Promotion of callus propagation by 5-aminolevulinic acid in *Laminaria japonica* sporophyte

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Abstract

The effects of 5-aminolevulinic acid (ALA) on the induction and growth of callus-like cells in *Laminaria japonica* were investigated in explants obtained from basal, middle and apical portions along the sporophyte. ALA treatment promoted the induction of callus-like cells in explants obtained from all portions, and the induction rate was higher when a concentration of 50-500 mg L^{-1} of ALA was used. The promotion was especially remarkable in apical explants, and the induction was 10-14 times higher in the 100-500 mg L^{-1} range than that in the 0 mg L^{-1}. The cell division rate of callus-like cells showed highest value in the explants cultured with 500 mg L^{-1} of ALA for 14 days. The promotion of the cell division rate by culturing with 500mg L^{-1} ALA was also observed under white, blue and red lights. The callus-like cells, which were cultured in 500 mg L^{-1} of ALA for 2 months, had many clear chloroplasts. After 3 months, young thalli occurred. These results suggest that the ALA treatment is effective for stable propagation of callus-like cells in *L. japonica*.

Introduction

The aquacultural production of *Laminaria japonica* is the highest of all the seaweeds in the world (Zemke-White and Ohno, 1999). Laminariales plants have been widely used in foodstuff, medicine, industrial materials, fodder and cosmetics. In addition, Laminariales plants have useful chemical components, such as alginate, fucoidan, etc., that allow us to produce individuals with useful characteristics such as high quality, high production, high resistance to disease, and high environmental responsibility. From this point of view, the development of techniques for plant breeding and the production of useful seed using biotechnology are desired.

Plant tissue culture is an important technique in both basic and applied studies. The technique is also useful and valuable in seaweeds. Therefore, many investigations have been carried out in many seaweed species. At present, the induction of callus-like cells and regeneration became possible in *L. japonica* (Saga et al., 1978; Yan, 1984; Wang et al., 1998). However, the problems remains that the induction level of callus and its growth rate are lower in seaweed than higher plants (Aguirre-Lipperheide et al., 1995). In addition, a reproducible and well-understood protocol for production of rapidly growing callus-like cells from sporophyte explants of Laminariales has not yet been achieved (Butler and Evans, 1990).

5-Aminolevulinic acid (ALA) is known as a precursor of chlorophyll *a*, and is naturally distributed in photosynthetic bacteria, algae and plants. This compound promotes higher plant growth at low concentrations (Hotta et al., 1997), and acts as an herbicide at high concentrations (Rebeiz et al., 1984). Additionally, this compound has been used to produce color improvement and tolerance to cold and high salt levels (Sakai et al., 2002). The purpose of this study is to evaluate the effect of ALA on the induction and growth rate of callus-like cells in the sporophyte of *L. japonica*. The effect of ALA on the production of callus-like cells in *L. japonica* is also discussed.

Materials and Methods

Preparation of young sporophytes

The mature sporophytes of *L. japonica* were collected in the coastal area near Hakodate and Hokuto cities in Hokkaido, Japan, from February 2006 and August 2007. These sporophytes were transported into our laboratory. The sorus parts were cut from the mature sporophyte using a dissecting knife and

stored at 4 °C in a refrigerator overnight after being wiped with a paper towel and wrapped with a newspaper. After 24 hr, the sorus part was then placed in 200 mL of seawater to release zoospores. The zoospores were then cultured in a polystyrene case (Square type, 8.5cm x 17.5cm x 3.5cm) at 4 °C, 60 μmol photons m^{-2} s^{-1} in a 12-h light: 12-h dark cycle until they developed to sporophytes through the gametophyte stage in the bottom of the culture case. Provasoli's enriched seawater (Provasoli, 1968) without vitamins was used as a medium and renewed once a week during the culture. When the sporophytes grew to *ca.* 1 mm in length, they were detached from the bottom of culture case. The detached sporophytes were cultured in a polyethylene terephthalate bottle (1L) with aeration and allowed to grow thalli with *ca.* 5 cm in blade length at 10 °C, 60 μmol photons m^{-2} s^{-1} (12L: 12D cycle). The young sporophytes were supplied to the explant collection. The seawater used as a medium was filtered through a glass fiber filter (GA-100, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and autoclaved at 121 °C for 20 min (ASV-2401, Tiyoda Manufacturing Co., Ltd., Tokyo, Japan).

Bacteriostatic treatment of explants

The young sporophytes were cut 0.5 cm from the blade-stipe transition and the apex, respectively, and the basal and apical parts were removed. The residual central portions of the thallus were subjected to bacteriostatic treatment. The segments were placed in 50 mL of seawater with fine sands, and they were shaken for 15 seconds. This washing procedure was repeated 3 times. The segments were then placed in seawater containing 0.1% of Tween 20, and were shaken for 10 seconds. After the segments were fully washed with seawater, the segments were placed in sterilized seawater including 0.1 % of Popiyodon (10% povidone-iodine solution, Yoshida Pharmaceutical Co. Ltd., Tokyo, Japan) and agitated. After discolored segments were removed, the living segments were placed in an antibiotic mixture (Druehl and Hsiao, 1969). After 12 hr, the explants (*ca.* 2mm x 2mm) were obtained. They were washed with seawater and their sterility was checked by the following procedure. The sterility test was carried out using 5 media: STP (Provasoli et al., 1957), ST3 (Iwasaki, 1965), ESS_{B1} (Saga, 1991), STM (Polne-Fuller and Gibor, 1984), and Zobell 2216 (Zobell, 1941). Five test tubes containing each medium with 0.4% agar were respectively autoclaved. After they were cooled at room temperature, the explants were inserted into each medium and respectively cultured at 20°C under 0 (dark) and 60 μmol photons m^{-2} s^{-1} for 2 weeks. These test tubes were observed under an inverted microscope (Leica, DMIL) to confirm the existence or absence of bacterial colonies.

