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Spiral breakage and photoinhibition of *Arthrospira platensis* (Cyanophyta) caused by accumulation of reactive oxygen species under solar radiation

Zengling Ma[a,b], Kunshan Gao[a,*]

[a] *State Key Laboratory of Marine Environmental Science, Xiamen University, Daxue Road, 361005 Xiamen, China*
[b] *Key and Open Laboratory of Marine and Estuary Fisheries (Ministry of Agriculture), East China Sea Fisheries Research Institute, Chinese Academy of Fisheries Science, 200090 Shanghai, China*

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ABSTRACT

Previous studies showed that exposure of *Arthrospira* spp. spirals to natural levels of solar radiation in the presence of UV radiation (UVR, 280–400 nm) led to the breakage of its spiral structure. However, the underlying mechanisms have not yet been explored. Here, we showed that associated accumulation of reactive oxygen species (ROS) resulted in the spiral breakage by oxidizing the lipids of sheath or cell membrane in *Arthospira platensis*, and presence of UVR brought about higher accumulation level of the ROS. Activities of superoxide dismutase (SOD) and catalase (CAT) were inhibited by high levels of solar PAR, addition of UVR led to further inhibition of CAT activity. High levels of ROS also decreased the content of photosynthetic pigments, damaged photosystem II (PSII) and inhibited the photosynthesis and growth. It is concluded that both UV and high PAR levels could generate higher amounts of ROS, which decreased the photosynthetic performances and led to spiral breakage of *A. platensis*.

1. Introduction

Solar radiation drives photosynthesis, but also harms photosynthetic machinery (Aro et al., 1993). The UV radiation (UVR, 280–400 nm), especially UV-B (280–315 nm), is known to damage DNA and protein molecules (Niyogi, 1999) and therefore to affect physiology of cyanobacteria and algae (Häder et al., 2007). Light can also generate oxidative stress by producing reactive oxygen species (ROS) as inevitable byproducts of photosynthesis: reduction of oxygen on the acceptor side of photosystem I (PSI), as a result of the photosynthetic transport of electrons, leading to the formation of the superoxide peroxide (O_2^-), which can be further converted to hydrogen peroxide (H_2O_2) and hydroxyl radical ($^\bullet OH$) (Asada, 1999). Transfer of excitation energy from excited chlorophylls to oxygen in the light-harvesting complexes can lead to the formation of singlet-state oxygen ($^1O_2$) (Zolla and Rinalducci, 2002). Under less stressful conditions, these ROS can be reduced to tolerable levels by intracellular ROS-scavenging enzymes, such as superoxide dismutase (SOD) and ascorbate peroxidase, as well as antioxidants, such as β-carotene and α-tocopherol (Asada, 1999; Havaux et al., 2005). However, under stressful levels of photosynthetic active radiation (PAR), production of ROS is accelerated, giving rise to oxidative stress (Asada, 1999). Exposure to ROS, such as $^1O_2$, O_2^-, H_2O_2 and $^\bullet OH$, results in specific cleavage of the D1 protein, an essential component of PSII (Chen et al., 1992; Miyao et al., 1995; Okada et al., 1996). Recent studies showed that ROS preferentially inhibits the repair of PSII rather than damaging it (Nishiyama et al., 2005).

Arthrospira platensis, an economically important cyanobacterium, has been commercially produced for more than 30 years under solar radiation in artificial ponds (Belay, 1997). Its photosynthetic performance is low at noontime during such production, especially when the temperature deviates from the optimal range (Torzillo et al., 1998). The O_2 concentration in the commercial ponds may exceed five times the saturation level, leading to inhibited photosynthesis and growth (Vonshak, 1997). The accumulation of O_2 in the cultures of microalgae may enhance the production of ROS, which damages lipids, proteins and DNA by oxidation (Halliwell and Gutteridge, 1999; Nishiyama et al., 2005). Exposing indoor cultures of *Arthrospira* spp. to high oxygen concentrations leads to lower growth rate and bleached pigments (Marquez et al., 1995; Singh et al., 1995).

