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Co-culture with *Cyperus alternifolius* induces physiological and biochemical inhibitory effects in *Microcystis aeruginosa*

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ABSTRACT

A semi-continuous co-culture system was established to explore the inhibitory activity of *Cyperus alternifolius* on *Microcystis aeruginosa* growth. The mechanisms underlying the allelopathic effect were also assessed, including oxidative damage and anti-oxidative responses in *M. aeruginosa*. The results demonstrated that *C. alternifolius* significantly inhibited the growth of *M. aeruginosa*. Interestingly, the activities of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) in plant-cyanobacteria group declined remarkably. Indeed relative activities of 26.3% and 37.3% were obtained for SOD and CAT, respectively, in the plant-cyanobacteria group, when compared to the control group at 132 h. In addition, malondialdehyde (MDA) content in the plant-cyanobacteria group (165.6 nmol $mgprot^{-1}$) was 3.6-fold higher compared with controls (37.3 nmol $mgprot^{-1}$) at 108 h, before decreasing sharply. The results indicated that the algal anti-oxidative system is vulnerable to *C. alternifolius*, which caused oxidative damage. These findings provide a basis for understanding the mechanisms involved in *C. alternifolius* inhibition of *M. aeruginosa*.

1. Introduction

As a result of algal overgrowth, harmful algal blooms (HABs) often take place in eutrophic water body. Cyanobacterial bloom is the most prominent episode in eutrophic freshwater (Kong and Song, 2011). Cyanobacteria can release toxic microcystins, which could threaten the survival of aquatic organisms and even humans (Oh et al., 2000). Therefore, it is imperative to control the growth of *Microcystis aeruginosa*.

Several methods for algal removal have been developed; however, most of them are difficult to use in practice. Though mechanical removal is an effective means, it potentially induces increased algal density due to fragmentation and rapid

Abbreviations: C. alternifolius, *Cyperus alternifolius*; CAT, catalase; CG, control group; COD_{Cr}, chemical oxygen demand; GSH, reduced glutathione; HABs, harmful algal blooms; *M. aeruginosa*, *Microcystis aeruginosa*; MDA, malondialdehyde; NH_3–N, ammonia nitrogen; PBS, phosphate-buffered saline; PC, plant-cyanobacteria group; RGI, relative growth inhibition; ROS, reactive oxygen species; SOD, superoxide dismutase; SP, water-soluble protein; TN, total nitrogen; TP, total phosphorus.

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re-growth. Chemical herbicides control algal growth efficiently, but inevitably exterminate harmless aquatic plants and animals within a short period of time (Caffrey and Monahan, 1999). The discovery of allelopathy in plants and microorganisms makes it possible for aquatic plants to control algal growth (Gross, 2003; Zhang et al., 2009b). On one hand, aquatic plants assimilate mass nutrients from the water body and accelerate the alteration of dominant species (Scheffer et al., 2001). On the other hand, aquatic plants exert an allelopathic impact on water environment. Many studies have demonstrated the feasibility of inhibiting algae by aquatic plants. Indeed, Nakai et al. (1999) reported that the submerged plants *Cabomba caroliniana* and *Myriophyllum spicatum* simultaneously inhibit *M. aeruginosa* growth in co-culture conditions. In addition, Zhang et al. (2009a) demonstrated that *Chara vulgaris* influenced the growth of *M. aeruginosa* through allelopathy. Furthermore, emerged plants including *Acorus tatarinowii* (He and Wang, 2001), *Stratiotes aloides* (Mulderij et al., 2005), *Phragmites communis* (Li and Hu, 2005) and *Thalia dealbata* (Zhang et al., 2011) can significantly inhibit the growth of *M. aeruginosa*. Emerged plants have many advantages over submerged species: huge biomass, a relatively large amount of secondary metabolite secretion and easy growth control (Hu and Hong, 2008). Therefore, it is crucial to discover new emerged plants for controlling cyanobacteria bloom.

Cyperus alternifolius (*C. alternifolius*) is a perennial plant with evergreen foliage in the genus *Cyperus* of the sedge family (Cyperaceae). Zhu et al. (2010) reported that *C. alternifolius* showed good efficiency in assimilating nitrogen and phosphorus with average removal rates of 45%, 72% and 55% for chemical oxygen demand (COD_{Cr}), ammonia nitrogen (NH_3–N) and total phosphorus (TP), respectively. However, studies describing the potential use of *C. alternifolius* allelopathy in the control of cyanobacterial bloom are scarce.

