

# Comparison of Sensitivity of Grasses (*Lolium perenne* L. and *Festuca rubra* L.) and Lettuce (*Lactuca sativa* L.) Exposed to Water Contaminated with Microcystins

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**Abstract** The effects of aqueous extracts from *Microcystis aeruginosa* strains (both microcystin-producers and non-microcystin producers) on germination and root growth were investigated for three economically important plant species: *Festuca rubra* L., *Lolium perenne* L., and *Lactuca sativa* L. There was a clear inhibition of root growth for *L. sativa* exposed to strains containing microcystins (5.9–56.4  $\mu\text{g L}^{-1}$ ). The strain that produced the most pronounced effects contained the lowest concentration of microcystin suggesting that other cellular compounds may also affect growth.

**Keywords** Microcystins · Germination inhibition · Grass · Lettuce

The demand for intensive agriculture from a rapidly increasing human population has lead to the eutrophication of aquatic ecosystems through the excess accumulation of nutrients, namely phosphorus and nitrogen. In many cases, these waterbodies are used for recreational activities as well as sources for potable water. Environmental

conditions such as increased temperature and radiation accompanied with low rates of vertical mixing of the water column and slow flow rates produce conditions conducive to the growth of many species of toxic cyanobacteria. These organisms can accumulate at the water surface forming scums that cause deoxygenation of the water column, changes in organoleptic characteristics, and the mass release of toxins. Among the most common hepatotoxic species found in the freshwater environment are *Microcystis*, *Anabaena*, *Nodularia*, *Oscillatoria*, *Nostoc* and *Cylindrospermopsis* (Carmichael 1992). The most common hepatotoxins found in freshwater lakes and rivers are the microcystins (MCs) and over 60 different chemical forms have been described (Sivonen and Jones 1999). These compounds have been implicated in several outbreaks of sickness and death in humans as well as the intoxication of domestic and wild animals (Kuiper-Goodman et al. 1999). The toxic effect of MCs is due to their inhibitory effects on protein phosphatases 1 and 2A (PP1 and PP2A) (Mackintosh et al. 1990). These enzymes are important for normal cell growth and metabolism. Although initial studies on the toxicity of MCs have focused on the effects of purified MCs on humans and other animals, it has also been suggested that these compounds might have much wider ecological impacts (Pflugmacher 2002).

Studies carried out by Mitrovic et al. (2004) showed that concentrations in the range from 10 to 15  $\mu\text{g MC-LR mL}^{-1}$  lead to reduced growth in *Lemna minor* L. and *Wolffia arrhiza* L. Kurki–Helasmo and Meriluoto (1998) showed that seed germination of *Sinapsis alba* L. was significantly reduced in the presence of MC-RR at a concentration of 5  $\mu\text{g mL}^{-1}$ . Higher concentrations, in the range of 20–40  $\mu\text{g mL}^{-1}$ , lead to a decrease in root growth. McElhiney et al. (2001) showed that photosynthesis in *Phaseolus*

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*vulgaris* L. was inhibited by three different MC variants at concentrations between 1.6 and 7.7  $\mu\text{g mL}^{-1}$ . Hamvas et al. (2003) reported that MC-LR caused reduced growth and productivity of *Sinapis alba* L. Tests carried out with the aquatic duckweed *Lemna minor* L. showed a reduction in total chlorophyll content as well as growth inhibition following exposure to MC-RR (Weiss et al. 2000). Pflugmacher (2002) reported that the growth rate of the macrophyte *Ceratophyllum demersum* L. was severely reduced in response to absorption of MC-LR. A decrease in the number and size of leaves of *Spirodela oligorrhiza* L. was found to result from exposure to MC (Romanowska-Duda and Tarczyska 2002). Exposure to MCs also been shown to cause reduced growth rates in some plant species cultivated for human consumption such as *Lactuca sativa* L. These compounds can accumulate in the tissues and be transferred along food chains (Codd et al. 1999).

