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Effects of Allelochemical Gramine on Metabolic Activity and Ultrastructure of Cyanobacterium *Microcystis aeruginosa*

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***Abstract*—**In this study, inhibition of *Microcystis aeruginosa* by antialgal alleochemical gramine, was studied by analyzing algal metabolic activity (represented by esterase and total dehydrogenase activities) and cell ultrastructure (showing morphological and ultrastructure alterations using transmission electron microscopy and DNA ladder analysis). After gramine exposure, esterase and total dehydrogenase activities were increased firstly but decreased later. In contrast with the controls, the cells exposed to gramine showed apparent ultrastructure alterations with thylakoids in breakage, phycobilins in decrease, lipid and cyanophycin granules abundant firstly but dissolved afterwards, DNA in fragementation. The occurrence of increase of metabolic activity and specific granules reflected that the resistance of cellular response to gramine was initiated. DNA fragementation associated with the increase of metabolic activity and specific granules hinted that gramine caused *M. aeruginosa* cells to initiate some morphotype of programmed cell death.

***Keywords*—**Allelochemical, gramine, metabolic activity, *Microcystis aeruginosa*, ultrastructure.

I. Introduction

ALLELOPATHY is definited to be the process in which allelochemicals excreted by plants including microorganisms influence the growth or physiology of other organisms [1]. Antialgal allelopathy or allelochemicals have many strongpoints compared with the traditional algicides (such as copper sulfate), for example, the secretion or allelochemicals could be easily degraded in natural environments [2], and allelopathic effects on algae were usually species-specific [3]. Therefore, antialgal allelopathy and allelochemicals are considered good alternatives for algal bloom control and the related study has become a great concern [4].

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Till now, several types of allelochemicals have been isolated and identified, including α-asarone and phenylpropane from *Myriophyllum verticillatum*, pyrogallic acid, ellagic acid, and nonanoic acid from *M. spicatum*, ethyl 2-methylacetoacetate (EMA) *from Phragmites communis*, N-phenyl-1-naphthylamine and N-phenyl-2-naphthylamine from *Eichhornia crassipes*, gramine (N,N-dimethyl-3-amino-methylindole) from *Arundo donax* [5]-[9]. Among them, gramine with 0.47 $mg \cdot L^{-1}$ of medium effective concentration to inhibit the growth of *Microcystis aeruginosa* was isolated and identified in our previous study [10]. Compared with other allelochemicals, gramine inhibiton on *M. aeruginosa* was one of the strongest [9].

There have been some research results about gramine inhibition on *M. aeruginosa*. Among them, gramine was found to induce large amount of reactive oxygen species to accumulate in cells, to suppress the activities of enzymatic antioxidants, to decrease the non-enzymatic antioxidant contents [11]. These findings pointed out the upstream point to make which imbalance would be the cause of gramine inhibition on *M. aeruginosa* at first. The growth inhibition always gradually became serious dependent on the downstream action. So we concentrated our following studies into clarifying the downstream point or final way for 'ripple effect' to go at last.

In this study, metabolic activity (reflected by esterase activity and totoal dehydrogenase activity) and algal ultrastructure alterations including appearance or disappearance of cellular inclusions and changes of DNA status were examined to judge if any cellular resistance appeared or some morphotype of programmed cell death was involved in.

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 [12]: $NaNO_3$ 1500 $mg \cdot L^{-1}$,$K_2HPO_4 \cdot 3H_2O$ 40 $mg \cdot L^{-1}$, Na_2CO_3 20 $mg \cdot L^{-1}$, $MgSO_4 \cdot 7H_2O$ 75 $mg \cdot L^{-1}$, $CaCl_2 \cdot 2H_2O$ 36 $mg \cdot L^{-1}$, Na2EDTA 1 $mg \cdot L^{-1}$, $C_6H_8O_7$ (citric acid) 6 $mg \cdot L^{-1}$, $Fe(NH_4)_3(C_6H_5O_7)_2$ (ferric ammonium citrate) 6 $mg \cdot L^{-1}$, $Co(NO_3)_2 \cdot 6H_2O$ 0.049 $mg \cdot L^{-1}$, H_3BO_3 2.86 $mg \cdot L^{-1}$,

$MnCl_2 \cdot 4H_2O$ 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.