UVR is known to affect photosynthetic performance (Wu et al., 2005; Ma and Gao, 2009a), bleach photosynthetic pigments (Gao and Ma, 2008) and damage DNA (Gao et al., 2008) of *A. platensis*. Presence of UVR led to broken and compressed spirals of *A. platensis* (Wu et al., 2005). We showed previously that a 52.0 kDa periplasmic proteins was responsible for the compression of the spiral (Ma and Gao, 2009b). The trichomes of *Arthrospira* were surrounded by a thin, diffluent sheath similar to Gram-negative bacteria cell

* Corresponding author. Tel.: +86 592 2187963; fax: +86 592 2187963.
E-mail address: ksgao@xmu.edu.cn (K. Gao).

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wall, playing a role in maintaining the integrity of the filaments (Tomaselli, 1997). It is known that the main components of the sheath are peptidoglycans and lipids (Glauner et al., 1988; Sugai et al., 1990; Tavares and Sellstedt, 2000). On the other hand, ROS are harmful to many cellular components including lipids, proteins and nucleic acids (Oda et al., 1989; Miyao et al., 1995). We hypothesize that accumulation of ROS may lead to spiral breakage of the cyanobacterium. UV-B can induce formation of ROS in the cyanobacterium *Anabaena* sp. (He and Häder, 2002a). However, whether the accumulation of ROS in *Arthrospira* spp. contribute to the spiral disintegration remains to be investigated. Here, we report our results on the accumulation of ROS under UVR and PAR and its impacts on the morphology and physiology of *A. platensis*.

2. Materials and methods

2.1. Experimental organism

A. platensis (D-0083) was obtained from Hainan DIC microalgae CO. LTD., Hainan, China. A single healthy spiral was chosen and all the trichomes were propagated from it and cultured in Zarrouk's medium (Zarrouk, 1966). The cultures were aerated with filtered (0.22 μm) air at 30 °C and 60 μmol m^{-2} s^{-1} of cool-white light (12L:12D). Cells grown in the exponential growth phase were used in the following sub-cultures or experiments.

2.2. Measurement of solar radiation

Solar irradiances were measured using a broadband ELDONET filter radiometer (Real Time Computer, Möhrendorf, Germany) that has 3 channels for photosynthetically active radiation (PAR, 400–700 nm), ultraviolet-A radiation (UV-A, 315–400 nm) and ultraviolet-B radiation (UV-B, 280–315 nm), respectively (Häder et al., 1999). This instrument has been calibrated with the help of the maker regularly.

2.3. Radiation treatments and determination of antioxidant enzyme activity

To investigate the changes in activity of superoxide dismutase (SOD) or catalase (CAT) in the cells exposed to intensive PAR or full solar radiation, the trichomes were collected on GF/F filters and diluted with fresh Zarrouk medium to a biomass density of 0.33 g l^{-1} before transferred to quartz tubes (Ø 4.9 cm, 16 cm long). The re-suspended cells, while aerated with ambient air at 0.4 l min^{-1}, were then irradiated under PAR (P treatment, 260.86 W m^{-2}, about 1200 μmol m^{-2} s^{-1}) or PAR + UVR (PAB treatment, PAR of 260.86 W m^{-2}, UVA of 60.72 W m^{-2}, UV-B of 1.95 W m^{-2}) under a solar simulator (Sol 1200W, Dr. Hönle, Martinsried, Germany) for 4 h. For the P treatment, the tubes were wrapped with 395 cut-off foil (UV Opak, Digefra, Munich, Germany); for the PAB treatment, they were covered with 295 nm cut-off filter (Ultraphan, Digefra, Munich, Germany) to screen off UV-C. Transmission of the cut-off foils (about 90%) have been published elsewhere (Zheng and Gao, 2009); these foils reflected 4% of PAR under water (Gao et al., 2007). The tubes were placed a water bath in which the water temperature was controlled at 25 °C by a circulating cryostat (Eyela, CAP-3000, Tokyorikakikai Co. Ltd., Tokyo, Japan) set.

The harvested cells were sonicated in 10 ml ice-cold phosphate buffer, a small aliquot (1 ml) was used for total protein determination (Bradford, 1976) and the resulting supernatant was used for the assay of the antioxidant enzymes.

