Original Papers Plant Tissue Culture Letters, **11**(1), 6-13 (1994)

Growth Characteristics and Morphogenesis of the Liverwort *Marchantia paleacea* Suspension Cells

Kanji ONO, Masaharu NAKAYAMA and Masayuki TAKAMIYA

Department of Biological Science, Faculty of Science, Kumamoto University, Kumamoto, 860 Japan

(Received October 2, 1992)
(Accepted October 2, 1993)

Suspension cultured cells of the liverwort *Marchantia paleacea* var. *diptera* (MpK) were investigated to clarify the relationship between morphogenesis and the mode of utilization of nitrogen sources in different media. MpK cells grew vigorously in a Murashige and Skoog's liquid medium with 4% glucose in the light condition. Thallus regeneration from cultured cells occurred in culture medium with 0. 5% glucose and 0. 1% $CaCO_3$ or 10 mM malic acid. Addition of $CaCO_3$ or malic acid to the medium was necessary to prevent the pH of the medium dropping rapidly to a very acidic value over the duration of the culture. The cause for the extreme acidification of the medium was that the cells regenerating thalli preferentially utilize ammonium as a nitrogen source without the depletion of nitrate in the early phase of culture.

Introduction

Cultured cells of bryophytes, especially in suspension culture, have recently been noted as ideal material for morphogenetic, physiological and phytochemical studies, because they grow abundantly with the retention of chloroplasts and possess characteristics that facilitate the simple control of morphogenesis[1] and produce secondary metabolites[2-4]. Most bryophyte cultured cells grow heterotrophycally requiring both carbon sources and light[5,6]. However, heterotrophic cultured cells capable of growing in the dark with chlorophyll synthesis[7,8] and photoautotrophic cultured cells capable of growing with only a supply of sufficient carbon dioxide have also been reported in a few bryophyte species[9,10]. Thus, bryophyte cultured cells are especially useful in the field of plant physiological studies.

As to morphogenesis in bryophyte cultured cells, regenerated thalli were obtained from calli by transferring them from an organic medium to an inorganic medium without carbon sources and phytohormones[1,5]. Previously, we presented that the liverwort, *Marchantia paleacea* var. *diptera* cultured cells did not regenerate any thalli under the above culture condition and the addition of $CaCO_3$ or malic acid to the inorganic medium was required for morphogenesis[6]. However, almost no explanation was given about the cause of this phenomenon at that time.

The present paper describes growth characteristics and the relationship between morphogenesis and the utilization of nitrogen sources in *M. paleacea* var. *diptera* cultured cells.

Materials and Methods

1. *Suspension culture*

Marchantia paleacea var. *diptera* suspension cell line (MpK) was used in this study. The MpK

suspension culture was established from callus tissues induced by culturing spores[1]. The suspension cells were maintained in the MSG 4 medium which consisted of major and minor salts and vitamins of Murashige and Skoog's medium (MS)[10] containing 4% glucose and no phytohormones. The suspension culture was routinely subcultured at 10-14 day-intervals by pouring about 2.0 m*l* of cell pellets into 100 m*l* of fresh medium in a 300 m*l* Erlenmeyer flask. The flasks were placed on a gyratory shaker at 120 rpm and 25°C in continuous light (3000 lux).

The solid medium for morphogenesis of suspension cells consisted of MS salts and vitamins, 0.5% glucose, 0.1% $CaCO_3$ and 0.3% gellangum. Various concentrations (0-4%) of glucose or mannitol were added to the media to investigate the effect of sugar concentration for morphogenesis. After autoclaving about 4 m*l* aliquots of media were solidified in a Falcon petridish (60×15 mm). One m*l* of cell suspension was plated on each medium and cultured in continuous light (2000 lux).

The liquid media for morphogenesis were MS medium containing 0.5% glucose (MSG 0.5) and MS medium containing 0.5% glucose with 0.1% $CaCO_3$ (MSG 0.5 CO_3) or 10 mM malic acid (MSG 0.5 Mal). These cells in a flask, were cultured under the same conditions as the suspension culture. The pH of all media was usually adjusted to 5.8 before autoclaving.

