Inhibitory effects of *Pontederia cordata* on the growth of *Microcystis aeruginosa*

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**ABSTRACT**

This study investigated the effect of *Pontederia cordata* on *Microcystis aeruginosa* growth in three different experimental settings: (i) co-cultivation, (ii) exposure of cyanobacteria to culture water of *P. cordata*, and (iii) exposure of *M. aeruginosa* to organic extracts of *P. cordata*. Results showed that the growth of *M. aeruginosa* was significantly inhibited by co-cultivation, with the highest inhibition rate of 61.9% within 5 days. Moreover, 95% culture water with *P. cordata* could markedly inhibit the growth of *M. aeruginosa*, with inhibition rate of 98.3% on day 6, indicating that most of the algal cells died. The organic extracts of fibrous root showed stronger inhibition effect than the leaf and stalk extracts. Acetone extract of fibrous root showed the strongest inhibitory effect on *M. aeruginosa*. Different components of 80% acetone extracts from fibrous root exhibited varied effects on the growth of *M. aeruginosa*. Ethyl acetate and water components had strong inhibition effects on *M. aeruginosa*. By contrast, n-butyl alcohol components had weak inhibition effects, and hexane components even promoted the growth of *M. aeruginosa*. Allelochemicals of *P. cordata* were primarily released into the water through the fibrous root. Results indicated that *P. cordata* can be applied in environmentally friendly algal inhibition.

**Key words** | allelopathy, inhibition rate, *Microcystis aeruginosa*, *Pontederia cordata*

**INTRODUCTION**

Water eutrophication is becoming a major environmental problem in China. Eutrophication results in adverse effects on ecosystems, such as reduction in biodiversity, extinction of submerged macrophytes, and harmful algal blooms (HABs; Qin et al. 2016). *Microcystis aeruginosa* is the most widely distributed cyanobacteria causing HABs in surface waters worldwide (Paerl et al. 2011). This species naturally produces toxins, such as microcystin-YR, microcystin-LR, and microcystin-RR (Kurmayer et al. 2015), which have substantial adverse impacts on flora, fauna, and aquatic ecosystems. The main impacts of these toxins include the following: i) production of hepatotoxins that cause mortalities in fish, seabirds, and mammals; ii) human illness or death via the bioaccumulation of algal toxins in the food web (Ibrahim 2013; Meneely & Elliott 2013); and iii) the cost of treating the polluted water. Therefore, controlling eutrophication and HABs are important concerns in environmental water treatment.

These negative impacts have led to increased studies that explore environmentally friendly and efficient treatment methods for eutrophication control, such as reducing nutrient input and promoting the growth of beneficial phytoplankton by optimizing hydrological conditions. When prevention of HABs is not successful, additional physical, chemical, and biological remediation techniques may be used to control HABs. Physical remediation mainly includes mechanical removal, ultraviolet removal and capping. However, these processes are not frequently used, because they are time-consuming and expensive. Additionally, some of the physical remediation techniques, such as ultrasound, have been proven not to be safe for non-target organisms; for example, ultrasound was acutely harmful to zooplankton (Lürling & Tolman 2014). Remediation with chemicals, such as using hydrogen peroxide, aluminum sulfate, or ferric sulfate, leads to secondary pollution and ecological risk, although this process may rapidly kill algae. For example, use of aluminum sulfate can cause increased accumulation of aluminum in biota, and some aluminum compounds are highly acidic and can cause unwanted ecological alterations (Boyd & Massaut 1999). To date, biological remediation is
the efficient treatment. This process is advantageous because of its low cost, low potential to generate pollution, and increased sustainability (Filzgerald 1969).

As a biological measure, using allelochemicals released by aquatic plants has been demonstrated to inhibit algal growth. Studies have primarily investigated submerged plants, such as Myriophyllum spicatum (Nakai et al. 2000; Zhu et al. 2010), Myriophyllum verticillatum (Dai et al. 2014), Myriophyllum aquaticum (Wang et al. 2017), and Vallisneria spiralis (Gette-Bouvarot et al. 2015). However, emergent plants have received less attention. Studies have mainly focused on Arundo donax (Hong et al. 2011; Abu-Romman & Ammari 2015), Iris pseudacorus (Chen et al. 2015), and Eichhornia crassipes (Shanab et al. 2010).

