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

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21 Abstract

22

23 Cyindrospermopsin (CYN) is a cyanobacterial toxin known as a eukaryotic protein synthesis
24 inhibitor. We aimed to study its effects on growth, stress responses and mitosis of a
25 eukaryotic model, *Vicia faba* (broad bean). Growth responses depended on exposure time (3
26 or 6 days), cyanotoxin concentration, culture conditions (dark or continuous light) and *V. faba*
27 cultivar (“Standard” or “ARC Egypt Cross”). At 6 days of exposure, CYN had a transient
28 stimulatory effect on **root system growth, roots being possibly** capable of detoxification. The
29 toxin induced nucleus fragmentation, blebbing and chromosomal breaks indicating double
30 stranded DNA breaks and programmed cell death. **Root necrotic tissue was frequently**
31 **observed at 0.1-20 $\mu\text{g mL}^{-1}$ CYN and at ~~10-20 $\mu\text{g mL}^{-1}$ CYN, two continuous necrotic cell~~**
32 **layers formed in main roots** that probably impeded toxin uptake into vascular tissue. Growth
33 and cell death processes observed were general stress responses. In lateral root tip meristems,
34 lower CYN concentrations (0.01-0.1 $\mu\text{g mL}^{-1}$) induced the stimulation of mitosis and distinct
35 mitotic phases, irrespective of culture conditions or the cultivar used. Higher cyanotoxin
36 concentrations inhibited mitosis. Short-term exposure of hydroxylurea- synchronized roots to
37 5 $\mu\text{g mL}^{-1}$ CYN induced delay of mitosis that might have been related to a delay of *de novo*
38 protein synthesis. CYN induced the formation of double, split and asymmetric preprophase
39 bands (PPBs), in parallel with the alteration of cell division planes, related to the interference
40 of cyanotoxin with protein synthesis, thus it was a plant- and CYN specific alteration.

41

42 Keywords: cyindrospermopsin, *Vicia faba*, growth stimulation, cell death, mitotic alterations,
43 preprophase band

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46 1. Introduction

47

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

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

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

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

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84 2. Materials and methods

85

86 2.1. The purification of cylindrospermopsin

87

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

95

96 2.2. Plant material and CYN treatments

97

98 Two cultivars of *Vicia faba* (broad bean, “Standard” /Hungarian cultivar/ and “ARC Egypt
99 Cross”) were used in order to see whether genetic differences between them can influence
100 their response to the cyanotoxin. Seeds were surface sterilized, pregerminated and plantlets
101 were grown as described previously for microcystin-LR treatments (Beyer et al., 2012),
102 except that Allen (1968) medium was used. CYN was used in a concentration range of 0.01-
103 20 $\mu\text{g mL}^{-1}$ (0.024-48.2 μM).

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

110

111 2.3. Microscopy, histochemical and immunohistochemical methods

112

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

119

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

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

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

136

137 2.4. The preparation of chromosome spreads

138

139 Lateral root tips were treated with 0.1 % (w/v) colchicine (Sigma- Aldrich) for 3 h, then fixed
140 for 20 min in 45 % (v/v) acetic acid. Fixed samples were hydrolyzed in 1 N HCl at 60 °C for
141 5 min, washed with water and stained with 5% (w/v) carmine-acetic acid for 30 min at 60 °C.
142 Chromosome squashes were examined by using the bright-field facilities of **the** Olympus
143 Provis AX-70 microscope. At least 100 cells containing metaphase chromosomes were
144 examined for each treatment per experiment.

145

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

147

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 ³⁵S-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, Massachusetts, USA).

163

164 2.6. Data analysis

165

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

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

172

173

174 3. Results

175

176 3.1. Effects of CYN on the growth and development of *V. faba* seedlings

177

178 At CYN treatments of *V. faba* cv. “Standard” under continuous light, no significant effects on
179 growth could be observed at 3 days of toxin exposure, except for main roots, where $5 \mu\text{g mL}^{-1}$
180 CYN had a stimulatory effect. Six days of cyanotoxin exposure ~~induced a dual effect of~~
181 ~~growth: it~~ had stimulatory effects on epicotyl and main root elongation at a concentration of
182 $0.1 \mu\text{g mL}^{-1}$, and on lateral root development (it increased the number of laterals developed
183 during exposure) at $2.5 \mu\text{g mL}^{-1}$. ~~At higher cyanotoxin concentrations had an opposite effect:~~
184 slight growth inhibition ~~was observed~~ for epicotyl and significant inhibition ~~was observed~~ for
185 main root ~~were observed~~ at $5\text{--}20 \mu\text{g mL}^{-1}$. Slight inhibition of lateral root development was
186 detected at $10\text{--}20 \mu\text{g mL}^{-1}$ (Fig. 1a-c). ~~Growth inhibition was visible on plant morphology.~~
187 High concentrations of CYN induced intensive browning of roots that indicated the presence
188 of necrotic tissue (Fig. 1f and section 3.2.). When seedlings were treated for 3 days in the
189 dark, stimulatory effects were not observed for epicotyl and main root elongation, but
190 inhibition of main root growth was characteristic at $5\text{--}10 \mu\text{g mL}^{-1}$ CYN. The toxin stimulated
191 lateral root development at $1 \mu\text{g mL}^{-1}$ and slightly inhibited lateral root formation at $10 \mu\text{g}$
192 mL^{-1} (Fig. 1d, e).