Allelopathy of aquatic plants is characterized by transient response, combinational effect and low effective dosage with species selectivity. It is challenging to develop a rational experiment precluding the restrictive factors such as light irradiation, nutrient and microorganism presence (Hu and Hong, 2008). The addition of fresh plant tissue homogenates to the target organism culture could provide a substrate for the potential heterotrophic bacteria and inhibit the algal growth (Gross et al., 2007). The use of extracts cannot give deliver ecologically relevant proof to demonstrate allelopathy between plant and algae. However, plant co-culture with algae in controlled experimental conditions can serve as classical set-up to assess allelopathy (Gross et al., 2007). To assess *C. alternifolius* allelopathy on *M. aeruginosa*, a semi-continuous co-culture experimental device was designed, which precluded several cyanobacterial growth inhibitory factors.

Previous reports have identified four main mechanisms for plant inhibition of algal growth, including cell structure damage, photosynthesis alteration, decreased respiration and alteration of enzymatic activities (Yang et al., 2011). The evaluation of allelopathy was carried out in this study by assessing selected physiological and biochemical parameters, including cell density, chlorophyll a content, MDA amounts, the activities of anti-oxidative enzymes SOD and CAT, and levels of glutathione (GSH), a non-enzymatic component (Hu and Hong, 2008). The current study aimed to (1) provide evidence for the allelopathic effect of *C. alternifolius* on *M. aeruginosa*, (2) explore the mechanism underlying the allelopathic effect of *C. alternifolius*, and (3) assess the feasibility of using emerged plants to control cyanobacteria.

2. Materials and methods

2.1. Material preparation

Unicellular strain *M. aeruginosa* FACHB-905 was acquired from the Institute of Hydrobiology, Chinese Academy of Sciences (China). *C. alternifolius* was collected from Peach Lake in the suburb of Hunan University (Hunan, China), at an initial height of 2 feet. Then, roots and leaves were washed three times with deionized water and soaked in 0.2% potassium permanganate to remove rhizosphere microorganisms.

2.2. Semi-continuous co-culture system design

The design of the semi-continuous co-culture system aimed to imitate an artificial aseptic condition. The system was placed in an artificial climate box; culture was carried out under a 12 h light/dark cycle, illuminated with cool-white fluorescent lamps at 50 $\mu molm^{-2}\ s^{-1}$ from four horizontal directions. The room was maintained at 25 ± 1 °C and relative humidity of 75%. The device consisted of a hollow cylinder of 12 cm in diameter within an acrylic box (20 cm × 20 cm × 20 cm), with a mobile tectum. An engraftment basket was placed on top of the hollow cylinder to allow a stable environment for plant growth. A valve was placed on the broadside to maintain a constant volume when additional medium was appended. Microfiltration membranes (0.45 μm) covered the three apertures on the hollow cylinder, preventing the circulation of algae and other microorganisms into the solution, and ensuring the exchange of nutrients and possible allelopathic substances. After daily sampling, 100 mL MA medium was discharged and replaced by equivalent amounts of fresh MA medium. The transmission was carried out at a uniform rate of 1 mL min^{-1} through a peristaltic pump, which supplied adequate oxygen and nutrients for growth.

2.3. Preliminary culture experiment

C. alternifolius and *M. aeruginosa* were enriched in 2000 mL MA medium, respectively, for 10 d under aseptic conditions. *C. alternifolius* biomass in the monoculture was 12.5 g fresh wt L^{-1}, which was similar to the density observed in the natural environment; the initial density of *M. aeruginosa* in monoculture was approximately 6×10^5 cells mL^{-1}. Nutrient consumption during the culture was determined by measuring total nitrogen (TN) and TP.

2.4. Semi-continuous culture experiment

The experiment comprised two treatment groups: 1) *C. alternifolius* co-existence with *M. aeruginosa* in semi-continuous culture (plant-cyanobacteria group, PC) and 2) plastic structures resembling macrophyte nurtured with *M. aeruginosa* in semi-continuous culture (control group, CG). The co-culture experiment was performed for 6 d as describe above for the preliminary culture experiment.

2.5. Determination of physiological and biochemical parameters

One mL cyanobacterial suspension was collected from PC and CG samples and used for the determination of algal density by a hemocytometer count. The chlorophyll a content was assessed according to the hot ethanol method (Pápista et al., 2002). Briefly, 25 mL of culture medium were passed through 0.45 μm filter membranes, which were cut into pieces and immersed into 5 mL 95% ethanol and heated at 75 °C for 5 min. After cooling to room temperature, the samples were centrifuged for 20 min at 2500 g, and absorbance was determined in supernatants at 665 and 649 nm with a UV-Vis spectrophotometer.

