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Time-dependent alterations in growth, photosynthetic pigments and enzymatic defense systems of submerged *Ceratophyllum demersum* during exposure to the cyanobacterial neurotoxin anatoxin-a

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ABSTRACT

Recently, aquatic macrophytes have been considered as promising tools for eco-friendly water management with a low running cost. However, only little information is available thus far regarding the metabolic capacity of macrophytes for coping with cyanobacterial toxins (cyanotoxins) in the aquatic environment. Cyanotoxins have become emerging contaminants of great concern due to the high proliferation of cyanobacteria (cyanobacterial bloom) accelerated by eutrophication and climate change. Anatoxin-a, one of the common and major cyanotoxins, is suggested as a high priority water pollutant for regulatory consideration owing to its notoriously rapid mode of action as a neurotoxin. In this study, the time-course metabolic regulation of the submerged macrophyte *Ceratophyllum demersum* (*C. demersum*) was investigated during exposure to anatoxin-a at an environmentally relevant concentration (15 μg/L). Biotransformation and antioxidative systems in *C. demersum* responded positively to anatoxin-a through the promoted synthesis of most of the involved enzymes within 8 h. Maximum enzyme activities were exhibited after 24 or 48 h of exposure to anatoxin-a. However, an apparent decline in enzyme activities was also observed at longer exposure duration (168 and 336 h) in company with high steady-state levels of cell internal H_2O_2, which showed its highest level after 48 h. Meanwhile, irreversible inhibitory influence on chlorophyll content (vitality) was noticed, whereas the ratio of carotenoids to total chlorophyll was increased with the increase in exposure duration. Consequently, the reduction in growth (biomass) of *C. demersum* was observed in sub-chronic exposure to anatoxin-a (8 weeks). Overall results clearly indicate, on the one hand, that anatoxin-a causes negative allelopathic effects on the macrophyte by inducing oxidative stress. On the other hand, the macrophyte might have interactions with anatoxin-a, based on the prompt reaction of its enzymatic defense systems to the toxin. The result obtained from the present study could contribute to the improvement of basic knowledge about the ecological impact of anatoxin-a and the environmental fate of the toxin in the aquatic environment.

1. Introduction

Macrophytes are organisms with fundamental functions in the maintenance of nutrient balances and dynamics as well as proper biological population structure in the aquatic environment (Carpenter and Lodge, 1986; Gross et al., 2007; Madsen et al., 2001; Sand-Jensen and Borum, 1991). Moreover, considering their adaptability and metabolic potential, macrophytes are involved in the removal or degradation of contaminants, thereby contributing to the natural management of water quality (Dhir et al., 2009; Hilt and Gross, 2008; Sandermann, 1994; Takamura et al., 2003). In this aspect, the application of aquatic macrophytes for sustainable water management (e.g. bioindicators of water quality status or phytoremediating agents of water pollutants) has attracted considerable public attention (Coops et al., 2007; Melzer, 1999; Qiu et al., 2001; Schaumburg et al., 2004).

Currently, freshwater bodies are under stress in relation with the mass development of cyanobacteria, so called cyanobacterial blooms, which are promoted by eutrophication and climate change (Carmichael, 2008; Paerl and Huisman, 2008). Many of the bloom-forming cyanobacteria are discovered to release secondary metabolites, which exhibit diverse types of biological and biochemical activities, into a surrounding medium (Cardozo et al., 2007; Nagle and Paul, 1999; Rastogi and Sinha, 2009). Some of these metabolites are identified as potent toxins, cyanotoxins, which cause adverse effects on human, animal and ecological health (Carmichael et al., 2001; Codd, 1995; Stewart et al., 2008). Anatoxin-a is one of the most commonly detected cynobacterial

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neurotoxins in several regions of the world and is defined as a powerful postsynaptic de-polarizing agent that can bind to nicotinic acetylcholine receptors (nAChRs) in neurons and at neuromuscular junctions by mimicking the action of acetylcholine (Ach) (Devlin et al., 1977; Thomas et al., 1993; WHO, 2003). Numerous efforts have been actively made to monitor the production or occurrence of anatoxin-a in water resources and to conduct toxicological studies required for establishing regulations to confront its risk (Fromme et al., 2000; Fawell and James, 1994; Hundnell, 2010; Osswald et al., 2007). However, information is still scarce on the interaction between anatoxin-a and the major members of the aquatic ecosystem, particularly macrophytes, which the toxin comes into contact first after its release (Mitrovic et al., 2004). Considering the function of macrophytes in the stabilization of aquatic environments and their potential to control anatoxin-a in an environmentally friendly manner, interest in how aquatic macrophytes adjust their metabolisms during exposure and acclimation to anatoxin-a has grown (Brix and Schierup, 1989; Engel, 1990).

