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Polyphenolic allelochemical pyrogallic acid induces caspase-3(like)-dependent programmed cell death in the cyanobacterium *Microcystis aeruginosa*

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ABSTRACT

The naturally occurring polyphenolic allelochemical pyrogallic acid (PA) is secreted by the aquatic vascular plant *Myriophyllum spicatum* and exhibits strong inhibitory effects on harmful cyanobacteria; however, the mechanisms underlying this inhibitory effect are largely unknown. In the current study, the biochemical and morphological changes that occurred during the PA-induced cell death of *Microcystis aeruginosa* and the mechanisms underlying these changes were investigated. PA exposure inhibited the growth of *M. aeruginosa* and significantly elevated caspase-3(-like) activities. The caspase-3 inhibitor aldehyde-terminated tetrapeptide (Ac-DEVD-CHO) abrogated the PA-induced caspase-3(-like) activities, which resulted in the alleviation of PA-induced growth inhibition. When *M. aeruginosa* cells were exposed to 14 mg L^{-1} PA for 72 h, nucleoid disintegration, photosynthetic lamellae rupture, pyknosis formation, vacuolation and DNA fragmentation were observed. The alignment of caspase domains showed that the key functional regions were conserved and showed remarkable orthology among different *M. aeruginosa* strains. Furthermore, a gas chromatography-mass spectrum analysis indicated that PA was gradually degraded. In addition, elevated intracellular reactive oxygen species (ROS) were detected. These results suggested that the production of ROS during the autoxidation of PA might act as a mediator that led to caspase-3(-like) activation and the subsequent initiation of programmed cell death in the cyanobacterium *M. aeruginosa*.

1. Introduction

Cyanobacterial blooms present a severe challenge to aquatic environments. The frequent occurrence of cyanobacterial blooms worldwide, which is aggravated by the increased eutrophication of water supplies [1,2], has led to the deterioration of water quality through the production of scum, toxins, and hypoxia as well as bad tastes and odors [3]. The accumulation of toxins in the aquatic food web threatens the health of human beings. Recent studies suggested a link between the neurotoxin β-methylamino-L-alanine (BMAA) and neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis [4]. Therefore, the control of cyanobacterial blooms is an important issue for the protection of aquatic environments.

A number of physical and chemical methods have been proposed to control cyanobacterial blooms, although most are not applicable because of the high cost and/or subsequent secondary pollution [5]. Naturally occurring allelochemicals are considered promising approaches for the control of harmful cyanobacterial blooms because of their environmentally friendly nature [6,7]. As early as 1949, Hasler and Jones [8] reported that submerged macrophytes could maintain phytoplankton densities at relatively low levels in natural water bodies. Nakai et al. [9] investigated the allelopathic effects produced by nine species of macrophytes and found that *Myriophyllum spicatum* showed the most significant inhibitory effects on *Microcystis aeruginosa* [10]. Four polyphenol allelochemicals, pyrogallic acid (PA), gallic acid (GA), ellagic acid (EA) and (+)-catechin (CA), were identified in the culture solution of *M. spicatum*. A number of studies have been conducted to explore the allelopathic mechanisms of these polyphenolic compounds and revealed that polyphenolic allelochemicals exerted their inhibitory effects on algae by suppressing alkaline phosphatase activities, attacking the photosystem II (PSII) of *M. aeruginosa* and leading to oxidative damage [10–13]. However, these mechanisms cannot fully explain the strong allelopathic activity observed in field investigations and laboratory studies.

Programmed cell death (PCD) is an irreversible genetically controlled suicide mechanism that promotes and maintains genetic

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stability [14]. Cells undergoing PCD display a series of typical features, including chromatin condensation, caspase activation and DNA cleavage. In many plant-pathogen interactions, hypersensitive responses triggered by pathogen infection involve the activation of a plant-encoded pathway for cell death known as PCD [15]. Babula et al. [16] demonstrated that allelochemical naphthoquinones triggered the PCD of tobacco BY-2 cells. In recent decades, increasing evidence has indicated that phytoplankton show PCD features in response to environmental stress [17]. Experimental evidence for PCD in cyanobacteria has been observed in three species: *Anabaena* sp. [18], *Trichodesmium* sp. [19], and *Microcystis* sp. [20]. Evidence from inhibitor tests and biochemical approaches suggests that caspase-like proteases may be involved in the control of cell death in phytoplankton [17]. Under oxidative stress, *M. aeruginosa* presents features of PCD. For instance, Ross et al. [20] detected an increase of caspase-3(-like) activities in *M. aeruginosa* exposed to hydrogen peroxide. When *M. aeruginosa* was cultivated under darkness, a slow increase in intracellular oxidative stress coincided with the activation of caspase 3-like proteins and an increase in TUNEL-positive cells [21]. Ding et al. [22] found that hydrogen peroxide induced apoptotic-like cell death in *M. aeruginosa* in a dose-dependent manner. Although a series of PCD marker have been observed in phytoplankton, whether allelochemicals inhibit the growth of the cyanobacterium *M. aeruginosa* by triggering the PCD pathway has remained unclear.