2. *Measurements of cell growth*

For measurement of growth, 2 g of cells (fresh weight) were pored into 500 m*l* Erlenmeyer flasks containing 200 m*l* of fresh medium and cultured under the same conditions as the subculture described above. For determination of cell dry weight, 5-10 m*l* of the cell suspension was put on a pre-dried and preweighed Miracloth disc. The samples on the discs were dried at 80°C for 24 hours and weighed. In cultures in a medium containing 0.1% $CaCO_3$, the measurement was achieved after dissolving the residual crystals by the addition of 1 N HCl solution to the medium. The pH of a medium after suspension culture was measured prior to the determination of cell dry weight.

3. *Determination of chlorophyll content*

Cells were collected by filtering the suspension culture two or three days preceding the stationary phase. The cells (200 mg in fresh weight) were ground in 0.8 m*l* of 100% acetone and small amounts of $CaCO_3$ with quartz sand using a mortar, followed by extracting the supernatant after centrifuging (30 sec. at 200×g). The residue was ground again with 80% acetone and then the supernatant was removed. This manipulation was repeated until the green color disappeared from the extract. All extracts collected were centrifuged again (3 minutes at 750×g) following filtration through filter paper (Toyo No. 2) and diluted to a certain concentration with 80% acetone. Absorbances at 645 and 663 nm of the acetone extracts were determined with a Shimadzu Spectrophotometer (Model-UV-3000) and chlorophyll contents were calculated by the equation of Arnon[12].

4. *Assay of ammonium and nitrate in the medium*

Ammonium was assayed by the phenol-sodium hypochlorite method and nitrate by high performance liquid chromatography (HPLC). HPLC was performed on Shimadzu LC-6A system and was run on Shim-pack CLC-ODS (M) (4.6×150 mm) column at 50°C with a flow rate of 1.5 m*l*/min. monitoring at 210 nm. The solvent system used was the mixture of KH_2PO_4 (10 mM) and tetra-*n*-butyl ammonium hydroxide (0.7 mM).

Results

1. *Growth characteristics and chlorophyll content*

As shown in **Fig. 1**, the chlorophyllous suspension cells of *M. paleacea* var. *diptera* (MpK) in light condition entered an exponential growth phase without going through a clear lag phase just after

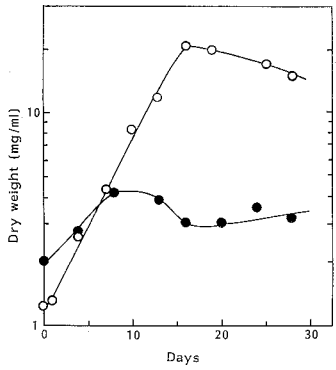

Fig. 1 Growth of *Marchantia paleacea* cells (MpK) cultured in MSG 4 medium in light (○) and dark (●) conditions.
Each value represents the mean of triplicate determinations.

Table. 1 Chlorophyll content of the MpK cells cultured in MSG 4 medium at 25°C in continuous light (3000 lux).

Days of culture	Chlorophyll content	
	μg/m*l* medium	mg/g dry weight
3	7. 27±0. 03	6. 04±0. 71
6	11. 05±0. 22	5. 45±0. 28
9	18. 56±1. 14	4. 14±0. 43
12	41. 47±1. 48	4. 34±0. 11
15	102. 87±4. 62	7. 12±0. 29
18	126. 10±0. 40	5. 57±0. 87
21	150. 35±5. 45	8. 45±0. 37
24	141. 65±19. 25	8. 29±0. 83