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

195 cyanotoxin had only inhibitory effects at both exposure times, at concentrations $\geq 5 \mu\text{g mL}^{-1}$.
196 For main root elongation, 3 days of exposure induced slight growth inhibition at $\geq 5 \mu\text{g mL}^{-1}$,
197 but 6 days of treatment resulted in a dual response similar to that observed for cv. “Standard”:
198 growth was stimulated at $1 \mu\text{g mL}^{-1}$ CYN and slightly inhibited at $\geq 5 \mu\text{g mL}^{-1}$ CYN (Fig. 1f,
199 g).

200

201 3.2. Histological and cytological effects of CYN in differentiated tissues and interphase 202 meristematic cells

203

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

216

217 3.3. Effects of CYN on chromosome morphology

218

219 In control lateral root meristems, most of chromosome preparations showed the typical one
220 metacentric and five acrocentric chromosome pairs of *V. faba* (Fig. 2i; see Fuchs et al., 1998
221 for comparison). A low percentage of metaphase chromosome spreads (3.44 ± 0.54 % for cv.
222 “Standard”) showed some chromosome breaks. CYN increased the occurrence of these
223 chromosome breaks at both cultivars, light conditions and exposure times (3 and 6 days)
224 studied (Fig. 2j). The occurrence of chromosome breaks was 2.7- fold higher than in controls-
225 9.3 ± 1.12 % for cv. “Standard” at $0.1-5 \mu\text{g mL}^{-1}$ CYN.

226

227 3.4. Effects of CYN on mitotic activity and preprophase band formation in non-synchronized
228 meristematic cells

229

230 For *V. faba* cv. “Standard”, mitotic activity of control meristematic cells was higher under
231 continuous light, than in dark (Fig. 3 a-c). At 3 days of CYN exposure, significant changes
232 occurred neither in total mitotic activity, nor in the percentage of particular mitotic phases
233 under both light conditions (Fig. 3a-c). At 6 days of cyanotoxin exposure, the stimulation of
234 mitosis and particular mitotic phases were detected at $0.01-1 \mu\text{g mL}^{-1}$ CYN and inhibitory
235 effects were detected at $2.5-20 \mu\text{g mL}^{-1}$ CYN. Metaphase stimulation was not significant (Fig.
236 3d-f).

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

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

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

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

258

259 3.5. Short-term effects of CYN on mitosis and protein synthesis

260

261 Synchronized *V. faba* control lateral root tip meristems showed maximal mitotic activity after
262 8-9 hours of HU washout. This coincided with the maximum percentage of cells in early and
263 late mitosis. Early mitosis index was the percentage of cells in prophase, prometaphase and
264 metaphase, while late mitosis index was the sum of the percentage of anaphase and telophase
265 cells (Fig. 5a-c). 5 $\mu\text{g mL}^{-1}$ CYN delayed mitosis in general and early mitotic activity: the
266 maximal activities were detected at 12-14 h after HU washout. The highest late mitotic
267 indices were at 12-18 after HU washout: CYN increased the duration of late mitosis (Fig. 5a-

268 c). We detected similar mitotic delay in synchronized *Sinapis alba* seedlings treated with 18
269 $\mu\text{g mL}^{-1}$ CYN.

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

278

279

280 4. Discussion

281

282 Growth responses of vascular plants to CYN are variable. It stimulates growth at low
283 concentrations ($0.1\text{-}0.4 \mu\text{g mL}^{-1}$) or it stimulates lateral root development in aquatic
284 macrophytes (Kinnear et al., 2008; Beyer et al., 2009) and in *Sinapis alba* (Máthé et al.,
285 2013a). In *Vicia faba* seedlings, we detected transient stimulation of growth parameters-
286 especially root growth- at 6 days of treatment with $0.1 \mu\text{g mL}^{-1}$ CYN under continuous light,
287 in both *V. faba* cultivars. This concentration is environmentally relevant: it can be found in
288 freshwaters, where blooming of CYN producing cyanobacteria occurs (Kinnear et al., 2008;
289 Kinnear, 2010). Higher cyanotoxin concentrations induced growth inhibition (Fig.1). CYN
290 induced growth stimulation can be considered as a stress response: increases in plant or organ
291 (root) biomass increase the capacity of CYN detoxification (Kinnear et al., 2008; Beyer et al.,
292 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 $\mu\text{g mL}^{-1}$, 0.1-1 $\mu\text{g mL}^{-1}$
305 being environmentally relevant concentrations), necrotic patches were characteristic for root
306 cortex. At higher CYN concentrations (10-20 $\mu\text{g mL}^{-1}$), 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