Among polyphenolic allelochemicals, PA shows the strongest inhibitory activity against *M. aeruginosa* with an EC_{50} of 0.65–2.97 mg L^{-1} [9, 13]. In natural aquatic environments, allelochemicals are continuously released into the surrounding water at low concentrations [9]. The concentrations of PA used in this study were higher than the concentrations secreted by aquatic macrophytes in natural water bodies. In the present study, PA was used as the model allelochemical compound and *M. aeruginosa* as the test species. Single exposure and acute toxicity test were performed to investigate whether the polyphenolic allelochemicals released by the submerged macrophyte *M. spicatum* could trigger PCD in *M. aeruginosa*, and if so, to explore how this process happened. Answering this question could provide further insights into the mechanisms of allelopathic interactions between aquatic plants and phytoplankton in aquatic ecosystems.

2. Materials and methods

2.1. Culture conditions and chemical source

The *M. aeruginosa* DIANCHI 905 strain was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. *M. aeruginosa* was cultured in BG11 medium [23] in 250-mL batch culture bottles (100 mL culture per bottle) at 25 ± 2 °C with a light:dark cycle of 12 h:12 h, an irradiance of 57 μmol photons m^{-2} s^{-1}, and agitation three times each day. PA identified in the *M. spicatum* culture solution [9] was commercially obtained (>99%, TCI, Tokyo, Japan).

2.2. Treatment of M. aeruginosa cells

The experiment was performed with a series of 250-mL conical flasks in which 100 mL BG11 culture solution was added and inoculated with *M. aeruginosa* at an initial concentration of 2000 cells/μL. The cell density of *M. aeruginosa* was examined using flow cytometry (BD Accuri C6, USA). Before exposure to the allelochemicals, the exponentially growing cells were pre-inoculated in autoclaved culture media at 27 μmol photons m^{-2} s^{-1} for 3 days. The tested organisms were treated in triplicate with 2, 7 and 14 mg L^{-1} of PA for 216 h. To test the role of caspase-3(-like) protease in PA-induced cell death, *M. aeruginosa* was treated with 14 mg L^{-1} PA in the presence of the reversible aldehyde caspase inhibitor aldehyde-terminated tetrapeptide (Ac-DEVDCHO) (50 mg L^{-1}). The addition of Ac-DEVDCHO (50 mg L^{-1}) without PA was used as a negative control.

2.3. Transmission electron microscopy assay

M. aeruginosa cell samples were collected via centrifugation of 35 mL culture solution at 1500 ×*g* for 15 min. The fixation and dehydration of cell samples and the preparation of ultrathin sections were performed according to the method described by [24]. The stained samples were examined using a Hitachi 7700 transmission electron microscope (Tokyo, Japan). Representative images under the different exposure concentrations were presented. The different morphologies were counted by analyzing three fields of view for different exposure concentrations.

2.4. Detection of caspase-3(-like) protease activity

The activity of caspase-3(-like) proteases was determined using the Enzchek® Caspase-3 Assay Kit #2 (Invitrogen) according to the method described by Ross et al. [20]. Briefly, 1.0×10^8 cells were collected by centrifugation at 3800 ×*g* for 5 min. The pellets were suspended in 1.5 mL of PBS (100 mM, pH 7.8), frozen with liquid N_2, sonicated on ice using a microtip sonicator until just thawed, and then immediately refrozen. This freezing/sonication cycle was repeated three times. The cell extract was then centrifuged (6600 ×*g*, 15 min) to remove any debris, and the supernatant was transferred to a clean microtube. The Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to determine the total protein concentration of the samples. The caspase-3(-like) activity was quantified using the Enzchek® Caspase-3 Assay Kit #2 according to the manufacturer's instructions. This assay exploits the specific proteolytic cleavage of the amino acid sequence Asp–Glu–Val–Asp (DEVD). The cell extracts (50 μL) were incubated with the caspase substrate *Z*-DEVD-R110 (25 μM, final concentration) for 1 h in the dark at room temperature. The appearance of fluorescent rhodamine 110 (R110) upon enzymatic cleavage of the nonfluorescent substrate Z-DEVD-R110 was subsequently assayed with a microplate reader (Molecular Device, M5, US) using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The background fluorescence signal from the negative controls without any enzyme was subtracted from the fluorescence of all of the samples.

2.5. DNA extraction and analysis

Total genomic DNA of the sample was prepared using Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China) following the manufacturer's instructions. The absorbance of 260 and 280 nm was measured to determine the quantity and quality of DNA extracts using a NanoDrop 8000 spectrophotometer (Thermo, USA). Equal amounts of DNA stained with Gel Red were separated by electrophoresis on a 1.5% TBE agarose gel at 60 V for 90 min, and the bands was visualized using the Bio-Rad Imaging System (Geldoc XR, CA, USA).

2.6. Flow cytometry analysis of phosphatidylserine (PS) externalization

PS externalization was performed as reported by Moharika et al. [25]. One milliliter of culture sample was centrifuged at 1500 ×*g* for 5 min and washed twice with 100 mM phosphate buffer (pH 7.4). Then, the pellet was suspended in 500 μL binding buffer and incubated with 5 μL PI and 5 μL Annexin V-FITC for 15 min in the dark according to Annexin V-FITC Apoptosis Detection Kit (Trevigen, MD, USA). The fluorescence detection was performed by high-speed sorting flow cytometry (FACSAria TMIII, BD, US) within 1 h. Green fluorescence of Annexin V-FITC was detected by channel FL1, and the red fluorescence of PI was detected by channel FL3. For every sample, 10,000 events were tested.