World Academy of Science, Engineering and Technology
International Journal of Environmental and Ecological Engineering
Vol:4, No:11, 2010

Effects of Allelochemical Gramine on Photosynthetic Pigments of Cyanobacterium *Microcystis aeruginosa*

Y. Hong, H. Y. Hu, A. Sakoda, and M. Sagehashi

***Abstract*—**Toxic and bloom-forming cyanobacterium *Microcystis aeruginosa* was exposed to antialgal allelochemical gramine (0, 0.5, 1, 2, 4, 8 mg·L^{-1}), The effects of gramine on photosynthetic pigments (lipid soluble: chlorophyll a and β-carotene; water soluble: phycocyanin, allophycocyanin, phycoerythrin, and total phycobilins) and absorption spectra were studied in order to identify the most sensitive pigment probe implicating the crucial suppression site on photosynthetic apparatus. The results obtained indicated that all pigment parameters were decreased with gramine concentration increasing and exposure time extending. The above serious bleaching of pigments was also reflected on the scanning results of absorption spectra. Phycoerytherin exhibited the highest sensitivity to gramine added, following by the largest relative decrease. It was concluded that gramine seriously influenced algal photosynthetic activity by destroying photosynthetic pigments and phycoerythrin most sensitive to gramine might be contributed to its placing the outside of phycobilins.

***Keywords*—**Absorption spectra, allelochemical, gramine, *Microcystis aeruginosa*, photosynthetic pigments.

I. Introduction

OUTBREAKS of cyanobacterial blooms have been great threats to aquatic ecological preservation, public utility management of drinking, and even economic development [1]. Allelochemicals synthesized by aquatic plants with strong antialgal activities have been considered good alternatives for algicides [2]. Till now, *Myriophyllum spicatum*, *Ceratophyllum demersum* (L.), *Phragmites communis* Trin, *Arundo donax* Linn., and *Acorus gramineus* were reported to suppress cyanobacterial growth and some allelcochemicals have been isolated and identified from them, such as polyphenols from *M. spicatum*, ethyl 2-methyl acetoacetate (EMA) from *P. communis* Trin, gramine from *A. donax* Linn., α-asarone from *A. gramineus* [3]-[7]. Among the allelochemicals, gramine (N,N-dimethyl-3-amino-methylindole) as an indole alkaloid was one of the strongest to inhibit toxic and bloom-forming cyanobacterium *Microcystis aeruginosa* [8].

In our previous report, gramine caused significant increase of reactive oxygen species (ROS), accumulation of malondialdehyde, stress responses of antioxidant defense system including superoxide dismutase, catalase, ascorbic acid, and glutathione. It is thus clear that the phytotoxicity of gramine on *M. aeruginosa* might be due to oxidative damage via oxidation of ROS [9]. The potential reason for gramine inhibition on *M. aeruginosa* was disclosed, but the attacking site for gramine on *M. aeruginosa* was still not well understood.

As to the antialgal mechanism of allelochemicals, four attacking sites have been summarized, including to destroy the cell structure of algae, to affect the photosynthesis of algal cells, to affect the respiration of algal cells, and to affect the enzymatic activity of algal cells [10]-[13]. Among them, photosynthesis as a key process for algal growth and reproduction is usually attacked by allelochemicals, especially photosynthetic pigments [14]-[15]. In this study, the effects of gramine on photosynthetic pigments of *M. aeruginosa* were investigated in order to assess the implication of gramine damage on algal photosynthetic apparatus and identify the most sensitive pigment probe.

II. Materials and Methods

A. Algal Species and Condition of Cultivation

Cyanobacterium *Microcystis aeruginosa* was obtained from FACHB (Freshwater Algae Culture of Hydrobiology Collection, China). The algae was cultivated in sterile media containing [16]: $NaNO_3$ 1500 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}, $ZnCl_2{\cdot}7H_2O$ 0.22 mg·L^{-1}, $CuCl_2{\cdot}5H_2O$ 0.079 mg·L^{-1}, $Na_2MoO_4{\cdot}2H_2O$ 0.039 mg·L^{-1} under an irradiance of 40-60 μmol photons·m^{-2}·s^{-1} and a photoperiod of 14 (light): 10 h (dark) at 24-25 ˚C.