Pontederia cordata, commonly known as pickerelweed, is a perennial emergent plant with green leaves and flowers with attractive colors. This plant has characteristics suitable for treatment of polluted waters. P. cordata can remove ammonium nitrogen, nitrate nitrogen, total nitrogen, and total phosphorus from polluted waters (Lu & Huang 2013). As a biological measure, using allelochemicals released by P. cordata on algal growth have not been determined. In this study, the inhibitory effects of culture water with P. cordata on the growth of M. aeruginosa were investigated. Moreover, the allelopathy of P. cordata and M. aeruginosa in co-cultivation was verified. The main positions in plants that release allelochemicals were explored, and the organic solvent most suitable to extract allelochemicals was determined. Further systematic studies, such as separation, purification, identification of allelochemicals, and their safety towards non-target organisms and human health will be beneficial in developing environmentally friendly algicides.

MATERIALS AND METHODS

Algal cultures

Microcystis aeruginosa (FACHB-905) was provided by the Freshwater Algae Culture Collection of the Chinese Academy of Sciences (Wuhan, China). The tested cyanobacteria were axenically cultivated in BG-11 medium (Rippka et al. 1979) at 25 ± 1 °C on a 12 h/12 h light/dark cycle with approximately 90 μmol photons m⁻² s⁻¹ to achieve exponential growth. Each flask was shaken thrice a day, and its position was randomly changed to ensure consistent irradiation. The biomass of M. aeruginosa was determined by measuring the spectroscopic absorbance of algal liquid at 650 nm (OD₆₅₀) at 24 h intervals.

Maintenance of pickerelweed

P. cordata was purchased from a horticulture company in Nanjing, China. In early April 2016, prior to the experiment, the selected plant was planted in a culture box, containing sediment and pond water, and cultivated in the campus of Nanjing Forestry University. Well-grown pickerelweed plants were washed with tap and Milli-Q water prior to the experiment.

Experiments with co-culture pickerelweed and M. aeruginosa

Four months later, 20 L of M. aeruginosa in exponential growth phase was placed in an incubator (80 cm × 40 cm × 50 cm); the initial OD₆₅₀ value was 0.06. Fresh pickerelweed of 50 g·L⁻¹ was added to culture boxes containing M. aeruginosa. To fix the macrophyte in place, P. cordata was loosely bundled at the middle with 5–10 cm of rope that was attached to a stone, acting as an anchor to hold the P. cordata in the center of each box. The stones and ropes were washed with sterile water thrice and loose enough to avoid injury to the plant. M. aeruginosa solution, without P. cordata, served as the control throughout the experiment. Triplicates of treatments and controls were performed. All culture boxes were placed at the same location, which was sunny with consistent light and temperature conditions.

Experiments with pickerelweed culture water

Pickerelweed (200 g) was cultured in the sterile culture box (80 cm × 40 cm × 50 cm), which contained 5 L of ultrapure water. Ultrapure water (10 L) was added to the incubator after 10 days. After 20 days of cultivation, the culture water was passed through filter paper to remove any large solids, and subsequently passed through a 0.22 μm microporous membrane to remove microorganisms. These steps were performed twice. Different amounts of culture water (0, 25, 50, 75, and 95 mL) were put into sterile 250 mL flasks, then supplemented with the required nutrients for BG-11 medium and the total experimental volumes were adjusted to 100 mL with ultrapure water. The resulting concentrations of culture water in solution were 0%, 25%, 50%, 75%, and 95%. M. aeruginosa was inoculated in the culture
solution so that the initial OD_{650} value was ensured to be 0.06. The tested cyanobacteria were axenically cultivated in an incubator and shaken thrice every day.