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.

C. Esterase Activity Analysis

The effects of gramine on esterase activity were investigated by flow cytometric analysis using fluorescein diacetate (FDA) (F7378, Sigma, USA). FDA is a classic dye which can diffuse into cells where it is cleaved by non-specific esterases to yield the fluorescent product fluorescein. Accumulation of fluorescein is therefore the result of intracellular esterase activity. The fluorescence signal detected is proportional to the esterase activity. The esterase activity was determined as described by Yoshida *et al.* [13]. The gramine-treated and control algal cells were harvested into 1 mL of phosphate buffered saline (PBS) solution (50 mM, pH 7.0) after repetitive suspensions. Twenty microlitres of 1 mg·mL^{-1} FDA was then added into the last incubation saline. Before measurements, staining was done in the dark for 20 min at 25 ˚C and then cells were washed twice with fresh PBS. Cells were analyzed by a FACS Calibur (Becton Dickinson, USA) to detect fluorescein fluorescence at the FL-1 parameter. The fluorescein fluorescence (emission wavelength of fluorescein: 525 nm) is excited with Argon-ion laser (excitation wavelength: 488 nm). The number of algal cells detected by flow cytometer in every curve of the same figure is the same. The event of detection is at least 2000 cells per treatment. The relative esterase activity (%) is calculated by the formula given below:

$$\text{Relative Esterase Activity (\%)} = \frac{\text{Mean Fluorescein Fluorescence Intensity[Gramine-added]}}{\text{Mean Fluorescein Fluorescence Intensity[Control]}} \times 100$$

D.2,3,5-Triphenyltetrazolium Chloride (TTC) Reduction Analysis

The measurement of TTC reduction is based on the formation of formazan. Algal cells were immersed in the incubation solution (50 mM phosphate solution, pH 7.4 with 0.05% (v/v) TritonX-100) containing 0.6% (w/v) TTC, and incubated at 25 ˚C for 24 h in darkness without shaking. The cells were rinsed twice with ultrapure water and extracted four times by 95% (v/v) ethanol (2 mL each time) for 5 min at 85 ºC. The extracts were combined and concentrated into 4 mL. The formazan formed in the algae was measured at 530 nm instead of 485 nm to avoid the interference of pigments [14]-[15]. The positive controls were the groups with algae treated with 0 mg·L^{-1} of gramine and appropriate reagents of TTC-reduction test. The negative controls were the groups with appropriate reagents of TTC-reduction test and the algae without gramine treatment that was boiled for 10 min to ensure that the enzymes were denatured. The experimental groups of TTC reduction were the groups with algae treated with appropriate concentration of gramine and reagents of TTC-reduction test.

E. Transmission Electron Microscopy (TEM)

The algal cells for TEM observations were taken from cultures inoculated with 1×10^6 cells·mL^{-1}, either with different exposure time to 2 mg·L^{-1} of gramine or without addition of gramine. All treatments were obtained from at least three cultures and processed separately. The samples were harvested from cultures by centrifugation at 4500 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 Spurr's 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) [16]-[17].

