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Effects of a Novel Allelochemical Ethyl 2-Methyl Acetoacetate (EMA) on the Ultrastructure and Pigment Composition of Cyanobacterium *Microcystis aeruginosa*

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Abstract Allelochemical ethyl 2-methyl acetoacetate (EMA) can significantly inhibit the growth of bloom-forming *Microcystis aeruginosa*. In order to assess the implication of the damage of EMA on the algal photosynthetic apparatus, the effects of EMA on the algal ultrastructure and pigment composition were investigated. At initial exposure time to EMA (0–40 h), algal allophycocyanin, phycoerythrin and carotenoid degraded firstly; chlorophyll a increased, especially by 47% in the algae exposed to 2 mg L^{-1} of EMA; phycocyanin was not significantly affected; lipid bodies increased remarkably. After 40 h of EMA exposure, chlorophyll a decreased gradually, especially by 45% in the algae exposed to 4 mg L^{-1} of EMA; lipid bodies greatly reduced but cyanophycin granules accumulated; thylakoid structures were dissolved or disappeared with the presence of numerous vacuoles. These results showed that all ophycocyanin, phycoerythrin and carotenoid were more sensitive to EMA than other pigments, the cells of *M. aeruginosa* was stressed by EMA with the occurrence of cyanophycin granules and the photosynthesis pigments and ultrastructure of *M. aeruginosa* were quickly destroyed by EMA with exposure time increasing.

Keywords Ethyl 2-methyl acetoacetate (EMA) · *Microcystis aeruginosa* · Photosynthetic pigments · Ultrastructure

Naturally allelopathic compounds with high allelopathic activity produced by macrophytes such as polyphenols from *Myriophyllum spicatum* (Nakai et al. 2005), α-asarone from *Acorns tatarinowii* (Pollio et al. 1993), polyphenols and ethyl 2-methyl acetoacetate (EMA) from *Phragmites communis* (Li and Hu 2005; Nakai et al. 2006) are good alternatives for novel algicides. The exploration of natural algicides greatly depends on understanding their inhibitory mechanisms on bloom-forming algae (Einhellig 1995a). However, the mechanisms of several natural compounds are not well understood (Einhellig 1995b).

Blooms of toxic *M. aeruginosa* are in frequent outbreaks in many districts, especially in China (Chen et al. 2004; Pan et al. 2006; Romanowska-Duda et al. 2002).The novel compound EMA is one of the primary effective allelochemicals in *P. communis* to inhibit *M. aeruginosa*. EMA has been reported to cause cells of *M. aeruginosa* to leak metal ions, decrease some antioxidant enzymes, and increase the proportion of unsaturated lipid fatty acids in cell membrane (Li and Hu 2005; Hong et al. 2008). The effects of EMA on the photosynthetic pigments and ultrastructure of *M. aeruginosa* have never been examined.

The objective of the current study was to assess the effects of EMA on photosynthetic pigments and ultrastructure of *M. aeruginosa* for deeper understandings about EMA inhibition on *M. aeruginosa*, especially its photosynthetic apparatus.

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Materials and Methods

Microcystis aeruginosa was purchased from FACHB (Freshwater Algae Culture of Hydrobiology Collection, China). The algae was cultured in autoclaved media containing: $NaNO_3$ 1,500 mg L^{-1},$K_2HPO_4{\cdot}3H_2O$ 40 mg L^{-1}, Na_2CO_3 20 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ 75 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 36 mg L^{-1}, Na_2EDTA 1 mg L^{-1}, $C_6H_8O_7$ (citric acid) 6 mg L^{-1}, $Fe(NH_4)_3(C_6H_5O_7)_2$ (ferric ammonium citrate) 6 mg L^{-1}, $Co(NO_3)_2{\cdot}6H_2O$ 0.049 mg L^{-1}, H_3BO_3 2.86 mg L^{-1}, $MnCl_2{\cdot}4H_2O$ 1.81 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.22 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.079 mg L^{-1}, $Na_2MoO_4{\cdot}2H_2O$ 0.39 mg L^{-1} under an irradiance of 40–60 μmol photons$\cdot m^{-2}$ s^{-1} and a photoperiod of 14 (light): 10 h (dark) at 24–25°C.

