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Effects of pyrogallic acid on *Microcystis aeruginosa*: oxidative stress related toxicity

Zhiying Lu [a,b], Yongyuan Zhang [a], Yunni Gao [a,c], Biyun Liu [a,*], Xuemei Sun [a,b], Feng He [a], Qiaohong Zhou [a,*], Zhenbin Wu [a]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei 430072, China
[b] University of Chinese Academy of Sciences, Beijing 100039, China
[c] College of Fisheries, Henan Normal University, Xinxiang, Henan 453007, China

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ABSTRACT

Pyrogallic acid (PA) is used in various industrial and consumer products. The molecular mechanisms underlying PA's toxicity was not fully understood. In this study, toxicity of PA on *Microcystis aeruginosa* with reactive oxygen species (ROS) generation as an end point was investigated. The results showed an increase in the percentage of cells with loss of membrane integrity and enhanced intracellular ROS production. Exposure to 50 mg L^{-1} PA for 48 h caused the highest percentage of loss of membrane integrity (56.7%), and a 2.54-fold higher intracellular ROS level compared to control. Further investigation revealed that PA caused a dose-dependent increase in DNA strand breaks (DSB) of *M. aeruginosa* at exposure concentration from 2 to 50 mg L^{-1}. The incubation of cells with ROS scavengers ascorbic acid, N-acetyl-L-cysteine (NAC) and tocopherol markedly alleviated the level of PA-induced DSB. Analysis of PA autoxidized products in culture solution showed that PA was quickly converted to purpurogallin (PG), and PG was further autoxidized to other polyphenolic compounds. PA and PG might participate a futile redox cycle, which mediated ROS production in *M. aeruginosa*. These results suggested DNA strands and cell membrane were two targets of ROS induced by PA, and oxidative damage was an important mechanism for the toxicity of PA against *M. aeruginosa*.

1. Introduction

As commonly used in many industrial and consumer products, pyrogallic acid (PA) was widely distributed in nature (Gilner et al., 1994). In biodiesel industry, PA was commonly used as an additive added to the biodiesel to increase the oxidation stability. In photographic industry, PA was widely applied as a photographic developing agent. In hair dying industry, PA was largely employed as an antiseptic (Upadhyay et al., 2010; Avase et al., 2015). Despite its beneficial properties, PA-mediated toxicity has been a major concern for almost all vital organs exposed to it. It was reported that PA showed mutagenic effect and liver, lung, kidney and gastrointestinal tract were its major target organs (Upadhyay et al., 2010). In addition, PA was also suggested to have bactericidal effects against both gram-negative and gram-positive bacteria. Tinh et al. (2016) reported that PA could inhibit *Vibrio parahaemolyticus* at concentrations of 32–64 mg L^{-1}.

Recently, PA as strong allelochemical attracted great attention. Release of the allelochemicals (secondary metabolite) by the submerged macrophytes was considered to be an approach to inhibit the growth of phytoplankton (Gross, 2003; Hilt and Gross, 2008). Nakai et al. (2000) reported that four polyphenolic allelochemicals, ellagic acid, gallic acid, pyrogallic acid (PA) and (+)-catechin were identified in the culture water of *Myriophyllum spicatum*. Among these polyphenolic compounds, PA showed the strongest inhibitory activity to *Microcystis aeruginosa* with 50% effect concentration (EC_{50}) of 0.65–2.97 mg L^{-1} (Nakai et al., 2000; Zhu et al., 2010). Previous studies proposed that the inhibitory effect of PA against *M. aeruginosa* may involve of oxidative stress (Wu et al., 2007), interruption of the electron transfer chain of PSII (Dziga et al., 2007; Zhu et al., 2010), and interfering the expression of antioxidative gene (Shao et al., 2009). However, the complete molecular mechanism underlying its toxicity on phytoplankton was not yet completely elucidated.

Reactive oxygen species (ROS), was a blanket term for a collection of partially reduced oxygen containing molecules, including superoxide ($O_2^{\bullet -}$), peroxides (H_2O_2 and ROOH) and free radicals ($HO^{\bullet}$ and $RO^{\bullet}$) (Dixon and Stockwell, 2014). Certain amounts of ROS were crucial for normal cell and organismal function (Dröge, 2002). However, increased level of intracellular

* Corresponding authors.
E-mail addresses: liuby@ihb.ac.cn (B. Liu), qhzhou@ihb.ac.cn (Q. Zhou).

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ROS was linked to the damage of biological macromolecule, such as DNA, proteins (enzymes), and lipids (membrane) etc. (Gill and Tuteja, 2010). Our previous studies indicated that the excessive production of ROS could be induced in *M. aeruginosa* exposed to PA (Wang et al., 2011). At present, it was unclear whether there is a cause-effect relationship between PA exposure and DNA or membrane damage.

To understand the cellular and molecular effects of PA-mediated toxicity on phytoplankton, *M. aeruginosa* was used as test species and the single dose acute toxic experiment was performed in present study: (a) to investigate if PA can induce DNA strand breaks and membrane damage, (b) to analyze the relationship between ROS and DNA and membrane damage, and (c) to explore the mechanism of ROS production induced by PA in *M. aeruginosa.*

2. Materials and methods

2.1. Reagents

2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), *N*-acetyl-L-cysteine (NAC), ascorbic acid, and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bis(trimethylsilyl) trifluoroacetamide (BSTFA, Derivatization Grade) was purchased from Supelco (Bellefonte, PA, USA). Bisbenzimide Hoechst 33258, tocopherol and lysozyme were obtained from Alfa Aesar (Heysham, Lancashire, UK). PA (AR, 99.5%, Chem Service, West Chester, PA, USA) was obtained from Invitrogen (CA, USA). DCFH-DA stock solution (2 mM) in DMSO and Hoechst 33258 stock solutions (60 μM) in phosphate buffer saline (PBS, 100 mM, pH 7.6) were prepared and stored at 4 °C in the dark.