F. DNA Ladder Analysis

DNA Ladder analysis was referred to Liu *et al.* and Ning *et al.* [18]-[19]. All algal groups include the cells treated with 0, 1.0, and 8.0 mg·L^{-1} of gramine for 1 d and 8 d. The cells with no gramine added for 1 d and 8 d were used for 'Control'. Algal cells with the quantity of 1×10^7 cells were collected from algal medium by centrifugation in 5000 rpm for 5 min at 4 ˚C. The algal pellet was immersed in the solution (20mM pH 8.0 EDTA, 100 mM pH 8.0 Tris, 0.8% (w/v) SDS) for 30 min at 50 ˚C. Then the supernatant was kept after centrifugation in 12000 rpm for 5 min. Ten μL of RNA enzyme A/T1 mixture (500 U·mL^{-1}/20000 U·mL^{-1}, produced by Ambion Inc.) was added in the supernatant and incubated for 2 h at 37 ˚C. Next, 10 μL of Protease K (20 mg·mL^{-1}, produced by Ambion Inc.) was added and kept for 2 h at 37 ˚C. Algal DNA was precipitated by 10 μL of 5M NaCl and 50 μL of isoamyl alcohol for 12 h at -20 ˚C. The precipitated DNA was immersed in 20 μL of the solution with 20 mM pH 8.0 EDTA and 100 mM pH 8.0 Tris and then mixed with 5 μL of 6×DNA loading buffer (30% glycerol, 0.25% bromophenol blue). The quality of DNA was examined on 1.5% agarose gel with voltage of 35 V for 4 h or when the dye reached to 2/3 of the gel length. The gel was staining with 1.0 μg·mL-1 ethidium bromide for 1 h after eletrophoresis.

G. 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 Metabolic Activity of M. aeruginosa

The metabolic activity of *M. aeruginosa* with gramine added is shown in Fig. 1.

Esterase activity was an important indicator to reflect cellular metabolic status. No activity changes after 2 h of exposure and activity increase after 24 h (P<0.05 at 1 mg·L^{-1} and 8 mg·L^{-1} of gramine) occurred following with activity decrease after 48 h (P>0.05 at high concentration of gramine versus the controls) (Fig. 1A). The dynamic show of results was seen in Fig. 2. From flow cytometer seeing, algal cells dyed with FDA when cleaved by intracellular esterases were in fluorescence and esterase activity increase caused fluorescence to intensify; Through recording the cell counting and cellular fluorescence and delineating them into a FCM figure, the visual results could dynamically reflect the detailed changes not being shown in Fig. 1A. The increase peak of esterase activity (Fig. 2B) hinted that there being a vigorous metabolic stage during gramine inhibition on *M. aeruginosa* and it might be due to the initiation of cellular resistance mechanism or programmed cell death. Franklin and Berges reported that dinoflagellate *Amphidinium carterae* was in an alternative morphotype of programmed cell death with increasing esterase activity after short-term exposure to darkness pressure [20]. Similar to the phenomena found in *A. carterae*, *M. aeruginosa* was verified to be exposed to the significant increase of ROS pressure caused by allelochemical gramine and the results of increase esterase activity found in this study inferred that its increase might be due to the starting of programmed cell death.

TTC-reduction test was used for the assay of dehydrogenase reduction potential, which is a vital index for cell vitality [21]. In Fig. 1B, relative TTC reduction to reflect total dehydrogenase activity in cells had no obvious increase or decrease with a concentration series of gramine exposure after 4 h. However, when the exposure time extended to 40 h, the changes happened. Increase of total dehydrogenase activity occurred when gramine concentration increased to 1 mg·L^{-1} (P<0.05). Its increase only lasted to 4 mg·L^{-1} of gramine (P<0.05) and then 8 mg·L^{-1} of gramine caused the total dehydrogenase activity in decrease. Its value dropped to that of the controls even with no significant difference (P>0.05). Cellular reduction decrease reflected that cells were at inferior position for growth and reproduction and even in damage exposed to some adverse factors [22]. In this study, gramine as a pressure factor suppressed the growth of *M. aeruginosa* and decrease total dehydrogenase activity finally. In addition, an increase of relative TTC reduction brought into correspondence with the increase of esterase activity and also supports the hypothesis that cellular resistance or programmed cell death was initiated in *M. aeruginosa* cells.

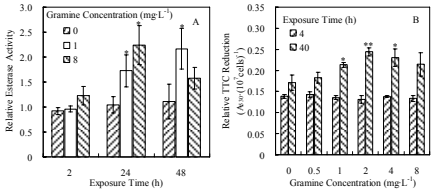

Fig. 1 Activities of esterase and total dehydrogenase in *M. aeruginosa* exposed to different concentrations of gramine. A, esterase activity reflected by the fluorescence intensity of fluorescein; B, total dehydrogenase activity reflected by the relative TTC reduction. Data are the means ± SD of three replicates. * (P<0.05), ** (P<0.01) and *** (P<0.001) indicate significant differences compared to the corresponding controls.

