Note

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Effect of Magnesium on the Division of Protoplasts of Brown Algae (*Undaria pinnatifida* and *Petalonia binghamiae*)

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Abstract

Protoplasts of *Undaria pinnatifida* and *Petalonia binghamiae* were cultured for two and one months, respectively, in the presence of various levels of Mg. Under Mg-free conditions in $ASP_{12}NTA$ medium all protoplasts died. The percentage of filaments per surviving protoplast of *U. pinnatifida* increased from 0.47% in 50 mM Mg to 5.33% in 200 mM Mg. Under these conditions the cell number per filament also increased from 154 to 513. The percentage of filaments per surviving protoplast of *P. binghamiae* increased from 77% in 50 mM Mg to 95% in 150 mM Mg, while the number of cells per filament increased from 5.8 to 60.8. At concentrations higher than 100 mM Mg, the Mg and chlorophyll a concentrations were almost equal to those in the thalli.

Keywords: brown algae, magnesium, protoplast division

Tissue and protoplasts of seaweeds have been cultured in synthetic media (ASP_1, ASP_2, ASP_6, ASP_7, and ASP_{12}) or media based on seawater (Provasoli, 1963). However, callus induction and redifferentiation are possible only in a limited number of species (Saga and Matsunaga, 1991). Four genera of brown algae, *Sphacelaria* (Ducreux *et al.*, 1988), Pilayella (Mejjad *et al.*, 1992), Laminaria (Sawabe *et al.*, 1997), and Petalonia (Wakabayashi *et al.*, 2000) have regenerated plantlets from protoplasts. However, the frequency of protoplast division was low and the growth was slower than in natural seaweeds. In order to improve division in tissue cultures of green and red algae, the effects of hormones (Fries, 1984; Bradley and Cheney, 1990; Amano and Noda, 1994), vitamins (Fries, 1984; Lawlor *et al.*, 1989), organic carbons (Lawlor *et al.*, 1989; Amano and Noda, 1994), and trace metals (Fries, 1982a, b) on growth have been investigated. However, these factors did not affect division of cultured tissue of brown algae.

In our preliminary experiments, Mg was more effective than Ca in a cell culture of *Petalonia binghamiae*. Tissue and protoplasts of all seaweeds have been cultured in light without a carbon source. Because culturing under light may be coupled with the presence or absence of Mg, which is a chlorophyll component, relates to chlorophyll synthesis, and activates RuBP carboxylase (Marschner, 1995), we have studied the effects of Mg on the division of protoplasts from *U. pinnatifida* and *P. binghamiae*.

Sporophytic thalli (3-5 cm in length) of *Undaria pinnatifida* were obtained from a local fishery cooperative (Motimune, Shizuoka, Japan) in December 2000. Gametophytic thalli (ca. 10 cm) of *Petalonia binghamiae* were collected from a beach in Shizuoka, Japan, in January 2001. The protoplasts were isolated by a method described in our previous paper (Wakabayashi *et al.*, 1999) with the exception that an enzyme solution now was prepared in 0.7 M NaCl; some antibiotics (50 mg l^{-1} streptomycin sulfate, 50 mg l^{-1} carbenicillin sodium, 50 mg l^{-1} cefotaxim sodium, and 10 mg l^{-1} gentamicin) were added, and the tissue was digested at 18℃-24℃. The digested solution was passed through a 3 μm nylon mesh, and then protoplasts were collected as pellets by centrifugation (90 g, 5 min). The protoplasts were resuspended in a Mg-free medium.

The Mg-free medium was $ASP_{12}NTA$ (Iwasaki, 1961), with the exception that $MgSO_4$ and $MgCl_2$ were removed and 23 mM Na_2SO_4 and 511 mM NaCl were added. Magnesium was added to the Mg-free medium in 50, 100, 150, 200 or 300 mM concentrations as $MgCl_2$. A different 50 mM Mg treatment with a high-osmolarity solution containing 736 mM NaCl resulted in an osmolarity equivalent to that of the 200 mM Mg treatment. The osmotic pressures in those media ranged from 1.28

to 2.18 osmol l^{-1} (**Table 1**).