Induction conditions on callus-like cells

Preliminary experiments were done to clarify the preferable water temperature, salinity, and light intensity in callus induction of *L. japonica*. Thirty explants (*ca.* 2 x 2 mm square) of the sporophyte were cultured in the basal conditions, which were 10 °C, 27.5 psu, and pH 7.8 in vitamin-free PES medium under 30 μmol photons m^{-2} s^{-1} (12L: 12D cycle) of illumination under a white fluorescent lamp (Parck cool, 18W, National) after the bacteriostatic treatment described above. The preferable conditions were determined by fluctuating of each factor in the basal conditions. The formation of callus-like cells was observed under an inverted microscope in 7 days of culture. The callus induction index was estimated according to our previous report (Mizuta et al., 2007). The callus occupation rate on a cut face of a square explant was divided into six levels (x) (0%, 0<x<25%, 25≤x<50%, 50≤x<75%, 75≤x<100%, 100%). We conveniently used the represented values of 0%, 12.5%, 37.5%, 62.5% 87.5% and 100%, which corresponded to six callus occupation levels, to calculate a callus induction index. The index (CI_n) was calculated using the following equation:

$$CI_n\ (\%) = \sum C_i / 4N$$

Where CI_n is the average callus index of total observed explants, N is the number of observed sporophyte explants and C_i expresses the sum of the callus coverage in each phase of a square explants. Accordingly, when a callus occupied four phase of the explants in whole, the callus induction index was defined to be 100% in C_1.

Effects of 5-aminolevulinic acid (ALA) on induction and growth of callus-like cells

To study the effects of ALA on callus induction in different collection portions of explants, the sporophyte (*ca.* 5 cm in length) was divided into apical, middle, and basal portions along the sporophyte axis. Forty explants (2mm x 2mm) obtained from each portion were cultured at the density of 20-explants/50 mL in the different ALA concentrations of 0, 1, 5, 10, 50, 100, and 500 mg L^{-1} under the basal conditions as described above. After 7 days, the callus induction index was estimated. The culture continued for about 3 months.

Other 20 explants (*ca.* 5 mm in length and *ca.* 250 μm in width), which were collected from the basal, middle and apical portions along the thallus, were cultured in vitamin-free PES medium containing 0, 10, 50, 100, 500 and 750 mg L^{-1} of ALA under the basal conditions to study the effect

of ALA on the growth of callus. After culturing for 4 and 14 days, the filamentous callus-like cells were observed under an inverted microscope to estimate the cell division rate. Fifteen filaments, which were mostly progressive advanced, were selected and the cell number of filaments was counted. In each condition, the cell division rate was expressed in the unit day^{-1}.

Moreover, other 50 explants were also cultured in vitamin-free PES medium with and without 500 mg L^{-1} of ALA under white (peaks at 450, 540 and 610nm, Parck, National), blue (400-530nm, peak at 470nm) and red light (600-700nm, peak at 660nm) (MIL – B18, MIL – R18, SANYO Electric Biochemical Co., Ltd.). After culturing for 14 days, the callus-like cells were observed under an inverted microscope, and the cell division rate was estimated.

All data were expressed as the mean $\pm$ standard deviation. The statistical differences in preliminary experiments were using a Kruskal-Wallis test followed by Scheffe's test, because these data did not show homologous variances. In the other experiments for evaluation of the effects of ALA on the induction and cell division rate of callus-like cells, the statistical analysis was performed using two-way or three-way analysis of variance (ANOVA). Results were considered significant at $P< 0.05$.

Results

There were no bacterial colonies in the sterility test tubes containing ST3, ESS and STM media incubated in both dark and light conditions. However, bacterial colonies were observed in one tube of STP medium and 3 tubes containing Zobell 2216 medium incubated in the light conditions, and in 2 tubes containing STP medium and 3 tubes containing Zobell 22 incubated in the dark.

The callus induction index was significantly influenced by water temperature, light intensity and salinity, respectively (Fig. 1). The callus induction index was highest at 10 ˚C. The high light intensities, which were more than 60 μmol photons m^{-2} s^{-1}, resulted in decrease of callus induction index. Callus-like cells were not induced in salinities lower than 25 psu.

