Cytotoxic effects of cylindrospermopsin in mitotic and non-mitotic *Vicia faba* cells

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Abstract

Cylindrospermopsin (CYN) is a cyanobacterial toxin known as a eukaryotic protein synthesis inhibitor. We aimed to study its effects on growth, stress responses and mitosis of a eukaryotic model, *Vicia faba* (broad bean). Growth responses depended on exposure time (3 or 6 days), cyanotoxin concentration, culture conditions (dark or continuous light) and *V. faba* cultivar ("Standard" or "ARC Egypt Cross"). At 6 days of exposure, CYN had a transient stimulatory effect on root system growth, roots being possibly capable of detoxification. The toxin induced nucleus fragmentation, blebbing and chromosomal breaks indicating double stranded DNA breaks and programmed cell death. Root necrotic tissue was ~~frequently~~ observed at 0.1-20 µg mL^{-1} CYN ~~and at 10-20 µg mL^{-1} CYN, two continuous necrotic cell layers formed in main roots~~ that probably impeded toxin uptake into vascular tissue. Growth and cell death processes observed were general stress responses. In lateral root tip meristems, lower CYN concentrations (0.01-0.1 µg mL^{-1}) induced the stimulation of mitosis and distinct mitotic phases, irrespective of culture conditions or the cultivar used. Higher cyanotoxin concentrations inhibited mitosis. Short-term exposure of hydroxylurea- synchronized roots to 5 µg mL^{-1} CYN induced delay of mitosis that might have been related to a delay of *de novo* protein synthesis. CYN induced the formation of double, split and asymmetric preprophase bands (PPBs), in parallel with the alteration of cell division planes, related to the interference of cyanotoxin with protein synthesis, thus it was a plant- and CYN specific alteration.

Keywords: cylindrospermopsin, *Vicia faba*, growth stimulation, cell death, mitotic alterations, preprophase band

1. Introduction

Cylindrospermopsin (CYN) is a worldwide occurring alkaloid-type toxin, a tricyclic guanidine derivative containing a hydroxymethyluracil group. Several CYN varieties have been described (Ohtani et al., 1992; Banker et al., 2000). It is produced by cyanobacterial strains of several species/ genera (*Cylindrospermopsis raciborskii*, *Anabaena*, *Aphanizomenon* sp., *Lyngbya wollei*, *Raphidiopsis* sp., *Umezakia* sp.). Its impacts on human and animal health and its potential effects on aquatic ecosystems have been described (Kinnear, 2010). CYN can modulate the level and expression of many proteins in a single organism: both stimulation and inhibition have been observed (Beyer et al., 2009; Puerto et al., 2011). Although it is thought to be a eukaryotic protein synthesis inhibitor, the understanding of its molecular targets needs further research (Metcalf et al., 2004; Froscio et al., 2008).

Research on CYN induced cytotoxicity is important for the understanding of its health and environmental impacts. It is genotoxic and induces cell death in animal and human cells and organisms (Bazin et al., 2010). Toxin-induced tissue necrosis has been known for a long time (Terao et al., 1994). CYN alters cytoskeletal organization in mammalian cells (Fessard and Bernard, 2003). Concerning plants, we have shown for the first time that CYN induces growth inhibition (Vasas et al., 2002). The cyanotoxin generates stress/ defence responses like lignification of cell walls or formation of a callus-like tissue, alterations that are thought to play a role in the inhibition of toxin uptake by plants (Beyer et al., 2009; Máthé et al., 2013a). It alters the organization of plant microtubules (MTs), probably by the inhibition of synthesis and/ or activity of microtubule associated proteins (Beyer et al., 2009).

In spite of the above studies on CYN toxicity at the cellular level, there is still a need for research on the mechanisms of its cytotoxicity (Máthé et al., 2013b). There are only a few

data on the effects of CYN on cell division (Lankoff et al., 2007; Beyer et al., 2009). However, we are far from understanding the interference of CYN with mitotic regulation. CYN can alter the mitotic activity of plant cells (Beyer et al., 2009). One of our aims was to study whether the cyanotoxin induces arrest in certain mitotic phases, since the hypothesis was that it interferes with the regulation of entry into or exit from mitosis. There are limited data on the cytotoxic effects of CYN on non-mitotic plant cells. Next, we intended to look for specific cellular markers of CYN toxicity. Overall, our aim was to contribute to the understanding of CYN toxicity by a detailed analysis of cyanotoxin induced cellular alterations in non-mitotic as well as mitotic cells of *Vicia faba* (broad bean), a well-known model system for plant and in general, eukaryotic cell biology. This study has potential applicability to cyanotoxin effects in natural environments.

2. Materials and methods

2.1. The purification of cylindrospermopsin

CYN was purified from *Aphanizomenon ovalisporum* (Forti) ILC 164 (BGSD 423 in our culture collection) isolated by Banker et al. (1997). The purification method was described previously (Vasas et al., 2002, 2004), involving methanol-ethanol extractions of cells repeatedly frozen-thawn, followed by size-exclusion chromatography on Toyopearl HW-40 (Tosoh, Tokyo, Japan). Further purification was performed with semi-preparative HPLC (Supercosyl TM SPLC-18 column, Supelco, Bellefonte, USA). The purity of toxin was $\geq$ 95% as checked by HPLC and capillary electrophoresis.

2.2. Plant material and CYN treatments

Two cultivars of *Vicia faba* (broad bean, "Standard" /Hungarian cultivar/ and "ARC Egypt Cross") were used in order to see whether genetic differences between them can influence their response to the cyanotoxin. Seeds were surface sterilized, pregerminated and plantlets were grown as described previously for microcystin-LR treatments (Beyer et al., 2012), except that Allen (1968) medium was used. CYN was used in a concentration range of 0.01-20 µg mL^{-1} (0.024-48.2 µM).

For growth analysis, cyanotoxin treatments lasted for 3 days both in dark and at continuous light (cool white fluorescent illumination) of 100 µmol m^{-2} s^{-1}. Six days' exposures were performed only under continuous light, since control seedlings did not survive under light-depleted conditions. Growth of CYN treated seedlings was assayed by means of increase in the length of epicotyls, main roots and number of lateral roots. These parameters were measured at the beginning and at the end of cyanotoxin treatments.

2.3. Microscopy, histochemical and immunohistochemical methods

For histological and cytological analysis, non-synchronized and synchronized roots were used. The non-synchronized system consisted of the seedlings treated with CYN for 3 and 6 days. For the analysis of synchronized cells, roots of cv. "ARC Egypt Cross" were treated with hydroxylurea (HU) for arresting cells in the S phase (Beyer et al., 2012). After HU washout, CYN treatments were performed for 24 h under continuous dim light of 3 µmol m^{-2} s^{-1} PFD ~~photon fluence rate~~.

Microscopical analyses were performed with an Olympus Provis AX-70/A fluorescence microscope (Olympus, Tokyo, Japan). Hand-made cross-sections of non-

synchronized main roots were used for histological analysis. Autofluorescence of tissues was detected at an excitation wavelength range of 320-360 nm. Histological samples used for the analysis of non-mitotic chromatin were fixed in 3.7 % (v/v) formaldehyde, followed by staining with 3 µg mL^{-1} 4',6'-diamidino-2-phenylindole (DAPI, Fluka, Buchs, Switzerland). Mitotic figures of lateral root tip meristems were analyzed both from non-synchronized and synchronized roots. The tips of lateral roots were fixed with 3.7 % (v/v) formaldehyde and cryosectioned with a Leica Jung Histoslide 2000 microtome (Leica, Nussloch, Germany). Labeling of MTs and of chromatin was performed with the aid of a Cy3-conjugated anti-β-tubulin antibody (Sigma-Aldrich, St. Louis, Mo., USA) and DAPI, by previously described methods (Beyer et al., 2009; Máthé et al., 2009). Excitation wavelength range was 540-580 nm for Cy3 and 320-360 nm for DAPI.

For the detection of mitotic figures, at least 30 sections of 5-6 lateral root tips per treatment were used. The quiescent center and cells giving rise to vascular tissue were excluded from analysis. Total mitotic index and indices for particular mitotic phases were calculated.

2.4. The preparation of chromosome spreads

Lateral root tips were treated with 0.1 % (w/v) colchicine (Sigma- Aldrich) for 3 h, then fixed for 20 min in 45 % (v/v) acetic acid. Fixed samples were hydrolyzed in 1 N HCl at 60 °C for 5 min, washed with water and stained with 5% (w/v) carmine-acetic acid for 30 min at 60 °C. Chromosome squashes were examined by using the bright-field facilities of the Olympus Provis AX-70 microscope. At least 100 cells containing metaphase chromosomes were examined for each treatment per experiment.

146 2.5. The assay of *de novo* protein synthesis in *Sinapis alba* seedlings

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148 The assay of protein synthesis inhibition was carried out in order to see whether mitotic
149 alterations induced by CYN are related to its effects on *de novo* protein synthesis. *Sinapis*
150 *alba* (white mustard) seeds were surface sterilized as described previously (Kós et al., 1995)
151 and placed into 90-mm Petri dishes in Allen medium. Half of Petri dishes contained Allen
152 medium with 18 µg mL^{-1} CYN. Samples for protein labeling were taken at regular time
153 intervals between 14-42 hours after cyanotoxin treatment was started. Roots of seedlings were
154 pulse labeled for 1 h with 4 MBq ml^{-1} ^{35}S-methionine (Institute of Isotopes, Budapest,
155 Hungary). Samples were then ground under liquid nitrogen and homogenized in a buffer
156 containing 60 mM Tris.HCl, pH 6.8 (Sigma-Aldrich), 10 % (v/v) glycerol (Molar, Budapest,
157 Hungary) and 4% (v/v) 2-mercaptoethanol (Sigma-Aldrich). Following centrifugation at
158 12,000 rpm with a Hettich (A. Hettich, Tuttlingen, Germany) microcentrifuge, proteins of
159 supernatants were precipitated with 10% (v/v) trichloroacetic acid (TCA, Sigma-Aldrich),
160 followed by repeated washes with 5% TCA dissolved in ethanol. Samples were then vacuum-
161 dried and redissolved in 0.2 M NaOH. Radioactivity was measured in Bray's solution in a PE
162 Wallac 1409 liquid scintillation counter (Perkin-Elmer, Waltham, Massachusets, USA).

