Photosynthetic performance of regenerated protoplasts from disintegrated cells of *Bryopsis hypnoides* (Chlorophyta)

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Abstract

The photosynthetic performances of regenerated protoplasts of *Bryopsis hypnoides*, which were incubated in seawater for 1, 6, 12, and 24 h, were studied using chlorophyll (Chl) fluorescence and oxygen measurements. Results showed that for the regenerated protoplasts, the pigment content, the ratios of photosynthetic rate to respiration rate, the maximal photosystem II (PSII) quantum yield (F_v/F_m), and the effective PSII quantum yield (Φ_{PSII}) decreased gradually along with the regeneration progress, indicated that during 24 h of regeneration there was a remarkable reduction in PSII activity of those newly formed protoplasts. We assumed that during the cultivation progress the regenerated protoplasts had different photosynthetic vigor, with only some of them able to germinate and develop into mature thalli. The above results only reflected the photosynthetic features of the regenerated protoplasts at each time point as a whole, rather than the actual photosynthetic activity of individual aggregations. Further investigation suggested a relationship between the size of regenerated protoplasts and their viability. The results showed that the middle-sized group (diameter 20–60 μm) retained the largest number of protoplasts for 24 h of growth. The changes in F_v/F_m and Φ_{PSII} of the four groups of differently sized protoplasts (*i.e.* < 20, 20–60, 60–100, and > 100 μm) revealed that the protoplasts 20–60 μm in diameter had the highest potential activity of the photosynthetic light energy absorption and conversion for several hours.

Additional key words: *Bryopsis hypnoides*; chlorophyll fluorescence; imaging-PAM; oxygen measurements; protoplasts; photosynthesis.

Introduction

B. hypnoides Lamouroux is a unicellular coenocytic green alga whose extruded cell organelles can aggregate spontaneously in seawater to form numerous new cells that can develop into mature algal thalli. Previous studies have revealed that from some unicellular organisms, such as *Acetabularia* ssp., to most of the multicellular organisms which have cell totipotency and can develop into new individuals, these processes rely on two prerequisites: (*1*) the cell membranes being intact and (*2*) the protoplasm also being intact, *i.e.* a part of the protoplasm cannot regenerate into a mature individual. However, *Bryopsis* sp., which is a multinucleate cell organism, when cut into several segments that, while only a part of one cell, can each develop into mature individuals. This distinctive regeneration phenomenon has provided an excellent system for the study of protoplast regeneration (Gibor 1965, Tatewaki and Nagata 1970, Kobayashi and Kanaizuka 1985, Menzel 1988, Pak *et al*. 1991, Kim *et al*. 2001, Ye *et al*. 2005a).

Protoplasts regeneration in *Bryopsis* involves two interesting issues. First, minutes after the protoplasm is extruded from the cell, organelles begin to aggregate from dissociated cell fractions into orderly restructured masses; this may be an important model system for the study of such processes as the interaction of various cell organelles, and formation of various hybrid cell types. Second, there is a *de novo* synthesis of cell membranes and walls, so *Bryopsis* can be used to study the formation

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Abbreviations: AOI – area of interest; Chl – chlorophyll; F_0 – intrinsic fluorescence in the dark-adapted state (excitation with measuring light only); F_m – maximum fluorescence in the dark-adapted state (measuring light plus saturation pulse); F_v/F_m – the maximal PSII quantum yield; NPQ – non-photochemical quenching; PAM – Pulse-Amplitude-Modulation; PAR – photosynthetic active radiation; P/R – ratio of photosynthetic rate to respiration rate; PSII – photosystem II; RLC – rapid light curve; Φ_{PSII} – the effective PSII quantum yield.
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process of cell membranes and walls in addition to the aggregation mechanism of organelles. In recent years much attention has focused on the morphology and mechanism of the regeneration process of protoplasts, and there has been little investigation of the physiological features of those regenerated individuals.