The protoplasts (ca. 10 μl) of *U. pinnatifida* ($10^4 \pm 8 \cdot 10^2$) or *P. binghamiae* ($10^5 \pm 1.4 \cdot 10^4$) were added to 1.5 ml of the above medium in the wells of a microculture plate (NUNC, 174926). They were then cultured for two and one months, respectively, in cycles of 10 h light (20 μmol $m^{-2}s^{-1}$) and 14 h dark at 15 ℃. In one case, protoplasts of *P. binghamiae* were cultured in cycles of light and dark for the first three weeks and then cultured in the dark after the third week. The colonies were scraped off the microculture plate after two months of culture for *U. pinnatifida* and after two, three, or four weeks of culture for *P. binghamiae*. The surviving protoplasts and colonies were counted with a hemocytometer. These measurements were conducted at least in duplicates.

Cultured cells were washed in a Mg-free medium and then collected by centrifugation. The cells were digested with HNO_3-$HClO_4$ (4:1), and then Mg was assayed by atomic absorption analysis (285.2 nm). Chlorophyll a and fucoxanthin were extracted from cells with 100% methanol overnight in a refrigerator. The chlorophyll a concentration was calculated from the absorbance at 665 nm (Holden, 1976). The amount of fucoxanthin obtained by purification on silica gel-TLC (Merck: petroleum ether· acetone 7:3) in N_2 gas was calculated from the absorbance at 446 nm (Jensen, 1961). The tests were conducted at least in duplicates.

Protoplasts of *U. pinnatifida* (**Fig. 1-A**) and *P. binghamiae* (**Fig. 1-D**) were cultured in various levels of Mg. In a Mg-free condition, all the protoplasts died (data not shown). About half of the protoplasts survived in the 50 to 150 mM Mg media. The protoplast survival rate decreased when the medium contained more than 200 mM Mg for *U. pinnatifida* (**Table 1**), while the protoplasts showed a tendency to lower survival rates for *P. binghamiae* under high-osmolarity conditions, which were 200 mM Mg or high-osmolarity 50 mM Mg treatments. These results suggest that the lower survival rate in 200 mM Mg is caused by a high Mg concentration in *U. pinnatifida* and by a high osmolarity in *P. binghamiae*.

The surviving protoplasts from thalli of *U. pinnatifida* (**Fig. 1-B, C**) and *P. binghamiae* (**Fig. 1-E, F**) germinated and grew into filaments. The thallus has been regenerated from a filament in a protoplast culture of *P. binghamiae* (Wakabayashi *et al.*, 2000) and from a filament in tissue culture of *Undaria* (Notoya and Aruga, 1992; Kimura and Notoya, 1997). The proportion of filaments to surviving protoplasts of *U. pinnatifida* increased from 0.47% in the 50 mM Mg medium to 5.33% in the