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164 2.6. Data analysis

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166 Growth measurements were performed in at least 3, histological and cytological analyses
167 were performed in at least 5 independent experiments. Mean ± SE values were calculated and
168 plotted with the SigmaPlot 10.0 software (Systat Software Inc., San Jose, CA, USA).
169 Statistical significances of CYN treatments vs. controls were estimated by t-test. Differences

were considered significant when $P<0.05$. ~~Representative experiments are presented in the Results section.~~

3. Results

3.1. Effects of CYN on the growth and development of *V. faba* seedlings

At CYN treatments of *V. faba* cv. "Standard" under continuous light, no significant effects on growth could be observed at 3 days of toxin exposure, except for main roots, where 5 µg mL^{-1} CYN had a stimulatory effect. Six days of cyanotoxin exposure ~~induced a dual effect of growth: it~~ had stimulatory effects on epicotyl and main root elongation at a concentration of 0.1 µg mL^{-1}, and on lateral root development (it increased the number of laterals developed during exposure) at 2.5 µg mL^{-1}. At higher cyanotoxin concentrations ~~had an opposite effect:~~ slight growth inhibition ~~was observed~~ for epicotyl and significant inhibition ~~was observed~~ for main root were observed at 5-20 µg mL^{-1}. Slight inhibition of lateral root development was detected at 10-20 µg mL^{-1} (Fig. 1a-c). ~~Growth inhibition was visible on plant morphology.~~ High concentrations of CYN induced intensive browning of roots that indicated the presence of necrotic tissue (Fig. 1f and section 3.2.). When seedlings were treated for 3 days in the dark, stimulatory effects were not observed for epicotyl and main root elongation, but inhibition of main root growth was characteristic at 5-10 µg mL^{-1} CYN. The toxin stimulated lateral root development at 1 µg mL^{-1} and slightly inhibited lateral root formation at 10 µg mL^{-1} (Fig. 1d, e).

Concerning cv. "ARC Egypt Cross", we studied the effects of CYN on light grown ~~plants and toxin treatments lasted for 3 and 6 days~~. In case of epicotyl elongation, the

cyanotoxin had only inhibitory effects at both exposure times, at concentrations ≥ 5 µg mL^{-1}. For main root elongation, 3 days of exposure induced slight growth inhibition at ≥ 5 µg mL^{-1}, but 6 days of treatment resulted in a dual response similar to that observed for cv. "Standard": growth was stimulated at 1 µg mL^{-1} CYN and slightly inhibited at ≥ 5 µg mL^{-1} CYN (Fig. 1f, g).

3.2. Histological and cytological effects of CYN in differentiated tissues and interphase meristematic cells

Control main roots showed normal anatomy, characteristic for dicot roots capable of secondary thickening (Fig. 2a). Three days of CYN exposure did not induce tissue necrosis in *V. faba* roots. After 6 days of exposure, necrosis could be observed at all concentrations studied. 0.1-5 µg mL^{-1} CYN induced the formation of necrotic patches in root cortical tissue (Fig. 2b), while 10-20 µg mL^{-1} CYN induced the formation of one or two continuous rings of necrotic tissue in root cortex and in rhizodermis (Fig. 2c). Necrotic effects of CYN were observed in both *V. faba* cultivars studied. Concerning interphase meristematic cells, controls showed normal nucleus morphology (Fig. 2d). All concentrations of CYN examined induced nuclear blebbing, irrespective of the duration of treatments (3 or 6 days), light conditions and the *V. faba* cultivar used (Fig. 2e). Control root hairs were characterized by normal chromatin organization (Fig. 2f). Higher CYN concentrations (10-20 µg mL^{-1}) induced partial, then total fragmentation of nuclear material after 6 days of exposure (Fig. 2g, h).

3.3. Effects of CYN on chromosome morphology

In control lateral root meristems, most of chromosome preparations showed the typical one metacentric and five acrocentric chromosome pairs of *V. faba* (Fig. 2i; see Fuchs et al., 1998 for comparison). A low percentage of metaphase chromosome spreads (3.44 ± 0.54 % for cv. "Standard") showed some chromosome breaks. CYN increased the occurrence of these chromosome breaks at both cultivars, light conditions and exposure times (3 and 6 days) studied (Fig. 2j). The occurrence of chromosome breaks was 2.7- fold higher than in controls- 9.3 ± 1.12 % for cv. "Standard" at 0.1-5 µg mL^{-1} CYN.

3.4. Effects of CYN on mitotic activity and preprophase band formation in non-synchronized meristematic cells

For *V. faba* cv. "Standard", mitotic activity of control meristematic cells was higher under continuous light, than in dark (Fig. 3 a-c). At 3 days of CYN exposure, significant changes occurred neither in total mitotic activity, nor in the percentage of particular mitotic phases under both light conditions (Fig. 3a-c). At 6 days of ~~cyanotoxin~~ exposure, the stimulation of mitosis and particular mitotic phases were detected at 0.01-1 µg mL^{-1} CYN and inhibitory effects were detected at 2.5-20 µg mL^{-1} CYN. Metaphase stimulation was not significant (Fig. 3d-f).

Concerning *V. faba* cv. "ARC Egypt Cross", mitotic activities were assayed only for plants grown under continuous light. Three days of cyanotoxin exposure induced no significant changes in total mitotic and early mitotic activities and induced stimulation of late mitotic activity at 1-10 µg mL^{-1} CYN (Fig. 3g, h). At 6 days of exposure, 0.1 µg mL^{-1} CYN induced significant stimulation of total mitotic and prophase activity. Metaphase and anaphase was slightly stimulated, while no effects were detected for telophase cells (Fig. 3 g,

i, j). 5-10 µg mL^{-1} CYN induced significant decrease of telophase index. Inhibition of total mitosis, prophase, metaphase and anaphase occurred only at 10 µg mL^{-1} CYN (Fig. 3 g, i, j).

Analysis of microtubular organization in late G2/ prophase cells showed normal organization of preprophase bands (PPBs) and perinuclear MT arrays giving rise to the mitotic spindle (Fig. 4a). CYN treatments induce the formation of abnormal PPBs (Fig. 4b-d): (i) double PPBs; (ii) split PPBs with a single array of MT bundles on one side and two arrays on the other side of the future cell division plane; (iii) asymmetrical PPBs consisting of a single array of MT bundles with an excentrical position relative to the nucleus. Abnormal PPBs were characteristic to 0.1-10 µg mL^{-1} CYN treatments of both *V. faba* cultivars, irrespective of exposure time and light conditions. They appeared even in synchronized cells (see section 3.5.) exposed for 18 h to CYN.

For control lateral root tips, meristematic regions giving rise to root cortex showed normal cell division patterns (Fig. 4e). In contrast, treatment with 5-10 µg mL^{-1} CYN resulted in altered cell division planes: adjacent mitotic cells showed a random pattern of anticlinal and periclinal divisions (Fig. 4f).

3.5. Short-term effects of CYN on mitosis and protein synthesis

Synchronized *V. faba* control lateral root tip meristems showed maximal mitotic activity after 8-9 hours of HU washout. This coincided with the maximum percentage of cells in early and late mitosis. Early mitosis index was the percentage of cells in prophase, prometaphase and metaphase, while late mitosis index was the sum of the percentage of anaphase and telophase cells (Fig. 5a-c). 5 µg mL^{-1} CYN delayed mitosis in general and early mitotic activity: the maximal activities were detected at 12-14 h after HU washout. The highest late mitotic indices were at 12-18 after HU washout: CYN increased the duration of late mitosis (Fig. 5a-

c). We detected similar mitotic delay in synchronized *Sinapis alba* seedlings treated with 18 µg mL^{-1} CYN.

Pulse labeling with ^{35}S-methionine of control non- synchronized young roots of *S. alba* seedlings revealed that *de novo* protein synthesis underwent two cycles (two peaks) in the time frame analyzed, with maximal levels of protein synthesis at 15-16 h and 38-39 h after the start of seed germination (Fig. 5d). 18 µg mL^{-1} CYN induces 50 % growth inhibition of *S. alba* seedlings (Vasas et al., 2002). It delayed protein synthesis in this time frame. Two peaks were observed as in controls. In the first cycle, protein synthesis was inhibited as compared to control, with maximal values at 16-18 h. In the second cycle, protein synthesis was stimulated, with maximal values at 39-40 h after the start of seed germination (Fig. 5d).

4. Discussion

Growth responses of vascular plants to CYN are variable. It stimulates growth at low concentrations (0.1-0.4 µg mL^{-1}) or it stimulates lateral root development in aquatic macrophytes (Kinnear et al., 2008; Beyer et al., 2009) and in *Sinapis alba* (Máthé et al., 2013a). In *Vicia faba* seedlings, we detected transient stimulation of growth parameters- especially root growth- at 6 days of treatment with 0.1 µg mL^{-1} CYN under continuous light, in both *V. faba* cultivars. This concentration is environmentally relevant: it can be found in freshwaters, where blooming of CYN producing cyanobacteria occurs (Kinnear et al., 2008; Kinnear, 2010). Higher cyanotoxin concentrations induced growth inhibition (Fig.1). CYN induced growth stimulation can be considered as a stress response: increases in plant or organ (root) biomass increase the capacity of CYN detoxification (Kinnear et al., 2008; Beyer et al., 2009). Growth effects of CYN depended on exposure time, light conditions and the plant

293 cultivar used for *V. faba*. Three days of exposure had less pronounced effects on growth, than
294 6 days of treatment. When plants were grown in darkness, the stimulatory effects of CYN
295 were not observed for epicotyl and mainroot of cv. "Standard" and were absent for light-
296 grown epicotyls of cv. "ARC Egypt Cross" (Fig. 1). Thus, growth effects of CYN can be
297 largely dependent on the genotypes used and growth conditions.

