ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XXXVII — nr 1
1968

Studies on the activity of kinetin in cultures of *Funaria hygrometrica*

I. Bud-inducing activity of kinetin and protein synthesis

A. SZWEYKOWSKA, W. RATAJCZAK and J. SCHNEIDER

One of the most distinct effects of kinetin on plants is induction of gametophore buds in the protenoma of mosses (cf. Bopp 1965; Szweykowska 1966) which makes moss cultures a suitable material for investigations on cytokinin action in plant cells.

In our previous paper (Szweykowska and Handszu 1965), the protein content and protein concentration in cultures of *Ceratodon purpureus* grown in inorganic and organic media with and without kinetin were investigated and the conclusion was reached that the bud-inducing activity of kinetin is not directly related to the intensity of protein synthesis. In the present study, the relationship between growth, bud induction and protein metabolism as affected by kinetin were investigated in *Funaria hygrometrica* cultures by way of quantitative protein N determinations in various culture conditions and treatment of cultures with specific inhibitors of protein synthesis.

MATERIAL AND METHODS

Liquid cultures of *Funaria hygrometrica* Hedw. were grown from spores in 100 ml Erlenmayer flasks containing 30 ml of nutrient solution. The cultures were kept in a culture room under light from white fluorescent tubes and at temp. 20°C. As nutrient solutions mineral Kofler medium alone (inorganic medium) or supplied with 0.25% glucose (organic medium) were used. The method of obtaining sterile cultures from spores, the composition of mineral medium and the method of protein N determination have been described earlier (Szweykowska and Handszu 1965). All determinations were made on 10—3 (depending on the stage of growth) parallel cultures.

RESULTS

In inorganic medium, a healthy growth proceeded during the whole culture time (75 days) (fig. 1). Differentiation was slow; numerous buds and gametophores appeared only after 40—60 days, although occasional

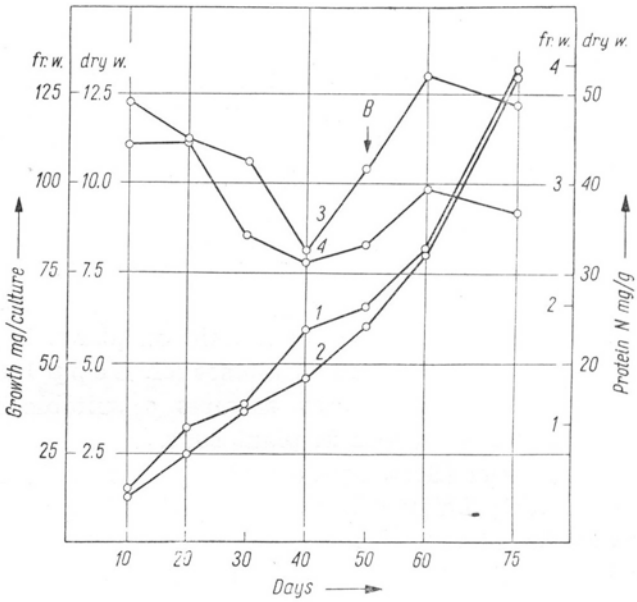

Fig. 1. Fresh (*1*) and dry (*2*) weight production, and protein content in fresh (*3*) and dry (*4*) weight in an inorganic culture. B — time of bud formation.

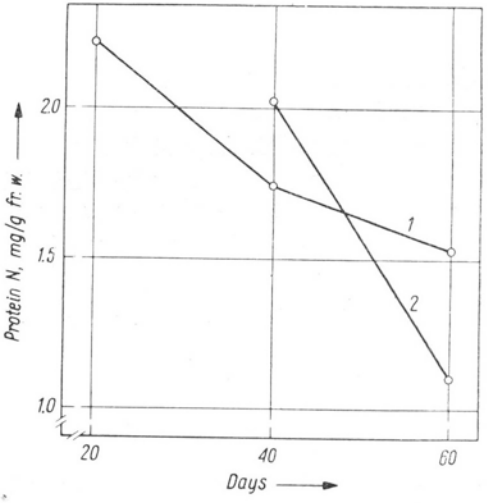

Fig. 2. Protein content in fresh weight of protonema (*1*) and gematophores (*2*) at various stages of culture development in inorganic medium.