Conical flasks (500 mL) were prepared and autoclaved, each of which contains 200 mL culture media. After sterilization, the initial concentration gradients of EMA added were designed as follows: 0, 0.25, 0.5, 1.0, 2.0, and 4.0 mg L^{-1}. The medium without any EMA was taken as the controls. The initial algal density in each flasks was 1×10^6 cells mL^{-1}. The cultures were incubated under the same condition mentioned above. The algal densities were determined by counting cell numbers using a hemocytometer. Ultraviolet–visible (UV–Vis) absorption of cell suspensions between 350 and 800 nm were measured using a Shimadzu UV-2401PC spectrophotometer and a quartz cuvette with a 1 cm path length.

About 200 mL of culture media (algal density: 1×10^6 cells mL^{-1}) was centrifuged at 4,500 rpm for 10 min. The pellet was washed twice with Na-phosphate buffer (50 mM, pH 7.2) and then resuspended in 2 mL of chilled 80% acetone at 4°C under dim light. Before sonication, the sample was vortexed for 1 min. Then the sample was lysed by an ultrasonic cell pulverizer (JY92-2D, Xinzhi Co., China) at 200 W with total time of 5 min (ultrasonic time, 2 s; rest time, 8 s). The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant, just cell-free extract was kept to following assays. A second extraction of the cell pellet was carried out as the above-mentioned procedure to ensure complete extraction. The total supernatants were collected and the volume was adjusted to 4 mL with 80% acetone. The absorbance of the supernatant was detected at 480, 645, and 663 nm for the estimation of chlorophyll a and carotenoid contents (Arnon 1949; Grobe and Murphy 1998).

About 200 mL of culture media (algal density: 1×10^6 cells mL^{-1}) was centrifuged at 4,500 rpm for 10 min. The pellet was washed twice with Na-phosphate buffer (50 mM, pH 6.8) and then resuspended in 3 mL of chilled Na-phosphate buffer (50 mM, pH 6.8) with 0.5% (w/v) lysozyme at 4°C under dim light. Before sonication, the sample was vortexed for 1 min. Then the sample was lysed by an ultrasonic cell pulverizer (JY92-2D, Xinzhi Co., China) at 200 W with total time of 5 min (ultrasonic time, 2 s; rest time, 8 s). The homogenate was incubated for 4 h at 25°C by continuously shaking at a rate of 100 rpm and then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant, just cell-free extract containing phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE) was kept to following assays. The absorbance of the supernatant was detected at 565, 620, and 650 nm for the estimation of phycobilinprotein contents (Abelson and Simon 1988; Padgett and Krogmann 1987).

The algal cells for TEM observations were taken from cultures inoculated with 1×10^6 cells$\cdot mL^{-1}$, either with different exposure time to 1 mg L^{-1} of EMA or without addition of EMA. All treatments were obtained from at least three cultures and processed separately. The samples were harvested from cultures by centrifugation at 4,500 rpm for 10 min. The samples were then washed twice using Na-phosphate buffer (50 mM, pH 7.2). The samples were fixed with 2% glutaraldehyde buffered with phosphate buffer (the same as above) for 2 h at room temperature. The samples were then washed in buffer three times, and post-fixed in 1% osmium tetraoxide in the same buffer for 2 h at room temperature. Subsequently, the samples were rinsed three times with buffer, dehydrated with an ethanol series, and then embedded in the epoxy resin. The ultrathin sections were cut with a LKB-V ultramicrotome (LKB, Bromma, Sweden), and then stained with 3% methanolic uranyl acetate and lead citrate. The observations and photographs of the algal fine structure were made with a Hitachi H-600 TEM (Tokyo, Japan) (Pollio et al. 1993; Wu et al. 1998). Representative transmission electron micrographs were selected from thousands of algal cells and revealed their typical ultrastructure characteristic.