**Experiments with different parts of pickerelweed**

The fibrous roots, stems, and leaves of *P. cordata* were washed by ultrapure water and placed in an oven at 40 °C to dry for 48 h, or until a constant weight. Then, samples were crushed using a small mill. Then, 4 g of powdered samples were put into the flasks with 100 mL of extraction solutions (petroleum ether, 80% acetone, or anhydrous ethanol). The flasks were placed on a shaking table and shaken for 48 h under constant temperature. Each extraction was performed thrice, and the three identical samples were combined. First, the extracts were passed through filter paper twice. Second, a rotary evaporator was used to remove excess solvent. The residual substance was dissolved in dimethyl sulphoxide (DMSO), and the total volume was brought to 25 mL. Third, the resulting solution was filtered through a 0.22 μm microporous membrane and stored at 4 °C until analysis.

To elucidate the effects of the extracts on *M. aeruginosa*, 0.2 mL of extracts was added to the sterile flasks with 100 mL of BG-11 medium. The control group contained 0.2 mL of DMSO and 100 mL of BG-11 medium. Flasks were inoculated with *M. aeruginosa* culture in exponential growth phase so that the initial OD_{650} value was 0.06. The experiments were performed in triplicate.

**Experiments with different chemical components of 80% acetone extracts of pickerelweed**

According to the method in the previous section, 10 g of milled pickerelweed powder were put into the flasks containing 400 mL of 80% acetone. The flasks were placed on a shaking table and shaken for 48 h under constant temperature. Extraction was performed thrice, and the three extracts were combined. Extracts were passed through a filter paper and a rotary evaporator was used to remove excess 80% acetone. The residual substance was successively extracted twice by n-hexane, ethyl acetate, and n-butyl alcohol. Third, a rotary evaporator was used to remove the excess solvent from each extraction, and solid extracts of the n-hexane phases, ethyl acetate phases, n-butyl alcohol phases, and water phases were obtained. Finally, a proper amount of DMSO was added to each chemical component to produce saturated solutions. The saturated solutions, which were passed through a 0.22 μm microporous membrane, were stored at 4 °C for further use.

Saturated extract solutions (1 mL) were added to sterile flasks with 100 mL of BG-11 medium. The control group contained 1 mL of DMSO and 100 mL of BG-11 medium. Flasks were inoculated with *M. aeruginosa* culture in exponential growth phase so that the initial OD_{650} value was 0.06. The experiments were performed in triplicate. The tested cyanobacteria were axenically cultivated in an incubator and shaken thrice every day.

**Statistics**

The inhibition rate was calculated as follows:

\[
IR_t(\%) = \left(1 - \frac{N_t}{M_t}\right) \times 100
\]

where \(t\) is time, \(IR_t\) is inhibition rate at day \(t\), \(N_t\) is mean value of OD_{650} of *M. aeruginosa* in the experimental group at day \(t\), and \(M_t\) is mean value of OD_{650} of *M. aeruginosa* in the control group at day \(t\).

SPSS 19.0 was used to analyze all data by analysis of variance (ANOVA), regression analysis, and Duncan’s tests for multiple comparisons of means.

**RESULTS AND DISCUSSION**

**Growth inhibition by the co-culture experiment**

The growth of *M. aeruginosa* was both enhanced and inhibited by *P. cordata* under the co-culture experiment, with algal inhibition continuously increasing with time. The algal inhibition rate was 61.9% on day 5 (\(p < 0.05\); Figure 1).

**Figure 1** | Inhibition rate of fresh *P. cordata* on *M. aeruginosa* growth. Note: Bars represent means ± SD with \(n = 3\). The lower case letters indicate significant difference at \(p < 0.05\), whereas the same letters indicate no significant difference. This representation is also applied to the other figures.
Co-culture in the first 3 days showed negative inhibition rates, indicating that *P. cordata* promoted the growth of *M. aeruginosa*. From day 4, algal density decreased continuously, and algal growth rate dropped considerably, indicating that *P. cordata* significantly inhibited the growth of *M. aeruginosa* (*p* < 0.05). Frequent visual evaluation indicated that water in the treatment group was clearer and more transparent. The water in the control was green and odorous.

