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CYLINDROSPERMOPSIN AND MICROCYSTIN-LR ALTER THE GROWTH, DEVELOPMENT AND PEROXIDASE ENZYME ACTIVITY OF WHITE MUSTARD (*SINAPIS ALBA* L.) SEEDLINGS, A COMPARATIVE ANALYSIS

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This work focuses on the comparative analysis of the effects of two cyanobacterial toxins of different chemical structure cylindrospermopsin (CYN) and microcystin-LR (MC-LR) on the white mustard (*Sinapis alba* L.) seedlings. Both cyanotoxins reduced significantly the fresh mass and the length of cotyledons, hypocotyls and main roots of seedlings in a concentration dependent manner. For various mustard organs the 50% inhibitory concentration values (IC_{50}) of growth were between 3–5 μg ml^{-1} for MC-LR and between 5–10 μg ml^{-1} for CYN, respectively. Cyanotoxins altered the development of cotyledons, the accumulation of photosynthetically active pigments and anthocyanins. Low MC-LR concentrations (0.01 and 0.1 μg ml^{-1}) stimulated anthocyanin formation in the cotyledons but higher than 1 μg ml^{-1} MC-LR concentrations strongly inhibited it. The CYN treated chlorotic cotyledons were violet coloured in consequence of high level of anthocyanins, while MC-LR induced chlorosis was accompanied by the appearance of necrotic patches. Necrosis and increases of peroxidase enzyme activity (POD) are general stress responses but these alterations were characteristic only for MC-LR treated mustard plants. These findings provide experimental evidences of developmental alterations induced by protein synthesis and protein phosphatase inhibitory cyanotoxins (CYN and MC-LR) in a model dicotyledonous plant.

Keywords: Cylindrospermopsin – Microcystin-LR – *Sinapis alba* L. – anthocyanin – POD

INTRODUCTION

Microcystin-LR (MC-LR) and cylindrospermopsin (CYN) are the most frequently detectable toxins produced by cyanobacteria in fresh waters [48]. They have different chemical structure and biochemical target molecules. Microcystin-LR is a cyclic heptapeptide, a potent and specific inhibitor of animal and plant protein phosphatases of type 1, 2A and 4 and as such, it interferes with essential biochemical and cellular events in animals and higher plants [14, 29–31]. Cylindrospermopsin is a water soluble cyclic guanidine alkaloid. It is known that CYN does not inhibit protein phosphatases [12] but interferes with glutathione metabolism, inhibits protein synthesis,

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Abbreviations: BGST, Blue-Green *Sinapis* Test; CYN, cylindrospermopsin; IC_{50}, fifty percent inhibitory concentration; MC, microcystins; MC-LR, microcystin-LR; POD, peroxidase enzyme.

disrupts the actin cytoskeleton and causes DNA damage in mammalian experimental systems [62]. However, only a few analyses studying biological effects of CYN on plants (e.g. alteration of growth) are available so far [7, 25, 34, 58, 61].

MC-LR and CYN can accumulate worldwide in fresh waters due to overproduction of potencial toxin producer cyanobacterial genera, including *Microcystis, Cylindrospermopsis, Aphanizomenon, Anabaena, Oscillatoria, Nostoc, Anabaenopsis, Hapalosiphon, Plantothrix, Umezakia* [62]. They could be harmful not only for the plants and animals belonging to water communities [62], but on terrestrial crops via spray irrigation as well [1, 13, 18]. The uptake of MC was demonstrated in crops like mustard [24, 26, 27, 33], salad lettuce [13], runner bean [33], rape and rice [11], alfalfa and wheat [43], which points to its potential hazard in human alimentation, too. In these species MC induced developmental, histological and biochemical alterations [18, 23, 26, 46]. In *Phaseolus vulgaris* (L.) MC inhibited the photosynthesis [1] and in *Brassica napus* (L. em. Metzer) they increased oxidative stress enzyme activities [11].

The growth inhibitory effect of MC-LR on plants was first obtained by our laboratory: white mustard (*Sinapis alba* L.) a sensitive *in vivo* plant bioassay, the Blue-Green *Sinapis* Test (BGST) has been introduced for monitoring and assaying of microcystins [26]. We established that MC-LR dramatically inhibits fresh mass, shoot and root growth and lateral root formation of toxin treated seedlings after 8 days of treatment [37]. Physiological studies on white mustard proved the inhibition of protein phosphatases [27] and the increase of single-stranded DNase activity [37].