Photosynthesis is the most important process in autotrophic plants and the chloroplast is a target for many biotic and abiotic stresses (Biswal 1999). Our previous studies have shown that in the process of protoplasts regeneration there are related changes in chloroplasts, as well as in photosynthetic properties (Ye 2005b). So far researchers have accumulated substantial information regarding photosynthetic activity of plant protoplasts. Senger and Krupinska (1986) demonstrated changes in the molecular organization of thylakoid membranes during ontogenetic development of *Scenedesmus obliquus*. Krupinska and Humbeck (1994) studied changes in the photosynthetic apparatus during chloroplast development. Goh *et al*. (2002) found that the photosynthetic capacity had some relationship with the locations of protoplasts in cells. Using scanning electrochemical microscopy, Shiku *et al.* (2005) investigated respiration and photosynthetic activities as a function of a protoplast size of *B. plumosa*, which revealed that the respiration rate was controlled by a protoplast volume, while photosynthetic rate was controlled by protoplast surface area. Conventional measurements of photosynthesis are restricted to giving averaged information on the whole samples. Owing to the special system of aggregated protoplasts for *Bryopsis*, there is a need for techniques to enable comparison of responses between selected regions of the specimen and to detect basic physiological heterogeneities. The development of pulse-amplitude-modulated (PAM) Chl fluorometry has made such measurements possible.

Chl variable fluorescence had proved to be a useful and powerful tool for studying photosynthetic parameters in both algae (Sagert and Schubert 2000, Beer *et al.* 2000) and higher plants (Beer *et al*. 1998, Roháček and Barták 1999, Roháček *et al.* 2002, Berger *et al*. 2004, Bonfig *et al*. 2006). It has been widely used as a diagnostic approach to estimate the physiological state, especially the photosynthetic efficiency, of photosynthetic organisms (Schreiber 2004). Parameters such as F_v/F_m and Φ_{PSII} are useful tools for plant bioassays because they indicate the capacity of light-energy transfer into chemical energy of plants (Bolhàr-Nordenkampf *et al.* 1989, Genty *et al.* 1989). Rapid light curves (RLCs) provide detailed information on the saturation characteristics of electron transport, as well as the overall photosynthetic performance of plants (Ralph and Gademann 2005). It is well accepted that photochemical quenching (q_P) and non-photochemical quenching (NPQ) are useful for monitoring of photosynthetic energy conversion processes (Schreiber *et al.* 1986, Seaton and Walker 1992). Since these parameters are linked to different events of the photosystem II, we used these parameters in the present study to investigate the photosynthetic activity response of the regenerated protoplasts over time following the onset of aggregation, and explore the correlations among size, viability, and photosynthetic performance of regenerated protoplasts of *B. hypnoides*. It should be noted that in this study, the term "sub-protoplast" refers specifically to the protoplasmic mass enclosed by a gelatinous envelope according to the definition of Kim *et al*. (2001), which is distinguished from the protoplast with a lipid-based membrane. "Regeneration" in present work refers to both of them; the term "protoplast" in this text contains also "sub-protoplast" unless specified.

Materials and methods

Algal materials and protoplast culture: *B. hypnoides* were collected from the intertidal zone of Huiquan Bay, Qingdao, China. Samples were rinsed with plenty of autoclaved seawater and a soft brush was used to remove the surface microbial and epiphytic organisms. Then the algal thalli were cut into small pieces and the protoplasm was squeezed into a clear Petri dish containing artificial seawater (100 ml distilled water, 2.63 g NaCl, 0.609 g $MgCl_2$, 0.193 g $MgSO_4$, 0.074 g KCl, 0.11 g $CaCl_2$, and 0.1 g Tris [pH 8.3]; Kim *et al.* 2001), and the dish was gently rocked to disperse the extruded cell components. Afterwards the aggregated protoplasmic masses were cultured at 25°C under circular cool-white fluorescent illumination of approximately 20 μmol m^{-2} s^{-1}.

All measurements were performed using the four parallel experiment samples which were randomly sampled at 1, 6, 12, and 24 h after cultivation. The samples at time 0 used in the oxygen exchange and Chl fluorescence measurements were the intact algae.

