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Programmed cell death in the cyanobacterium *Microcystis aeruginosa* induced by allelopathic effect of submerged macrophyte *Myriophyllum spicatum* in co-culture system

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Abstract This investigation demonstrates that programmed cell death (PCD) in a cyanobacterium, *Microcystis aeruginosa*, resulting from allelopathic stress induced by a submerged macrophyte, *Myriophyllum spicatum*, in a co-culture system. The hallmarks of PCD, caspase-3-like protease activity, DNA fragmentation, and destruction of cell ultrastructure, as well as intracellular PCD signaling radicals, reactive oxygen species (ROS), and nitric oxide (NO), were measured in *M. aeruginosa* cells co-cultured with *M. spicatum* for 7 days. The results showed a dose–response relationship between *M. spicatum* biomass and *M. aeruginosa* mortality. A caspase-3-like protease was activated and elevated from day 3. Thylakoid disintegration, cytoplasmic vacuolation, and fuzzy nuclear zone were observed by transmission electron microscopy, and distinct DNA fragmentation was detected in *M. aeruginosa* cells at a *M. spicatum* biomass of 6.0 g fresh weight (FW) L^{-1} during the 7 days. Allelochemicals of total phenolic compounds (TPCs) were determined in co-culture water, and the concentration increased with increasing of *M. spicatum* biomass and co-culture time. Compared with the level of ROS production in the control group, a significant overproduction of ROS was detected in *M. aeruginosa* cells in the treatment group, and this was positively correlated with TPC concentration. Furthermore, the level of intracellular NO increased with the percent mortality of *M. aeruginosa*. The results indicated that a PCD pathway was induced in the cyanobacterium *M. aeruginosa* when co-cultured with the submerged macrophyte *M. spicatum*.

Keywords *Microcystis aeruginosa* · *Myriophyllum spicatum* · Allelopathic effect · ROS/NO · Caspase-3-like activity · Programmed cell death

Introduction

Submerged macrophytes play a key role in stabilizing the clear-water state in shallow eutrophic lakes (Scheffer et al. 1993). Inhibition of phytoplankton by allelopathy mediated by allelochemicals released from submerged macrophytes is one of the mechanisms that stabilize the clear-water state in shallow lakes (Hilt and Gross 2008). Ma et al. (2009) reported that transformation of Yuehu Lake, a shallow eutrophic lake in China, from turbid algae green to a transparent state, was achieved by restoring the submerged macrophyte community. The macrophytes *Myriophyllum*, *Ceratophyllum*, *Elodea*, and *Najas* were found to have potential allelopathic activity toward phytoplankton, with *Myriophyllum* displaying the stronger growth inhibition (Hilt and Gross 2008; Nakai et al. 1999).

Allelochemicals are likely to influence multiple physiological processes in phytoplankton (Zhu et al. 2010; Einhellig et al. 2004). One of primary responses of phytoplankton to allelopathic stress is overproduction of reactive oxygen species (ROS), including the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH). Wang et al. (2011) showed that excessive ROS production could be induced in *Microcystis aeruginosa* and *Pseudokirchneriella subcapitata* by exposure to an acutely toxic level of the allelochemicals (+)-catechin acid and pyrogallic acid, respectively. ROS may

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act as chemical oxidizer causing macromolecular damage or as a signaling radical to trigger programmed cell death (PCD) in phytoplankton and higher plants (Bhattacharjee 2005; Ross et al. 2006). ROS also works in concert with nitric oxide (NO) as signaling radical in response to biotic and abiotic stresses (Arasimowicz-Jelonek et al. 2012; Zhao et al. 2001). NO, a highly reactive gaseous radical, was initially described as a toxic compound and later recognized as a key signaling radical for regulating cellular function in both animals and plants (Wendehenne et al. 2001). Vardi et al. (2008) studied the diatom gene and proposed that it was involved in controlling fundamental cellular processes including photosynthesis, oxidative stress, and PCD, via management of NO signaling.

PCD, a genetically controlled form of cell suicide, is irreversible. It plays a crucial role in cellular homeostasis by selectively eliminating certain individuals in a population. For metazoans, PCD is described primarily as a developmental strategy or safeguard, a process in which proteolytic enzymes (caspases) and endonucleases are activated. The process is accompanied by several morphological hallmarks, including chromatin condensation and DNA fragmentation (Zuppini et al. 2007). It has been shown that PCD occurred not only in multicellular organisms but also in unicellular eukaryotes and prokaryotes, such as yeasts, bacteria, and phytoplankton (Ameisen 1996; Yarmolinsky 1995; Zuo et al. 2012), and it has been speculated that the origin of the self-destruct capacity may be as ancient as the origin of the very first cell (Ameisen 2002). Recent work has also shown evidence of PCD activation, in both eukaryotic and prokaryotic phytoplankton, in response to the adverse environmental stress (nutrient deprivation, cell aging, temperature, and UV radiation) leading to massive cell death (Warhurst 2014). In aquatic ecosystems, growth inhibition and antioxidant enzyme activity (SOD, CAT, and POD) in *Acutodesmus* (*Scenedesmus) obliquus* and *M. aeruginosa* were observed when co-cultured, respectively, with *Potamogeton malaianus* (Wu et al. 2007) and *Cyperus alternifolius* (Zhu et al. 2014). This implies that overproduction of intracellular ROS in *A. obliquus* and *M. aeruginosa* is induced by allelochemicals secreted from these macrophytes. The allelopathic effect of macrophytes may be viewed as an adverse environmental stress applied to chlorophytes and cyanobacteria. If PCD does occur in phytoplankton, triggered by ROS and NO signaling radical due to allelochemicals secreted by submerged macrophytes, it could play an important role in succession and the regulation of phytoplankton populations in aquatic ecosystems, and there are important implications for stabilizing water quality. However, there is currently little information available in the literature.

