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Synergistic and antagonistic interactions among five allelochemicals with antialgal effects on bloom-forming *Microcystis aeruginosa*

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ABSTRACT

In this study, we investigated the allelopathic interactions among five representative allelochemicals at different proportions using bloom-forming *Microcystis aeruginosa* as the test receptor. Binary or ternary mixtures of allelochemicals obtained three types of allelopathic interactions, i.e., synergistic, antagonistic, and additive effects. However, combinations of four or five allelochemicals only yielded antagonistic effects. Interestingly, the algal inhibition gradually increased with the tested period for all treatments. For instance, the synergistic interaction occurred with the mixture including coumarin + ρ-hydroxybenzoic acid at 80%:20%; the mixture on the 10th day showed 0.80 of algal inhibition. While the mixture comprised protocatechuic acid + stearic acid + ρ-aminobenzene-sulfonic acid at 33.3%:33.3%:33.3%, the additive interactions occurred and then the maximum algal inhibition (0.83) was acquired at the end of tested period. In conclusion, the joint effects of different allelochemicals depend on various factors such as the chemicals used, their respective proportions, the total concentration of the mixture, and the receptor species. Thus, it is necessary to consider the complexity of allelopathic interactions and the field conditions during the control and management of noxious cyanobacteria.

1. Introduction

Allelopathy is a chemical cross-talk between plants or microorganisms in their vicinity (Kato-Noguchi and Ino, 2013). For example, the aquatic macrophytes have been shown to suppress phytoplankton growth, but the cyabacterium *Microcystis aeruginosa* by itself also produces cyanotoxins that have allelopathic effects on the hydrophytes, algae and diatoms (Ger et al., 2016; Nakai et al., 2014). Thus, allelopathic interactions have attracted considerable attention from researchers.

At the species level, allelopathic antagonistic interactions are widespread in nature, such as the allelopathic interactions between crops and weeds, or those between woody plants and crops, or macrophytes and water-bloom algae (Oliveros-Bastidas et al., 2014; Kumar and Vimala, 2008; Mulderij et al., 2007). Thus, Kato-Noguchi and Ino (2013) discovered that momilactone B in rice can induce and increase allelopathy in barnyard grass; however, barnyard grass also produces defensive chemical(s) in response to allelopathic rice. In addition, Nupur and Trivedi (2011) found that the roots, stems and leaves of *Parthenium hysterophorus* Linn., *Cassia tora* Linn., and *Croton bonplandianum* Baill. have allelopathic potential, where they have allelopathic effects on each other. Mulderij et al. (2009) discovered that allelopathic interactions between *Stratiotes aloides* and filamentous algae, mainly Cladophera Kutzing and Spirogyra Link, did occur under natural conditions, but nutrient competition between the two can also be an important influence factor. Moreover, Kirpenko (2009) elucidated the allelopathic interactions between algae in various ecological mixtures, which showed that the allelopathic interactions were influenced by the intensity of growth and photosynthesis by the algae. Therefore, to remove harmful weeds from agricultural ecosystems and noxious algae from eutrophic lakes, it would be useful to consider the specific efficiency of bio-control agents based on allelopathic antagonistic interactions.

Many studies have detected allelopathic synergistic interactions in natural ecosystems. For example, Zuo et al. (2015) discovered that predatory zooplankton can enhance the algal inhibition of allelopathic aquatic macrophytes. It resulted from phenotypic and genotypic adaptation and improving tolerance of the zooplankton while increasing exposure to blooms (Ger et al., 2014). In another study, Zuo et al. (2014) performed field and laboratory tests, which showed that many co-existing aquatic plants have synergistic effects on algal inhibition. In addition, Barto et al. (2010) found that arbuscular mycorrhizal fungi can protect the native

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plant *Impatiens pallida* from the allelopathic effects of an invader *Alliaria petiolata*. Synergistic interactions may be related to the combined effect of various functional chemicals (Singh et al., 2003), but no previous studies have focused on the additive effects of allelopathic species.

In fact, the allelopathic interactions among organisms are actually due to the interactions among allelochemicals. At the allelochemical level, it is necessary to consider the active chemicals produced by species and their allelopathic synergistic interactions. For example, Chugh and Bharti (2014) demonstrated that the combined fractions collected from *Emblica officinalis* were more effective against two test pathogens (*Fusarium oxysporum* and *Rhizoctonia solani*) than each separate fraction. Thus, the isolated bioactive constituents had a synergistic effect when combined with other chemical constituents present in the fraction. Zhu et al. (2010) found that the submerged macrophyte (*Myriophyllum spicatum*) could produce allelopathic polyphenols, i.e., pyrogallic acid, gallic acid, ellagic acid, and (+)-catechin, which exhibited synergistic interactions as well as additive interactions in suppressing cyanobacteria. Park et al. (2006) detected a synergistic effect on algal growth inhibition when two or three phenolic compounds from rice straw were added.