The most effective plant defense mechanisms associated with tolerance to xenobiotics are biotransformation and antioxidative systems. In the process of biotransformation, the structure and toxicity of exogenous compounds are modified in vivo by various enzymes. The biotransformation of xenobiotics in plants proceeds in three phases: (i) phase I (functional transformation) generally conducted by cytochrome P-450 monooxygenases; phase II (conjugation) predominantly performed by glutathione S-transferases (GSTs); and phase III (compartmentalization) mainly undergone in cell wall fractions or in vacuoles for depositing phase I and phase II metabolites in the absence of excretory function (Coleman et al., 1997; Marrs, 1996; Sandermann, 1992). Owing to this biotransformation capacity, plants are considered as the 'green-liver' in ecosystems, by which they can eliminate environmental pollutants through naturally occurring biological action (Burken, 2003; Nimptsch et al., 2008). In unfavorable environmental conditions, to some degree several metabolites, formed partially as a result of the above described biotransformation process, and also the biotransformation reactions themselves, can enhance the production of reactive oxygen species (ROS) in plants, which leads to oxidative stress resulting in damage to all components of the cell (Gill and Tuteja, 2010; Sies, 1997). In order to keep a steady level of cellular ROS that is essential for normal physiological and biochemical functioning, plants possess several antioxidative mechanisms modulated by a wide range of enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR), together with free-radical traps (antioxidants) (Asada, 1992; Mittler, 2002; Noctor and Foyer, 1998).

Therefore, this study examined time-course responses of the aquatic macrophyte in terms of biotransformation and antioxidative metabolisms during exposure to anatoxin-a at an environmentally realistic concentration (i.e. the highest level of anatoxins-a in German fresh water bodies, which is the worst case scenario). The rootless, submerged aquatic macrophyte, *Ceratophyllum demersum* is found in a variety of habitats with moderate to high nutrient levels such as eutrophic conditions, and has a world-wide distribution (Jeppesen et al., 1999; Johnson et al., 1995). This macrophyte has high biomass production and good capability of absorbing environmental contaminants (e.g. metals and industrial radionuclides) (Bolsunovskii et al., 2002; Keskinkan et al., 2004; Ornes and Sajwan, 1993). Hence, *C. demersum* was chosen as an appropriate model organism in the current study. Changes in the activity of biotransformation (GST) and antioxidative enzymes (SOD, POD, APX, GR and monodehydroascorbate reductase (MDAR)) were investigated in parallel to the analysis of the formation of ROS, especially H_2O_2. In addition to biotransformation and oxidative stress status, the vitality and biomass of *C. demersum* under anatoxin-a stress was also assessed through photosynthetic pigment contents (i.e. chlorophylls and carotenoids) and growth (i.e. fresh weight and shoot length), respectively.

This study aimed to evaluate the metabolic capacity and resistance of *C. demersum* against anatoxin-a and the subsequent consequences of observed responses at higher levels of biological organization in order to provide insight on aquatic macrophytes coping with the toxin.

2. Materials and methods

2.1. Plant material

C. demersum was provided by Extraplant (Extragroup GmbH, Münster, Germany). Macrophyte cultures were maintained in a glass tank (60 cm × 60 cm × 60 cm) under cool white fluorescent light (35 μE m^{-2} s^{-1} irradiation) with a 14:10 h light:dark photoperiod. Macrophytes were grown non-axenically in modified Provasoli's medium containing de-ionized water, $CaCl_2$ [0.2 g/L], $NaHCO_3$ [0.103 g/L] and sea-salts [0.1 g/L] at a temperature of 22–24 °C.

2.2. Cyanotoxin treatments

(±) Anatoxin-a fumarate was obtained from Tocris Bioscience (Strathmore Road Natick, MA, USA). The exposure concentration of anatoxin-a was set to 15 μg/L based on the highest concentration (the sum of cell-bound and dissolved toxins) recorded in the monitoring studies of German freshwater bodies (Bumke-Vogt et al., 1999; Sächsisches Staatsministerium für Soziales, 2009). The toxin was dissolved in methanol (70%) and further diluted with culture medium to the final experimental concentration. *C. demersum* with a fresh weight of 4.5 ± 0.5 g was introduced in 100 mL of the exposure medium under the controlled culture conditions described above, excluding the experiment for determination of macrophyte growth. Sampling was performed after 4, 8, 12, 24, 48, 168, and 336 h of exposure. The exposure medium in each test was re-filled daily with culture medium up to the initial volume to prevent any change in exposure concentration caused by evaporation. To consider the influence of normal experimental condition on macrophytes under different treatment times, controls (water medium without anatoxin-a) were prepared in parallel with each toxin exposure. The macrophyte directly taken from a culture tank was used as 0 h control. Both controls and toxin treatments were independently replicated five times. After exposure, macrophytes were washed twice with 100 mL of de-ionized water to remove any surface-associated toxin and were shock-frozen in liquid nitrogen. The frozen macrophytes were stored at −80 °C for further biological experiments.

2.3. Hydrogen peroxide (H_2O_2) determination

H_2O_2 levels were determined by monitoring the formation of titanium peroxide, which is indicated by a red-orange coloration according to Jana and Choudhuri (1982). H_2O_2 was extracted by homogenizing 0.4 g of macrophyte tissue with 3 mL of 50 mM sodium phosphate buffer (pH 7.0). The homogenate was spun down at 20,800 × g for 15 min at 4 °C, and the transparent supernatant was collected. Supernatant amounting to 750 μL was mixed with 250 μL of 0.1% titanium chloride in 20% (v/v) H_2SO_4. Then, the absorbance of the reaction mixture in three replicates was recorded at 410 nm The content of H_2O_2 was calculated using the extinction coefficient 0.28 l $mmol^{-1}$ cm^{-1} and expressed as μmol/gFW.