Ten mL cyanobacterial suspension of each sample was sampled from the PC and CG and submitted to centrifugation at 2500 g for 20 min. Then, algal pellets were washed twice with sterile phosphate-buffered saline (PBS) (0.1 M, pH 7.4) and resuspended in 2 mL PBS (0.1 M, pH 7.4). The resulting suspensions were homogenized using an ultrasonic cell pulverizer at 300 W for a total time of 5 min (ultrasonication, 2 s; rest, 6 s) on ice. After the homogenates were centrifuged twice at 16,000 g for 10 min at 4 °C, supernatants were collected for the assessment of enzymatic antioxidant, non-enzymatic antioxidant and soluble protein amounts. SOD and CAT activities, GSH and MDA contents, water-soluble protein (SP) amounts in each sample were determined using ELISA kits purchased from Jiancheng Biotech, (Nanjing, China), following the manufacturer's instructions.

2.6. Statistical analysis

The inhibitory effect of *C. alternifolius* on the algal growth was estimated by relative growth inhibition (RGI), which was defined as follows:

$$RGI\ (\%) = (1 - N/N_0) \times 100$$

where N_0 and N represent the cell numbers in the CG and PC groups, respectively.

All experimental data were expressed as mean ± SD. Graphical works were carried out using the software Origin 8.0 (OriginLab, USA). Data were analyzed by SPSS 19.0 (SPSS, USA). One-way analysis of variance (ANOVA) and LSD test were used to assess the differences between individual means (Zhang et al., 2010). $P < 0.05$ was considered statistically significant. All experiments were run in triplicate with randomized blocks.

3. Results

3.1. C. alternifolius effects on M. aeruginosa growth

The growth of *M. aeruginosa* was significantly inhibited by *C. alternifolius* in a time dependent manner (Fig. 1). The RGI of *M. aeruginosa* was 81.7% in the PC group compared to the CG after 132 h of co-culture (Fig. 1). Interestingly, the cell density increased rapidly to 60 h, before decreasing remarkably after 84 h of incubation in the PC group. After 132 h of culture, the cell density in the PC group decreased to 49.5% of its initial value (Fig. 1). In contrast, the growth of *M. aeruginosa* in CG increased with time and the green color of the culture medium gradually deepened; the culture medium in the PC group became transparent with yellow sediment at the bottom after 132 h.

3.2. Chlorophyll a content of M. aeruginosa

Chlorophyll a contents increased to 36 h and then decreased linearly. Interestingly, 0.059 mg L^{-1} chlorophyll a was found in the PC group at 132 h, which was 12.2% of that of the CG. At 84 h chlorophyll a content in the PC group was 0.143 mg L^{-1}, 2.4-fold higher than what observed in the same group at 132 h (Fig. 2A). The chlorophyll a amounts were not correlated with cell density in PC ($R^2 = 67.6\%$) (Fig. 2B).

3.3. MDA content of M. aeruginosa

The MDA content was determined to assess the oxidative damage on algal cells. The MDA contents in PC slightly increased before 84 h, and displayed a much more dramatic alteration after 84 h compared to the CG. MDA levels in PC were lower than those of CG till 36 h. However, MDA levels in PC were enhanced after 36 h, exceeding those in CG. Specifically, the MDA content in PC rose to 165.6 nmol mgprot^{-1} at 108 h, which was 3.6-fold higher than that of CG. At experiment end, MDA levels in PC were 78.9 nmol mgprot^{-1}, 2.1-fold the values obtained for the CG (Fig. 3A).

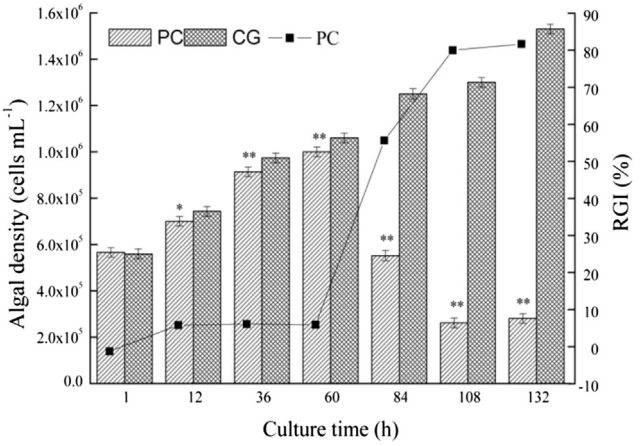

Fig. 1. Effects of *C. alternifolius* on *M. aeruginosa* growth. (1) Algal density during the culture period. (2) Relative growth inhibition curve compared to the corresponding CG from 1 h to 132 h. Data are mean ± SD, $n = 3$. * ($p < 0.05$) and ** ($p < 0.01$) indicated significant differences compared to the CG.