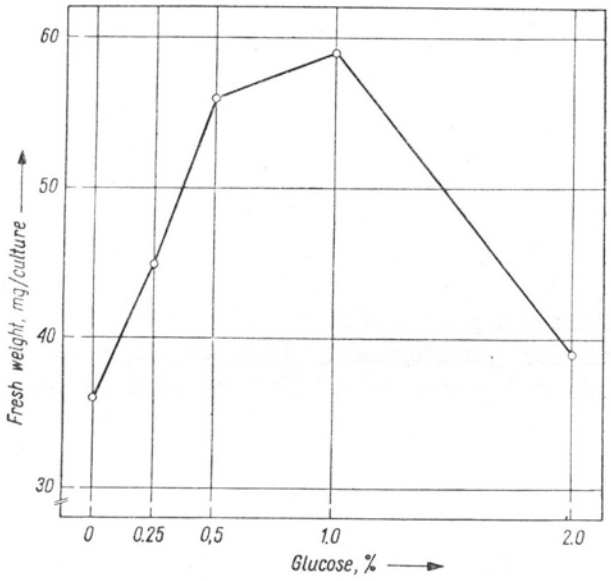

Fig. 3. Effect of glucose on the fresh weight of *Funaria* in liquid cultures after 35 days.

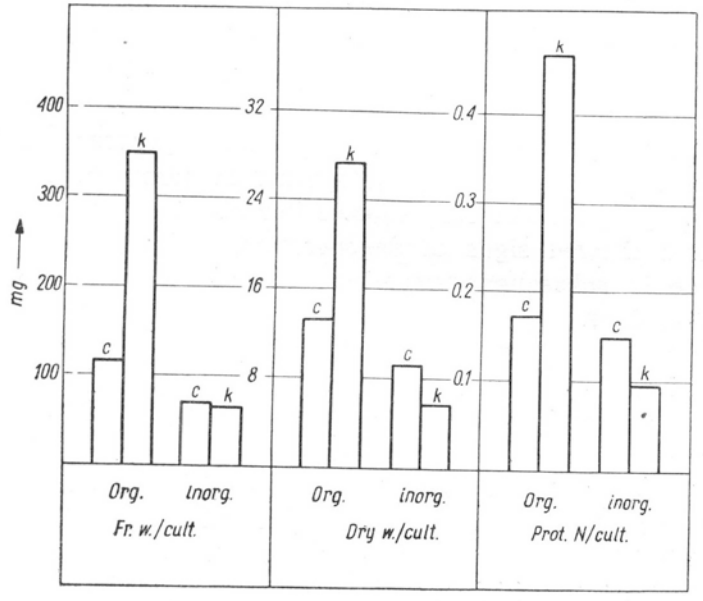

Fig. 4. Effect of kinetin on growth and protein synthesis in inorganic (after 60 days) and organic (after 40 days) cultures. *C* — control *K* — kinetin added

buds were observed after 30 days. The protein concentration in cultures was decreasing during the protonema stage of growth, increasing at the time of bud formation and decreasing afterwards again (fig. 1). The decrease in protein concentration may represent the process of ageing of the culture, the time of bud induction being a period of culture rejuvenation manifested by a temporary increase in relative protein content. The data in fig. 1 concern the culture as a whole. To prove if actually the increase in protein concentration in the culture at the time of gametophore formation is due to a relatively higher protein content in cells of young gametophores, another experiment was carried out in which gametophores were picked from cultures at various stages of their development and protein nitrogen was estimated separately for the protonema and for gametophores, after 20 days of culture (young protonema, buds and young gametophores), 40 days (older protonema, buds and young gametophores) and 60 days (old protonema, gametophores and buds). The results presented in fig. 2 showed a relatively high protein content in young protonemas and young gametophores which diminished during their growth and differentiation. Protonema and gametophores thus appear as two different forms of the gametophyte in which stages of juvenility and ageing occur in some way separately.

Glucose promoted growth of the cultures, its effect reaching a maximum at 1 per cent (fig. 3). At this concentration, however, the cultures browned soon owing to the brown-pigmented cell walls and the morphology of gametophores (leaf development) was abnormal (cf. Szweykowska and Maćkowiak 1962). Therefore, 0,25 per cent of glucose was used for experiments with organic media. As compared with inorganic medium, glucose at 0.25 per cent — besides stimulating growth of the cultures — increased their dry matter content per unit fresh weight (14 per cent as compared with 8 per cent in inorganic medium) and enhanced their development in terms of earlier (after 15—20 days) gametophore formation. After about 60 days the cultures browned and showed signs of degeneration, and for this reason the culture time in subsequent experiments with organic medium was reduced to 40 days.