The effects of ALA concentration on the callus induction of the explants obtained from different portions along the sporophyte are shown in Table 1. The collection portion significantly influenced the callus induction index (Table 2), and the explants obtained from the basal portion had highest indexes ($\geq$99.2%) and the lowest indexes were observed in the explants obtained from the apical portion. ALA concentrations greatly influenced the induction index in the explants obtained from the apical and middle portions, and the index increased with elevated levels of ALA. The promotion of the induction of callus-like cells by the addition of ALA was remarkable in the explants obtained from apical portion,

and higher indexes were observed in the 50, 100, and 500 mg L^{-1} ALA concentrations. During the culture, the survival rate of basal segments was 100% regardless of the ALA concentration for the first 7 days of culture. However, there were some bleached explants from the middle and apical portions. In particular, this tendency was strong in explants obtained from the apical portion, and the lowest survival rate (25-32.5%) was observed in low ALA concentrations less than 10 mg L^{-1}.

The effects of ALA concentration on the cell division rate of callus-like cells are shown in Fig. 2. The explant collection portions significantly influenced the cell division rate (Table 3) as similar to the case of the callus induction. The cell division rate of callus-like cells was lower (0.14-0.51 d^{-1}) in the explants obtained from apical portions than those (0.21-1.04 d^{-1}) from the middle and basal portions through the culture period. The cell division rate in the first 4 days of culturing was increased with increasing ALA, and was highest in 500 or 750 mg L^{-1} regardless of the collection portion of the explants. The higher cell division rate in 500 mg L^{-1} was maintained for 14 days of culturing, particularly in the explants obtained from the middle portion of the thallus. In contrast, the cell division rate of callus-like cells cultured in 750 mg L^{-1} decreased to the same level as in the 0 mg L^{-1} after 14 days, and the color changed to greenish and some dead cells were observed.

The features of callus-like cells cultured for 2 weeks in different ALA concentrations are shown in Fig. 3. The callus-like cells grew elongated and branched with increasing ALA concentration except under the condition of 750 mg L^{-1}. The callus-like cells continued to grow and the filament reached more than 300 μm in length from the section after 2 weeks of being cultured in 500 mg L^{-1}. When the explants were cultured in 500 mg L^{-1} for one month, the callus-like cells were unpigmented and they were often bleached (Fig. 4A). In 2 months of culture, the number and size of chloroplasts increased (Fig. 4B). The chloroplasts became clear after 3 months (Fig. 4C). After 3 months, regenerated thalli were often observed (Fig. 4D-F). In addition, there are often two types of thalli. The types were characterized by the site of rhizoidal cells. One was the thallus that had rhizoidal cells attached to the apical cells of a callus-like filament (Fig. 4D, E). Another site of rhizoidal cells did not attach to the callus-like filament, but the apex of the thallus attached directly to the filament (Fig. 4F).

The effects of light quality and ALA treatment on the cell division rate of callus-like cells in the explants obtained from different portions of sporophyte are shown in Fig. 5. The three-way ANOVA result shows statistically significant effects of light quality and ALA treatment on the cell division rate of callus-like cells (Table 4). The cell division rate was high under red light and lower under blue light in all explants regardless of the collection portions. Moreover, ALA treatment promoted the cell division rate under all light qualities. In particular, the rate increased 32-104% in explants from basal

and apical portions. The explants obtained from the basal portion reached a cell division rate of 1.0 day^{-1} after the addition of ALA under white and red light conditions.

Discussion

The results of a sterility test showed that bacterial colonies were detected in a part of the media. This means that the pre-treatment of explants was not under completely bacteria-free conditions but rather under disinfected conditions.

The preferable water temperature for callus induction was consistent with that for growth in the whole sporophyte (Okada et al., 1985). Exposure to salinity lower than 20 psu resulted in discoloration and erosion of the cells. This means that the callus-like cells show the same response as the whole plant (Mizuta, pers. commun.). However, the callus induction was inhibited in 60 and 120 μmol m^{-2} s^{-1}, which the growth of whole plants is not inhibited. The results showed a negative effect of high light intensity on the induction of callus-like cells, although the reason for this was not clear. In addition, callus induction occurred even in the dark, but the subsequent growth was inhibited. This result indicates that callus induction does not require light irradiation, and that the subsequent growth requires light. It is also suggested that the callus-like cells are able to grow autotrophically.

In this study, ALA promoted the induction and propagation of callus-like cells at concentrations of more than 50 mg L^{-1}. Particularly, the cell division rate was maintained at the high concentration of 500 mg L^{-1}, which was the same concentration to stimulate the growth of *Spirulina platensis* (Sasaki et al. 1995). In higher plants, ALA promotes plant growth at low concentrations (Hotta et al., 1997) and acts as an herbicide at high concentrations (Rebeiz et al., 1984). In this study, the culturing in 750 mg L^{-1} for 14 days inhibited the propagation of callus-like cells regardless of the explant collection portion along the thallus. The growth inhibition by high ALA level is considered to be due to the herbicidal effect of ALA. The herbicidal effect also seems to become remarkable with culture time. In addition, Bindu and Vivekanandan (1998) reported in the callus of *Vigna unguiculata* that low concentrations promoted growth and high concentrations promoted root formation, showing that ALA may have hormone-like activity. Therefore, the optimal concentration of ALA treatment might be different among species.