This study investigated the relationship between the biomass of submerged macrophytes and the production of ROS/NO, as well as the hallmarks of PCD in *M. aeruginosa* co-cultured with *M. spicatum* in laboratory. The goal was to ascertain whether or not a PCD event was induced in *M. aeruginosa* co-cultured with *M. spicatum* and to provide experimental evidence for further study of allelopathic effects of submerged macrophytes in shallow lakes.

Materials and methods

Macrophyte and cyanobacteria cultures

Myriophyllum spicatum was collected in May 2014 from Donghu Lake in Wuhan, China. The fresh macrophytes were rinsed with tap water to remove zooplankton and phytoplankton, planted in 80-L containers with 20–30 cm of lake sediment on the bottom, and grown outdoors for at least 1 month to facilitate adaptation to laboratory conditions. Before experiments, top tender leafy shoots were selected and rinsed with tap water again to adequately remove attached zooplankton and phytoplankton and maintained in aquaria containing 5 L of MIII nutrient solution (Nicklisch 1992).

Unicellular *M. aeruginosa* (toxic FACHB 905) was identified and provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The cyanobacteria were cultured with sterilized BG11 medium (Rippka et al. 1979) under controlled conditions (12:12-h light:dark photocycle with a light intensity of 25 μmol photons m^{-2} s^{-1} at 25 ± 1 °C) and were manually swirled twice daily during incubation. *M. aeruginosa* cells in the exponential growth phase were used in all experiments.

Co-culture experimental design

Pre-incubated *M. spicatum* were rinsed with sterile water thoroughly to remove zooplankton and plant fragments. The fresh weight (FW) of *M. spicatum* was measured after blotting with filter paper. An appropriate mass of macrophyte (0.5, 1.0, 3.0, and 6.0 g FW) was placed into 2000-mL flasks containing 1000-mL sterilized MIII medium. *M. aeruginosa* was inoculated with 1×10^6 cells mL^{-1}, and flasks without macrophyte were used as the control. At day 1 and day 2 of an experiment, 0.25-mL MIII medium (concentrated ×100) was added to the flasks to avoid nutrient limitation (Körner and Nicklisch 2002). Co-culture experiment duration was 7 days, and samples were collected for assays on days 1, 3, 5, and 7.

Measurement of growth and mortality of *M. aeruginosa*

During co-culture experiments, the growth and percent mortality of *M. aeruginosa* were determined by counting cells using flow cytometry (BD Accuri™ C6). The growth or percent mortality of each group was calculated as follows:

$$\mu(\%) = \left[(N_t - N_0) \Big/ N_0\right] \times 100$$

where μ is the growth percentage ($\mu > 0$) or percent mortality ($\mu \leq 0$), N_0 is the initial number of cells which were inoculated at day 0, and N_t is the number of cells at time t (from 1 to 7 days).

Assay of caspase-3-like activity

To quantify caspase-3-like activity in *M. aeruginosa* cells, the Enzchek® Caspase-3 Assay Kit no. 2 (Invitrogen) was used as described by Ross et al. (2006). Briefly, 100-mL suspensions of *M. aeruginosa* cells were collected by centrifugation at 3800×*g* for 5 min. The cell pellets were resuspended in 1.5-mL phosphate-buffered saline (PBS; 100 mM, pH 7.8), frozen with liquid N_2, sonicated until just thawed on ice using a microtip sonicator (Scientz-IID Ultrasonic Processor, China), and then immediately refrozen. The freezing/sonication cycle was repeated three times. The cell extract was then centrifuged (6600×*g*, 15 min) to remove debris, and the supernatant was transferred to a clean microtube. The caspase-3-like activity was then quantified using the Enzchek® Caspase-3 Assay Kit no. 2 according to manufacturer's instructions. This assay exploits the specific proteolytic cleavage of the amino acid sequence Asp–Glu–Val–Asp (DEVD). Cell-free supernatant (50 μL) was incubated with the caspase substrate Z-DEVD-R110 in final concentration of 25 μM for 60 min in the dark at room temperature. The appearance of fluorescent rhodamine 110 (R110) upon enzymatic cleavage of the non-fluorescent substrate Z-DEVD-R110 was subsequently assayed with a microplate reader (Molecular Device, M5, US) with excitation at 485 nm and emission at 538 nm. Many of the recently described caspase-3-like proteases, including caspase-3, caspase-7, and caspase-8, cleave poly(ADP-ribose) polymerase (PARP) at the amino acid sequence DEVD-G. Peptide aldehyde Ac-DEVD-CHO based on this sequence, which contains tetrapeptide recognition motif for caspase-3-like, was used to confirm the activity of caspase-3-like proteases (Kim et al. 1997). To verify the caspase-3-like activity of the experiments, the caspase-3-like inhibitor (Ac-DEVD-CHO) was applied according to kit instructions. The Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to determine the total protein concentration of the supernatants (Bradford 1976). Processing for protein quantification assay was as follows: 20 μL of cell free supernatant was added to 200 μL of Coomassie Brilliant Blue. After 5 min of incubation, the absorbance of blue complex with the proteins was measured at 595 nm. The caspase-3-like activity was calculated from the slope as fluorescence units per milligram of protein per minute of reaction.

