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Table: 1, Figs : 4

# Inhibition of photosynthesis-related gene expression by berberine in *Microcystis aeruginosa*

X. D. BI*, W. DAI, S. L. ZHANG*, Y. PANG, K.Z. XING. and D.J. ZHANG

Key Laboratory for Aquatic Ecology and Aquaculture of Tianjin,
Department of Fisheries Science, Tianjin Agricultural University,
Jinjing Road 22, Tianjin 300384, P. R. China
E. Mail: hy_101018@yahoo.com

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## ABSTRACT

The effects of berberine on the expression of six key photosynthesis-related genes in *M. aeruginosa* was examined. Berberine induced the expression of *psaA* and *psaB* at 1 mg·L$^{-1}$, *psbA* at 2 mg·L$^{-1}$, but repressed at higher concentrations. It inhibited the transcription of *psbD1*, *rbcL* and *rbcX* significantly ($p$ <0.05). This inhibition of transcription of key photosynthesis-related genes by berberine led to disturbed photosynthesis, specially by blocking the electron transport chain, affecting carbon assimilation and photo-respiratory carbon oxidation.

**Keywords:** Allelopathic mechanism, berberine, gene expression, *Microcystis aeruginosa*; photosynthesis.

## INTRODUCTION

Cyanobacterial blooms result in water anoxia, secretion of algal toxins and several other adverse effects (17). *Microcystis aeruginosa* is one of the most common bloom-forming cyanobacterium in eutrophic freshwaters and poses a great threat to freshwater aquaculture world wide (24, 25). It is a photosynthetic prokaryote with a large quantity of regular thylakoids in the cytoplasm (21). It has the ability to survive, grow and dominate over other phytoplanktons under unsuitable conditions owing to high photosynthetic activity (8).

Allelopathy, any direct or indirect, inhibitory or stimulatory effects produced by an organism on another organism through the release of chemical compounds into the environment**,** is a new effective method to control water blooms (11, 12). Some aquatic plants such as lotus (*Nelumbo nucifera*) contain berberine (9), which has been found to inhibit the growth of *M. aeroginosa* (15) under light but not in dark (26), suggesting that this alkaloid affects photosynthesis in *M. aerogunosa.* Although there is no evidence of berberine release into the aquatic system from such plants, decay of old plant tissue may release this alkaloid into the aquatic system and control the photosynthetic algal blooms.

To elucidate the allelopathic inhibitory mechanism of berberine on *M. aeruginosa*, we employed real-time qPCR technology to analyze the changes in photosynthesis-related gene expression in *M. aeruginosa,* when exposed to berberine. The expression of six key

*Correspondence author

photosynthesis-related genes, including the synthesis of photosystem I core proteins PSaA and PSaB (*psaA* and *psaB*), photo system II core proteins D1 and D2 (*psbA* and *psbD*), large subunit of Rubisco (*rbcL*) and Rubisco activase (*rbcX*), was examined to elucidate the response of *M. aeruginosa* to berberine stress at the gene expression level and to provide insight into the mechanism of berberine on cyanobacteria (26).

## MATERIALS AND METHODS

### Strain and culture conditions

*M. aeruginosa* FACHB469 was provided by Institute of Hydrobiology, Chinese Academy of Sciences. Berberine was purchased from Tianjin Phytomarker Ltd. (Shenyang, China), and dissolved in hot distilled water to a concentration of 100 $mg \cdot mL^{-1}$ as the stock solution. Experiments were done in 5 L conical flasks containing 3 L BG11 liquid sterile medium (1) under 12:12 hour LD cycle with a light density of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 25±1 °C. All flasks were shaken for 5 min, four times everyday.