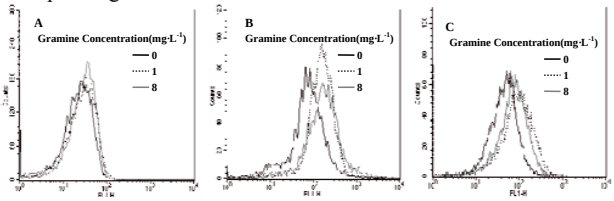

Fig. 2 Effects of gramine on esterase activity in *M. aeruginosa*. Y-axis (counts): the number of algal cells detected by flow cytometer; X-axis (FL1-H): the fluorescence intensity under the emission wavelength of fluorescein (525 nm); The number of algal cells detected by flow cytometer in every curve of the same figure is the same. The event of detection is at least 2000 cells per treatment. The value used for evaluating effects of gramine on esterase activities with different treatments is fluorescence intensity, and the changes of curves represent changes of algal populations in the relevant value of fluorescence with statistical significance. A, 2 h of gramine exposure; B, 24 h of gramine exposure; C, 48 h of gramine exposure.

B. Effects of Gramine on Ultrastructure of M. aeruginosa

For deeper understandings on how *M. aeruginosa* responded to gramine, algal ultrastructure was investigated. Using TEM sections to reveal different characteristics changing with exposure time is favorable for gramine effect on cellular ultrastructure. The 'stage by stage' collapse of *M. aeruginosa* caused by gramine is clearly shown (Fig. 3). In Fig. 3A, the control cells were regular with integral cell wall outside; the gap between cell membrane and cell wall was in uniformity; no redundant granules accumulated in cells; thylakoids were abundant and well-distributed; the phycobilisomes were regularly distributed on thylakoids. In comparison with the controls, the cells with the exposure of gramine were altered in cellular ultrastructure significantly. Different alterations occurred with the exposure time extending. After 12 h, the density of thylakoids gradually decreased with some in breakage; the phycobilisomes on thylakoids also were reduced, accompanying with the occurrence of abundant lipid granules (Fig. 3B). After 18 h, lipid granules were preserved at a great quantity, and further, a mass of cyanophycin granules appeared in these cells (Fig. 3C); From 24 h to 60 h, the cell wall began to be destroyed with cellular inclusions in leakage, moreover, polyhedral bodies and lipid granules as well as cyanophycin granules were reduced gradually with only polyphosphate bodies in almost no changes at all. At last, algal cells lost the fundamental structure into disappearance (Fig. 3D-3F).

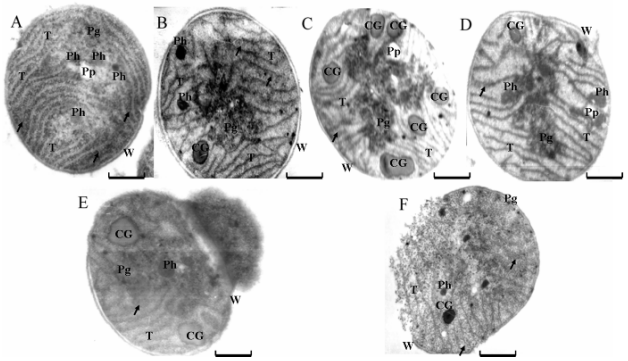

Fig. 3 Effects of gramine on fine structures of *M. aeruginosa* cells. (A) Cell without treatment, showing layered cell wall (W), electron-dense phycobilinsomes (arrow) on thylakoid membranes (T), plastoglobuli (Pg), polyhedral bodies (Ph), and polyphosphate bodies (Pp). (B-G) Cells with 2 mg·L^{-1} of gramine treatments for 12h, 18h, 24h, 48h, 60h, 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 of thylakoid membranes, leakage of cellular inclusions and/or the changes in other cell structure. Scale bar = 0.5 μm.