The induction and propagation of callus-like cells are often influenced by the explant collection portion from the plant (Notoya, 1988) and the collection time (Fries, 1980; Kawashima and Tokuda, 1990). In previous studies, the explants obtained from meristem show higher induction and growth. In *L. japonica*, the induction was lower in the explants from the apical portion than those in the middle

and basal portions. The apical portion is sited far from the meristematic region between stipe and lamina. Therefore, the difference of induction of callus-like cells among collection portions is considered to be due to age and cell division activity of the explant cells. In addition, the nutritional status of explants may influence to be the induction and growth of callus (Aguirre-Lipperhaide et al., 1995). Interestingly, the difference in cell division rate of callus-like cells among the portions of the thallus seemed to be smaller than that in the induction of callus-like cells. These results suggest that the callus-like cells divided independently from the mother explants. In other words, the contribution of mother plant to the propagation of callus-like cells seems to be low compared to its contribution to the induction of callus-like cells.

It has been reported that the morphological form of callus-masses raised from explants of seaweeds can be divided into two types, pigmented and unpigmented filaments (Notoya and Aruga 1987; Kawashima and Tokuda 1990; Lowlor et al. 1988). In this study in *L. japonica*, the callus-like cells cultured with ALA showed typical unpigmented filaments early in the culturing period. These filaments often branched. However, the unpigmented cells changed to pigmented filaments after 2 months of culturing. Chloroplast changes in both size and number in the callus-like cells were also observed in *Eckloniopsis radicosa* (Notoya 1997). The pigmented cells re-differentiated to the thallus through unicellular embryos after 3 months. These observations indicate that the development of callus-like cells progresses in the following order: unpigmented cells, pigmented cells, embryogenesis and thallus formation. When an embryo developed to a 2-celled bladelet, different types of thalli were observed. These thalli were divided into 2 types based on the site of the rhizoidal cells (Fig. 4). One type of thallus had rhizoidal cells located on the far side of the callus-like cells. The rhizoidal cells in this type showed good growth. Rhizoidal cells of other type were located on the near side of the callus-like cells.

Light quality is an important factor in the induction, propagation and re-differentiation of algal callus-like cells. The effects of different wave-lengths of light on the callus have been reported in *Ecklonia radiata* (Lowlor et al., 1989) and *L. japonica* (Mizuta et al. 2007), in which it was found that the growth of the callus was promoted by red light. Lawlor et al. (1989) found that the photosynthetic activity was promoted when the wave-length of red light corresponded to the maximum absorption wave-length of chlorophyll *a*. Interestingly, ALA led to callus-like cell growth that was more rapid than the growth observed under the irradiation of red light.

The doubling rate has been used as an indicator of the propagation of single cells in higher plants. In this study, the doubling time in the early period of callus-like cell formation was estimated to be

about 1-1.4 days, although the time was influenced by environmental conditions. This doubling time was shorter than that (5 days) of free-living gametophytes of *L. digitata* (Martino et al., 2000). Thus ALA treatment promotes the vegetative propagation of callus-like cells, but it took about three months to obtain in the re-differentiated sporophytes of *L. japonica*. Wang et al. (1998) also reported that the one callus differentiated into sporophyte of *L. japonica* after five months of culture. Probably, there is a different mechanism between vegetative propagation and re-differentiation of the callus-like cells.

In conclusion, ALA treatment of explants of *L. japonica* sporophyte is useful to produce and propagate callus-like cells stably. The ALA treatment will be a good tool for the investigation of callus induction and formation. In addition, the thallus originated from callus-like cells often showed an abnormal form. Therefore, the development of techniques for obtaining the normal morphogenesis from callus-like cells is a future aim.

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Figure legends

Figure legends

Fig. 1. Effects of water temperature (A), light intensity (B) and salinity (C) on the callus induction of explants of *Laminaria japonica* sporophyte. Data show mean + s.d. (n=30). Different letters represent statistically significant differences at P=0.05 by Scheffe's test (P=0.05). n.d. represents no data.

Fig. 2. Effect of external ALA concentration on the cell division rate of callus-like cells in the explants obtained from apical (A), middle (B) and basal (C) portions of *Laminaria japonica* sporophyte. White and black columns show the cell division rate of callus-like cells after 4 and 14 days of culturing, respectively. Data show mean + s.d. (n=15).

Fig. 3. Callus-like cells in explants obtained from the basal parts of *Laminaria japonica* sporophyte. The explants were cultured in different ALA concentrations for 14 days. (A) ALA 0 mg L^{-1}, (B) ALA 10 mg L^{-1}, (C) ALA 50 mg L^{-1}, (D) ALA 100 mg L^{-1}, (E) ALA 500 mg L^{-1}, (F) ALA 750 mg L^{-1}. Scale bar: 100 μm for all.

Fig. 4. Morphological changes of callus-like cells in basal *Laminaria japonica* explants cultured in ALA 500 mg L^{-1}. (A) After one month, filamentous callus-like cells with undeveloped chloroplasts are shown. (B) After two months, filamentous callus-like cells with developing chloroplasts are shown. (C) After three months, filamentous and swelling callus-like cells with developed chloroplasts are shown. (D-F) After three months, regenerated thalli are shown. Scale bars: 100μm for all.

