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Reduction in germination rate and elevation of peroxidase activity in *Zea mays* seedlings due to exposure to different microcystin analogues and toxic cell free cyanobacterial crude extract

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Summary

Agricultural crop plants may come into contact with cyanobacterial toxins *via* spray irrigation with water contaminated with cyanobacteria/ cyanobacterial toxins. Many of the bloom forming cyanobacteria are known to produce toxins amongst those the group of the microcystins, cyclic heptapeptides are the best known once. In this study the germination of *Zea mays* under exposure to different microcystins and cell free cyanobacterial crude extract containing microcystin-LR was investigated. The concentration used for all microcystins in this study was 5.0 µg L^{-1} which is well in the environmental range. The inhibition of germination was shown as well as the inhibition of root and shoot length by toxin exposure. As a sign for the generation of oxidative stress promoted by the toxins taken up, guajacol peroxidase was measured showing in most toxin exposures an elevation of peroxidase activity. This study showed that there is a potential concern a reduction in crop yield and also to human health if agriculturally important crop plants were exposed to cyanobacterial toxins *via* spray irrigation.

Introduction

Modern agriculture is dependent on spray irrigation of the crops to ensure the crop yield necessary. In many countries, especially in semi-arid and arid regions this water is taken from water bodies in which bloom-forming cyanobacteria might be present. From many of these cyanobacteria it is well known that they can produce a variety of different secondary metabolites, so called cyanobacterial toxins (cyanotoxins) and it was estimated that 25 - 75 % of these cyanobacterial blooms are toxic (LAWTON and CODD, 1991). Under these toxins, hepatotoxins, neurotoxins and cytotoxins can be found. The microcystins, cyclic heptapeptides of five relatively invariant D-amino acids and two most variable L-amino acids, are the most well known group from which about 60 analogues were known (SIVONEN and JONES, 1999). Spray irrigation with water contaminated with cyanobacteria respectively their toxins showed to have negative effects on terrestrial plants. In most cases this studies were performed in hydroponic culture using the most common hepatotoxin, microcystin-LR. Effects monitored were decrease in root length (KOS et al., 1995; MCELHINEY et al., 2001; KURKI-HELASMO and MERILUOTO, 1998), inhibition of photosynthesis (ABE et al., 1996), inhibition of sucrose transport (SIEGL et al., 1990), inhibition of protein phosphatases (MCKINTOSH et al., 1990), indicating the uptake of toxin *via* the root system. A previous study showed the uptake of two microcystins (MC-LR and MC-LF) in different agricultural important crop plants, amongst those corn (*Zea mays*) by PEUTHERT et al. (in press) giving toxin values in roots of *Z. mays* of 12.7 (MC-LR), 14.5 (MC-LF) and shoots of *Z. mays* of 26.5 (MC-LR), 18.0 (MC-LF) after 24 h exposure time.

From cyanobacterial toxins it is known that they can generate oxidative stress after being taken up by plant cells (GEHRINGER et al., 2003; PFLUGMACHER, 2004; PFLUGMACHER et al., 2006). Oxidative stress is always generated from a rapid and transient production of high quantities of reactive oxygen species (ROS). Unless the production of ROS in plants is a natural phenomenon *via* the photosynthesis in the chloroplasts, excessive ROS formation could also be triggered by quite a number of external factors amongst those exposures to xenobiotics or cyanobacterial toxins (COSSU et al., 1997; PFLUGMACHER et al., 2006). Oxidative damage has been found in DNA, proteins, carbohydrates and lipids (CADENAS, 1995). To prevent oxidative damage from ROS a protective system has been evolved based on small molecular antioxidants such as glutathione (GSH), ascorbate or tocopherols and antioxidative enzymes such as superoxide dismutase, catalase, peroxidase, glutathione S-transferase and glutathione reductase.

Aim of this study was to examine the response of corn seedlings when exposed to different microcystin variants and furthermore to cell-free cyanobacterial crude extract containing microcystin-LR.

