Effects of microcystin-LR, cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture on growth, oxidative stress and mineral content in lettuce plants (*Lactuca sativa* L.)

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A B S T R A C T

Toxic cyanobacterial blooms are documented worldwide as an emerging environmental concern. Recent studies support the hypothesis that microcystin-LR (MC-LR) and cylindrospermopsin (CYN) produce toxic effects in crop plants. Lettuce (*Lactuca sativa* L.) is an important commercial leafy vegetable that supplies essential elements for human nutrition; thus, the study of its sensitivity to MC-LR, CYN and a MC-LR/CYN mixture is of major relevance. This study aimed to assess the effects of environmentally relevant concentrations (1, 10 and 100 μg/L) of MC-LR, CYN and a MC-LR/CYN mixture on growth, an-tioxidant defense system and mineral content in lettuce plants.

In almost all treatments, an increase in root fresh weight was obtained; however, the fresh weight of leaves was significantly decreased in plants exposed to 100 μg/L concentrations of each toxin and the toxin mixture. Overall, GST activity was significantly increased in roots, contrary to GPx activity, which decreased in roots and leaves. The mineral content in lettuce leaves changed due to its exposure to cyanotoxins; in general, the mineral content decreased with MC-LR and increased with CYN, and ap-parently these effects are time and concentration-dependent. The effects of the MC-LR/CYN mixture were almost always similar to the single cyanotoxins, although MC-LR seems to be more toxic than CYN. Our results suggest that lettuce plants in non-early stages of development are able to cope with lower concentrations of MC-LR, CYN and the MC-LR/CYN mixture; however, higher concentrations (100 μg/L) can affect both lettuce yield and nutritional quality.

Keywords: Microcystin-LR Cylindrospermopsin GST; GPx; Minerals; *Lactuca sativa*

Introduction

Toxic cyanobacterial blooms have become increasingly widespread in aquatic ecosystems, potentially as a consequence of eutrophication and climate change (Elliott, 2012; O'Neil et al., 2012). Among cyanobacteria, *Microcystis* is recognized as the most common bloom forming genus, and microcystin-LR (MC-LR), primarily produced by *Microcystis aeruginosa*, is the predominant variant of microcystins (MCs). Nevertheless, the tricyclic alkaloid cylindrospermopsin (CYN) has been recognized to be of increased concern due to the invasive nature of its main producer, *Cylindrospermopsis raciborskii* (Kinnear, 2010; Poniedziałek et al., 2012). The use of irrigation water containing toxic cyanobacterial blooms can be hazardous to the agricultural sector because several studies have reported that cyanotoxins negatively impact the yield, quality and safety of crop plants. The primary mechanism of the toxicity of MC-LR in both animals and higher plants is well recognized and consists of the irreversible inhibition of serine/threonine protein phosphatases 1 and 2A (PP, PP1 and PP2A) by covalent binding (Mackintosh et al., 1990). Potentially associated with this mechanism, several studies have shown that MCs, including MC-LR,

Abbreviations:
AAS, atomic absorption spectroscopy;
APX, ascorbate peroxidase;
BSA, bovine serum albumin;
CAT, catalase;
CYN, cylindrospermopsin;
GSH, glu-tathione;
GPx, glutathione peroxidase;
GST, glutathione-S-transferase;
ICP-MS, inductively coupled plasma-mass spectrometry;
MeOH, methanol;
MC-LR, micro-cystin-LR;
MCs, microcystins;
PDA, photoelectric diode array;
PP, protein phos-phatases;
PPO, polyphenoloxidase;
POD, peroxidase;
ROS, reactive oxygen species;
SPE, solid phase extraction;
SOD, superoxide dismutase;
TFA, trifluoroacetic acid

inhibit germination, decrease plant growth and crop yield and alter chlorophyll content and photosynthesis (Chen et al., 2004; El Khalloufi et al., 2011; Gehringer et al., 2003; McElhiney et al., 2001; Mitrovic et al., 2005; Pereira et al., 2009; Pflugmacher, 2002; Pflugmacher et al., 2006, 2007; Pietsch et al., 2001; Saqrane et al., 2009). The induction of oxidative stress by the production of reactive oxygen species (ROS) seems to be another important biochemical mechanism of MC-LR toxicity in plants. Several studies have been performed on the oxidative stress generated in plants due to MCs exposure, and changes in the antioxidant mechanisms (enzymatic and non-enzymatic components) have been reported (Corbel et al., 2014; El Khalloufi et al., 2013; Lahrouni et al., 2013; Pflugmacher et al., 1999, 2001, 2006, 2007; Saqrane et al., 2009). Among the antioxidant enzymes, glutathione-S-transferase (GST) has been successfully employed to assess the oxidative stress promoted by MC-LR in plants. This strategy was developed because the described pathway of MC-LR detoxification is by its conjugation with the tripeptide glutathione (GSH), catalyzed via GST (Pflugmacher et al., 1998, 2001). Nevertheless, Gehringer et al. (2003) and Stüven and Pflugmacher (2007) have obtained a significant increase in glutathione peroxidase (GPx) activity in seedlings of *Lepidium sativum* exposed to MC-LR either purified or from extracts, suggesting that GPx may play an important role to mitigate the negative effects of ROS generated by MC-LR in plants. However, if the antioxidant mechanisms are not efficient to scavenge the enhanced amount of ROS promoted by cyanotoxins, extensive cellular damage can occur, which may lead to potential negative effects on plant nutrient uptake and translocation. Minerals are essential to plant growth and development; they are intrinsic components in their structure and normal metabolism and function (Taiz and Zeiger, 2002). Interestingly, Saqrane et al. (2009) have reported that the exposure of *Triticum durum*, *Zea mays*, *Pisum sativum* and *Lens esculenta* plants to MC-containing extracts resulted in changes in the mineral content in roots in a concentration-dependent manner. More recently, El Khalloufi et al. (2012) and Lahrouni et al. (2013) have also demonstrated that cyanobacterial bloom extracts containing MCs induced changes in mineral assimilation and content in tomato (*Lycopersicon esculentum*) and faba bean (*Vicia faba*).

Although the effects of *CYN* in plants have been studied to a much lesser extent than MC-LR, this toxin is expected to become increasingly recurrent and thus enhancing the knowledge of its impact on crop plants is of critical importance. So far, the molecular mechanism of toxicity of CYN has not yet been established; however, CYN is known to inhibit protein synthesis with similar intensities in plant and mammalian cell extracts (Froscio et al., 2008). The few studies that have arisen regarding the effects of CYN on plants indicate that CYN results in the induction of oxidative stress (Prieto et al., 2011), the reduction of pollen germination (Metcalf et al., 2004) and the inhibition of growth (Beyer et al., 2009; Vasas et al., 2002). According to our knowledge, there are no studies reporting the effects of CYN in the mineral content of plants.