### Inhibitory effects of berberine on the growth of *M. aeruginosa*

Berberine was added to algal culture at 0, 1, 2 and 4 $mg \cdot L^{-1}$ concentrations, based on our earlier observation that 1.25 $mg \cdot L^{-1}$ was the lowest concentration effective against *M. aeruginosa* (7). Each treatment was replicated thrice and the initial density of *M. aeruginosa* was $1.033 \times 10^6$ $cell \cdot mL^{-1}$. Algal cell number was determined microscopically using a phytoplankton counting chamber. A 100-µL sample was taken from 1 cm below the water surface at 24 h intervals (0, 24, 48, 72, and 96 h) by a transfer pipette to determine the extent of growth inhibition rate [IR (%)] as given below and the relative gene expression level.

$$IR\ (\%) = (N_0 - N_S)/N_0 \times 100,$$

Where, IR: Inhibition rate, $N_0$: Algal cell density ($ind \cdot L^{-1}$) in control; *N*s: Algal cell density ($ind \cdot L^{-1}$) in treated.

### RNA extraction and reverse transcription

*M. aeruginosa* cells from 1000 mL algal culture were harvested by centrifuging at 7000 rpm for 5 min 4 °C. Pelleted cells were suspended in Trizol reagent (TOYOBO, Japan) and ground under liquid nitrogen. Total RNAs were extracted following the Trizol reagent manual (TOYOBO, Japan), then digested with RNase-free DNase (TOYOBO, Japan). For reverse transcription, 1 µg of total RNA was mixed with random primers $p(dN)_9$ and reverse transcriptase as per the instructions of the reverse transcriptase kit (Generay, China).

### Primer design and real-time qPCR

The primers of *psa*A and *rbc*X were designed by Prmier Premier 5.0 (23) in present study and the other 5 pairs of primers, including 4 photosynthesis-related genes and 16S rRNA gene, were referred (19) from previous research report (Table 1). Real-time

Table 1. Primers designed for real-time PCR in present study (19)

| Gene name | Forward primer (5'-3') | Reverse primer (5'-3') | Reference NO. |
|---|---|---|---|
| *16S* | GCCGCRAGGTGAAAMCTAA | AATCCAAARACCTTCCTCCC | 19 |
| *psaA* | TGGCGTCGTGGTAGCAGT | GGCAGGGGAAACGGAAACCGAGATT | * |
| *psaB* | CGGTGACTGGGGTGTGTATG | ACTCGGTTTGGGGATGGA | 19 |
| *psbA* | GGTCAAGARGAAGAAACCTACAAT | GTTGAAACCGTTGAGGTTGAA | 19 |
| *psbD1* | TCTTCGGCATCGCTTTCTC | CACCCACAGCACTCATCCA | 19 |
| *rbcL* | CGTTTCCCCGTCGCTTT | CCGAGTTTGGGTTTGATGGT | 19 |
| *rbcX* | GAACATCTAGCCGAACAGGTTT | ATTCCGATGTCTCTGGTTGACT | * |

* Present study

qPCR was performed with 25 μL THUNDERBIRD SYBR® qPCR Mix (TOYOBO, Japan), 1.5 μL forward primer and reverse primer (10 pmol·μL$^{-1}$), respectively, 1 μL cDNA, and 21 μL distilled water to a final volume of 50 μL. The amplification reactions were performed by a MyiQ™ 5 Real time RT-PCR (Bio-Rad, USA) using the following conditions: one cycle at 94 °C for 2 min followed by 40 cycles at 94 °C for 10s, 57 °C for 30s, 72°C for 30 s. Gene expression data from real-time qPCR were evaluated using $C_t$ value (16), and the 16S rRNA gene was used as the housekeeping gene to normalize the expression levels of target gene since the expression of 16S rRNA is stable under various conditions (4, 5). The induction ratio was calculated as under using $2^{-\triangle\triangle Ct}$

$$\text{Where } \triangle\triangle Ct = (C_{t,\ \text{target gene}} - C_{t,\ 16S\ rrn})_{stress} - (C_{t,\ \text{target gene}} - C_{t,\ 16S\ rrn})_{control} \text{ (16).}$$

**Statistical analysis**

Values were normalized to gene expression levels of 16S rRNA, and represented the mean mRNA expression value ±SD (n=3) relative to the control (without berberine). One-way ANOVA test by SPSS 13.0 was used to evaluate whether there were any significant differences among treatments. Statistical significance was established at $p < 0.05$.