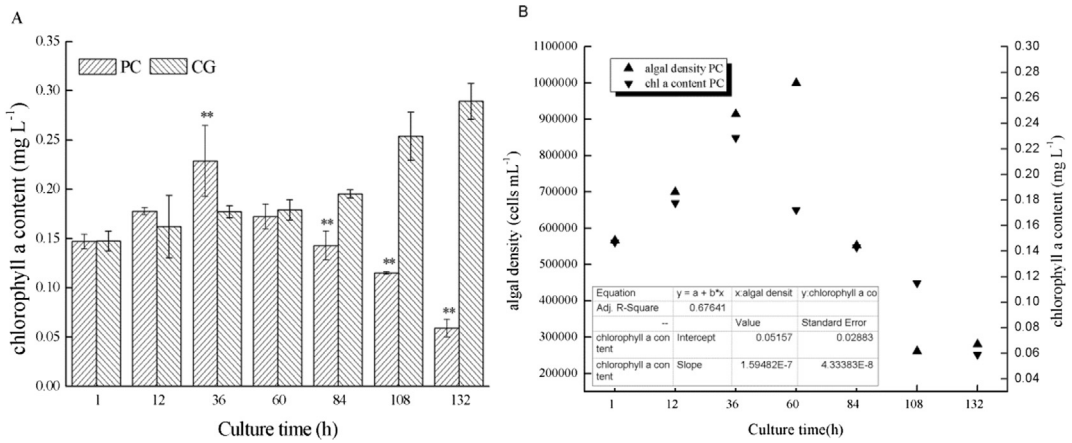

Fig. 2. Effects on the chlorophyll a content of *M. aeruginosa*. (A) Fluctuation curve of chlorophyll a content with culture time. (B) Correlation between algal density and chlorophyll a content. Data are mean ± SD, $n = 3$. * ($p < 0.05$) and ** ($p < 0.01$) indicated significant differences compared to the CG.

3.4. Effects of C. alternifolius on M. aeruginosa enzymatic antioxidants activities

To investigate the fluctuation in activity of the cellular defense system, the activities of enzymatic antioxidants including SOD and CAT were examined. As shown in Fig. 4A, SOD activity rapidly increased after 36 h in PC, reaching a maximum value

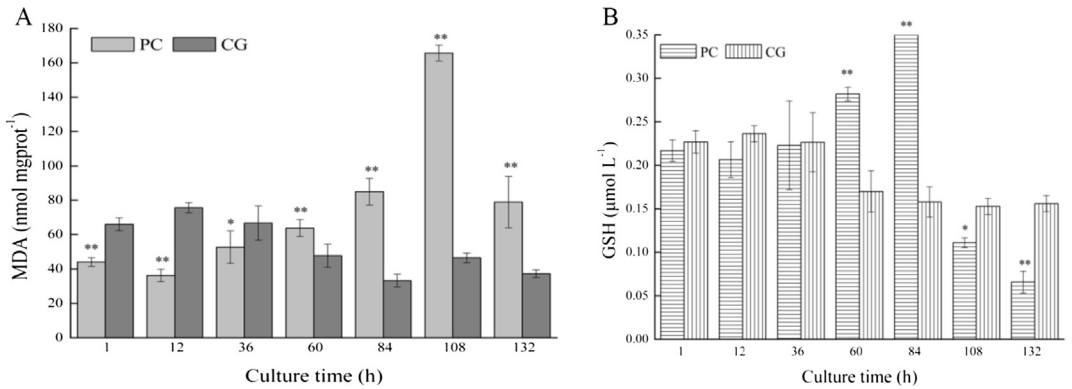

Fig. 3. (A) Effects of *C. alternifolius* on MDA content in *M. aeruginosa*. (B) Effects of *C. alternifolius* on the activities of GSH in *M. aeruginosa*. Data are mean ± SD, $n = 3$. * ($p < 0.05$) and ** ($p < 0.01$) indicated significant differences compared to the CG.