In the majority of the studies performed on the effects of cyanotoxins in plants, the concentrations of the cyanotoxins used did not take into account their ecological relevance (e.g., 1500–20,000 μg/L in Mitrovic et al. (2005) and 2220–22,240 μg/L in El Khalloufi et al. (2011, 2012)), and almost all plants were tested in early stages of development. Furthermore, in the aquatic environment, the simultaneous occurrence of different cyanotoxins can be highly expectable; inclusively, the co-occurrence of MC-LR and CYN has already been reported (Brient et al., 2008). In laboratory studies, synergistic effects have been suggested on the oxidative stress response (GST activity) of rice plants (*Oryza sativa*) exposed to cyanobacterial extracts containing CYN (0.13 μg/L) and MC-LR (50 μg/L) (Prieto et al., 2011). Thus, a study of the effects of the mixture of these two prevalent cyanotoxins (MC-LR and CYN) at environmentally relevant concentrations is of major significance to predict the potential impact of their interaction in crop plants.

Lettuce (Lactuca *sativa* L.) is a leafy vegetable widely used for human consumption due to its extensive production, convenience and nutritional value. Among other nutrients, lettuce provides an important source of minerals for the human diet (Pinto et al., 2014).

The aim of this study was to assess the effects of environmentally relevant concentrations (1, 10 and 100 μg/L) of MC-LR, CYN and a mixture of MC-LR and CYN on growth, antioxidant defense systems and mineral content in lettuce plants (*Lactuca sativa* L.) in non-early stages of development.

Materials and methods

Cyanobacterial culture and toxin purification and quantification

Culture of Microcystis aeruginosa and Cylindrospermopsis raciborskii

M. aeruginosa (LEGE 91094) and *C. raciborskii* (LEGE 97047) were grown to exponential phase in Z8 medium (Kotai, 1972) (6-L flasks) under fluorescent light with a light/dark cycle of 14/10 h and a temperature of 25 ± 1 °C. The cultured cells were gathered by centrifugation (20 min, 4 °C, 4495*g*), frozen at −80 °C and then freeze-dried. As CYN is highly hydrophilic, the culture medium of *C. raciborskii* was also freeze-dried. The lyophilized material was stored at room temperature in the dark until toxin extraction and purification. In this study, purified toxins were chosen for the experiments to find the specific effects of the MC-LR/CYN mixture, avoiding interferences of other potentially toxic metabolites (e.g., lipopolysaccharides) deriving from cyanobacterial crude extracts.

MC-LR extraction, purification and quantification by HPLC–PDA

MC-LR was extracted from *M. aeruginosa* cells according to Ramanan et al. (2000) with some modifications. Briefly, the lyophilized *M. aeruginosa* biomass was extracted with 75% (v/v) methanol (MeOH) (Fisher Scientific, UK) by continuous stirring for 20 min at room temperature. The sample was then ultrasonicated five times on ice at 60 Hz for 1 min (Vibra-Cell 50-sonics and Material Inc. Danbury, CT, USA). The homogenate was centrifuged at 10,000*g* for 15 min, and the resulting supernatant was collected and stored at 4 °C. The pellet was re-extracted with an equal volume of solvent, and the pooled supernatants were subjected to solid phase extraction (SPE) with a Water Sep-Pak® Vac 6-mL C18 cartridge preconditioned with 100% MeOH and distilled water at a flow rate of 1 mL/min. The loaded column was washed with 20% MeOH, and the MC-LR was then eluted using 80% MeOH. The MC-LR fraction was evaporated by rotary evaporation at 35 °C to remove the entire MeOH portion. The concentrated MC-LR was thereafter purified and quantified by a Waters Alliance e2695 HPLC system coupled with a photoelectric diode array (PDA) 2998. The MC-LR semi-preparative assay was performed using a reversed-phase column (Phenomenex Luna RP-18 (250 mm × 10 mm, 10 μm) maintained at 35 °C. The gradient elution was performed with MeOH and water, both acidified with 0.1% trifluoroacetic acid (TFA), with a flow rate of 2.5 mL/min. The injection volume was 500 μL. The peak purity and percentage of purified MC-LR were calculated at 214 and 238 nm. The fraction with purified MC-LR was then evaporated with nitrogen air for one day until all of the solvent was removed. Then, the residue was resuspended in distilled water. The chromatographic purity of MC-LR was 97%. The purified fractions of MC-LR were then quantified in the same HPLC system on a Merck Lichrospher RP-18

endcapped column (250 mm × 4.6 mm, 5 μm) equipped with a guard column (4 × 4 mm^2, 5 μm), both maintained at 45 °C. The PDA range was 210–400 nm with a fixed wavelength of 238 nm. The linear gradient elution consisted of (A) MeOH+0.1% TFA and (B) H_2O+0.1% TFA (55% A at 0 min, 65% A at 5 min, 80% A at 10 min, 100% A at 15 min, and 55% A at 15.1 and 20 min) with a flow rate of 0.9 mL/min. The injected volume was 20 μL. The MC-LR was identified by a comparison of its spectra and retention time with that of the MC-LR standard (batch number 018K1209, 10.025 μg/mL in MeOH, 98% purity, Cyano Biotech GmbH, Berlin, Germany). The system was calibrated using a set of seven dilutions of the MC-LR standard (0.5–20 μg/mL) in 50% MeOH. Each vial was injected in duplicate, and every HPLC runs series of 10 samples included a blank and two different standard concentrations. The Empower 2 Chromatography Data Software was used for calculating and reporting the peak information.The retention time of the MC-LR peak was 10.44 min (data from method validation not published).

CYN extraction, purification and quantification by HPLC–PDA

CYN was extracted from *C. raciborskii* cells and culture medium following a modified version of the method described by Welker et al. (2002). Briefly, the freeze-dried material (0.7 g) was first sonicated in a bath for 15 min in 5 mL of 0.1% (v/v) TFA (spectrophotometric grade) and then subjected to five cycles of ultrasonication with a Vibra-Cell at 60 Hz for 1 min. The homogenate was stirred for 1 h at room temperature and centrifuged (20,000*g*, 4 °C, 20 min). The supernatant was collected and the pellet was subjected to a second extraction. The supernatants were then pooled and stored at −20 °C. CYN was thereafter purified in the same HPLC–PDA system on a semi-preparative Gemini C18 column (250 mm × 10 mm, 5 μm) from Phenomenex that was maintained at 40 °C. The isocratic elution utilized a 5% MeOH solution containing 2 mM sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 2.5 mL/min and an injection volume of 1000 μL. Working solutions of CYN (0.08–5.0 μg/mL) were prepared in water. Standard CYN was supplied by Alexis (San Diego, CA, USA). The purified CYN fractions were then quantified in an HPLC system on an Atlantis® HILIC phase column (250 mm × 4.6 mm, 5 μm) from Waters maintained at 40 °C. The PDA range was 210–400 nm with a fixed wavelength of 262 nm. The isocratic elution was also a 5% MeOH solution containing 2 mM sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 0.9 mL/min and an injection volume of 10 μL. The system was calibrated using a set of seven dilutions of the CYN standard (25, 20, 10, 5, 2, 1 and 0.5 μg/mL) in ultrapure water. Each vial was injected in duplicate, and every HPLC run consisting of a series of 10 samples included a blank and two different standard concentrations. The chromatographic purity of CYN was 98%. The Empower 2 Chromatography Data Software was used for the calculation and reporting the peak information. The retention time of the CYN peak was 7.35 min (data from method validation not published).