## RESULTS AND DISCUSSION

**Inhibitory effects of berberine on *M. aeruginosa***

Low concentration berberine (1 mg·L$^{-1}$) slightly promoted the growth of *M. aeruginosa*, while higher concentrations (2 and 4 mg·L$^{-1}$) were inhibitory (Fig. 1). The IR % at 24 h exceeded 15% in 4 mg·L$^{-1}$ berberine-added groups and at 96 h it reached to 70.5%.

**The quality of total RNA extracted from *M. aeruginosa***

It is common for nucleic acid samples to be contaminated with other molecules (i.e. proteins, organic compounds, other). The ratio of the absorbance at 260 and 280 nm ($OD_{260}$/ $OD_{280}$) was used to assess the purity of nucleic acids. Total RNA extracted was of good quality as seen by agarose gel electrophoresis (Fig. 2), and the $OD_{260}$/$OD_{280}$ ratios were all within the range of 1.8-2.0.

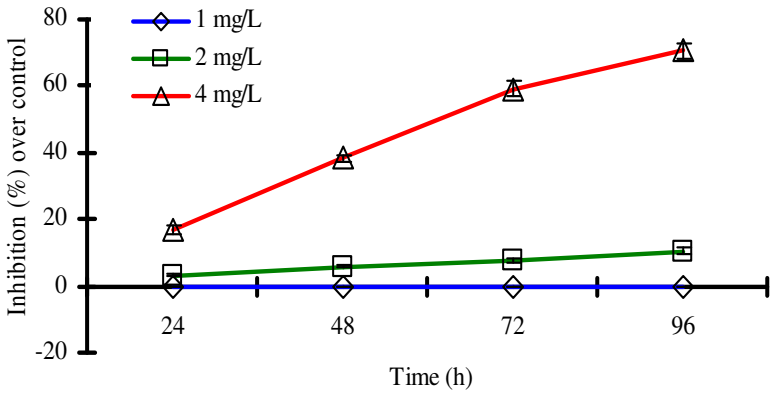


Figure 1. Concentration dependant inhibition of *M. aeruginosa* by berbrine.

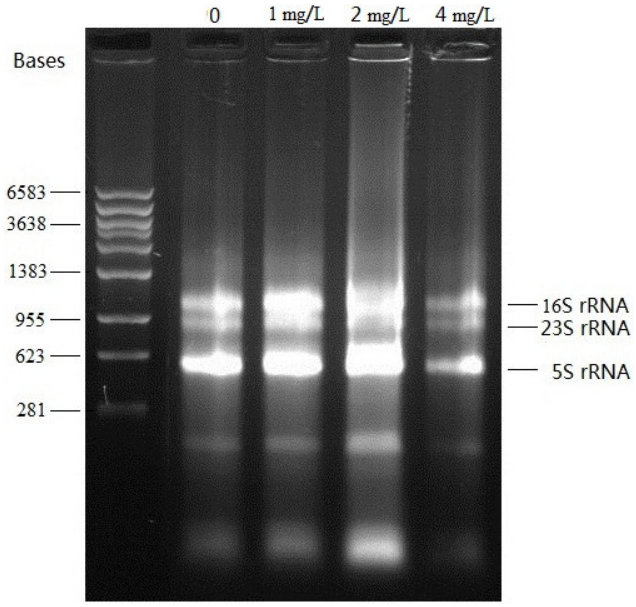


Figure 2. Partial results of total RNA extracted from *M. aeruginosa* stressed by berberine at 24 h. Total RNA run along with RNA Marker on a 1.5% agarose gel. The 16S and 23S rRNA bands are clearly visible in the intact RNA sample.