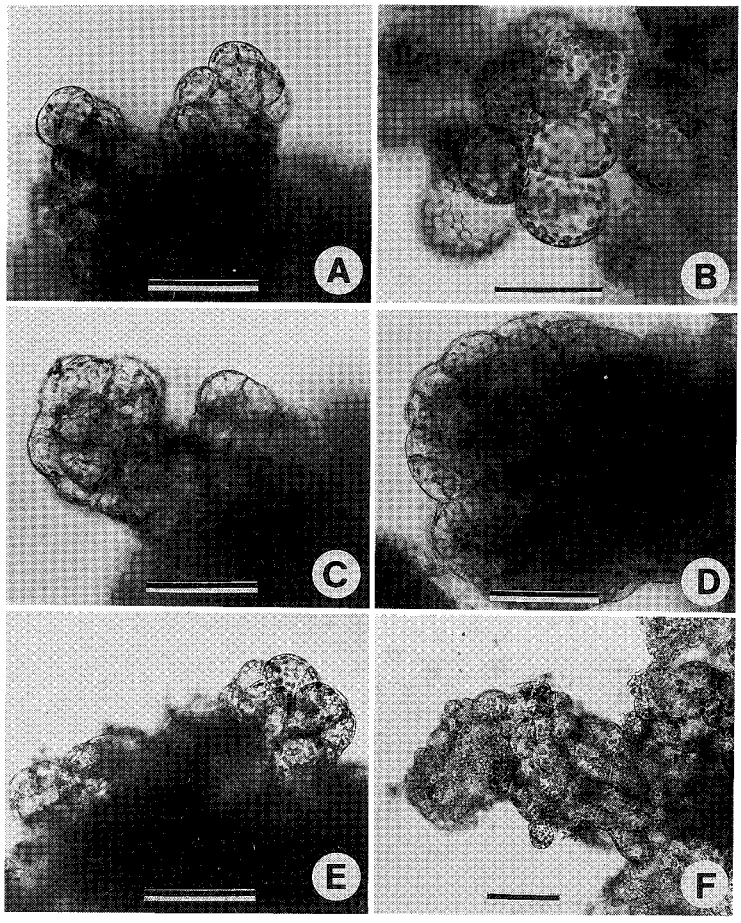

Fig. 2 Morphogenesis of the MpK cells cultured in liquid media with or without $CaCO_3$ or malic acid in the light condition.
A: A protuberance developed 10 days after transfer to MSG 0. 5 medium. **B**: A cell cluster that did not developed into thalli in MSG 0. 5 medium after 30 days. **C** and **D**: Young thalli developed in MSG 0. 5 CO_3 medium after 10 days (C) and after 30 days (D). **E** and **F**: Young thalli developed in MS 0. 5 Mal medium after 10 days (E) and after 30 days (F). Bars indicate 50 μm in A-E and 100 μm in F.

transferring to MSG 4 medium. They reached a stationary phase after 16 days, showing about a 17- fold increase over the initial value for dry weight. On the other hand, their growth in the dark was very slow from the start of culture. They reached a stationary phase after 7 days, showing only about 3-fold increase and ceased to grow in the subsequent culture. Thus, MpK cells revealed heterotrophic growth requiring light even in the presence of glucose. **Table 1** shows changes of chlorophyll content of MpK cells during culture in the light condition. MpK cells synthesized chlorophyll throughout the growth cycle. Chlorophyll content per dry weight decreased up to 12 days from the start of culture (mid-log phase), but it began to increase after 15 days (late-log phase) and reached a maximum of 8. 45 mg/g dry weight after 21 days (stationary phase). Thus, MpK cells were found to synthesize a large amount of chlorophyll at a late-log phase to stationary phase.