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B. Gramine Treatments on M. aeruginosa

Conical flasks (500 mL) were prepared and sterilized, each of which contains 200 mL culture media. After disinfection, the initial concentration gradients of gramine added were designed as follows: 0, 0.5, 1.0, 2.0, 4.0, and 8.0 mg·L^{-1}. The media without any gramine were 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 nm and 800 nm were measured using a Shimadzu UV-2401PC spectrophotometer and a quartz cuvette with a 1 cm path length.

C. Determination of Chlorophyll a and β-carotene Contents

About 200 mL of culture media (algal density: 1×10^6 cells·mL^{-1}) was centrifugated at 4500 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 centrifugated 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 β-carotene contents [17]-[18].

D. Determination of Phycobilinprotein Contents

About 200 mL of culture media (algal density: 1×10^6 cells·mL^{-1}) was centrifugated at 4500 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 centrifugated 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 [19]-[20].

E. Data Analysis

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. Student's *t* test was used to evaluate the dose-response relationships of the algae to gramine.

III. Results and Discussion

A. Effects of Gramine on Lipid-soluble Photosynthetic Pigments of M. aeruginosa

Cyanobacterium is a kind of autotrophs. Strong photosynthetic function keeps up its growth and reproduction. Photosynthetic pigments responsible for absorbing, transferring, and transforming luminous energy into biomass are very important in photosynthesis process [21]. Photosynthetic pigments are divided into two parts: I, lipid soluble pigments; II, water soluble pigments. Chlorophyll a and β-carotene are representatives of lipid soluble pigments in cyanobacterium.

Cyanobacterium has only one kind of Chlorophylls, i.e. Chlorophyll a [22]. Chlorophyll a is involved in light absorption, transmission, and majority of Chlorophyll a participates in light transformation [21]. The effects of gramine on the contents of chlorophyll a are shown in Fig. 1. After 4 h of exposure, gramine with all concentrations in the study almost had no effects on the content of Chlorophyll a (P>0.05). When exposure time extended to 40 h, the content of Chlorophyll a was significant decrease from 100% to 55% of the controls (1 mg·L^{-1} of gramine, P<0.05), to 36% of the controls (8 mg·L^{-1} of gramine, P<0.001) (Fig. 1A). In Fig. 1B, the concentration of gramine was fixed at 2 mg·L^{-1} and chlorophyll a content decreased with the exposure time extending. Starting from 16 h of exposure, the content of chlorophyll a decreased significantly (P<0.05).

In carotenoids, β-carotene is the most abundant part of it and plays not only photosynthetic pigments but also is one important non-enzymatic antioxidant to protect algal cells from destroying by external or internal adverse factors [23]. The effects of gramine on the content of β-carotene are shown in Fig. 2. Similar to Chlorophyll a, after 4 h of exposure, gramine had no effect on the content of β-carotene. After 40 h of exposure, its decrease was more significant than that of Chlorophyll a, from 42% exposed to 1 mg·L^{-1} of gramine (P<0.05) to 24% exposed to 8 mg·L^{-1} of gramine (P<0.05) (Fig. 2A). Likewise, the significant decrease of β-carotene started from 8 h of exposure (P<0.05). The loss of antioxidant defense function might initiate the loss of other primary physiological functions. The earlier occurrence of damage on β-carotene than chlorophyll might be due to β-carotene double functions.

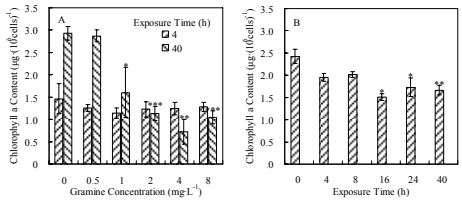

Fig. 1 Effects of gramine on chlorophyll a content of *M. aeruginosa* cells. (A) Cells treated with a concentration series of gramine for 4 h and 40 h, respectively. (B Cells treated with 2 mg·L^{-1} of gramine for a series of exposure time, respectively. Vertical bars indicate standard deviations of three replicates in each treatment group. *(P<0.05), ** (P<0.01) and *** (P<0.001) indicate significant differences compared to the corresponding controls.

