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Cylindrospermopsin induces alterations of root histology and microtubule organization in common reed (*Phragmites australis*) plantlets cultured *in vitro*

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ABSTRACT

We aimed to study the histological and cytological alterations induced by cylindrospermopsin (CYN), a protein synthesis inhibitory cyanotoxin in roots of common reed (*Phragmites australis*). Reed is an ecologically important emergent aquatic macrophyte, a model for studying cyanotoxin effects. We analyzed the histology and cytology of reed roots originated from tissue cultures and treated with 0.5–40 μg ml^{-1} (1.2–96.4 μM) CYN. The cyanotoxin decreased root elongation at significantly lower concentrations than the elongation of shoots. As general stress responses of plants to phytotoxins, CYN increased root number and induced the formation of a callus-like tissue and necrosis in root cortex. Callus-like root cortex consisted of radially swollen cells that correlated with the reorientation of microtubules (MTs) and the decrease of MT density in the elongation zone. Concomitantly, the cyanotoxin did not decrease, rather it increased the amount of β-tubulin in reed plantlets. CYN caused the formation of double preprophase bands; the disruption of mitotic spindles led to incomplete sister chromatid separation and disrupted phragmoplasts in root tip meristems. This work shows that CYN alters reed growth and anatomy through the alteration of MT organization.

1. Introduction

Cylindrospermopsin (CYN) is a tricyclic guanidine derivative containing a hydroxymethyluracil group (Ohtani et al., 1992). It is a cyanobacterial toxin produced by strains of *Cylindrospermopsis raciborskii, Umezakia natans, Anabaena bergii, Raphidiopsis curvata, Lyngbya wollei* and of several *Aphanizomenon* species. The toxin is of wide interest, because it was found in freshwaters of the tropical and temperate climate as well, and had impact on the environment and on human health (Ohtani et al., 1992; Harada et al., 1994; Banker et al., 1997; Li et al., 2001; Seifert et al., 2007; Mihali et al., 2008). It is an inhibitor of eukaryotic protein synthesis, although the molecular mechanism is yet to be established (Metcalf et al., 2004; Froscio et al., 2008). In addition, the

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inhibition of pyrimidine nucleotide synthesis by CYN was reported in mouse liver cell-free extracts (Reisner et al., 2004).

It is known that CYN induces necrosis of liver tissue (Harada et al., 1994; Terao et al., 1994; Lankoff et al., 2007) in animal systems. Cell death caused by the cyanotoxin is preceded by the decrease of reduced glutathione (GSH) level. GSH is involved in the formation of conjugates with cellular metabolites or toxins. Because its level is reduced, CYN is not inactivated effectively, and therefore it can exert its toxic cellular effects (Runnegar et al., 1994). CYN is known to inhibit division of animal cells (Lankoff et al., 2007). Based on their cytokinesis-block micronucleus assay, Humpage et al. (2000) showed that the cyanotoxin induces chromosome loss during mitosis in a lymphoblastoid cell line, which leads to the formation of micronuclei. Other studies proved that the CYN induced growth inhibition of CHO-K1 cells is related to both microtubule and actin filament reorganization (Fessard and Bernard, 2003; Gácsi et al., 2009). Despite CYN is known to be a cytotoxin, there is still a need for a detailed study of its effects on animal cells.

In contrast to animal organisms, there are only a limited number of studies on the effects of CYN on the growth of higher plants. Vasas et al. (2002) proved that this cyanotoxin inhibits the growth of white mustard (*Sinapis alba*) seedlings, with an 50% inhibitory concentration (IC_{50}) of 18.2 $\mu g\,ml^{-1}$. Kinnear et al. (2008) demonstrated that whole-cell extracts of *C. raciborskii* containing 0.4 $\mu g\,ml^{-1}$ CYN induced a general growth stimulation of *Hydrilla verticillata*, an aquatic plant, and this effect was concomitant with the decrease of chlorophyll content. To date, our knowledge on the effects of CYN on the histological and cytological organization of higher plants is limited, and its effects on plant cell cytoskeleton are unknown.