Size distribution assays: At each time point ten fields of vision (× 200) were selected randomly and all of the protoplasts with different sizes were determined with an eyepiece micrometer. Based on their diameter, we divided these protoplasts into four groups: XL, L, M, and S (> 100, 60–100, 20–60 and < 20 μm, respectively). The size distribution rate of S group at time y (y = 1, 6, 12, or 24 h) was calculated using the equation % = (number of S group at y/ number of all the four groups at y) × 100%.

Chl concentration was measured using the spectrophotometric assay based on the method of Arnon (1949). The algal samples were lyophilized and ground to a fine powder in liquid nitrogen, and then 8 ml of 80 % acetone solution was added and then kept in darkness at 4°C for 12 h. The acquired mixture was centrifuged at 10,000 × *g*

and 4°C for 10 min, the supernatant was adjusted to 10 ml for further analyses. The extractions were then analyzed with a spectrophotometer (*UV757CRT, Shanghai Precision & Scientific Instrument Co. Ltd.*, China) using scanning absorption spectrum (350–700 nm) with 80% acetone solution as the blank. Absorbance was recorded at 663 nm (the absorption wavelengths of Chl *a*) and 645 nm (the absorption wavelengths of Chl *b*). The contents of Chl *a* (C_a) and Chl *b* (C_b) within the thalli samples were calculated according to formula $C_a = 12.7\ OD_{663} - 2.69\ OD_{645}$, $C_b = 22.9\ OD_{645} - 4.68\ OD_{663}$; while the content of total Chl (C_T) was calculated following formula $C_T = C_a + C_b = 8.02\ OD_{663} + 21.21\ OD_{645}$. Total Chl content was standardized to fresh mass. In each experiment triplicate samples were used.

Oxygen evolution and respiration measurements: The photosynthetic oxygen production was determined using a Clark-type electrode (*Hansatech Instruments Ltd.*, King´s Lynn, UK). All measurements were made at ambient temperature of 20°C controlled by a thermostatic water bath. During the experiment, the rotational speed of magnetic stirrer in the electrode chamber was set at slow to avoid damaging the aggregated protoplasts.

Oxygen evolution: The pretreatment of the samples: before oxygen evolution measurement 1 ml of measuring protoplast suspension was maintained at 20°C by a recirculating water bath (*Thermo, CoolTech320*), for 15 min. Firstly, the electrode was stabilized for several minutes with 2 ml of artificial seawater in the chamber to determine the baseline. Then 1 ml of artificial seawater was removed from the chamber and 1 ml of measuring protoplast suspension was inserted instead. Oxygen production rates were measured under predetermined saturating irradiances (500 μmol m^{-2} s^{-1}).

Respiration: Before respiration measurements 1 ml of measuring protoplast suspension was maintained at 20°C by a recirculating water bath for 15 min and then dark-adapted for 20 min; then the respiration rate was measured. Both oxygen evolution and respiration rates were replicated three times to obtain a reliable estimate of the apparent quantum efficiency. The rates of oxygen evolution and respiration were standardized to Chl biomass.

Chl fluorescence measurements: The photosynthetic parameters were determined using an *Imaging-PAM Chlorophyll Fluorometer* (*Walz*, Effeltrich, Germany). First all samples (on Petri dishes as they were cultured) were dark-adapted for 10–15 min and then several regions in fluorescence images of the samples were selected [*i.e.* AOI (area of interest)] after which fluorescence kinetics was measured. Intrinsic fluorescence (F_0) was determined after maintaining the samples in darkness for 15 min under weak measuring light (7 μmol m^{-2} s^{-1}). A saturating actinic light pulse [SP (intensity 6,000 μmol m^{-2} s^{-1}; duration 0.8 s)] was applied to obtain maximum fluorescence (F_m) of the dark-adapted samples. The maximum fluorescence yield in illuminated samples was denoted by F_m. The variable fluorescence (F_v) was obtained by the difference between F_m and F_0. F_v/F_m was noted as the maximal PSII quantum yield (Schreiber *et al.* 1986). The effective PSII quantum yield, Φ_{PSII}, was calculated according to Genty *et al.* (1989) by the formula: $(F_m' - F)/F_m'$, where F is the fluorescence yield obtained by averaging the fluorescence readings within 0.2 s, the maximum fluorescence yield (F_m') was detected when the samples were illuminated by actinic light (intensity 150 μmol m^{-2} s^{-1}) which was normally lower than F_m due to non-photochemical quenching (*i.e.* heat dissipation). The above-mentioned parameters were extracted directly from the *ImagingWin* software.