DNA extraction and fragmentation analysis

Total DNA was extracted from *M. aeruginosa* cells using the TIANamp Bacteria DNA Kit (Tiangen, China) according to manufacturer's instructions. The DNA was then stained with GelRed (Biotium, catalog no. 41003, 0.5 mL) and analyzed by gel electrophoresis with 0.8 % agarose prepared in 0.5× TBE buffer (10× TBE was 0.89 M Tris, 0.89 M boric acid, and 20 mM Na_2EDTA, pH 8.3) at 100 V for 30 min (Ding et al. 2012).

Examination of cell ultrastructure by transmission electron microscopy

Cell ultrastructure was examined according to Berman-Frank et al. (2004). Cell samples of *M. aeruginosa* were washed three times with PBS, fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), and then placed in 1 % OsO_4 solution in 0.1 M phosphate buffer (pH 7.0) for 3 h at room temperature. After graded ethanol dehydration, the samples were embedded in epoxy resin (Epon-812) and polymerized at 45 °C for 48 h. Sections were obtained with an ultramicrotome, then stained with uranyl acetate in ethanol for 30 min at room temperature, and examined with a Hitachi H-7000 electron microscope.

Determination of total phenolic compounds in co-culture water

Total phenolic compounds in the co-culture water were determined by the Folin-Ciocalteau colorimetric method (Ben El Hadj Ali et al. 2014). Briefly, 2 mL Folin-Ciocalteau reagent was added to 2 mL of culture water, mixed well, and incubated at room temperature for 3 min. Then, 2 mL 7.5 % Na_2CO_3 (*w*/*v* in purified water) was mixed, and after 90 min, the absorbance was measured at 760 nm and results were expressed as gallic acid equivalents.

Detection of intracellular ROS and NO

The ROS level in *M. aeruginosa* was measured using the cell permeable indicator 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma) according to Hong et al. (2009). *M. aeruginosa* cells were collected by centrifugation and resuspended in 1 mL of PBS (50 mM, pH 7.0). DCFH-DA was added to give a concentration of 10 μM, and the cell resuspension was placed in the dark for 60 min at 25 °C and washed twice with fresh PBS. Sample fluorescence was measured with a BD Accuri C6 flow cytometer using the FL 1 channel, and 10,000 events were collected per sample.

The intracellular NO level was measured with the NO-specific fluorescent probe diaminofluorescein-FM diacetate (DAF-FM DA) as described by Tang et al. (2011). *M. aeruginosa* cells were collected by centrifugation and incubated in 10 μM DAF-FM DA for 60 min in the dark at 30 °C. Sample fluorescence was measured using BD Accuri C6 flow cytometer with the FL 1 channel.

Test for effect of exogenous NO on *M. aeruginosa*

Microcystis aeruginosa (1×10^6 cells mL^{-1}) were incubated in BG11 medium containing the exogenous NO donor, sodium nitroprusside (SNP), at concentrations of 25, 100, and 200 μmol L^{-1}. Each test group was cultured for 7 days, and the NO level and caspase-3-like activity in *M. aeruginosa* were detected at days 1, 3, 5, and 7.

Statistics

All experiments were carried out in triplicate. Results are expressed as mean values ± standard deviation (SD) of the mean. Significant differences among experimental results were determined by one-way analysis of variance (ANOVA) followed by the least significance difference (LSD). Data were assessed for normality (Shapiro-Wilk test) and for homogeneity of variance (Levene test) at the 0.05 significance level. When non-normality or non-homogeneity of variance was found, non-parametric Jonckheere-Terpstra test was conducted. Differences were considered significant at $P < 0.05$. Statistical analyses were carried out with the SPSS 13.0 software (Version 19.0, SPSS Inc., USA).

Results

Growth and mortality percentages of *M. aeruginosa* for each treatment group over the 7 days of co-culturing with *M. spicatum* are shown in Fig. 1. In the control group, *M. aeruginosa* continued to grow exponentially with a doubling time of approximately 2 days. In the treatment groups, growth of *M. aeruginosa* was inhibited significantly and mortality increased with increasing *M. spicatum* biomass. Significant cell death of *M. aeruginosa* in the high biomass group (6.0 g FW L^{-1}) occurred by day 3 and in all treatment groups by day 5. Mortality percentages were 45.23, 71.27, 96.74, and 97.41 % for groups co-cultured with 0.5, 1.0, 3.0, and 6.0 g FW L^{-1} *M. spicatum* ($P < 0.05$), respectively, at day 7.

For study of the cell death pathway, the caspase-3-like activity in *M. aeruginosa* was detected during the co-culture experiment (Fig. 2) and the activity was directly proportional to *M. spicatum* biomass. On day 3, activation of caspase-3-like activity was observed in the treatment group and it increased with co-culture time and reached a maximum at day 7 (Fig. 2a). To verify caspase-3-like activity in the present experiment, the samples were pre-incubated with the caspase-3-like inhibitor (Ac-DEVD-CHO), and a significant decrease in activity was detected (Fig. 2b), which indicated that the caspase-3-like activity was credible. In our co-culture system, the activation of caspase-3-like activity in *M. aeruginosa*, caused by *M. spicatum*, suggested that the cell mortality observed resulted from PCD.

