Generation of reactive oxygen species in cyanobacteria and green algae induced by allelochemicals of submerged macrophytes

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Abstract

Inhibition of phytoplankton by allelochemicals released by submerged macrophytes is reported to be one of the mechanisms that maintain a clear-water state in shallow lakes. In order to elucidate this mechanism, the ability of six polyphenols and two long-chain fatty acids to induce the generation of reactive oxygen species (ROS) in phytoplankton was studied using the ROS sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The results showed that only (+)-catechin (CA) and pyrogallic acid (PA) could induce ROS formation in Microcystis aeruginosa and Pseudokirchneriella subcapitata. 25 mg L\(^{-1}\) CA caused 1.2, 1.4 and 1.8 times increase of ROS levels in M. aeruginosa at 1, 2 and 4 h exposure, respectively, and, correspondingly in P. subcapitata cells, these values were 3.7, 6.2 and 7.7, respectively. PA also significantly increased the levels of intracellular ROS in P. subcapitata (\(P < 0.01\)); however, significant ROS generation in M. aeruginosa was observed at only 4 h exposure (\(P < 0.01\)). Light enhanced ROS generation in CA treated cells, but not in the cells treated with PA. CA and PA may act as redox cyclers after uptake by test organisms and produce ROS successively. These results suggest that the oxidative stress induced by the redox cycling property of allelochemicals may be one of the important causes for the inhibitory effect of some submerged macrophytes towards undesired phytoplankton in natural aquatic ecosystems.

Keywords: Pyrogallic acid (+)-Catechin Reactive oxygen species Phytoplankton Redox cycling

1. Introduction

Human activities have accelerated eutrophication in water bodies, especially in developing countries. Eutrophication results in the frequent occurrence of algal blooms which cause ecological and aesthetic problems, and algal toxins can pose serious threats to animal and human health. Suppressing the growth of undesired phytoplankton species is crucial for controlling algal blooms. Recently, research dealing with the natural and natural-based allelochemicals (cyanobactericides) has gained more and more attention due to the negative attributes of currently available algicides (Schrader, 2005). As far back as 1949, Hasler and Jones (1949) reported that submerged macrophytes could reduce phytoplankton densities in ponds. Mjelde and Faafeng (1997) investigated 24 lakes in Norway and indicated that the low population of phytoplankton species and the clear-water state were only found in the lakes with high biomass density of submerged macrophytes, even the lakes contained high concentrations of phosphorus (above 30 mg m\(^{-3}\)). Later studies showed that the submerged macrophytes significantly inhibited the growth of phytoplankton by the excretion of some second metabolites (Körner and Nicklisch, 2002; Nakai et al., 2005; Mulderij et al., 2006), and this phenomenon is termed allelopathy (Rice, 1984). It is a new possibility for the effective control of algal blooms (Nakai et al., 1999).

Members of the genus Myriophyllum (Haloragaceae) are highly competitive submerged macrophytes with strong allelopathic potential (Hilt and Gross, 2008). Myriophyllum spicatum L. (Haloragaceae) contains high concentrations of phenolics (Smolders et al., 2000), especially gallo- and ellagitannins (Gross et al., 1996; Gross, 1999). Planas et al. (1981) reported that the secretion of M. spicatum contained 14 kinds of phenols and polyphenols, such as caffeic, ellagic, and gallic acids, which could significantly inhibit the growth of phytoplankton. Nakai et al. (2000, 2005) found that four polyphenols—pyrogallic acid, (+)-catechin, ellagic and gallic acids, and three fatty acids—nonanoic, cis-6-octadecenoic, and cis-9-octadecenoic acids in the culture solution of Microcystis aeruginosa have significant inhibitory effects on Microcystis aeruginosa. However, except for the 50% growth inhibitory concentrations (EC\(_{50}\) values) of most reported allelochemicals to phytoplankton, only a little information about physiological and biochemical responses to allelochemicals stress is available.

