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).

Graphical abstract

Although the effects of the mixture were stronger than those of CYN alone, in both experiments they seem to have been concentration-dependent and proteins involved in photosynthesis/carbon metabolism and stress/defense response were the most differentially changed in abundance.
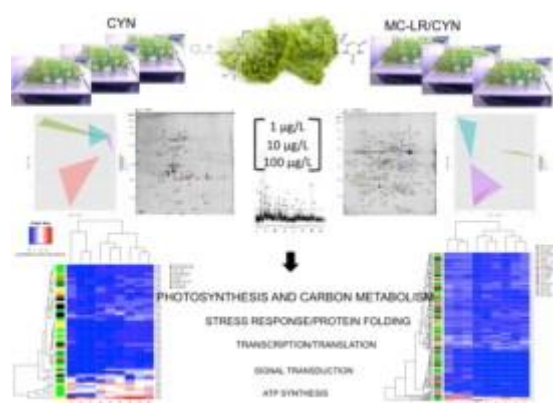
Abbreviations

- 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

Keywords

Lactuca sativa; Proteomics; Cylindrospermopsin; Microcystin-LR; Mixture

1. Introduction

The progressive occurrence and global expansion of harmful cyanobacteria blooms have been forecasted as consequences of eutrophication and climate change (Elliott, 2012 and 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 and 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 and 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, Pflugmacher et al., 2007 and 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 and El Khalloufi et al., 2011), chlorophyll content (McElhiney et al., 2001 and Pflugmacher, 2002), and antioxidative response (Pflugmacher et al., 1999, Pflugmacher et al., 2001, Stüven and Pflugmacher, 2007, Pflugmacher et al., 2007, Gehringer et al., 2003, Pflugmacher, 2004, Pflugmacher et al., 2006, Saqrane et al., 2009, El Khalloufi et al., 2011 and 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 and 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 and 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 and 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 and 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 and 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 scarce information on the lettuce genome, a high rate of proteins was successfully identified by the combination of 2-DE, MALDI-TOF/TOF MS, and lettuce expressed sequence tag (EST) databases (NCBI). A functional characterization of the differentially abundant leaf-lettuce proteins was performed to better understand the physiological response of this important crop plant to CYN and MC-LR/CYN exposure.
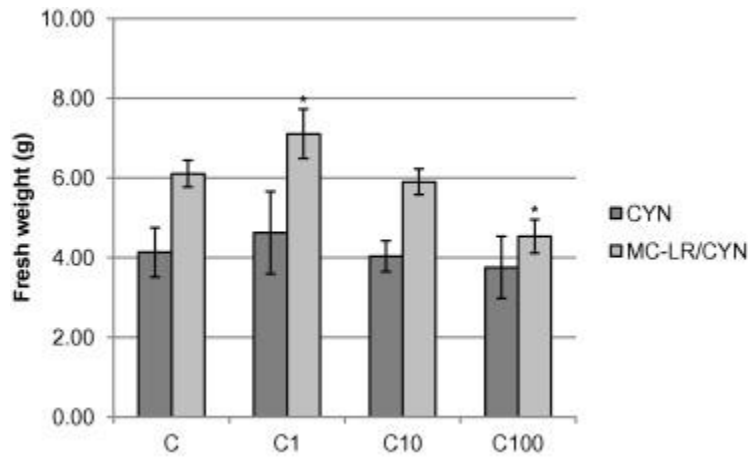
Fig. 1. Fr. wt (g) of leaves of lettuce plants exposed to CYN and MC-LR/CYN. The values are expressed as the means ± standard deviation (SD) (n = 10). The asterisk (∗) indicates significant differences (P < 0.05) between the control and exposed groups. C1: 1 μ