The appearance and disappearance of specific granules inferred that the algal cells responded to gramine. Cyanophycin granules usually appeared when cyanobacterial cells was stressed by external or internal pressure factors [23]-[24]. In this study, the accumulation of cyanophycin granules demonstrated that *M. aeruginosa* was seriously stressed by gramine. The accumulation of lipid granules also supported the above statement [25]. Polyhedral bodies are the places in which carbon dioxide (CO_2) was fixed by algal cells and a large amount of ribulose-1,5-bisphosphate carboxylase/oxygenase and carbonic anhydrase were encapsulated for enquired for CO_2 fixation [26]. The fact that gramine decreased polyhedral bodies of *M. aeruginosa* implied that algal carbon assimilation process might be destroyed. Accompanying with the decrease of polyhedral bodies, algal cells would be completely disabled following with loss of lipid granules and cyanophycin granules.

The above results including the peak increase of metabolic activity and accumulation of cyanophycin granules reflected that algal cells were in stress to initiate short-term resistance mechanism, and even programmed cell death. To offer further proof for the hypothesis, DNA status was assayed using DNA ladder method.

The same number of algal cells no matter in the controls or the treatment groups was collected for DNA ladder assay. After 1 d of exposure, DNA content was found to be increased by 1 mg·L^{-1} of gramine with the proof of improved ultraviolet absorbance; nevertheless, the decrease of DNA content appeared when algal cells were exposed to 8 mg·L^{-1} of gramine; and DNA fragmentation was less pronounced (the left arrow in Fig. 4). When the exposure time extended to 5 d, significant DNA fragmentation occurred (right gray arrows shown in Fig. 4) and almost no DNA was preserved integrity.

These data indicated that gramine treatment caused DNA 'fragmentation-like' degradation in *M. aeruginosa* cells. Fragmentation of nuclear DNA is the biochemical hallmark of programmed cell death in eukaryotes. In prokaryotes, programmed cell death is presented as "proapoptosis' [27]. Ning *et al.* demonstrated that salt stress induced programmed cell death in prokaryotic cyanobacterium *Anabaena* sp. PCC7120 with 'fragmentation-like' degradation [19]. Therefore, on the basis of 'DNA ladder' results in this study, we deduced that gramine might lead the cells of *M. aeruginosa* to programmed cell death.

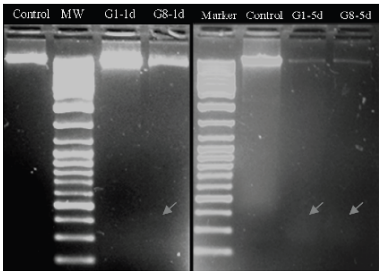

Fig. 4 Effects of gramine on DNA structures of *M. aeruginosa* cells. From the left to the right of the above label, cells without treatment, showing 'control', cells with 1 mg·L^{-1} of gramine treatments for 1 d, showing 'G1-1d', 8 mg·L^{-1} for 1 d, showing 'G8-1d', 1 mg·L^{-1} for 5 d, showing 'G1-5d', 8 mg·L^{-1} for 5 d, showing 'G8-5d'. DNA small fragmentation was arrowed.

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She now is working at College of Environmental Science and Engieering, Beijing Forestry University in Beijing City of P.R. China, is major in Environmental Science and Engineering from 2008. Till now, she has published more than thirty scientific journal articles, including eleven SCI-cited articles. The research directions include: environmental toxicology and safety evaluation of environmental pollutants, ecological restoration techniques, algal growth control theory and technology, theory and technique of forest waste comprehensive utilization, algal and aquatic plant allelopathy.

Dr. Hong now serves as membership of International Water Association (IWA), membership of Chinese Environmental Sciences Association, life member of Asia Allelopathy Association, and the member of China organizing committee of the Sixth World Congress on Allelopathy.