Materials and methods

Plant material and exposure experiments

Corn (*Zea mays* L. cv Badischer Gelber) seeds were purchased from Kiepenkerls (Norken, Germany). Seeds were soaked in running tap water overnight. The seeds were germinated non-aseptically on the surface of wet filter papers (Whatman No. 1, Roth, Karlsruhe, Germany) in the dark at 13 °C in plastic plates with 24 wells. In each well one seed was placed in exposure medium (1 ml). For each toxin treatment five plastic plates were used which are 100 seeds per treatment. Concentration of the different microcystins in the exposure medium was 5.0 µg L^{-1} and in the used cyanobacterial cell-free crude extract was equivalent to a microcystin-LR concentration of 5.0 µg L^{-1}.

Microcystin variant and cyanobacterial crude extract

The different microcystin analogues (Tab. 1) were purchased from Axxora (Lörach, Germany) and in case of MCHCyR was a gift from Prof. Dr. G.A. Codd (University of Dundee). The cyanobacterial bloom material (mainly *Microcystis aeruginosa* and *Aphanezomenon flos-aqua*) was collected in June 2000 from the shore of Lake Müggelsee, Berlin (Germany). To obtain the cell-free crude extract, 20 g dry weight of bloom material was suspended in 500 mL Milli-Q water and stirred on ice for 15 min. After ultrasonication on ice, centrifugation of the resulting slurry was done at 22,000 x g for 15 min. Supernatant was collected and stored on ice. The pellet was reprocessed in the same manner as described above five times and the extracts were combined thereafter. The extract was stored in the deep freezer (-80°C) before use. Toxin analysis of the crude extract revealed the presence of microcystin-LR (MC-LR) and not quantifiable traces of microcystin-RR (MC-RR). The extract was diluted to a final concentration of 5.0 µg L^{-1} MC-LR.

Determination of cyanobacterial secondary metabolites

Analyses were performed as described in (PFLUGMACHER et al., 2006) using a Waters HPLC system (Waters, Eschborn, Germany) with

Tab. 1: Microcystin variants used in this study and their structural differences concerning the amino acid composition and the molecular weight.

Analogue	Structure	Molecular weight	Reference
Microcystin-LR	Cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	994	Botes et al. (1985)
Microcystin-RR	Cyclo (-D-Ala-L-**Arg**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1037	Kusumi et al. (1987)
Microcystin-YR	Cyclo (-D-Ala-L-**Tyr**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1044	Botes et al. (1985)
Microcystin-LF	Cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Phe**-Adda-D-Glu-Mdha-)	985	Azevedo et al. (1994)
Microcystin-LA	Cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Ala**-Adda-D-Glu-Mdha-)	909	Botes et al. (1982)
Microcystin-LW	Cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Trp**-Adda-D-Glu-Mdha-)	1024	Lawton et al. (1994)
[D-Asp3, (*Z*)-Dhb7]microcystin-HtyR	Cyclo (-D-Ala-L-Hty-D-**Asp**-L-Arg-Adda-D-Glu-(**Z**)-**Dhb**-)	1044	Sano et al. (1998)

photodiode array detector (waters 2996) detection. Separation was carried out on a Symmetry 5 µm C18 column (3.9 x 150 mm). The mobile phase consisted of solvent A: Milli-Q water and solvent B: acetonitrile (Rathburn, Walkerburn, UK) both containing 0.1 % (v/v) trifluoracetic acid. Solvent B was linearly increased from 30 % to 45 % over 10 min at a flow rate of 1 mL min^{-1}. Column temperature was maintained at 40 °C and the injection volume was set to 80 µl.

Enzyme preparation

Enzyme preparation from *Z. mays* seedlings roots and shoots was carried out according to (Pflugmacher et al., 2006). Shoots and roots were cut of with a scalpel and were frozen in liquid nitrogen, ground to a powder with mortar and pestle and suspended in ice-cold 0.1 M sodium phosphate buffer pH 6.5 containing 14 mM dithioerythritol and 1 mM ethylenediaminetetraacetic acid. After removing cell debris at 5000 x *g*, the supernatant was centrifuged at 100,000 x *g* for 60 min to collect the microsomal fraction. The supernatant (soluble fraction) was precipitated twice with solid ammonium sulphate (0-30 % and 30-80 % saturation). The pellet from the last precipitation step was suspended in 20 mM sodium phosphate buffer (pH 7.0) and the resulting extract (soluble fraction) was desalted using NAP-10 columns (Amersham Pharmacia, Uppsala, Sweden).