The light response of photosynthetic electron transport was assessed with the help of a preprogrammed sequence of illumination steps at increasing photosynthetically active radiation (PAR 7, 30, 50, 69, 106, 150, 199, 263, 341, 422, 501, and 597 μmol m^{-2} s^{-1}) values (light curves). The illumination time at each step was 30 s. At the end of each illumination step, a saturation pulse was applied for the assessment of fluorescence parameters, and these data were plotted to create the RLC.

Statistics: Chl fluorescence data often do not exhibit a Gaussian distribution (Lazár and Nauš 1998). Thus, we presented the curves of Chl fluorescence variables using box plots with median, minimum, maximum, 25, and 75% quantiles to indicate the distribution of the data. Values of other measurements were presented as mean ± standard deviation. Statistical analysis was performed using *Excel 2003* (*Microsoft Corporation*, USA) and *SPSS 13.0 for Windows* (*SPSS*, Chicago, IL, USA). Differences between the means were detected using a one-way analysis of variance, in conjunction with the nonparametric *Kruskal–Wallis* test.

Results

The distribution of different sizes of protoplasts: When the extruded cell organelles were transferred into artificial seawater (pH 8.3), the organelles immediately aggregated into numerous balls and fine strands of different sizes. The newly formed sub-protoplasts were mostly spherical in shape, fewer were oval and rod-like, and their diameter range was 5–200 μm. All sub-protoplasts > 100 μm disintegrated after 12 h of cultivation and then divided into several small sub-protoplasts or degenerated. The sub-protoplasts of the other three groups also dramatically decreased in number after 12 h of cultivation (Table 1). We compared the distribution

Table 1. The absolute numbers of protoplasts in each size class. Means ± SD (n = 5). S – < 20 μm; M – 20–60 μm; L – 60–100 μm; XL – > 100 μm.

Time after disintegration [h]	Number of protoplasts 1	6	12	24
Size class				
S	35.8±1.79	20.0±1.58	12.6±1.67	6.8±0.84
M	114.0±3.16	72.4±3.05	53.4±2.07	49.0±2.35
L	46.2±3.03	24.2±2.39	16.8±1.10	11.6±1.14
XL	4.2±0.84	1.2±0.84	0	0

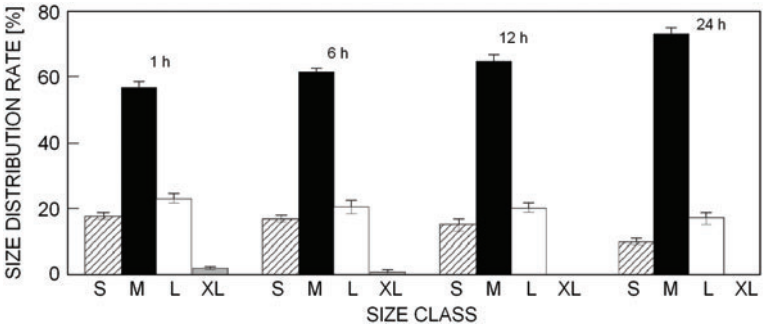

Fig. 1. The relative size distribution of protoplasts (*B. hypnoides*) determined at different time-points in each size class after disintegration. The absolute numbers of protoplasts in each size class are presented in Table 1. Means ± SD (n = 5). S – <20 μm; M – 20–60 μm; L – 60–100 μm; XL – >100 μm.

