Aquatic Botany 92 (2010) 179–184

Contents lists available at ScienceDirect

Aquatic Botany

journal homepage: www.elsevier.com/locate/aquabot

Cytoskeletal and developmental alterations in *Ceratophyllum demersum* induced by microcystin-LR, a cyanobacterial toxin

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ARTICLE INFO

Article history:
Received 11 May 2009
Received in revised form 29 October 2009
Accepted 11 November 2009
Available online 18 November 2009

Keywords:
Ceratophyllum demersum
Microcystin-LR
Growth inhibition
Cell elongation
Microtubule reorientation
Shoot tip development

ABSTRACT

The aim of the present work was the investigation of microtubule organization related to developmental processes of *Ceratophyllum demersum*, a submergent aquatic macrophyte, as affected by microcystin-LR (MCY-LR), a cyanobacterial toxin. We studied the time- and dose-dependent effects of the cyanotoxin in a concentration range of 0.01–20 μg mL^{-1} (0.01–20.1 μM) at exposure times of 2–16 d. At short term (4 d) of MCY-LR exposure we observed the inhibition of *C. demersum* shoot tip elongation. This phenomenon was already observed at 0.01 μg mL^{-1} MCY-LR (reduction of shoot tip length to 56 ± 3.6% of controls) and correlated with the induction of cortical microtubule (CMT) reorientation from transverse to longitudinal known to induce radial expansion of meristematic cells instead of normal elongation. Concomitantly we detected the increase of the percentage of cells in early mitosis in shoot tip meristems, from 1.17 ± 0.2% for controls to 1.93 ± 0.3 at 0.01 μg mL^{-1} MCY-LR and 6.19 ± 0.5 at 10 μg mL^{-1} MCY-LR. Cyanotoxin exposure induced the inhibition of general shoot elongation that was more pronounced than inhibition of the increase of leaf whorl number or fresh weight in the treatment period and this was observable at as short as 2–4 d of 2.5 μg mL^{-1} MCY-LR exposure. This observation further proved that the MCY-LR-induced inhibition of cell elongation is responsible mainly for growth inhibition in *C. demersum*. Concomitantly with developmental and growth changes MCY-LR decreased protein and chlorophyll content at 16 d of exposure: at 20 μg mL^{-1} of cyanotoxin, protein content was reduced to 53.3 ± 2.8%, while total chlorophyll content to 46.53 ± 2.7% of controls. This is the first study showing that MCY-LR inhibits the growth of *C. demersum* through cytoskeletal reorganization. This plant proved to be a powerful model system for toxicological as well as plant cell biology studies.

1. Introduction

Ceratophyllum demersum L. is a submerged freshwater macrophyte of major importance in aquatic ecosystems for at least two reasons: (i) it is known to produce allelochemicals that influence the development of phytoplankton (Hilt and Gross, 2008; van Donk and van de Bund, 2002); (ii) it is an indicator of environmental pollutants such as heavy metals or phytotoxins (Lewis, 1993). *C. demersum* develops a well-defined defence response against heavy metals by producing phytochelatins and conjugating those metals to glutathione by the glutathione-S-transferase (GST) enzyme (Mishra et al., 2009). *C. demersum*, as *Phragmites australis*, a well-known emergent aquatic macrophyte, is frequently used as a test organism in ecotoxicological studies (Armstrong and Armstrong, 2001; Lewis, 1993; Máthé et al., 2007; Pflugmacher, 2004).

Microcystin-LR (MCY-LR) is a toxin that occurs frequently in freshwaters. It is produced by several cyanobacterial genera including *Microcystis*, *Anabaena* or *Oscillatoria* (Wiegand and Pflugmacher, 2005). MCY-LR has important implications in animal and human health and it affects severely aquatic plant communities including those dominated by *C. demersum* (see for example, Carmichael, 1992; Wiegand and Pflugmacher, 2005). Growth inhibition as well as biochemical alterations in submerged and emergent freshwater aquatic plants have been reported for this cyanotoxin (Máthé et al., 2007; Pflugmacher, 2002, 2004; Romanowska-Duda and Tarczyńska, 2002; Romanowska-Duda et al., 2002; Saqrane et al., 2007). Its natural concentrations in

Abbreviations: CMT, cortical microtubule; MCY-LR, microcystin-LR; MT, microtubule; PPB, preprophase band.

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doi:10.1016/j.aquabot.2009.11.003

eutrophic freshwaters are frequently reported to be 10 μg L^{-1} (0.01 μg mL^{-1}) and in case of toxic cyanobacterial mats and scums can reach 25 μg mL^{-1} (Codd et al., 2005; Falconer et al., 1999). Although our principal aim was to study the mechanism of cyanotoxin action at the cellular level, these concentrations were taken into consideration when we designed the concentration range of MCY-LR used throughout our experiments.

MCY-LR is a cyclic heptapeptide, a potent inhibitor of eukaryotic type 1, 2A and 4 serine-threonine protein phosphatases and as such, it interferes with essential biochemical and cellular events in higher plants (Luan, 2003; MacKintosh and Diplexcito, 2003). MCY-LR is one of the most frequently occurring phytotoxins in freshwaters. Besides its importance in affecting aquatic ecosystems, MCY-LR is currently used as a powerful tool in the study of the influence of protein dephosphorylation on eukaryotic cellular and biochemical events (MacKintosh and MacKintosh, 1994; MacKintosh and Diplexcito, 2003). Regarding *C. demersum*, Pflugmacher and his coworkers made comprehensive uptake, biochemical and plant growth studies on the effects of MCY-LR (Pflugmacher, 2002, 2004; Pflugmacher et al., 1998a,b, 1999). Those studies have shown the following physiological/biochemical changes: (i) MCY-LR changes chlorophyll a/b ratio and inhibits the rate of photosynthesis; (ii) the cyanotoxin induces the accumulation of phenolic compounds known to be involved in the promotion of defence responses in higher plants; (iii) it increases the activity of enzymes involved in oxidative stress defense; (iv) as a consequence of biochemical changes, MCY-LR induces significant growth inhibition of *C. demersum* plants; (v) this aquatic macrophyte is able to detoxify MCY-LR by binding it to glutathione with the aid of the enzyme glutathione-S-transferase. The changes presented above indicate the need for further study of MCY-LR induced cellular alterations in submerged aquatic macrophytes and among them, *C. demersum*.