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

323 CYN induced stimulation of mitosis and distinct mitotic phases at low,
324 **environmentally relevant** concentrations (0.01-0.1 $\mu\text{g mL}^{-1}$) at long-term exposure (6 days)
325 and inhibited cell division at higher concentrations (2.5-20 $\mu\text{g mL}^{-1}$) in *V. faba* (Fig. 3). This
326 suggests that mitosis is affected by CYN in general, without arresting cells in certain mitotic
327 phases. Transient stimulation of mitosis was detected in *Phragmites australis* (Beyer et al.,
328 2009), but not observed in *Sinapis alba* (Máthé et al., 2013a). **In spite of mitotic stimulatory**
329 **effects, the toxin induces abnormal mitosis at such low concentrations (Beyer et al., 2009 and**
330 **this study)**. In animal cells, both CYN induced mitotic stimulation and inhibition were
331 observed (Kinnear et al., 2007; Lankoff et al., 2007). Multiple mitotic effects of CYN indicate
332 that the cyanotoxin acts on cell division by complex mechanisms. In *V. faba*, mitotic
333 stimulation is similar in both cultivars studied and might involve the stimulation of entry into
334 mitosis, probably by the inhibition of protein activities regulating correct timing of cell
335 division. At shorter (3 days) of exposure, the cyanotoxin does not have significant effects on
336 mitotic activity (Fig. 3), but it delays mitosis in synchronized cells at a higher concentration
337 (5 $\mu\text{g mL}^{-1}$) at short-term (24 h) exposures. This effect can be observed in *Sinapis alba*
338 seedlings as well (data not shown), where it delays *de novo* protein synthesis in the time
339 frame studied (43 h) as shown by pulse labeling (Fig. 5). Thus, one of the possible cause of
340 mitotic changes induced by CYN is its protein synthesis inhibitory/ delaying effect. CYN
341 increases the duration of late mitosis in *V. faba* (Fig. 5), therefore it can delay mitotic exit.
342 The above data show that CYN could be a powerful tool in the study of plant and in general,

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

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

361 PPB can determine the site of future cell division. Perturbations of PPB formation lead
362 to abnormal cytokinesis, cell plate formation and division plane orientation (Wright et al.,
363 2009). Granger and Cyr (2001) detected multiple PPB disorders in tobacco cell line BD2-5,
364 including double, split and asymmetrical PPBs. Double PPBs were correlated with
365 cytokinesis anomalies. Indeed, we observed misorientation of cell division planes in CYN
366 treated *V. faba* root tips (Fig. 4f).

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

389

390

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392

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404

405

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509

510 Figure captions

511

512 **Fig. 1.** The effect of CYN on the growth of 3 and 6 days old *Vicia faba* seedlings. **(a, b)**
513 Effects on epicotyl and mainroot growth and **(c)** on lateral root development in light grown
514 seedlings of *V. faba* cv. “Standard”. **(d)** effects on epicotyl and mainroot growth and **(e)** on
515 lateral root development in 3 days old dark grown seedlings of *V. faba* cv. “Standard”. **(f)**
516 Morphology of light grown *V. faba* cv. “Standard” seedlings: controls and 10 $\mu\text{g mL}^{-1}$ CYN,
517 6 d of exposure. Scalebar = 10 mm. **(g)** Effects on epicotyl and **(h)** mainroot growth in light
518 grown *V. faba* cv. “ARC Egypt Cross”. Differences between control and CYN treatments
519 were considered to be significant at $P < 0.05$ (*).

520

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

535

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

544

545 **Fig. 4.** The effects of CYN on the organization of preprophase bands (PPBs) and perinuclear
546 microtubule (MT) arrays as well as on the formation of mitotic division planes. **(a-d)** labeling
547 of microtubules (MTs) with anti- β - tubulin antibody and of chromatin with DAPI; **(e, f)**
548 labelling of chromatin with DAPI. **(a)** Normal PPB organization from a control lateral root
549 (cv. “Standard”) meristematic cell grown for 3 days in dark; **(b)** double PPB, treatment for 3
550 days in dark of cv. “Standard” with $1 \mu\text{g mL}^{-1}$ CYN; **(c)** split PPB (arrowhead shows the site
551 of splitting), treatment for 6 days under continuous light with $0.1 \mu\text{g mL}^{-1}$ CYN; **(d)** two cells
552 with asymmetric PPBs, treatment for 3 days under continuous light of cv. “Standard” with 0.1
553 $\mu\text{g mL}^{-1}$ CYN; **(e)** control meristematic tissue from a lateral root (cv. “ARC Egypt Cross”)
554 grown for 6 days; **(f)** altered division planes in meristems of cv. “ARC Egypt Cross” (see
555 arrowheads), treatment for 6 days under continuous light with $5 \mu\text{g mL}^{-1}$ CYN. Scalebars: 5
556 μm (a-d), 10 μm (e, f).

