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Inhibitory effects of sanguinarine against the cyanobacterium *Microcystis aeruginosa* NIES-843 and possible mechanisms of action

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ABSTRACT

Sanguinarine showed strong inhibitory effect against *Microcystis aeruginosa*, a typical water bloom-forming and microcystins-producing cyanobacterium. The EC50 of sanguinarine against the growth of *M. aeruginosa* NIES-843 was 34.54 ± 1.17 μg/L. Results of chlorophyll fluorescence transient analysis indicated that all the electron donating side, accepting side, and the reaction center of the Photosystem II (PS II) were the targets of sanguinarine against *M. aeruginosa* NIES-843. The elevation of reactive oxygen species (ROS) level in the cells of *M. aeruginosa* NIES-843 upon exposure indicated that sanguinarine induced oxidative stress in the active growing cells of *M. aeruginosa* NIES-843. Further results of gene expression analysis indicated that DNA damage and cell division inhibition were also involved in the inhibitory action mechanism of sanguinarine against *M. aeruginosa* NIES-843. The inhibitory characteristics of sanguinarine against *M. aeruginosa* suggest that the ecological- and public health-risks need to be evaluated before its application in cyanobacterial bloom control to avoid devastating events irreversibly.

1. Introduction

Eutrophication is a widespread problem in aquatic ecosystems around the world (Bennett et al., 2001). Cyanobacteria frequently dominate in eutrophic waters, and cause severe water quality problems including scum formation, toxin production, hypoxia, bad taste and odors. *Microcystis aeruginosa* is a typical water bloom-forming and microcystins-producing cyanobacterium. Microcystins can inhibit the eukaryotic protein phosphatases PP1 and PP2A, and cause liver cancer and tumors in humans and wild animals (Carmichael, 1995). Therefore, control of harmful *M. aeruginosa* based bloom is crucial for maintenance of safe water supplies and also ecological health.

Some chemicals, such as $CuSO_4$ and herbicides have been used to control harmful algal blooms (HABs) (Anderson, 1997). Though these chemicals can rapidly control cyanobacterial blooms from appearing visually, they also cause secondary pollution of the aquatic environments due to chemical residues and stability in the environment. Biologically derived substances (BDSs), e.g. polyphenols (Nakai et al., 2000), fatty acid (Nakai et al., 2005) and L-lysine (Yamamoto et al., 1998) are known to inhibit the growth of aquatic bloom-forming cyanobacteria. Selective BDSs have no- or low-toxicity to non-target aquatic animals and humans. As substances originated from natural sources, most of them are easily biodegraded in aquatic environments. These characteristics make BDSs one of the most promising substances in control of HABs.

Though many BDSs have been identified, few of them are applicable in field conditions. One major reason for this is that most BDSs only show weak inhibitory effects against cyanobacteria. For example, the EC_{50} of β-ionone, ellagic acid, linoleic acid against the growth of *M. aeruginosa* was 21,200 μg/L (Shao et al., 2011), 5100 μg/L (Nakai et al., 2000), 3670 μg/L (Ni et al., 2011), respectively. All of them are far higher than synthetic algicidal chemicals used currently. Pyrogallic acid and ethyl 2-methylacetoacetate are two mostly reported BDSs against *M. aeruginosa*. But the EC_{50} values of each of them are all 650 μg/L (Nakai et al., 2000; Li and Hu, 2005), which is far higher than the EC_{50} of synthetic algicidal chemicals, e.g. $CuSO_4$ (EC_{50} at approximately 80 μg/L, Qian et al., 2010), cyanazine (EC_{50} 37.6 μg/L, Ma et al., 2010). Sanguinarine is a benzophenanthridine alkaloid derived from the root extract of *Sanguinaria canadensis* and other poppy-fumaria species, and it has natural antibacterial activities (Kim et al., 2008). Our previous

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results showed that the EC_{50} of sanguinarine against the growth of *M. aeruginosa* was 34.54 ± 1.17 μg/L. Among the reported BDSs, it stands out as the strongest one against *M. aeruginosa*.

PS II of photosynthetic organisms has been found to be sensitive to environmental changes (Demmig-Adams and Adams, 1992). Chlorophyll fluorescence can indicate the photosynthetic efficiency and provide useful information on the physiological condition of the organisms as well as the relationship between structure and function of PS II (Christen et al., 2007), and it has been widely used in evaluation of toxic effect and toxic targets of various stresses on cyanobacterial photosystem. Analysis of targeting site on molecular level is also a useful tool in the evaluation of toxic effects and elucidating toxic mechanisms of environmental stress against microorganisms (Qian et al., 2010).

In order to elucidate the inhibitory effects and the possible mechanisms of toxicity of sanguinarine against *M. aeruginosa*, growth characteristics, chlorophyll fluorescence transients, cellular ultrastructure, and gene expression profiles of *M. aeruginosa* NIES-843 under sanguinarine stress were studied in this investigation.