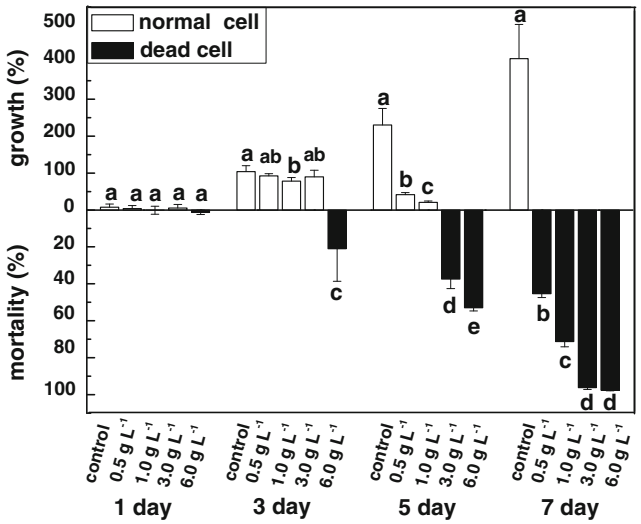

Fig. 1 *M. aeruginosa* growth and mortality percentages with *M. spicatum* biomass and co-culturing time. *Lowercase letters* a, b, c, and d indicate statistically significant differences ($P < 0.05$). One-way analysis of variance (ANOVA) followed by the least significance difference (LSD) was used, and non-parametric Jonckheere-Terpstra test was applied where there was an absence of normality or homogeneity of variance. Data and error bars represent means ± SD ($n = 3$)

Another hallmark of PCD is DNA fragmentation caused by activation of endonuclease during PCD. In this study, DNA fragmentation in *M. aeruginosa* was examined on days 0, 1, 3, 5, and 7 using agarose gel electrophoresis for the co-culture group with 6.0 g FW L^{-1} *M. spicatum* (Fig. 3). The degradation of DNA to lower-molecular-weight fragments was visible on day 3 and was much pronounced by day 7. The DNA fragments observed on the agarose gel were in the size range of 1000–15,000 bp, and there was an obvious increase in the degree of DNA fragmentation with the increase of co-culturing time. The DNA fragmentation observed is consistent with feature of PCD.

The cell ultrastructure of *M. aeruginosa* in the 6 g FW L^{-1} *M. spicatum* group was investigated, and the changes in cell ultrastructure are shown in Fig. 4. The normal nucleoid zone, dense cytoplasm, well-defined thylakoid, and intact plasma membranes were clearly visible in the cells of the control group (Fig. 4a, b). Compared with the control, partial disintegration of thylakoids and loss of cytoplasm density in test group *M. aeruginosa* cells were observed on day 3 (Fig. 4c), and on day 5 (Fig. 4d–f), the changes were amplified, a fuzzy nuclear zone, cytoplasmic vacuolation, and disintegration of thylakoids.

In this study, the allelochemical polyphenols secreted by *M. spicatum* were measured as total phenolic compounds (TPCs), which were used to evaluate the inhibitory effect of *M. spicatum* on *M. aeruginosa*. During the co-culture period, the concentration of TPC in the culture medium increased

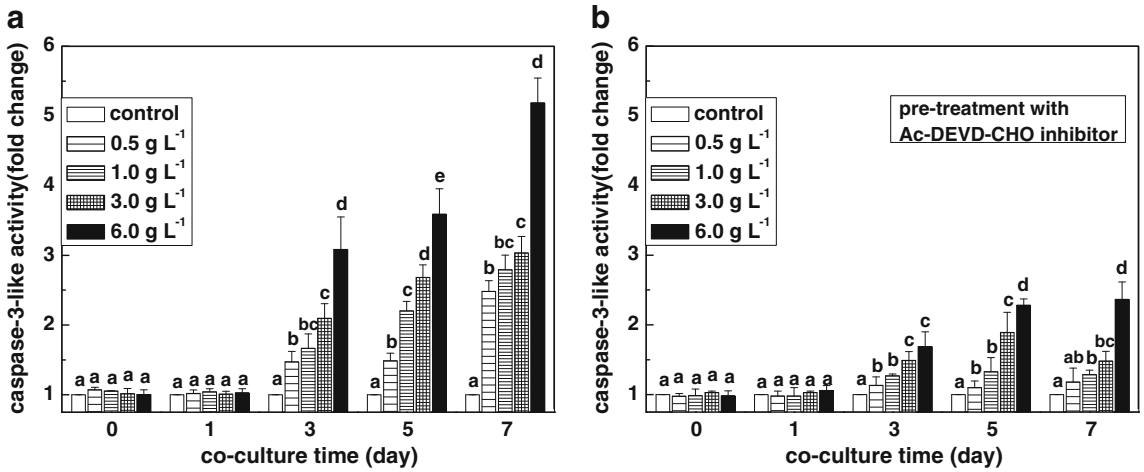

Fig. 2 Caspase-3-like activity of *M. aeruginosa* (**a**) and caspase-3-like activity of *M. aeruginosa* when pre-incubated with the caspase inhibitor Ac-DEVD-CHO (**b**). *Lowercase letters* a, b, c, d, and e indicate statistically significant differences ($P < 0.05$). One-way analysis of variance (ANOVA) followed by the least significance difference (LSD) was used, and non-parametric Jonckheere-Terpstra test was applied where there was an absence of normality or homogeneity of variance. Caspase-3-like activity was expressed as percent enzyme activity compared to control group. Data and error bars represent means ± SD ($n = 3$)

with both the biomass of *M. spicatum* and the co-culture time (Fig. 5). TPC was not detected in culture media without submerged macrophytes. The relationship between TPC content and macrophyte biomass indicated that these secondary metabolites were released to the culture medium by *M. spicatum*, leading to the growth inhibition of *M. aeruginosa* cells. There also was a positive correlation between the concentration of TPC in co-culture water and the activity of caspase-3-like in *M. aeruginosa*.

The ROS levels measured in *M. aeruginosa* cells during the co-culture experiment are shown in Fig. 6. The ROS production increased in all test groups by day 3. The levels of ROS in the 0.5, 1.0, 3.0, and 6.0 g FW L^{-1} *M. spicatum* groups were, respectively, 1.30, 1.38, 1.86, and 2.09 times higher than that in the control group on day 5 and were higher still on day 7. Overproduction of ROS in *M. aeruginosa* increased with *M. spicatum* biomass and was attributed to the increased TPC in co-culture water (Fig. 5).