**Gene expression of core proteins of photosystem under berberine stress**

Changes in the relative genes expression values for the photosystem core proteins of *M. aeruginosa* are shown in Fig. 3. Berberine at 1 mg·L$^{-1}$ increased the relative values of *psaA* in 48 h, but decreased it after 72 h. At 2 and 4 mg·L$^{-1}$ berberine, the relative values

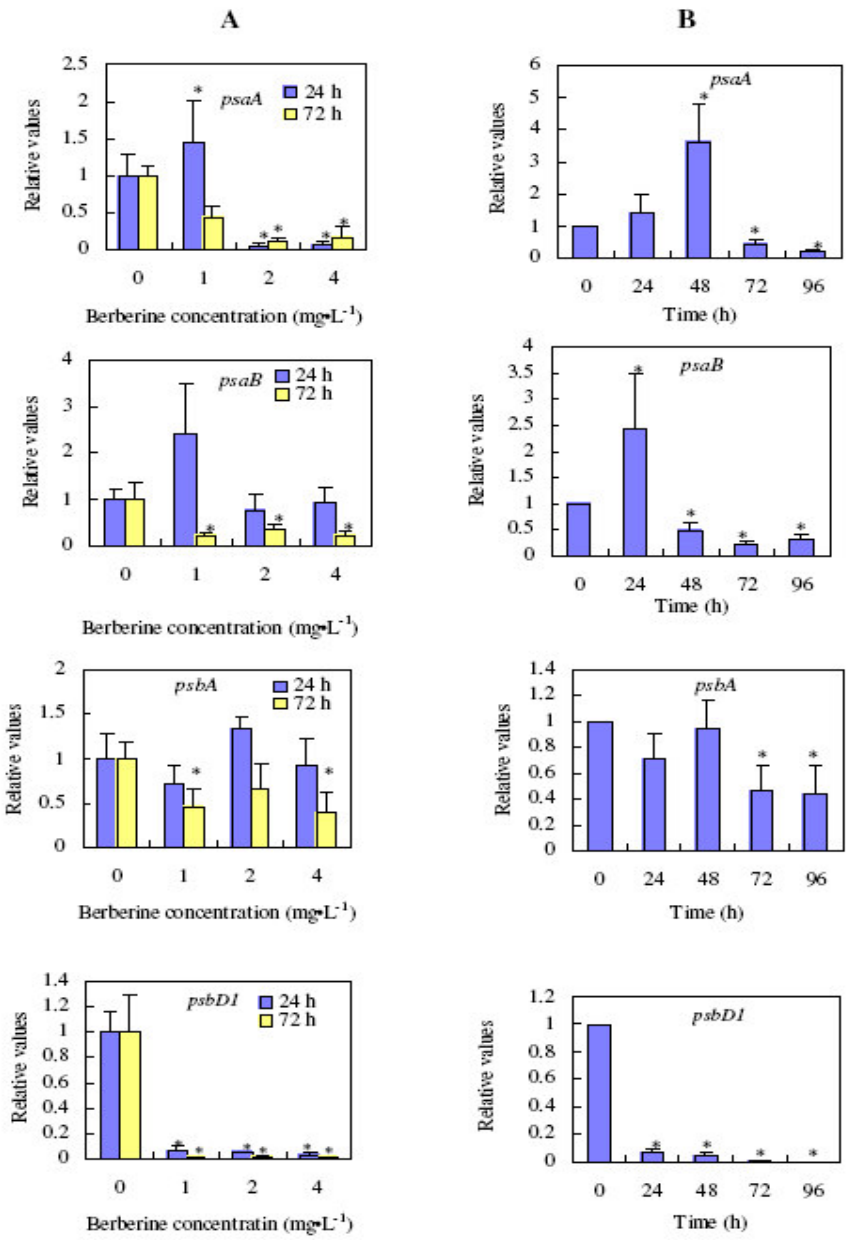


Figure 3. Effect of berberine on the relative gene expression values for the photosystem core proteins of *M. aeruginosa* . Cells treated with different conc of berberine for 24 h and 72 h. (B) Cells treated with 1 mg·L$^{-1}$ berberine for various exposure times. Values normalized to levels of 16S rRNA and represent mean mRNA value ±SD (n=3) over control at times. * ($P$ <0.05) indicates significant differences than corresponding controls without berberine for the same time.

of *psaA* were significantly decreased ($P < 0.05$). The relative values of *psaB* also decreased significantly ($P < 0.05$) except at 1 mg·L$^{-1}$ berberine at 24 h. With increasing concentrations of berberine, the relative value of *psbA* first increased and then decreased and the maximum values of both was at 2 mg·L$^{-1}$ at 24 h and 72 h, respectively. The relative value of *psbD1* was also significantly decreased ($P <0.05$) when *M. aeruginosa* was exposed to 2 mg·L$^{-1}$ berberine at 24 h.