2. Materials and methods

2.1. Cyanobacterial strain, culture conditions and chemicals

M. aeruginosa NIES-843 was kindly provided by the National Institute of Environmental Science, Japan. It was grown in CT liquid medium (pH 8.5) (Ichimura, 1979), under a 12 h:12 h (light/dark) cycle with a light intensity of 30 μmol photons/(s m^2) provided by cool white fluorescent tubes at 25 ± 1 °C. Sanguinarine (≥98% purity) (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium chloride; molecular weight, 367.78) was purchased from Sigma (Sigma, St. Louis, MO, USA).

2.2. Inhibitory effects of sanguinarine on the growth of M. aeruginosa NIES-843

Experiments were carried out in 250 mL Erlenmeyer flasks containing 94.9 mL CT liquid medium which were sterilized by autoclaving, and 100 μL of membrane-sterilized sanguinarine solutions in dimethyl sulfoxide (DMSO). Prior to these experiments, we found that 0.2% (v/v) DMSO had no obvious effects on the growth and photosynthetic processes of *M. aeruginosa* NIES-843. Five milliliters of exponential-phase cultures of *M. aeruginosa* NIES-843 (in CT medium) were inoculated aseptically into each Erlenmeyer flasks in order to reach a final volume of 100 mL. The final sanguinarine concentrations were set at 0, 10, 20, 40 and 80 μg/L for five different treatments. The initial cell concentration of *M. aeruginosa* NIES-843 was 7.5×10^5 cells/mL at exponential phase as inoculum. Each treatment was replicated three times. All treatments were cultured under the identical conditions as mentioned above. The cell densities of *M. aeruginosa* NIES-843 were counted on day 1, 3 and 5 using a Hemacytometer.

2.3. Determination of photosynthetic pigments

Chl *a* and carotenoid contents were determined according to the method described by Wang (2000). Cultures of the different treatments were sampled on day 1, 3 and 5 and enumeration was carried out immediately. At the same time, cell culture was centrifuged at 8000 × *g* for 10 min, and the supernatant was discarded and the pellet was re-suspended in 90% ethanol for the extraction of Chl *a* and carotenoids for analysis. The whole procedure was carried out in the darkness at 4 °C.

2.4. Measurement of chlorophyll fluorescence transients

The chlorophyll fluorescence transients were measured on a Handy-Plant Efficiency Analyser (Handy-PEA, Hansatech, King's Lynn, Nor-folk, UK) with an actinic light of 3000 μmol photons/(m^2 s). All samples were dark-adapted for 15 min before measurements were made. Parameter changes of chlorophyll fluorescence transients were calculated as follows (for detail, see Christen et al., 2007):

$$V_J = (F_{2ms} - F_0)/(F_M - F_0)$$

$$\varphi P_0 = 1 - (F_0/F_M)$$

$$\varphi D_0 = F_0/F_M$$

$$\psi_0 = 1 - V_J$$

$$\varphi E_0 = (1 - F_0/F_M)(1 - V_J)$$

2.5. Reactive oxygen species (ROS) of M. aeruginosa NIES-843 under the stress of sanguinarine

The intracellular ROS levels in the cells of *M. aeruginosa* NIES-843 were determined using 2,7-dichlorofluorescein diacetate (Sigma, St. Louis, MO, USA) based on the method described by Hong et al. (2008). The fluorescence intensity of 2,7-dichlorofluorescein was obtained using a microplate reader (Molecular Device, M2, Union City, CA, USA). Excitation and emission wavelengths were set at 485 and 530 nm, respectively.

2.6. Transmission electron microscopic observations

M. aeruginosa NIES-843 was inoculated into CT medium containing 0, 10 and 80 μg/L sanguinarine. Cells were harvested, after 72 h of exposure to sanguinarine, and fixed for 2 h in 2.5% glutaraldehyde buffered with phosphate (pH 7.0) at room temperature. These fixed samples were post-fixed in 1% osmium tetraoxide, followed by dehydration by gradual increase in ethanol concentrations to 100% absolute alcohol, embedded in Spurr's resin. Sectioning and staining followed those described by Ozaki et al. (2009). The stained samples were then examined using a transmission electron microscope (TEM, FEI Tecnai G^2 20 TWIN, Eindhoven, The Netherlands).

2.7. Determination of gene expressions

Six genes (recA, mcyB, ftsZ, psbA, psbO and prx) were selected for measurements in this study. *recA*, *mcyB* and *ftsZ* are a set of genes involved in the repairing of DNA, synthesis of microcystins, and the cell division, respectively. psbA and psbO are two genes encoding key proteins in the oxygen evolution complex while *prx* is a gene involved in the synthesis of a anti-oxidant protein, peroxiredoxin (Prx). The expression of the six genes in *M. aeruginosa* NIES-843, under sanguinarine stress was determined by qPCR on day 1 and 3. Total RNA extraction and reverse transcription were performed according to the method described previously by Shao et al. (2009). The PCR amplification reactions were performed using a MyiQ™ qPCR Detection System (Bio-Rad, Hercules, CA, USA) under the following conditions: initially at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 30 s, 72 °C for 15 s. PCR primers used in this study are listed in Table 1. Gene expression data from qPCR were evaluated using *Ct* values. The16S rRNA gene (16S *rrn*) was used as housekeeping gene to normalize the expression levels