These results indicated that the inhibition effect of allelochemicals was related to ‘low-promotion, high-inhibition’ phenomenon (hormesis effect) (Nakai et al. 1999). The biological and allelopathic effects originated from a chemical substance released by aquatic plants that had to accumulate prior to inhibition of the growth of algae. At the beginning of co-culture, relatively low amounts of chemical substances were released, and the culture solution could provide sufficient nutrients for algal growth. Allelochemicals would accumulate with increasing time, resulting in relatively higher concentrations of solutions. This phenomenon can account for the inhibited growth of *M. aeruginosa*, leading to reduced algal density.

**Growth inhibition of *M. aeruginosa* by the culture water**

As expected, the algal densities in control samples increased with time continuously (Figure 2). Algal density of *M. aeruginosa* in the treatments increased on day 1 but decreased on days 2–6. Algal density decreased with increased concentration of culture water.

Multiple comparisons of the main effects (Table 1) and interaction effects (Figure 3) showed that the different concentrations of culture water inhibited the growth of *M. aeruginosa*. Significant differences were found between the cultures with different concentrations (*p* < 0.05). Within days 1–6, the inhibition of algal growth was significantly enhanced with prolonged processing time (*p* < 0.05). The inhibitory rates of all treatments were greater than 50% on day 3. In addition, the inhibition rates of the culture water with concentrations of 95%, 75%, 50%, and 25% on day 6 were 98.3%, 96.2%, 92.9%, and 85.8%, respectively. The cultures were essentially transparent, and most of the algae died. The color of the controls was deeper than that of the treatments. Microscopic visual evaluation showed that more algal cells were found in the controls than in the treatments. This result indicated that the *P. cordata* culture water inhibited the algal growth, and the inhibitory effects increased with incubation time and the concentration of *P. cordata* culture water.

Secondary metabolites released by plants inhibited the growth of algae by dissolving in water (Nakai et al. 1999). In addition, algal inhibition varied according to the percentage of culture water, indicating a dose-dependent process (Liu et al. 2012).

As observed in previous studies, average algal biomass in the treated pond declined rapidly after the unfiltered *Radix astragali* decoction was added. Moreover, after remaining at a low level for 33 days, the algal biomass began to recover, and the level of algal bloom almost reached that of the control pond after 68 days (Yan et al. 2011). It is of practical significance to explore the action period of allelochemicals on algae, which may guide future large-scale popularization and utilization of allelochemicals to inhibit algal outbreak.

**Growth inhibition by different parts and extracts of *P. cordata***

Allelopathic effects of the different tissues from *P. cordata* (i.e. fibrous roots, leaf, and stem) on the same algae also varied. Multiple comparison of the main effects (Table 2) showed significant differences in the effects of the fibrous root, stem, and leaf of *P. cordata* on the growth of

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**Table 1 | Main effects of concentration and treatment time on the growth of *M. aeruginosa***

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Inhibition rate (%)</th>
<th>Treatment time (d)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95%</td>
<td>77.8 ± 4.3 a</td>
<td>1</td>
<td>20.9 ± 2.2 f</td>
</tr>
<tr>
<td>75%</td>
<td>71.8 ± 4.3 b</td>
<td>2</td>
<td>55.2 ± 4.2 c</td>
</tr>
<tr>
<td>50%</td>
<td>66.8 ± 4.4 c</td>
<td>3</td>
<td>69.6 ± 3.8 d</td>
</tr>
<tr>
<td>25%</td>
<td>55.8 ± 4.5 d</td>
<td>4</td>
<td>82.4 ± 2.3 c</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>5</td>
<td>87.3 ± 2.1 b</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>6</td>
<td>93.0 ± 1.9 a</td>
</tr>
</tbody>
</table>

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**Figure 2 | Effects of *P. cordata* culture solutions on the growth of *M. aeruginosa***
M. aeruginosa \((p < 0.05)\), within inhibition decreasing from fibrous root > leaf > stem. Different extracts also showed significantly different inhibitory effects on the growth of algae \((p < 0.05)\), with inhibition greatest for 80% acetone extracts, followed by petroleum ether and anhydrous ethanol extracts.