298 Necrotic cell death is induced by many abiotic and biotic stress factors: CYN induced
299 necrosis is a non-specific response, because it is not related directly to the biochemical effects
300 of the cyanotoxin. It does induce tissue necrosis in *Phragmites australis* and *V. faba* (Beyer et
301 al., 2009 and this study), that is not detectable in *Sinapis alba* (Máthé et al., 2013a). Necrosis
302 is a defence response in general- e.g. it serves the isolation of pathogens and/ or their toxins
303 from healthy tissues (Kosslak et al., 1997). For *V. faba*, the pattern of necrosis formation was
304 CYN dose dependent. At lower cyanotoxin concentrations (0.1-5 µg mL^{-1}, 0.1-1 µg mL^{-1}
305 being environmentally relevant concentrations), necrotic patches were characteristic for root
306 cortex. At higher CYN concentrations (10-20 µg mL^{-1}), continuous necrotic rings were
307 formed in root cortical parenchyma and rhizodermis (Fig. 2b, c). Thus, it is possible that CYN
308 induced necrosis serves for the inhibition of cyanotoxin uptake into vascular (inner) tissue of
309 roots, being a defence mechanism. Alterations of chromatin organization – nuclear blebbing
310 and fragmentation – added further proof to CYN induced cell death in *V. faba* (Fig. 2e, g, h).
311 These alterations are associated with a process different to necrosis - plant programmed cell
312 death (PCD) (Drew et al., 2000). However, plant cells can undergo PCD and necrosis
313 subsequently (Kosslak et al., 1997). The fragmentation of nucleus during PCD involves
314 dsDNA cleavage that is an inducer of chromosomal aberrations (chromosome breaks,
315 translocations) as well (van Gent et al., 2001). We have observed CYN induced chromosomal
316 breakage in *V. faba* cells (Fig. 2j), raising the possibility that the cyanotoxin can induce
317 dsDNA breaks. CYN induces PCD in mammalian and human cells (Bazin et al., 2010; Štraser

et al., 2011). The induction of chromosomal aberrations by CYN is controversial. In CHO-K1 cells it did not induce such aberrations (Lankoff et al., 2007), while there is indirect evidence for their occurrence in the human HepG2 cell line, involving possible rearrangements or telomere end-fusions (Štraser et al., 2011). Chromosome loss has also been reported (Humpage et al., 2000).

CYN induced stimulation of mitosis and distinct mitotic phases at low, environmentally relevant concentrations (0.01-0.1 µg mL^{-1}) at long-term exposure (6 days) and inhibited cell division at higher concentrations (2.5-20 µg mL^{-1}) in *V. faba* (Fig. 3). This suggests that mitosis is affected by CYN in general, without arresting cells in certain mitotic phases. Transient stimulation of mitosis was detected in *Phragmites australis* (Beyer et al., 2009), but not observed in *Sinapis alba* (Máthé et al., 2013a). In spite of mitotic stimulatory effects, the toxin induces abnormal mitosis at such low concentrations (Beyer et al., 2009 and this study). In animal cells, both CYN induced mitotic stimulation and inhibition were observed (Kinnear et al., 2007; Lankoff et al., 2007). Multiple mitotic effects of CYN indicate that the cyanotoxin acts on cell division by complex mechanisms. In *V. faba*, mitotic stimulation is similar in both cultivars studied and might involve the stimulation of entry into mitosis, probably by the inhibition of protein activities regulating correct timing of cell division. At shorter (3 days) of exposure, the cyanotoxin does not have significant effects on mitotic activity (Fig. 3), but it delays mitosis in synchronized cells at a higher concentration (5 µg mL^{-1}) at short-term (24 h) exposures. This effect can be observed in *Sinapis alba* seedlings as well (data not shown), where it delays *de novo* protein synthesis in the time frame studied (43 h) as shown by pulse labeling (Fig. 5). Thus, one of the possible cause of mitotic changes induced by CYN is its protein synthesis inhibitory/ delaying effect. CYN increases the duration of late mitosis in *V. faba* (Fig. 5), therefore it can delay mitotic exit. The above data show that CYN could be a powerful tool in the study of plant and in general,

eukaryotic cell cycle regulation. One hour pretreatment with cycloheximide (CH, 2.5 µg mL^{-1}), a potent eukaryotic protein synthesis inhibitor exerted effects different to CYN in synchronized *V. faba* cells: after CH washout, there was a significant and reversible inhibition of protein synthesis and metaphase-anaphase transition was delayed (Olszewska et al., 1990), phenomena not observed for CYN.

There was one structural mitotic alteration induced by CYN independent of exposure time, cultivar and light conditions in *V. faba*. This was abnormal PPB development (Fig. 4). Moreover, we have observed this in *P. australis* as well (Beyer et al., 2009). CYN induced formation of double, split and asymmetrical PPBs could be universal in plants, and it might be directly related to its influence on protein synthesis, hence a specific effect of CYN in plants (animal cells do not possess such structures). PPB disorders including double PPBs are generally related to alterations in the synthesis and/or functioning of proteins in general or of particular proteins. Double PPBs are formed during CH treatment in onion root tip cells (Mineyuki, 1999). Alteration of auxin transport involving specific transport proteins lead to double PPB development in tobacco BY-2 cells, causing changes in cell division plane orientation (Dhonukshe et al., 2005). Asymmetrical PPBs are normally formed during unequal division e.g. of guard mother cells (Mineyuki, 1999). However, we could not detect abnormal unequal divisions in *V. faba* meristems.

PPB can determine the site of future cell division. Perturbations of PPB formation lead to abnormal cytokinesis, cell plate formation and division plane orientation (Wright et al., 2009). Granger and Cyr (2001) detected multiple PPB disorders in tobacco cell line BD2-5, including double, split and asymmetrical PPBs. Double PPBs were correlated with cytokinesis anomalies. Indeed, we observed misorientation of cell division planes in CYN treated *V. faba* root tips (Fig. 4f).

In conclusion, CYN induced transient growth stimulation (increase of biomass, especially in roots) and the induction of root necrosis are probably general stress responses of *V. faba* serving for detoxification and as a defence against transport of CYN towards healthy tissues that are not in direct contact with the toxin during exposure. These alterations are not CYN-specific, since they can be induced by a large variety of stressors. The co-occurrence of chromatin alterations (partial and total nuclear fragmentation) with necrosis indicated that PCD and necrosis could be subsequent events in CYN treated *V. faba* cells. The toxin induces transient stimulation of mitosis at long-term exposure and delays cell division at short-term exposures. Mitotic alterations could be partially attributed to the alteration of protein synthesis. Alterations of PPB development may be related to the direct/ specific effect of CYN (protein synthesis inhibition) in plants. Therefore, this type of cellular structure could be used as an indicator of CYN effects in natural water bodies where this cyanotoxin occurs as the sole protein synthesis inhibitor. Environmentally relevant CYN concentrations (≤ 1 µg mL^{-1}) affect growth and have cytotoxic effects like necrosis, non-mitotic chromatin alterations, chromosome aberrations and the induction of abnormal PPB formation. These observations raise the possibility of CYN cytotoxicity on plants in natural environments. To our best knowledge, we have shown for the first time (i) that CYN induces chromatin alterations that are markers of PCD in plants; (ii) that there is a direct evidence for CYN induced chromosomal aberrations; (iii) that CYN induces mitotic delay that may be related to the delay of protein synthesis; (iv) that CYN induces the formation of asymmetrical PPBs and PPB anomalies can induce alterations in cell division planes. ~~This is a toxin- and plant-specific alteration induced by the cyanotoxin.~~

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Figure captions

Fig. 1. The effect of CYN on the growth of 3 and 6 days old *Vicia faba* seedlings. **(a, b)** Effects on epicotyl and mainroot growth and **(c)** on lateral root development in light grown seedlings of *V. faba* cv. "Standard". **(d)** effects on epicotyl and mainroot growth and **(e)** on lateral root development in 3 days old dark grown seedlings of *V. faba* cv. "Standard". **(f)** Morphology of light grown *V. faba* cv. "Standard" seedlings: controls and 10 µg mL^{-1} CYN, 6 d of exposure. Scalebar = 10 mm. **(g)** Effects on epicotyl and **(h)** mainroot growth in light grown *V. faba* cv. "ARC Egypt Cross". Differences between control and CYN treatments were considered to be significant at $P<0.05$ (*).

Fig. 2. (a-h) CYN induces cell death in *V. faba* cv. "Standard" roots as revealed by fluorescence microscopy (excitation wavelength: 320-360 nm). **(a-c)** Cross sections of main roots grown for 6d under continuous light. **(a)** Control root; **(b)** main root treated with 1 µg mL^{-1} CYN, necrotic tissues (arrows); **(c)** main root treated with 20 µg mL^{-1} CYN showing continuous rings of necrotic tissue in rhizodermis, the outer and inner layers of cortex. **(d, e)** Apical meristematic cells of lateral root tips grown for 3d under darkness, stained for DNA with DAPI. **(d)** Controls; **(e)** treatment with 5 µg mL^{-1} CYN, blebbing of interphase nuclei (arrowheads). **(f-h)** Root hairs of laterals grown for 6d under continuous light. **(f)** Control root hairs with intact nuclei; **(g)** degradation of nuclei induced by 10 µg mL^{-1} CYN and **(h)** fragmentation of a nucleus induced by 20 µg mL^{-1} CYN. **(i, j)** Chromosome squashes from lateral root tip meristems of *V. faba* cv. "Standard". **(i)** Control; **(j)** meristematic cell at treatment for 3 d with 5 µg mL^{-1} CYN in the dark, chromosome break (arrow). This cell contains only 11 chromosomes, even though aneuploidy is generally not induced by CYN. Scalebars: 300 µm (a-c), 5 µm (d, e, i, j), 100 µm (f-h).