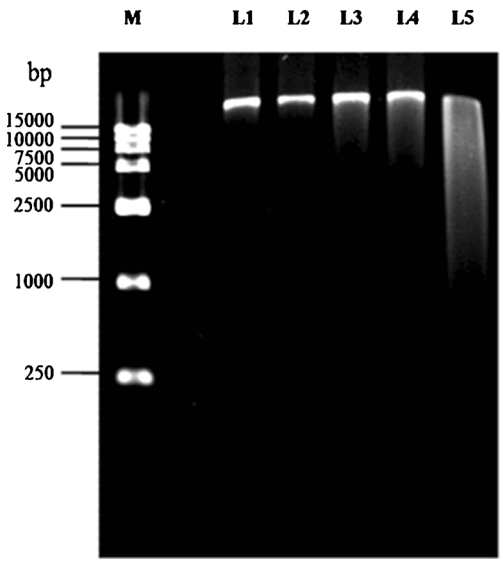

Fig. 3 DNA fragmentation in *M. aeruginosa* cells co-cultured with *M. spicatum* at 6 g FW L^{-1}. L1, L2, L3, L4, and L5 represent the DNA condition of *M. aeruginosa* cells on days 0, 1, 3, 5, and 7, respectively. *M* DNA marker (DS 15000, Dongsheng, China)

NO levels in the *M. aeruginosa* cells were also measured (Fig. 7). In the groups with 3.0 and 6.0 g FW L^{-1} of macrophyte, NO generation was 1.25 and 1.31 times higher than the control group on day 5 and higher on day 7. A positive control for NO staining of cell was performed by exposure of *M. aeruginosa* to exogenous NO donor SNP (see Fig. 8a). The results showed a dose–response and time-dependent relationship between treatment concentration of SNP and the level of intracellular NO. It indicated that the result of NO generation in cyanobacteria was credible. Comparing Figs. 2, 6, and 7, the positive relationship between ROS/NO level and caspase-3-like activity can be seen.

To verify the role of NO signaling on the initiation of PCD in *M. aeruginosa*, the intracellular NO level and activation of caspase-3-like proteases were investigated by addition of SNP, an exogenous NO donor, to the culture media with *M. aeruginosa*. The results showed that SNP produced a dose-dependent rise in intracellular NO (Fig. 8a). The caspase-3-like activity in *M. aeruginosa* was triggered, and activity increased significantly with increase of SNP concentration (Fig. 8b). To verify the caspase-3-like activity in *M. aeruginosa* cells, samples were pre-incubated with the caspase-3-like inhibitor Ac-DEVD-CHO and caspase-3-like activity was significantly lower than in untreated cells, which indicated that the activity of caspase-3-like in the samples of *M. aeruginosa* cells was credible (data not shown). This exogenous NO donor experiment suggested