Fig. 5. Effects of light quality and ALA treatment on the cell division rate in explants obtained from apical (A), middle (B) and basal (C) parts of *Laminaria japonica* sporophyte. The explants were cultured with and without 500 mg L^{-1} ALA under white, blue and red light for 14 days. Data show mean + s. d. (n=50).

Table 1. Effect of external ALA concentration on callus-like cells induction in the explants obtained from apical, middle and basal portions of *Laminaria japonica* sporophyte

ALA concentration ($mg\ L^{-1}$)	Portion of thallus		
	Apical	Middle	Basal
0	4.14 ± 9.87 (25.0)	26.5 ± 41.4 (35.0)	99.2 ± 4.94 (100)
1	8.36 ± 18.8 (27.5)	36.6 ± 46.3 (37.5)	100 ± 0.00 (100)
5	7.27 ± 16.6 (25.0)	34.8 ± 45.5 (40.0)	100 ± 0.00 (100)
10	7.11 ± 14.7 (32.5)	44.4 ± 46.5 (50.0)	100 ± 0.00 (100)
50	35.7 ± 38.9 (77.5)	50.0 ± 50.6 (50.0)	100 ± 0.00 (100)
100	58.2 ± 40.7 (75.0)	94.1 ±22.4 (95.0)	100 ± 0.00 (100)
500	43.8 ± 41.4 (77.5)	89.3 ± 30.4 (85.0)	100 ± 0.00 (100)

Data are mean induction index (%) ± standard deviations (n=40). The numbers in parentheses indicate the survival rate (%) of explants cultured for a week.

Table 2. Two-way ANOVA results for the effect of aminolevulinic acid (ALA) concentration and the collection portion of explants on the callus induction in *Laminaria japonica* sporophyte.

Source of variation	df	MS	F	*P* -value
ALA concentration	6	29928.192	30.77	<0.001 *
Collection portion	2	415432.7	427.16	<0.001 *
ALA conc. x Portion	12	8211.127	9.47	<0.001 *
Error	819	866.481		
Total	839			

Asterisks indicate the statistically significant differences at the 1% level.

Table 3. Two-way ANOVA results for the effect of aminolevulinic acid concentration and the explant collection portion on the cell division rate of callus-like cells in *Laminaria japonica* sporophyte cultured for 4 and 14 days.

Source of variation	df	MS	F	*P* -value
a) Culture for 4 days				
ALA concentration	2	1.504	290.69	<0.001 **
Collection portion	5	0.245	47.28	<0.001 **
ALA conc. x Portion	10	0.027	5.13	<0.001 **
Error	72	0.005		
Total	89			
b) Culture for 14 days				
ALA concentration	2	0.297	19.65	<0.001 **
Collection portion	5	0.249	16.45	<0.001 **
ALA conc. x Portion	10	0.030	1.97	0.0490 *
Error	72	0.015		
Total	89			

Asterisks indicate the statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 4. Three-way ANOVA results for the effect of explant collection portion and light quality on cell division rate of callus-like cell of explants in *Laminaria japonica* sporophyte cultured with and without aminolevulinic acid (500mg L^{-1}).

Source of variation	df	MS	F	*P* -value
Collection portion	2	8.052	1893.4	<0.0001 *
Light quality	2	2.283	528.26	<0.0001 *
ALA treatment	1	7.608	1760.7	<0.0001 *
Portion x Light	4	0.564	130.56	<0.0001 *
Portion x ALA tr.	2	0.532	123.05	<0.0001 *
Light x ALA tr.	2	0.348	80.53	<0.0001 *
Portion x Light x ALA tr.	4	0.079	18.39	<0.0001 *
Error	894	0.009		
Total	899			

* Asterisks indicate the statistically significant differences at the 1% level.