Kinetin at 1 mg/l strongly promoted bud formation. In the organic control, the buds began to appear after 15—20 days. With kinetin added, they appeared as early as after 10 days, and after 15 days they predominated over protonema. The fresh and dry weight productions as well as protein synthesis were distinctly higher in the presence of kinetin (fig. 4), however, the average dry weight per unit fresh weight was lower (8 per cent in kinetin cultures as compared with 14 per cent in organic controls), indicating a higher water content in cells grown in the presence of kinetin. Also the protein contents per unit fresh weight were lower in kinetin cultures (fig. 5).

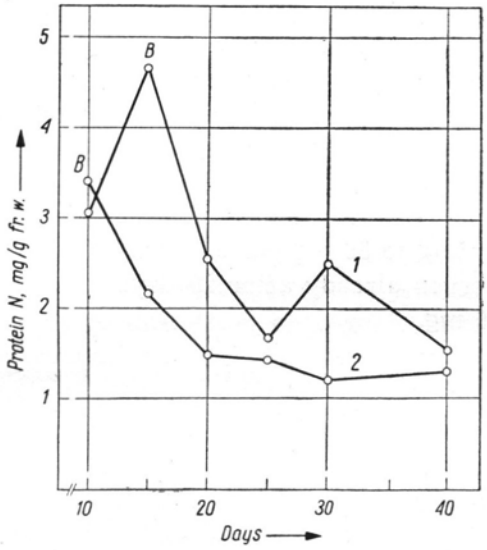

Fig. 5. Protein content in fresh weight of cultures in organic control (*1*) and kinetin-containing (*2*) media. *B* — time of bud formation.

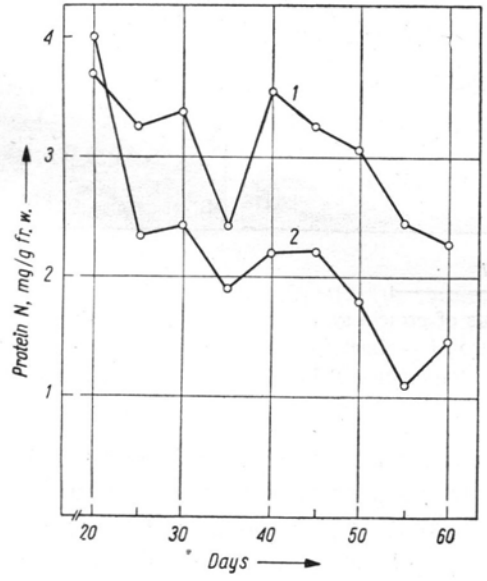

Fig. 6. Protein content in fresh weight of cultures grown in inorganic control (*1*) and kinetin containing (*2*) media.

In inorganic medium 0.1 mg/l, kinetin was used, as higher concentrations were inhibitory for growth. At 0,1 mg/l, the inhibition of growth was only slight (especially in fresh weight production) and marked only after about 50 days (fig. 4). The average dry weight per unit fresh weight was not affected and amounted to 10 per cent in both control and kinetin cultures, however, similarly as in organic cultures, a marked decrease of protein concentration in cells occurred under the influence of kinetin as shown in fig. 6.

In controls, numerous buds appeared as late as after 45 days with fresh weight amounting to 36 mg per culture. With kinetin, vigorous bud formation was observed already after 20 days of culture when its fresh weight was only 16 mg.

