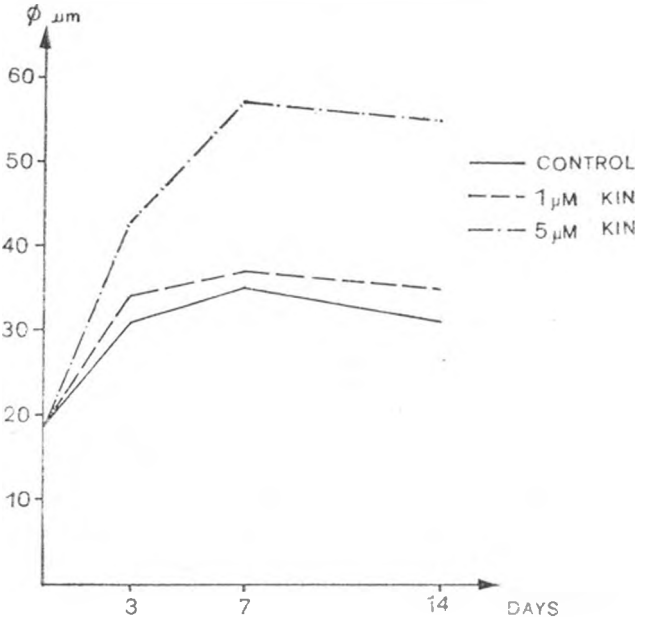

Fig. 2. Increase in diameter of spherical cells regenerating from *F. hygrometrica* protoplasts (average for 100 cells).

On the other hand protoplasts from 5 µM kinetin medium rapidly increased their diameters up to the third day, with spherical cells reaching maximal dimensions on the 7th day of culture. Diameters of these cells were three times greater than those of protoplasts freshly isolated with no greater changes taking place to the end of the observations.

3. Initial divisions of cells

Observations of *Funaria* protoplast cultures showed regenerating cells of different shape and different stage of growth: spherical, elipsoidal, dividing, budding cells, cell forming aggregates and developing into protonema. Figs 3 and 4 show the percental composition of these specific forms in individual tests.

Changes in the shape of cells were observed after three days of culture. Elipsoidal and budding cells appeared in the control material. The first divisions was found to take place on the 7th day of culture. Very few cells showed asymmetric division (Fig. 6b), with such cells then indicating a tendency to form protonema filaments. The number of divided cells increased with the advancement of culture, reaching their peak after 14 days (Fig. 3).

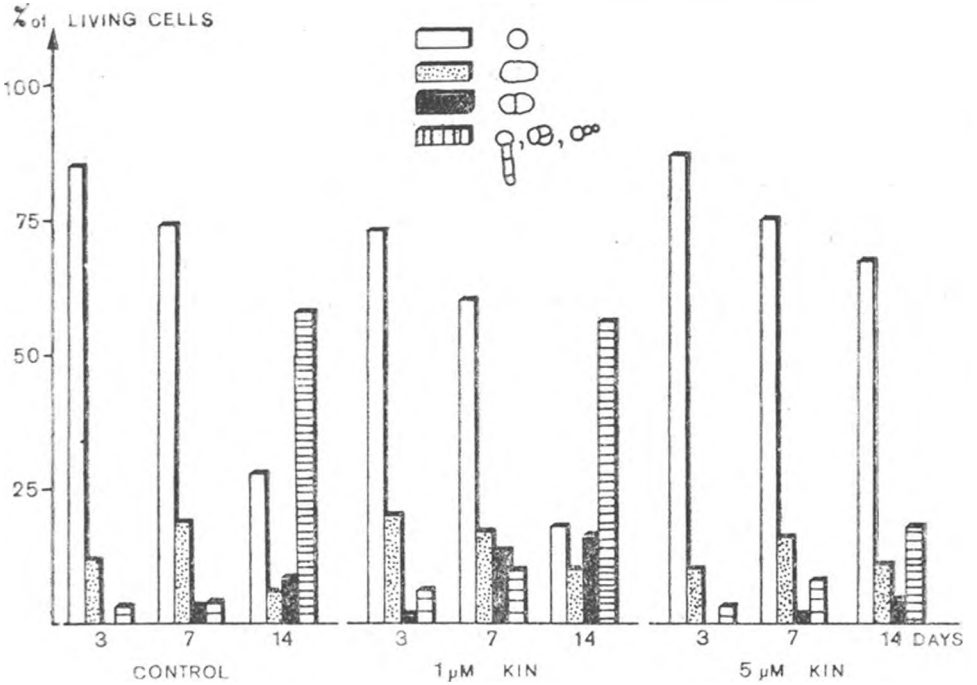

Fig. 3. Shape and arrangement of cells regenerating from *F. hygrometrica* protoplasts (average for 500 cells).

