Effect of environmental factors on allelopathic inhibition of *Microcystis aeruginosa* by berberine

Shulin Zhang, Wei Dai, Xiangdong Bi, Dajuan Zhang and Kezhi Xing

ABSTRACT

To understand how environmental conditions affect the allelopathic inhibition of toxic *Microcystis aeruginosa* by berberine, the independent effects of some environmental factors, including temperature, light, and aeration, on the growth and extracellular microcystin (MC) content of *M. aeruginosa* (FACHB 905) treated with 0.000 and 0.001% (w/v) berberine were investigated. The results showed that higher temperature and light density, and aeration in daytime were beneficial for the growth of *M. aeruginosa* under the measured environmental conditions. The allelopathic effects of berberine on *M. aeruginosa* were closely associated with the environmental conditions. Berberine had the best inhibitory effects when temperature, light and aeration were more optimal for growth. In darkness, no changes in the density of *M. aeruginosa* were observed with the prolongation of culture time and berberine could hardly exhibit algicidal effects. Disturbance in the photosynthesis process might be one of the main reasons responsible for algicidal function. Berberine could increase extracellular MC contents significantly via killing and lyzing algal cells. Other treatments coupled with berberine needed to be carried out to degrade or remove MC released from berberine-killed algal cells.

Key words | allelopathic inhibition, berberine, environmental conditions, *Microcystis aeruginosa*

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INTRODUCTION

Microcystis aeruginosa is a bloom-forming harmful cyanobacterium distributed in freshwater and estuarine ecosystems throughout the world (Hong *et al.* 2009). In eutrophic waters, toxic and non-toxic strains of *M. aeruginosa* usually coexist (Lürling 2003), and scientific interests have focused especially on toxic *M. aeruginosa* for its microcystin (MC)-producing ability (Pan *et al.* 2008). MCs, a type of cyclic heptapeptide hepatotoxins, can pose hazards to environmental safety and public health (Rinehart *et al.* 1994).

Up to now, several measures have been proposed to control cyanobacterial blooms, such as physical methods (e.g. clay adsorption), chemical ones (e.g. inorganic or organic algaecide) and biological ones (e.g. fish and algicidal bacteria). However, only a few of them are applicable due to high cost, secondary pollution, or impracticability. With specific biodegradable characteristics, plant allelochemicals offer an environmentally friendly method for algal blooms control (Mulderij *et al.* 2005; Nakai *et al.* 2006; Park *et al.* 2006). Chinese herbal medicine has been used in disease control and prevention for centuries in China, and using it as a novel and safe method for algal-bloom control enables a new prospect. Zhou *et al.* (2007) studied the inhibitory activity of five Chinese herbs on red-tide causing alga, *Alexandrium tamarense*. The results showed that golden thread (*Rhizoma coptidis*) and areca seed (*Semen arecae*) had the best inhibitory effects. In our previous study, we found that golden thread could inhibit the growth of non-toxic *M. aeruginosas* effectively. As the major component of golden thread, berberine was the main allelochemical implementing the inhibitory effects (Zhang *et al.* 2010, 2011).

The growth of the *M. aeruginosa* population is to a large extent affected by environmental factors, such as water stability, temperature, light, and nutrient availability (Jacoby *et al.* 2000; Cao *et al.* 2006). These environmental factors might also influence the algicidal effects of an allelochemical. Understanding interactions between allelopathy and the environment has implications for the application strategies of allelochemicals in bloom control. In the case of cyanobacterial blooms, toxigenic species often dominate cyanobacterial blooms, with estimates suggesting that

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more than 50% of cyanobacterial blooms typically produce cyanotoxins (Graham *et al*. 2004).

Different cyanobacterial species or strains might have different sensitivities to allelochemicals. The allelopathic effects of exudates from the aquatic macrophyte *Stratiotes aloides* on the growth of two cyanobacteria (toxic and non-toxic *M. aeruginosa*) were strain-specific (Mulderij *et al*. 2005). To obtain the best algicidal effects in practical algal-bloom control, we studied the independent effects of some environmental factors, including temperature, light, and aeration, on the growth and extracellular MC content of toxic *M. aeruginosa* (FACHB 905). Moreover, the independent effects of these environmental factors on allelopathic inhibition of *M. aeruginosa* 905 by berberine were investigated.