Vasas et al. [58] described that plant growth was inhibited by cylindrospermopsin at least in the case of etiolated *Sinapis alba* (L.) seedlings. Their slightly modified Blue-Green *Sinapis* Test, used microtiter plates for the assay, the improved method reduced the amount of sample and time needed for the assay.

Under natural conditions CYN and MC-LR may be produced in the same cyanobacterial bloom [9], their toxicity has been compared only in animal bioassay systems [17, 35, 56]. Our aim was a comparative approach to understand possible effects of CYN and MC-LR, well-known cyanobacterial toxins with basically different mode of action on model crops, by adopting the procedure of Vasas et al. [58].

MATERIALS AND METHODS

The purification of cyanotoxins

Cylindrospermopsin (CYN, Mol. mass: 415 Da) was purified from the cultures of *Aphanizomenon ovalisporum* (Forti) strain ILC-164 (isolated from Lake Kinneret in 1994, Israel) [6]. For CYN purification cyanobacterial filaments were harvested by centrifugation and kept at –20 °C until use. The thawed cell pellet was extracted by 90% methanol and after rotary evaporation, the extract was loaded onto Toyopearl HW-40 size exclusion column and further purified by semi-preparative HPLC (Supercosil TM SPLC-18) [57, 58].

Microcystin-LR (MC-LR, Mol. mass: 994 Da) was purified from *Microcystis aeruginosa* (Kütz.) strain BGSD 243 isolated from Lake Velencei (Hungary) in 1991 as it described earlier [26, 37, 57]. Briefly, cells of *M. aeruginosa* were harvested by centrifugation (7000 × g, 10 min) and extracted with 5% (v/v) acetic acid which was followed by DEAE-cellulose (DE-52, Whatman) ion-exchange chromatography. The purity and identity of cyanotoxins were checked by HPLC, capillary electrophoresis and NMR [57].

Blue-Green Sinapis *Test and plant material*

Blue-Green *Sinapis* Test (BGST) was performed as described earlier by our laboratory [26] with minor modifications [37, 58]. White mustard (*Sinapis alba* L. convar. 'Budakalászi sárga', Budakalász, Hungary) was used. Surface sterilized mustard seeds (3 × 10 min in 5% H_2O_2 and 3× washing in sterile H_2O) were imbibed and germinated (16 h) in Petri dishes in dark. Germinating seeds were transferred and grown under axenic conditions in wells of microtiter plates on the surface of 1% plant nutrient supplemented agar (Bacto) complemented with cyanotoxin-containing solutions [58]. The investigated cyanotoxin concentration regimes were between 0.01 and 40.0 μg ml^{-1} (0.024–96.38 μM for CYN and 0.01–40.20 μM for MC-LR, respectively). The volume of the assay system was 200 μl/well/seed. The microtiter plates were placed into a sterile glass container covered with Saran wrap. The seedlings were grown under 14/10-hour photoperiods in a Conviron (Canada) type Phytotron at light intensity of 200 ± 50 μmol m^{-2} s^{-1}, temperature 22/18 ± 0.5 °C and humidity 45 ± 5%. Three independent experiments were performed. In each treatment at least 15 seedlings were cultivated under the above axenic conditions. Seedlings were studied after four days of toxin exposure. Such axenic conditions assured a well-controlled experimental system, where only the influences of cyanotoxins were used as variable parameters.

Measurements of seedling growth

To characterize the extent of growth inhibition, fresh mass and the lengths of cotyledons, hypocotyls and roots from 10 seedlings were measured and the fifty percent inhibition of plant growth (IC_{50}) was calculated.

Measurement of pigment content of cotyledons

Total chlorophyll contents of cotyledons were assayed using the method of Arnon [5], and the total anthocyanin contents of cotyledons were measured spectrophotometrically (Shimadzu 1601A spectrophotometer) as we described earlier [37]. The total chlorophyll and anthocyanin content of control and cyanotoxin treated cotyledons

were calculated as μg chlorophyll mg^{-1} fress mass of cotyledon and μmol total anthocyanin g^{-1} fress mass of cotyledon, and were expressed in the percentage of control.