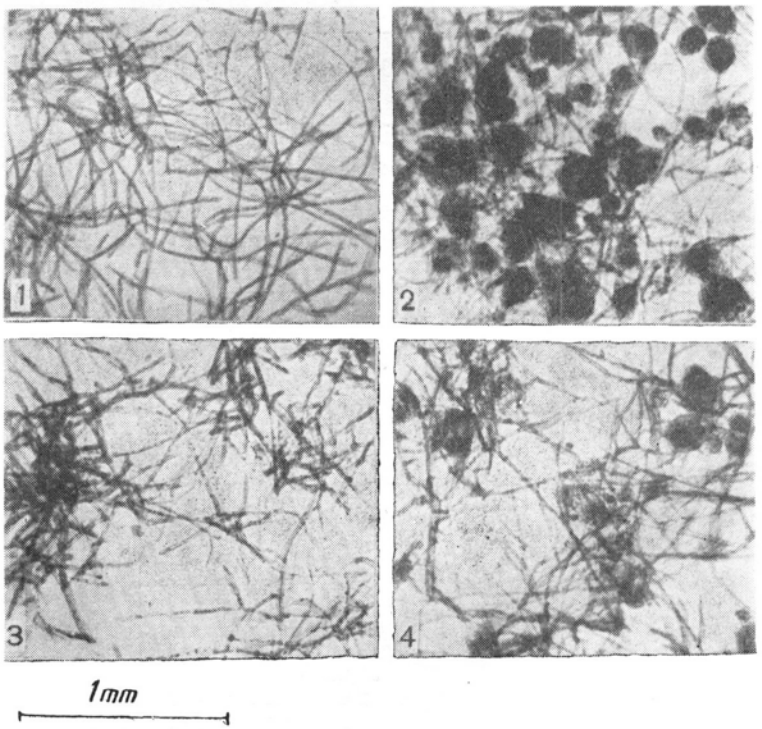

Fig. 7. Effect of inhibitors of protein synthesis on the bud-inducing activity of kinetin. *1* — Basal control, *2* — Kinetin control, *3* — Kinetin + fluorophenylalanine 0.1 mM, *4* — Kinetin + 5-methyltryptophane 0.5 mM + tryptophane 1.0 mM.

The results showed that in favourable conditions (organic medium) kinetin activated various processes of growth and development resulting in increased protein synthesis, fresh and dry weight production, water uptake, induction of bud formation and enhancement of culture ageing expressed in a lowered protein content per unit fresh weight. In conditions unfavourable for growth (inorganic medium), the effects of

kinetin were reduced to bud induction and enhancement of ageing. Bud induction seems to be a process most directly related to the primary action of kinetin, and inorganic cultures are most suitable for investigating its mechanism. No stimulation of protein synthesis could be observed in connection with bud-inducing activity of kinetin in inorganic media, and even some inhibition could be noticed after a certain time. On the other hand, many data suggest (cf. Parthier 1965) a participation of kinetin in protein synthesis. To obtain evidence whether or not protein synthesis plays an essential role in the bud-induction process and in the bud-inducing activity of kinetin, a set of experiments was carried out with the use of inhibitors of protein synthesis. In these experiments, spores were inoculated on organic medium and grown for 14 days. The protonema was then filtered, washed and transferred to inorganic media containing 1 mg/l kinetin and various inhibitors. As controls, inorganic medium alone (basal control) and inorganic medium with kinetin only (kinetin control) were used. As inhibitors, chloramphenicol and amino acid analogues β-2-thienylalanine, m-fluorophenylalanine and 5-methyltryptophane in concentrations of 0.01, 0.05, 0.1, 0.5, 1, 2 and 5 mM/l were used. The cultures were observed for ten days and their development was recorded. During this time, no buds or only few (1—10) appeared in basal controls. In the kinetin control, a great number of buds appeared as early as after 2 days (fig. 7).

Chloramphenicol showed an inhibitory effect on kinetin-induced bud formation at 0.1 and 0.5 mM. In the presence of kinetin with these concentrations of inhibitor, the protonema did not differ from that in the basal control (fig. 7). No inhibition was observed at 0.01 mM. In 1—5 mM, the protonema soon lost its green colour and died after 2—4 days. Also the amino acid analogues strongly inhibited bud formation. In 0.01 mM of thienylalanine buds appeared after 4 days, in 0.05 mM only after 10 days, and in higher concentrations no buds at all appeared during 10 days. Beginning with 1 mM, some abnormalities were found in the protonema cells. Some of them were distended and with brown and aggregated chloroplasts.

Fluorophenylalanine showed a similarly strong inhibition. At concentrations higher than 0.05 mM no buds were observed during 10 days. Few small buds appeared on the 10th day in 0.05 mM and no inhibition occurred in 0.1 mM. 5-methylhyltryptophane proved toxic beginning with 1 mM. In 5 mM, the protonema was dead already after 4 days. In 0.5 mM a distinct inhibition of the bud-forming activity of kinetin occurred, the cultures resembling basal controls. A delay in bud appearance (6th day) was caused by 0.1 mM concentration, and almost no inhibition was observed beginning with 0.05 mM. Experiments were also carried out on the reversion of the inhibition caused by amino acid analogues by means of corresponding amino acids or transfer to

analogue-free medium. In the first set of experiments, the amino acid analogue in the lowest concentration nacessary for complete inhibition of kinetin-induced bud formation was accompanied by a corresponding amino acid in twice as high concentration. Thienylalanine (0.1. mM) + alanine (0.2 mM), fluorophenylalanine (0.1 mM) + phenylalanine (0.2 mM), and 5-methyltryptophane (0.5 mM) + tryptophane (1.0 mM) were used.