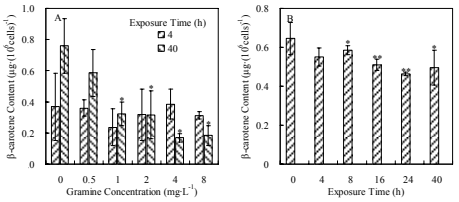

Fig. 2 Effects of gramine on β-carotene content of *M. aeruginosa* cells. (A) Cells treated with a concentration series of gramine for 4h and 40h, respectively. (E) Cells treated with 2 mg·L^{-1} of gramine for a series of exposure time, respectively. Vertical bars indicate standard deviations of three replicates in each treatment group. * (P<0.05), ** (P<0.01) and *** (P<0.001) indicate significant differences compared to the corresponding controls.

B. Effects of Gramine on Water-soluble Photosynthetic Pigments of M. aeruginosa

In cyanobacterium, water soluble photosynthetic pigments primarily are composed of phycocyanin, allophycocyanin, phycoerythrin. The three antenna pigments are particular pigments of cyanobacterium and form the large pigment total phycobilins. Total phycobilins are assembled in phycobilisomes and are attached to the thylakoid surface involved in photosynthesis [15]. The effects of gramine with different concentrations and different exposure times on the water soluble pigments are shown in Fig. 3.

On phycocyanin, gramine had almost no effect after 4 h and 40 h but only 1 mg·L^{-1} of gramine decreased it after 40 h of exposure (Fig. 3A). With 2 mg·L^{-1} of gramine added, phycocyanin content had almost no change in the algal cells exposed from 0 h to 40 h. Phycocyanin was the largest part of total phycobilins. It stayed in the outside of allophycocyanin but inside of phycoerythrin. The favorable site of phycocyanin decided that it might be protective from being destroying first by gramine.

After 4 h of exposure, the decrease of allophycocyanin content occurred from 2 mg·L^{-1} of gramine (P<0.05) (Fig. 3B). When the exposure time increased to 40 h, the content of allophycocyanin with 2 mg·L^{-1} of gramine decreased to 57% of the controls (P<0.01) and 8 mg·L^{-1} of gramine decreased to 38% of the controls (P<0.01). The damage of gramine in constant concentration on allophycocyanin with exposure time increasing was seen in Fig. 3E. Significant decrease occurred after 8 h. As the attachment status of total phycobilins on thylakoids was destroyed by gramine, allophycocyanin in total phycobilins would be exposed to gramine. The results support the hypothesis of damage on allophycocyanin. And in our other work, we also verified the hypothesis with the results of transmission electron microscopy sections [24].

Phycoerythrin was the minimum part of total phycobilins with only about 10%. Similar to allophycocyanin, its significant decrease with constant concentration of gramine (2 mg·L^{-1}) also occurred after 8 h. However, phycoerythrin was the most sensitive to gramine. After 4 h of exposure, 8 mg·L^{-1} of gramine decreased phycoerythrin content to only 18.3% of the controls (P<0.05). With the exposure time extending, the damage effect was more serious. After 40 h, the decrease was changed into 5.2% of the controls (P<0.05) (Fig. 3C). In the total phycobilins, phycoerythrin was the exterior component of the 'three-bundled polymer'. Its sensitivity (first and most serious 'being-destroyed') might be directly related with its space position exposed to gramine.

The total phycobilins function through the cooperation among phycocyanin (absorbing light energy), phycocyanin (transporting light energy to allophycocyanin), and allophycocyanin (transferring excitation energy to chlorophyll a) [25]. So the effect of gramine on total phycobilins was the synthesized reflection of all three functional subunits. Its content was beginning to significantly decrease after 4 h when exposed to 8 mg·L^{-1} of gramine (P<0.05) (Fig. 3D) and after 24 h when exposed to 2 mg·L^{-1} of gramine (P<0.05) (Fig. 3E).