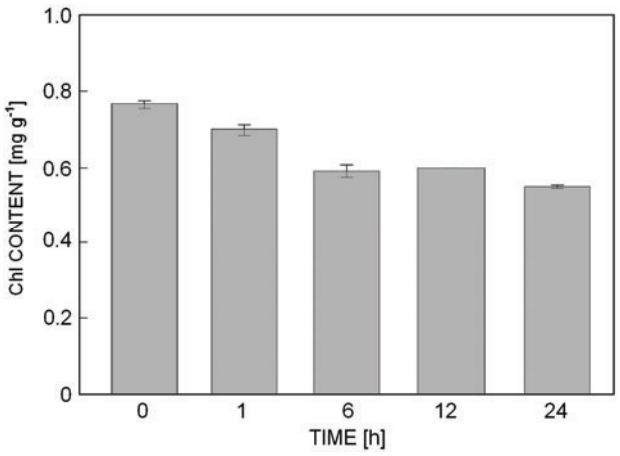

Fig. 2. The chlorophyll (Chl) content of the regenerated protoplasts at different time-points (1, 6, 12, and 24 h after onset of culture) normalised to fresh mass. Vertical bars represented the means ± SD (n = 3).

of different sizes of sub-protoplasts after 1, 6, 12, and 24 h of cultivation. The middle-sized sub-protoplasts group (diameter 20–60 μm) maintained the largest remaining number of sub-protoplasts (Fig. 1), its size distribution rate was 57% in the first hour of development, and this increased to about 72% after 24 h of cultivation (Fig. 1).

Chl concentration of regenerated protoplasts decreased gradually, reaching values significantly lower than the intact algae (time 0) until the end of the 24 h of culture (0.72 of controls, P<0.05; Fig. 2).

Rate and efficiency of photosynthetic oxygen evolution: All treatments showed regular photosynthetic

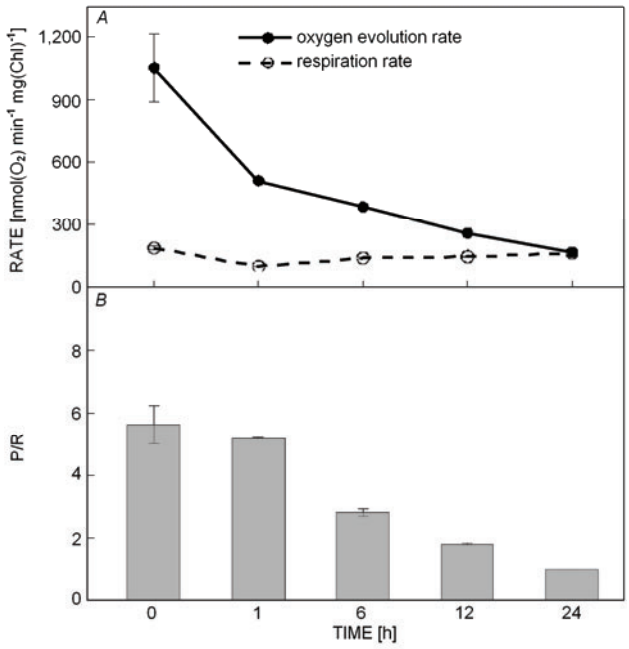

Fig. 3. The oxygen-exchange parameters of the regenerated protoplasts at different time-points (1, 6, 12, and 24 h after onset of culture). The oxygen evolution rates and respiration rates were normalised to chlorophyll basis. Vertical bars represented the means ± SD (n = 3).

and respiration curves (data not shown). Compared to intact algae (time 0), regenerated protoplasts showed a significant reduction in the oxygen evolution rate and respiration rate, as shown by the oxygen exchange parameters (Fig. 3*A*). The oxygen evolution rate dropped dramatically in the first hour after disintegration, and then