Total SOD activity was assayed by monitoring the inhibition of reduction of nitro blue tetrazolium (NBT) according to Giannopolitis and Ries (1977). 6 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT (nitroblue tetrazolium), 2 μM riboflavin, 0.1 mM EDTA and 1 ml of the enzyme extract. The reaction mixture was illuminated for 20 min with 80 μmol m^{-2} s^{-1} of PAR. Two blanks were prepared with the reaction mixture without the lysate, one was kept in light and the other in darkness. The former was taken as reference for calculating absorbance for one unit SOD and the latter was used as blank. One unit of the enzyme activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of nitro blue tetrazolium reduction measured at 560 nm.

The activity of CAT was determined according to Cohen et al. (1996). Reagents and buffer for catalase assay were held at 0 °C in an ice-water bath, except for the 0.6N H_2SO_4 which is held at ambient temperature. The reaction kinetics was conducted at 0 °C with 6 mM H_2O_2. The reaction mixture (5 ml) contained 1 mM phosphate buffer (pH 7.0), 0.02N sulfuric acid 0.08 mM ferrous sulfate, 48 mM H_2O_2, 0.15 mM potassium thiocyanate (KSCN) and 0.5 ml enzyme extract. The samples were covered with aluminum foil to shield them from light and their absorbances at 492 nm (A_1) were measured after 20 min incubation. For the control, the enzyme extract was replaced with 0.5 ml phosphate buffer and its absorbance (A_0) was determined at same reaction time. The enzyme activity was determined as follows: U/mg protein = $(A_0 - A_1)$/protein.

2.4. Determination of ROS (H_2O_2)

To measure the diurnal variation of the ROS level under solar radiation, the cells at a concentration of 0.33 g l^{-1} got from linear of absorbance and dry weight were exposed to PAR or PAR + UVR (full solar spectrum) on a sunny day (21st June, 2007). The content of H_2O_2 was determined according to Rosenkranz et al. (1992) using a membrane-permeable non-fluorescent, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA can be deacetylated by esterase in the membrane and the hydrolyzed form, 2′,7′-dichlorodihydrofluorescein (DCFH), is very sensitive to oxidant species and can be oxidized to a highly fluorescent one 2′,7′-dichloroflurescein (DCF) *in vivo*. An aliquot of 20 μl DCFH-DA solution (5 mg ml^{-1}) was added in 4 ml algal suspension after the radiation treatments. The emission fluorescence at 538 nm was measured with a spectrofluorimeter (RF-5310PC, Shimadzu, Kyoto, Japan) with the excitation at 485 nm after 2 min dark incubation. The concentration of ROS was derived on the basis of the relationship of the fluorescence levels with the standard H_2O_2 concentrations determined in the same way.

2.5. Measurement of chlorophyll fluorescence

In order to investigate whether phycobilisome (PBS) and chlorophyll–protein complexes was damaged by ROS, we examined the changes in room-temperature Chl fluorescence in the cells treated with H_2O_2 at levels of 0.01, 0.1, 1 and 5 mmol g^{-1}-cells of dry mass. The Chl fluorescence emission spectra were measured with the spectrofluorimeter as mentioned above. The excitation wavelength was set at 436 for Chl *a* or 580 nm for PBS (Wen et al., 2005).

2.6. Determination of photosynthetic activity

To examine the effects of H_2O_2 on the photosynthetic capacity, the effective quantum yield (F'_v/F'_m) and relative electron transport rate (rETR) were determined with a portable pulse amplitude modulated fluorometer (WATER-ED, Walz, Effeltrich, Germany). The actinic light was 80 with the measuring light of 0.3 and the saturating pulse of 5600 μmol m^{-2} s^{-1}.

Parameters of the rETR vs. *E* curves were analyzed according to Eilers and Peeters (1988) as follows: $rETR = E/(aE^2 + bE + C)$,

where E is the irradiance ($\mu mol\ m^{-2}\ s^{-1}$), a, b and c are the adjustment parameters. The initial slope (i.e. α), the maximum rETR ($rETR_{max}$) and the light saturation parameters (E_k) are expressed as a function of the parameters a, b and c: $E_k = (c/a)^{1/2}$, $\alpha = 1/c$, $rETR_{max} = 1/[b + 2(ac)^{1/2}]$.