Peroxidase enzyme activity measurement

For the determination of POD activity control and cyanotoxin treated plants were collected, pulverized in liquid nitrogen and homogenized in a buffer containing 150 mM NaCl (Reanal, Budapest, Hungary) and 14.6 mM 2-mercaptoethanol (Sigma-Aldrich, Budapest, Hungary) in 100 mM Tris-HCl (Sigma-Aldrich) pH 8.0. Homogenates were centrifuged (15,000 g, 10 min, Beckman Avanti J-25 centrifuge) at 4 °C and supernatant was used as crude protein extract. The protein contents of extracts were assayed according to Bradford [8]. The oxidation of pyrogallol (20 mM, ε_{420}: 2.47 mM^{-1} cm^{-1}, Reanal, Budapest, Hungary) in the presence of 10 mM H_2O_2 (Reanal, Budapest, Hungary) was measured spectrophotometrically (Shimadzu 1601A spectrophotometer) in a reaction medium containing 50 mM potassium-phosphate buffer (pH 7.5). The reaction was initiated by addition of protein sample (6 μg protein in 10 μl crude extract to 990 μl reaction mixture) and was measured for 60 sec. The enzyme activity was calculated as mM oxidized pyrogallol min^{-1} mg^{-1} protein and were expressed in the percentage of control.

Statistical analysis

Data were analyzed using the statistical and graphical functions of Sigma Plot 11.0 (USA). In data sets the significant differences between control and treatment's means were calculated with one-way ANOVA. The mean values ±SE are reported in the figures.

RESULTS AND DISCUSSION

Growth inhibition of Sinapis alba *L. seedlings by CYN and MC-LR*

Cylindrospermopsin and microcystin-LR exposure influenced the growth of white mustard seedlings (Fig. 1a–d). In both cases, they reduced the fresh weight (Fig. 2a), and the length of cotyledon (Fig. 2b), hypocotyl (Fig. 2c) and main root (Fig. 2d) of seedlings in a concentration dependent manner. However, there was a difference in the effects of CYN and MC-LR. Under the applied conditions CYN significantly inhibited the fresh mass, hypocotyl and main root length of seedlings at a concentration of 0.1 μg ml^{-1} and above. The 50% inhibitory concentrations (IC_{50}) of CYN were between 5–10 μg ml^{-1} for the investigated organs (Fig. 1a and Fig. 2a, c–d). As shown in Fig. 1a and Fig. 2a, c–d, the exposure of mustard seedlings to MC-LR resulted in

significant growth inhibition only above 0.5 μg ml^{-1}. The IC_{50} values of MC-LR were between 3–5 μg ml^{-1} for the studied plant organs. Our results are in accordance the studies made on *Sinapis alba* (L.) [27], *Phaseolus vulgaris* (L.), *Brassica napus* (L. em. Metzger), *Lepidium sativum* (L.), *Oryza sativa* (L.) seedlings, *Solanum tuberosum* (L.) shoot cultures, callus-derived *Phragmites australis* (Cav. Trin.) plantlets and