Fig. 3. The effects of CYN on mitotic activities of *V. faba* lateral root tip meristematic cells. **(a-f)** *V. faba* cv. "Standard": **(a-c)** 3 days of cyanotoxin treatment ; **(d-f)** 6 days of cyanotoxin treatment under continuous light. **(g-j)** *V. faba* cv. "ARC Egypt Cross" treated with CYN under continuous light: **(g)** total mitotic indices at 3 and 6 d of CYN exposure; **(h-j)** indices for particular mitotic phases: **(h)** 3 days of cyanotoxin treatment; **(i, j)** 6 days of cyanotoxin treatment. Abbreviations: P-prophase; PM- prometaphase; M- metaphase; A- anaphase; T-telophase. Differences between control and CYN treatments were considered to be significant at $P<0.05$ (*).

Fig. 4. The effects of CYN on the organization of preprophase bands (PPBs) and perinuclear microtubule (MT) arrays as well as on the formation of mitotic division planes. **(a-d)** labeling of microtubules (MTs) with anti- β- tubulin antibody and of chromatin with DAPI; **(e, f)** labelling of chromatin with DAPI. **(a)** Normal PPB organization from a control lateral root (cv. "Standard") meristematic cell grown for 3 days in dark; **(b)** double PPB, treatment for 3 days in dark of cv. "Standard" with 1 µg mL^{-1} CYN; **(c)** split PPB (arrowhead shows the site of splitting), treatment for 6 days under continuous light with 0.1 µg mL^{-1} CYN; **(d)** two cells with asymmetric PPBs, treatment for 3 days under continuous light of cv. "Standard" with 0.1 µg mL^{-1} CYN; **(e)** control meristematic tissue from a lateral root (cv. "ARC Egypt Cross") grown for 6 days; **(f)** altered division planes in meristems of cv. "ARC Egypt Cross" (see arrowheads), treatment for 6 days under continuous light with 5 µg mL^{-1} CYN. Scalebars: 5 µm (a-d), 10 µm (e, f).

Fig. 5. The effect of CYN on mitotic and protein synthesis cycles. **(a-c)** Time courses of mitotic indices in *V. faba* (cv. "ARC Egypt Cross") lateral root tip meristems synchronized

with hydroxylurea (HU). **(d)** Time course of pulse labeling of *Sinapis alba* seedlings with ^{35}S methionine.

Cytotoxic effects of cylindrospermopsin in mitotic and non-mitotic *Vicia faba* cells

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Abstract

Cylindrospermopsin (CYN) is a cyanobacterial toxin known as a eukaryotic protein synthesis inhibitor. We aimed to study its effects on growth, stress responses and mitosis of a eukaryotic model, *Vicia faba* (broad bean). Growth responses depended on exposure time (3 or 6 days), cyanotoxin concentration, culture conditions (dark or continuous light) and *V. faba* cultivar ("Standard" or "ARC Egypt Cross"). At 6 days of exposure, CYN had a transient stimulatory effect on root system growth, roots being possibly capable of detoxification. The toxin induced nucleus fragmentation, blebbing and chromosomal breaks indicating double stranded DNA breaks and programmed cell death. Root necrotic tissue was observed at 0.1-20 µg mL^{-1} CYN that probably impeded toxin uptake into vascular tissue. Growth and cell death processes observed were general stress responses. In lateral root tip meristems, lower CYN concentrations (0.01-0.1 µg mL^{-1}) induced the stimulation of mitosis and distinct mitotic phases, irrespective of culture conditions or the cultivar used. Higher cyanotoxin concentrations inhibited mitosis. Short-term exposure of hydroxylurea- synchronized roots to 5 µg mL^{-1} CYN induced delay of mitosis that might have been related to a delay of *de novo* protein synthesis. CYN induced the formation of double, split and asymmetric preprophase bands (PPBs), in parallel with the alteration of cell division planes, related to the interference of cyanotoxin with protein synthesis, thus it was a plant- and CYN specific alteration.

Keywords: cylindrospermopsin, *Vicia faba*, growth stimulation, cell death, mitotic alterations, preprophase band

1. Introduction

Cylindrospermopsin (CYN) is a worldwide occurring alkaloid-type toxin, a tricyclic guanidine derivative containing a hydroxymethyluracil group. Several CYN varieties have been described (Ohtani et al., 1992; Banker et al., 2000). It is produced by cyanobacterial strains of several species/ genera (*Cylindrospermopsis raciborskii*, *Anabaena*, *Aphanizomenon* sp., *Lyngbya wollei*, *Raphidiopsis* sp., *Umezakia* sp.). Its impacts on human and animal health and its potential effects on aquatic ecosystems have been described (Kinnear, 2010). CYN can modulate the level and expression of many proteins in a single organism: both stimulation and inhibition have been observed (Beyer et al., 2009; Puerto et al., 2011). Although it is thought to be a eukaryotic protein synthesis inhibitor, the understanding of its molecular targets needs further research (Metcalf et al., 2004; Froscio et al., 2008).

Research on CYN induced cytotoxicity is important for the understanding of its health and environmental impacts. It is genotoxic and induces cell death in animal and human cells and organisms (Bazin et al., 2010). Toxin-induced tissue necrosis has been known for a long time (Terao et al., 1994). CYN alters cytoskeletal organization in mammalian cells (Fessard and Bernard, 2003). Concerning plants, we have shown for the first time that CYN induces growth inhibition (Vasas et al., 2002). The cyanotoxin generates stress/ defence responses like lignification of cell walls or formation of a callus-like tissue, alterations that are thought to play a role in the inhibition of toxin uptake by plants (Beyer et al., 2009; Máthé et al., 2013a). It alters the organization of plant microtubules (MTs), probably by the inhibition of synthesis and/ or activity of microtubule associated proteins (Beyer et al., 2009).

In spite of the above studies on CYN toxicity at the cellular level, there is still a need for research on the mechanisms of its cytotoxicity (Máthé et al., 2013b). There are only a few

data on the effects of CYN on cell division (Lankoff et al., 2007; Beyer et al., 2009). However, we are far from understanding the interference of CYN with mitotic regulation. CYN can alter the mitotic activity of plant cells (Beyer et al., 2009). One of our aims was to study whether the cyanotoxin induces arrest in certain mitotic phases, since the hypothesis was that it interferes with the regulation of entry into or exit from mitosis. There are limited data on the cytotoxic effects of CYN on non-mitotic plant cells. Next, we intended to look for specific cellular markers of CYN toxicity. Overall, our aim was to contribute to the understanding of CYN toxicity by a detailed analysis of cyanotoxin induced cellular alterations in non-mitotic as well as mitotic cells of *Vicia faba* (broad bean), a well-known model system for plant and in general, eukaryotic cell biology. This study has potential applicability to cyanotoxin effects in natural environments.

2. Materials and methods

2.1. The purification of cylindrospermopsin

CYN was purified from *Aphanizomenon ovalisporum* (Forti) ILC 164 (BGSD 423 in our culture collection) isolated by Banker et al. (1997). The purification method was described previously (Vasas et al., 2002, 2004), involving methanol-ethanol extractions of cells repeatedly frozen-thawn, followed by size-exclusion chromatography on Toyopearl HW-40 (Tosoh, Tokyo, Japan). Further purification was performed with semi-preparative HPLC (Supercosyl TM SPLC-18 column, Supelco, Bellefonte, USA). The purity of toxin was $\geq$ 95% as checked by HPLC and capillary electrophoresis.

95 2.2. Plant material and CYN treatments

96

97 Two cultivars of *Vicia faba* (broad bean, "Standard" /Hungarian cultivar/ and "ARC Egypt
98 Cross") were used in order to see whether genetic differences between them can influence
99 their response to the cyanotoxin. Seeds were surface sterilized, pregerminated and plantlets
100 were grown as described previously for microcystin-LR treatments (Beyer et al., 2012),
101 except that Allen (1968) medium was used. CYN was used in a concentration range of 0.01-
102 20 μg mL^{-1} (0.024-48.2 μM).

103 For growth analysis, cyanotoxin treatments lasted for 3 days both in dark and at
104 continuous light (cool white fluorescent illumination) of 100 μmol m^{-2} s^{-1}. Six days'
105 exposures were performed only under continuous light, since control seedlings did not survive
106 under light-depleted conditions. Growth of CYN treated seedlings was assayed by means of
107 increase in the length of epicotyls, main roots and number of lateral roots. These parameters
108 were measured at the beginning and at the end of cyanotoxin treatments.

109

110 2.3. Microscopy, histochemical and immunohistochemical methods

111

112 For histological and cytological analysis, non-synchronized and synchronized roots were
113 used. The non-synchronized system consisted of the seedlings treated with CYN for 3 and 6
114 days. For the analysis of synchronized cells, roots of cv. "ARC Egypt Cross" were treated
115 with hydroxylurea (HU) for arresting cells in the S phase (Beyer et al., 2012). After HU
116 washout, CYN treatments were performed for 24 h under continuous dim light of 3 μmol m^{-2}
117 s^{-1} PFD.

118 Microscopical analyses were performed with an Olympus Provis AX-70/A
119 fluorescence microscope (Olympus, Tokyo, Japan). Hand-made cross-sections of non-

synchronized main roots were used for histological analysis. Autofluorescence of tissues was detected at an excitation wavelength range of 320-360 nm. Histological samples used for the analysis of non-mitotic chromatin were fixed in 3.7 % (v/v) formaldehyde, followed by staining with 3 μg mL^{-1} 4',6'-diamidino-2-phenylindole (DAPI, Fluka, Buchs, Switzerland). Mitotic figures of lateral root tip meristems were analyzed both from non-synchronized and synchronized roots. The tips of lateral roots were fixed with 3.7 % (v/v) formaldehyde and cryosectioned with a Leica Jung Histoslide 2000 microtome (Leica, Nussloch, Germany). Labeling of MTs and of chromatin was performed with the aid of a Cy3-conjugated anti-β-tubulin antibody (Sigma-Aldrich, St. Louis, Mo., USA) and DAPI, by previously described methods (Beyer et al., 2009; Máthé et al., 2009). Excitation wavelength range was 540-580 nm for Cy3 and 320-360 nm for DAPI.

For the detection of mitotic figures, at least 30 sections of 5-6 lateral root tips per treatment were used. The quiescent center and cells giving rise to vascular tissue were excluded from analysis. Total mitotic index and indices for particular mitotic phases were calculated.