From the literature, it can be found that the concentrations of allelochemicals secreted into natural water bodies by submerged macrophytes are much lower than the EC\(_{50}\) values of the individual allelochemicals (Nakai et al., 2000, 2001, 2005). For example, the...
concentrations of polyphenols and fatty acids released by *M. spicatum* were 5.2–76.6 μg L$^{-1}$ at the density of 100 g fw L$^{-1}$ after 3 d cultivation in the laboratory (Nakai et al., 2000, 2005). The concentrations of allelochemicals in the natural condition will be much lower than that in the laboratory condition, since the biomass density of macrophytes in natural environment was only about 10 g fw L$^{-1}$ (Duarte and Kalf, 1990; Körner and Nicklisch, 2002). However, the EC$_{50}$ values of individual polyphenols and fatty acids were reported to range from 0.5 to 9.77 mg L$^{-1}$ for different strains of *M. aeruginosa* (Nakai et al., 2000, 2001, 2005; Zhu et al., 2010). So that, further study on the toxicological mechanisms of the allelopathy is necessary that may be helpful to explain that low concentrations of allelochemicals exhibit significant inhibitory effects on the growth of phytoplankton in natural aquatic environment.

One of the early responses of phytoplankton to algicides stress is the excess production of reactive oxygen species (ROS) (Hirata et al., 2004; Schrader et al., 2005). Wu et al. (2007) reported that the antioxidant enzymes activities viz. superoxide dismutase (SOD), catalase and peroxidase as well as the malondialdehyde were stimulated in *Scenedesmus oblilquis* when co-existence with *Potamogeton malaisianus*. Shao et al. (2009) also reported that oxidative damage as shown by lipid peroxidation might be one of the causes for the allelopathic effect of pyrogallic acid on *M. aeruginosa*. However, direct evidence for oxidative stress, namely, ROS formation induced by the allelochemicals in phytoplankton is scarce to be found in the literature.

The aims of this study are (1) to screen the active substances which can induce ROS formation in the cells of *M. aeruginosa* and *Pseudokirchneriella subcapitata* from six allelopathic polyphenols and two long-chain fatty acids that are excreted by *M. spicatum*; (2) to discuss the possible mechanism involved in ROS generation which may possibly take place in the cells of phytoplankton.

**2. Materials and methods**

**2.1. Culture conditions**

*M. aeruginosa* (toxic FACHB 942) and *P. subcapitata* (FACHB 271) were purchased from Freshwater Algae Culture Collection of the Institute of Hydrobiology, the Chinese Academy of Sciences. The unialgal *M. aeruginosa* were axenically cultivated in BG-11 medium (Rippka et al., 1979) under an irradiance of 22.5 μmol m$^{-2}$ s$^{-1}$ and *P. subcapitata* cells were grown axenically in ISO 8692 medium (ISO 8692, 2004) under an irradiance of 60 μmol m$^{-2}$ s$^{-1}$; all cultures were cultivated at 25 ± 2 °C in an incubator with a 12/12 h light/dark cycle, and shaken twice each day for 30 s by hand at 12 h intervals. Exponentially growing cultures were used for all experiments.

**2.2. Chemicals**

In order to evaluate ROS production by allelochemicals in *M. aeruginosa* and *P. subcapitata*, six polyphenols and two long-chain fatty acids were commercially obtained, namely pyrogallic acid (PA, 99.5%, Chem Service, West Chester, PA, USA), gallic acid (GA, >96.0%, Acros Organics, Morris Plains, NJ, USA), ellagic acid (EA, >96.0%, Fluka Biochemika, Buchs, Switzerland), (++)-catechin (CA, 98%, TCI, Tokyo, Japan), caffeic acid (CFA, Sigma–Aldrich, St. Louis, MO, USA), protocatechuic acid (PCA, 97%, Alfa Aesar, Heysham, Lancashire, UK), nonanoic acid (NA, Alfa Aesar, Heysham, Lancashire, UK) and cis-9-octadecenoic acid (C0A, Sigma–Aldrich, St. Louis, MO, USA). 2’7’-Dichlorodihydrofluorescein diacetate (DCFH-DA) and dimethyl sulfoxide (DMSO, >99.5%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Analyticalgrade hydroperoxide (H$_2$O$_2$, 30%) was obtained from Shanghai Biochemical Reagent (Shanghai, China).