2.7. Determination of biomass and pigments

Biomass densities of the cultures were measured by filtering 30 ml culture on a pre-dried Whatman GF/F glass fiber filter (diameter 25 mm), drying in an oven at 80 °C for 24 h, and cooling off in a desiccator, weighting on an electronic balance and subtracting the known weight of the dried filter. While the samples were filtered, residual salts were removed using acidified ($4 < pH < 5$) distilled water.

To measure the contents of chlorophyll *a* (Chl *a*) and carotenoids (Car), the cells filtered on the Whatman GF/F glass fiber filter (Ø 25 mm) were extracted with absolute methanol overnight in the dark at 4 °C. The absorbance spectrum of the centrifuged ($5000 \times g$ for 5 min) supernatant was measured with a spectrophotometer (Shimadzu, UV 2501-PC). The content of Chl *a* was estimated according to Porra (2002) and the concentration of Car was determined according to Parsons and Strickland (1963).

To extract phycocyanin (PC) and allophycocyanin (APC), the harvested cells were re-suspended into sodium phosphate buffer of pH 6.7 containing 0.2 mM sodium chloride, sonicated with ultrasonic homogenizer (CPX600, Cole-Parmer, USA) while cooled with icy water. Contents of PC and APC were obtained on the basis of the absorbances of the supernatant at 615 and 652 nm (Bennet and Bogorad, 1973).

2.8. Determination of malondialdehyde (MDA)

To investigate the oxidation of ROS, malondialdehyde (MDA) content was estimated based on the modified procedure described by Heath and Packer (1968). The harvested *Arthrospira* cells were homogenized in 10% trichloroacetic acid (TCA) and then centrifuged at $5000 \times g$ for 10 min. 2 ml supernatant was mixed with 2 ml 0.6% thiobarbituric acid (TBA), heated in boiling water for 15 min, and then re-centrifuged after the cooling. The absorbance of the supernatant was measured with a spectrophotometer (Shimadzu, UV 2501-PC), and then the concentration (C) of TBA was determined as follows: $C(\mu M) = 6.45 \times A_1 - 0.56 \times A_2$, where A_1 and A_2 denote the absorbance at 532 and 450 nm, respectively.

2.9. Morphological examination

The morphological change of *A. platenssis* D-0083 was examined with a microscope (Zeiss Axioplan 2; Carl Zeiss, Germany). Digital images were recorded with a Zeiss Axicam HRC color camera (Carl Zeiss, Jena, Germany) and analyzed with a Vision Analysis system (Axio Vision 3.0).

2.10. Statistical analysis

Data were analyzed by One-Way ANOVA followed by a multiple comparison using Tukey-test. A confidence level of 95% was used in all analyses.

3. Results

The activity of SOD decreased significantly ($p < 0.05$) after the cells were shifted from indoor low PAR ($60\ \mu mol\ m^{-2}\ s^{-1}$) to an intensive level of PAR ($1200\ \mu mol\ m^{-2}\ s^{-1}$) in the absence or presence of UVR ($62.67\ W\ m^{-2}$) and had been cultured for 4 h under the solar simulator (Fig. 1A). Presence of UVR decreased the activity of SOD by 3.4%, which was insignificant ($p > 0.05$). In contrast, the activity of CAT decreased significantly ($p < 0.01$) under the high PAR, and was further reduced with the addition of UVR ($p < 0.01$) (Fig. 1B). The activity of CAT decreased by 35% and 57% under the PAR and PAR + UVR treatments, respectively.