One of the most dramatic changes in MCY-LR treated animal cells is the alteration of cytoskeleton organization (see for example, Gácsi et al., 2009). Among the determinants of plant cell shape and growth, the organization of actin and microtubule (MT) cytoskeleton and its regulation by protein phosphorylation/dephosphorylation plays a crucial role (Baskin and Wilson, 1997; Camilleri et al., 2002; Mathur, 2004).

For the reasons mentioned above, we aimed to study the effects of MCY-LR on the organization of microtubule (MT) cytoskeleton and its consequences in the growth of *C. demersum*. We designed an experimental system using axenic *C. demersum* plants in order to exclude any additional cytological effects of algal, fungal or bacterial epiphyton. Our experiments show for the first time, that MCY-LR, a protein phosphatase inhibitor induces longitudinal cortical microtubule reorientation and consequent radial expansion of meristematic cells instead of normal elongation, leading to growth inhibition in *C. demersum*.

2. Materials and methods

2.1. Plant material and culture

C. demersum L. plants were collected from a small pond located in the Botanical Garden of University of Debrecen, Hungary. Plants were thoroughly washed with tap water, then cut to obtain 50–60 mm segments containing shoot apical meristem. Shoots were washed with 70% (v/v) ethanol, followed by surface sterilization with 0.01 M $KMnO_4$ (Reanal, Budapest, Hungary) for 6 min. After three washes with sterile water, shoots were ready for culturing. Part of shoots were transferred to standard liquid Luria Broth and Murashige-Skoog (MS) media, the latter containing 2% (w/v) sucrose as carbon source (Murashige and Skoog, 1962) to check them for bacterial and fungal contamination. *C. demersum* axenic cultures were established and maintained on half-strength liquid Allen (1968) medium at 20 ± 1 °C under continuous dim light (3 μmol m^{-2} s^{-1} photon fluence rate). The results presented below were similar when *C. demersum* plants were cultivated under a 16/8 h photoperiod.

2.2. The purification of microcystin-LR

Microcystin-LR (MCY-LR) was purified from *Microcystis aeruginosa* strain BGSD243 as described previously and involved ion-exchange chromatography and desalting with Waters Sep-Pak cartridges (Kós et al., 1995). Its purity was checked by HPLC and capillary electrophoresis (CE) methods as described by Vasas et al. (2004) and proved to be ≥97%.

2.3. Cyanotoxin treatment for cytological and growth studies

For the start of cyanotoxin treatments, we used *C. demersum* shoots with apical meristems. Shoots were of 24.5 ± 0.4 (SE) mm length and contained 5–6 leaf whorls. *C. demersum* was treated with microcystin-LR for 16 d. After 5 d of preculture, cyanotoxin exposures were made in test tubes containing 5 mL half-strength Allen medium. The concentration regime used was 0.01–20 μg mL^{-1} (0.01–20.1 μM) MCY-LR.

For cytological studies, we used shoot tips cut from control and MCY-LR treated plants after 4 d of exposure. After fixation with 4% (v/v) formaldehyde (Reanal, Budapest, Hungary) in phosphate buffered saline (PBS), 10 μm thick sections were made by cryosectioning with a Leica Jung Histoslide 2000 microtome. Microtubules (MTs) were labelled with a Cy3-conjugated anti-β tubulin antibody (Sigma–Aldrich, Budapest, Hungary), followed by staining of chromatin with 4′6′-diamidino-2-phenylindole (DAPI, Fluka, Buchs, Switzerland). Cytological procedures were performed according to Zhang et al. (1992) as modified in our laboratory (Máthé et al., 2009). The microscopes used were an Olympus Provis AX-70/A conventional fluorescence microscope equipped with an Olympus Camedia 4040 digital camera and a Zeiss LSM 510 confocal laser scanning microscope. For the conventional fluorescence microscope, microtubules were detected by a 540–580 nm excitation filter, while nuclear DNA was observed with a 320–360 nm excitation filter. For the confocal microscope, excitation wavelengths were 543 nm and 351/364 nm, respectively. Cytological studies included the measurement of shoot tip meristem length defined as the distance between shoot dome and the fifth internode of shoot tip. We have also calculated total mitotic index and the indices for particular mitotic phases: we have counted the number of cells in certain mitotic phases per total number of meristematic cells and we expressed indices in percentages. Six shoot tips per treatment were analyzed in each experiment.

The percentage of increase in shoot length, fresh weight and number of leaf whorls of *C. demersum* plants were analyzed during a 16 d of MCY-LR treatment period. Therefore, at the start of experiments, all values of those growth parameters were expressed as zero. Chlorophyll content was assayed using the method of Arnon (1949) and protein content was assayed by the Bradford (1976) method at the end of treatment period. The standard for protein assay was bovine serum albumin (BSA, Merck Hungary Ltd., Budapest, Hungary).

All experiments were repeated at least three times and representative experiments are presented in figures. For a given experiment, the number of samples per treatment was $n \geq 6$. The mean ± SE values are shown in figure graphs. The significance of differences between controls and MCY-LR treatments was analyzed by one-way ANOVA. Results were presented with the aid of graphical and statistical facilities of Sigma Plot 8.0 software.