To date, there are no conclusive studies demonstrating the role of cyanotoxins such as MC, although there have been some reports indicating that they provide chemical defence against herbivory and have allelopathic effects on other aquatic organisms. Pflugmacher (2002) showed that MCs display allelopathic effects against the aquatic macrophyte *C. demersum* L. causing growth inhibition and reduced photosynthetic oxygen production. Leblanc et al. (2005) found that MC-LR produced by *M. aeruginosa* did not inhibit growth of the macrophyte *Lemna gibba* L., however, the authors demonstrated a wide variation in responses to MC between different species of aquatic macrophytes. There seems to be a difference on species sensitivity to microcystins and there are suspicions that other compounds from cyanobacteria apart from the toxins may have negative effects on germination and growth of many plant species.

In this work we investigate the effects of cell extracts from MC-producing and non-MC-producing strains of *Microcystis aeruginosa* on germination and root growth of two grass species (*Festuca rubra* L. and *Lolium perenne* L.) and lettuce (*Lactuca sativa* L.).

## Materials and Methods

Cyanobacteria extracts were prepared using *Microcystis aeruginosa* strains isolated from several different lakes and reservoirs in Portugal (Table 1). Test solutions were prepared with 1 mg of lyophilised cyanobacteria in 10 mL of distilled water. The mixture was homogenized and ultrasonicated on ice for 15 min to break the cell walls and facilitate the release of MCs. Extracts were then centrifuged at 4,000g for 8 min to remove cell debris, before filtering (0.45  $\mu\text{m}$ ).

**Table 1** Total microcystin content (MC-LR eq g DW<sup>-1</sup>) of the *M. aeruginosa* strains used in this study

Strains	MC-LR eq ( $\mu\text{g g DW}^{-1}$ )	MC variants
IZANCYA 4	ND	ND
IZANCYA 41	ND	ND
IZANCYA 38	59.4	U (100%)
IZANCYA 7	313.7	LR (100%)
IZANCYA 33	329.2	LR (100%)
IZANCYA 2	494.1	LR (95%), LA (5%)
IZANCYA 5	515.2	LR (83%), LA (14%), AR (3%)
IZANCYA 3	563.8	LR (92%), LA (1%), U (7%)

U unknown MC variant, ND not detected (data from Vasconcelos et al. 1995)

Germination and root growth assays were carried out using seeds from two species of grass (*Festuca rubra* L. and *Lolium perenne* L.) and lettuce (*Lactuca sativa* L.). The two grass species included in the study are commonly used in lawns both on golf courses and in recreational areas. Lettuce represents an important agricultural species. Firstly, seeds were washed in a 10% bleach solution for 20 min to prevent fungal growth during the incubation, and then rinsed with ultrapure water. The seeds were placed in plastic Petri dishes containing absorbent paper disks. Ten seeds were added to each Petri dish and the paper disks moistened with either 2 mL of the cyanobacteria extract solution or 2 mL of water (for control samples). Petri dishes were then sealed with Parafilm® to prevent evaporation. Five replicate Petri dishes were used for each of the *Microcystis* treatments and the control. A total of eight *Microcystis* strains were included in the study (Table 1). Petri dishes containing lettuce seeds were placed in the dark, which is a requirement for the germination of this species. Petri dishes containing grass seeds were positioned under white fluorescent light at an intensity of approximately 10  $\mu\text{m m}^{-2} \text{s}^{-2}$  and photoperiod of 14 h, at a temperature of  $24 \pm 1^\circ\text{C}$ . The number of germinated seeds in each Petri dish was recorded after 5 days. Germination was considered to have occurred when the root had a length of at least 2 mm. Root length was recorded for each specimen.

Detection and quantification of MCs in the pure extracts was carried out using the ELISA assay (EnviroGard® Microcystin Plate Kit), with a detection limit of 0.1 ng mL<sup>-1</sup>. For each of the *Microcystis* strains, 5 mg of freeze-dried material was sonicated in 2 mL of ultrapure water, and then frozen and thawed twice to facilitate the release of MCs into solution. Solutions were then filtered (0.45  $\mu\text{m}$ ) and diluted if necessary.