MATERIALS AND METHODS

Organisms and cultivation

M. aeruginosa (FACHB-905) was provided by the Institute of Hydrobiology, Chinese Academy of Sciences. Before the experiment, the alga was pre-cultured in conical flasks containing sterilized BG11 medium under 12:12 light–dark cycle with a light density of 3,000 lx at 25 °C without aeration. Cultures were used till algae were in the exponential growth phase.

Experimental design

Berberine (the Northeast General Pharmaceutical Factory, China) was dissolved in heated distilled water to prepare the stock solution (10%, w/v), then stored at 4 °C until use. *M. aeruginosa* was cultured in 500 mL conical flasks containing 300 mL BG11 medium with the initial algal cell density of 1.0×10^6 cells/mL; 0.001% berberine could inhibit *M. aeruginosa* 905 (1.0×10^6 cells/mL) growth effectively with 96 h inhibitory rate (IR) reaching up to 90% (Dai *et al*. 2013), so it was selected as the final concentration of berberine added. The media prepared without berberine were taken as the control group. Experiments were carried out at three temperatures (25, 30 and 35 °C), four light intensities (0 lx (darkness), 1,500, 3,000 and 6,000 lx), and four aeration modes (with (continuous, diurnal, overnight aeration) and without aeration) independently. Other environmental conditions were the same as those used in the algae pre-cultivation. All treatments were done in triplicate flasks. The experiment was run for 8 d. To reduce any effects related to minor differences in photon irradiance, the flasks were shaken slightly three times each day and rearranged randomly.

Inhibitory effects on growth

The algal densities were tested per day using a hemacytometer under microscope. Each sampling was replicated three times and averaged. IR was calculated with the following formula:

$$\mathrm{IR}(\%) = (N_0 - N_S)/N_0 \times 100$$

where N_0 = algal cell density in control (10^6 cells/mL); N_S = algal cell density in berberine-added treatment (10^6 cells/mL).

Extracellular MC quantification

MC content in medium was measured every 4 d. Two millilitres culture was centrifuged at 9,500 rpm for 6 min at 4 °C, and the supernatant was collected for MC analysis using a competitive Enzyme-Linked Immunosorbent Assay Kit (Beacon Analytical Systems Inc., USA) following the protocols supplied by the manufacturer.

Statistical analysis

The result data were expressed as mean ±SD and subjected to one-way analysis of variance (ANOVA, SPSS version 10.0) to determine significant differences among treatments. Significant differences ($p < 0.05$) between treatments on the same day were determined by least significant difference multiple-range test.

RESULTS

As compared to 25 °C, higher temperatures of 30 and 35 °C had stimulative effects on the growth of *M. aeruginosa* (Figure 1(a)). As shown in Figure 1(b), *M. aeruginosa* growth increased in a light intensity-dependent manner. With the prolongation of culture time, algal cell densities increased in all the light-offered groups on a light–dark rhythm of 12–12 h, and they remained almost invariable in darkness without light–dark alternation. In Figure 1(c), aeration decreased *M. aeruginosa* growth in the first 2 d while it increased algal growth in the last 2 d.

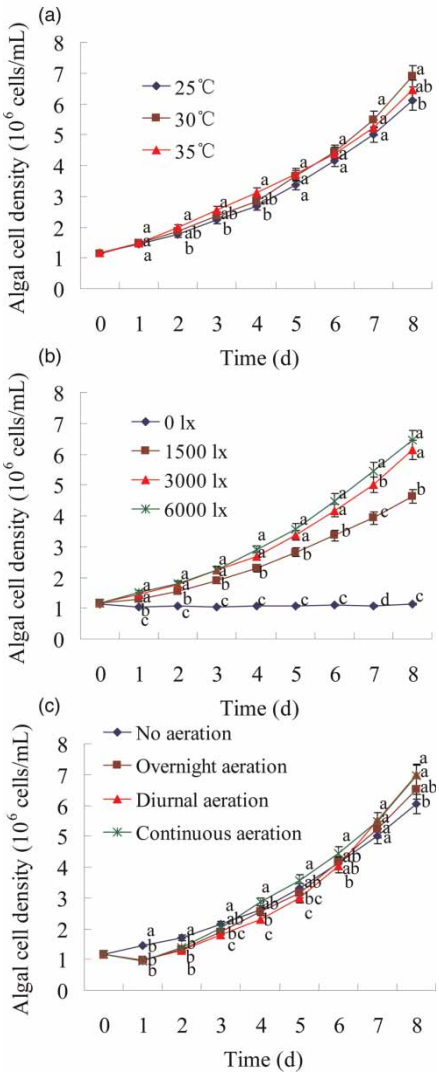

Figure 1 | Effects of environmental factors on the growth of *M. aeruginosa* 905. (a), Temperature; (b), light intensity; (c), aeration mode. Values are represented as means ±SD. Values sharing different letters differ significantly (p <0.05), whereas those with same letters are not significantly different (p >0.05).