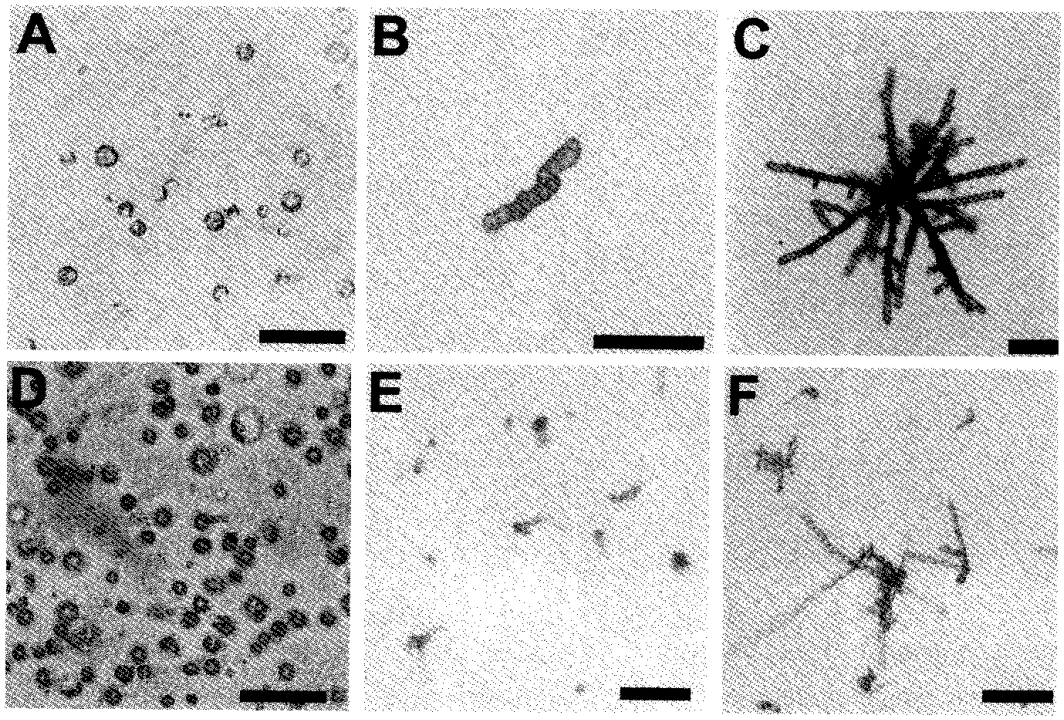

Fig. 1 Protoplasts and cultures of *U. pinnatifida* (A-C) and *P. binghamiae* (D-F). (A, D) Isolated protoplasts, (B) germinating protoplast in 200 mM Mg after 48 days of culture, (C) a filament from a protoplast in 200 mM Mg after 2 months of culture, (E) filaments from protoplasts in 50 mM Mg after 3 weeks of culture, and (F) filaments from protoplasts in 150 mM Mg after 3 weeks of culture. Scale bars represent 50 μm on (A) and (D), and 100 μm on (B), (C), (E) and (F).

Table 1 The effect of Mg on the culture of U. pinnatifida and P. binghamiae after two months and two weeks, respectively.

Mg treatment (mM)	Osmotic pressure (Osmol l^{-1})	*U. pinnatifida*			*P. binghamiae*		
		Survival of protoplasts (%)	Filaments / surviving protoplasts (%)	Cells / filament	Survival of protoplasts (%)	Filaments / surviving protoplasts (%)	Cells / filament*
50	1.43	56.1 ± 19.0	0.47 ± 0.11	154 ± 54	40.6 ± 3.9	76.9 ± 6.0	5.8 ± 1.7
100	1.58	59.1 ± 3.1	0.76 ± 0.16	128 ± 20	41.4 ± 6.6	88.7 ± 3.0	9.8 ± 0.6
150	1.73	n.m.	n.m.	n.m.	42.6 ± 3.9	95.4 ± 0.6	60.8 ± 17.4
200	1.88	24.7 ± 12.4	5.33 ± 3.89	513 ± 17	31.5 ± 7.6	91.6 ± 3.6	14.0 ± 4.2
300	2.18	0.1 ± 0.1	100.0**	35**	n.m.	n.m.	n.m.
50	1.88	57.0 ± 3.3	0.80 ± 0.17	183 ± 9	29.3 ± 9.1	77.4 ± 5.1	3.8 ± 0.4

Value of *U. pinnatifida* was mean ± range and of *P. binghamiae* was mean ± s.d.
n.m.; not measured *; measurement after four weeks **; only one plate