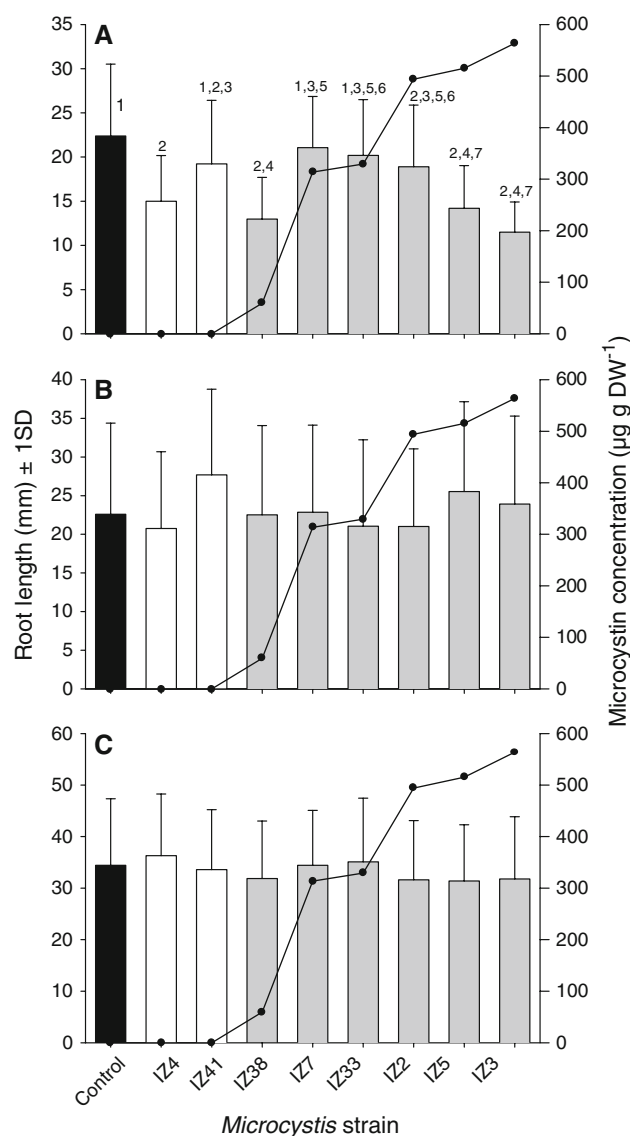
The results obtained for root length and germination in each of the treatments were compared statistically using ANOVA and the Tukey test for comparison of means (Statistica version 6.0). Correlations between MC concentration and root inhibition used only data from those strains that had the MC profile characterized (all but IZ38) with Statistica version 6.0.

## Results and Discussion

Exposure of *L. sativa* L. seeds to extracts of MC-producing and non-MC-producing strains of *M. aeruginosa* for 5 days did not have any observable effects on germination ( $F = 1.741$ ,  $df = 8$ ,  $p = 0.118$ ). The results obtained for the root length of *L. sativa* L. following exposure to extracts of *M. aeruginosa* are shown in Fig. 1. The *M. aeruginosa* treatments resulted in reduced root growth compared with the control samples ( $F = 20.938$ ,  $df = 8$ ,  $p = 0.00$ ). The Tukeys test for comparison of means also showed that there were significant differences between strains that produced MC and others that did not (Fig. 1a).

For the grass species *F. rubra* L., there was much lower variability in terms of differences in the inhibition of germination and root growth between the different strains of *M. aeruginosa*. No significant difference was found in the germination rates in the eight treatments ( $F = 1.212$ ,  $df = 8$ ,  $p = 0.316$ ) compared with the control samples, suggesting an absence of acute toxic effects. In addition, there were no significant differences obtained for the root length measurements for MC-producing and non-MC-producing strains ( $F = 1.626365$ ,  $df = 8$ ,  $p = 0.115584$ ) (Fig. 1b). For the other grass species, *Lolium perenne* L., no significant differences were found between MC-producing and non-MC-producing strains with respect to the effects on germination ( $F = 0.672$ ,  $df = 8$ ,  $p = 0.713$ ). Neither was root growth inhibited by any of the treatments ( $F = 1.048$ ,  $df = 8$ ,  $p = 0.399$ ) (Fig. 1c).