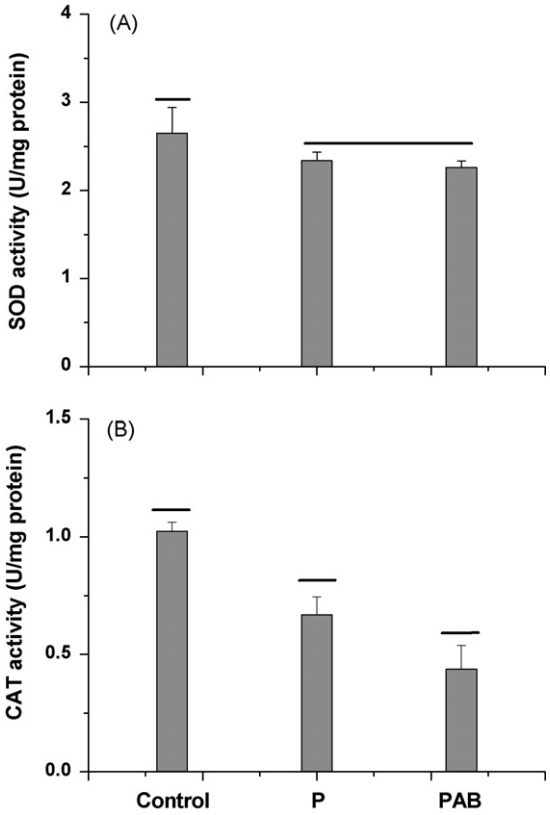

Fig. 1. Changes in SOD and CAT activity of *A. platensis* D-0083 cells when irradiated with PAR (P, $260.87\ W\ m^{-2}$) and PAR + UV-A + B (PAB, PAR + UV-A + UV-B = 260.78 + 60.72 + 1.95 $W\ m^{-2}$) from a solar simulator at 25 °C. The means and standard errors were based on triplicate incubations. Horizontal bars at different levels above the columns indicate significant ($p < 0.05$) differences among the treatments.

When the cells were exposed to incident solar radiation, the content of H_2O_2 increased with increased solar radiation and reached a peak at 16:00 (Fig. 2A). Presence of UVR significantly ($p < 0.05$) increased the content of H_2O_2 during the day. At 16:00, the H_2O_2 content increased by 61% and 120% under the PAR and PAR + UVR treatments, respectively.

To examine the effects of ROS on photosynthesis, the spirals of *A. platensis* were exposed to different levels of H_2O_2. Adding of H_2O_2 to the levels more than 1 $mmol\ g^{-1}$-cell DW had significant effect on the PSII photochemical efficiency (Table 1). Furthermore,

Table 1
Photosynthetic parameters derived from the rETR-I curves (Fig. 3) for *A. platensis* D-0083 cells treated with different H_2O_2 levels ($mmol\ g^{-1}$-cell DW) for 2 h at 25 °C. $rETR_{max}$, the maximum relative electron transfer rate; α, the relative electron transfer efficiency; I_k, light saturation point ($\mu mol\ m^{-2}\ s^{-1}$); F_v/F_m, optimal quantum yield. The means and standard errors were based on triplicate incubations.

ROS	$rETR_{max}$	α	I_k	F_v/F_m
0	197.95 ± 4.25^a	0.211 ± 0.009^a	2070 ± 59^a	0.504 ± 0.017^a
0.1	197.10 ± 1.92^a	0.201 ± 0.004^b	2260 ± 167^a	0.501 ± 0.027^a
1	106.07 ± 15.52^b	0.175 ± 0.014^c	869 ± 49^b	0.470 ± 0.017^b
5	20.18 ± 1.16^c	0.094 ± 0.012^d	415 ± 23^c	0.406 ± 0.011^c

The different superscript letters in the same column indicate significant ($p < 0.05$) differences among the treatments.

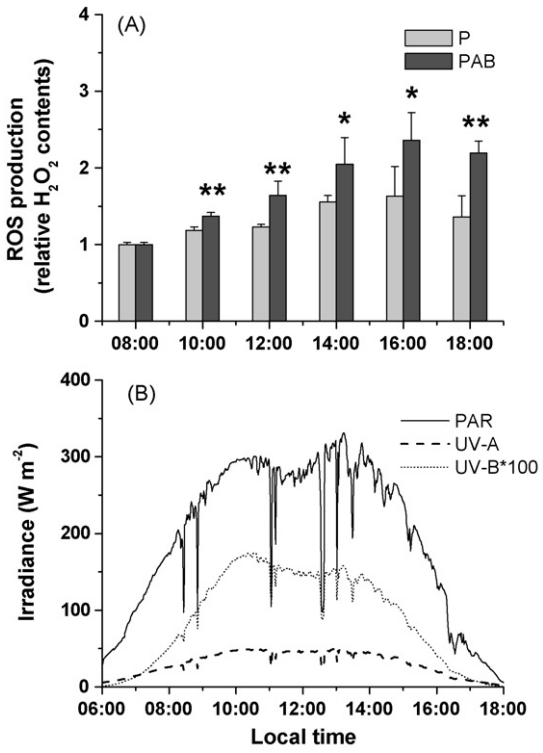

Fig. 2. Diurnal variation of relative abundance of the ROS (H_2O_2) in *A. platensis* D-0083 cells (A) irradiated with solar PAR and PAR in addition to UVR on June 21st, 2007 (B) at 25 °C. The initial content of H_2O_2 estimated from fluorescence intensity was set as 1 and those at other time were normalized to the initial value. The means and standard errors were based on triplicate incubations. The symbols "*" and "**" indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively.