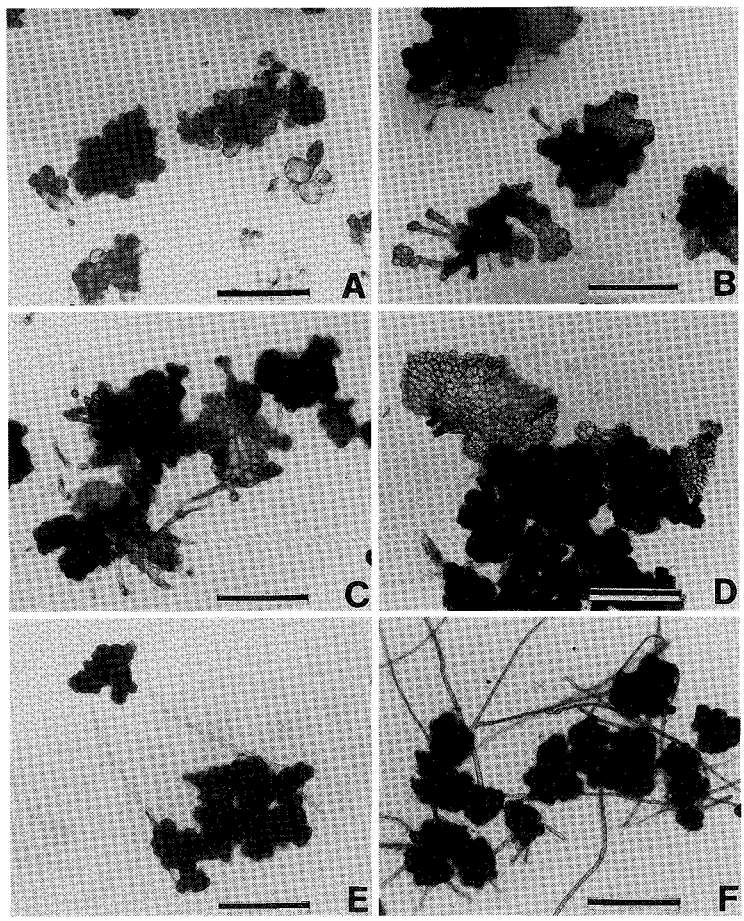

Fig. 3 Morphogenesis of the MpK cells cultured on agar media with different concentrations of glucose or mannitol in the light condition.
A: The MpK cells in suspension culture in MSG 4 medium without $CaCO_3$. **B**: Young thalli developed 10 days after transfer to $MSCO_3$ medium without a sugar and with 0. 1% $CaCO_3$. **C** and **D**: Young thalli developed on MSMan 0. 5 CO_3 medium with 0. 5% mannitol and 0. 1% $CaCO_3$ (C) and MSG 0. 5 CO_3 medium with 0. 5% glucose and 0. 1% $CaCO_3$ (D) after 30 days. **E** and **F**: Cell clusters cultured on MSMan 4 CO_3 medium with 4% mannitol and 0. 1% $CaCO_3$ (E) and MSG 4 CO_3 medium with 4% glucose and 0. 1% $CaCO_3$ (F) after 30 days. Bars. indicate 200 μm.

2. *Morphogenesis*

In order to examine the morphogenesis of MpK cells, they were cultured in the liquid media of MSG 0.5, MSG 0.5 CO_3 and MSG 0.5 Mal. After 10 days, many protuberances arose from a periphery of cell clusters in all media (**Fig. 2-A, C** and **E**). However, protuberances which arose from culture on MSG 0.5 medium maintained the state of dedifferentiated cell clusters and soon died (**Fig. 2-B**). After 30 days, they developed into young thalli only in media with $CaCO_3$ (MSG 0.5 CO_3) or malic acid (MSG 0.5 Mal) (**Fig. 2-D** and **F**). Thallus regeneration of MpK cells also occurred in the medium which 50 mM HEPES buffer was added to MSG 0.5 medium (data not shown).

Morphogenesis of MpK cells was also affected by sugar concentrations (**Fig. 3-A**). Thallus regeneration occurred in cultures on the solid media containing 0.1% $CaCO_3$ with 0-2% glucose or mannitol (**Fig. 3-B, C** and **D**). In MSG 4 or MSMn 4 medium with $CaCO_3$, MpK cells did not regenerate any thalli and maintained the state of undifferentiated cell clusters, but regenerated rhizoids (**Fig. 3-E** and **F**). Cells cultured in MSMn 4 medium grew slowly and soon died. Thus, thallus regeneration of MpK cells was induced only under conditions of the low osmotic potential and the presence of $CaCO_3$ or malic acid.