557

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

560 with hydroxylurea (HU). **(d)** Time course of pulse labeling of *Sinapis alba* seedlings with ³⁵S
561 methionine.

1

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

3

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20

21 Abstract

22

23 Cylindrospermopsin (CYN) is a cyanobacterial toxin known as a eukaryotic protein synthesis
24 inhibitor. We aimed to study its effects on growth, stress responses and mitosis of a
25 eukaryotic model, *Vicia faba* (broad bean). Growth responses depended on exposure time (3
26 or 6 days), cyanotoxin concentration, culture conditions (dark or continuous light) and *V. faba*
27 cultivar (“Standard” or “ARC Egypt Cross”). At 6 days of exposure, CYN had a transient
28 stimulatory effect on root system growth, roots being possibly capable of detoxification. The
29 toxin induced nucleus fragmentation, blebbing and chromosomal breaks indicating double
30 stranded DNA breaks and programmed cell death. Root necrotic tissue was observed at 0.1-20
31 $\mu\text{g mL}^{-1}$ CYN that probably impeded toxin uptake into vascular tissue. Growth and cell death
32 processes observed were general stress responses. In lateral root tip meristems, lower CYN
33 concentrations (0.01-0.1 $\mu\text{g mL}^{-1}$) induced the stimulation of mitosis and distinct mitotic
34 phases, irrespective of culture conditions or the cultivar used. Higher cyanotoxin
35 concentrations inhibited mitosis. Short-term exposure of hydroxylurea- synchronized roots to
36 5 $\mu\text{g mL}^{-1}$ CYN induced delay of mitosis that might have been related to a delay of *de novo*
37 protein synthesis. CYN induced the formation of double, split and asymmetric preprophase
38 bands (PPBs), in parallel with the alteration of cell division planes, related to the interference
39 of cyanotoxin with protein synthesis, thus it was a plant- and CYN specific alteration.

40

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

43

44

45 1. Introduction

46

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

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

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

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

81

82

83 2. Materials and methods

84

85 2.1. The purification of cylindrospermopsin

86

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

94

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 $\mu\text{g mL}^{-1}$ (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 $\mu\text{mol m}^{-2} \text{s}^{-1}$. 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 $\mu\text{mol m}^{-2}$
117 s^{-1} PFD.

118

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

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

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

135

136 2.4. The preparation of chromosome spreads

137

138 Lateral root tips were treated with 0.1 % (w/v) colchicine (Sigma- Aldrich) for 3 h, then fixed
139 for 20 min in 45 % (v/v) acetic acid. Fixed samples were hydrolyzed in 1 N HCl at 60 °C for
140 5 min, washed with water and stained with 5% (w/v) carmine-acetic acid for 30 min at 60 °C.
141 Chromosome squashes were examined by using the bright-field facilities of the Olympus
142 Provis AX-70 microscope. At least 100 cells containing metaphase chromosomes were
143 examined for each treatment per experiment.

144

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 $\mu\text{g mL}^{-1}$ 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, Massachusetts, 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 \pm 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 $P < 0.05$.

170

171 3. Results

172

173 3.1. Effects of CYN on the growth and development of *V. faba* seedlings

174

175 At CYN treatments of *V. faba* cv. “Standard” under continuous light, no significant effects on
176 growth could be observed at 3 days of toxin exposure, except for main roots, where $5 \mu\text{g mL}^{-1}$
177 CYN had a stimulatory effect. Six days of cyanotoxin exposure had stimulatory effects on
178 epicotyl and main root elongation at a concentration of $0.1 \mu\text{g mL}^{-1}$, and on lateral root
179 development (it increased the number of laterals developed during exposure) at $2.5 \mu\text{g mL}^{-1}$.

180 At higher cyanotoxin concentrations slight growth inhibition for epicotyl and significant
181 inhibition for main root were observed at $5\text{-}20 \mu\text{g mL}^{-1}$. Slight inhibition of lateral root
182 development was detected at $10\text{-}20 \mu\text{g mL}^{-1}$ (Fig. 1a-c). High concentrations of CYN induced
183 intensive browning of roots that indicated the presence of necrotic tissue (Fig. 1f and section
184 3.2.). When seedlings were treated for 3 days in the dark, stimulatory effects were not
185 observed for epicotyl and main root elongation, but inhibition of main root growth was
186 characteristic at $5\text{-}10 \mu\text{g mL}^{-1}$ CYN. The toxin stimulated lateral root development at $1 \mu\text{g}$
187 mL^{-1} and slightly inhibited lateral root formation at $10 \mu\text{g mL}^{-1}$ (Fig. 1d, e).