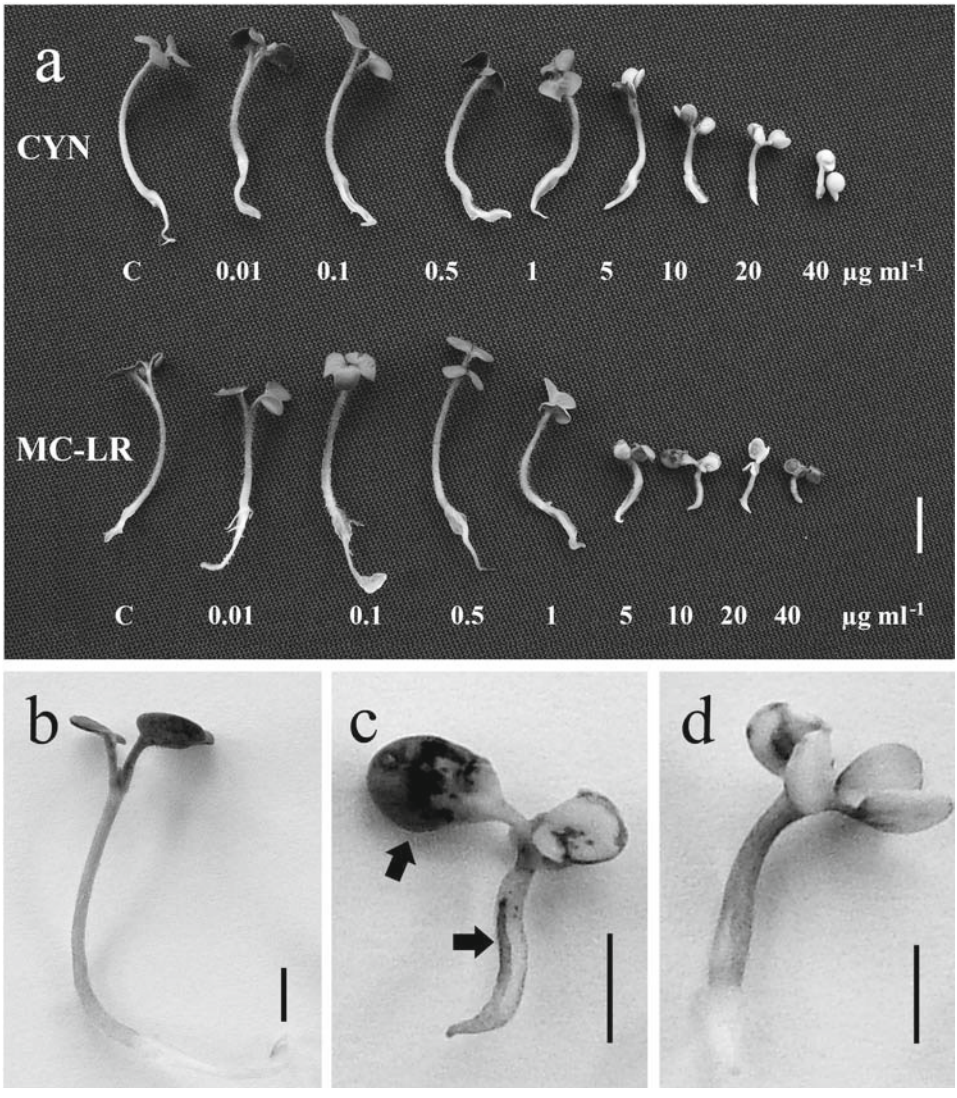

Fig. 1. Cylindrospermopsin (CYN) and microcystin-LR (MC-LR) induced growth inhibition and morphological changes of *Sinapis alba* seedlings. a: CYN and MC-LR concentration (0.01–40.0 μg ml^{-1}) dependent growth and morphology of cyanotoxin exposed mustard plants. Bar: 10 mm. b: control mustard seedling, c: necrosis (arrows) on seedling treated with 10 μg ml^{-1} MC-LR, d: 20 μg ml^{-1} CYN induced chlorosis of mustard seedlings. Bars for b–d: 5 mm

Lemna minor (L.) [1, 11, 18, 32, 33, 59] where microcystin supported the knowledge on growth inhibitory effects in plant systems. Of course the MC-LR concentrations with the effect of 50% growth inhibition (IC_{50}) depend on the sensitivity of plant species and the circumstances of tests (continuous light, photoperiod or dark, longevity of treatment, etc.). In a relevant experiment Gehringer et al. [18] established, that their *Lepidium sativum* (L.) bioassay is more sensitive, than our Blue-Green *Sinapis* Test [36], where the IC_{50} of MC-LR was 14.7 μg ml^{-1} in etiolated plants and 19.9 μg ml^{-1} in photoperiodically grown plants after 8 days, or the *Sinapis alba* bioassay of McElhiney [33] in which three commonly occurring variants of microcystin (MC-LR, -RR and -LF) caused 50% growth inhibition after 7 days in 1.9, 1.6 and 7.7 μg ml^{-1} concentrations, respectively. Indeed, *Lepidium sativum* (L.), another species