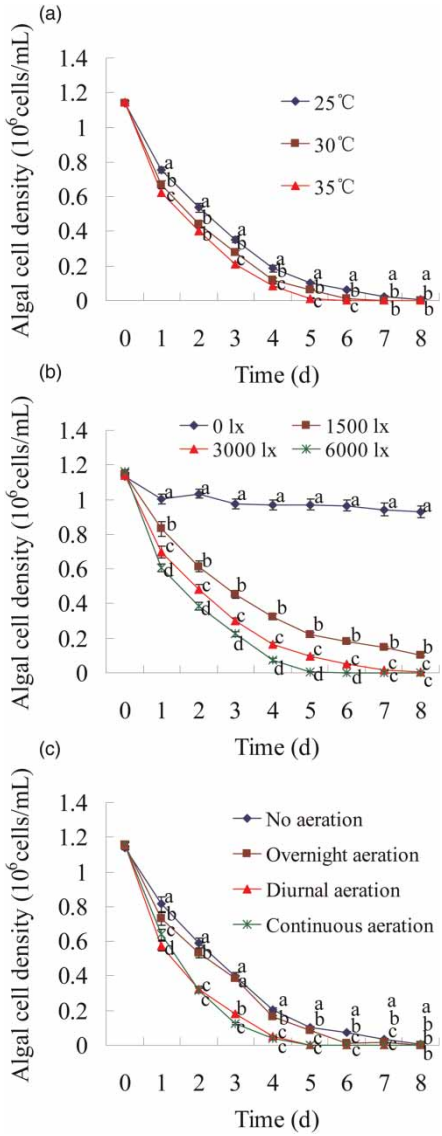

Figure 2 | Effects of environmental factors on allopathic inhibition of berberine on *M. aeruginosa* 905. (a), Temperature; (b), light intensity; (c), aeration mode. Values are represented as means ±SD. Values sharing different letters differ significantly (p <0.05), whereas those with same letters are not significantly different (p >0.05).

As shown in Figure 2, under different environmental conditions, *M. aeruginosa* densities in all the berberine-treated groups decreased with the prolonging culture time except that no changes of density were observed in group cultured in darkness, meaning algal exponential growth was inhibited. In Figure 3, IR increased in a contrary manner for all the groups except that little changes were observed in groups cultured in darkness. At day 4, except for the less light-offered one, IR in all the berberine-added treatments reached up to 90%, and they reached nearly 100% at the end of the experiment. Berberine inhibited algal growth in a temperature-dependent manner (Figure 2(a)), and stimulatory effects of irradiance on berberine-induced algicidal function were light intensity dependent (Figure 2(b)). Better inhibitory effects of berberine on the growth of *M. aeruginosa* were observed under continuous and diurnal aeration as compared to no aeration and overnight aeration (Figure 2(c)).

As shown in Figure 4, extracellular MC contents increased with the prolonging culture time in all the berberine-untreated groups except for the no light-offered group. The same MC changes were observed in berberine-treated groups. Under the same environmental conditions, berberine treatment significantly increased extracellular MC