**2.3. Allelochemicals exposure**

Because the ROS sensitive probe DCFH-DA autoxidizes readily, concentrations of allelochemicals higher than the reported EC$_{50}$ values as well as the short exposure time were used in this study to permit more rapid formation of ROS (Bais et al., 2003; Schrader et al., 2005). Therefore, the nominal concentrations of 25, 50, 75, 100 mg L$^{-1}$ were set for all allelochemicals. To create a positive control, oxidative activity was provided with H$_2$O$_2$.

**2.4. Detection of reactive oxygen species**

ROS formation was measured by using the cell permeable indicator 2’7’-dichlorodihydrofluorescein diacetate (DCFH-DA). The DCFH-DA is hydrolyzed by cellular esterases to form the nonfluorescent 2’7’-dichlorodihydrofluorescein (DCFH) after penetrating into the cell of the test organisms, and then DCFH is immediately transformed to highly fluorescent 2’7’-dichlorofluorescein (DCF) in the presence of ROS, such as hydrogen radical (OH$^-$) and H$_2$O$_2$-peroxidase, but not superoxide radical anion (O$_2^-$) or H$_2$O$_2$ only (Halliwell and Gutteridge, 1999; He and Häder, 2002). The stock solution of DCFH-DA was prepared in DMSO at a concentration of 10 mM and stored at −20 °C. The ROS level was determined as the method described by Schrader et al. (2005) and Knauret and Knauer (2008).

For the determination of ROS, exponentially growing *M. aeruginosa* and *P. subcapitata* cells were harvested by centrifugation (5000 rpm, 5 min and 7 min, respectively), and the cell pellets were washed once with phosphate buffer saline (PBS) solution (50 mM, pH 7.0) and resuspended in PBS with 10 μM DCFH–DA to adjust the cell density to 5 × 10$^6$ cells mL$^{-1}$ and 10$^6$ cells mL$^{-1}$ for *M. aeruginosa* and *P. subcapitata*, respectively. Pre-incubation was carried out on a shaker at 25 °C in the dark for 1 h. Then, the samples were washed and resuspended in respective culture medium. 125 μL cell suspension and 125 μL allelochemical solution were transferred into a 96-well microplate. Control wells received only phytoplankton cells and DCFH–DA in respective culture medium. Solvent controls were performed similarly. DMSO concentrations did not exceed 0.25% (v/v) in solvent control and treated wells, and the results indicated that the concentrations of DMSO added had no effect on ROS generation of the test organisms. The background fluorescence formation was determined with the mixture of BG-11/ISO medium, cells of test phytoplankton without loading the DCFH–DA and different concentrations of the allelochemicals. Four replicates for each test concentration and background were performed.

The experiments were performed simultaneously under light and dark conditions. As the fluorescence probe and its oxidation product are light-sensitive, the experiments under light conditions were run for 2 h with illumination of 22.5 and 60 μmol m$^{-2}$ s$^{-1}$ for *M. aeruginosa* and *P. subcapitata*, respectively, and detected the fluorescence of DCF at the end of exposure. For the dark conditions, the microplates loaded with the reaction system were wrapped in aluminum foil and the ROS formation was determined after exposure 1, 2 and 4 h, respectively. The fluorescence of DCF was measured with a microplate reader (Molecular Device, M2) at room temperature, with excitation and emission filters of 485 and 530 nm, respectively. The total DCF fluorescence data were corrected by subtracting the background fluorescence. Each experiment was repeated three times.
2.5. Statistical analysis

The normality of the data was verified using the Kolmogorov–Smirnov test, and the homogeneity of the variance was checked using Levene’s test. One-way ANOVA followed by Dunnet’s post-test was used to compare ROS production in exposure groups between dark and light conditions. *P*-values less than 0.05 were considered statistically significant. All the statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL).