However, there has been little previous research into the allelopathic interactions among four or more allelochemicals. Furthermore, allelochemical interactions depend on many factors such as the receptor, the respective proportions of allelochemicals, and the abiotic or biotic conditions. All of these factors mean that allelochemical interaction profiles are rather complex. Nakai et al. (2012) reported that the addition of the polyphenols and fatty acids would inhibit the growth of *M. aeruginosa*, and the interaction of the polyphenols and fatty acids was additive. Moreover, when the collective activity of a mixture of the polyphenols, i.e., ellagic, gallic and pyrogallic acids and (+) – catechin, was examined, the synergistic growth inhibition of *M. aeruginosa* occurred (Nakai et al., 2000).

In the present study, we assessed the allelopathic interactions among five typical allelochemicals based on their effects on algal inhibition. In particular, we compared the allelopathic interactions between two, three, four, and five allelochemicals. We also considered the test receptor, the total concentration of the mixture of allelochemicals, the respective proportions of allelochemicals, and the types of allelochemicals.

2. Materials and methods

2.1. Algal species and typical allelochemicals

An axenic strain of *Microcystis aeruginosa* was provided by the Freshwater Algae Culture of Hydrobiology Collection, China. The unialgal inoculants was cultured in sterilized *942* Medium (See the Supplementary material) under irradiance at 70 μmol photons/m^2/s, with a photoperiod of 12 h light/12 h dark and a temperature cycle of 25 °C light/20 °C dark in a temperature-conditioned growth chamber. All of the flasks containing microalgae, with the lid by Millipore filter, were shaken manually twice each day at a set time. The microalgae were cultured to the exponential phase before subsequent inoculation. The initial *M. aeruginosa* cell density used in the experiments was approximately 5.5×10^5 cells/mL. The culture conditions in the following experiments were the same as those described by Ye et al. (2014) unless stated otherwise.

In this study, we tested five typical allelochemicals that we identified previously in the allelopathic exotic plant *Alternanthera philoxeroides* (Mart.) Griseb. (Zuo et al., 2012), i.e., coumarin, CO; ρ-hydroxybenzoic acid, HA; protocatechuic acid, PA; stearic acid, SA; ρ-aminobenzene-sulfonic acid; AA). We obtained these five allelochemicals from Bangcheng Chemical Co. Ltd, Shanghai, China.

2.2. Algal treatments with allelochemicals

Exponential phase *M. aeruginosa* was subjected to the following five treatments: (1)–(5). After adding the allelochemical or mixture of allelochemicals only once, the algal density was recorded every 2 days, i.e., 2, 4, 6, 8, and 10 days. No chemical addition was set as the control. All treatments repeat thrice.

(1) We added each allelochemical to the algal culture medium, where the concentrations were set at: 0.1, 0.2, 0.4, 0.8, or 1.0 mg/L for CO or HA; and 1, 2, 4, 8, or 10 mg/L for PA, SA, or AA. A positive inhibitory effect of concentration (a dose-response relationship) was detected in the present study. The half maximal inhibitory concentration (IC_{50}) was 0.4 mg/L for CO and HA, whereas the IC_{50} was 4 mg/L for PA, SA, and AA.
(2) Two of the five allelochemicals were combined into a mixture, i.e., four mixtures that comprised CO + HA, PA + SA, PA + AA, and SA + AA were tested. Each mixture was tested at five ratios.
(3) Three of the five allelochemicals were combined into a mixture, where only one mixture was tested, i.e., PA + SA + AA. This mixture was tested at seven ratios.
(4) Four of the five allelochemicals were combined into a mixture, where only one mixture was tested, i.e., CO + HA + SA + AA. The mixture was tested at five ratios.
(5) All five allelochemicals were combined into a single mixture, i.e., CO + HA + PA + SA + AA. The mixture was tested at four ratios.

All five treatments above mentioned were shown in Table 1.

2.3. Statistical treatments

2.3.1. Actual and theoretical algal inhibition rates

The effects of different treatments on the growth of the test microalgae were expressed as the algal inhibition rate (IR), which is defined by the following equation:

$$1-\frac{N}{N_0} \quad (1)$$

where N and N_0 are the cell density (cells/mL) in the treatment and the control, respectively.

For treatment (1), IR was calculated using Eq. (1).

For treatment (2), the actual IR value was calculated using the IR formula and the theoretical value was determined with Eq. (2):

$$\sum_{i=1}^{2} IR_{i,k} \times Percentage_i \quad (2)$$

$$\sum_{i=1}^{2} Percentage_i = 1$$

where $IR_{i,k}$ is the actual IR value at concentration k for allelochemical i. In Eq. (3), k = 0.4 for CO + HA, and k = 4 for PA + SA, SA + AA, or AA + PA.

For treatment (3), the actual IR value was calculated using the IR formula and the theoretical value was determined with Eq. (3):

$$\sum_{i=1}^{3} IR_{i,4} \times Percentage_i \quad (3)$$

$$\sum_{i=1}^{3} Percentage_i = 1$$

where $IR_{i,4}$ is the actual IR value at a concentration of 4 g/L for allelochemical i. In Eq. (3), the theoretical value was determined for PA + SA + AA.