2.1. Differential leaf-proteome profiles related to CYN and MC-LR/CYN exposure

The analysis of the leaf-proteome profiles of lettuce plants exposed to CYN and MC-LR/CYN revealed a total of 68 and 286 protein spots with significant abundance variations, respectively ($p < 0.05$, spot intensity variation of at least two-fold). The comparisons were made between the control group (C) and the groups with different exposure concentrations (C1, C10, and C100), as follows: C/C1, C/C10, and C/C100 (Fig. 2A and B). Although some differentially abundant protein spots were common in the groups exposed to different concentrations, each group (C1, C10, and C100) exhibited a specific response pattern. The multivariate principal component analysis (PCA) (Fig. 3 and Fig. 4) allowed an accurate classification of the different experimental groups ($p < 0.05$, ANOSIM test) (Supporting information Figs. 2 and 3). In the CYN experiment, the first component explains 40% of the variation and separates groups C and C1 from groups C10 and C100. The second and third components explain 14% and 9% of the proteome variation, respectively, and these separate group C from groups C1, C10, and C100. In the MC-LR/CYN experiment, the experimental groups were also well separated by the first three components. PC1 explains 33% of the proteome variation and separates groups C, C10, and C100 from group C1. Groups C10 and C100 are in the same component. PC2 explains 25% of the variation and separates groups C and C100 from groups C1 and C10, whereas the third component, which explains 11% of the variation, clearly separates group C from group C100.
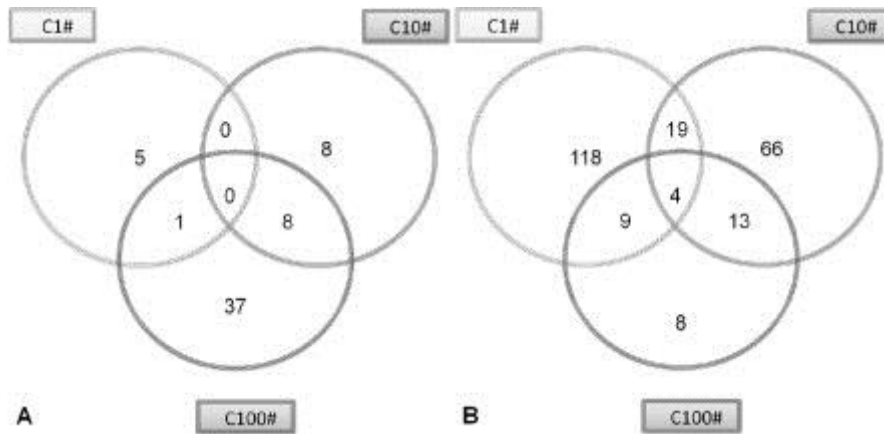
Fig. 2. Venn diagram of the protein spots that exhibit differential abundance on leaf-lettuce plants exposed to CYN (A) and MC-LR/CYN (B) compared with the control group. The intersections also show the number of common proteins spots between different groups.

Fig. 3. PCA diagrams representing the first and second components and the second and third components of the differential protein abundance (spot intensity) on the 2-DE gel of leaf-lettuce plants exposed to CYN. C1: 1 µg/l; C10: 10 µg/l; and C100: 100 µg/l.
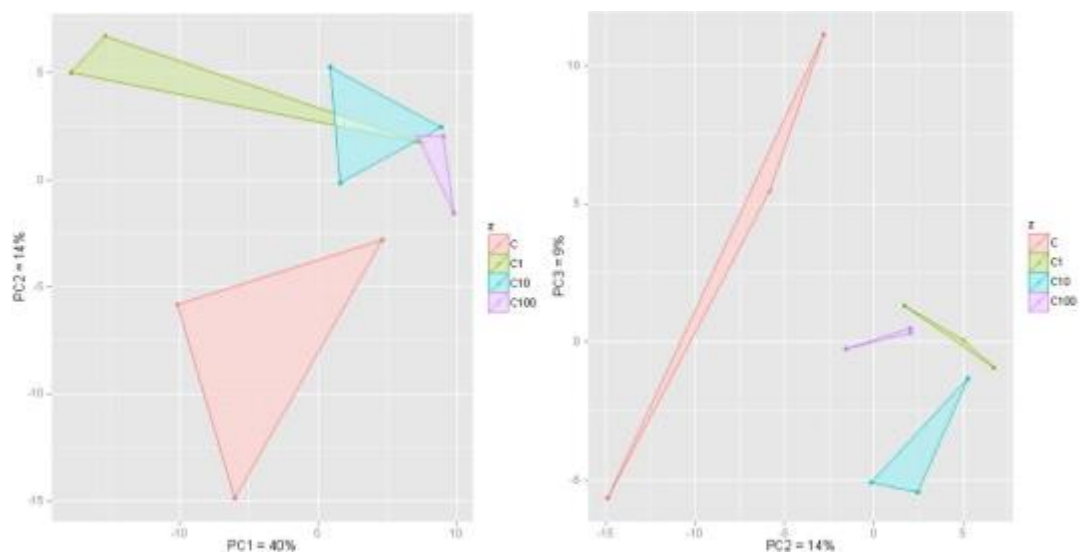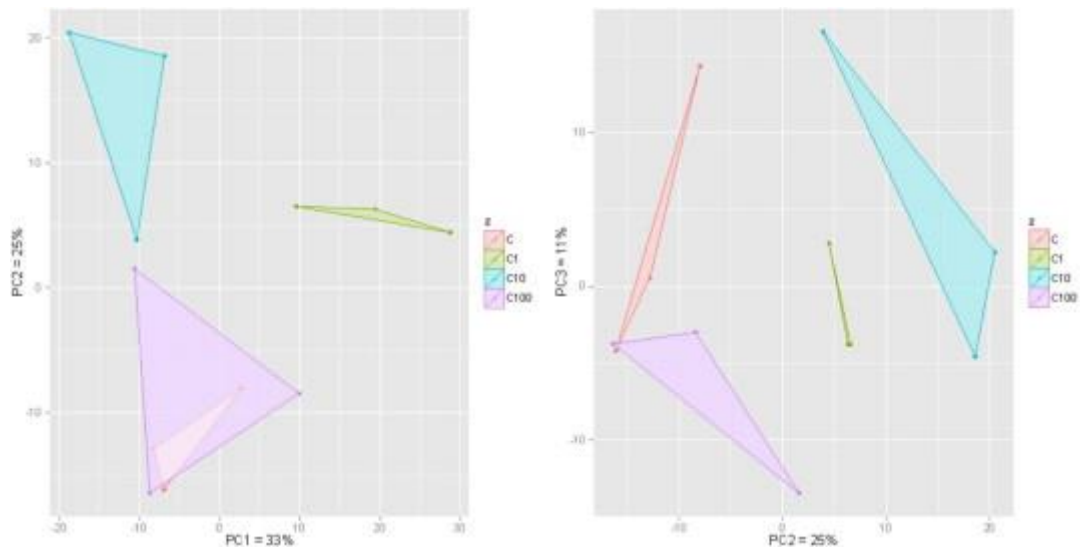
Fig. 4. PCA diagrams representing the first and second components and the second and third components of the differential protein abundance (spot intensity) on the 2-DE gel of leaf-lettuce plants exposed to MC-LR/CYN. C1: 1 μg/l; C10: 10 μg/l; and C100: 100 μg