Plant material and exposure to MC-LR, CYN and MC-LR/CYN mixture

The lettuce plants (*Lactuca sativa* L. var. 'Susybel') were purchased in a commercial soil substrate at four to five weeks' maturity. The roots were carefully washed with tap water until complete soil removal, and 20 lettuce plants were then transferred to the holes of plastic boards (PVC), which were placed on black glass trays (35 × 25 × 5 cm^3 deep). The roots were completely immersed in 3 L culture medium (adapted from Jensen and Malter (1995), (Table 1)), pH 6.5, which was continuously aerated. The lettuce plants were acclimated for one week with fluorescent white light (light/dark cycle of 14/10 h) and a temperature of 21 ± 1 °C. After acclimation, the culture medium was renewed and the purified toxins were diluted to the ecologically relevant concentrations of 1, 10, and 100 μg/L, in which the lettuce plants were exposed for five days. The concentration used in the mixture was 1, 10 and 100 μg/L for each toxin. After this time, 10 lettuce plants were harvested and the remnant ones were exposed again to the same conditions for five days further. Three independent trials were performed (exposure to MC-LR, CYN and a MC-LR/CYN mixture) in triplicate in a total of 12 trays for each assay (control group and the three concentrations of exposure). To monitor the stability of the cyanotoxins, samples of 500 μL were taken every day, during five days, and the analysis confirmed that MC-LR and CYN were stable throughout the experiments (data not shown).

Table 1
Detailed information of the composition (constituents and concentration) of culture medium used to expose lettuce plants to MC-LR, CYN and MC-LR/CYN in a closed hydroponic system. Adapted from Jensen and Malter (1995).

Chemical compound	Principal element	Concentration
Macronutrients (g/L)		
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Mg	0.5
Monopotassium phosphate (KH_2PO_4)	K, P	0.27
Potassium nitrate (KNO_3)	K, N	0.2
Potassium sulfate (K_2SO_4)	K	0.1
Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$)	N, Ca	0.5
Iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Fe	0.025
Ethylenediaminetetraacetic acid (EDTA)		0.03
Micronutrients (mg/L)		
Boric acid (H_3BO_3)	B	4.17
Manganous chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Mn	3.75
Cupric chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	Cu	0.21
Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	Mo	0.14
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Zn	0.66

Determination of plant growth

At the end of five *days* of exposure, 10 lettuce plants were randomly harvested and the roots and leaf tissues were separated. The fresh weight (fw) was determined, and tissues were then stored at −80 °C for further analysis. This procedure was repeated for the 10-remainder lettuce plants exposed for 10 days. Plant growth was expressed as the mean fresh weight (fw) ± standard deviation (SD) of $n = 10$.

Enzyme activity measurement

The measurements of soluble (cytosolic) GST and GPx activities were performed in the roots and leaves of two lettuce plants (in a total of six plants, $n = 3$). Tissues were ground in liquid nitrogen to a powder with a pestle and mortar and then homogenized in phosphate buffer, (100 mM) pH 6.5, in a ratio of 1.5 g/1 mL (tissue/buffer). The homogenates were centrifuged at 4495*g* for 20 min at 4 °C, and the supernatants were recovered. Aliquots were stored at −80 °C until further analysis. Protein content was determined according to the method of Bradford (1976), in which bovine serum albumin (BSA) was used as a standard. The sGST activity was determined according to the method of Habig et al. (1974), whereas GPx activity was determined according to the method of Lawrence and Burk (1976). GST and GPx activities were performed with 0.1 mg protein/mL, and the enzymatic activities were calculated according to Azevedo et al. (2014) and expressed in nkat/mg protein.

Determination of mineral content in lettuce leaves

Leaf tissues of three lettuce plants from the MC-LR, CYN and MC-LR/CYN experiments were analyzed regarding to their content of calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P), sodium (Na), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu) and molybdenum (Mo). The digestion and analysis of lettuce samples were performed according to Pinto et al. (2014). Freeze-dried leaves of lettuce plants were digested in an MLS 1200 Mega high performance microwave digestion (Milestone, Sorisole, Italy) unit. Samples were weighed into the PTFE vessels and 5 mL of HNO_3 (65% w/w, *Trace*SELECT® Ultra) and 2 mL of 30% H_2O_2 (30% v/v, *Trace*SELECT®Fluka) were added to each vessel. Afterwards, the mixture was submitted to the following microwave heating programs (power/time): 250 W/1 min, 0 W/2 min, 250 W/5 min, 400 W/5 min and 600 W/5 min. Sample solutions were then analyzed by a 3100 flame atomic absorption spectroscopy (AAS) instrument (Perkin Elmer, Überlingen, Germany) and by inductively coupled plasma-mass spectrometry (ICP-MS) (VG Elemental PlasmaQuad 3, Winsford, UK) for total metal content. For AAS analysis, multi-element calibration standards were prepared from 1000 mg/L single-element standard solutions (Sigma, MO) of Ca, Mg, K and Na. For ICP-MS analysis, calibration standards were prepared from AccuStandard® (New Haven, CT) 10 μg/mL multi-element ICP-MS standard solution (ICP-MS-200.8-CAL1-1). All solutions were prepared using ultrapure water (> 18.2 M Ω cm at 25 °C) obtained with a Milli-Q (Millipore, Billerica, MA) water purification system.

The ICP-MS instrument was equipped with a glass concentric nebulizer (Meinhard® Type A), a water-cooled glass spray chamber with impact beads, a standard quartz torch and nickel skimmer and sampling cones. For sample introduction, a Minipuls 3 (Gilson, Villiers le Bel, France) peristaltic pump was used. Argon of 99.9% purity (Alphagaz 2™, supplied by Air Liquide, Maia, Portugal) was used as the plasma source. The ICP-MS analysis was performed under the following conditions: argon flow rate (13 L/min); auxiliary argon flow rate (0.7 L/min); nebulizer flow rate (0.8 L/min); RF power (1350 W); scan regions dwell time (200 ms); and detection mode (pulse counting). The elemental isotopes (*m/z* ratios) ^{55}Mn, ^{56}Fe, ^{65}Cu, ^{66}Zn and ^{95}Mo were monitored for analytical determination and ^{45}Sc, ^{89}Y and ^{115}In were used as internal standards. The instrument was tuned daily for maximum signal sensitivity and stability using ^{115}In as the target isotope. Phosphorus was determined according to Murphy and Riley (1962). Results were expressed on a dry weight (dw) basis.