2.4. The preparation of chromosome spreads

Lateral root tips were treated with 0.1 % (w/v) colchicine (Sigma- Aldrich) for 3 h, then fixed for 20 min in 45 % (v/v) acetic acid. Fixed samples were hydrolyzed in 1 N HCl at 60 °C for 5 min, washed with water and stained with 5% (w/v) carmine-acetic acid for 30 min at 60 °C. Chromosome squashes were examined by using the bright-field facilities of the Olympus Provis AX-70 microscope. At least 100 cells containing metaphase chromosomes were examined for each treatment per experiment.

145 2.5. The assay of *de novo* protein synthesis in *Sinapis alba* seedlings

146

147 The assay of protein synthesis inhibition was carried out in order to see whether mitotic
148 alterations induced by CYN are related to its effects on *de novo* protein synthesis. *Sinapis*
149 *alba* (white mustard) seeds were surface sterilized as described previously (Kós et al., 1995)
150 and placed into 90-mm Petri dishes in Allen medium. Half of Petri dishes contained Allen
151 medium with 18 µg mL^{-1} CYN. Samples for protein labeling were taken at regular time
152 intervals between 14-42 hours after cyanotoxin treatment was started. Roots of seedlings were
153 pulse labeled for 1 h with 4 MBq ml^{-1} ^{35}S-methionine (Institute of Isotopes, Budapest,
154 Hungary). Samples were then ground under liquid nitrogen and homogenized in a buffer
155 containing 60 mM Tris.HCl, pH 6.8 (Sigma-Aldrich), 10 % (v/v) glycerol (Molar, Budapest,
156 Hungary) and 4% (v/v) 2-mercaptoethanol (Sigma-Aldrich). Following centrifugation at
157 12,000 rpm with a Hettich (A. Hettich, Tuttlingen, Germany) microcentrifuge, proteins of
158 supernatants were precipitated with 10% (v/v) trichloroacetic acid (TCA, Sigma-Aldrich),
159 followed by repeated washes with 5% TCA dissolved in ethanol. Samples were then vacuum-
160 dried and redissolved in 0.2 M NaOH. Radioactivity was measured in Bray's solution in a PE
161 Wallac 1409 liquid scintillation counter (Perkin-Elmer, Waltham, Massachusets, USA).

162

163 2.6. Data analysis

164

165 Growth measurements were performed in at least 3, histological and cytological analyses
166 were performed in at least 5 independent experiments. Mean ± SE values were calculated and
167 plotted with the SigmaPlot 10.0 software (Systat Software Inc., San Jose, CA, USA).
168 Statistical significances of CYN treatments vs. controls were estimated by t-test. Differences
169 were considered significant when $P<0.05$.

3. Results

3.1. Effects of CYN on the growth and development of *V. faba* seedlings

At CYN treatments of *V. faba* cv. "Standard" under continuous light, no significant effects on growth could be observed at 3 days of toxin exposure, except for main roots, where 5 μg mL^{-1} CYN had a stimulatory effect. Six days of cyanotoxin exposure had stimulatory effects on epicotyl and main root elongation at a concentration of 0.1 μg mL^{-1}, and on lateral root development (it increased the number of laterals developed during exposure) at 2.5 μg mL^{-1}. At higher cyanotoxin concentrations slight growth inhibition for epicotyl and significant inhibition for main root were observed at 5-20 μg mL^{-1}. Slight inhibition of lateral root development was detected at 10-20 μg mL^{-1} (Fig. 1a-c). High concentrations of CYN induced intensive browning of roots that indicated the presence of necrotic tissue (Fig. 1f and section 3.2.). When seedlings were treated for 3 days in the dark, stimulatory effects were not observed for epicotyl and main root elongation, but inhibition of main root growth was characteristic at 5-10 μg mL^{-1} CYN. The toxin stimulated lateral root development at 1 μg mL^{-1} and slightly inhibited lateral root formation at 10 μg mL^{-1} (Fig. 1d, e).

Concerning cv. "ARC Egypt Cross", we studied the effects of CYN on light grown seedlings. In case of epicotyl elongation, the cyanotoxin had only inhibitory effects at both exposure times, at concentrations ≥ 5 μg mL^{-1}. For main root elongation, 3 days of exposure induced slight growth inhibition at ≥ 5 μg mL^{-1}, but 6 days of treatment resulted in a dual response similar to that observed for cv. "Standard": growth was stimulated at 1 μg mL^{-1} CYN and slightly inhibited at ≥ 5 μg mL^{-1} CYN (Fig. 1f, g).

195 3.2. Histological and cytological effects of CYN in differentiated tissues and interphase
196 meristematic cells
197
198 Control main roots showed normal anatomy, characteristic for dicot roots capable of
199 secondary thickening (Fig. 2a). Three days of CYN exposure did not induce tissue necrosis in
200 *V. faba* roots. After 6 days of exposure, necrosis could be observed at all concentrations
201 studied. 0.1-5 µg mL^{-1} CYN induced the formation of necrotic patches in root cortical tissue
202 (Fig. 2b), while 10-20 µg mL^{-1} CYN induced the formation of one or two continuous rings of
203 necrotic tissue in root cortex and in rhizodermis (Fig. 2c). Necrotic effects of CYN were
204 observed in both *V. faba* cultivars studied. Concerning interphase meristematic cells, controls
205 showed normal nucleus morphology (Fig. 2d). All concentrations of CYN examined induced
206 nuclear blebbing, irrespective of the duration of treatments (3 or 6 days), light conditions and
207 the *V. faba* cultivar used (Fig. 2e). Control root hairs were characterized by normal chromatin
208 organization (Fig. 2f). Higher CYN concentrations (10-20 µg mL^{-1}) induced partial, then total
209 fragmentation of nuclear material after 6 days of exposure (Fig. 2g, h).
210
211 3.3. Effects of CYN on chromosome morphology
212
213 In control lateral root meristems, most of chromosome preparations showed the typical one
214 metacentric and five acrocentric chromosome pairs of *V. faba* (Fig. 2i; see Fuchs et al., 1998
215 for comparison). A low percentage of metaphase chromosome spreads (3.44 ± 0.54 % for cv.
216 "Standard") showed some chromosome breaks. CYN increased the occurrence of these
217 chromosome breaks at both cultivars, light conditions and exposure times (3 and 6 days)
218 studied (Fig. 2j). The occurrence of chromosome breaks was 2.7- fold higher than in controls-
219 9.3 ± 1.12 % for cv. "Standard" at 0.1-5 µg mL^{-1} CYN.

220

221 3.4. Effects of CYN on mitotic activity and preprophase band formation in non-synchronized
222 meristematic cells

223

224 For *V. faba* cv. "Standard", mitotic activity of control meristematic cells was higher under
225 continuous light, than in dark (Fig. 3 a-c). At 3 days of CYN exposure, significant changes
226 occurred neither in total mitotic activity, nor in the percentage of particular mitotic phases
227 under both light conditions (Fig. 3a-c). At 6 days of exposure, the stimulation of mitosis and
228 particular mitotic phases were detected at 0.01-1 µg mL^{-1} CYN and inhibitory effects were
229 detected at 2.5-20 µg mL^{-1} CYN. Metaphase stimulation was not significant (Fig. 3d-f).
230 Concerning *V. faba* cv. "ARC Egypt Cross", mitotic activities were assayed only for
231 plants grown under continuous light. Three days of cyanotoxin exposure induced no
232 significant changes in total mitotic and early mitotic activities and induced stimulation of late
233 mitotic activity at 1-10 µg mL^{-1} CYN (Fig. 3g, h). At 6 days of exposure, 0.1 µg mL^{-1} CYN
234 induced significant stimulation of total mitotic and prophase activity. Metaphase and
235 anaphase was slightly stimulated, while no effects were detected for telophase cells (Fig. 3 g,
236 i, j). 5-10 µg mL^{-1} CYN induced significant decrease of telophase index. Inhibition of total
237 mitosis, prophase, metaphase and anaphase occurred only at 10 µg mL^{-1} CYN (Fig. 3 g, i, j).
238 Analysis of microtubular organization in late G2/ prophase cells showed normal
239 organization of preprophase bands (PPBs) and perinuclear MT arrays giving rise to the
240 mitotic spindle (Fig. 4a). CYN treatments induce the formation of abnormal PPBs (Fig. 4b-d):
241 (i) double PPBs; (ii) split PPBs with a single array of MT bundles on one side and two arrays
242 on the other side of the future cell division plane; (iii) asymmetrical PPBs consisting of a
243 single array of MT bundles with an excentrical position relative to the nucleus. Abnormal
244 PPBs were characteristic to 0.1-10 µg mL^{-1} CYN treatments of both *V. faba* cultivars,

irrespective of exposure time and light conditions. They appeared even in synchronized cells (see section 3.5.) exposed for 18 h to CYN.

For control lateral root tips, meristematic regions giving rise to root cortex showed normal cell division patterns (Fig. 4e). In contrast, treatment with 5-10 µg mL^{-1} CYN resulted in altered cell division planes: adjacent mitotic cells showed a random pattern of anticlinal and periclinal divisions (Fig. 4f).

3.5. Short-term effects of CYN on mitosis and protein synthesis

Synchronized *V. faba* control lateral root tip meristems showed maximal mitotic activity after 8-9 hours of HU washout. This coincided with the maximum percentage of cells in early and late mitosis. Early mitosis index was the percentage of cells in prophase, prometaphase and metaphase, while late mitosis index was the sum of the percentage of anaphase and telophase cells (Fig. 5a-c). 5 µg mL^{-1} CYN delayed mitosis in general and early mitotic activity: the maximal activities were detected at 12-14 h after HU washout. The highest late mitotic indices were at 12-18 after HU washout: CYN increased the duration of late mitosis (Fig. 5a-c). We detected similar mitotic delay in synchronized *Sinapis alba* seedlings treated with 18 µg mL^{-1} CYN.