Enzymatic measurements

Photometric determination of peroxidase (POD, E.C. 1.11.1.9) activity was done according to (Putter, 1965) using guajacol as a substrate. The assay contained 01.mM sodium phosphate buffer pH 6.0, 0.3 mM guajacol, 0.12 mM H_2O_2, 40 µL of protein extract to a total volume of 1200 µL and increase in absorption was measured at 436 nm. Enzyme activities are given in nkat mg^{-1} protein (SI unit: katal = conversion rate of one mol substrate per second). Protein content of the samples was determined according to (Bradford, 1976) using bovine serum albumin fraction V as calibration standard at 595 nm.

Analysis of Data

The statistical significance was calculated by one-way analysis of variance (ANOVA) followed by Newman-Keuls test, $p < 0.05$ (SPSS 9.0 for Windows, Chicago, USA).

Results

Seeds from *Z. mays* were exposed to different microcystin analogues in a concentration of 0.5 µg L-1 for 7 days. Tab. 2 showed the germination rate of *Z. mays* seeds. The highest reduction in germination rate was seen using the cell free cyanobacterial crude extract (90 %) and MC-LA (88 %). For all other microcystin variants the inhibition

Tab. 2: Germination rate of *Z. mays* seeds exposed to different microcystin variants.

treatment	germinated seeds (n = 100)	reduction in germination [%]
Control	100	0
MC-LR	68	32
MC-RR	93	17
MC-YR	73	27
MC-LF	74	26
MC-LA	12	88
MC-LW	89	11
MCHCyR	72	28
Crude extract (MC-LR)	10	90

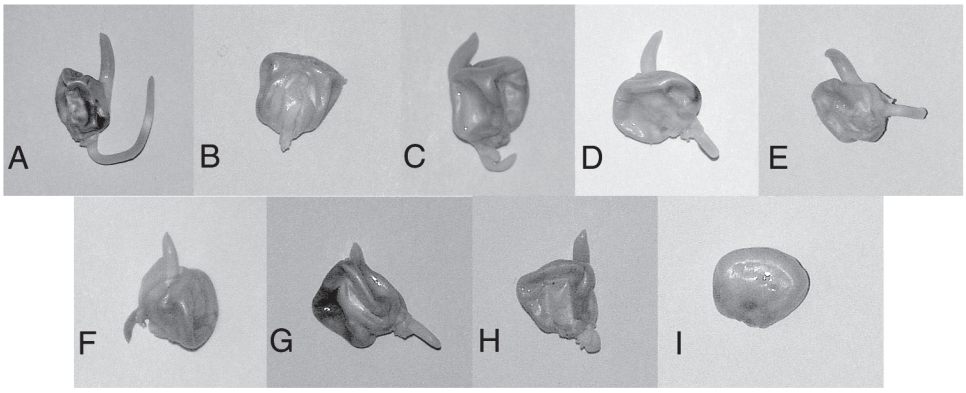

Fig. 1: *Z. mays* seeds exposed to different microcystin variants A) control, B) MC-LR, C) MC-RR, D) MC-YR, E)MC-LF, F) MC-LA, G) MC-LW, H) Htyr-MC, I) crude extract