Analysis of the data was done using Origin 7.0 software (OriginLab Corporation). The means and standard deviations (SD) of all data were determined and graphed. One-way ANOVA was used to evaluate the dose-response relationships of the algae to EMA. All assays were done with at least three replications each time. The whole experiment was done twice, and similar results were obtained.

Results and Discussion

Chlorophylls are the basic pigments involved in light absorption and photochemistry in algae, photosynthetic bacteria and plants (Eullaffroy and Vernet 2003). Chlorophyll a is the principal chlorophyll in the cyanobacteria where it is generally associated with phycobilins (Papageorgiou 1996). To examine the dynamic changes of

chlorophyll a in *M. aeruginosa* exposed to EMA, the content of chlorophyll a in the algal cells exposed to a concentration series of EMA for a series of time were quantified. From 0 to 60 h of EMA exposure, chlorophyll-a content changed from increase to decrease (Fig. 1). When the algal cells exposed to a concentration series of EMA, chlorophyll a was not significantly changed at 4 h of exposure, closely increased as EMA concentration increased at 40 h, and decreased as EMA concentration increased at 60 h. The maximal value of chlorophyll a, about 147% of the control, occurred at 40 h. Interestingly, chlorophyll-a content was not in a positive correlation with the concentration of EMA at 40 h. Chlorophyll a at 4 mg L^{-1} of EMA was less than those at the lower concentrations of EMA and in no significant difference with the controls.

Carotenoids as accessory pigments in cyanobacteria serve as light-harvesting pigments during photosynthesis and protective molecules against photo-oxidative damage (Bryant 1996). To examine the dynamic changes of carotenoids in *M. aeruginosa* exposed to EMA, the content of carotenoids in the algal cells exposed to a concentration series of EMA for a series of time were quantified. From 0 to 60 h of EMA exposure, carotenoid content gradually decreased (Fig. 2). When the algal cells exposed to a concentration series of EMA, carotenoids like chlorophyll a was not significantly changed at 4 h of exposure. The significant difference between treatment groups and control groups just occurred after 40 h of exposure. After 40 h, carotenoid decreased remarkably as EMA concentration increased to 4 mg L^{-1}. When exposure time extended to 60 h, the carotenoids in the algae exposed to EMA with concentration above 1 mg L^{-1} decreased. The minimal

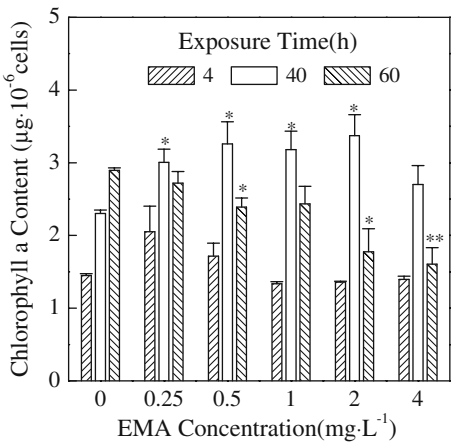

Fig. 1 Effects of EMA on chlorophyll a content of *M. aeruginosa* cells. *Vertical bars* indicate SD of three replicates in each treatment group. * ($p < 0.05$) and ** ($p < 0.01$) indicate significant differences compared to the corresponding controls

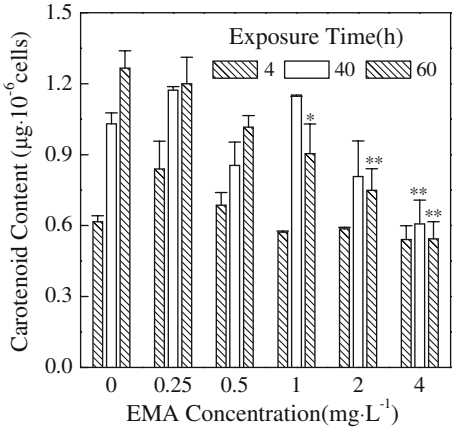

Fig. 2 Effects of EMA on carotenoid content of *M. aeruginosa* cells. *Vertical bars* indicate SD of three replicates in each treatment group. * ($p < 0.05$) and ** ($p < 0.01$) indicate significant differences compared to the corresponding controls

value of carotenoids decreased to about 50% of the control after 60 h.