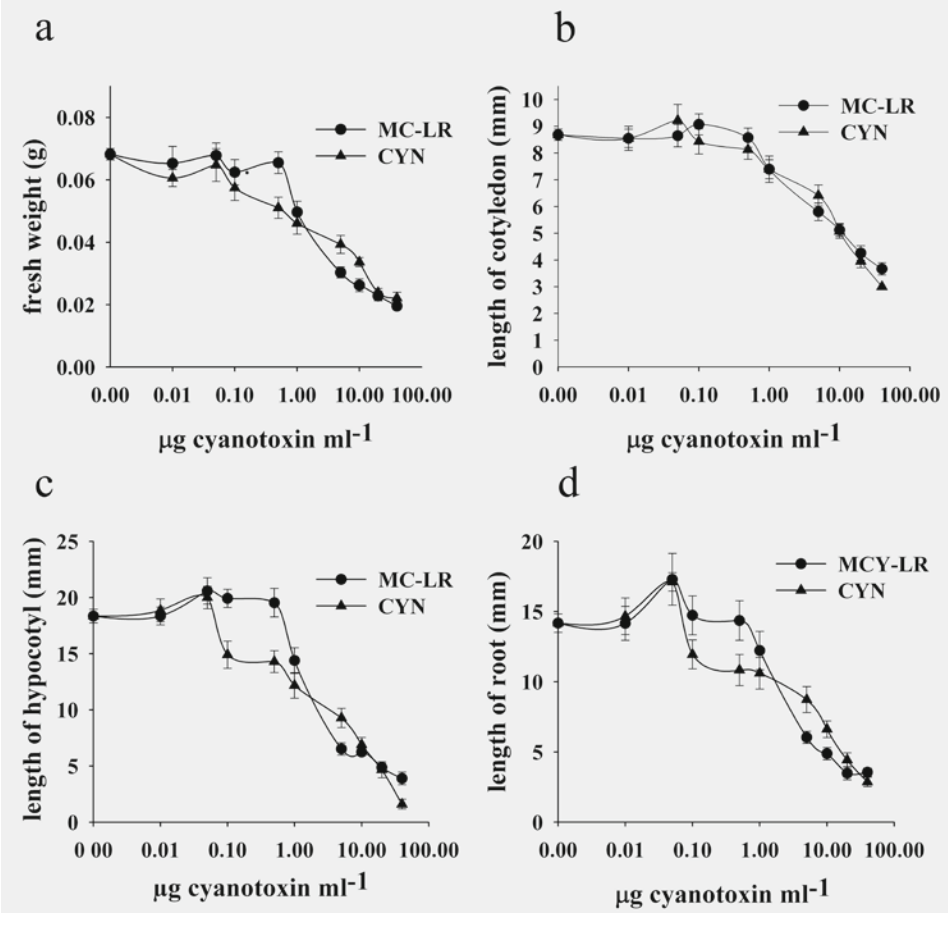

Fig. 2. The effects of microcystin-LR (MC-LR) and cylindrospermopsin (CYN) on the growth of mustard seedlings. a: fresh weight, b: length of cotyledons, c: length of hypocotyls, d: length of primary roots of seedlings on the fourth day of cyanotoxin treatment

belonging to same family, germinated under continuous light demonstrated high sensitivity to MC-LR. In 0.01 μg ml^{-1} concentration it caused significant reduction in growth characteristics of seedlings on the second day of the treatment. In this study we used microtiter plates so we were able to reduce the time and toxin required for responses and the one seedling per one well ratio brought the decrease of IC_{50} values. Although *Sinapis alba* (L.) is not the most sensitive species to MC-LR, it is sensitive to CYN as well [58]. It is a well-known crop plant [47], good model-system for morphological [39], biochemical [49] and plant biotechnological studies [51]. In the same test on the basis of growth parameters *S. alba* (L.) seedlings showed more sensitivity to MC-LR than to CYN, especially in ≥1 μg ml^{-1} concentration regimes (Fig. 1 and Fig. 2a, c, d). CYN is less effective in growth inhibition on μg ml^{-1} basis, however on molar bases these differences are about fourfold (see for molecular masses in Materials and methods).