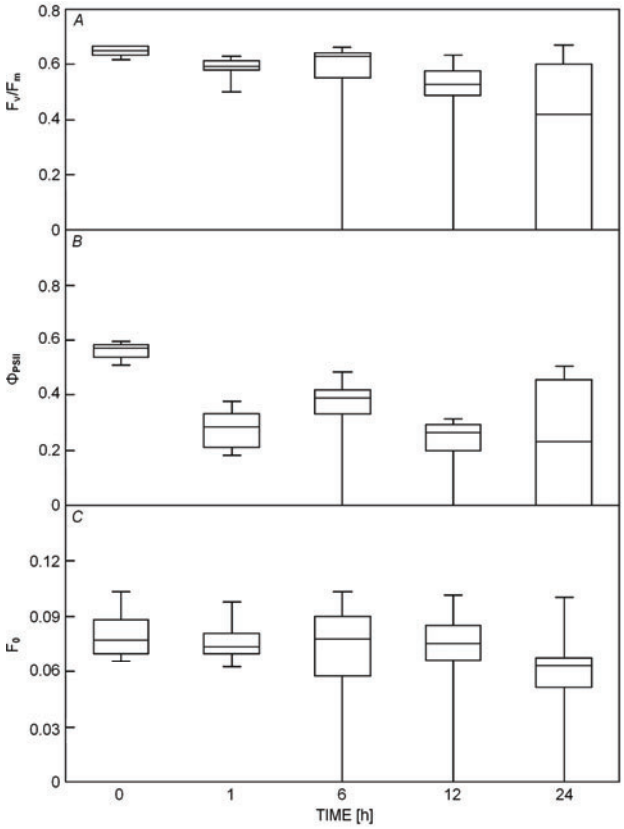

Fig. 4. The chlorophyll fluorescence parameters, F_v/F_m, Φ_{PSII}, and F_0 of the regenerated protoplasts at different time-points (1, 6, 12, and 24 h after the onset of the culture). Data from 4–8 separate experiments are shown as a box and whisker plot. The median, 25th, and 75th percentiles of measurements are represented by the *center line* and the *upper* and *lower edges* of the box. The minimum and maximum values are shown as whiskers below and above the box.

declined steadily during the first 24 h; the respiration rate showed distinct behavior in which an initial reduction occurred within 1 h after disintegration, followed by increase over the next few hours. As the ratio of photosynthetic rates to respiration rates (P/R) is a dimensionless number which proved to be useful because it normalizes biomass and size differences among specimens and allows comparisons independent of absolute levels of metabolic activity (Coles and Jokiel 1977), we suggest that in the present study P/R ratio would be a better indicator of the cell metabolism, considering the variety of the size and composition of protoplasm in the different time points. During the regeneration progress, the P/R ratio decreased slightly after 1 h of onset of culture, and then dropped significantly, with a loss of about 50% in relation to the healthy algae, and decreased observably afterwards (Fig. 3*B*).

Chl fluorescence: The F_v/F_m declined steadily from its initial value of close to 0.66, to about 0.42 after 24 h of cultivation. The F_0 value followed a similar pattern. The value of Φ_{PSII} showed initial decrease (0–1 h) and only then slight increase after 6 h of development, with a peak of 0.39, and then dropped to < 0.24 after 24 h (Fig. 4).

The time course of changes in Chl fluorescence for the different sizes of protoplasts is shown in Fig. 5. Comparative investigations on the photosynthetic parameters showed that the changes were quite complex. F_v/F_m ratio showed slight increase for all the groups in the initial phase of aggregation (6 h), but declined markedly after 12 h. Then all sub-protoplasts > 100 μm disappeared, presumably divided into several small sub-protoplasts or degenerated; also some sub-protoplasts in the L and S groups died and so no intrinsic fluorescence signals were detected from them. Afterwards, the F_v/F_m of S and L groups kept dropping, while for sub-