the relative electron transport rate (rETR) was significantly reduced at the levels of H_2O_2 equal to or higher than 1 mmol g^{-1}-cell DW (Fig. 3). The maximal quantum yield (F_v/F_m) decreased by 20% in 2 h when the added H_2O_2 reached 5 mmol g^{-1}-cell DW (Table 1). The $rETR_{max}$, relative electron transfer efficiency (α) and light saturation point (I_k) also decreased with increased H_2O_2 concentrations (Table 1).

When the cells were treated at 5 mmol H_2O_2 g^{-1}-cell DW for different durations, the biomass mass density and contents of pigments all decreased in 2–4 h (Table 2). In 6 h, the biomass density decreased by 68.8%, and the contents of APC, PC, Chl *a* and Car

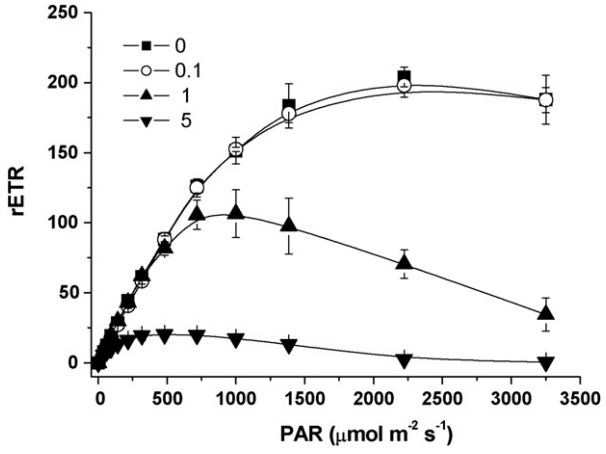

Fig. 3. Variation in relative electron transfer rate (rETR) of *A. platensis* D-0083 cells treated with different H_2O_2 levels (mmol g^{-1}-cell DW) for 2 h at 25 °C. The means and standard errors were based on triplicate incubations.

Table 2
Changes in cell density and pigments contents of *A. platensis* D-0083 treated with H_2O_2 (5 mmol H_2O_2 g^{-1}-cell DW) for 2, 4 and 6 h at 25 °C. The means and standard errors were based on triplicate incubations.

Duration (h)	0	2	4	6
DW	0.32 ± 0.04^a	0.31 ± 0.04^a	0.28 ± 0.03^b	0.10 ± 0.17^c
Chl*a*	11.95 ± 0.53^a	10.49 ± 0.29^b	10.48 ± 0.61^b	1.57 ± 0.11^c
PC	29.81 ± 1.96^a	29.16 ± 1.03^a	23.85 ± 2.10^b	0.32 ± 0.15^c
APC	103.38 ± 4.82^a	95.03 ± 1.06^b	80.23 ± 10.29^c	0.64 ± 0.58^d
Car	3.08 ± 0.09^a	2.78 ± 0.08^b	2.74 ± 0.12^b	0.34 ± 0.21^c

declined by 99.4%, 89.9%, 86.7% and 90.4%, respectively. The accessory pigments (APC and PC) were more bleached by the ROS than Chl *a* and Car.

When the Chl *a* and PC were respectively excited at 436 and 580 nm, influence of the ROS on the pigments was also evidenced. The emitted fluorescence of Chl *a* increased by 192.4% in 4 h after being exposed to H_2O_2 at 5 mmol g^{-1}-cell DW (Fig. 4A). The emitted fluorescence of PC also increased significantly and the emission peak shifted by 11 nm to shorter wavelength (from 649 to 638 nm) in 4 h (Fig. 4B), reflecting a damage and structure change in the phycobilisome.