The antenna pigments, phycobilins included phycocyanin, allophycocyanin, and phycoerythrin. They are assembled in phycobilisomes and are attached to the surface of the thylakoids for photosynthesis. The contents of phycocyanin, allophycocyanin, and phycoerythrin of the algae exposed to EMA were measured respectively. In Fig. 3, phycocyanin was tested to be the most abundant part of the total phycobilins, about 60% in the controls without EMA treatments. During the exposure time and the exposure concentration tested above, phycocyanin decreased slightly but was not be remarkably influenced by EMA. Allophycocyanin which was about 30% of the total phycobilins in the controls without EMA treatments, was the first one of the total phycobilins to decrease after 4 h of exposure, especially significantly at 4 mg L^{-1} of EMA. As the exposure time extended to 40 h, allophycocyanin in the algae exposed to 4 mg L^{-1} of EMA decreased by 67%. Phycoerythrin was the least part in the total phycobilins of the tested algae. Under a concentration series of EMA exposure, phycoerythrin was hardly changed after 4 h but decreased after 40 h when EMA was above 0.5 mg L^{-1}. As the ratios of three compositions differed greatly, so total phycobilins were just significantly decreased after 40 h with EMA concentration above 0.5 mg L^{-1}.

In accord with the results above, the color of *M. aeruginosa* was observed to be from blue green in the beginning (4 h), yellow green in the medium stage (4 d) to colorlessness in the last stage examined (8 d). Moreover, the adsorption spectra of cell suspensions of *M. aeruginosa* after 3 d and 4 d of exposure were examined (Fig. 4). The maximum peaks of chlorophyll a were at 436 and 678 nm; c-phycocyanins at 626 nm; carotenoids at 464 nm with a