3. *Changes of the pH and depletion of nitrogen sources in media*

MS basal medium contains both the ammonium and nitrate as nitrogen sources. Therefore, one-sided utilization of two nitrogen sources by cultured cells affects changes of the pH of the medium. **Fig. 4** shows the pH changes and depletion of ammonium and nitrate in MSG 4 medium during a growth cycle of MpK cells. The pH of the medium was decreased rapidly from its initial value of 4.5 to 3.5 within 4 days from the start of culture. Then, it began to rise gradually after 5 days, reached to a maximum of 5.4 after 16 days and hereafter was maintained at about 5 (**Fig. 4-A**). The amount of nitrate and ammonium in the medium decreased gradually over the period

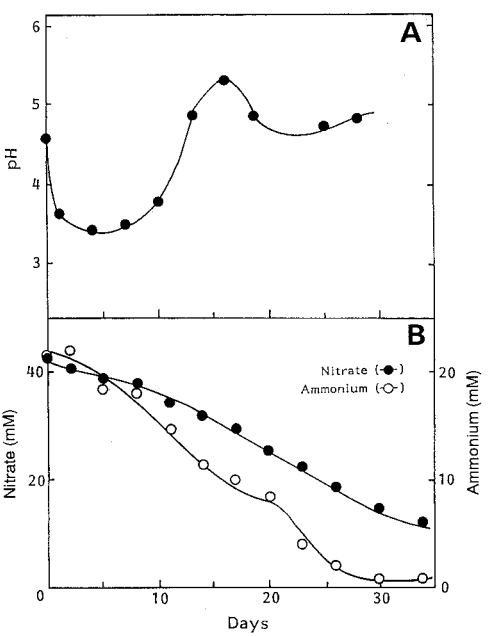

Fig. 4 Changes of pH and depletion of nitrogen sources in MSG 4 medium in the light condition.
A: pH in the medium. **B**: Nitrate (●) and ammonium (○) in the medium. Each value represents the mean of triplicate determinations.

of culture, though a significant decrease in ammonium began after 12 days when compared with that of nitrate (**Fig. 4-B**).

Fig. 5 shows the pH changes in MSG 0. 5, MSG 0. 5 CO_3 and MSG 0. 5 Mal media and the mode of depletion of the two nitrogen sources in MSGCO_3 medium. In the case of MSG 0. 5 medium, the pH of the medium dropped rapidly from its initial value of 4. 8 to 3. 1 within 4 days after transfer and once rose to 4. 2 after 13 days, then dropped again to 3. 2 (**Fig. 5-A**). Such a very acidic condition in the early phase of culture had a highly inhibitory action on the growth and morphogenesis of cells. Therefore, the cells cultured under such a condition showed no thallus differentiation and died changing from green to brown within a month. The pH of the MSG 0. 5 CO_3 medium with 0. 1% $CaCO_3$ maintained at the 6. 3-7. 2 level throughout a growth cycle, while that of the MSG 0. 5 Mal medium with 10 mM malic acid dropped gradually from its initial value 5. 8 to 4. 3 with the passage of culture (**Fig. 5-A**). Cells were capable of surviving and differentiating thalli in these media.

The mode of depletion of nitrogen sources in MSG 0. 5 CO_3 medium for thallus differentiation was

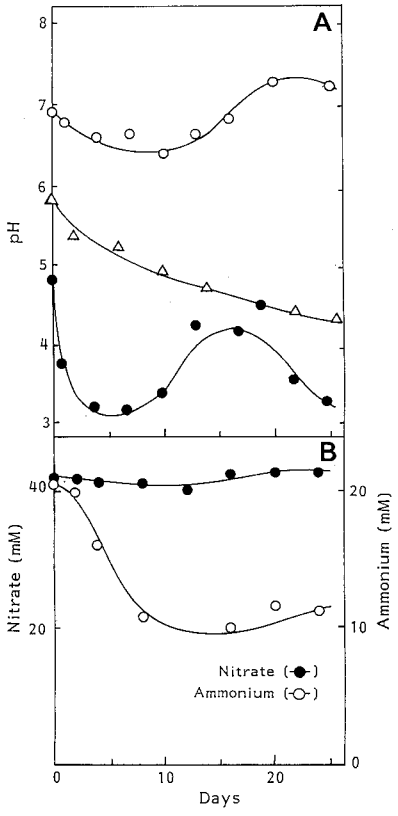

Fig. 5 Changes of pH in different media for morphogenesis and depletion of nitrogen sources in MSG 0. 5 CO_3 medium in the light condition.
A: pH in MSG 0. 5 (●), MSGCO_3 (O) and MSG 0. 5 Mal (△) media. **B**: Nitrate (●) and ammonium (○) in MSG 0. 5 CO_3 medium. Cells were pre-cultured in each medium for 10 days after transfer from MSG 4 medium. Each value represents the mean of triplicate determinations.