Berberine exhibited more serious inhibitory effects on transcription of *psbD1* than other tested genes. With increased berberine concentrations, relative values of *psbD1* decreased at 24 h and an exposure time-dependent decrease in *psbD1* transcription was noticed.

**Gene expression of Rubisco and Rubisco activase under breberine stress**

The berberine inhibited the transcription of *rbc*L throughout the experimental period (Fig. 4). The relative values of *rbcL* first decreased and then increased with increasing berberine concentrations at 24 h while opposite changes were observed at 72 h. When *M. aeruginosa* was exposed to 1 mg·L$^{-1}$ berberine, the minimal relative value was at 48 h. The relative value of *rbcX* decreased with increasing berberine concentrations at 24 h and 72 h, respectively. When *M. aeruginosa* was exposed to 1 mg·L$^{-1}$ berberine, the maximal relative value was at 24 h while the minimal value was at 72 h.

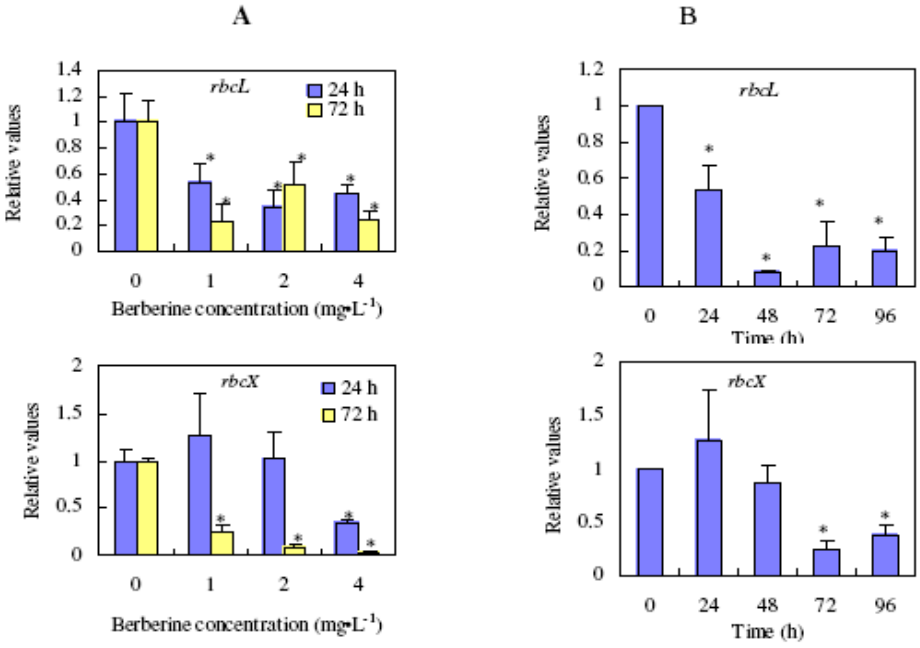


Figure 4. Effects of berberine on the relative gene expression values for Rubisco and Rubisco activase of *M. aeruginosa* . (A) Cells treated with different concentrations of berberine for 24 h and 72 h. (B) Cells treated with 1 mg·L$^{-1}$ berberine for a series of exposure times. Values were normalized to levels of 16S rRNA and represent the mean mRNA expression value ±SD (n=3) relative to the control at different times. * ($P$<0.05) indicates significant differences compared to the corresponding controls without berberine for the same time.

## DISCUSSION

Photosynthetic cyanobacteria convert solar energy into chemical energy with the aid of two large protein complexes located in the thylakoid membranes: photosystem I (PSI) and photosystem II (PSII). PSI contains 9 protein subunits featuring transmembrane α-helices (PsaA, PsaB, and other 7 subunits) and 3 stromal subunits. PsaA and PsaB are related by a pseudo-$C_2$ axis located at the centre of the PSI monomer (13). The organic cofactors of the electron transfer chain are arranged in 2 branches along the pseudo-$C_2$ axis and most of the antenna Chla molecules, the carotenoids and the lipids are bound to PsaA and PsaB (14).