Alterations in cotyledon morphology, chlorophyll and anthocyanin accumulation

Both cyanotoxins inhibited the growth of cotyledons at ≥1 μg ml^{-1} approximately in the same manner (Fig. 2b). It is well known that during the photoregulated development of cotyledons the cell number remains unchanged. The intensive elongation of cells and biosynthesis of chlorophylls parallel with utilisation of storage material from cotyledons leads to the expanded green form of cotyledons with photosynthethic activity [16, 36, 40]. The light-induced anthocyanin synthesis is a characteristic feature of photomorphogenesis [39]. Our experiments provided evidences that CYN and MC-LR had a pronounced inhibitory effect on photomorphogenesis of *S. alba* (L.) seedlings on the fifth day of germination (Fig. 1a–d, Fig. 2b and Fig. 3a–d). Both cyanotoxins inhibited the synthesis of chlorophylls in a concentration dependent manner (Fig. 3c), the cotyledons of cyanotoxin treated plants were obviously chlorotic and smaller (Fig. 3a, b). At 20 μg ml^{-1} CYN and MC-LR concentration 30–40% and 20–30% of cotyledons remained closed in the testa, respectively. At higher concentration (40 μg ml^{-1}) these values increased to 90–100% and 50%, consequently the cotyledons were isolated from light. *S. alba* seedlings accumulate anthocyanins in the epidermis cells of cotyledons, which is a light regulated process involving phytochromes [40]. The anthocyanin synthesis triggered by light is a very rapid process [28]. We measured the highest anthocyanin concentration on the 2nd day of germination of control seeds, and parallel with the expansion of cotyledons a decrease in it was detected (data not shown). In our earlier work we demonstrated that MC-LR significantly reduced the anthocyanin content in the cotyledons on the 8th day of treatment, which was opposite to the effects induced by environmental stress, like lack of mineral nutrients, too heavy light, UV, cold stress or fungal infection. The above-mentioned stresses induced anthocyanin accumulation in the investigated plants [15, 21, 60]. In this study, on the 4th day of treatment we established, that lower MC-LR concentrations (0.01 and 0.1 μg ml^{-1}) could stimulate anthocyanin formation

in cotyledons but the IC_{50} and higher MC-LR concentrations strongly inhibited it (Fig. 1c and Fig. 3b, d). Cotyledons exposed to 5 μg ml^{-1} and higher MC-LR concentrations showed ≥80% inhibition in chlorophyll and ≥90% in anthocyanin accumulation. Seedlings germinated on the medium completed with concentrations of CYN higher than 5 μg ml^{-1} had smaller, yellow and violet coloured cotyledons (Fig. 1d, 2b and 3a). The chlorophyll accumulation was significantly inhibited, so the protective function of anthocyanin content might be more pronounced (Fig. 3a, c, d). At 40 μg ml^{-1} CYN concentration the cotyledons remained under the testa and etiolated (Fig. 3a). These data are in good agreement with results published by other laboratories studying MC interference with photosynthetic pigment alterations and photosynthesis [1, 33, 50, 59]. Here we report that CYN reduces the chlorophyll content of