Addition of H_2O_2 also affected the spiral structure of *A. platensis* D-0083 (Fig. 5). In 220 min, morphology of the spirals was altered, and 20 min later they became disintegrated. The color of the cultures changed from green to yellowish in 360 min when only single cells or protoplasts existed. On the other hand, the content of malondialdehyde (MDA) increased with time by 37%, 116% and 425% times in 3, 4 and 6 h, respectively (Fig. 6).

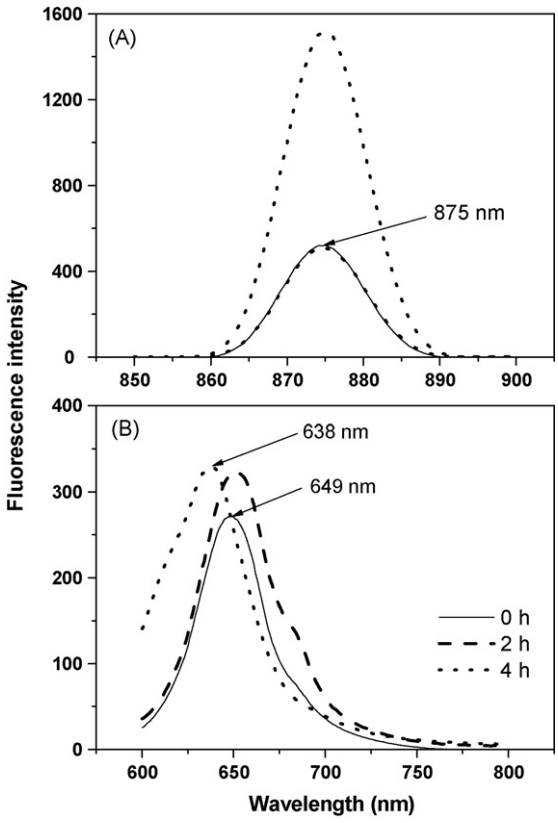

Fig. 4. Changes in fluorescence emission of Chl *a* (A) and phycocyanin (B) in *A. platensis* D-0083 cells treated with H_2O_2 (5 mmol g^{-1}-cell DW) for 2 and 4 h, respectively. The excitation wavelength for Chl *a* and phycocyanin was 436 and 580 nm, respectively. The means were based on triplicate incubations.

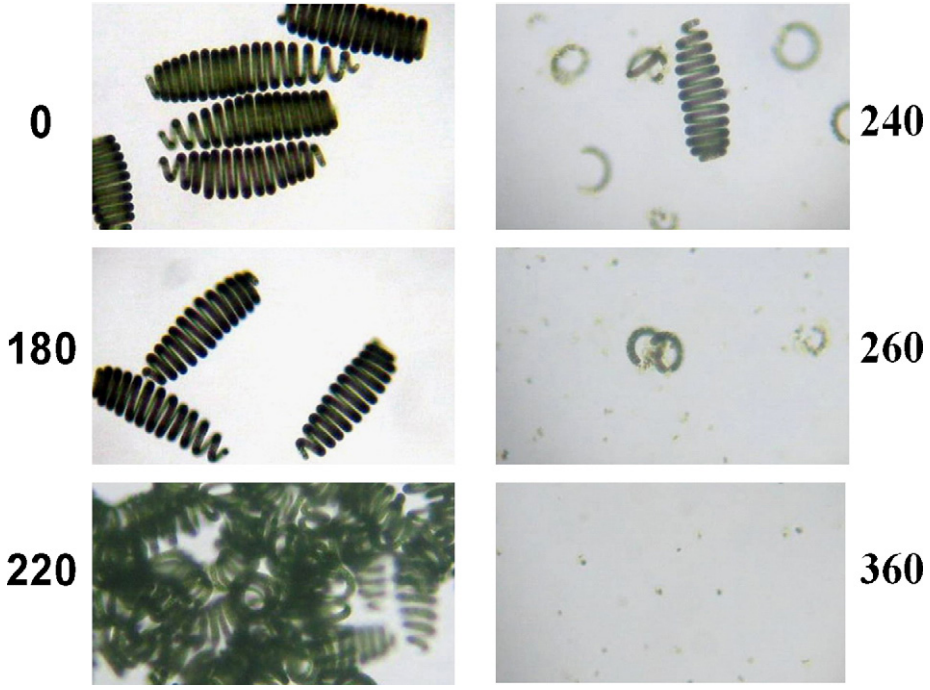

Fig. 5. Morphological change of *A. platensis* D-0083 treated with H_2O_2 at 5 mmol g^{-1}-cell DW with increased time (min).