Statistical analysis

The statistical analysis of lettuce growth and enzymatic activity was performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The homogeneity of variance was checked with the Levene test, and when it was not observed, data were transformed to achieve this assumption. Statistical analysis of mineral content was conducted using the Mann–Whitney *U* test (IBM® SPSS® *Statistics version 21.0 for Mac OS X*). The significance level was set at $P < 0.05$.

Results and discussion

Effects of MC-LR, CYN and MC-LR/CYN mixture on lettuce growth

Overall, at morphological levels, the treatments applied in this study did not produce perceptible deleterious effects in lettuce plants (e.g., chlorosis or necrosis). The effects of MC-LR, CYN and MC-LR/CYN mixture on lettuce growth were studied by comparing the mean fresh weight of the control and treated plants (Fig. 1A). The exposure of lettuce plants to 1, 10 and 100 μg/L of MC-LR led to a significant increase in root fresh weight ($P < 0.05$). Interestingly, the fresh weight of lettuce leaves was also significantly increased ($P < 0.05$), after exposure to low concentrations (1 and 10 μg/L) of MC-LR. In contrast, the highest concentration of MC-LR (100 μg/L) produced a significant decrease ($P < 0.05$) in fresh weight of lettuce leaves after five days of exposure; however, this effect attenuated after 10 days of exposure. Unlike our results, lettuce root growth has been inhibited by extracts of *M. aeruginosa* containing MCs in concentrations ranging from 5.9 to 56.4 μg/L, following five days of exposure (Pereira et al., 2009). Furthermore, several

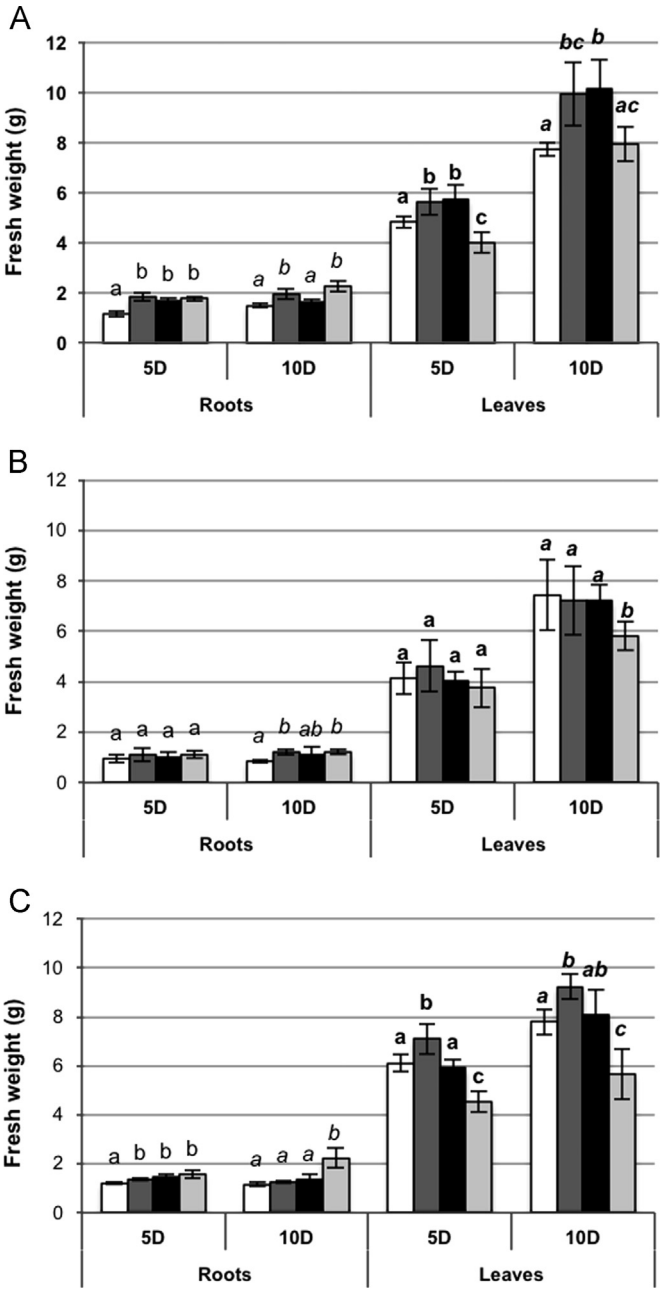

Fig. 1. The fresh weight of lettuce plants (roots and leaves) after being exposed 5 and 10 days (5D and 10D, respectively) to MC-LR (A), CYN (B) and a MC-LR/CYN mixture (C). Control: white bars; 1 μg/L: dark grey bars; 10 μg/L: black bars; 100 μg/L: light grey bars. Values are expressed as mean ± SD ($n = 10$). Different letters (a, b and c) means significant differences ($p < 0.05$).

studies have reported the growth inhibition of roots and leaves of plants as result of cyanobacterial extracts containing MCs exposure (Chen et al., 2004; El Khalloufi et al., 2011; Gehringer et al., 2003; McElhiney et al., 2001; Mitrovic et al., 2005; Pflugmacher, 2002; Pflugmacher et al., 2006, 2007; Saqrane et al., 2009). However, as evidenced by these studies, various factors can influence the toxicological effects. These include plant species, stage of plant development, the use of purified toxins or crude extracts, time of exposure and the range of concentrations studied. In most of these studies, the toxic effects of MC-LR were produced in plants in early stages of development and/or by high concentrations, approximately 100- to 1000-fold higher than those used in this study. The contradictory results obtained by Pereira et al. (2009) could be explained by the differences in the stage of development of lettuce plants, the parameter used to assess the growth (root length) and the use of crude extracts instead of purified toxin. Interestingly, in the latter study, the strain of *M. aeruginosa* that produced the most pronounced root growth inhibition contained the lowest concentration of MCs. This suggests that at ecologically relevant concentrations, other components of the extracts of *M. aeruginosa* may induce inhibitory effects on lettuce root growth, but these findings may not be a result of MC-LR by itself.