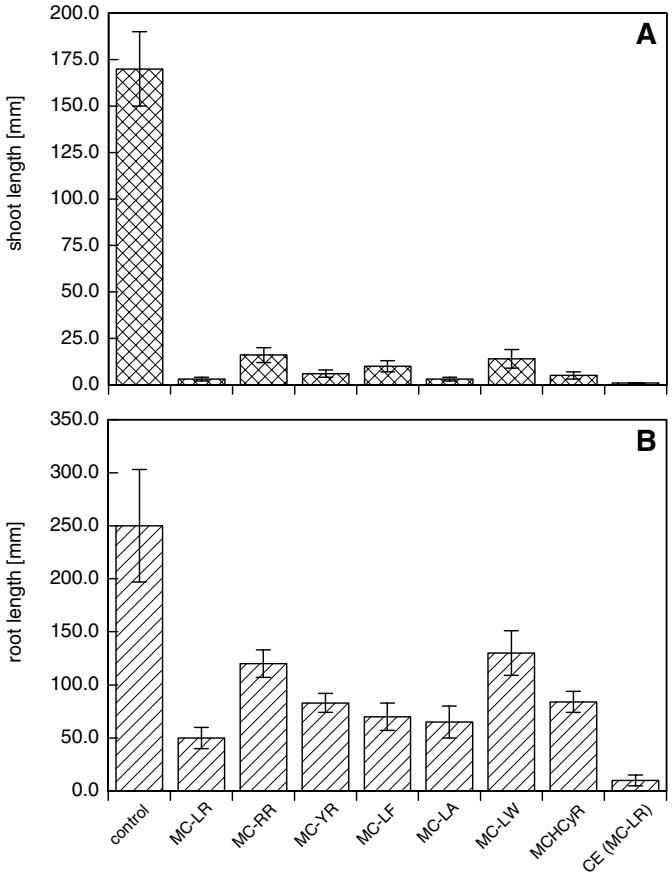

Fig. 2: Shoot length (A) and root length (B) of *Z. mays* seedlings after exposure to different microcystin variants.

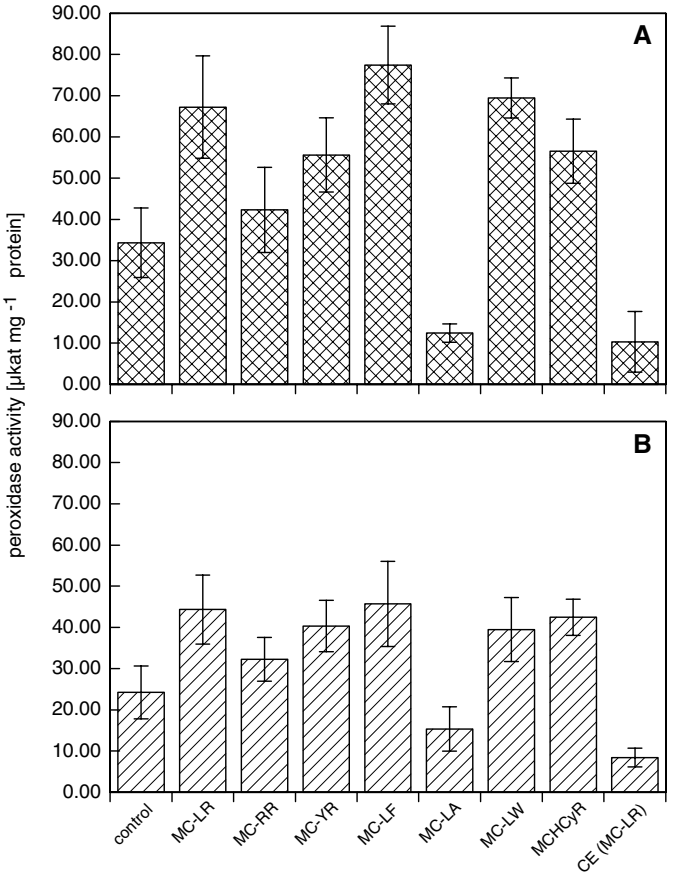

Fig. 3: Peroxidase activity in shoots (A) and roots (B) of *Z. mays* seedlings after exposure to different microcystin variants.

of germination was between 17 - 32 % compared to control seeds. The exposure to microcystins and to cell free cyanobacterial crude extract had significant overall effects on primary root length and shoot length (Fig. 1). The shoot length of the *Z. mays* seeds were significantly reduced in all exposures but highest reduction was detected in exposures using the MC-LR containing crude extract (Fig. 2A). A similar picture was obtained looking at the root length. Also here all exposures reduced the primary root length significantly from control up to a factor of 18 (crude extract containing MC-LR) compared to controls (Fig. 2B).