Multiple comparison of the interaction effect (Figure 4) showed that the root organic extracts of \(P.\) cordata significantly inhibited growth of \(M.\) aeruginosa \((p < 0.05)\). The inhibition rates of the extracts of 80% acetone and petroleum ether significantly increased on days 3 and 4, respectively, and reached corresponding rates of 67.3% and 79.3% on day 6. The inhibition rates of the extracts of ethanol first increased and then decreased as time increased. This phenomenon showed the promotion of the growth of \(M.\) aeruginosa on day 3. The removal efficiencies of \(M.\) aeruginosa rose significantly and reached 60.8% on day 6.

The results showed that the allelopathic effects of the leaf organic extracts of \(P.\) cordata varied (Figure 5). Petroleum ether extract showed almost no effects on the
growth of *M. aeruginosa* during the first 3 days but began to exhibit significant inhibitory effect on day 5 (*p* < 0.05) and reached 12.3% on day 6. The inhibition rates of the 80% acetone extracts first increased and then decreased with time (*p* < 0.05). This extract slightly promoted the growth of *M. aeruginosa* on day 3. However, the inhibitory rate significantly increased on day 4 and reached the highest rate of 65.9% on day 6. Ethanol extract inhibited the growth of *M. aeruginosa* on days 1 and 2, but the highest inhibition rate was only 11.2%. Afterward, this extract promoted algal growth, with inhibition rate of 1.8% on day 6.

The stem organic extracts of *P. cordata* almost significantly promoted the growth of *M. aeruginosa* (Figure 6). The promotion rates of petroleum ether, 80% acetone, and anhydrous ethanol extracts reached 17.9%, 30.9%, and 14.8% on days 4, 5, and 5, respectively.

The fibrous root organic extracts of *P. cordata* showed the strongest inhibition effect compared with the leaf and stalk extracts. Methanol and acetone extracts of the different parts of *E. crassipes* inhibited *M. aeruginosa* to various extents, and the methanol abstract of root of *E. crassipes* showed the greatest inhibition (Hu et al. 2015). Chen et al. (2015) found that *A. donax* had better inhibitory capacity than *Nymphaea tetragona*, and different tissues of *A. donax* and *N. tetragona* exerted different inhibitive effects on *M. aeruginosa*. Inhibition effect from the haulm of *A. donax* was stronger than that of the leaves, while the leaves of *N. tetragona* exerted stronger inhibition than the stems. The inhibition effects varied greatly, probably because organ-level differences in secretion of secondary metabolites and their quantities may vary, and inhibition effects are consequences of synergistic effects of the different allelochemicals. The results show that the allelochemicals of *P. cordata* responsible for algae inhibition are mainly released by the fibrous root.

**Growth inhibition by different chemical components of 80% acetone extracts**

The growth of *M. aeruginosa* in the control was sustainable, and the algal density was 2.88 times of the initial value on day 6 (Figure 7). The n-butyl alcohol phases promoted the growth of *M. aeruginosa*, but the increment was less than that of the control (CK). The n-hexane phases promoted
the growth of *M. aeruginosa*, and the increment was more than that of CK, except on days 5 and 6. In addition, algal density decreased with increasing time under treatment of ethyl acetate phases and water phases.

Multiple comparison of the main effect (Table 3) and interaction effect (Figure 8) showed that the different chemical components of 80% acetone extracts with *P. cordata* inhibited the growth of *M. aeruginosa* (*p* < 0.05). Except for the n-hexane phases, the other phases inhibited algal growth, with inhibitory effects as follows: ethyl acetate phases > water phases > n-butyl alcohol phases > n-hexane phases. The inhibitory ability increased with time.