In the second set, the protonema kept for 6 days in the presence of kinetin + analogue was transferred for another 6 days to media with kinetin alone.

In the case of thienylalanine, no reversal of the inhibition could be obtained. On the contrary, the inhibitions caused by chloramphenicol, fluorophenylalanine and 5-methyltryptophane were at least partly prevented by the presence of the corresponding amino acids or reversed by transfer to medium not containing the analogue (fig. 7).

The above experiments showed that, in the presence of inhibitors of protein synthesis in concentrations of 0.1 mM for chloramphenicol, thienylalanine and fluorophenylalanine, and 0.5 mM for methyltryptophane, kinetin at 0.005 mM (1 mg/l) had no bud-inducing effect. In the case of chloramphenicol, fluorophenylalanine and metyltryptophane the inhibition was reversible by appropriate amino acids or by a transfer to inhibitor-free medium. The results point to an involvement of protein synthesis in the kinetin action and probably also in the bud-forming process itself. As kinetin induces bud formation even in conditions drastically unfavourable for intensive protein synthesis, its action probably involves activation of synthesis of some specific proteins responsible for the transition from protonema to the gametophore form.

DISCUSSION

The hypothesis of Hotta and Osawa (1958) that the transition from protonemal to the prothallial form of growth in ferns is dependent on the rapid synthesis of protein and increase of its concentration in cells, has not been confirmed by Kasimir and Mohr (1965). Also in a study on bud differentiation in the protonema of the moss *Ceratodon purpureus* (Szweykowska and Handszu 1965), the intensity of protein synthesis and protein content in cells appeared in no direct relationship to the form of growth. In the present experiments carried out on another moss species —*Funaria hygrometrica*, similar results were obtained. Kinetin which is a very active bud inducer showed its effect also in conditions unfavourable for growth (liquid inorganic culture) and eventually brought about a decrease in protein synthesis and protein concentration in cells. Brandes (1967) investigating the uptake and distribution of ^{14}C-phenylalanine in the moss protonema showed that the availability of protein-building materials was not a limiting factor

for the bud-inducing activity of kinetin. However, the inhibition of bud induction by inhibitors of protein synthesis demonstrated in this paper for *Funaria* cultures and also obtained in cultures of *Ceratadon purpureus* (Szweykowska and Schneider 1967) point to an involvement of protein synthesis in this process. This might be an instant general stimulation of protein synthesis followed very soon after bud induction in inorganic medium by a depletion of the materials necessary for continuing protein synthesis, and resulting in an inhibition and enhancement of ageing.

An alternative, more specific way of kinetin action might be the synthesis of a specific protein (or proteins) necessary in minute amounts for changing the course of development, in the first place the orientation of cell divisions. Changes in protein composition have been found during morphological differentiation in lower plants (cf. Cantino and Goldstein 1962) and are likely to occur also during differentiation in mosses. Brandes and Bopp (1965) obtained an inhibition of both spontaneous and kinetin-induced formation of gametophores with actinomycin D and concluded that kinetin activates synthesis of DNA--dependent RNA. Brandes (1967 a and b) showed that RNA accumulated in pro-bud cells and that this accumulation was stimulated by kinetin. Activation of DNA-dependent RNA synthesis followed by formation of new specific proteins seems thus to be involved in bud induction in mosses and possibly also in the cytokinin activity in this process.

SUMMARY

1. During the growth of the protonema of the moss *Funaria hygrometrica* in liquid culture a steady decrease in protein content per unit weight (protein concentration) occurs which is probably the sign of progressive ageing of the protonema. The protein concentration increases in young gametophores, their formation being thus a kind of rejuvenation of the culture, and in older gamethopores it decreases again.

2. Glucose promotes growth and enhances the appearence of gametophore buds.

3. In the presence of glucose, kinetin stimulates growth and protein synthesis and induces an early appearance of a large number of buds.

4. In inorganic medium, kinetin has no stimulatory effect on growth and protein synthesis, however, its bud-inducing activity remains very strong.

5. In both inorganic and organic medium kinetin lowers the protein concentration in cells which may be regarded as an enhancement of ageing.

6. Inhibitors of protein synthesis (chloramphenicol, thienylalanine, flourophenyloalanine, methyltryptophane) abolish the bud-inducing activity of kinetin. The effect of inhibitors may be reversed by the appropriate amino acids or by transfer to an inhibitor-free medium.