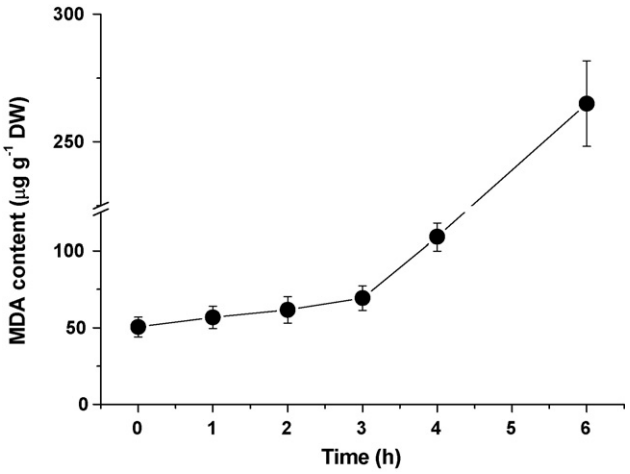

Fig. 6. Change in total MDA of *A. platensis* D-0083 cells treated with H_2O_2 at 5 mmol g^{-1}-cell DW. The means and standard errors were based on triplicate incubations.

4. Discussion

The accumulation of ROS is known to be enhanced under high levels of PAR as well as UVR (He and Häder, 2002a). Free radical scavengers and antioxidants can reduce generation of ROS and membrane lipid peroxidation (Asada, 1999; He and Häder, 2002b; Havaux et al., 2005). In the present study, the activities of scavenging enzymes, SOD and CAT, were suppressed under high levels of PAR and UVR (Fig. 1A and B), while the accumulation of ROS increased (Fig. 2). ROS are known to impair cells (Chen et al., 1992; Miyao et al., 1995) and to inhibit the repair process of damaged components (Nishiyama et al., 2005). In *A. platensis*, presence of UVR increased the accumulation of ROS (Fig. 2), reflecting that UVR can lead to additional harms indeed by enhancing production of ROS. Shorter UV wavelength was shown to increase photosynthetic inhibition and decrease cell division of a calcifying phytoplanktonic alga (Guan and Gao, 2009), which might be related to the accumulation of ROS enhanced at the shorter UV band.

During commercial production of *Arthrospira* spp., accumulation of oxygen during daytime leads to enhanced generation of ROS (Marquez et al., 1995). Increase of intracellular ROS following addition of H_2O_2 was shown with spin resonance (ESP) spectroscopy in *Arthrospira* cells (Ganesh et al., 2007). In the present study, addition of H_2O_2 led to disintegration of the spirals and to inhibition of growth and electron transfer rate (Table 1; Fig. 3). It is known that the H_2O_2 penetrated in cells can be transformed to $^{\bullet}OH$ by reacting with metal ions (Fenton-type reaction) (Neyens and Baeyens, 2003). The increased emission fluorescence of Chl *a* (Fig. 4A) and blue shift emission fluorescence of phycocyanin (Fig. 4B) indicates the damage of H_2O_2 to the photochemical components in the electron transport systems. The decreased photosynthetic pigments content with increased emission fluorescence proved the degradation of chlorophyll and phycobilisomes.

The trichomes of *Arthrospira* are composed of multiple cylindrical cells and enveloped in a thin, diffluent sheath. Propagation of *Arthrospira* spirals multiplication occurs via fragmentation with formation of hormogonia (Tomaselli et al., 1981). The peroxidation of components in the spiral sheath or cell membrane caused by ROS could lead to loss of trichome rigidity and lysis of spirals (Figs. 5 and 6). The spiral breakage was enhanced with accumulated MDA (Figs. 5 and 6), a product of oxidized lipids. The sudden increased breakage of spirals was accompanied a burst of MDA production. The filaments of *Arthrospira* spp. broke when they were irradiated with intensive PAR or PAR combined with UVR (Wu et al., 2005), and such breakage of *Arthrospira* coincided with increased damaged DNA molecules (Gao et al., 2008). Generation of ROS during exposure to UVR is the most important cue leading to the disintegration of the spirals.

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