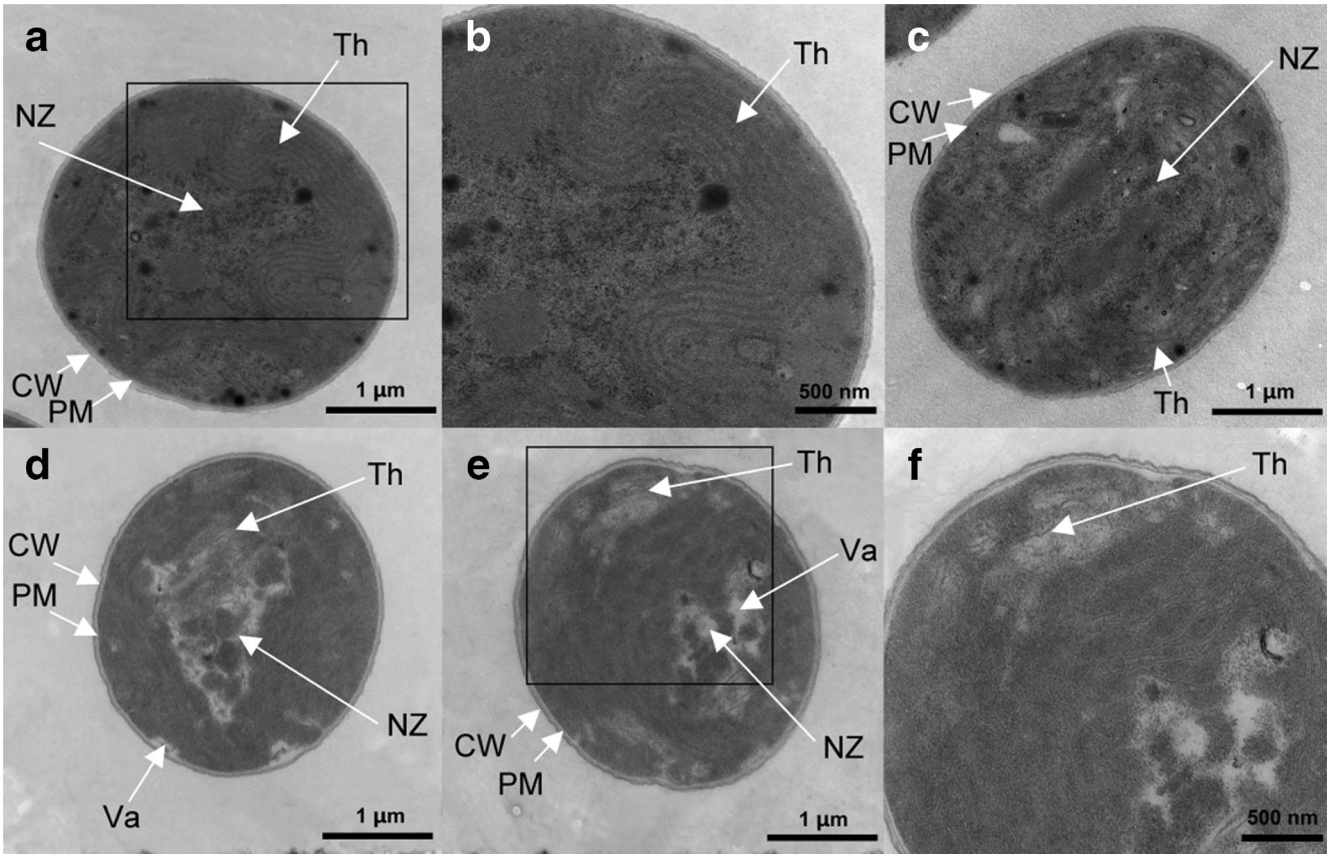

Fig. 4 Ultrastructure changes in *M. aeruginosa* cells co-cultured with *M. spicatum* of 6 g FW L^{-1} at different co-culture time. **a** Control cells. **b** The magnified thylakoid membrane of designated window in **a. c** *M. aeruginosa* cells on day 3. **d**, **e** *M. aeruginosa* cells on day 5. **f** The magnified thylakoid membrane of designated window in **e**. *NZ* nucleoid zone, *Th* thylakoid, *Va* vacuolation, *CW* cell wall, *PM* plasma membrane

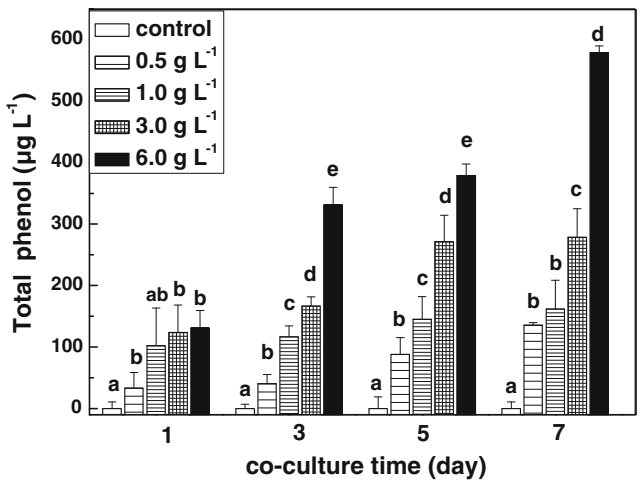

Fig. 5 Total phenolic compounds (TPCs) in the co-culture water with *M. spicatum* biomass and co-culturing time. *Lowercase* letters a, b, c, d, and e indicate statistically significant differences ($P < 0.05$). One-way analysis of variance (ANOVA) followed by the least significance difference (LSD) was used, and non-parametric Jonckheere-Terpstra test was applied where there was an absence of normality or homogeneity of variance. Data and error bars represent means ± SD ($n = 3$)

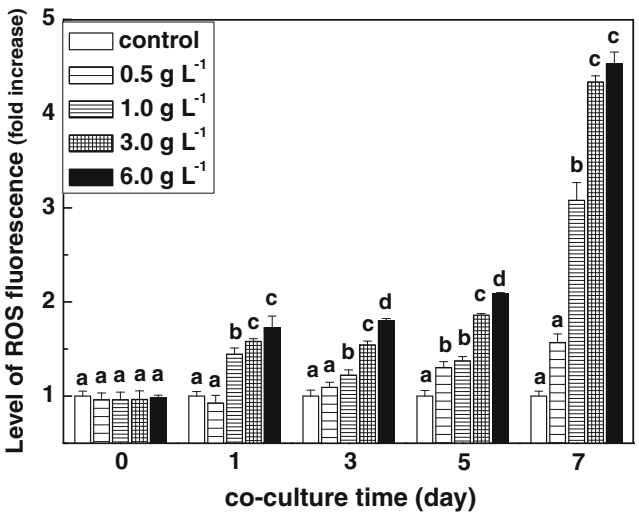

Fig. 6 ROS generated in *M. aeruginosa* co-cultured with *M. spicatum* biomass and co-culturing time. *Lowercase letters* a, b, c, and d indicate statistically significant differences ($P < 0.05$). One-way analysis of variance (ANOVA) followed by the least significance difference (LSD) was used, and non-parametric Jonckheere-Terpstra test was applied where there was an absence of normality or homogeneity of variance. Data and error bars represent means ± SD ($n = 3$)

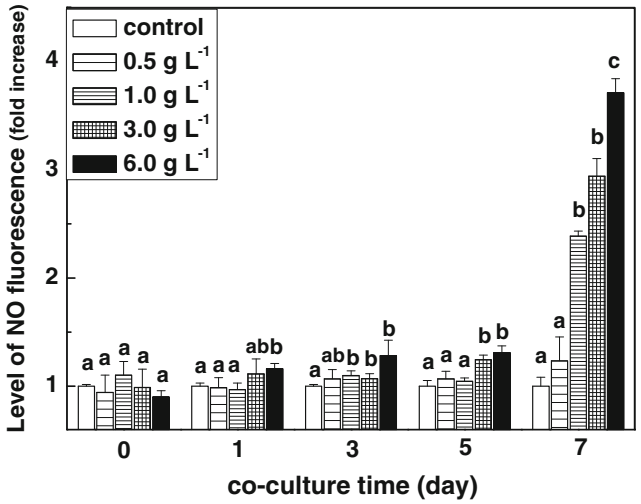

Fig. 7 NO generated in *M. aeruginosa* with *M. spicatum* biomass and co-culturing time. *Lowercase letters* a, b, and c indicate statistically significant differences ($P < 0.05$). One-way analysis of variance (ANOVA) followed by the least significance difference (LSD) was used, and non-parametric Jonckheere-Terpstra test was applied where there was an absence of normality or homogeneity of variance. Data and error bars represent means ± SD ($n = 3$)

that the intracellular NO had the potential to trigger PCD in the test organism.