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

194

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 $\mu\text{g mL}^{-1}$ CYN induced the formation of necrotic patches in root cortical tissue
202 (Fig. 2b), while 10-20 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$) 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 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$ CYN and inhibitory effects were
229 detected at 2.5-20 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$ CYN (Fig. 3g, h). At 6 days of exposure, 0.1 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$ CYN induced significant decrease of telophase index. Inhibition of total
237 mitosis, prophase, metaphase and anaphase occurred only at 10 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$ CYN treatments of both *V. faba* cultivars,

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

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

251

252 3.5. Short-term effects of CYN on mitosis and protein synthesis

253

254 Synchronized *V. faba* control lateral root tip meristems showed maximal mitotic activity after
255 8-9 hours of HU washout. This coincided with the maximum percentage of cells in early and
256 late mitosis. Early mitosis index was the percentage of cells in prophase, prometaphase and
257 metaphase, while late mitosis index was the sum of the percentage of anaphase and telophase
258 cells (Fig. 5a-c). 5 $\mu\text{g mL}^{-1}$ CYN delayed mitosis in general and early mitotic activity: the
259 maximal activities were detected at 12-14 h after HU washout. The highest late mitotic
260 indices were at 12-18 after HU washout: CYN increased the duration of late mitosis (Fig. 5a-
261 c). We detected similar mitotic delay in synchronized *Sinapis alba* seedlings treated with 18
262 $\mu\text{g mL}^{-1}$ CYN.

263 Pulse labeling with ^{35}S -methionine of control non- synchronized young roots of *S.*
264 *alba* seedlings revealed that *de novo* protein synthesis underwent two cycles (two peaks) in
265 the time frame analyzed, with maximal levels of protein synthesis at 15-16 h and 38-39 h after
266 the start of seed germination (Fig. 5d). 18 $\mu\text{g mL}^{-1}$ CYN induces 50 % growth inhibition of *S.*
267 *alba* seedlings (Vasas et al., 2002). It delayed protein synthesis in this time frame. Two peaks
268 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 ($0.1-0.4 \mu\text{g mL}^{-1}$) 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 $0.1 \mu\text{g mL}^{-1}$ 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

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

316 CYN induced stimulation of mitosis and distinct mitotic phases at low,
317 environmentally relevant concentrations ($0.01-0.1 \mu\text{g mL}^{-1}$) at long-term exposure (6 days)
318 and inhibited cell division at higher concentrations ($2.5-20 \mu\text{g mL}^{-1}$) 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 ($5 \mu\text{g mL}^{-1}$) 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, $2.5 \mu\text{g mL}^{-1}$),
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

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

354 PPB can determine the site of future cell division. Perturbations of PPB formation lead
355 to abnormal cytokinesis, cell plate formation and division plane orientation (Wright et al.,
356 2009). Granger and Cyr (2001) detected multiple PPB disorders in tobacco cell line BD2-5,
357 including double, split and asymmetrical PPBs. Double PPBs were correlated with
358 cytokinesis anomalies. Indeed, we observed misorientation of cell division planes in CYN
359 treated *V. faba* root tips (Fig. 4f).

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

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

381

382

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384

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396

397

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500

501

502 Figure captions

503

504 **Fig. 1.** The effect of CYN on the growth of 3 and 6 days old *Vicia faba* seedlings. **(a, b)**
505 Effects on epicotyl and mainroot growth and **(c)** on lateral root development in light grown
506 seedlings of *V. faba* cv. “Standard”. **(d)** effects on epicotyl and mainroot growth and **(e)** on
507 lateral root development in 3 days old dark grown seedlings of *V. faba* cv. “Standard”. **(f)**
508 Morphology of light grown *V. faba* cv. “Standard” seedlings: controls and 10 $\mu\text{g mL}^{-1}$ CYN,
509 6 d of exposure. Scalebar = 10 mm. **(g)** Effects on epicotyl and **(h)** mainroot growth in light
510 grown *V. faba* cv. “ARC Egypt Cross”. Differences between control and CYN treatments
511 were considered to be significant at $P < 0.05$ (*).