Pulse labeling with ^{35}S-methionine of control non- synchronized young roots of *S. alba* seedlings revealed that *de novo* protein synthesis underwent two cycles (two peaks) in the time frame analyzed, with maximal levels of protein synthesis at 15-16 h and 38-39 h after the start of seed germination (Fig. 5d). 18 µg mL^{-1} CYN induces 50 % growth inhibition of *S. alba* seedlings (Vasas et al., 2002). It delayed protein synthesis in this time frame. Two peaks were observed as in controls. In the first cycle, protein synthesis was inhibited as compared to

269 control, with maximal values at 16-18 h. In the second cycle, protein synthesis was
270 stimulated, with maximal values at 39-40 h after the start of seed germination (Fig. 5d).
271
272
273 4. Discussion
274
275 Growth responses of vascular plants to CYN are variable. It stimulates growth at low
276 concentrations (0.1-0.4 µg mL^{-1}) or it stimulates lateral root development in aquatic
277 macrophytes (Kinnear et al., 2008; Beyer et al., 2009) and in *Sinapis alba* (Máthé et al.,
278 2013a). In *Vicia faba* seedlings, we detected transient stimulation of growth parameters-
279 especially root growth- at 6 days of treatment with 0.1 µg mL^{-1} CYN under continuous light,
280 in both *V. faba* cultivars. This concentration is environmentally relevant: it can be found in
281 freshwaters, where blooming of CYN producing cyanobacteria occurs (Kinnear et al., 2008;
282 Kinnear, 2010). Higher cyanotoxin concentrations induced growth inhibition (Fig.1). CYN
283 induced growth stimulation can be considered as a stress response: increases in plant or organ
284 (root) biomass increase the capacity of CYN detoxification (Kinnear et al., 2008; Beyer et al.,
285 2009). Growth effects of CYN depended on exposure time, light conditions and the plant
286 cultivar used for *V. faba*. Three days of exposure had less pronounced effects on growth, than
287 6 days of treatment. When plants were grown in darkness, the stimulatory effects of CYN
288 were not observed for epicotyl and mainroot of cv. "Standard" and were absent for light-
289 grown epicotyls of cv. "ARC Egypt Cross" (Fig. 1). Thus, growth effects of CYN can be
290 largely dependent on the genotypes used and growth conditions.
291 Necrotic cell death is induced by many abiotic and biotic stress factors: CYN induced
292 necrosis is a non-specific response, because it is not related directly to the biochemical effects
293 of the cyanotoxin. It does induce tissue necrosis in *Phragmites australis* and *V. faba* (Beyer et

al., 2009 and this study), that is not detectable in *Sinapis alba* (Máthé et al., 2013a). Necrosis is a defence response in general- e.g. it serves the isolation of pathogens and/ or their toxins from healthy tissues (Kosslak et al., 1997). For *V. faba*, the pattern of necrosis formation was CYN dose dependent. At lower cyanotoxin concentrations (0.1-5 µg mL^{-1}, 0.1-1 µg mL^{-1} being environmentally relevant concentrations), necrotic patches were characteristic for root cortex. At higher CYN concentrations (10-20 µg mL^{-1}), continuous necrotic rings were formed in root cortical parenchyma and rhizodermis (Fig. 2b, c). Thus, it is possible that CYN induced necrosis serves for the inhibition of cyanotoxin uptake into vascular (inner) tissue of roots, being a defence mechanism. Alterations of chromatin organization – nuclear blebbing and fragmentation – added further proof to CYN induced cell death in *V. faba* (Fig. 2e, g, h). These alterations are associated with a process different to necrosis - plant programmed cell death (PCD) (Drew et al., 2000). However, plant cells can undergo PCD and necrosis subsequently (Kosslak et al., 1997). The fragmentation of nucleus during PCD involves dsDNA cleavage that is an inducer of chromosomal aberrations (chromosome breaks, translocations) as well (van Gent et al., 2001). We have observed CYN induced chromosomal breakage in *V. faba* cells (Fig. 2j), raising the possibility that the cyanotoxin can induce dsDNA breaks. CYN induces PCD in mammalian and human cells (Bazin et al., 2010; Štraser et al., 2011). The induction of chromosomal aberrations by CYN is controversial. In CHO-K1 cells it did not induce such aberrations (Lankoff et al., 2007), while there is indirect evidence for their occurrence in the human HepG2 cell line, involving possible rearrangements or telomere end-fusions (Štraser et al., 2011). Chromosome loss has also been reported (Humpage et al., 2000).

CYN induced stimulation of mitosis and distinct mitotic phases at low, environmentally relevant concentrations (0.01-0.1 µg mL^{-1}) at long-term exposure (6 days) and inhibited cell division at higher concentrations (2.5-20 µg mL^{-1}) in *V. faba* (Fig. 3). This

319 suggests that mitosis is affected by CYN in general, without arresting cells in certain mitotic
320 phases. Transient stimulation of mitosis was detected in *Phragmites australis* (Beyer et al.,
321 2009), but not observed in *Sinapis alba* (Máthé et al., 2013a). In spite of mitotic stimulatory
322 effects, the toxin induces abnormal mitosis at such low concentrations (Beyer et al., 2009 and
323 this study). In animal cells, both CYN induced mitotic stimulation and inhibition were
324 observed (Kinnear et al., 2007; Lankoff et al., 2007). Multiple mitotic effects of CYN indicate
325 that the cyanotoxin acts on cell division by complex mechanisms. In *V. faba*, mitotic
326 stimulation is similar in both cultivars studied and might involve the stimulation of entry into
327 mitosis, probably by the inhibition of protein activities regulating correct timing of cell
328 division. At shorter (3 days) of exposure, the cyanotoxin does not have significant effects on
329 mitotic activity (Fig. 3), but it delays mitosis in synchronized cells at a higher concentration
330 (5 µg mL^{-1}) at short-term (24 h) exposures. This effect can be observed in *Sinapis alba*
331 seedlings as well (data not shown), where it delays *de novo* protein synthesis in the time
332 frame studied (43 h) as shown by pulse labeling (Fig. 5). Thus, one of the possible cause of
333 mitotic changes induced by CYN is its protein synthesis inhibitory/ delaying effect. CYN
334 increases the duration of late mitosis in *V. faba* (Fig. 5), therefore it can delay mitotic exit.
335 The above data show that CYN could be a powerful tool in the study of plant and in general,
336 eukaryotic cell cycle regulation. One hour pretreatment with cycloheximide (CH, 2.5 µg mL^{-1}),
337 a potent eukaryotic protein synthesis inhibitor exerted effects different to CYN in
338 synchronized *V. faba* cells: after CH washout, there was a significant and reversible inhibition
339 of protein synthesis and metaphase-anaphase transition was delayed (Olszewska et al., 1990),
340 phenomena not observed for CYN.
341 There was one structural mitotic alteration induced by CYN independent of exposure
342 time, cultivar and light conditions in *V. faba*. This was abnormal PPB development (Fig. 4).
343 Moreover, we have observed this in *P. australis* as well (Beyer et al., 2009). CYN induced

formation of double, split and asymmetrical PPBs could be universal in plants, and it might be directly related to its influence on protein synthesis, hence a specific effect of CYN in plants (animal cells do not possess such structures). PPB disorders including double PPBs are generally related to alterations in the synthesis and/or functioning of proteins in general or of particular proteins. Double PPBs are formed during CH treatment in onion root tip cells (Mineyuki, 1999). Alteration of auxin transport involving specific transport proteins lead to double PPB development in tobacco BY-2 cells, causing changes in cell division plane orientation (Dhonukshe et al., 2005). Asymmetrical PPBs are normally formed during unequal division e.g. of guard mother cells (Mineyuki, 1999). However, we could not detect abnormal unequal divisions in *V. faba* meristems.

PPB can determine the site of future cell division. Perturbations of PPB formation lead to abnormal cytokinesis, cell plate formation and division plane orientation (Wright et al., 2009). Granger and Cyr (2001) detected multiple PPB disorders in tobacco cell line BD2-5, including double, split and asymmetrical PPBs. Double PPBs were correlated with cytokinesis anomalies. Indeed, we observed misorientation of cell division planes in CYN treated *V. faba* root tips (Fig. 4f).

In conclusion, CYN induced transient growth stimulation (increase of biomass, especially in roots) and the induction of root necrosis are probably general stress responses of *V. faba* serving for detoxification and as a defence against transport of CYN towards healthy tissues that are not in direct contact with the toxin during exposure. These alterations are not CYN-specific, since they can be induced by a large variety of stressors. The co-occurrence of chromatin alterations (partial and total nuclear fragmentation) with necrosis indicated that PCD and necrosis could be subsequent events in CYN treated *V. faba* cells. The toxin induces transient stimulation of mitosis at long-term exposure and delays cell division at short-term exposures. Mitotic alterations could be partially attributed to the alteration of protein

synthesis. Alterations of PPB development may be related to the direct/ specific effect of CYN (protein synthesis inhibition) in plants. Therefore, this type of cellular structure could be used as an indicator of CYN effects in natural water bodies where this cyanotoxin occurs as the sole protein synthesis inhibitor. Environmentally relevant CYN concentrations (≤ 1 μg mL^{-1}) affect growth and have cytotoxic effects like necrosis, non-mitotic chromatin alterations, chromosome aberrations and the induction of abnormal PPB formation. These observations raise the possibility of CYN cytotoxicity on plants in natural environments. To our best knowledge, we have shown for the first time (i) that CYN induces chromatin alterations that are markers of PCD in plants; (ii) that there is a direct evidence for CYN induced chromosomal aberrations; (iii) that CYN induces mitotic delay that may be related to the delay of protein synthesis; (iv) that CYN induces the formation of asymmetrical PPBs and PPB anomalies can induce alterations in cell division planes.