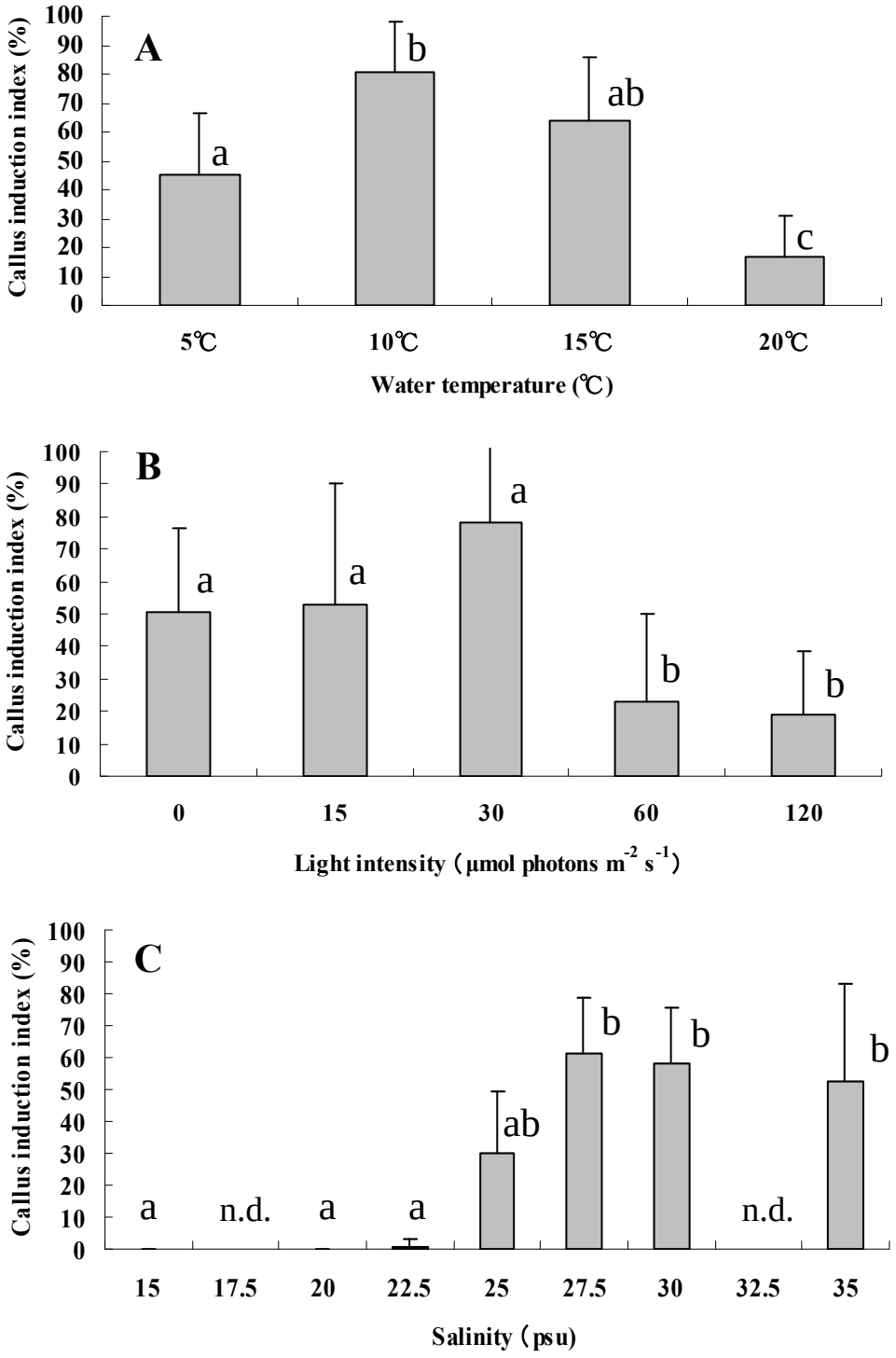
A
Callus induction index (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
ab
c
5℃
10℃
15℃
20℃
Water temperature (℃)
B
Callus induction index (%)
a
a
a
b
b
0
15
30
60
120
Light intensity (μmol photons m-2 s-1)
C
Callus induction index (%)
a
n.d.
a
a
ab
b
b
n.d.
b
15
17.5
20
22.5
25
27.5
30
32.5
35
Salinity (psu)

Fig. 1

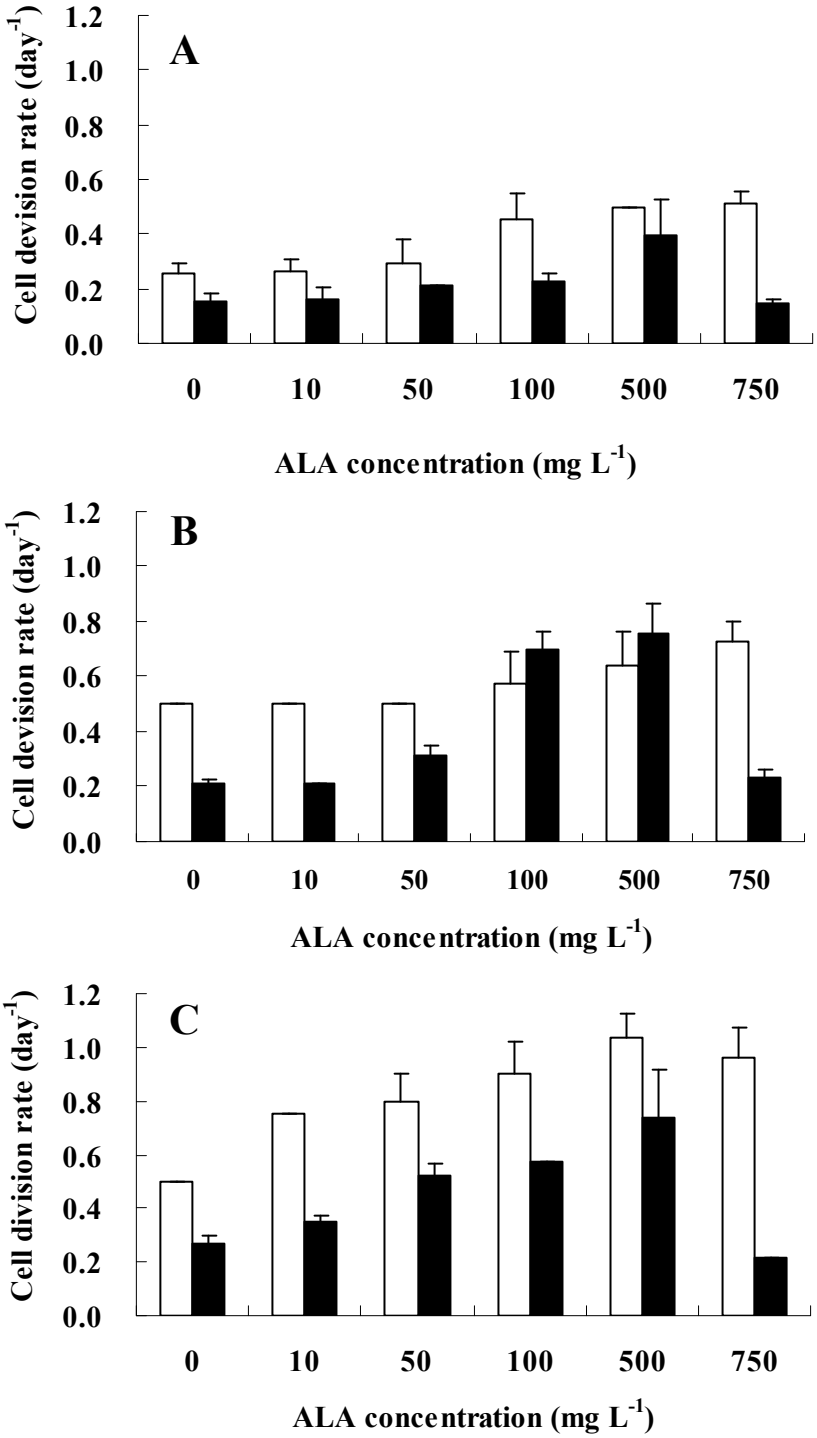
A
B
C
Cell devision rate (day-1)
Cell division rate (day-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
50
100
500
750
ALA concentration (mg L-1)