512

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

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

527

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

536

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

542 days in dark of cv. “Standard” with $1 \mu\text{g mL}^{-1}$ CYN; (c) split PPB (arrowhead shows the site
543 of splitting), treatment for 6 days under continuous light with $0.1 \mu\text{g mL}^{-1}$ CYN; (d) two cells
544 with asymmetric PPBs, treatment for 3 days under continuous light of cv. “Standard” with 0.1
545 $\mu\text{g mL}^{-1}$ 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 $5 \mu\text{g mL}^{-1}$ 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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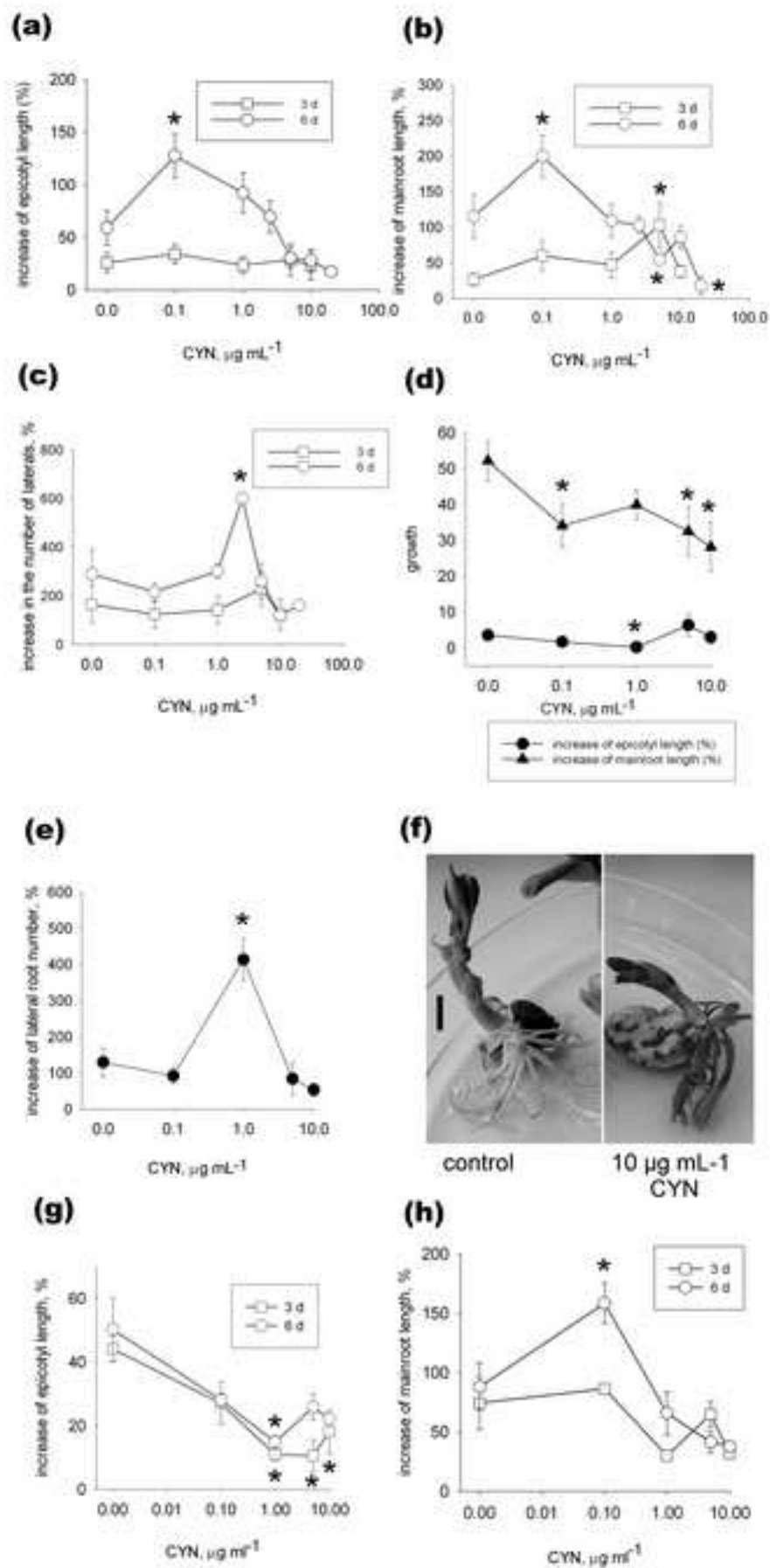