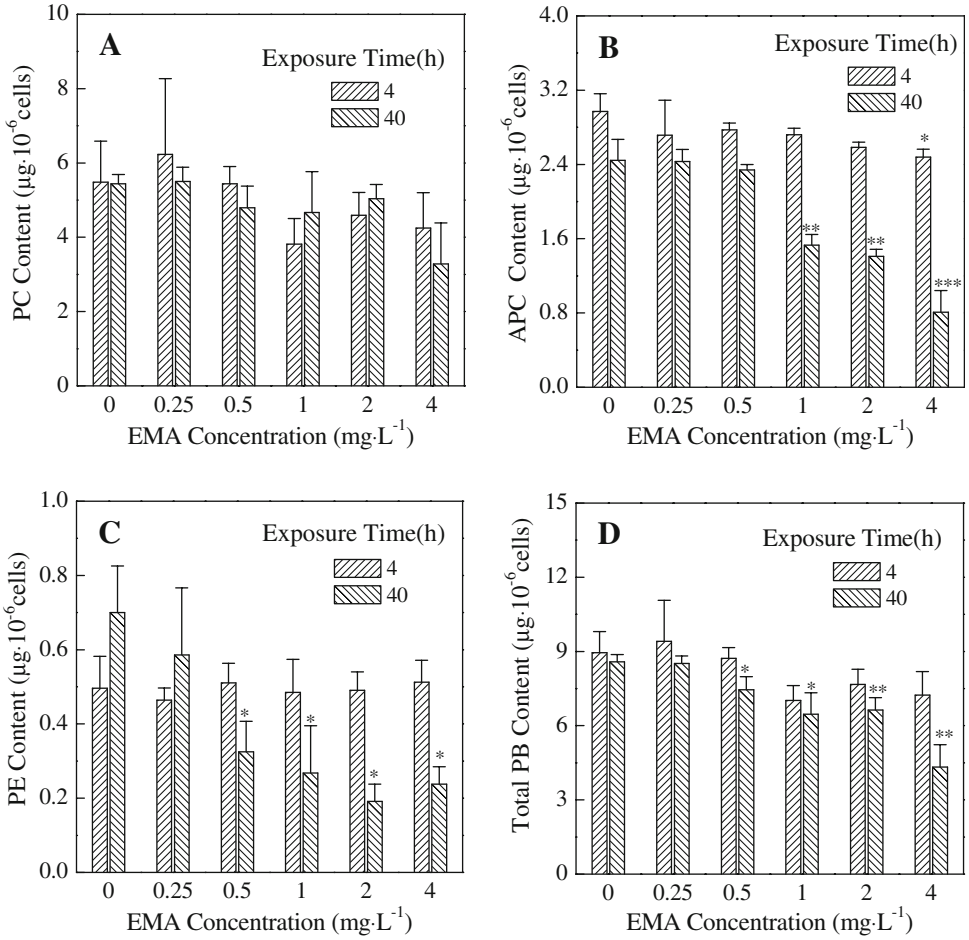

Fig. 3 Effects of EMA on phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE) and total phycobilins (PB) contents of *M. aeruginosa* cells. **a–d** Cells treated with a concentration series of EMA for 4 h and 40 h, respectively, showing PC, APC, PE and PB contents, respectively. *Vertical bars* indicate SD of three replicates in each treatment group. * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) indicate significant differences compared to the corresponding controls

shoulder at 490 nm (Gerasimenko et al. 1972). In Fig. 4, 4 mg L^{-1} of EMA caused more serious bleaching of pigments at 4 d than 3 d, including chlorophyll a, c-phycocyanins and carotenoids. However, the decrease in the algae exposed to 0.5 mg L^{-1} of EMA was not significant after 4 d.

The investigation above has pointed out that the pigment composition of *M. aeruginosa* was influenced by EMA. For deeper understandings on how EMA affect *M. aeruginosa*, especially the photosynthetic apparatus, so the ultrastructure of *M. aeruginosa* was investigated. Regular and abundant thylakoids were distributed in the algae without EMA exposure (Fig. 5a). During the exposure time to 1 mg L^{-1} of EMA, the ultrastructure of *M. aeruginosa* was gradually changed. After 12 h of exposure, the density of algal thylakoids decreased and some shapes of thylakoids

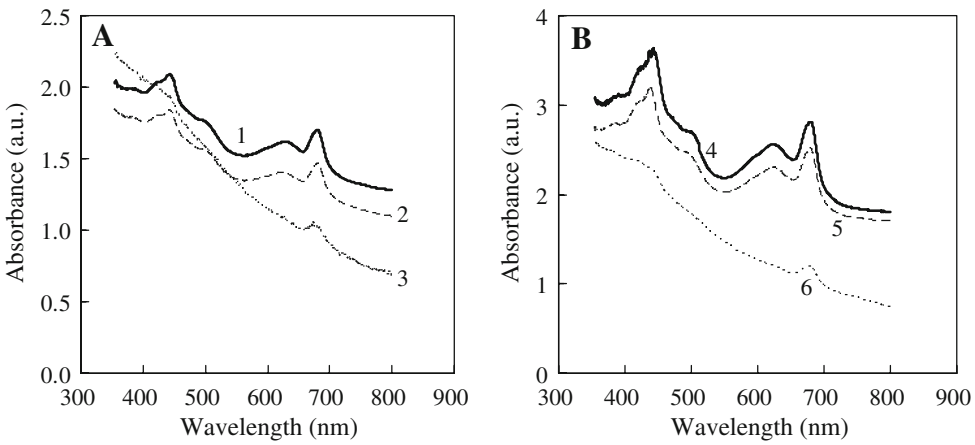

Fig. 4 Effects of EMA on absorption spectra of *M. aeruginosa* suspension. **a** Exposure to EMA for 3 d; **b** Exposure to EMA for 4 d. (1, 4) Cells without addition of EMA; (2, 5) Cells exposed to 0.5 mg L^{-1} of EMA; (3, 6) Cells exposed to 4 mg L^{-1} of EMA

became irregular (Fig. 5b). After 18 h, the thylakoids in the algae treated with 1 mg L^{-1} of EMA became more sparse and the phycobilins attached to the membrane of thylakoids decreased, moreover, many plastoglobuli (lipid bodies), polyphosphate bodies, and cyanophycin granules occurred in the cells (Fig. 5c). When the exposure time extended to 24 h, the shapes of thylakoids became blurring (Fig. 5d). The damage on the cells of *M. aeruginosa* was more distinctive after 36 h, as the shapes of thylakoids fully disappeared (Fig. 5e). The accumulation of cyanophycin granules and polyphosphate bodies still existed in the cells after 48 h (Fig. 5f). After 60 h, more serious breakdown of cellular structure occurred. Large clear areas (i.e. numerous vacuoles) appeared in the *M. aeruginosa* cells and the multiple-layered cell wall turned into unclear (Fig. 5g).