The effects of time of exposure were more evident in the CYN trial (Fig. 1B). The fresh weight of lettuce plants was only affected after 10 days of exposure, whereas the growth of roots was stimulated in all concentrations of exposure, and the leaf fresh weight decreased at 100 μg/L of CYN ($P < 0.05$). Similar results in leaf fresh weight of rice plants exposed nine days to extracts of *A. ovalisporum* containing 2.5 μg/L of CYN have been reported (Prieto et al., 2011). *Hydrilla verticillata* has been reported to exhibit an increase in root growth following 14 days of exposure to extracts of *C. raciborskii* containing 400 μg/L of CYN (Kinnear et al., 2008). According to Kinnear et al. (2008), several benefits could result from the increased root production, such as the potential production of exudates, allelochemicals or phytochelatins, which might scavenge CYN, preventing its uptake by plant cells. In all of the experiments in our study, we observed a gradual abundance of

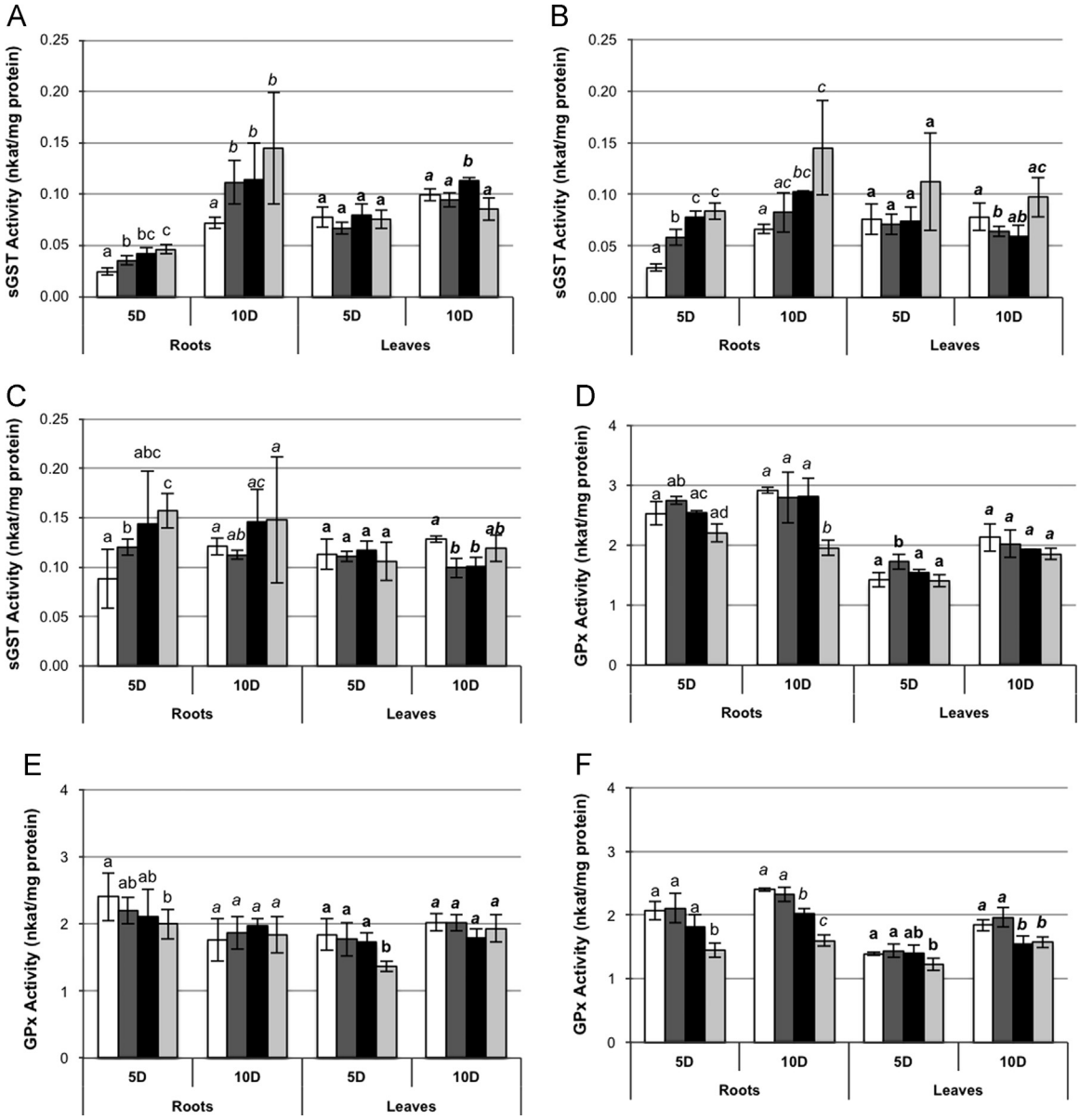

Fig. 2. GST (A), (B) and (C) and GPx (D), (E) and (F) activities in lettuce plants (roots and leaves) after being exposed 5 and 10 days to MC-LR, CYN and a MC-LR/CYN mixture, respectively. Control: white bars; 1 μg/L: dark grey bars; 10 μg/L: black bars; 100 μg/L: light grey bars. Values are expressed as mean ± SD ($n=3$). Different letters (a, b, c and d) means significant differences ($p < 0.05$).

exudates in the culture medium that were proportional to the toxin concentration (data not shown). Although whether these compounds are effective to protect lettuce plants to cyanotoxins is not known, their potential contribution should be studied further. However, the increased metabolic activity in roots required for growth, production of exudates and potential detoxification could be hypothesized to compromise the leaf water content and the growth of the lettuce plants exposed to 100 μg/L concentrations.

The exposure of lettuce plants to a MC-LR/CYN mixture promoted an increase in root growth in all concentrations after five days of exposure and at the highest concentration (100 μg/L) after 10 days of exposure (Fig. 1C). The fresh weight of leaves significantly increased with 1 μg/L and decreased with 100 μg/L of MC-LR/CYN after both five and 10 days of exposure ($P < 0.05$). Apparently, the MC-LR/CYN mixture produces similar effects of the single toxins, not enabling the assumption of additive, synergistic or antagonistic effects on the lettuce fresh weight. Prieto et al. (2011) have studied the effects of *A. ovalisporum* and *M. aeruginosa* cell extracts containing CYN and MCs, respectively, on rice plants and neither extracts nor its mixture produce significant changes in fresh and dry weight of roots and leaves after 48 h of exposure. In general, lettuce plants seem to be susceptible of homeostatic compensation with low concentrations of MC-LR, CYN and MC-LR/CYN (e.g., 1 μg/L and 10 μg/L); however, high concentrations (e.g., 100 μg/L) may cause adverse effects on leaf yield, which suggests that these cyanotoxins promote an hormetic response in lettuce. Nevertheless the potential interaction between the mechanisms of action of MC-LR (inhibition of serine/threonine PP) and CYN (inhibition of protein synthesis) should be further studied at cellular level.

