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Growth and physiological responses of freshwater green alga *Selenastrum capricornutum* to allelochemical ethyl 2-methyl acetoacetate (EMA) under different initial algal densities

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Abstract

Most natural algicides including macrophytic allelochemicals are known to selectively inhibit algal growth. The investigations on the modes of action about the species-specific algicides are little. In this study, the effects of allelochemical ethyl 2-methyl acetoacetate (EMA) identified from reed (*Phragmites communis*) on the growth, physiological, and biochemical processes of green alga *Selenastrum capricornutum* were investigated. The results showed that EMA had multiple effects on the growth of *S. capricornutum* under different initial algal densities (IADs). The algal growth was inhibited by EMA at low IADs, but stimulated at high IADs. Further, the potential modes of action of EMA on *S. capricornutum* were explored from ultrastructure, metabolic activity, reactive oxygen species level, and lipid peroxidation to trace the microenvironment changes in the algal cells. Damage in cell structure occurred at low IAD, but cells were well developed with increased metabolic activity at high IAD. The reactive oxygen species (ROS) levels were increased under both conditions. The increase of ROS level was acute at low IAD but slow at high IAD. EMA caused significant lipid peroxidation, i.e. oxidative damage on membrane lipids at low IAD but not at high IAD. Based on these results, the initial algal density is considered an important factor to influence algal growth and physiological and biochemical responses to EMA, the effects of EMA on *S. capricornutum* may be "hormesis-like", and different ROS increase ratio may be directly related with different responses of *S. capricornutum* to EMA.

Keywords: Algicide; Allelochemical; Ethyl 2-methyl acetoacetate (EMA); Hormesis; Physiology; *Selenastrum capricornutum*

1. Introduction

Allelopathy was defined to be the process in which secondary metabolites (called allelochemicals) [1] secreted by plants, bacteria, algae, etc. can affect other organisms [2]. Natural algicides including macrophytic allelochemicals can be easily degraded in natural environments. So they have been considered environment-friendly and dealt with greatly in recent years [3]. The studies on the allelopathy between macrophytes and algae hinted that the allelochemical effects on algae were species-specific. Derivatives of natural compound 9,10-anthraquinone from plant tannin extracts [4] selectively inhibited the growth of cyanobacterium *Oscillatoria perornata* but had no inhibitory effects on green algae and diatoms [5]. Sophorolipid, a kind of antialgal compound, exhibited much different effects on the growth of three common harmful marine algae *Alexandrium tamarense*, *Heterosigma akashiwo*, and *Cochlodinium polykrikoides* [6]. Ethyl 2-methyl acetoacetate (EMA) identified from reed (*Phragmites communis*) significantly inhibited the growth of *Chlorella pyrenoidosa* and *Microcystis aeruginosa* with no significant effect on *Chlamydomonas reinhardtii* but with stimulation on *Chlorella*

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vulgaris [7,8]. The aqueous acetone extracts of *Myriophyllum spicatum* shoots were reported to exhibit strong inhibition on various cyanobacteria, but to a lesser extent on chlorophytes and diatoms [9]. In addition, the extracts of the harvested and dried biomass of barley straw (*Hordeum vulgare*), a terrestrial plant were considered as highly effective natural algicides to inhibit the growth of algae such as *Synura petersenii*, *Dinobyron* sp., and *M. aeruginosa* when employed in water bodies, although the effect of barley straw was not a sort of allelopathy [10–13]. But Ferrier et al. reported that the extracts of barley straw could significantly facilitate the growth of *Selenastrum capricornutum*, *Spirogyra* sp., *Oscillatoria lutea* var. contorta, and *Navicula* sp. [12].

Considering the effect complexity between allelochemicals and algae, to learn much about the modes of action of allelochemicals may be favorable to understand the underlying processes of chemical interaction. It was pointed out by Einhellig that the barrier to block the studies on the allelochemical modes of action was due to unavailability of sufficient allelochemicals [14]. The technical improvements on the isolation, purification, identification, and even synthesis of allelochemicals overcame the problem. Many studies to disclose the potential modes of action of allelochemicals were reported in forestry and agriculture but still little on macrophytic allelopathy [15]. Moreover, the comparative studies on the inhibitory and stimulative effects of allelochemicals on algae are in deficiency.