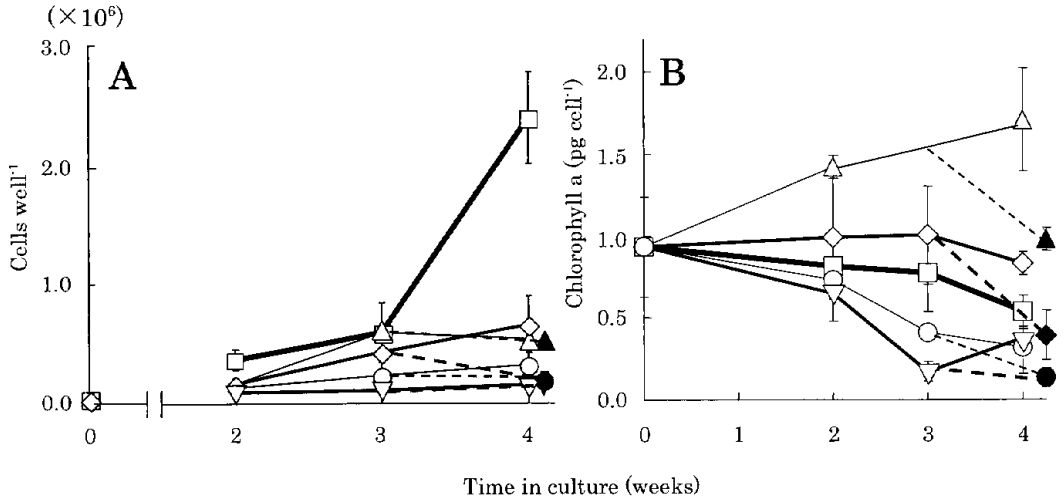

Fig. 2 The effect of Mg on the number (A) and chlorophyll a concentration (B) of cells in a protoplast culture of *P. binghamiae* . The symbols " ○ , △ , □ , ◇ , and ▽ " indicate Mg treatments with 50, 100, 150, 200 mM and high-osmolarity 50 mM concentrations, respectively. Protoplasts were cultured in cycles of 10 h light and 14 h dark (open symbols), with the exception of the period between the third and fourth week where they were maintained in darkness (solid symbols). The values in week zero are the same as the number of surviving protoplasts in the second week. A bar represents the standard deviation, except that the bar at the fourth week is a range of duplicates.

200 mM Mg medium (**Table 1**). The filament number per well was highest with the 200 mM Mg treatment. The cell number per filament increased 3.3 fold when the Mg concentration was increased from 50 mM to 200 mM. In *P. binghamiae*, the filament formation was slightly larger at Mg concentrations higher than 100 mM than that it was at a 50 mM Mg concentration (**Table 1**). The cell number per filament increased tenfold when the Mg concentration was increased from 50 mM to 150 mM. The total number of *P. binghamiae* cells was higher in a Mg concentration of 100 mM than it was in one of 50-mM ($ASP_{12}NTA$ medium) (**Fig. 2-A**). Especially, the cell number in the 150 mM Mg medium was the highest because of the large filament. However, the number of cells did not increase in the absence of light at any Mg concentration. Thus, a high level of Mg was necessary for further division in both species, but a high osmolarity was not. This may be related to the high Mg content of the meristematic zone of *U. pinnatifida* (Ito and Miyoshi, 1990).

The Mg concentration of thalli of P. binghamiae was 1.01 ± 0.34 μg Mg in one million cells. The Mg

concentrations in one million cells after three weeks of culture in 50, 100, 150, and 200 mM Mg or in the high-osmolarity medium containing 50 mM Mg was 0.19, 0.59, 1.17, 1.58 and 0.72 μg , respectively. Thus, the cultured cells in a 150 mM Mg medium contained the same level of Mg as natural thalli. On the other hand, the chlorophyll a concentrations of the cultured cells decreased in 50 mM Mg, increased in 100 mM Mg, and remained unchanged in 150 mM and 200 mM Mg in a cycle of light and dark (**Fig. 2B**). The chlorophyll a concentrations after one month of culture were 40, 183, 58, 89, and 34 % of those of thalli (chlorophyll a = 0.93 $\pm$ 0.30 pg cell^{-1}) in treatments with 50, 100, 150, 200 mM Mg, and high-osmolarity 50-mM Mg, respectively. When the cells were cultured in darkness between the third and fourth week, the chlorophyll a concentrations decreased significantly. No difference was found in the fucoxanthin concentrations in the cells except in the 100 mM Mg treatment, in which the content increased (data not shown).

The number of cell divisons as well as the amount of chlorophyll a per well were highest (1.32 μg) in the 150 mM Mg treatment of protoplasts from *P. binghamiae*. However, in the same treatment, the amount of chlorophyll a per cell was not high. The Mg concentration in cells at the 150 mM Mg treatment was equal to that in thalli. Therefore, we suggest that the cellular Mg concentration is important for the division of protoplast from *P. binghamiae*. The cellular Mg and chlorophyll a concentrations decreased considerably in the $ASP_{12}NTA$ medium (50 mM Mg conditions). In order to compensate for this decrease, treatment with a high Mg concentration would be necessary. The intracellular Mg concentration also would be important for the cell division of protoplast from other brown algae, including *U. pinnatifida*. However, the suitable concentrations for the survival and division of the protoplasts were slightly different in *U. pinnatifida* and *P. binghamiae*. Hence, we suggest that the most suitable Mg concentration varies among species. In the present study, we found that the protoplast cultures of brown algae required a higher Mg concentration in the medium than did the thalli.