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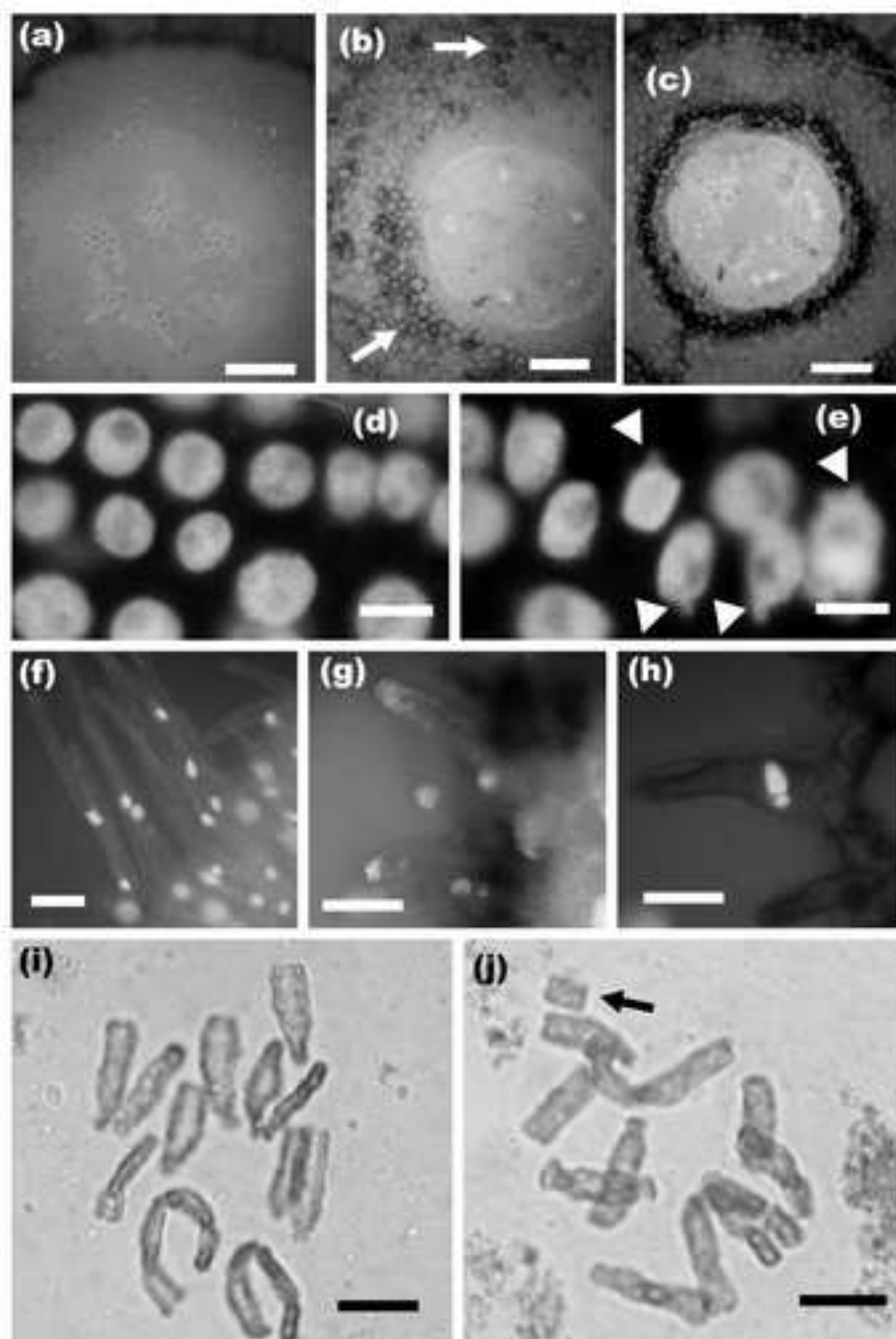


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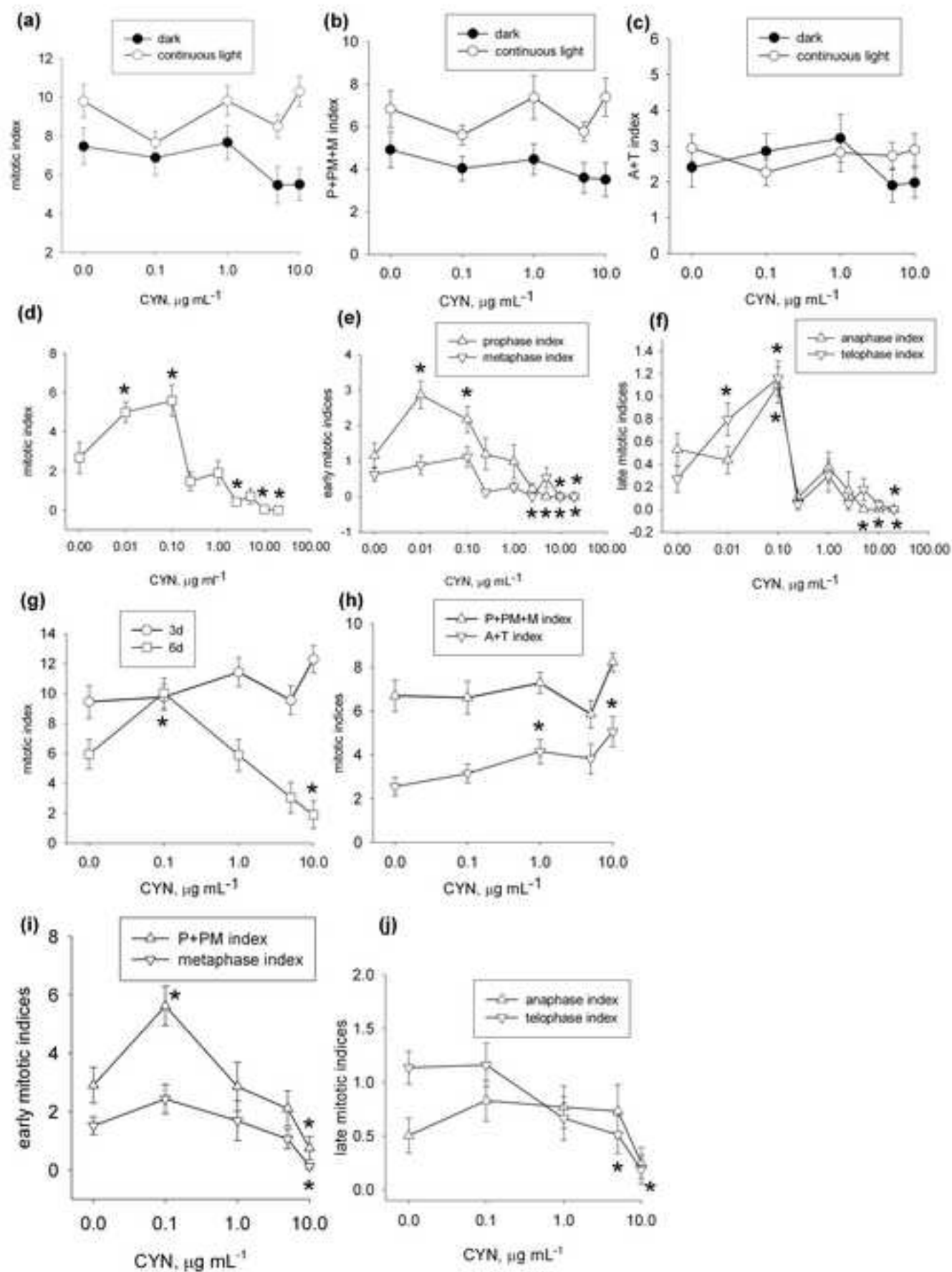