In the previous work, Men et al. discovered that the effect of EMA on algae is species-specific [8]. Under 3×10^4 cell mL^{-1} (unpublished data) and 1×10^5 cell mL^{-1} of the initial algal densities, EMA significantly inhibited the growth of *S. capricornutum*. The study stopped at these algal densities without further considerations about the effects of different algal densities. In the present work, we designed algal density-gradients to investigate whether the inhibitory effect attenuates with density increasing or not. Besides the growth effects, algal physiological responses to EMA including metabolic activity (reflected by esterase activity), cell ultrastructure (reflected by the section of TEM), redox status (reflected by reactive oxygen species (ROS) level), and lipid peroxidation (reflected by malondialdehyde (MDA) content) were investigated to help the understanding of the potential effects and modes of action of natural algicides (especially allelochemicals) on algae.

2. Materials and methods

2.1. Materials

The axenic culture of freshwater green alga *S. capricornutum* was provided by the FACHB collection (Freshwater Algae Culture of Hydrobiology Collection, China, collection number: 271). The allelochemical EMA (CAS No.: 609-14-3) was purchased from Sigma–Aldrich Company.

2.2. Algal incubation

The alga was incubated using sterilized culture medium as reported by Trainor (1964) [16]. The alga was pre-incubated in 200 mL of culture medium in 500 mL conical flasks under an irradiance of 40–60 μmol photons m^{-2} s^{-1} (14 h light/10 h dark), at 24–25 °C before the experiment. During the late exponential phase of growth, the algal cultures were diluted with culture medium and counted with a haemacytometer (YA-XQ100, Improved Neubauer Counting Chamber, China) to adjust IADs.

2.3. Algal growth assay at different initial algal densities

The effects of EMA on *S. capricornutum* growth were measured under different IADs to investigate whether the inhibitory effect attenuates with density increasing or not. EMA solution (solvent: ethanol; the final concentration of the stock solution: 40 g L^{-1}) was added into 500 mL conical flasks, each of which contained 5 mL of algal inoculant and culture medium to fill up to 200 mL. The initial concentration gradients of EMA added were designed as follows: 0, 0.5, 1.0, 2.0, 4.0, and 8.0 mg L^{-1}. IAD gradients were 5×10^4, 15×10^4, and 5×10^5 cells mL^{-1}. All treatments were done in triplicate samples. The alga was incubated under an irradiance of 40–60 μmol photons m^{-2} s^{-1} (14 h light/10 h dark) at 24–25 °C for 7 days. The algal densities were tested per day during incubation. The algal densities were measured using a haemacytometer.

2.4. Esterase activity analysis

Fluorescein diacetate (FDA) (F7378, Sigma) was a classic dye diffusing into cells and then being cleaved by non-specific esterases to yield the fluorescent product fluorescein. Accumulation of fluorescein reflected intracellular esterase activity. Enzymatic hydrolysis of FDA released fluorescein with emitting intense fluorescence upon light excitement. The fluorescence signal detected was proportional to the esterase activity. The EMA-treated and control algal cells were harvested into 1 mL of phosphate buffered saline (PBS) solution (50 mM, pH 7.0) after repetitive suspensions. Then 20 mL of 1 mg mL^{-1} FDA was added into the last incubation saline. Before measurements, staining was done in the dark for 20 min at 25 °C and then cells were washed twice with fresh PBS. Cells were analyzed by flow cytometer (FACS Calibur; Becton Dickinson) to detect fluorescein fluorescence at the FL-1 parameter [17,18]. The fluorescein fluorescence (emission wavelength of fluorescein: 525 nm) was excited with Argon–ion laser (excitation wavelength: 488 nm). The number of algal cells detected by flow cytometer in every curve of the same figure was the same. The event of detection was at least 2000 cells