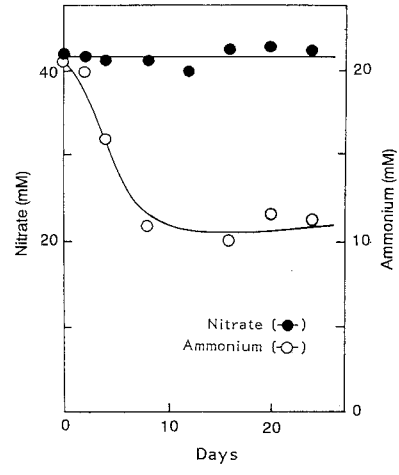

Fig. 6 Depletion of nitrate (●) and ammonium (○) in MSG 0. 5 Mal medium in the light condition.
Cells were pre-cultured in MSG 0. 5 Mal medium for 10 days after transfer from MSG 4 medium. Each value represents the mean of triplicate determinations.

remarkably different from that of MSG 4 medium for suspension culture. As shown in **Fig. 5-B**, the amount of ammonium in the medium decreased rapidly from the early phase of culture, though that of nitrate little changed throughout a growth cycle. Similar results were also obtained in culture in MSG 0.5 Mal medium (**Fig. 6**). Thus, when MpK cells were transferred into the medium for morphogenesis, they preferentially utilized ammonium as a nitrogen source without utilization of nitrate.

Discussion

The chlorophyllous cultured cells (MpK) of the liverwort, *Marchantia paleacea* var. *diptera* grew vigorously in MSG 4 medium in the light, while they could not grow in the dark even in the presence of glucose. Such heterotrophic growth was also reported in cell lines of several liverworts, *M. polymorpha*[9], *Calypogeia granurata*[2], *Heteroscyphus bescherellei*[13] and in those of several mosses, *Polytrichum commune*[1], *Hedwigia ciliata*[13].

Katoh[9] reported that *M. polymorpha* cultured cells performed not only photoassimilation of external glucose but also photosynthetic carbon dioxide fixation. The MpK cells are also considered to grow heterotrophycally in the same manner as *M. polymorpha* cells. On the other hand, a cell line which grows actively with concomitant synthesis of chlorophyll either in the light or in the dark has been established in the same species, *M. paleacea*, as the MpK cells[8]. This phenomenon was found also in cell lines of a liverwort, *Rebouria hemisphaerica*, and a moss, *Barbula unguiculata*[7]. It is desirable to clarify the reason MpK cells established from the same species can not grow in the dark. In the culture of a liverwort, *Jungermannia sublata* cells, Ohta *et al.*[14] reported that the addition of $CaCO_3$ or organic acids of the TCA cycle supported the growth of cells by preventing the abrupt drop in the pH of the medium during the earliest phase of growth, because the cultured cells of this species preferentially utilized ammonium and did not take up nitrate as long as ammonium was present in the medium.

We previously observed similar growth behavior as *J. sublata* cells in several liverworts, *J. infusca*, *Plagiochilla ovarifolia*, *Pellia endiviifolia* and *P. neesiana*[6]. when another *J. sublata* cell line (JsO), established in our laboratory, was cultured in the medium containing nitrate as the sole nitrogen source the cells were capable of growing and the pH of the medium stayed approximately constant throughout a culture period[6]. The reason these cells preferentially utilized ammonium in the medium containing both ammonium and nitrate is unknown. In contrast, another liverwort, *Marchantia polymorpha* utilized these two nitrogen sources at a balanced rate and the pH of the medium was maintained within the physiological range[9]. In the present study, MpK cells in suspension culture were found to use nitrogen sources in a manner similar to *M. polymorpha*.