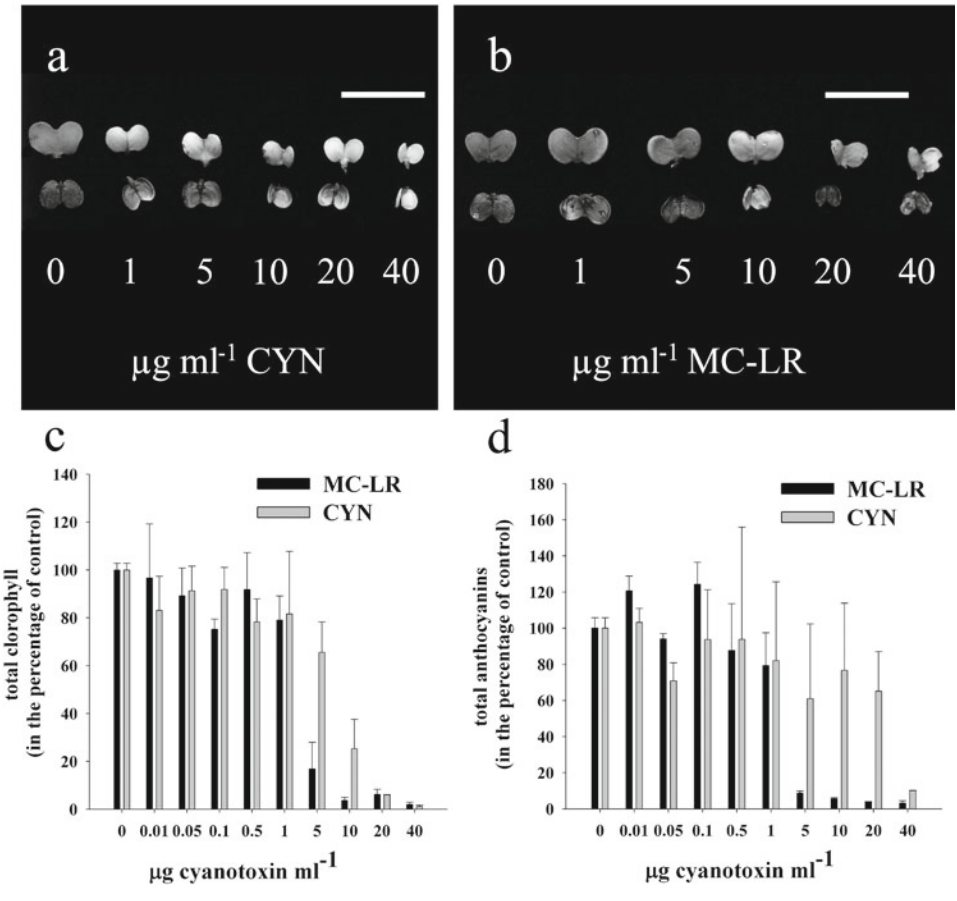

Fig. 3. The effects of microcystin-LR (MC-LR) and cylindrospermopsin (CYN) on the photoregulated development of the cotyledons. a: cotyledons of CYN treated seedlings, b: cotyledons of MC-LR treated mustard seedlings (Bar: 15 mm), c: chlorophyll (a+b) and d: anthocyanin contents of cotyledons on the fourth day of cyanotoxin treatment

mustard seedlings as well. Chlorophyll and anthocyanin contents not change parallel in CYN treated plants. It is not surprizing, since Reiss et al. [49] found similar tendencies in herbicide exposed *S. alba* (L.) plants. Herbicide induced photooxidative destruction resulted in damaged chloroplasts where the expression of plastid genes seemed impossible, though the biogenesis of anthocyanins proceeded. It is believed that the increase of anthocyanin pool in plants is a well-recognized indicator of environmental stresses [10, 15]. Lack of phosphorous, nitrogen, sulfur, effect of UV-B, heavy metals, pathogen attack etc. induce anthocyanin accumulation [20, 21, 60].

Contrary to CYN, higher concentrations of MC-LR greatly reduced anthocyanin accumulation in mustard cotyledons [37 and this study, Fig. 3]. Therefore the kinetics and regulation of anthocyanin accumulation appears to be different in CYN and MC-LR exposed cotyledons. It is tempting to speculate that MC-LR as a specific inhibitor of protein phosphatases may inhibit the regulatory processes of anthocyanin synthesis and metabolism which are dependent on protein phosphorylation and dephosphorylation. Indeed, the anthocyanin synthesis is regulated by gene products (enzymes) and regulatory proteins [55], which directly or indirectly are subjects of phosphorylation and dephosphorylation [2, 52]. In most cases, the regulatory genes encoding transcriptional factors to control the expression of anthocyanin biosynthetic enzymes, like MYC-, MYB proteins, phenylalanine ammonia lyase (PAL), chalcone synthetase etc. are phosphorylated and dephosphorylated [3, 19, 22, 52].

In an earlier study our laboratory provided evidence for the necrosis of etiolated cotyledon tissues of MC-LR treated mustard seedlings, a characteristic consequence of MC-LR exposure [37]. Necrotic patches on the cotyledons, on the hypocotyl and mainroot, especially at the transition zone appeared on the 2nd day of treatment and became day by day more characteristic (Fig. 1c). We obtained that CYN treatment of mustard seedlings resulted in a dose dependent reduction of growth but tissue necrosis did not occur (Fig. 1a, d and Fig. 3a). McElhiney et al. [33] observed necrosis in MC-LR treated potato shoot cultures, runner bean leaves and roots. Plant tissue necrosis might be induced by biotic and abiotic stresses such as anoxia, phytotoxins or plant pathogens [4]. However, tissue necrosis which was always characteristic in MC-LR exposed mustard seedlings did not occur in the presence of CYN (Fig. 1d), suggesting a basically different mode of action.