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Figure captions

Fig. 1. The effect of CYN on the growth of 3 and 6 days old *Vicia faba* seedlings. **(a, b)** Effects on epicotyl and mainroot growth and **(c)** on lateral root development in light grown seedlings of *V. faba* cv. "Standard". **(d)** effects on epicotyl and mainroot growth and **(e)** on lateral root development in 3 days old dark grown seedlings of *V. faba* cv. "Standard". **(f)** Morphology of light grown *V. faba* cv. "Standard" seedlings: controls and 10 µg mL^{-1} CYN, 6 d of exposure. Scalebar = 10 mm. **(g)** Effects on epicotyl and **(h)** mainroot growth in light grown *V. faba* cv. "ARC Egypt Cross". Differences between control and CYN treatments were considered to be significant at $P<0.05$ (*).

Fig. 2. (a-h) CYN induces cell death in *V. faba* cv. "Standard" roots as revealed by fluorescence microscopy (excitation wavelength: 320-360 nm). **(a-c)** Cross sections of main roots grown for 6d under continuous light. **(a)** Control root; **(b)** main root treated with 1 µg mL^{-1} CYN, necrotic tissues (arrows); **(c)** main root treated with 20 µg mL^{-1} CYN showing

continuous rings of necrotic tissue in rhizodermis, the outer and inner layers of cortex. **(d, e)** Apical meristematic cells of lateral root tips grown for 3d under darkness, stained for DNA with DAPI. **(d)** Controls; **(e)** treatment with 5 µg mL^{-1} CYN, blebbing of interphase nuclei (arrowheads). **(f-h)** Root hairs of laterals grown for 6d under continuous light. **(f)** Control root hairs with intact nuclei; **(g)** degradation of nuclei induced by 10 µg mL^{-1} CYN and **(h)** fragmentation of a nucleus induced by 20 µg mL^{-1} CYN. **(i, j)** Chromosome squashes from lateral root tip meristems of *V. faba* cv. "Standard". **(i)** Control; **(j)** meristematic cell at treatment for 3 d with 5 µg mL^{-1} CYN in the dark, chromosome break (arrow). This cell contains only 11 chromosomes, even though aneuploidy is generally not induced by CYN. Scalebars: 300 µm (a-c), 5 µm (d, e, i, j), 100 µm (f-h).

Fig. 3. The effects of CYN on mitotic activities of *V. faba* lateral root tip meristematic cells. **(a-f)** *V. faba* cv. "Standard": **(a-c)** 3 days of cyanotoxin treatment ; **(d-f)** 6 days of cyanotoxin treatment under continuous light. **(g-j)** *V. faba* cv. "ARC Egypt Cross" treated with CYN under continuous light: **(g)** total mitotic indices at 3 and 6 d of CYN exposure; **(h-j)** indices for particular mitotic phases: **(h)** 3 days of cyanotoxin treatment; **(i, j)** 6 days of cyanotoxin treatment. Abbreviations: P-prophase; PM- prometaphase; M- metaphase; A- anaphase; T-telophase. Differences between control and CYN treatments were considered to be significant at $P<0.05$ (*).

Fig. 4. The effects of CYN on the organization of preprophase bands (PPBs) and perinuclear microtubule (MT) arrays as well as on the formation of mitotic division planes. **(a-d)** labeling of microtubules (MTs) with anti- β- tubulin antibody and of chromatin with DAPI; **(e, f)** labelling of chromatin with DAPI. **(a)** Normal PPB organization from a control lateral root (cv. "Standard") meristematic cell grown for 3 days in dark; **(b)** double PPB, treatment for 3

542 days in dark of cv. “Standard” with 1 µg mL^{-1} CYN; **(c)** split PPB (arrowhead shows the site
543 of splitting), treatment for 6 days under continuous light with 0.1 µg mL^{-1} CYN; **(d)** two cells
544 with asymmetric PPBs, treatment for 3 days under continuous light of cv. “Standard” with 0.1
545 µg mL^{-1} CYN; **(e)** control meristematic tissue from a lateral root (cv. “ARC Egypt Cross”)
546 grown for 6 days; **(f)** altered division planes in meristems of cv. “ARC Egypt Cross” (see
547 arrowheads), treatment for 6 days under continuous light with 5 µg mL^{-1} CYN. Scalebars: 5
548 µm (a-d), 10 µm (e, f).
549
550 **Fig. 5.** The effect of CYN on mitotic and protein synthesis cycles. **(a-c)** Time courses of
551 mitotic indices in *V. faba* (cv. “ARC Egypt Cross”) lateral root tip meristems synchronized
552 with hydroxylurea (HU). **(d)** Time course of pulse labeling of *Sinapis alba* seedlings with ^{35}S
553 methionine.

To: Dr. Joop de Boer
Editor-in chief of Chemosphere
Institute for Environmental Studies
Vrije Universiteit
Amsterdam, The Netherlands

Dear Dr. J. de Boer!

Please find enclosed our revised manuscript (originally manuscript no. CHEM31187 entitled „ **Cytotoxic effects of cylindrospermopsin in mitotic and non-mitotic *Vicia faba* cells",** subject area: **Environmental Toxicology and Risk Assessment**). We would like to thank the comments and suggestions of Editor and Reviewers, that helped in improving our manuscript. All the corrections suggested have been made and marked in the revised paper. We attach a detailed answer to Reviewers' comments to this letter as well. The manuscript contains five figures. This material has not been published and it is not considered for publication elsewhere. All authors have been named in the manuscript and all of them approved its final version.

Conflict of interest statement:
The authors declare that there are no conflicts of interest.

Yours sincerely,

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Figure 1

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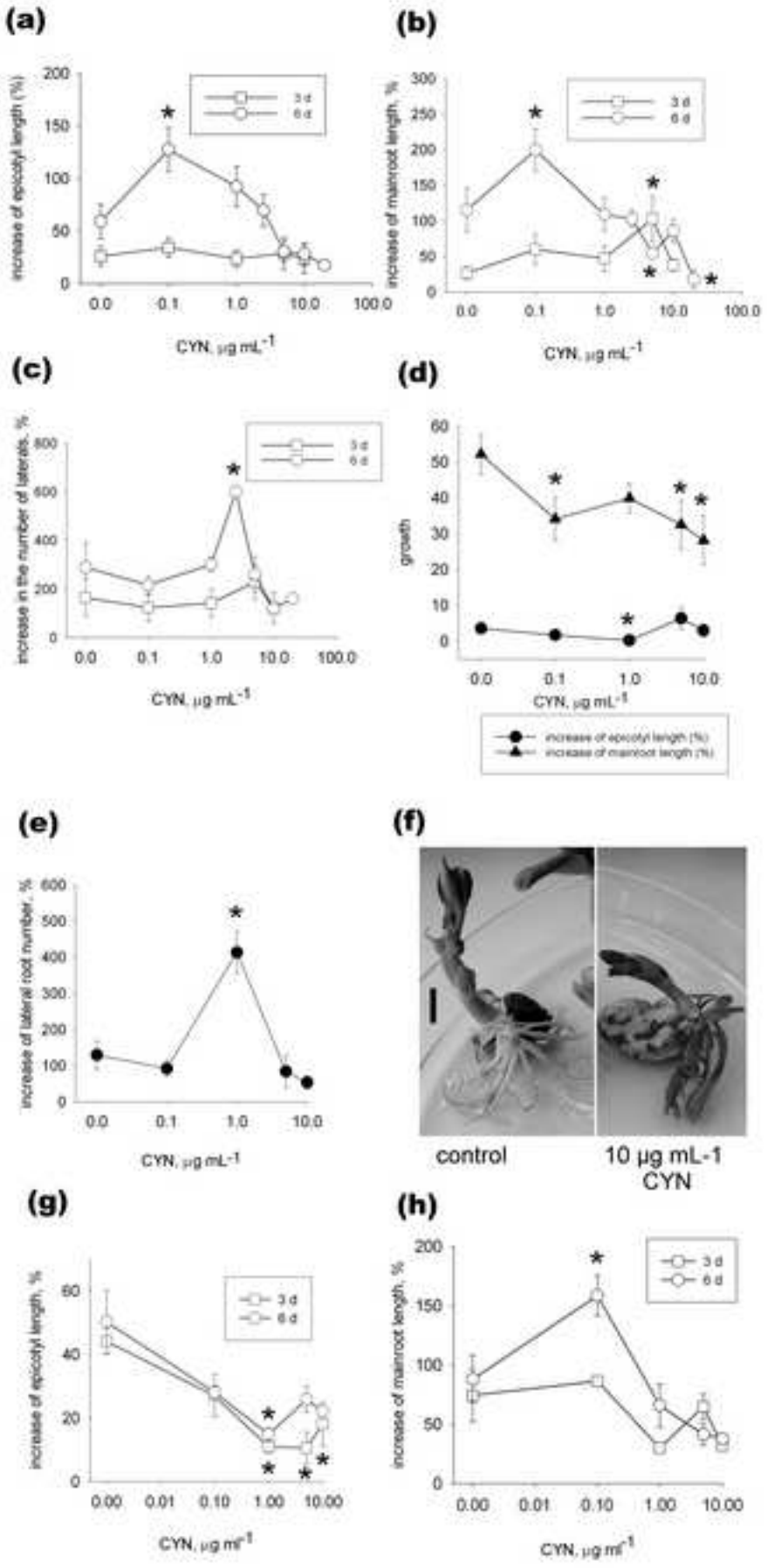