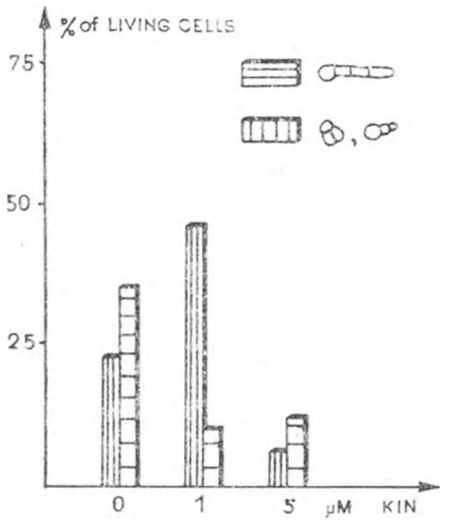

Fig. 4. Forms of growth after first division on the 14th day of culture. Particular column pairs in the diagram constitute the sum of corresponding checkered columns from the 14th day of observations presented in Fig. 3.

The 1 μM kinetin medium was observed to be the most favourable for transformation of spherical cells into elipsoidal ones, which later most frequently divided. First division in this medium was observed already on the third day of culture (Fig. 1d). The number of divided cells on the 7th and 14th day was considerably higher than in the control material. Apart from elipsoidal and budding cells likewise cells were observed showing atypical division taking place 2 and 3 times in various planes, and forming groups of connected cells (Figs. 5 and 6d). After some time later protonema could regenerate from such aggregates of cells.

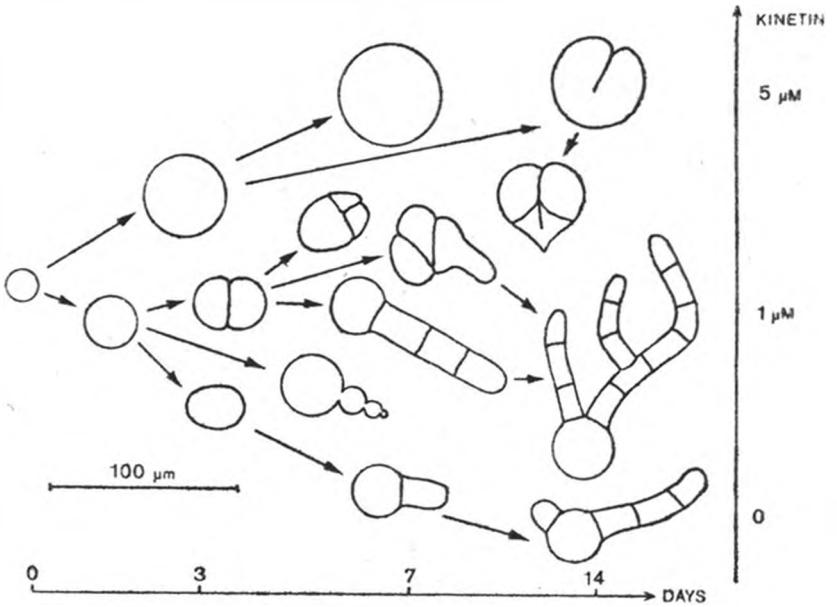

Fig. 5. Development of regenerated *F. hygrometrica* cells on various media.

Development of protoplasts on the 5 μM kinetin medium showed a different picture. Growth was rapid (Fig. 2), with minimal differences in shape of cells on the 3 and 7 day of culture. First division was observed on the 7th day, similary as was the case in the control material, but a higher number of cells showed incomplete division of the transversal cell wall (Fig. 6f). Furthermore, there was no such abundance of forms as in the material from the 1 μM kinetin medium, and — as can be seen in Fig. 3 — as many as 65% of the cells maitained a spherical shape on the 14th day, while the number of divided cells was more or less the same as on the 7th day of the control culture.