Effects of MC-LR, CYN and MC-LR/CYN mixture on oxidative stress response

The phase II *detoxification* enzymes GST and GPx have been widely used as indicators of oxidative stress promoted by cyanotoxins in several plant species. In this study, the effects of MC-LR, CYN and a MC-LR/CYN mixture on the GST and GPx activities in roots and leaves of lettuce plants were also assessed (Fig. 2A-F). The GST activity was significantly increased ($P < 0.05$) in the roots of plants exposed to MC-LR and CYN (Fig. 2A and B), and this increase seems to be time- and concentration-dependent. The increase in the GST activity in plants due to MCs exposure was also reported by other studies, performed either with cyanobacterial extracts and purified MCs (Gehringer et al., 2003; Pflugmacher et al., 1999, 2001, 2007; Stüven and Pflugmacher, 2007). Likewise, the MC-LR/CYN mixture promoted a significant increase ($P < 0.05$) of GST activity in roots after five days of exposure, and the high activity obtained (≈ 0.15 nkat/mg) when compared to MC-LR or CYN alone led us to suppose that synergistic effects may have occurred (Fig. 2C). After 10days of exposure to the MC-LR/CYN mixture, the GST levels in roots remained high, although no significant differences in comparison to control groups were found. The GST activity in leaves was similar to the corresponding control groups and in some cases was found to be even lower. These results suggest that roots were more affected than leaves by the exposure to cyanotoxins, most likely due to the direct contact with the cyanotoxins and higher uptake/accumulation. However, this differentiated response could be related to a replacement in leaves by other antioxidant components of the defense system (enzymatic and/or non-enzymatic). Chloroplasts, where photosynthesis takes place, are the major cellular compartments of ROS production because of their photoactive nature (Gill and Tuteja, 2010). Lettuce leaves are rich in antioxidant compounds (e.g., carotenoids and phenolics) that play an important role to protect the photosynthetic apparatus against oxidative stress. Inclusively, the non-enzymatic antioxidants, such as phenolic compounds and α- and β-tocopherol, have been reported to be enhanced in plants after their exposure to MCs (El Khalloufi et al., 2012; Lahrouni et al., 2013; Stüven and Pflugmacher, 2007). This raises attention to the impact of cyanotoxins in the nutritional value of lettuce leaves because the content of antioxidants can be enhanced as a physiological mechanism of response.

According to our results, GST activity in the *roots* of lettuce plants showed that it was a good marker of stress induced by MC-LR, CYN and its mixture. Similarly, Prieto et al. (2011) have reported that the exposure of rice plants to a mixture of *A. ovalisporum* and *M. aeruginosa* cell extracts containing CYN and MC-LR, respectively, resulted in a significant increase in GST activity in roots, also suggesting that a synergistic effect of both extracts may exist; however, no changes in GPx activity have been observed in rice plants after 48 h of exposure.

In this study, the GPx activity was significantly decreased ($P < 0.05$) in the roots of lettuce plants exposed for 10 days to 100 μg/L of MC-LR, whereas it was significantly increased ($P < 0.05$) in leaves of plants exposed to 1 μg/L for five days (Fig. 2D). GPx activity decreased in both roots and leaves of lettuce plants exposed to 100 μg/L of CYN for five days (Fig. 2E). A similar pattern was found for the MC-LR/CYN trial, and in this experiment, the GPx activity significantly decreased ($P < 0.05$) in roots and leaves exposed for 10 days to 10 and 100 μg/L of the mixture (Fig. 2F). Although the GPx activity has been successfully used to assess the effects of MC-LR in *Lepidium sativum* (Gehringer et al., 2003; Stüven and Pflugmacher 2007), GPx may not be the best biomarker of these cyanotoxins in lettuce plants. The activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) increased in *M. sativa* seedlings after exposure to ecologically relevant concentrations of MCs (Pflugmacher et al., 2006). Recently, El Khalloufi et al. (2013) also reported that peroxidase (POD), polyphenoloxidase (PPO) and CAT activities were significantly increased in leaves, roots and nodules of *M. sativa* exposed to cyanobacterial extract containing MCs. The enzyme GPx scavenges peroxides, especially hydrogen peroxide. However, CAT and APX can also convert hydrogen peroxide to water (Gill and Tuteja, 2010); thus, these enzymes should be further studied as potential indicators of oxidative stress generated in lettuce plants due to the exposure to cyanotoxins.

Effects of MC-LR, CYN and MC-LR/CYN mixture on mineral content in lettuce leaves

The impact of cyanotoxins on mineral content in crop plants has been barely studied. In this work we explored the effects of ecologically relevant concentrations of MC-LR, CYN and a MC-LR/CYN mixture on the mineral content in lettuce leaves, the edible portion of this plant. The basal levels of the minerals analyzed in lettuce leaves in each experiment at the end of five and 10 days (control groups) are shown in Table 2. Overall, the exposure of lettuce plants to MC-LR, CYN and a MC-LR/CYN mixture resulted in significant changes in mineral contents in their leaves. The ratios of the mean concentration of minerals in lettuce leaves from treated groups *vs* control groups are shown in Table 3. The exposure of lettuce plants to MC-LR produced, in general, a decrease in the mineral content in leaves, and the effects were more pronounced at the highest time and concentration of exposure. Significant differences ($P < 0.05$) were observed for the macronutrients Ca, Mg, K and P after five days of exposure and for all macro and micronutrients (except for Na) after 10 days of exposure. Minerals, after being absorbed by the roots, are translocated to various parts of the plant where they are used in numerous biological functions (Taiz and Zeiger, 2002). In this study, in general, the antioxidant response to stress promoted by MC-LR

Table 2
Mineral concentration in lettuce leaves of the control groups (without cyanotoxin) at the end of 5 and 10 days (5D and 10D, respectively) of the three experiments. Values are expressed as mean ± SD ($n=3$).

Mineral concentration		Control group of MC-LR		Control group of CYN		Control group of MC-LR/CYN	
		5D	10D	5D	10D	5D	10D
(mg/Kg DW)	Ca	694.1 ± 1.2	721.7 ± 6.8	599.6 ± 34.8	524.9 ± 59.7	595.1 ± 42.3	532.1 ± 79.0
	Mg	236.1 ± 3.2	227.9 ± 11.1	207.4 ± 3.1	202.8 ± 26.5	238.21 ± 14.4	193.16 ± 31.6
	K	4257.4 ± 40.6	4868.9 ± 420.6	4500.4 ± 278.1	5626.6 ± 113.5	4795.1 ± 323.1	4535.7 ± 163.5
	P	447.4 ± 4.4	343.5 ± 20.6	544.2 ± 15.3	548.9 ± 20.9	494.2 ± 18.7	457.5 ± 17.2
	Na	55.3 ± 3.3	100.4 ± 13.2	115.7 ± 10.5	64.5 ± 2.4	184.1 ± 6.8	80.9 ± 2.4
	Mn	28.4 ± 2.3	39.5 ± 3.5	26.3 ± 1.6	14.1 ± 1.9	16.8 ± 2.2	13.6 ± 0.2
	Fe	9.4 ± 1.2	7.4 ± 0.4	8.6 ± 1.2	3.2 ± 0.4	3.0 ± 0.7	1.97 ± 0.6
	Zn	8.6 ± 0.8	7.9 ± 0.2	5.3 ± 0.3	2.0 ± 0.1	2.7 ± 0.3	2.1 ± 0.1
(μg/Kg DW)	Cu	1439.4 ± 114.7	1190.5 ± 65.5	1112.1 ± 89.2	447.4 ± 35.6	624.3 ± 40.8	413 ± 57.2
	Mo	275.6 ± 6.1	264.9 ± 12.4	267.9 ± 11.0	89.5 ± 7.9	73.9 ± 9.9	61.3 ± 2.0