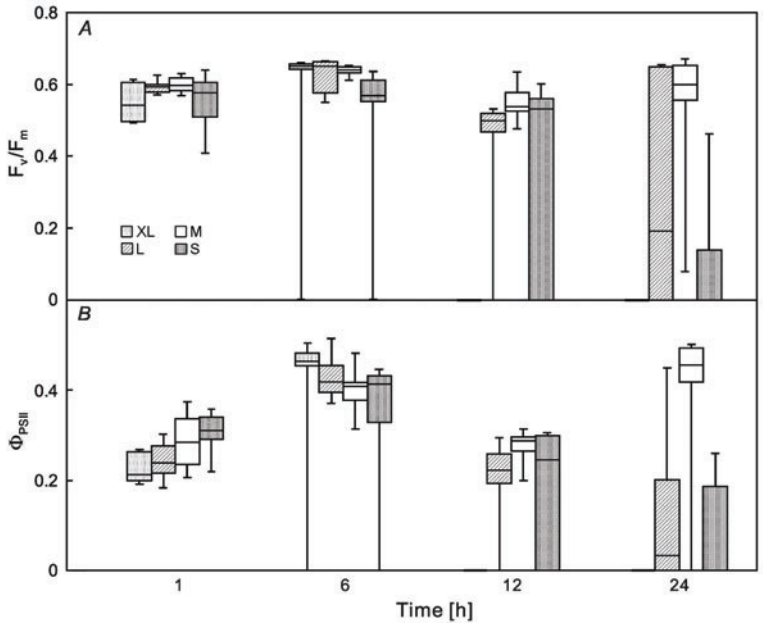

Fig. 5. The chlorophyll fluorescence parameters, F_v/F_m and Φ_{PSII}, of the different size groups of regenerated protoplasts at different time-points (1, 6, 12, and 24 h after the onset of the culture). Data from 8–12 separate experiments are shown as a box and whisker plot.

protoplasts of M group there was a slight rise after 24 h; the parameters of each group at this stage fluctuated much more dramatically. The Φ_{PSII} value followed a similar pattern of variation; remarkably higher in the middle-sized group than for the others, and with increasing time this trend became more distinct.

Discussion

When the *Bryopsis* thalli were cut and cytoplasmic contents squeezed out of the cells, the organelles immediately aggregated in seawater, forming distinct masses of irregular shape. Within 15–20 min, a gelatinous envelope composed of polysaccharides and lipids developed around the aggregates. Cell membranes and cell walls subsequently formed around the aggregates after 6 and 12 h of wounding. After 1–2 d of cultivation some of them could germinate and develop into fully differentiated *Bryopsis* (Pak *et al*. 1991, Kim *et al*. 2001, Ye *et al*. 2005a). Based on these studies on the protoplast regeneration of *Bryopsis,* we focused on the early stage of protoplasts regeneration (before protoplast germination), and chose four representative stages (1, 6, 12, and 24 h after onset of cultivation) to carry out our investigations.

From the optical microscopy image observation (data not shown), the color of the aggregated protoplasmic masses seemed unchanged during cultivation. Chl is the dominant photosynthetic pigment and is closely associated with photosynthetic performance of plants, so we measured the pigments content. The absorption spectra of extracted pigments (data not shown) showed no significant difference between the pigment compositions of the newly formed protoplasts through the 24 h of cultivation; however, the pigment content of the protoplasts showed a permanent downward trend (Fig. 2), which we assumed was due to the partial decomposition of the pigments with time. This assumption was confirmed by the F_0 parameter, measured by the imaging-PAM (Fig. 4*C*), which showed a similar variation pattern.

The P/R ratio is an appropriate indicator of the physiological state of the cells, which was 4:1 in the normal algae (Melis and Melnicki 2006). The healthy intact algae had a high photosynthetic capacity, giving a relative high P/R of around 5.6 (Fig. 3*B*). By the first hour after disintegration, the P/R decreased to around 5.2, and after that the P/R value declined steadily with time, and by 24 h of culture the P/R was only around 1.0. This suggested that the photosynthetic activity of newly formed protoplasts had significantly degenerated compared to fresh thalli, which was further confirmed by the photosynthetic parameters (Fig. 4). The F_v/F_m of protoplasts after 24 h of culture decreased to about 53% of that in fresh algae, and Φ_{PSII} was no more than 44% of that of wild algae. All these indicated that after 24 h of cultivation there was a remarkable reduction in PSII activity of the newly formed protoplasts.