Discussion

During the past decade, it has been recognized that PCD occurs virtually in all organisms, including metazoans, plants, fungi, bacteria, and protists. Caspases are cysteine-dependent, aspartate-directed proteases and play a critical role in the regulation of PCD in metazoans. In prokaryotes and most unicellular eukaryotes, metacaspases are found associated with PCD rather than caspases. Cyanobacteria contain a conserved p20-like domain but uniformly lack the p10-like domain in metacaspase. These were termed metacaspase-like proteins (Choi and Berges 2013). The first experimental characterization of biochemical properties of the caspase homolog gene MaOC1 from the toxic bloom-forming cyanobacterium *M. aeruginosa* PCC 7806 was expressed in *Escherichia coli* (Klemenčič et al. 2015). However, little molecular biological information is available on PCD in *M. aeruginosa*. Experimental evidence for caspase-3-like proteases in cyanobacteria has been obtained primarily from the studies on the effect of adverse environmental factors thought to induce PCD such as treatment of the cyanobacterium *M. aeruginosa* with H_2O_2 (Ding et al. 2012), exposure of the freshwater cyanobacterium *Anabaena* sp. to univalent cation salts (Ning et al. 2002), and subjection of *Trichodesmium* sp. to iron deprivation and excessive light intensity (Berman-Frank et al. 2004).

In this study, the effect of allelopathic stress on *M. aeruginosa* was studied during co-culture with *M. spicatum*. Caspase-3-like activity of cyanobacteria in the co-culture groups was significantly higher than in the control group and was drastically reduced when *M. aeruginosa* cell samples were pre-incubated with a reversible caspase inhibitor (Ross et al. 2006), which was accompanied by DNA fragmentation, disintegration of ultrastructure, and high-percent mortality of *M. aeruginosa*. Results indicated that PCD in *M. aeruginosa* was induced by the allelopathic stress from *M. spicatum*. Similar results have been reported for the green algae *Dunaliella tertiolecta* placed in darkness (Segovia et al. 2003) and *Chlorella saccharophila* upon heat shock (Zuppini et al. 2007).

DNA fragmentation in *M. aeruginosa* was observed during co-culture, indicating that endonuclease was activated along with caspase-3-like proteases consistent with a previous report

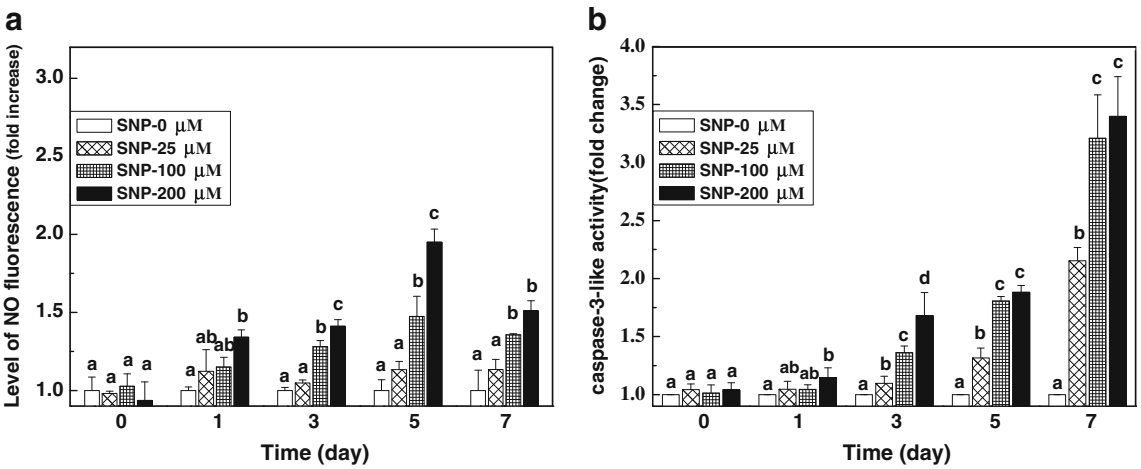

Fig. 8 Intracellular NO level (**a**) and caspase-3-like activity in *M. aeruginosa* (**b**) over 7 days after treatment with sodium nitroprusside (SNP). *Lowercase letters* a, b c, and d indicate statistically significant differences ($P < 0.05$). One-way analysis of variance (ANOVA) followed by the least significance difference (LSD) was used, and non-parametric Jonckheere-Terpstra test was applied where there was an absence of normality or homogeneity of variance. Data and error bars represent means ± SD ($n = 3$)

(Aleksandrushkina and Vanyushin 2009). PCD in eukaryotes leads to DNA fragmentation that gives a ladder-like pattern on the agarose gel. Examples include the PCD and DNA laddering observed in green algae subjected to stress, i.e., heat-stressed *Chlamydomonas reinhardtii* (Nedelcu 2006) and NaCl/KCl-stressed *Micrasterias denticulata* (Affenzeller et al. 2009). However *M. aeruginosa* is a prokaryote lacking chromosomal histones and nucleosome units (Makino and Tsuzuki 1971), and the DNA fragments give bands rather than a ladder-like pattern. Similar results have been reported for the cyanobacteria *Anabaena* sp. PCC7120 (Ning et al. 2002) and *M. aeruginosa* (Ding et al. 2012).