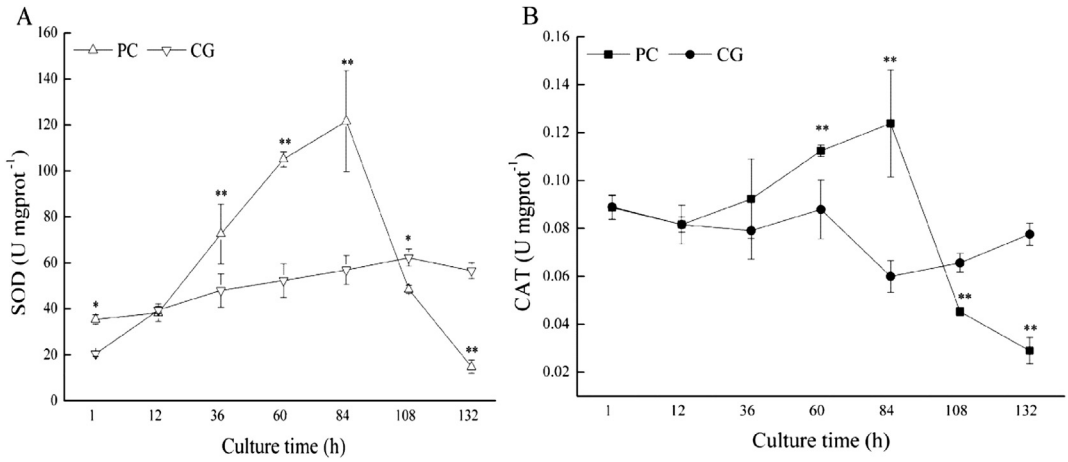

Fig. 4. Effects of *C. alternifolius* on the activities of enzymatic antioxidants in *M. aeruginosa*. (A) SOD activity fluctuation with culture time. (B) CAT activity fluctuation with culture time. Data are mean ± SD, $n = 3$. * ($p < 0.05$) and ** ($p < 0.01$) indicated significant differences compared to the CG.

of 121.6 U mgprot^{-1} (2.1-fold of that in CG) when algal cells were cultured with *C. alternifolius* for 84 h. This was followed by a sharp SOD activity decrease. At the end of the experiment, SOD activity in PC was 14.9 U mgprot^{-1}, 26.3% that in CG at 132 h.

The trend observed for CAT activity was similar to that of SOD activity in PC (Fig. 4B). There was a CAT activity increase from 60 to 84 h in PC, with 0.124 U mgprot^{-1} obtained at 84 h, a value 2.1-fold higher than that of CG. Compared with the CG at 132 h, CAT activity was remarkably decreased, eventually dropping to 0.029 U mgprot^{-1} in PC. Taken together, the activity of SOD and CAT rose slightly in CG but showed a biphasic curve in PC during the culture period.

3.5. Effects of *C. alternifolius* on *M. aeruginosa* non-enzymatic antioxidant activity

The effects of *C. alternifolius* on GSH pools were quantified and the results are shown in Fig. 3B. The GSH gradually increased to the 84 h time point, with the maximum concentration in PC of 0.36 μmol L^{-1}, which was 1.6-fold that in CG at 84 h. There was a sharp decrease after co-culture for 84 h in PC. At experiment end GSH levels in PC were 0.065 μmol L^{-1}, 42% of the value found in CG at 132 h (Fig. 3B). The GSH content had a moderate drop during the entire experimental period in CG.

4. Discussion

The decrease of algal density indicated that *C. alternifolius* significantly inhibited the growth of *M. aeruginosa*. However, the factors responsible for the sharp *M. aeruginosa* growth alteration are not known. Parameters such as light, nutrient and microorganism limitation had been excluded in the experimental design. Therefore, it could be concluded that *C. alternifolius* affects the algal growth likely through allelopathy.

The prominent inhibitory effect also demonstrates the potential feasibility of the use of *C. alternifolius* for *M. aeruginosa* growth inhibition. These findings provide a basis for the use of *C. alternifolius* to control cyanobateria under actual water environment, especially in shallow lake, river and other still water environment. When *C. alternifolius* was cultivated in shallow lake, river and artificial plant floating bed, the eco-restoration effect was overtly observed. Not only could *C. alternifolius* reduce nutrient supply of *M. aeruginosa* (Zhu et al., 2010), it also inhibited the growth of *M. aeruginosa* by allelopathy.

