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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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ABSTRACT

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, antioxidant 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 apparently 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.

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1. 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, glutathione; GPx, glutathione peroxidase; GST, glutathione-S-transferase; ICP-MS, inductively coupled plasma-mass spectrometry; MeOH, methanol; MC-LR, microcystin-LR; MCs, microcystins; PDA, photoelectric diode array; PP, protein phosphatases; PPO, polyphenoloxidase; POD, peroxidase; ROS, reactive oxygen species; SPE, solid phase extraction; SOD, superoxide dismutase; TFA, trifluoroacetic acid

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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.

2. Materials and methods

2.1. Cyanobacterial culture and toxin purification and quantification

2.1.1. 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.

2.1.2. 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

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