Activity of the POD in the shoots showed, that in most seeds exposed to microcystin analogues a significant elevation of enzyme activity was measured, which was highest in exposures using MC-LF, MC-LW or MC-LR. The elevation of POD activity was not significant in exposures using MC-RR. In two exposures (MC-LA and CE) the activity of the POD was decreased under control levels (Fig. 3A).

A similar activity pattern was measured in the roots with the exception of MC-RR exposures all POD activity were significantly different from control. In most cases an elevation was seen also in the shoots. In exposures using MC-LA and CE a decrease of POD activity was determined (Fig. 3B).

Discussion

During spray irrigation events, water contaminated with cyanobacterial toxins could be transferred to the field and the crop plants growing there.

In this experiment germination rate of the *Z. mays* seeds were inhibited by exposure to the different microcystin analogues. The highest inhibition was detected using the MC-LA one of the more lipophilic microcystin analogues and cyanobacterial cell-free crude extract containing MC-LR. Comparing the crude extract with the pure toxin a big difference in the potency to reduce germination is visible. Whereas the crude extract inhibits the germination more or less completely (90 %) the purified toxin gives only a reduction of 32 % from control. This led to the hypothesis that more bioactive compounds are present in the crude extract leading to such an effect as also suggested by PIETSCH et al. (2001).

Inhibition of germination by cyanobacterial toxins was also shown by METCALF et al. (2004) in pollen of *Nicotiana tabacum* exposed to cylindrospermopsin, another cyanobacterial toxin produced by cyanobacteria such as *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum* or *Rhaphidiopsis curvata*. OBERMEYER et al. (1998) correlated the inhibition of pollen tube growth in *Lilium longiorum* with the inhibition of protein phosphatases by okadaic acid. Also microcystins are known to inhibit the activity of protein phosphatases (MCKINTOSH et al., 1990) and therefore interfere with plant growth.

Previous work showed, that the growth and lateral root development of *Sinapis alba* was inhibited by exposure to the cyanobacterial toxin MC-LR in a concentration of 3.0 µg L^{-1}. A complete inhibition of root formation was reported by MCKINTOSH et al. (1990) in *S. alba* exposed to 5 mg L^{-1} MC-LR. Also in *Medicago sativa* a significant reduction in root growth was monitored when plants were exposed to two different microcystins (MC-LR, MC-LW) and to cyanobacterial crude extract containing MC-LR in a concentration of 5.0 µg L^{-1} (PFLUGMACHER et al., 2006). In this experiment the growth inhibition was more effective in the shoots of *Z. mays* than in the roots. Highest

inhibition was achieved using the cell free crude extract containing MC-LR again pointing out, that there might be more compounds included counting for the measured effect.

The generation was oxidative stress by cyanobacterial toxins was shown in *Lepidium sativum* and *Medicago sativa* yielding to an elevation of antioxidative enzymes such as glutathione peroxidase, catalase or glutathione S-transferase (GEHRINGER et al., 2003; PFLUGMACHER et al., 2006). Activity determination of the guajacol peroxidase in *Z. mays* root and shoots showed also an elevation of this enzyme by exposure to the different microcystin analogues. With MC-LA and the crude extract containing MC-LR a decrease of POD activity was measured. A possible explanations would be; an inhibition of the protein (enzyme) by an excessive amount of reactive oxygen species as known for protein phosphatases (HUBER, 2007).

These experiments have shown that *Z. mays* seedlings are sensitive to exposure of cyanobacterial toxins such as microcystins. The contact of *Z. mays* to these toxins might come via spray irrigation. After uptake of the toxin in the seeds germination, root and shoot development were negatively affected. This might lead to a decrease in crop yield in two ways: first by the reduced germination of the seeds and second by morphological damages leading to e. g. smaller plants. Because of the uptake of toxin in the plants (JÄRVENPÄÄ et al., 2007; PEUTHERT et al., in press) this could pose a possible risk for human consumption after such practices too.

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