Peroxidase enzyme activities of CYN and MC-LR treated Sinapis alba *L. seedlings*

The induction of oxidative stress by microcystins is known in vascular plants [11, 45, 46, 53, 63]. Plants have a number of different defence mechanisms by which they can respond to oxidative stress. These include the production of both non-enzymatic and enzymatic antioxidants such as ascorbate and glutathione and enzymatic antioxidants such as catalase, superoxide dismutase and POD. MC-LR caused significant increase in POD activity in a concentration dependent manner (Fig. 4), which confirmed our earlier observations [41]. CYN increased POD activity only at 0.05 μg ml^{-1}, but this

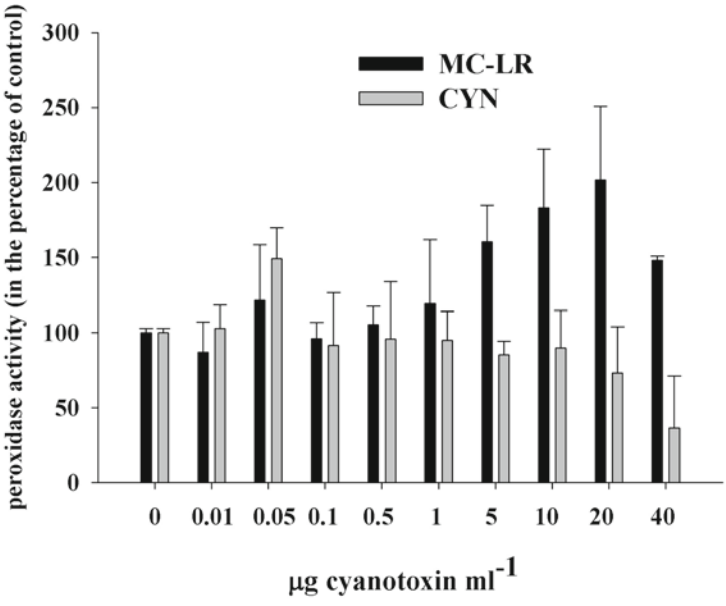

Fig. 4. The effects of cyanotoxins on the pirogallol peroxidase activity of mustard seedlings (mM oxidized pyrogallol min^{-1} mg^{-1} protein)

effect seemed to be a transient one. At 0.1–20 μg ml^{-1} CYN concentration regimes there were no significant differences between POD activities of control and CYN treated seedlings. Higher than 20 μg ml^{-1} cyanotoxin concentration caused a decrease in POD activity.

Pflugmacher et al. [44] established that glutathione S-transferase is a sensitive measure of plant stress caused by MC on aquatic plants. Mitrovic et al. [38] measured significant increase in soluble glutathione S-transferase-, catalase- and POD activities in *Lemna minor* (L.) treated with anatoxin-a a neurotoxin produced by cyanobacteria. Significant elevation of soluble glutathione S-transferase activity after 24 h cyanotoxin exposure indicated the start of the biotransformation in the plants, caused formation of reactive oxygen species in the cells, which would be detoxified during peroxidase and catalase reactions.

POD, glutathione S-transferase and catalase activity can be used as a sensitive measure of stress [38], and POD is one of the principle enzymes oxidizing xenobiotics in plants with wide spectrum of substrate specifity. When these defences fail to protect the plant from reactive oxygen species, cell death will be the result. For example plants that were exposed to stresses such as ozone or UV-B irradiation, each resulting in increased levels of reactive oxygen species, caused premature senescence followed by necrotic cell death [42].

CONCLUSIONS

This work is the first comparative study of the growth inhibitory and morphological effects of two well-known but structurally unrelated cyanobacterial toxins on a crop plant. The four days long exposure of mustard seedlings to 0.01–40.0 μg ml^{-1} cylindrospermopsin and microcystin-LR induced the inhibition of cotyledon-, hypocotyl- and root growth and characteristic developmental alterations. There were differences in the effects of cyanotoxins, e.g. we obtained changes in the 50% inhibitory concentrations, stimulation of hypocotyl-, root growth and anthocyanin content at low MC-LR concentrations (0.01–0.1 μg ml^{-1}), but a strong inhibition of these at ≥1 μg ml^{-1} MC-LR concentrations, which otherwise is not characteristic for CYN. The CYN-treated cotyledons were chlorotic but violet coloured in consequence of high anthocyanin content. The MC-LR caused chlorosis was accompanied by an appearance of necrotic patches. It is believed, necrosis and increase of peroxidase enzyme activity (POD) are general stress responses but these alterations were characteristic only for MC-LR treated mustard plants. Some other general stress responses like growth inhibition and decrease in chlorophyll content were induced by both cyanotoxins. There is nothing unusual, since it was shown that both protein phosphorylation/dephosphorylation and the synthesis of certain regulatory proteins are involved in the regulation of biotic and abiotic stress induced defence mechanisms in plants [29, 54].

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