7. The synthesis of minute amounts of specific proteins seems to be involved in both bud induction in mosses and in the cytokinin activity in this process.

Department of General Botany,
A. Mickiewicz University,
Stalingradzka 14, Poznań, Poland

(Entered: June 24, 1967.)

LITERATURE

Bopp M., 1965, Die Morphogenese der Laubmoose als Beispiel eines morphogenetischen Systems, Ber. d. D. Bot. Ges. 78:44—54.

Brandes H., 1967 a, Fluorescenzmikroskopische Analyse der Knospenanlagen von Moosprotonemen nach Anfärbung mit Acridinorange, Planta 74:45—54.

Brandes H., 1967 b, Der Wirkungsmechanismus des Kinetins bei der Induktion von Knospen am *Protonema* der Laubmoose, Planta 74:55—71.

Brandes H., und M. Bopp, 1965, Spezifische Hemmung der Kinetinwirkung durch Actinomycin D, Naturwiss. 52:521.

Cantino E. C., and A. Goldstein, 1962, Protein changes during morphological differentiation and its reversal in synchronized single generations of *Blastocladiella emersonii*, Amer. Jour. Bot. 49:642—646.

Hotta Y., and S. Osawa, 1958, Control of differentiation in the fern gametophyte by amino acid analogs and 8-azaguanine, Exp. Cell Res. 15:85—94.

Kasimir H., und H. Mohr, 1965, Die Regulation von Chlorophyll und Proteingehalt in Farnvorkeimen durch sichtbare Strahlung, Planta 67:33—43.

Parthier B., 1965, Die Wirkungen verschiedener Antibiotika auf RNS- und Protein-synthesen in Kinetin-behandelten Blattgeweben, Flora (A) 156:344—358.

Szweykowska A., 1966, Growth regulators in the development of gametophores in mosses, Zesz. Nauk. UMK Toruń Biol. VIII:75—80.

Szweykowska A., and A. Handszu, 1965, The effect of kinetin on gametophyte development and protein synthesis in the moss *Ceratodon purpureus*, Acta Soc. Bot. Pol. 34:73—81.

Szweykowska A., and T. Maćkowiak, 1962, On the development of gametophytes in *Funaria hygrometrica* nad *Ceratodon purpureus* in liquid cultures, Acta Soc. Bot. Pol. 31:269—274.

Szweykowska A., and J. Schneider, 1967, Action of 8-azaguanine and of inhibitors of protein synthesis on bud inducing activity of kinetin in the moss *Ceratadon purpureus*, Acta Soc. Bot. Pol. 36:735—738.

Badania nad aktywnością kinetyny w kulturach Funaria hygrometrica

1. Pączkotwórcza aktywność kinetyny a synteza białka

Streszczenie

1. W trakcie wzrostu splątka mchu *Funaria hygrometrica* w płynnej kulturze następuje stały spadek zawartości białka na jednostkę masy (koncentracja białka), będący prawdopodobnie objawem starzenia się splątka. Koncentracja białka wzrasta w młodych gametoforach, których pojawienie się stanowi więc niejako odmłodzenie kultury, następnie w starszych gametoforach znów spada.

2. Glukoza stymuluje wzrost i przyśpiesza pojawienie się gametoforów.

3. W obecności glukozy kinetyna stymuluje wzrost i syntezę białka oraz indukuje bardzo wczesne pojawienie się wielkiej liczby pączków gametoforów.

4. W pożywce nieorganicznej brak jest stymulacji wzrostu i syntezy białka przez kinetynę, a nawet występuje zahamowanie tych procesów, natomiast indukcja procesu pączkotwórczego pozostaje bardzo silna.

5. Zarówno w nieorganicznej, jak i organicznej pożywce kinetyna obniża koncentrację białka w komórkach, w tym sensie więc przyśpiesza starzenie się kultury.

6. Inhibitory syntezy białka (chloramfenikol, tienyloalanina, fluorofenyloalanina, metylotryptofan) blokują pączkotwórczą aktywność kinetyny. Efekt inhibitorów (z wyjątkiem tienyloalaniny) jest odwracalny za pomocą odpowiednich aminokwasów lub przeniesienia do pożywki nie zawierającej inhibitora.

7. Aktywacja syntezy niewielkiej ilości specyficznego białka wydaje się znajdować u podstawy zjawiska różnicowania gametoforów oraz aktywności kinetyny w tym procesie.