2.2. Culture conditions and treatments of test organism

The *M. aeruginosa* FACHB 905 strain was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, the Chinese Academy of Sciences. *M. aeruginosa* was cultured in the BG11 medium (Rippka et al., 1979) at 25 ± 2 °C using fluorescent lamps as illuminant with a light: dark cycle of 12 h: 12 h and a irradiance of 57 μmol photons $m^{-2} s^{-1}$ and shaken three times each day. All of the experiments were conducted with exponentially growing cells.

The exposure experiment was performed in 250 mL conical flasks in which 100 mL BG11 culture solution was inoculated with initial density of 5×10^6 cell mL^{-1}. PA was added with the concentration of 2, 10, 25 and 50 mg L^{-1}. The cultures without PA were used as the control. The *M. aeruginosa* culture samples were taken from each flask at exposure of 6, 12, 24 and 48 h, and the cells were collected for the detection of membrane integrity, intracellular ROS level and DSB. To prove the role of ROS in the PA-induced DNA strand breaks, the cells were treated with 50 mg L^{-1} PA for 12 h in the presence of different ROS scavengers ascorbic acid (1, 5 and 10 mM), NAC (1, 5 and 10 mM) and tocopherol (1, 5 and 10 mM), then DSB in test organism was detected.

2.3. Assessment of the membrane integrity by flow cytometry

Propidium iodide (PI) was excluded from normal cells but can enter into cells with a leaky membrane, where it bound to nucleic acids to make these cells highly fluorescent. The PI staining procedure was performed as previously described (Xiao et al., 2011). The fluorescence of the samples was detected by flow cytometer (BD accuri C6, US) with an excitation/emission wavelength of 485/610 nm. Ten thousand events were recorded for each sample. The cells treated in boiling water for 10 min and the unstained cells were used as positive control (100% loss of membrane integrity) and negative control (background signal), respectively. The ratio of loss of membrane integrity was expressed as the percentage of stained cells (PI-positive) relative to the control group.

2.4. Detection of intracellular ROS (peroxides and hydroxyl) level

ROS formation was measured by using the cell permeable indicator 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA was hydrolyzed by cellular esterases to form the non-fluorescent 2′, 7′-dichlorodihydrofluorescein (DCFH) after penetrating into the cell of the test organisms, and then DCFH is immediately transformed to highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of $HO^{\bullet}$, H_2O_2 and H_2O_2-peroxidase (Gutteridge and Halliwell, 2010). The method to detect intracellular ROS level was referred to Hong et al. (2009). Briefly, one milliliter of culture sample was centrifuged at 1500 × *g* for 15 min and washed twice with 100 mM PBS (pH = 7.0). Then the pellet was suspended in 500 μL PBS containing 10 μM DCFH-DA, and it was placed in the dark for 60 min at 25 °C. The flow cytometric measurements were performed on a flow cytometer (BD accuri C6, US) with excitation/emission wavelength of 485/535 nm. Ten thousand events were tested for every sample. The relative ROS level (%) was calculated by the formula given below:

$$\text{Relative ROS level}\,(\%) = \frac{\text{DCF fluorescence}_{\,[\text{treated sample}]}}{\text{DCF fluorescence}_{[\text{control}]}} \times 100\%$$

2.5. Measurement of DNA strand breaks

The DNA strand breaks (DSB) were determined by the fluorometric analysis of DNA unwinding (FADU), as described by He and Häder (2002) with a some modification in the cell lysis procedure according to Kanto et al., (2005). Briefly, 10 mL samples of *M. aeruginosa* cells were harvested by centrifugation at 1500 × *g* for 15 min. The pellet was washed with TE (Tris-ethylenediaminetetraacetic acid, pH 8.0) buffer and resuspended in lysis buffer (40 mM EDTA, 400 mM NaCl, and 50 mM Tris-hydrochloride, pH 9.0) to a volume of 415 μL. Then, 50 μL of 10 mg mL^{-1} lysozyme was added, and the mixture was incubated for 20 min at 37 °C to destroy the cell walls completely. A 25 μL of 10% sodium dodecyl sulfate (SDS) and 10 μL of 10 mg mL^{-1} proteinase K were added to a final volume of 500 μL and incubated at 50 °C for 2 h to lyse the cells. Then, 500 μL of 0.1 M NaOH was added to the sample. The following steps were operated as the method of He and Häder (2002). The strand scission factor (SSF) was calculated as follows:

$$\text{SSF} = -\ln\left(F_t/F_c\right)$$

where the F_t and F_c were the double-stranded DNA fraction of the treated and untreated cells, respectively. SSF = 1 was equivalent to the amount of 16 000 free DNA ends per cell in the FADU (Christa et al., 2000; Junk et al., 2014).

2.6. Analysis of PA and its autoxidation products

PA was unstable and was easily autoxidized by dissolved oxygen in the aqueous solution. To better understand the toxicological effect of PA on phytoplankton, it was necessary to investigate the change of PA and its autoxidized products during autoxidation. With this purpose, PA with concentration of 50 mg L^{-1} was added to BG11 culture media, and the mixtures were incubated as culture conditions mentioned above. The parent compound and its autoxidized products were determined by Gas chromatography-Mass spectrum (GC–MS), briefly as follows: 10 mL sample was collected and acidified to pH = 2 and extracted with ethyl acetate. The extracts were passed through an anhydrous sodium sulfate column