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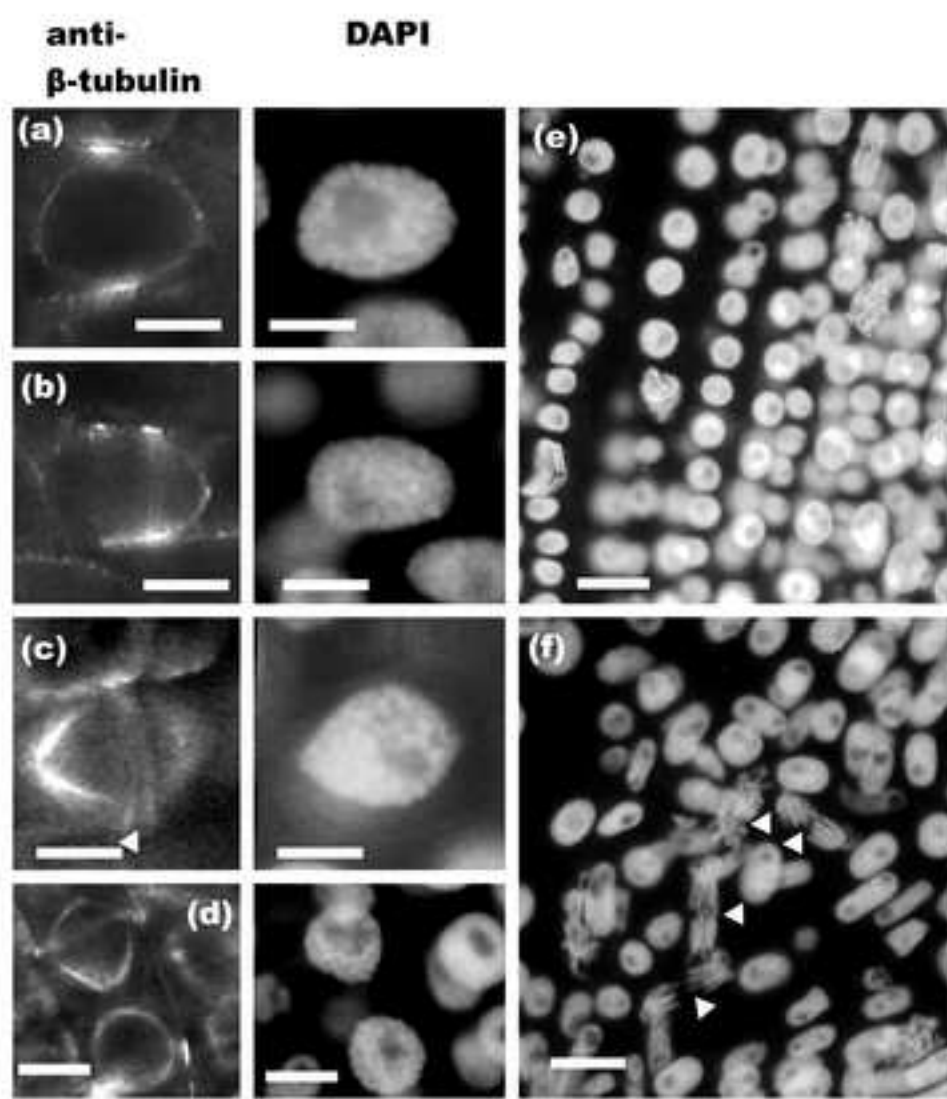


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