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Lettuce (*Lactuca sativa* L.) leaf-proteome profiles after exposure to cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture: A concentration-dependent response

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ABSTRACT

The intensification of agricultural productivity is an important challenge worldwide. However, environmental stressors can provide challenges to this intensification. The progressive occurrence of the cyanotoxins cylindrospermopsin (CYN) and microcystin-LR (MC-LR) as a potential consequence of eutrophication and climate change is of increasing concern in the agricultural sector because it has been reported that these cyanotoxins exert harmful effects in crop plants. A proteomic-based approach has been shown to be a suitable tool for the detection and identification of the primary responses of organisms exposed to cyanotoxins. The aim of this study was to compare the leaf-proteome profiles of lettuce plants exposed to environmentally relevant concentrations of CYN and a MC-LR/CYN mixture. Lettuce plants were exposed to 1, 10, and 100 μg/l CYN and a MC-LR/CYN mixture for five days. The proteins of lettuce leaves were separated by two-dimensional electrophoresis (2-DE), and those that were differentially abundant were then identified by matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF/TOF MS). The biological functions of the proteins that were most represented in both experiments were photosynthesis and carbon metabolism and stress/defense response. Proteins involved in protein synthesis and signal transduction were also highly observed in the MC-LR/CYN experiment. Although distinct protein abundance patterns were observed in both experiments, the effects appear to be concentration-dependent, and the effects of the mixture were clearly stronger than those of CYN alone. The obtained results highlight the putative tolerance of lettuce to CYN at concentrations up to 100 μg/l. Furthermore, the combination of CYN with MC-LR at low concentrations (1 μg/l) stimulated a significant increase in the fresh weight (fr. wt) of lettuce leaves and at the proteomic level resulted in the increase in abundance of a high number of proteins. In contrast, many proteins exhibited a decrease in abundance or were absent in the gels of the simultaneous exposure to 10 and 100 μg/l MC-LR/CYN. In the latter, also a significant decrease in the fr. wt of lettuce leaves was obtained. These findings provide important insights into the molecular mechanisms of the lettuce response to CYN and MC-LR/CYN and may contribute to the identification of potential protein markers of exposure and proteins that may confer tolerance to CYN and MC-LR/CYN. Furthermore, because lettuce is an important crop worldwide, this study may improve our understanding of the potential impact of these cyanotoxins on its quality traits (e.g., presence of allergenic proteins).

Abbreviations: 2-DE, two-dimensional electrophoresis; APX, ascorbate peroxidase; CHAPS, 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate; CYN, cylindrospermopsin; EST, expressed sequence tag; FAS, fatty acid synthesis; GSH, glutathione; GST, glutathione-S-transferase; HPLC, high-performance liquid chromatography; HSP, heat shock protein; IPG, immobilized pH gradient; IEF, isoelectric focusing; LEA, embryogenesis abundant protein; MALDI-TOF/TOF MS, matrix-assisted laser desorption/ionization time of flight-mass spectrometry; MC-LR, microcystin-LR; MeOH, methanol; PCA, principal component analysis; PDA, photoelectric diode array; PP, protein phosphatases; PPIase, peptidyl-prolyl cis–trans isomerase; PR, pathogenesis-related; PRK, phosphoribulokinase; PS, photosystem; ROS, reactive oxygen species; RuBisCO, ribulose bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate carboxylase/oxygenase; SB, solubilization buffer; SBPase, sedoheptulose-1,7-bisphosphatase; SD, standard deviation; SOD, superoxide dismutase; TCA, tricarboxylic acid; TFA, trifluoroacetic acid.