During short-term exposure to EMA, chlorophyll-a content increased firstly and then decreased at lower concentrations. The changes of chlorophyll a reflected the regulation of chlorophyll synthesis. The regulation of chlorophyll biosynthesis appears to be intimately associated with thylakoid development and photosynthetic activity. From TEM sections, the gradual damage on the thylakoids as exposure time extended was observed. Although the shapes of thylakoids almost disappeared at 36 h, an increase of chlorophyll a was still probable on the fragments of thylakoids. Additionally, the synthesis of chlorophyll a is generally based on the level of δ-aminolevulinic acid (ALA) formation (Goodwin 1971). The increase of chlorophyll a hinted that the precursor of chlorophyll a, i.e., ALA perhaps increased. Moreover, the increase of chlorophyll a also might be derived from the cellular responses to the stress of EMA. As the antenna pigments including carotenoids and phycobilins decreased, so the algal cells increased the reaction center pigment chlorophyll a to remedy the photochemical loss. The changes of chlorophyll a hinted that the photosynthetic activity of the algal cells was affected and chlorophyll-a increase might be a positive regulation for cellular photosynthesis. Further, chlorophyll-a increase did not appear at high concentration of EMA (4 mg L^{-1}) and not as the exposure time extended. As the exposure time extended, the decrease of chlorophyll a occurred. The decrease showed that cellular resistance disappeared, and pigment composition was deteriorated further.

The characteristic carotenoids of *M. aeruginosa* are β-carotene, zeaxanthin, echinenone, and etc. (Stransky and Hager 1970). All of the carotenoids are important antenna pigments for harvesting light. After exposure to EMA, the carotenoids gradually decreased. The decrease of carotenoids showed that EMA caused cellular light-harvesting activity in damage. Additionally, carotenoids are well-known antioxidants to removal toxic oxygen species. Therefore, the decrease of carotenoids also showed that EMA destroyed the antioxidant defense system.