3. Results and discussion

3.1. Validation of the method by H2O2

H2O2 is a well-known compound with strong oxidizing capability and is suggested as a promising selective agent against the growth of toxic cyanobacterial species (Kay et al., 1982; Drábková et al., 2007). Therefore, H2O2 was used as a positive control in this study to verify the reliability of DCFH-DA as a probe for intracellular ROS detection in *M. aeruginosa* and *P. subcapitata*. The results of DCF fluorescence generation induced by H2O2 are exhibited in Figs. 1a and 2a. A time-dependent rise in DCF fluorescence levels were observed in both species under dark conditions (Fig. 1a). H2O2 enhanced the DCF fluorescence generation significantly compared to respective controls in the two cultures (*P* < 0.01). DCF fluorescence generation in the cells of *P. subcapitata* induced by H2O2 was approximately twice higher than that in *M. aeruginosa* at all exposure concentrations at 4 h. When *M. aeruginosa* and *P. subcapitata* cells were exposed to different concentrations of H2O2 under illuminated conditions, DCF fluorescence generation was significantly accelerated compared with dark conditions (*P* < 0.01), and dose-response was provided with high linearity under both dark and light conditions (light condition \( R^2 = 0.9805, 0.9986 \), dark condition \( R^2 = 0.9926, 0.9757 \), respectively) (Fig. 2a). The results indicated that DCF fluorescence method for measuring of cellular ROS levels in *M. aeruginosa* and *P. subcapitata* under the experiment conditions was reliable.

In these short-term exposure experiments, initial cell densities of \( 5 \times 10^6 \) cells mL\(^{-1} \) for *M. aeruginosa* and \( 2 \times 10^6 \) cells mL\(^{-1} \) for *P. subcapitata* represented approximately equal biomass levels according to the method described by Drábková et al. (2007), but considerable difference in DCF fluorescence production was found between the two species in response to H2O2. This might attribute partly to the different extents of DCFH-DA penetrating into the two species (He and Häder, 2002; Schrader et al., 2005). However, species difference may possibly exist due to the distinction of antioxidant defense systems of the two test organisms that are responsible for scavenging ROS and adapting to oxidative stress (Schrader et al., 2005).

When the two test organisms were exposed to H2O2 under illuminated conditions, higher DCF fluorescence levels were observed than under dark incubation conditions, which indicated that light could enhance H2O2-induced DCF fluorescence generation. This is presumably due to the fact that more hydroxyl radicals are generated under illuminated conditions through the photo-Fenton reaction of H2O2 in the presence of Fe\(^{2+} \) ions (Zepp et al., 1992). Fe\(^{3+} \) ions are present in both culture mediums and commonly found in the aquatic organisms, so this reaction is possible and may lead to higher DCF fluorescence levels under light conditions.

3.2. ROS formation induced by CA

The results of ROS generation in *M. aeruginosa* and *P. subcapitata* induced by CA are displayed in Figs. 1b and 2b. Under dark conditions, the exposure of CA caused a time-dependent rise in ROS production in both species and the levels of ROS generation in *P.
subcapitata were about twice higher than that in M. aeruginosa (Fig. 1b). CA-induced ROS generation (Fig. 2b) was obviously enhanced by the illuminated conditions in both test organisms after 2 h exposure ($P < 0.01$), and the extents of increase were linearly correlated with the test concentrations.