4. Regeneration of protonema and gametophores

Of the total number of protoplasts about 70% maintained vitality after 2 weeks of culture. The number of spherical and elipsoidal cells declined in all of the nutrient media, whereas the number of divided cells and of various multi-cell forms, as well as of cells developing into protonema, increased (Fig. 4). Earliest protonema filaments were noticed in the 1 μM kinetin medium between the 4th and 7th day of culture (Fig. 6c). The protonema was formed both after symmetrical as well as after asymmetrical division. One of the sister cells, frequently a smaller one, began to divide initiating formation of the protonema filament. In cases where multi-plane division took place, leading to the formation of cell aggregates, the protonema developed from one or another edge placed cell. Only several short and bulky cells in the 5 μM kinetin medium formed protonema. A typical protonema occured in the control, however with only few branchings. The best developed, and frequently branched protonema, were noted in the 1 μM kinetin medium (Figs 4 and 5).

After three months of culture only the 1 μM kinetin medium showed numerous normally developing, foliated gametophores (Fig. 7a). After the same time of cultures on the 5 μM kinetin medium showed only degenerated protonema (Fig. 7b), whereas normal ones were observed in the control, but only with few retarded gametophores.

DISCUSSION

Apart from their very small dimensions *Funaria hygrometrica* protoplasts do not differ externally from typical protoplasts of the mesophyll of leaves of higher plants. During the first days of culture on various media they form their walls and grow intensively. A strong and well developed wall is already formed after 7 days, hence much earlier than observed by Binding (1966). Growth of protoplasts was of extreme importance for further development of cells, and especially for regeneration of protonema. Cell divisions on the control medium without kinetin began between the 4th and 7th day of culture, at which time the sperical diameter of cells increased by almost twofold as compared to freshly isolated protoplasts (Fig. 2). This was thus much earlier than observed by others: Stumm et al. (1975) after 8-10 days in *Physcomitrella patens*, Gay (1976) in *Polytrichum juniperinum* after 18 days, Gwóźdź and Waliszewska (1979) in *Funaria hygrometrica* after 10-12 days. Differences in the cited studies referred only to molarity and composition of the sugars used and to the mineral nutrient

media. The larger amount of glucose used in our experiments (0.1 M glucose) as compared to the study by Gwóźdź and Waliszewska (0.03 M glucose), as also the differences in lighting, might have been the cause for accelerated division in the control material and high level of vitality (70%).

Kinetin stimulated cell division. The time for transformation from spherical to elipsoidal forms undergoing division was shortened in the presence of kinetin. No differences were noted, however, in regeneration of the cell wall in protoplasts from the kinetin and control media. Likewise, no characteristic concentration of chloroplasts around the nucleus before division, as takes place in higher plants, was observed (Ohyama, Nitsh, 1972; Nagata, Takebe, 1970).

Protoplasts on the 1 μM kinetin medium reached an almost twofold growth of diameter already on the third day, the "optimal" stage for entering the phase of division, whereas this same stage was reached by protoplasts on the control medium 3 to 4 days later. In comparing diagrams for the control after 7 days of culture with the 1 μM kinetin medium after 3 days (Fig. 3), a striking conformity can be noted in the numbers of specific types of regenerating cells. This indicates distinctly that development of the control cells was retarded by several days. Almost the same number of budding and repeatedly dividing cells was noted from the 1 μM and from the control media on the 14th day of culture. From among these forms greatest numbers of protonema were noted on the kinetin medium (Fig. 4), in which case branching was likewise more evident (Fig. 5).

Higher kinetin concentrations (5 μM) stimulated growth of protoplasts but did not promote further development of regenerated cells. Only few of the cells formed protonema, and these consisted of a small number of very short cells.

It can be said that isolated protoplasts of the moss protonema of *Funaria hygrometrica* regenerated cell walls, divided, formed protonema and gametophores, and that all of these processes, with the exception of formation of cell walls around protoplasts, were stimulated by specifc concentrations of kinetin.

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Wpływ kintetyny na regenerację protonemy z izolowanych protoplastów mchu Funaria hygrometrica L. Sibth.

Streszczenie

Nie stwierdzono stymulującego wpływu kinetyny na tworzenie się ściany komórkowej wokół izolowanych protoplastów mchu *Funaria hygrometrica*, natomiast kinetyna w stężeniu 1 μM wyraźnie przyspieszała podziały komórek otrzymanych z protoplastów oraz stymulowała tworzenie się splątka i gametoforów. Przy wyższym stężeniu kinetyny (5 μM) protoplasty, a później komórki powiększały głównie swoją objętość, natomiast procesy regeneracyjne przebiegały znacznie wolniej niż w kontroli.