Upon algal cell damage, algal growth was inevitably decreased, and this was accompanied by a decrease in chlorophyll a levels. Chlorophyll a is a photosynthetic pigment in the blue-green algae, which captures and transfers luminous energy through photosynthesis (Kong and Song, 2011). However, the trend of cell density decline was not in line with the decrease of chlorophyll a, suggesting that the main mechanism of *M. aeruginosa* growth inhibition might be related to chlorophyll a synthesis.

One possibility of such inhibitory mechanisms is the exploitation of the enzymatic antioxidative system. Reactive oxygen species (ROS), including superoxide radical (O_2^-), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) are generated in a cell metabolic process (Mallick and Mohn, 2000). Excessive ROS can be scavenged by the enzymes of the antioxidative system, which maintain a dynamic equilibrium in the intracellular oxygen metabolism under normal circumstances (Yang et al., 2011). This ROS equilibrium is crucial for preventing the generation of the highly toxic hydroxyl radicals via the metal-dependent Haber-Weiss or the Fenton reactions (Mittler, 2002). When the dynamic equilibrium is broken, excessive reactive oxygen free radicals react with unsaturated fatty acids and cause cellular peroxidation reaction, which in turn induces lipid peroxidation and the subsequent leakage of cellular contents, rapid autolysis and algal cell death (Ali and Alqurainy, 2006).

MDA is a biomarker of lipid peroxidation (Hong et al., 2009), of which increased levels indicate an oxidative damage in algal cell membrane (Ahmad et al., 2008). The MDA content in PC increased by 3.6-fold compared to CG value at 108 h, which

represents a dramatic accumulation of lipid peroxidation in algal cells. However, the MDA level in PC decreased remarkably after 108 h, which was not consistent with increased MDA levels expected after growth in harsh environment (Zhang et al., 2011). These contradictory results suggest that a certain amount of MDA was possibly diffused across biological membranes, and then dissolved in the extracellular environment. The lysis of the algal cells might have also contributed to the observed decrease of MDA at later time points.

As the first line of defense ROS, SOD is active in catalyzing the conversion of the very active O_2^- into H_2O_2 and O_2 (Mishra et al., 1993; Qureshi et al., 2007). In response to environmental stress, the activity of SOD increases rapidly, before dropping to a certain level (Hong et al., 2009). In our study, the growth conditions were continuously deteriorated following the accumulation of *C. alternifolius* metabolites. This improved the activity of SOD in eliminating O_2^-. The activity of SOD in PC was 2.1-fold that of CG at 84 h, which was associated with stress tolerance in the organisms (Mishra et al., 1993). The sharp decline in SOD activity might cause enzyme protein damage due to excessive production of free radicals and peroxides (Zhang et al., 2007).

As a subsequent ROS scavenging agent, CAT showed a similar trend to that of SOD. CAT catalyzes the degradation of H_2O_2 into H_2O and O_2 without consumption of cellular reducing equivalents (Mallick and Mohn, 2000). Oxygen free radicals generated in harsh environment could be scavenged by the combined action of SOD and CAT (Mallick and Mohn, 2000). Deleterious substances, especially some secondary metabolic products released by *C. alternifolius*, began to accumulate after 84 h, leading to severe imbalance of production and scavenging of reactive oxygen species. In response to environmental stress, the concentration of dissociative O_2^-, H_2O_2 and ·OH are enhanced (Scandalios, 1993). Peroxyl radical (·OOH), which could be reversibly converted from O_2^-, readily diffuses across and damage algal membranes (Hume et al., 1983). H_2O_2 damages algal cells since it can readily spread from the membranes to the extracellular environment (Bowler et al., 1992). H_2O_2 can also oxidize cysteine (-SH) or methionine residues (-SCH_3) and inactivate enzymes by oxidizing their thiol groups (Pallavi et al., 2012). ·OH is the most reactive ROS and is hard to eliminate by enzymatic reactions in cells. When excess production of ·OH occurs, indiscriminate and rapid attacks on virtually all macromolecules are induced, which result in damage of cellular components and DNA, and eventually algal growth cessation or killing of algal cells (Pallavi et al., 2012). In other words, upon destruction of the antioxidative enzymatic system, the algal cells were gradually lysed or died. Changes in antioxidative responses of *M. aeruginosa* suggested that the algal antioxidative defense system might be vulnerable to *C. alternifolius*. Moreover, induced oxidative damage might be one of the mechanisms underlying the inhibitory effect of *C. alternifolius* on *M. aeruginosa*.

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