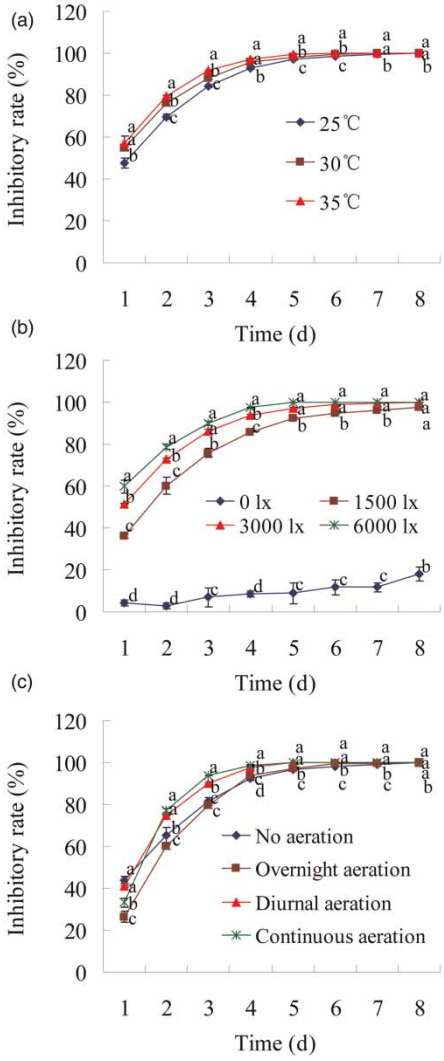

Figure 3 | Variations in the IR of berberine on *M. aeruginosa* 905 under different environmental factors. (a), Temperature; (b), light intensity; (c), aeration mode. Values are represented as means ±SD. Values sharing different letters differ significantly (p <0.05), whereas those with the same letters are not significantly different (p >0.05).

contents which reached a maximum value when all the algal cells were killed.

DISCUSSION

As *M. aeruginosa* is a photoautotroph, it is well known that light has a direct effect on its growth. Below light saturation point, the photosynthetic efficiency of *M. aeruginosa* enhanced with the increasing light intensity, and it would decrease with further increasing irradiance to above saturation point (Liu *et al.* 2006). The positive effects of irradiance on *M. aeruginosa* growth between 0 and 6,000 lx was consistent with previous findings (Wu *et al.* 2010). The density of *M. aeruginosa* remained almost invariable in darkness, suggesting that no algal cell lost its viability and no cell proliferation was supported without irradiance in 8 d. The shading light method is not recommended in the practical control of *M. aeruginosa* bloom. Aeration decreased *M. aeruginosa* growth in the first 2 d, which might be a result of algal inadaptability for the sudden environmental changes. Aeration could change the O_2/CO_2 ratio, as well as oxygen transfer and diffusion in water. During daylight hours, algae photosynthesis could lead to saturated dissolved oxygen and deficient CO_2 in water. Aeration-induced elevated CO_2 contents could accelerate algal photosynthesis and stimulated its growth, which might be the reason why stimulatory effects on the growth of *M. aeruginosa* were observed under continuous and diurnal aeration. Under natural conditions, microcystis bloom always broke out in eutrophic systems during the warmest periods of the year, and it was found that microcystis had an optimal temperature for growth and photosynthesis at, or above, 25 °C (Paerl & Huisman 2008). Different species and strains varied in the optimal growth temperature.

Allelopathy is strongly coupled with a biotic and an abiotic environment. The data indicated that crops were more sensitive to allelopathy when moisture, temperature, or nutrient conditions were less than optimal (Einhellig 1996). In a previous study, the changes in photosynthesis-gene expression of *M. aeruginosa* exposed to 1.25 mg/L berberine were analyzed using RNA-seq technology, and we found that the expression of key photosynthesis-genes, such as *psb*D, *psa*A and *psa*B, were repressed (data unpublished). It was suggested that berberine-induced disturbance in the photosynthesis process of *M. aeruginosa* might be one of the main reasons responsible for its algicidal function. In the present study, we found that *M. aeruginosa* was not inhibited by berberine in the darkness because *M. aeruginosa* could not perform photosynthesis and no sites of action for the berberine were provided. With the increasing light intensity, photosynthesis strengthened and more action sites were offered, and algicidal effects of berberine increased with the increasing irradiance. It was why berberine exhibited the best allelopathic inhibitory effects when light was more optimal for growth. Aeration gave more contacting opportunities for berberine and algae cells through infusing air and simultaneously stirring up the water. Only aeration in daytime was beneficial to allopathic inhibitory effects because berberine might implement algicidal function via inhibiting photosynthesis. Berberine, as a potent