Table 3
Ratio of mineral content in lettuce leaves exposed for 5 and 10 days (5D and 10D, respectively) to MC-LR, CYN and MC-LR/CYN mixture. Values express the ratio between the mean concentrations obtained in each condition by the mean concentration obtained in control group. Values are expressed as mean ± SD ($n=3$). Values in bold represent the concentrations that exceeded the screening value (control group, Table 2).

Mineral	Condition	MC-LR		CYN		MC-LR/CYN	
		5D	10D	5D	10D	5D	10D
Ca	1 μg/L	1.14 ± 0.16	1.03 ± 0.03	1.14 ± 0.10	1.03 ± 0.08	0.94 ± 0.09	1.19 ± 0.06
	10 μg/L	1.00 ± 0.09	0.92 ± 0.06*	1.28 ± 0.08*	1.08 ± 0.07	0.91 ± 0.05	1.14 ± 0.04
	100 μg/L	0.84 ± 0.12*	0.83 ± 0.03*	1.25 ± 0.02*	1.10 ± 0.03	1.06 ± 0.11	0.68 ± 0.02*
Mg	1 μg/L	0.93 ± 0.13	0.98 ± 0.01	1.18 ± 0.13	0.99 ± 0.05	0.95 ± 0.07	1.25 ± 0.06*
	10 μg/L	0.87 ± 0.09*	0.90 ± 0.04*	1.27 ± 0.09*	1.06 ± 0.09	0.84 ± 0.06	1.17 ± 0.11
	100 μg/L	0.83 ± 0.05*	0.82 ± 0.08*	1.23 ± 0.04*	1.04 ± 0.02	1.14 ± 0.11	1.04 ± 0.15
K	1 μg/L	1.16 ± 0.07*	0.97 ± 0.01	1.14 ± 0.06	0.96 ± 0.06	0.91 ± 0.01	0.95 ± 0.04
	10 μg/L	1.10 ± 0.07	0.99 ± 0.02	1.16 ± 0.10	0.93 ± 0.02	0.94 ± 0.03	1.01 ± 0.11
	100 μg/L	0.94 ± 0.07*	0.81 ± 0.06*	1.10 ± 0.13	0.95 ± 0.05	0.79 ± 0.07*	0.64 ± 0.11*
P	1 μg/L	0.95 ± 0.03*	1.18 ± 0.06	1.18 ± 0.04*	0.67 ± 0.01*	1.03 ± 0.07	1.05 ± 0.04
	10 μg/L	0.85 ± 0.05*	0.94 ± 0.12	1.23 ± 0.02*	0.69 ± 0.03*	0.97 ± 0.03	1.07 ± 0.09
	100 μg/L	0.86 ± 0.07*	0.74 ± 0.11*	1.17 ± 0.01*	0.60 ± 0.01*	1.16 ± 0.09*	0.91 ± 0.07
Na	1 μg/L	1.82 ± 0.01*	0.93 ± 0.01	0.85 ± 0.07	0.97 ± 0.13	0.74 ± 0.04*	2.01 ± 0.11*
	10 μg/L	2.29 ± 0.40*	0.82 ± 0.12	1.34 ± 0.04*	1.18 ± 0.16*	0.81 ± 0.04	1.75 ± 0.17*
	100 μg/L	1.14 ± 0.35	1.11 ± 0.06	1.31 ± 0.04*	1.21 ± 0.17*	1.16 ± 0.001	1.19 ± 0.04
Mn	1 μg/L	1.42 ± 0.20*	0.95 ± 0.04	1.41 ± 0.21*	1.77 ± 0.33	0.82 ± 0.12	1.04 ± 0.12
	10 μg/L	1.16 ± 0.07*	0.84 ± 0.09	1.78 ± 0.02*	2.77 ± 0.41*	0.71 ± 0.02	1.20 ± 0.15
	100 μg/L	1.00 ± 0.02	0.63 ± 0.04*	1.71 ± 0.01*	3.68 ± 0.29*	0.98 ± 0.14	0.54 ± 0.02*
Fe	1 μg/L	1.19 ± 0.13*	1.10 ± 0.02	1.15 ± 0.12	2.08 ± 0.08*	1.44 ± 0.01	2.02 ± 0.08*
	10 μg/L	0.94 ± 0.10	0.66 ± 0.03*	1.41 ± 0.11	3.20 ± 0.98*	0.74 ± 0.19	1.94 ± 0.17*
	100 μg/L	0.89 ± 0.03	0.68 ± 0.07*	1.14 ± 0.11	2.05 ± 0.25*	1.23 ± 0.21	1.35 ± 0.03
Zn	1 μg/L	0.99 ± 0.10	0.98 ± 0.003	1.13 ± 0.06	2.10 ± 0.09*	0.73 ± 0.07*	1.18 ± 0.12
	10 μg/L	1.03 ± 0.02	0.81 ± 0.07	1.11 ± 0.07	2.26 ± 0.17*	0.84 ± 0.10	1.00 ± 0.10
	100 μg/L	0.67 ± 0.06*	0.63 ± 0.03*	1.13 ± 0.07	2.14 ± 0.03*	1.05 ± 0.03	0.66 ± 0.07*
Cu	1 μg/L	0.94 ± 0.08	1.07 ± 0.02	1.51 ± 0.02*	1.79 ± ± 0.02*	1.03 ± 0.03	1.41 ± 0.14*
	10 μg/L	0.94 ± 0.02	0.88 ± 0.08	1.12 ± 0.13	2.01 ± 0.04*	1.02 ± 0.06	1.75 ± 0.03*
	100 μg/L	0.87 ± 0.08*	0.82 ± 0.05*	1.10 ± 0.01	1.96 ± 0.07*	1.22 ± 0.11	1.10 ± 0.04*
Mo	1 μg/L	1.48 ± 0.06*	1.01 ± 0.10	1.03 ± 0.07	1.88 ± 0.11*	1.51 ± 0.06*	0.91 ± 0.05
	10 μg/L	1.38 ± 0.03	0.89 ± 0.08	1.01 ± 0.07	2.24 ± 0.17*	0.78 ± 0.04	1.53 ± 0.02*
	100 μg/L	0.80 ± 0.02	0.73 ± 0.05*	0.94 ± 0.01	1.84 ± 0.06*	1.37 ± 0.10	0.37 ± 0.05*

