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Responses of enzymatic antioxidants and non-enzymatic antioxidants in the cyanobacterium *Microcystis aeruginosa* to the allelochemical ethyl 2-methyl acetoacetate (EMA) isolated from reed (*Phragmites communis*)

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Summary

Macrophytic allelochemicals are considered an environment-friendly and promising alternative to control algal bloom. However, studies examining the potential mechanisms of inhibitory allelochemicals on algae are few. The allelochemical ethyl 2-methyl acetoacetate (EMA), isolated from reed (*Phragmites communis*), was a strong allelopathic inhibitor on the growth of *Microcystis aeruginosa*. EMA-induced antioxidant responses were investigated in the cyanobacterium *M. aeruginosa* to understand the mechanism of EMA inhibition on algal growth. The activities of enzymatic antioxidants superoxide dismutase (SOD) and catalase (CAT), and the contents of non-enzymatic antioxidants reduced glutathione (GSH) and ascorbic acid (AsA) of *M. aeruginosa* cells were analyzed after treatments with different concentrations of EMA. Exposure of *M. aeruginosa* to EMA caused changes in enzyme activities and contents of non-enzymatic antioxidants in different manners. The decrease in SOD activity occurred first after 4 h of EMA exposure, and more markedly after 40 h. CAT activity did not change after 4 h of EMA exposure, but increased

Abbreviations: AGC, ascorbate–glutathione cycle; APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; DHAR, dehydroascorbate reductase; DHAsA, dehydroascorbate; DTNB-GR, 5, 5′-dithiobis-(2-nitrobenzoic acid)-glutathione reductase; EMA, ethyl 2-methyl acetoacetate; GPX, guaiacol peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide (i.e. oxidized glutathione); H_2O_2, hydrogen peroxide; MDHAR, NAD(P)H-dependent monodehydroascorbate reductase; NADPH, nicotinamide adenine dinucleotide phosphate; O_2, molecular oxygen; $O_2^{\bullet -}$, superoxide anion; $^1O_2$, singlet oxygen; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TNB, 5-thio-2-nitrobenzoic acid.

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obviously after 40 h. The contents of AsA and GSH were increased greatly by EMA after 4 h. After 60 h, low EMA concentrations still increased the CAT activity and the contents of AsA and GSH, but high EMA concentrations started to impose a marked suppression on them. EMA increased dehydroascorbate (DHAsA) and oxidized glutathione (GSSG) contents during all exposure times. After 60 h, the regeneration rates of AsA and GSH (represented by the AsA/DHAsA ratio and GSH/GSSG ratio, respectively) were reduced by high EMA concentrations. These results suggest that the activation of CAT and the availability of AsA and GSH at early exposure are important to counteract the oxidative stress induced by EMA, and the inactivation of SOD may be crucial to the growth inhibition of *M. aeruginosa* by EMA.

Introduction

In the past few decades, allelochemicals from macrophytes that suppress microalgal growth have gained great interest owing to their environmental potential as algicides to control water blooms (Nakai et al., 1999). Many allelochemicals have been identified, including γ-linolenic acid from *Typha latifolia* (Aliotta et al., 1990), α-asarone from *Acorus tatarinowii* (Pollio et al., 1993), 9, 10-dihydrophenanthrenoid from *Juncus acutus* (Dellagreca et al., 2002), tellimagrandin II and nonanoic acid from *Myriophyllum spicatum* (Leu et al., 2002; Nakai et al., 2005), and 2-ethyl-3-methylmaleimide from *Vallisneria spiralis* (Xian et al., 2006). It was noted by Einhellig (1995a) that the exploration of allelopathic herbicides depends greatly on understanding the mechanisms of action of these compounds and their behavior in the environment. Similarly, studies of allelopathic algicides are considered to need substantial understanding on their mechanisms of action. Harvested barley straw has been reported to be used in suppressing the growth of bloom-forming algae in field studies for several years (Ridge et al., 1999). The mechanisms of action of several allelochemicals and extracts from macrophytes, however, are not well understood (Einhellig, 1995b; Leu et al., 2002).

Several allelochemicals from higher plants have been reported to induce responses of the cellular antioxidant defense system (Yu et al., 2003; Gniazdowska and Bogatek, 2005; Singh et al., 2006). Singh et al. (2006) showed that activities of the enzymatic antioxidants including superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT), and glutathione reductase (GR) of the roots of *Cassia occidentalis* were significantly elevated under exposure of α-pinene. Yu et al. (2003) showed that root peroxidase and SOD activities of cucumber (*Cucumis sativus*) were significantly increased after exposure to allelochemicals. However, there is a general lack of investigations of allelochemical-induced antioxidant responses in algae.

The well-developed cellular antioxidant defense system includes enzymatic antioxidants and reduced non-enzymatic components (Mallick and Mohn, 2000). In enzymatic antioxidants, SOD conversion of superoxide anion ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) and CAT involvement in direct removal of H_2O_2 are very important. Ascorbic acid (AsA) and reduced glutathione (GSH) are crucial non-enzymatic antioxidants for H_2O_2 removal in the ascorbate–glutathione cycle (AGC). In the AGC, GSH is generally used as an electron donor to be oxidized into glutathione disulfide (i.e. oxidized glutathione, GSSG) for reducing dehydroascorbate (DHAsA) to generate AsA. The balance of reduced and oxidized non-enzymatic components is accurately regulated in normal cells.

The novel allelochemical ethyl 2-methyl acetoacetate (EMA) was reported to be one of the primary suppressive compounds in reed (*Phragmites communis* or *Phragmites australis* (Cav.) Trin. Ex Steudel, Poaceae) (Li and Hu, 2005). Growth was significantly inhibited by EMA in *Chlorella pyrenoidosa* and *Microcystis aeruginosa*, but not in *Chlorella vulgaris*. The medium effective concentrations (EC_{50}) of EMA on *C. pyrenoidosa* and *M. aeruginosa* were 0.49 and 0.65 mg L^{-1}, respectively. To date, the mechanisms of EMA inhibition on microalgal growth have mainly focused on leakage of metal ions from cells, decreases in SOD and peroxidase activities, and increases in the proportion of unsaturated lipid fatty acids in the cell membrane (Li and Hu, 2005). The general effects of EMA on the antioxidants, especially non-enzymatic antioxidants of algae, have not yet been examined.

The objective of the current study was to assess the effects of EMA on the enzymatic antioxidants (SOD and CAT) and non-enzymatic antioxidants (AsA and GSH) of *M. aeruginosa*, a prevalent