Previous studies have proposed that the diameter of newly formed sub-protoplasts range is 10–100 μm that only some of them can regenerate into mature organisms, and that the survival rate of sub-protoplast was about 40% after 12 h of development (Kim *et al*. 2001). Under optimal conditions about 15% of the developing protoplasts were able to regenerate into mature algal thalli (Ye *et al*. 2005a). Our size-distribution assays presented here showed a relationship between size of regenerated sub-protoplasts and their viability. No sub-protoplasts > 100 μm in size survived to 12 h; during cultivation for 24 h, the middle-sized group was always the dominant group (Fig. 1), consistent with results of Kim *et al*. (2001). Both the oxygen evolution measurement and Chl concentration determination reflected the features of the sub-protoplasts at each time point as a whole. However, this may not reflect the actual photosynthetic activity of each aggregation, as the newly formed sub-protoplasts grow asynchronously and many protoplasts died with increased time of culture. While, with *Imaging-PAM* the measurements can be made on single aggregation and even on a specific location. The technique makes it possible to evaluate the Chl fluorescence distribution across the protoplasmic masses and obtain information from certain protoplasts.

Image analysis of F_v/F_m showed a different response in the differently sized protoplasts (Fig. 5*A*). Protoplasts in the middle-size group had the highest value of F_v/F_m throughout the 24 h of cultivation, indicating that the photosynthetic activity of PSII was the highest. With time, F_v/F_m showed a slight increase in the first 6 h and then tended to decrease at different rate; the greatest decrease was in the XL group, where all sub-protoplasts disappeared by 6 h. Φ_{PSII} is related to the quantum yield of noncyclic electron transport at any given light intensity, and is widely used to estimate the operating quantum efficiency of PSII electron transport. The changes in Φ_{PSII} (Fig. 5*B*) also revealed that the M group sub-protoplasts had the highest potential activity of the photosynthetic light energy absorption and conversion after cultivation for several hours in the laboratory, consistent with the survival rate assays determined by the optical microscopy. F_v/F_m and Φ_{PSII} are both forms of quantum yield. F_v/F_m indicates photochemical efficiency of PSII and residual photoinhibitory stress, whereas Φ_{PSII} measures photosynthetic activity. F_v/F_m is determined with a maximally oxidized photosystem (dark-adapted with open PSII reaction centers), while Φ_{PSII} is measured under actinic light. Φ_{PSII} should be lower than F_v/F_m, due to the closure of reaction centers (decreased photochemical quenching) and stimulated heat dissipation (increased non-photochemical quenching) during actinic illumination (Schreiber 2004). The ratio F_v/F_m is a good indicator of the photosystems' state (Maxwell and Johnson 2000) but Φ_{PSII} provided an indication of the

amount of energy used in photochemistry (photosynthesis). It is noteworthy that the F_v/F_m ratio remained relatively constant for all the size groups in the initial phase of aggregation (6 h), however Φ_{PSII} exhibited a slight increase during the same period (Fig. 5*B*); thus, we suppose that during the early stage of protoplasts regeneration, the disorganized cellular material needed to restructure, or to restore its original function, especially there was a *de novo* synthesis of cell membranes and walls (Kim *et al*. 2001, Ye *et al*. 2005a), this biological process required a large number of carbohydrates, so this energy requirement may lead to the increased activity of PSII.

In conclusion, compared to conventional methods for studying the photosynthetic performance of plants, the fluorescence imaging technique provides more detailed information on localized changes in the photosynthetic process and permits detection of the early stages of many different types of development processes. Consequently, it was possible to identify important physiological conditions of the newly formed protoplasts long before they showed the typical growth characters.

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