Common reed (*Phragmites australis*/Cav./Trin. Ex Steud.) is an emergent aquatic macrophyte of major ecological importance (Ostendorp, 1989; Ye et al., 1998; Pflugmacher, 2002; Mészáros et al., 2003). In a previous study we have proved, that tissue cultures of common reed can be used as a model system for the study of the effects of microcystin-LR, a well-known cyanotoxin, on growth and histology of higher plants (Máthé et al., 2007). However, the cytological alterations induced by phytotoxins have not been studied in detail in this plant.

Cytoskeleton organization plays an important function in the determination of plant cell shape and growth (Mathur, 2004). Cyanotoxins, like microcystin-LR or CYN are known to influence plant growth (see for example, Pflugmacher, 2002; Vasas et al., 2002). Therefore, the study of their effects on microtubular cytoskeleton in higher plant cells is of particular interest. Differentiating and interphase meristematic cells are characterized by cortical microtubules (CMTs). Their orientation determines the pattern and direction of plant cell growth (Barlow and Baluška, 2000; Mathur, 2004). Cell division starts with the formation of preprophase bands (PPBs). Their organization influences the site of cytokinesis: together with phragmoplasts, they probably determine the position of the maturing cell plate (Mineyuki, 1999). The normal microtubule (MT) pattern of mitotic cells directs correct chromatid separation and cytokinesis and it is influenced by many factors including the synthesis of certain proteins (Mineyuki, 1999).

Our aim was to investigate the CYN induced growth and histological alterations in reed roots, in the light of the alterations induced at the cellular level. This experimental design contributes to the better understanding of the mechanisms of the effects of a well-known cyanotoxin and its possible impacts on aquatic ecosystems. The effects of CYN on the microtubular cytoskeleton and mitotic chromatin in plant cells are analyzed in detail for the first time in this study.

2. Materials and methods

2.1. Purification of cylindrospermopsin

Cylindrospermopsin (CYN, MW: 415 Da) was purified from the cultures of *Aphanizomenon ovalisporum* (Forti) strain ILC-164 isolated from Lake Kinneret, Israel in 1994 (Banker et al., 1997). The purification method was described previously by Vasas et al. (2002, 2004). Briefly, cyanobacterial filaments were harvested by centrifugation and frozen at −20 °C. After repeated freezing-thawing cycles, cell pellet was extracted with 90% (v/v) methanol. After rotary evaporation, the extract was resuspended in 50% (v/v) ethanol, and was loaded onto Toyopearl HW-40 (Tosoh, Tokyo, Japan) size exclusion column and further purified by semi preparative HPLC (Supercosyl TM SPLC-18 column, Supelco, Bellefonte, USA).

2.2. Plant material and CYN treatments

Common reed (*P. australis*/Cav./Trin. Ex Steud.) plantlets were regenerated from stem nodal embryogenic calli, according to Máthé et al. (2000). Plantlets were cultivated in liquid MS medium (Murashige and Skoog, 1962) supplemented with Gamborg's vitamins (Gamborg et al., 1968), 2% (w/v) sucrose (Reanal, Budapest, Hungary), 2.7 μM α-naphthalene acetic acid (Reanal). All experiments were carried out using 2 ml media in test tubes (15 mm wide × 160 mm high) as described by Máthé et al. (2007). CYN was added at the concentrations of 0.5–40 $\mu g\,ml^{-1}$ (1.2–96.4 μM). Plantlets with two to three primary roots were exposed to toxin for ten days. After CYN treatments the increase in the length of shoots and roots was measured and the number of newly formed roots was counted to detect alterations in root growth. Newly formed roots were the roots developed after the start of cyanotoxin treatment. Standard errors were calculated and data were plotted using Sigma Plot 8.0 data analysis software.

2.3. Microscopical analysis

Labelling of microtubules (MTs) with an anti-β-tubulin antibody was performed according to the manufacturer's instructions (Sigma-Aldrich, Budapest, Hungary) applied for plant cells (Zhang et al., 1992) and modified in our laboratory. The procedure was as follows. For microscopical analysis of MTs and chromatin structures, 4 mm long root tips were excised and fixed overnight in 4% (v/v) formaldehyde in phosphate buffered saline (PBS). After fixation,