PSII complex is composed of over 25 different proteins, and at the heart of this multi-subunit complex is the reaction centre, which is composed of D1 and D2 proteins. Surrounding the D1 and D2 proteins, are the other PSII subunits (10). In the present study, berberine was found the rinduce the expression of *psbA* at 2.0 mg·$L^{-1}$, and *psaA*, *psaB* under at 1 mg·$L^{-1}$ suggesting that the algal cells were accelerating *psbA*, *psaA* and *psaB* transcription in response to the stress of berberine. Qian *et al* (18) found that the synthesis of *psbA* mRNA was up-regulated at a concentration of 4.0 mg·$L^{-1}$ pyrogallol. PSII is very sensitive to environmental changes (20) and the degree of the repair of PSII is determined by the rate of synthesis for the D1 protein (3). Berberine repressed the expression of *psaA* and *psaB* at high concentration (2 and 4 mg·$L^{-1}$), which is consistent with our previous results that the expression of *psaA* and *psaB* were repressed when exposed to 1.25 mg·$L^{-1}$ berberine (7).

Distinct from the above three genes, the expression of *psbD1* was repressed to a greater extent throughout the experiment, suggesting that there was a decrease in the content of D2. For the important role in assembling the photosystem, a decrease in the content of PsaA, PsaB and D2 would damage PSI and PSII of algal cells, and then severely destroy the thylakoid lamella (6). This would then block the electron transport chain and disturb the photosynthetic process. Qian *et al* (18) found that decreased gene transcription of *psaB* and *psbD1* would block electron transport, which was an important mechanism involved in the inhibitory effect of N-phenyl-2-naphthylamine(18) and streptomycin (19) on *M. aeruginosa* (18).

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is a bi-functional enzyme, that catalyzes the initial step of photosynthetic carbonic reduction and photo-respiratory carbon oxidation cycles by combining $CO_2$ and $O_2$, respectively. Rubisco contains of 8 large subunits (RbcL) and 8 small subunits (RbcS) that assemble into a $L_8S_8$ holoenzyme in many cyanobacteria, algae, eubacteria, and higher plants (22). Rubisco activase encoded by nuclear genome modifies the conformation of Rubisco, releases inhibitors from active sites and increases enzymatic activity (2). Qian *et al* (18) found that both N-phenyl-2-naphthylamine and streptomycin could decrease *rbc*L gene transcription with an increase in concentration and the exposure time (18, 19). In the present study, berberine significantly inhibited transcription of *rbcL* throughout the experiment, leading to a decrease in the content of Rubisco in *M. aeruginosa*. The normal processes of photosynthetic carbon assimilation and photo-respiratory carbon oxidation must be disturbed for the evident decrease in the content of Rubisco, which might be involved in the inhibitory mechanism of berberine in *M. aeruginosa*. Berberine up-regulated the

expression level of *rbcX* at low concentration (1 mg·$L^{-1}$) while down-regulating it at higher concentrations (2 and 4 mg·$L^{-1}$) and the relative value of *rbcX* transitioned from an increase to a decrease with the prolongation of exposure time when it was was exposed to 1 mg·$L^{-1}$ berberine. A decrease in the Rubisco activase content would directly reduce the activity of Rubisco and then sharply disturb the photosynthetic process in *M. aeruginosa* (2).

## CONCLUSIONS

Consistent with our previous results (6, 7), it was presumed that berberine disturbs the photosynthetic process in *M. aeruginosa*, by blocking the electron transport chain and affecting carbon assimilation and photo-respiratory carbon oxidation by repressing the photosynthesis-related genes transcription, The results of this study provide a basis for the screening of anti-algal substances, in aquatic plants and the development of targeted and complex anti-algal agents to control the harmful Cyanobacterial blooms. Although there is no evidence at present that the toxic alkaloids are leached from the aquatic plants, it is likely that decaying tissue may release these alkaloids and thereby help in controlling the undesirable blooms. More research on this aspect is needed.

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