CA is unstable at the cytoplasm pH level (Chobot et al., 2009). When CA is taken up by the target organisms, it can be expected, according to the electrochemical character, that the autoxidation or enzymatic oxidation will easily form the semiquinone radical and the O$_2$ is simultaneously reduced to O$_2^-$(C5/C0) (Janeiro and Brett, 2004). The reactive semiquinone radical is able to react with O$_2$ to produce o-quinone form of CA accompanying the production of O$_2^-$(C5/C0), and the above-mentioned reactions are proved to be reversible over a wide pH range (Janeiro and Brett, 2004). Chobot et al. (2009) also determined the electrochemical potential of CA at the pH level characteristic for the cytoplasm, and suggested classifying CA as a redox cycler. All of these results provide the evidence that CA possesses the redox cycling property in the intracellular environment of phytoplankton. Namely, in the presence of intracellular reductants such as NAD(P)H, redox-active and electrophilic o-quinone undergoes a two-electron reduction to reform the CA or a one-electron reduction to reform the corresponding semiquinone radical (Cohen and d’Arcy Doherty, 1987). Consequently, a possible pathway of ROS generation in the phytoplankton cells induced by CA could be proposed as shown in Fig. 3. The events in Fig. 3 establish futile redox cycles, which amplify the generation of O$_2^-$ with the expense of NAD(P)H, and enhance greatly the toxicity to the target organisms. O$_2^-$ is a precursor of a variety of other reactive oxygen species (Richter and Schweizer, 1997). Dismutation of O$_2^-$ produces H$_2$O$_2$, a relatively long-lived molecule which can diffuse across biological membranes and cause damage far from its site of production (Dat et al., 2000). H$_2$O$_2$ can be reduced via the Fenton-type reaction in the presence of transition metals to form the OH$^-$, which is the most reactive oxygen species with very high affinity for biological macromolecules at the site of production (Dat et al., 2000) to cause oxidative damage of the target organisms.

In the cells of test organisms incubated under light conditions, O$_2$ is continuously produced from water, as well as the reducing power is generated and stored as NAD(P)H in the cells via photosynthesis. Both of these processes can accelerate the redox cycling of CA. However, under dark incubation, the quantities of intracellular O$_2$ and NAD(P)H were not as adequate as that in light. This may result in the lower ROS levels under dark conditions in both species compared with light conditions.

Oxidative damage to isolated and cellular DNA in human leukemia cells induced by CA has been reported by Furukawa et al. (2003). While in this study, CA-induced ROS production was observed in cells of cyanobacteria and green algae. These results suggest the potential for oxidative stress in phytoplankton induced by the redox cycling of active polyphenol released by submerged macrophytes.

3.3. ROS formation induced by PA

The results of ROS formation in the two species induced by PA are shown in Figs. 1c and 2c. Under dark conditions, a time-dependent increase in ROS production could be found in P. subcapitata cells when exposure to PA (Fig. 1c). In M. aeruginosa, the levels of ROS generation were not different between controls and treated groups at 1 and 2 h exposure ($P > 0.05$) (Figs. 1c and 2c); only at 4 h were significantly higher levels of ROS detected in treated groups ($P < 0.01$) (Fig. 1c). This obvious difference between the two species may closely relate to the species difference. From Fig. 2c, it could also be seen that unlike CA, light did not significantly enhance PA-induced ROS generation in the two species. This result indicates that some differences in the mode of action be-
between the two polyphenols may exist in test organisms, although both of them can induce ROS generation.