It is known that bryophyte cultured cells usually regenere thalli when cultured on a medium containing no carbon sources or low concentration of them and no phytohormones under low osmotic potential[1,7,15,16]. This differs from the higher plant cultured cells whose morphogenesis is usually controlled by combinations and concentrations of phytohormones. In order to regenerate thalli from MpK cells, however, the addition of $CaCO_3$ or malic acid to the medium was necessary to prevent the rapid drop in the pH of the medium at the early phase of culture, because the cells which encountered a very acidic condition (below 3.1 of the pH) grew weak and soon died without thallus regeneration.

The preferential utilization of ammonium as nitrogen source may cause the drastic drop in pH in the medium. On the other hand, the MpK cells cultured in MSG 4 medium utilized ammonium together with nitrate from the start of culture. This phenomenon results in maintenance of the pH

of the medium within a physiological range (above 3.5 of the pH) and seems to be conducive to the growth of cells. The drastic drop in pH of the medium was caused by the preferential utilization of ammonium as nitrogen source. This result suggested that the MpK cells altered the manner of utilization of two nitrogen sources in the medium when they proceeded from the state of dedifferentiation to that of thallus differentiation. This phenomenon is characteristic of MpK cells and has not yet been reported in other cultured cells of bryophytes.

We are now engaged in experiments on establishing a photoautotrophic cell line from MpK cells and investigating its photosynthetic activity and nitrogen metabolism.

References

1) Ono, K., Y. Murasaki, M. Takamiya, 1988. J. Hattori Bot. Lab., **65**: 391-401.
2) Takeda, R., K. Katoh, 1981. Planta, **151**: 525-530.
3) Ono, K., M. Toyota, Y. Asakawa, 1992. Phytochem., **31**: 1249-1250.
4) Becker, H., 1990. In "Bryophytes, Their Chemistry and Chemical Taxonomy" (eds. by Zinsmeister, H. D., R. Mues), p. 339-347, Clarendon Press, Oxford.
5) Ohta, Y., M. Ishikawa, S. Abe, K. Katoh, Y. Hirose, 1981. Plant Cell Physiol., **22**: 1533-1540.
6) Harashima, S., K. Ono, 1991. J. Hattori Bot. Lab., **69**: 171-184.
7) Takio, S., M. Kajita, S. Takami, S. Hino, 1986. J. Hattori Bot. Lab., **60**: 407-417.
8) Takio, S., S. Takami, S. Hino, 1988. J. Plant Physiol., **132**: 195-198.
9) Katoh, K., 1983. Physiol. Plant., **59**: 242-248.
10) Ngumi, V. W., S. Takio, S. Takami, 1990. J. Plant Physiol., **137**: 25-28.
11) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15**: 473-497.
12) Arnon, D. I., 1949. Plant Physiol., **24**: 1-15.
13) Takami, S., S. Takio, 1987. J. Plant Physiol., **130**: 267-272.
14) Ohta, Y., Y. Hirose, 1982. J. Hattori Bot. Lab., **53**: 239-244.
15) Ono, K., 1976. Jap. J. Genet., **51**: 11-18.
16) Kajita, M., S. Takio, S. Takami, S. Hino, 1987. Physiol. Plant., **70**: 21-26.

《和文要約》

苔類フタバネゼニゴケ培養細胞の成長特性と形態形成

小野莞爾・中山正晴・高宮正之

熊本大学理学部生物科学科

苔類フタバネゼニゴケ (*Marchantia paleacea* var. *diptera*) の培養細胞 (MpK) は，MS+2% グルコースの培地，明所の培養条件で盛んに成長した．しかし，暗所ではほとんど成長できず，いわゆる光混合栄養成長を示した．葉状体を再分化させるために，培養細胞を MS+0.5% グルコースの培地に植え替えたところ，培養初期に培地の pH が極端に下がり細胞は分化せずやがて枯死した．そこで，pH の低下を抑えるため，培地に 0.1% $CaCO_3$ か 10 mM リンゴ酸を加えて培養したところ，葉状体が再分化した．MpK 細胞は，脱分化した状態では培地中の硝酸塩とアンモニウム塩の両方を利用したが，再分化に向かう状態では，培養初期からアンモニウム塩を優先的に利用し，硝酸塩をほとんど消費しなかった．このように，MpK 細胞では脱分化の状態と再分化に向かう状態で，窒素源の利用の仕方に違いが見られた．