Fig. 2

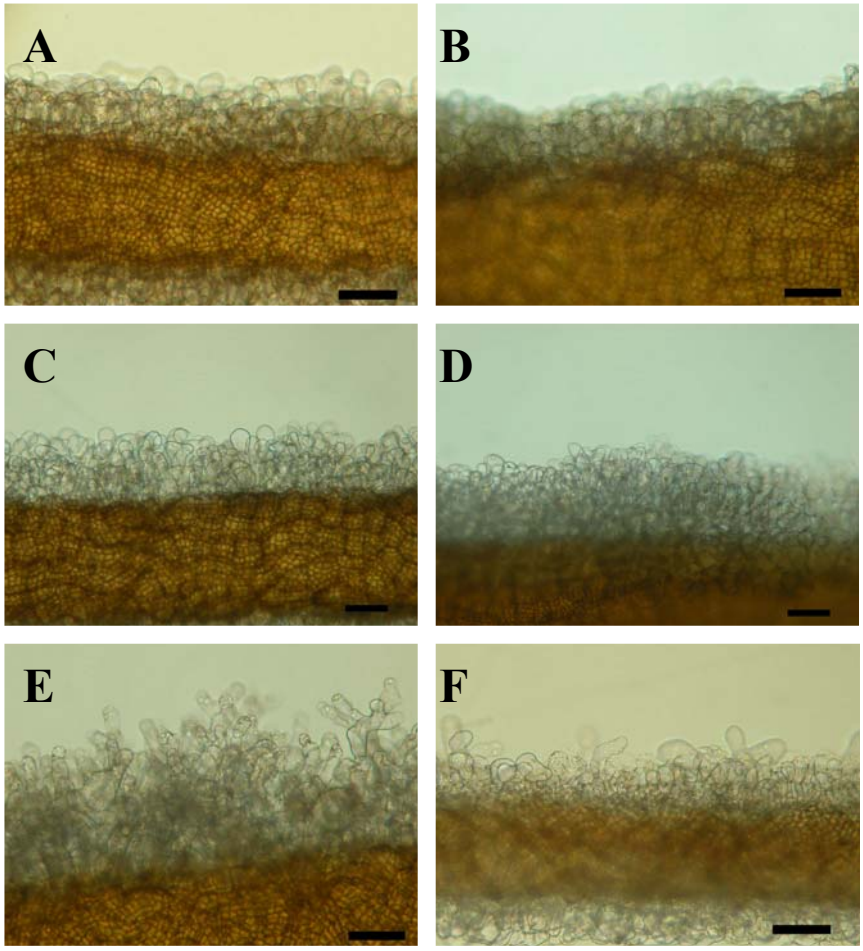

Fig. 3

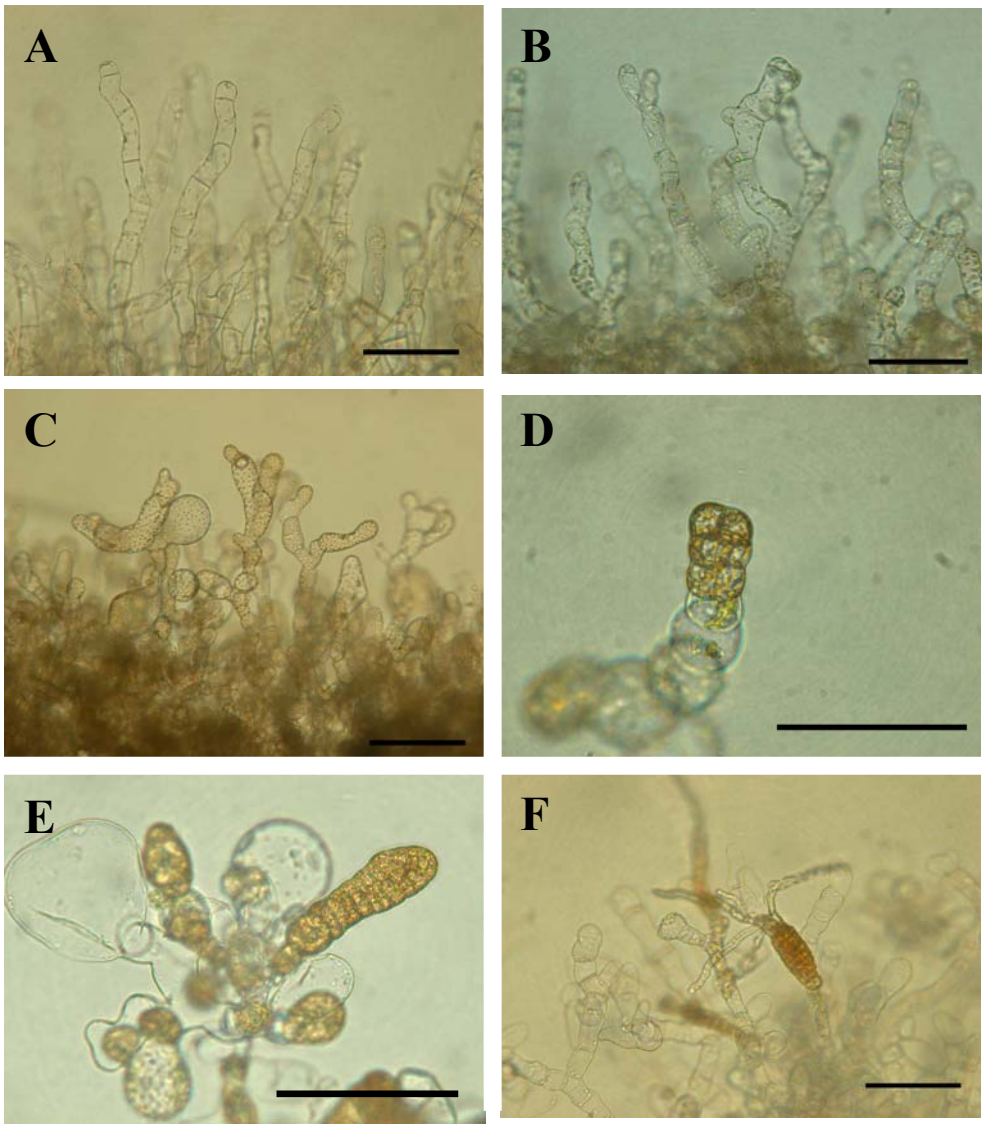

Fig. 4

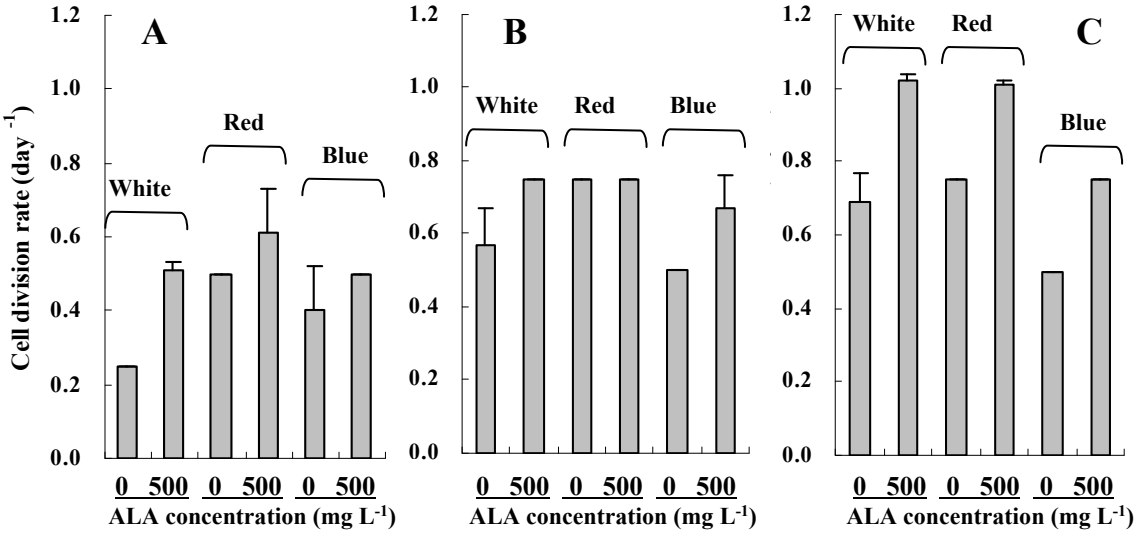
A
B
C
White
Red
Blue
Cell division rate (day -1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 500 0 500 0 500
ALA concentration (mg L-1)

Fig. 5