* Significant differences between control and exposed groups ($p < 0.05$).

was more pronounced in roots than in leaves. Thus, the oxidative stress and cellular damage potentiated in roots may have considerably affected the uptake and/or translocation of nutrients and water to the edible tissues of lettuce plants. The mineral content in the roots of *T. durum*, *Z. mays*, *P. sativum* and *L. esculenta* (Ca, Na, K, P and N), as well as *L. esculentum* (Ca, Na, K), increased in a concentration-dependent manner after 30 days of exposure to *M. aeruginosa* extract containing MCs (500–4200 μg/L; 2220–22,240 μg/L, respectively) (El Khalloufi et al., 2012; Saqrane et al., 2009). However, similar to our results and the range of concentrations used in this study, the K and Ca content in the shoots of *V. faba* have been recently reported to decrease, whereas Na increased after two months of exposure to *M. aeruginosa* extract containing 50 and 100 μg/L of MCs (Lahrouni et al., 2013).

Contrary to the MC-LR, the exposure of lettuce plants to CYN produced an enhancement in leaf mineral content at almost all concentrations after five days of exposure. Moreover, after 10 days of exposure to CYN, the content of the minerals Mn, Fe, Zn, Cu and Mo in the leaves was significantly increased at all concentrations of exposure ($P < 0.05$). In the CYN experiment only P was significantly decreased at all concentrations after 10 days of exposure ($P < 0.05$). However, generally, crop plants need small amounts of micronutrients; thus the excessive increase of these elements in the leaves of lettuce plants exposed to CYN for 10 days could be an indication of some physiological disorders, the minerals can be hypothesized to have been used as a defense mechanism. Exploring the mechanisms underlying the enhancement of the uptake of mineral nutrients after five days of exposure to CYN is important. The lettuce plants were able to retain almost all of the minerals (especially the macronutrients) in a concentration higher than the corresponding control value, and this can be an indication of the potential tolerance of lettuce plants to CYN. However, studies on the effects of cyanotoxins in crop plants have been primarily focused on yield losses; yet, the physiological stress promoted by cyanotoxins seems to alter the chemical composition of plants and can therefore change its nutritional quality. The ability of crop plants to cope with abiotic stress and also maximize its nutritional quality is of major relevance for food security. The leaves of lettuce plants exposed to CYN demonstrate the ability to retain a higher content of minerals; however other quality parameters should be assessed, such as antioxidants, proteins, non-structural carbohydrates, lipids and sensory quality traits, which are particularly prone to be changed due to exposure to abiotic stress (Wang and Frei, 2011).

The mixture of MC-LR/CYN resulted in a general decrease of nutrient content after five days of exposure. However, this tendency was reverted with the increase in time of exposure, and after 10 days only the 100 μg/L concentration resulted in lower concentrations of the minerals in comparison to the correspondent control group. Once again, apparently, the MC-LR/CYN mixture did not produce additive, synergistic or antagonistic effects on mineral content in lettuce leaves.

Plants require Ca, Mg, K and P in relatively high amounts, and these nutrients are essential to ensure its life cycle. Although each mineral contributes to several metabolic reactions, some general functions in plant metabolism could be affected due to the disturbance of mineral uptake and translocation promoted by MC-LR, CYN and its mixture in lettuce plants. Mg supports other functions related to respiration and the synthesis of DNA and RNA and is also a central atom of the chlorophyll molecule; thus, it plays an important role in the light-dependent reactions of photosynthesis. Additionally, both K and P are crucial for respiration and photosynthesis. Mn also plays an important role in the structure of photosynthetic proteins and enzymes, as well as ATP synthesis, as it activates several enzymes involved in tricarboxylic acid metabolism. Likewise, Zn is required for many enzymes and also for chlorophyll biosynthesis (Taiz and Zeiger, 2002). The decline of these minerals in lettuce leaves as was found after MC-LR exposure, could compromise the chlorophyll content and photosynthetic rates; thus, the chronic exposure of lettuce to MC-LR could result in loss of productivity. Indeed, the photosynthesis impairment by MC-LR is well recognized and has already been reported by several authors (El Khalloufi et al., 2011; McElhiney et al., 2001; Pietsch et al., 2001; Pflugmacher, 2002; Saqrane et al., 2009). Although other biological functions could be affected by mineral imbalances, including antioxidant activity and cell growth (for example, Fe and Cu are associated with enzymes involved in redox reactions; and Ca and Na are involved in the synthesis of new cell walls, cell division and cell expansion (Taiz and Zeiger, 2002)), photosynthesis, which plays an important role as energy source, and thus crop yield, could be one of the most affected processes by mineral imbalance due to exposure to MC-LR and CYN.

Conclusions

In the present study, the effects of ecologically relevant concentrations of MC-LR, CYN and a MC-LR/CYN mixture were investigated regarding to growth, oxidative stress and mineral content in lettuce plants. This is of particular relevance, facing a tendency towards an increasing occurrence of toxic cyanobacterial blooms and the challenge of the intensification of agricultural productivity. Our results suggest that lettuce plants are able to cope with lower concentrations (1 and 10 μg/L) of MC-LR, CYN and MC-LR/CYN mixture by ensuring the maintenance and even increasing the fresh weight and mineral content and controlling oxidative stress, as was indicated by the significant increase of the GST activity in roots. Furthermore, the enhancement of the mineral content in the leaves of plants exposed to CYN for five days may provide an indication of the potential tolerance of lettuce plants to this cyanotoxin. However, the concentration and the time of exposure are preponderant factors for the toxic effects of these cyanotoxins in lettuce plants. The exposure of lettuce plants to 100 μg/L of cyanotoxins resulted in a significant decrease in fresh weight of leaves and its mineral content, especially for MC-LR and the MC-LR/CYN mixture, highlighting the potential implications of these toxins for lettuce yield and nutritional quality. It is however important to point out that, in this study, experiments were carried out in a free-soil system. In a soil–plant approach these effects would most likely be attenuated by the potential reduction of the availability of these cyanotoxins due to biodegradation and especially adsorption onto sediments. Further studies should be developed with lettuce growing in soil substrate to better understand the implications of the utilization of water contaminated with MC-LR and CYN in agricultural production. Furthermore, the potential tolerance of lettuce plants to CYN raises the possibility of its accumulation in edible tissues, and this issue should also be further studied to avoid risks in human health.

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