Disintegration of ultrastructure is another hallmark of PCD and includes vacuolization and degradation of internal cellular components, although plasma membranes remain intact in prokaryotic cells (Berman-Frank et al. 2004). Typical inclusion bodies of cyanobacteria are the thylakoids, polyhedral bodies, glycogen granules, lipid inclusions, cyanophycin granules, polyphosphate bodies, ribosomes, and a visible nuclear zone with clumps of DNA (Jensen 1993). After co-culturing with *M. spicatum*, it was observed that external configuration of *M. aeruginosa* cells remained intact while the interior structures changed, in which inclusions collapsed, DNA dispersed, and the nuclear zone boundary became less distinct or even disappeared. Similar ultrastructure alterations were previously reported for *M. aeruginosa* exposed to the alleochemical gramine (0.5–8.0 mg L^{-1}) (Hong et al. 2010), *M. denticulata* treated with H_2O_2 (Darehshouri et al. 2008), and *D. tertiolecta* deprived with light (Segovia et al. 2003). In this study, these morphological changes, DNA fragmentation, and activation of caspase-3-like activity detected are in accordance with PCD hallmarks observed under adverse conditions by others (Moharikar et al. 2006; Berman-Frank et al. 2004; Zuppini et al. 2007).

The concentration of TPCs was in the μg L^{-1} range in the co-culture medium and increased with co-culture time. These compounds are degradable in water, implying that they are secreted continuously by submerged macrophytes and result in long-term stress to the phytoplankton. Sustained exposure to TPC leads to formation of excess ROS/NO that triggers PCD in *M. aeruginosa*. Ross et al. (2006) reported that caspase-3-like activity was elicited in *M. aeruginosa* in the wild environment treated with H_2O_2, and Ding et al. (2012) contributed to our understanding of PCD in *M. aeruginosa* by describing the diverse PCD markers that appeared after exposure to H_2O_2. It is well known that ROS can directly react with biomolecules and damage DNA, proteins (enzymes), lipids (membranes), etc. (Hemnani and Parihar 1998), as well as can act as signaling radical that activate the intracellular caspases and trigger PCD (Simon et al. 2000). Moreover, studies indicate that the level of ROS required for triggering PCD is significantly lower than the level that causes direct irreversible oxidative damage to *M. aeruginosa* cells (Lu, unpublished paper, 2014).

TPC secreted by *M. spicatum*, including pyrogallic acid, (+)-catechin acid, gallic acid, and ellagic acid (Nakai et al. 2000), have hydroquinone structures and autoxidize rapidly forming the corresponding semiquinone and *o*-quinone, which are able to participate in the futile redox cycle in the cell of organisms. In this process, the quinone is reduced to corresponding semiquinone radical by consumption of intracellular reduction power (i.e., DADPH) and the electron of the radical transfers to oxygen forming superoxide radicals ($O_2 \cdot^-$), and the quinone is reformed again in the cell of target organism (Kondo et al. 1999; O'Brien 1991; Long et al. 2000; Saeki et al. 2000; Janeiro and Brett 2004; Wang et al. 2011). This is suggested as the biochemical mechanism by which phenolic compounds, acting as allelochemicals, induce the excessive formation of ROS that triggers PCD.

NO is a reactive nitrogen that functions as signaling radical and is prevalent in phytoplankton (Vardi et al. 2008). This paper is the first report that the NO is detected in *M. aeruginosa* under co-culture with *M. spicatum*. As with H_2O_2, NO can react directly with biomolecules to produce deleterious effects and also act as a signal to trigger PCD (Brockhaus and Brüne 1998; Clarke et al. 2000). Paradoxically, NO is also reported to suppress cell death by scavenging ROS, suppressing superoxide and H_2O_2-mediated cytotoxicity, and thereby preventing PCD (Wink et al. 1993). In this study, NO levels increased with ROS and were followed by mass mortality of *M. aeruginosa*, suggesting that the role of NO under the experimental conditions was primarily as a signaling radical. The exogenous NO donor experiment using SNP showed that intracellular NO led to activation of caspase-3-like activity and *M. aeruginosa* cell death, thereby implying PCD. In aquatic ecosystems, unsaturated aldehydes produced by the diatom *Phaeodactylum tricornutum* act as infochemicals to induce overproduction of NO that activate a gene cascade and involved in PCD (Casotti et al. 2005; Vardi et al. 2006, 2008). To date, no relationship between the signaling radical, NO, and allelopathic effect has been reported. The elucidation of more detailed mechanisms for NO and ROS production in *M. aeruginosa* prior to the PCD caused by allelochemicals secreted by submerged macrophytes will require further research.

In conclusion, our results indicate that (1) PCD in *M. aeruginosa* was induced by allelopathic effects of the submerged macrophyte *M. spicatum* in a co-culture system. (2) A dose–response relationship between ROS/NO level and caspase-3-like activity was found in *M. aeruginosa*. The exogenous NO donor experiment showed that NO has potential to trigger caspase-3-like activity in *M. aeruginosa*. (3) Effective inhibition of phytoplankton by *M. spicatum* was demonstrated; the allelochemicals (measured as TPC) were continuously released from *M. spicatum* with low concentration causing the excess production of ROS/NO, which activated caspase-3-like proteases and PCD in the cyanobacterium, *M. aeruginosa*.

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