A clear correlation was found between the root growth inhibition of *L. sativa* L. and MC concentration ( $r^2 = 0.87$ ) for the strains that produced identified variants of MC (Table 2). However, even the two strains of *M. aeruginosa* lacking detectable concentrations of MC caused a reduction in root growth of between 15–30% compared with the control samples. *M. aeruginosa* strain IZ38 which contained a relatively low toxin content (Table 1) had a significant effect on root growth, resulting in more than 40% reduction in root length compared with the control samples. Exposure of *L. sativa* L. to strains of *M. aeruginosa* resulted in an average root length reduction of 25.7% relative to the control treatment. For *Lolium perenne* L. the average reduction was only 3.4%. *F. rubra* L. actually showed an increase in root growth of 2.5% relative to the



**Fig. 1** Root length of *Lactuca sativa* L. (a), *Festuca rubra* L. (b) and *Lolium perenne* L. (c) following a 5-days exposure to extracts of MC-producing and non-MC-producing strains of *M. aeruginosa* (open bars – Non-MC-producing strains). Line represents MC concentration. Bars with the same number are not significantly different

**Table 2** Maximum inhibition of root growth (%) and correlation coefficients between MC concentration ( $\mu\text{g/L}$ ) and inhibition of root growth (%) of the three plant species used in the assay (*L. sativa*, *L. perene* and *F. rubra*)

Species	$r^2$	Maximum inhibition (%)
<i>L. sativa</i>	0.87	49.4
<i>F. rubra</i>	−0.48	8.6
<i>L. perene</i>	0.28	9.6

control treatment following exposure to *M. aeruginosa* extracts and a negative correlation with toxin concentration of −0.48 (Table 2).

Previous works have shown that MC can affect seed germination in some terrestrial plants. Kurki–Helasmo and Meriluoto (1998) showed that germination of mustard seeds (*Sinapsis alba* L.) is affected by exposure to MC-RR, with an  $IC_{50}$  of  $800 \mu\text{g L}^{-1}$ . Kós et al. (1995) obtained an  $IC_{50}$  of  $3,000 \mu\text{g L}^{-1}$  in assays using *S. alba* L. and unidentified MC variants. McElhiney et al. (2001) showed that the toxicity of MCs to *S. alba* L. may be dependent on the hydrophilicity of the toxins. MC-RR and -LR showed more pronounced toxic effects with  $IC_{50}$  values of 1,600 and  $1,900 \mu\text{g L}^{-1}$ , respectively, whereas for the more lipophylic MC-LF,  $IC_{50}$  values were three times higher. In this study, we found that ecologically relevant concentrations of MC ( $5.9$ – $56.4 \mu\text{g L}^{-1}$ ), much lower than those used in many other studies, did not affect seed germination of *L. sativa* L., *L. perenne* L. or *F. rubra* L.

The inhibition of the root development is a useful indicator of toxicity because it can have a direct effect on the plant survival and growth. The results obtained using extracts of *M. aeruginosa* cultivated in the laboratory showed that studies carried out using purified microcystins might underestimate the toxicity of natural blooms. Our results with *L. sativa* L. showed that values close to  $IC_{50}$  were obtained with a MC concentration of  $50$ – $60 \mu\text{g L}^{-1}$ . This value is  $10$ – $30$  times more toxic than the values presented by other authors to *S. alba* L. using purified MCs. The concentration of MC-LR used in this our study ( $5.9$ – $56.4 \mu\text{g L}^{-1}$ ) commonly occur during cyanobacterial blooms in the natural environment. Although high concentrations such those found in Australia ( $1,800 \mu\text{g L}^{-1}$ ) (Jones and Orr 1994) or Japan ( $1,300 \mu\text{g L}^{-1}$ ) (Ueno et al. 1996), may occur, lower concentrations such as those reported for Portugal ( $37 \mu\text{g L}^{-1}$ ) (Ueno et al. 1996) or the UK ( $131 \mu\text{g L}^{-1}$ ) (Codd et al. 1995) are more common. Accumulation of MC in the agricultural fields or lawns is unlikely because MC degrades rapidly with UV irradiation (Rositano and Nicholson 1994).

Microcystins may affect growth of terrestrial plant species but other substances may also be implicated in reduced growth rate when these plants are watered with cyanobacteria contaminated water.

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