References

Amano, H., Noda, H., 1994. Effects of growth regulators, organic acids, and sugers on growth and chemical constituents in the tissue culture of sea lettuce *Ulva pertusa*. Fisheries science, **60**: 449-454.

Bradley, PM., Cheney, DP., 1990. Some effects of plant growth regulators on tissue cultures of the marine red alga *Agardhiella subulata* (Gigartinales, Rhodophyta). Hydrobiologia, **204/205**: 353-360.

Ducreux, D., Maillard, P., Sihachakr, D., 1988. Patterns of differentiation and regeneration in single cells and protoplasts of *Sphacelaria* (Phaeophyceae). In: Stadlar, T. *et al.* (Eds.): Algal Biotechnology, 129-137. Elsevier Applied Science, London and New York.

Fries, L., 1982a. Selenium stimulates growth of marine macroalgae in axenic culture. J. Phycol., **18**: 328-331.

Fries, L., 1982b. Vanadium an essential element for some marine macroalgae. Planta, **154**: 393-396.

Fries, L., 1984. Induction of plantlets in axenically cultivated rhizoids of *Fucus spiralis*. Can. J. Bot., **62**: 1616-1620.

Holden, M. 1976. Chlorophylls. In: Goodwin, T. W. D (Eds.): Chemistry and Biochemistry of Plant Pigments (2nd Edition), Vol.2., 2-37. Academic Press, London New York.

Ito, S., Miyoshi, T., 1990. Determination of Mg and Zn contents of Naruto "Wakame" (*Undaria pinnatifida*). Jpn. J. Hyg., **45**: 795-800.

Iwasaki, H., 1961. The life-cycle of *Porphyra tenera in vitro*. Biol. Bull., **121**: 173-187.

Jensen, A., 1961. Algal carotenoids I. fucoxanthin monoacetate. Acta Chem. Scand., **15**: 1604-1605.

Kimura, H., Notoya, M., 1997. Tissue culture of *Undaria undariodes* (Yendo) Okamura (Laminariales, Phaeophyta). J. Mar. Biotechnol., **5**: 100-102.

Lawlor, HJ., McComb, JA., Borowitzka, MA., 1987. The development of filamentous and callus-like growth in axenic tissue cultures of *Ecklonia radiata* (phaeophyta). In: Stadler T, Mollion J, Verdus MC, Karamanos Y, Morvan H, Christiaen D (Eds.): Algal biotechnology, 139-150. Elsevier Applyde Science, London and New York.

Marschner, H., 1995. Mineral nutrition of higher plants, Second edition., Institute of Plant Nutrition, University of Hohenheim Germany, Academic Press.

Mejjad, M., Loiseaux-de-Goër, S., Ducreux, G., 1992. Protoplast isolation, development, and regeneration in different strains of *Pilayella littoralis* (L.) Kjellm. (Phaeophyceae), Protoplasma, **169**: 42-48.

Notoya, M., Aruga, Y., 1992. Tissue culture of *Undaria pinnatifida* (Harvey) Suringar (Laminariales, Phaeophyta). Jpn. J. Phycol., **40**: 393-395.

Provasoli, L., 1963. Growing marine seaweeds. Proc. Int. Seaweed Symp., **4**: 9-17.

Saga, N., Matsunaga, T., 1991. Labo-manual Marine Biotechnology, Shokabo, Tokyo.

Sawabe, T., Ezura, Y., Yamamoto, H., 1997. Plant regeneration from protoplasts of *Laminaria japonica* Areschoug (Laminariales, Phaeophyceae) in a continuous-flow culture system. Plant Cell Reports, **17**: 109-112.

Wakabayashi, T., Kuboi, T., Tuboi, T., Kaji, M., Hara, M., 1999. Preparation of high yields of algal protoplasts using buccal juice of sea hare and commercial cellulase. Mar. Biotechnol., **1**: 407-410.

Wakabayashi, T., Tuboi, T., Hara, M., Kuboi, T., 2000. Protoplast development of *Petalonia binghamiae* (Phaeophyta, Scytosiphonaceae). Bull. Fac. Agric. Shizuoka Univ., **50**: 1-5