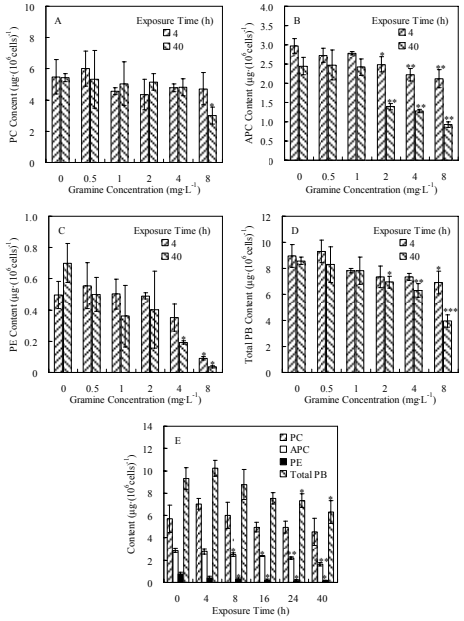

Fig. 3 Effects of gramine on phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE) and total phycobilins (PB) contents of *M. aeruginosa* cells. (A-D) Cells treated with a concentration series of gramine for 4 h and 40 h, respectively, showing PC, APC, PE and PB contents, respectively. (E) Cells treated with 2 mg·L^{-1} of gramine for a series of exposure time, respectively. Vertical bars indicate standard deviations 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.

C. Effects of Gramine on Absorption Spectra of M. aeruginosa

The above results about the effects of gramine on lipid soluble and water soluble pigments were from short-term studies but not long-term. For more visualized understandings of gramine effects on photosynthetic pigments of *M. aeruginosa*, the adsorption spectra of cell suspension was investigated.

In this study, algal adsorption spectra analysis was based on the maximum peaks of various pigments. Chlorophyll-a maximum peaks were at 436 nm and 678 nm; Phycocyanin maximum peak was at 626 nm; β-carotene maximum peak was at 464 nm with a shoulder at 490 nm [26]. In Fig. 4, the

bleaching of pigments exposed to gramine no matter 1 mg·L^{-1} or 8 mg·L^{-1} was serious. Especially, 8 mg·L^{-1} of gramine caused almost loss of peak absorption. The pigment bleaching was more significant after 4 d than after 3 d.

Additionally, the color of *M. aeruginosa* cultures could be observed to from blue green in the short-term stage (0-40 h) (After 40 h, still a large amount of phycocyanin kept in the algal cells and it was the determinant of blue green of *M. aeruginosa*), yellow green in the long-term stage (3-4 d) (decrease or even loss of peak absorption of pigments including chlorophyll a, phycocyanin, and β-carotene) to color fading in the last stage (7-8 d) (full loss of peak absorption of pigments verified by naked eyes).

Several studies about allelochemical effects on photosynthetic pigments have already been reported. Wu *et al.* investigated the allelopathic inhibitory effect and eco-physiological mechanism of *M. aquaticum* culture water on *M. aeruginosa* and found that phycobiliprotein would be the antialgal target of *M. aquaticum*. Moreover, they put forward the question which components of phycobilinprotein be most sensitive to the culture water and pointed out more specific evidence should be provided by future research [14]. The above study was focused on culture water but not pure allelochemicals.

In our previous study, the effects of allelochemical ethyl 2-methylacetoacetate (EMA) on photosynthetic pigment composition has been investigated and the results pointed out that allophycocyanin, phycoerythrin, and carotenoid were sensitive to EMA than other pigments [15]. Unfortunately, the results of EMA effects on photosynthetic pigments were not very specific and did not give a sound answer about which component is most sensitive. The results in this study clearly told that phycoerythrin as one component of phycobilinprotein was the most sensitive to antialgal allelochemical gramine. And in combination with the reports about the structure model of phycobiliprotein, it was concluded that phycoerythrin most sensitive to gramine might be contributed to its placing the outside of phycobilins.

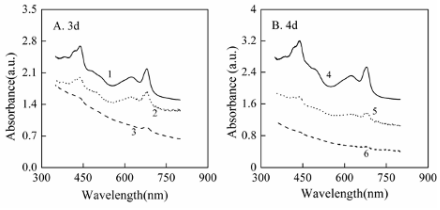

Fig. 4 Effects of gramine on absorption spectra of *M. aeruginosa* suspension. (A) Exposure to gramine for 3 d; (B) Exposure to gramine for 4 d. (1, 4) Cells without addition of gramine; (2, 5) Cells exposed to 1 mg·L^{-1} of gramine; (3, 6) Cells exposed to 8 mg·L^{-1} of gramine.

ACKNOWLEDGMENT

This study was supported by National Natural Science Foundation for the Youth (No. 30900128), Beijing Forestry University Young Scientist Fund (No. BLX2008025), Fundamental Research Funds for the Central Universities (No. 200-1240631) and National Science Fund for Distinguished Young Scholars (No. 50825801).

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