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1. Introduction

The progressive occurrence and global expansion of harmful cyanobacteria blooms have been forecasted as consequences of eutrophication and climate change (Elliott, 2012; O'Neil et al., 2012). Among freshwater cyanobacteria, *Microcystis aeruginosa* is the most common bloom former (O'Neil et al., 2012). However, the invasive species *Cylindrospermopsis raciborskii* has shown a substantial widespread distribution, including into temperate zones (Kinnear, 2010; Poniedziałek et al., 2012). The use of irrigation water from sources that contain toxic cyanobacterial blooms of *C. raciborskii* and *M. aeruginosa* may pose a threat on the agricultural sector because their cyanotoxins (CYN and MC-LR, respectively) appear to generate phytotoxic effects on crop plants. CYN is a tricyclic alkaloid, and although the molecular mechanism of its toxicity has not yet been established, it is known that CYN inhibits eukaryotic protein synthesis with similar intensity in plant and mammalian cell extracts (Froscio et al., 2008). The few studies that have analyzed the toxic effects of CYN indicate that it results in the reduction of pollen germination (Metcalf et al., 2004), inhibition of plant growth (Vasas et al., 2002), induction of abnormal mitosis, alteration of microtubule organization, inhibition of root and shoot elongation (Beyer et al., 2009), and increase in oxidative stress (Prieto et al., 2011). MC-LR, the most studied structural variant of microcystins, is a cyclic heptapeptide that irreversibly inhibits, by covalent binding, serine/threonine protein phosphatases (PP; PP1 and PP2A), and this is the main mechanism of its toxicity in both animals and higher plants (Mackintosh et al., 1990). The induction of oxidative stress by the production of reactive oxygen species (ROS) appears to be another important biochemical mechanism of MC-LR toxicity that may cause serious oxidative damage (Pflugmacher, 2004; Stüven and Pflugmacher, 2007). The toxic effects of MC-LR on plants have also been characterized. It has been reported that MC-LR results in the inhibition of germination, growth and development (McElhiney et al., 2001; Pflugmacher, 2002; Gehringer et al., 2003; Chen et al., 2004; Mitrovic et al., 2005; Pflugmacher et al., 2006, 2007; El Khalloufi et al., 2011), alteration of microtubule organization (Máthé et al., 2009), and induction of changes in photosynthesis (Pietsch et al., 2001; Pflugmacher, 2002; El Khalloufi et al., 2011), chlorophyll content (McElhiney et al., 2001; Pflugmacher, 2002), and antioxidative response (Pflugmacher et al., 1999, 2001, 2006, 2007; Gehringer et al., 2003; Pflugmacher, 2004; Stüven and Pflugmacher, 2007; Saqrane et al., 2009; El Khalloufi et al., 2011; Pichardo and Pflugmacher, 2011). Nevertheless, the effects of these cyanotoxins on plants seem to vary depending on the (1) use of purified toxins or crude extracts, (2) the plant species, (3) the stage of plant development, (4) the time of exposure, and (5) the range of concentrations studied. Therefore, it is important to note the ecological relevancy of these studies because few have confirmed the effects at environmentally relevant concentrations (Gehringer et al., 2003; Pflugmacher, 2002; Pflugmacher et al., 2007; Pichardo and Pflugmacher, 2011). The concentrations required to exhibit effects in a wider range of species appear to be non-environmentally realistic because these are 10–1000-fold higher than those usually found in ecosystems (McElhiney et al., 2001; Mitrovic et al., 2005; Beyer et al., 2009; Saqrane et al., 2009). It has been reported that exposure concentrations of pure CYN below 100 μg/l appear to have no significant harmful effects on a wide range of species (e.g., floating macrophytes and green algae) (Kinnear, 2010), leading to the hypothesis that plants have developed appropriate protective mechanisms to tolerate CYN. Otherwise, it can be questioned whether the traditional endpoints used to assess toxicity exhibit sufficient sensitivity to evaluate understated biochemical alterations. Recently, Azevedo et al. (2014) reported the lack of sensitivity of the conventional parameters for the analysis of the toxicity of *M. aeruginosa* extract on rice (*Oryza sativa*) plants (MC-LR concentrations of 0.26–78 μg/l); however, significant alterations were observed through proteomic analyses. The inhibition of protein synthesis by CYN and the inhibition of PP1/PP2A activities by MC-LR appear to interfere with a wide range of molecular processes in plants (Máthé et al., 2013). Although the conventional biochemical biomarkers of stress induced by CYN and MC-LR (antioxidative enzymes and nonenzymatic substances) appear to be suitable, because proteins are the main targets of these cyanotoxins, it is particularly important to investigate how these operate in plant systems at the protein level. Proteomics is a field of growing interest in the agricultural sector because it has contributed to a better understanding of the specific functions of the proteins involved in plant responses to environmental stresses (Afroz et al., 2011; Kosová et al., 2011; Abreu et al., 2013). A proteomic approach may enable the identification of protein biomarkers of the plant stress response and the discovery of the biological processes underlying stress tolerance, which may be used to enhance agricultural productivity (Kosová et al., 2011; Abreu et al., 2013). Moreover, some secreted proteins with defensive or protective functions on stress factors are recognized to also have allergenic potential (Abreu et al., 2013). From the health risk point of view, proteomics data associated with allergen identification may provide new insights into the protein composition, quality, and safety of edible plants exposed to environmentally relevant concentrations of cyanotoxins. Nevertheless, in aquatic ecosystems, single species of cyanobacteria are almost never found; hence, the existence of mixtures of cyanotoxins in the water column is likely and it was already reported for MC-LR and CYN (Brient et al., 2008). Simultaneous exposure to CYN and MC-LR may lead to changes in the response capability of crop plants, triggering potential synergistic or antagonistic effects. Recently, Prieto et al. (2011) suggested a synergistic effect on the oxidative stress response of rice plants due to its exposure to cyanobacterial extracts containing low concentrations of both CYN (0.13 μg/l) and MC-LR (50 μg/l). Proteomics studies investigating the effects of CYN and MC-LR have been mainly performed on bivalves, including mixtures with other environmental pollutants (e.g., herbicides) (Martins et al., 2009; Puerto et al., 2011; Malécot et al., 2013). As above mentioned, more recently, Azevedo et al. (2014) successfully applied a proteomic approach to assess the early physiological and biochemical responses of rice seedlings to environmentally relevant concentrations of MC-LR.

This work aimed to use a 2-DE proteomic approach and MALDI-TOF/TOF MS to investigate the leaf-proteome profiles of lettuce (*Lactuca sativa* L.) plants exposed to environmentally relevant concentrations (1, 10, and 100 μg/l) of a CYN and MC-LR/CYN mixture.

2. Results and discussion

In the present study, well-defined differences were observed in the leaf-proteome profiles of lettuce plants exposed for five days to ecologically relevant concentrations (1, 10 and 100 μg/l) of CYN and MC-LR/CYN. The proteomics approach was found to be a suitable tool that is sufficiently sensitive to recognize changes in the plant physiological responses that are not perceptible at the morphological level. Overall, the treatments applied in this study did not affect lettuce plants at the morphological level, with the exception of the leaf fr. wt of plants exposed to 1 μg/l and 100 μg/l MC-LR/CYN, which was significantly higher and lower than that of the control group ($p < 0.05$), respectively (Fig. 1). Although there is

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