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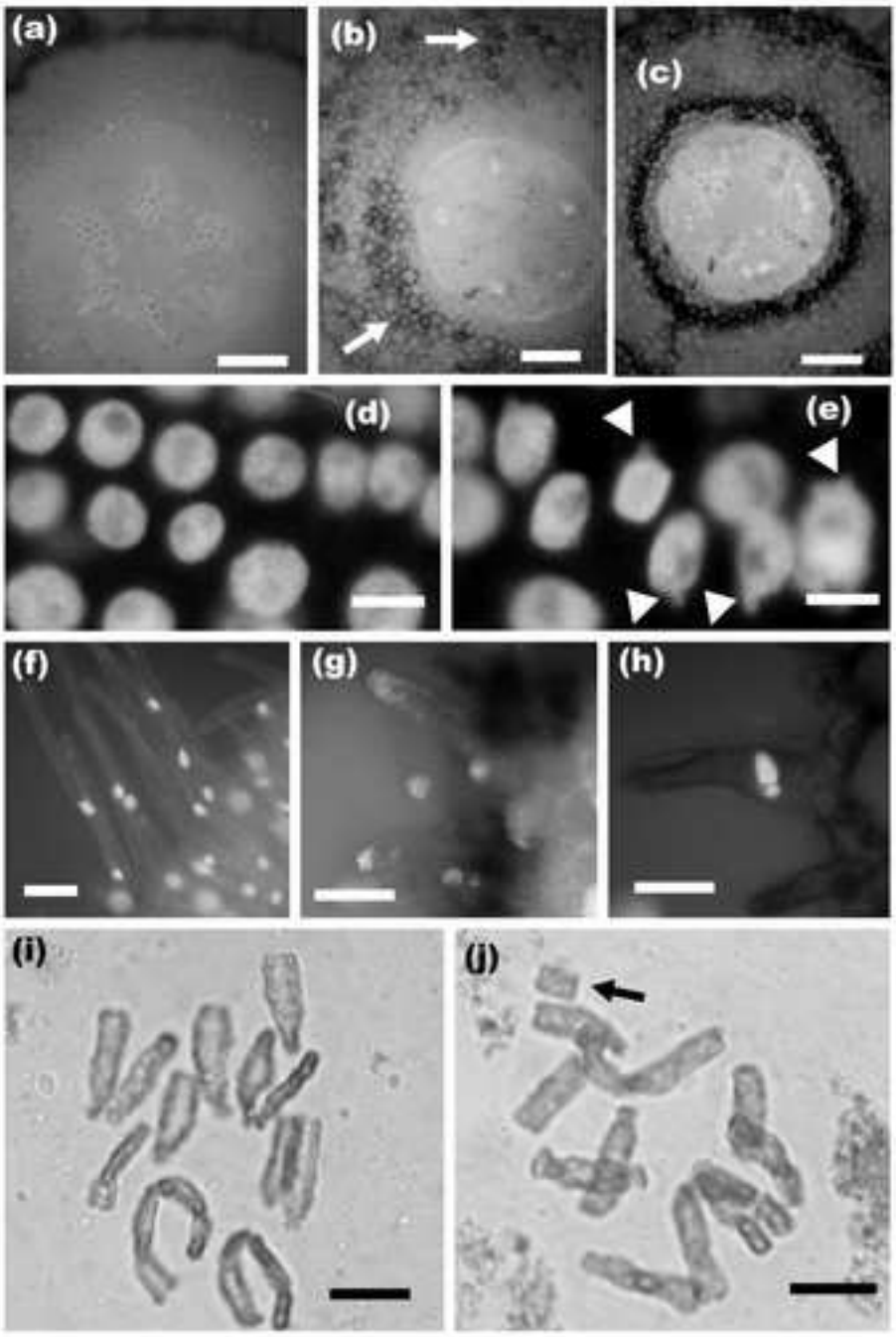

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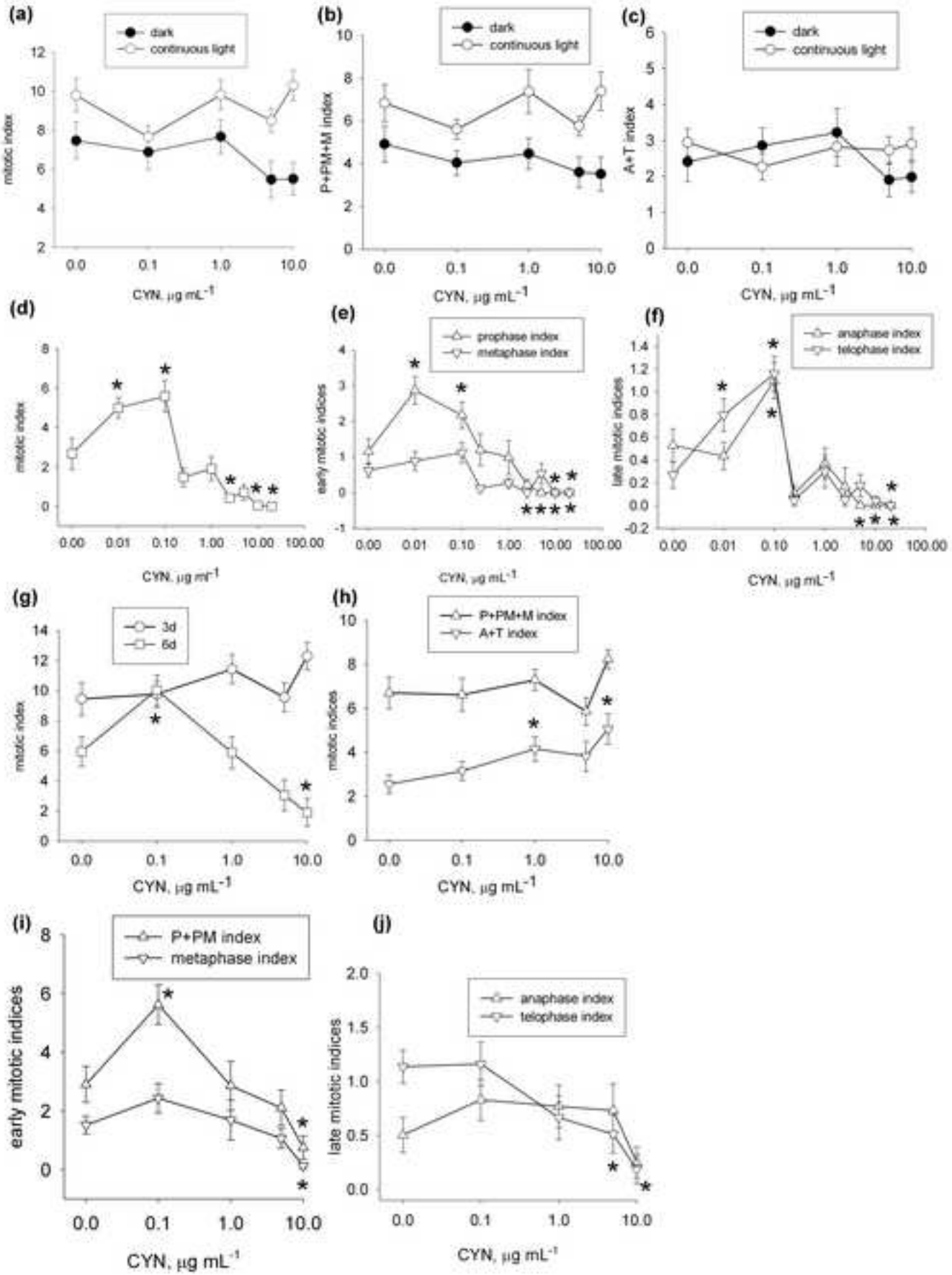

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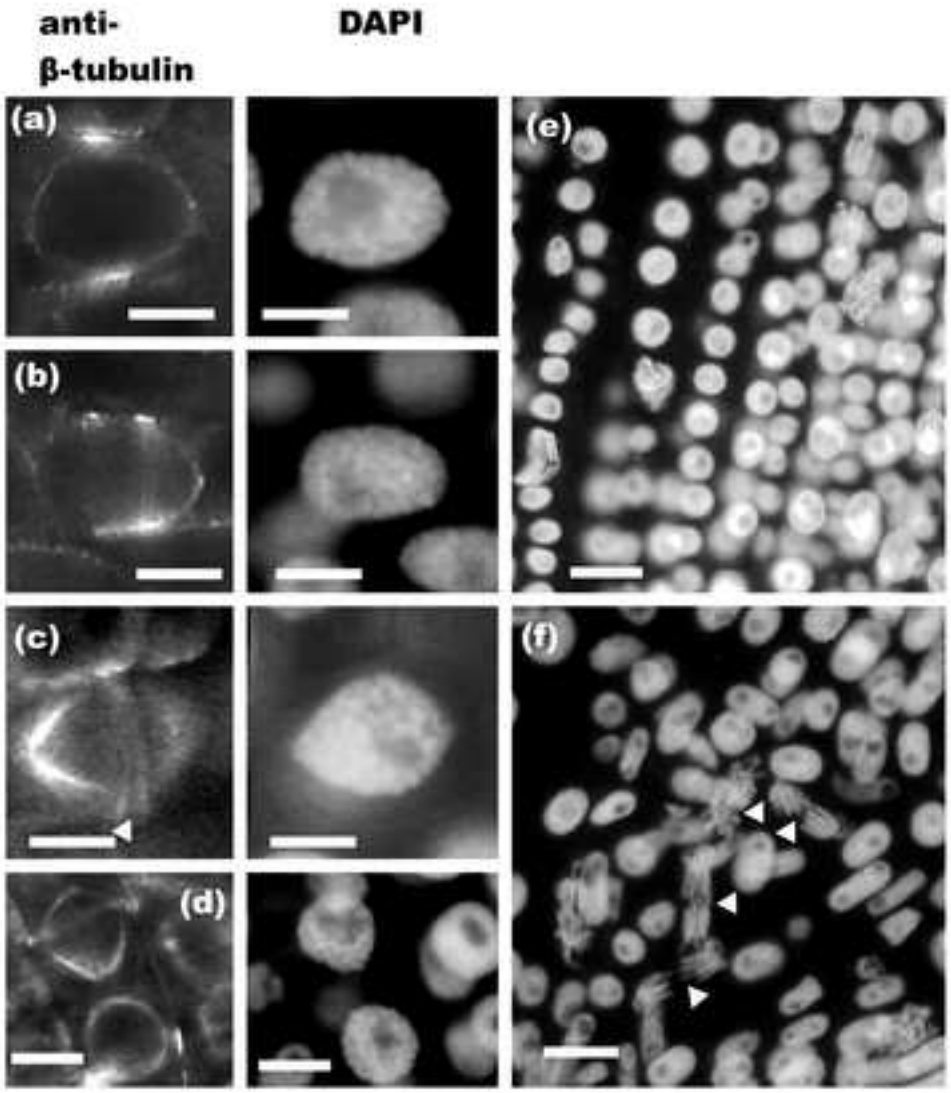

Figure 5

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