Ethyl acetate and water phases showed remarkable allelopathic effects on algae, and the inhibition rates reached 98.5% and 95.8% (*p* < 0.05) on day 6, respectively. After the addition of ethyl acetate phases, microscopic visual evaluation showed that the color of *M. aeruginosa* was less intense on day 2. The cells assembled and agglomerated on day 3. On day 6, the dead cyanobacteria were deposited at the bottoms of the experimental flasks. The inhibitory ability of n-butyl alcohol phases increased with time and reached 38.0% (*p* < 0.05) on day 6. N-hexane phases promoted the growth of *M. aeruginosa* at the beginning of the treatment but had a weak inhibition effect on days 5 and 6.

Compounds extracted from natural materials are composed of complex chemical components, such as alkaloids, flavonoids, phenolic acid, and fatty acids. Most of these compounds are secondary metabolites, which are released to the environment in multiple manners, such as by penetration, volatilization, and secretion. The 80% acetone extract of fibrous root showed the strongest inhibitory effect on *M. aeruginosa*. This result indicated that the allelochemicals of *P. cordata* was highly polar, facilitating their dissolution in 80% acetone. Different components of 80% acetone extract from the fibrous root of pickerelweed had diverse effects on the growth of *M. aeruginosa*. The ethyl acetate and water components had strong inhibition effects on *M. aeruginosa*, n-butyl alcohol component had weak inhibition effects, while, hexane component can promote
the growth of \textit{M. aeruginosa}. This phenomenon indicated that the allelochemicals mainly existed in the ethyl acetate and water extracts. Extracts of ethyl acetate and water material were mainly some alkaloids, glycosides, proteins and amino acids, which are probably the allelochemicals of \textit{P. cordata}. In the future, we will study the specific species and structure of allelochemicals by gas chromatography–mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC), and explore the effects of physiological and biochemical, molecular and gene levels of algal mechanism. Additionally, we should analyze the microcystins concentration after incubation with macrophyte and its extracts to make sure the concentration is within the safe range, otherwise it will cause harm to the aquatic environment and health.

\textit{P. cordata} inhibited the growth of \textit{M. aeruginosa} under laboratory conditions (limited light, temperature, moisture and nutrients), while actual growing conditions are expected to be different from the indoor simulation conditions. The species and quantities of allelochemicals released by \textit{P. cordata} may vary. Rzymski et al. (2014) found that cylindrospermopsin is a compound which can not only decrease growth of \textit{M. aeruginosa} but also likely inhibit the production of microcystins. However, this compound is known to be cytotoxic, it excludes its direct application in water environments. So this is a precaution that the allelochemicals isolated from plants should evaluate their safety towards non-target organisms and human health before an application to the environment. Nevertheless, the results are important for practical application. To ensure the original habitat of urban water, synergistic effects could be used to arrange \textit{P. cordata} with other aquatic plants. This process may improve water quality, inhibit algal growth and maintain the long-term stability of the water ecological system to realize the ecological and landscape effect.

**CONCLUSIONS**

The three cultural modes, namely, \textit{P. cordata} co-cultivation with \textit{M. aeruginosa}, \textit{P. cordata} culture water and organic extracts, inhibited the growth of \textit{M. aeruginosa}, with inhibition rates as high as 61.9\%, 98.3\% and 98.5\%, respectively. The inhibition effect of allelochemicals was related to hormesis effect, and increased with time in the co-culture and culture water treatments during the experiment. Fibrous root organic extracts of \textit{P. cordata} showed the strongest inhibition effect when compared to leaf and stalk extracts, and the highest inhibition rate reached 79.3\%. Different components of 80\% acetone extracts from fibrous root showed diverse effects on the growth of \textit{M. aeruginosa}. Ethyl acetate and water components showed strong inhibition effects on \textit{M. aeruginosa}, and their highest inhibition rates were 98.5\% and 95.8\%, respectively. By contrast, n-butyl alcohol components had weak inhibition effects, and hexane components even promoted the growth of \textit{M. aeruginosa}.

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