PA, as an O$_2^-$ generator, has been often used to investigate the role of ROS in the biological system (Park et al., 2007). It is a strong reducing agent and has long been known to autoxidize rapidly forming the corresponding semiquinone radical and o-quinone (Marklund and Marklund, 1974). Moreover, it was reported that pyrogallic acid-containing flavonoids were able to form the O$_2^-$ from O$_2$ accompanying the process of autoxidation (Kondo et al., 1999), and the o-quinone formed by the loss of two electrons from PA moiety in flavonoids might participate in redox cycling with the formation of O$_2^-$ and subsequently H$_2$O$_2$ (Kondo et al., 1999; Long et al., 2000). Therefore, after uptake by the test organisms, it is reasonable to expect that PA may undergo redox cycling in the presence of intracellular reductants such as NAD(P)H (O’Brien, 1991; Monks et al., 1992) and induce ROS production in M. aeruginosa and P. subcapitata as shown in Fig. 3. Increased patterns in intracellular levels of O$_2^-$ by PA were also detected in the As4.1 juxtaglomerular cells (Park et al., 2007) by using a O$_2^-$ highly selective fluorescence probe; however, no significant alteration of intracellular H$_2$O$_2$ level (using DCFH-DA as probe) and obvious inhibition of SOD activity in test cells were observed simultaneously (Park et al., 2007). In this study, it was noticeable that DCF fluorescence in PA-treated cells was not as strong as that in CA-treated cells (Fig. 1c), which may indicate that the dismutation rate from O$_2^-$ to H$_2$O$_2$ was inhibited by PA and the allelopathic effect on M. aeruginosa and P. subcapitata was due to the increase of the intracellular O$_2^-$ levels. On the other hand, DCFH-DA does not react with O$_2^-$, but can produce fluorescence by reacting with H$_2$O$_2$ and OH$^-$ (He and Häder, 2002). Therefore, when exposed to PA, more significant DCF fluorescence observed in P. subcapitata than that in M. aeruginosa may be due to more tolerant of the SOD in P. subcapitata, which maintains the conversion from O$_2^-$ to H$_2$O$_2$ and subsequently OH$^-$. As discussed previously, light enhanced CA-induced ROS formation in the two species, but this effect was not found for PA (Fig. 2c). This phenomenon may related with the levels of the intracellular O$_2$ and reductant NAD(P)H. Zhu et al. (2010) reported that PA could obviously reduce photosystem II and whole electron transport chain activities of M. aeruginosa. It can be expected that the production of both intracellular O$_2$ and reductant NAD(P)H, which are necessary for the redox cycling reactions of PA in the test organisms, decreases due to the inhibition of the photosynthesis, which results in no enhancing effect of light on PA-induced ROS generation observed in this study.

3.4. Exposure to other allelochemicals

ROS formation was also determined in M. aeruginosa and P. subcapitata after exposure to the other six allelochemicals. All showed no significant increase in ROS formation compared to respective controls under light and dark conditions (data not shown) ($P>0.05$). The results demonstrate that oxidative stress is not the cause for the allelopathic effect of these six allelochemicals on test phytoplankton.

The allelopathic mechanism of submerged macrophytes towards phytoplankton has been studied for many years and oxidative damage is suggested as one of the important toxicological mechanisms based upon changes of specific single antioxidants in the antioxidant defense system or the endpoint of the oxidative damage such as lipid peroxidation (Wu et al., 2007; Shao et al., 2009); however, the present study is the first to provide direct evidence of the ROS generation for oxidative stress in cyanobacteria and green algae induced by CA and PA that are excreted by M. spicatum. In addition, ROS generation induced by redox cycling of allelochemicals may be one of the important factors for allelochemicals to exhibit algicidal activity in natural aquatic ecosystems. For preferable explanation of the allelopathy in natural environment, further study is needed such as by using more sensitive analytical method and lower exposure concentration to observe the relationship between the formation of ROS and the response of antioxidant enzymes activities as well as the end point of oxidative damage in the cells of phytoplankton.

4. Conclusions

CA and PA can induce ROS generation in M. aeruginosa and P. subcapitata, which suggests that oxidative stress is an important cause for the allelopathic effects of submerged macrophytes against undesired phytoplankton. ROS generation induced by redox cycling of certain allelochemicals may be one factor for the algicidal activities in natural ecosystems. Light enhanced the CA-induced ROS generation in the two species, but this effect was not observed for PA, and this indicates a difference in the mode of action between the two polyphenols.

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