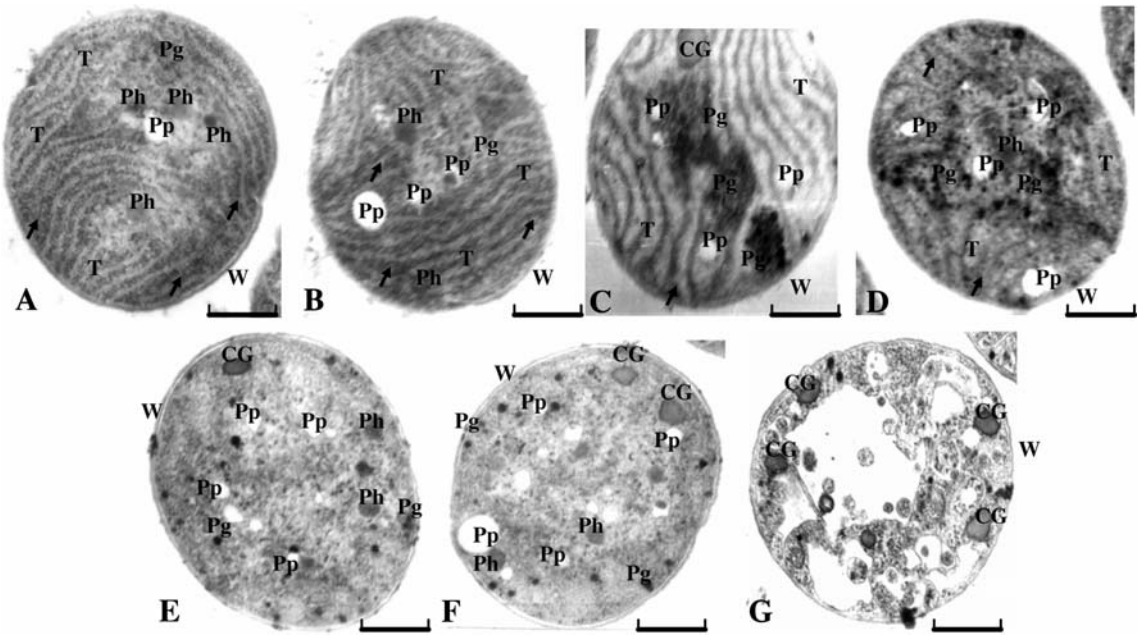

Fig. 5 Effects of EMA on fine structures of *M. aeruginosa* cells. **a** Cell without treatment, showing layered cell wall (W), electron-dense phycobilinsomes (*arrow*) on thylakoid membranes (T), plastoglobuli (Pg), polyhedral bodies (Ph), and polyphosphate bodies (Pp). **b–g** Cells with 1 mg L^{-1} of EMA treatments for 12, 18, 24, 36, 48, and 60 h, respectively, showing layered cell wall (W), electron-dense phycobilinsomes (*arrow*) on thylakoid membranes (T), plastoglobuli (Pg), polyhedral bodies (Ph) polyphosphate bodies (Pp), and/or cyanophycin granules (CG); Cells showing the distortion, collapse, and/or disappearance of thylakoid membranes and the changes in other cell structure. *Scale bar* = 0.5 μm

As the absorbency of chlorophyll a and carotenoids is poor at 550–650 nm and drops off in green, so the cyanobacteria evolved a set of phycobilin pigments to give good light absorption across the visible spectrum. Moreover, the color of *M. aeruginosa* is decided by the contents of phycobilins. The results of phycobilins showed that allophycocyanin and phycoerythrin of the algae were the most sensitive to EMA during short-term exposure (0–40 h); phycocyanin of the algae was sensitive to EMA after medium-term exposure to EMA. The decrease of phycocyanin might directly result in the bleaching of blue green in the algae, so the yellow green algae was observed at 4 d. Basically, Fig. 4 showed that longer exposure to EMA diminished more of the algal optical density at the absorption bands of pigments above.

In normal cells, the reaction center pigment, chlorophyll a and the accessory antenna pigments, carotenoids are attached to membrane-bound proteins of the thylakoids. Additionally, the accessory antenna pigments, phycobilins are attached to the cytosol face of the thylakoids and extend into the cytosol (Sarcina et al. 2001). In the cells exposed to EMA, the thylakoids were destroyed with decrease of phycobilins (Fig. 5). When exposed to EMA, the cells of *M. aeruginosa* also showed some peculiarities in cellular inclusions, such as an increase of cyanophycin granules. Cyanobacteria generally produced cyanophycin granules when environmentally stressed (Allen et al. 1980; Huang et al. 2002). So the occurrence of cyanophycin granules hinted that the cells of *M. aeruginosa* was stressed by EMA. The investigation of the ultrastructure at different exposure time to EMA revealed different characteristics caused by EMA, which could not be obtained by other methods. The gradual breakage of *M. aeruginosa* cells by EMA was clearly demonstrated by TEM sections after a series of exposure time. The disappearance and appearance of some inclusions during EMA exposure hinted that the responses of *M. aeruginosa* to EMA were complicated. The elucidation of the chemical nature of the inclusions and their relations to EMA damage on *M. aeruginosa* should be done further.

In sum, the exposure of allelochemical EMA may be an environmental stress to *M. aeruginosa*. The photosynthetic pigments destroyed by EMA may be lethal sites to *M. aeruginosa*. Additionally, although the addition of EMA caused the growth inhibition of *M. aeruginosa*, the cellular resistance or responses to the stress still existed at the initial exposure time. The exploration of the cellular regulation is important to help in-depth understandings of the allelopathic inhibitory actions.

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