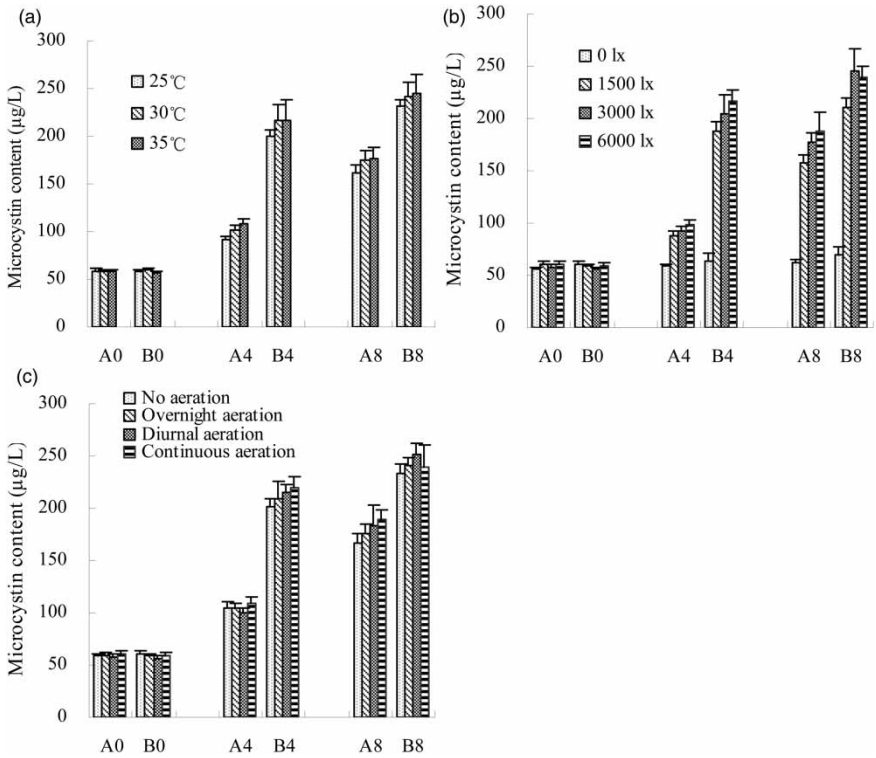

Figure 4 | Variations in the extracellular microsystin content of *M. aeruginosa* 905 under different environmental factors with or without berberine supplementatiom in medium. (a), Temperature; (b), light intensity; (c), aeration mode. Values are represented as means ±SD. Note: 0, 4 and 8 in the *x*-coordinate means day; A and B in the *x*-coordinate means 0.000 and 0.001% berberine, respectively.

antibacterial agent, is biologically a very active compound with many cell targets. *M. aeruginosa* belongs to the prokaryotes and has a similar structure to bacteria. Therefore, its algicidal mechanism might also be connected to many aspects involved in antibacterial activity, such as inhibiting DNA duplication, disrupting enzyme activity and destroying cell surface structure (Jin *et al.* 2010).

Environmental factors not only affect *M. aeruginosa* growth but also play an important role in the production of MC (Wiedner *et al.* 2003). However, optimal conditions for growth did not coincide with those for toxin production (Westhuizen & Eloff 1985). Inconsistent results for the effects of the same factor were always reported for the differences in methodology, such as different strains and culture modes (Westhuizen & Eloff 1985; Utkilen & Gjølme 1992). The environmental factors could affect extracellular MC concentrations in two different ways: via changing its release from live intact cells and dead broken cells. Low relative proportions of extracellular MC were always reported in culture experiments and field studies without breakdown of MC-producing cyanobacterial populations (Rapala *et al.* 1997; Wiedner *et al.* 2002). In this study, higher concentrations of extracellular MC were observed in berberine-treated groups as a result of dead cell lysis. MCs are hepatotoxic secondary metabolites of toxic cyanobacterial strains, and stress always triggers their production (Carmichael 1997). Berberine posed a stress to *M. aeruginosa* cells, which might also contribute to the increasing extracellular MCs. It is worth noting that berberine could perform as an algicide but could not degrade or remove MC released from the broken cells.

In conclusion, in laboratory, growth of *M. aeruginosa* depended to a great extent on associated environmental conditions, and algicidal effects of berberine were also closely related to environmental conditions. Naturally occurring cyanobacterial species differ from cultivated species in many aspects, and their reactions to allelopathic impacts might be much more complex and unpredictable. Moreover, in a natural ecosystem, many environmental factors, and not just temperature, light and aeration, play a crucial role in the allelopathic inhibition. The allelopathic effects of berberine on natural algal species need further investigation. Berberine-induced disturbance in the photosynthesis process might be involved in its allelopathic mechanisms. In practical application of berberine in bloom control, treatment of MC released from killed algal cells must be posed as a crucial problem.

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