The number of protein spots with significant abundance variation appears to be concentration-dependent in the CYN exposure experiment (Table 1). A low number of significantly different protein spots (6) were found in group C1, and almost all of these proteins decreased in abundance (5) compared with that observed in group C. In contrast, exposure to 10 and 100 μg/l CYN resulted in 11 and 39 differentially protein spots, respectively, that increased in abundance (Table 1). The number of differentially abundant protein spots in the MC-LR/CYN experiment also appears to be concentration-dependent but following a reverse trend, i.e., a high number of proteins increased in abundance in group C1 (150), and 61 and 17 protein spots absent in the gels of groups C10 and C100, respectively (Table 1).

Table 1. Quantitative description of the differentially abundant protein spots of the lettuce leaf-proteome profile obtained after treatment with ecologically relevant concentrations of CYN and the MC-LR/CYN mixture.

Exposure conditions	CYN			MC-LR/CYN		
	C1	C10	C100	C1	C10	C100
Spots with increased relative protein abundance	0	11	39	138	26	7
Spots with decreased relative protein abundance	5	1	0	1	11	5
Protein spots absent in C	1	4	7	7	4	5
Protein spots absent in C1	–	–	–	4	–	–
Protein spots absent in C10	–	–	–	–	61	–
Protein spots absent in C100	–	–	–	–	–	17
Differentially abundant protein spots (identified proteins)	6 (6)	16 (12)	46 (38)	150 (127)	102 (74)	34 (25)

C: control; C1: 1 μg/l; C10: 10 μg/l; and C100: 100 μg/l.

For a broader visualization of these extensive results, individual heat maps were generated for the CYN and MC-LR/CYN experiments. Associated with each heat map, two hierarchical clusterings were performed to display the similarities between the experimental groups and to determine the distribution pattern of the differentially abundant proteins (Fig. 5 and Fig. 6). In both the CYN and MC-LR/CYN exposure experiments, the clusters above the plots show two major groups (C1 and C10/C100). These clusters are corroborated by PCA (first component in the CYN experiment and first and second components in the MC-LR/CYN experiment) and suggest that the concentration of 1 µg/l in both experiments produced distinctive effects relative to the concentrations of 10 and 100 µg/l. The clustering of proteins by their abundance variation also generated two major groups. The first small group encloses the proteins that displayed major differences in abundance comparatively to group C (absence of spots in the gels or high-fold variation). In the CYN experiment, the color gradient shows a common pattern of the proteins that decreased in abundance in group C1 (dark blue/grey pattern). Group C100 is adjacent to group C10 and is clustered away from group C1 because all of the protein spots increased in abundance (light blue/grey pattern). With respect to the MC-LR/CYN experiment, the trend is reversed, and the majority of proteins identified in group C1 exhibited an increase in abundance (light blue/grey pattern), whereas the proteins in groups C10 and C100 decreased in abundance or were absent in the gels (dark blue/grey pattern).

Figura 5. Heat map of the proteins identified from the differentially abundant protein spots of leaf-lettuce plants exposed to CYN. The values shown were normalized and standardized using the control group as the reference. Two hierarchical clusterings were ma.
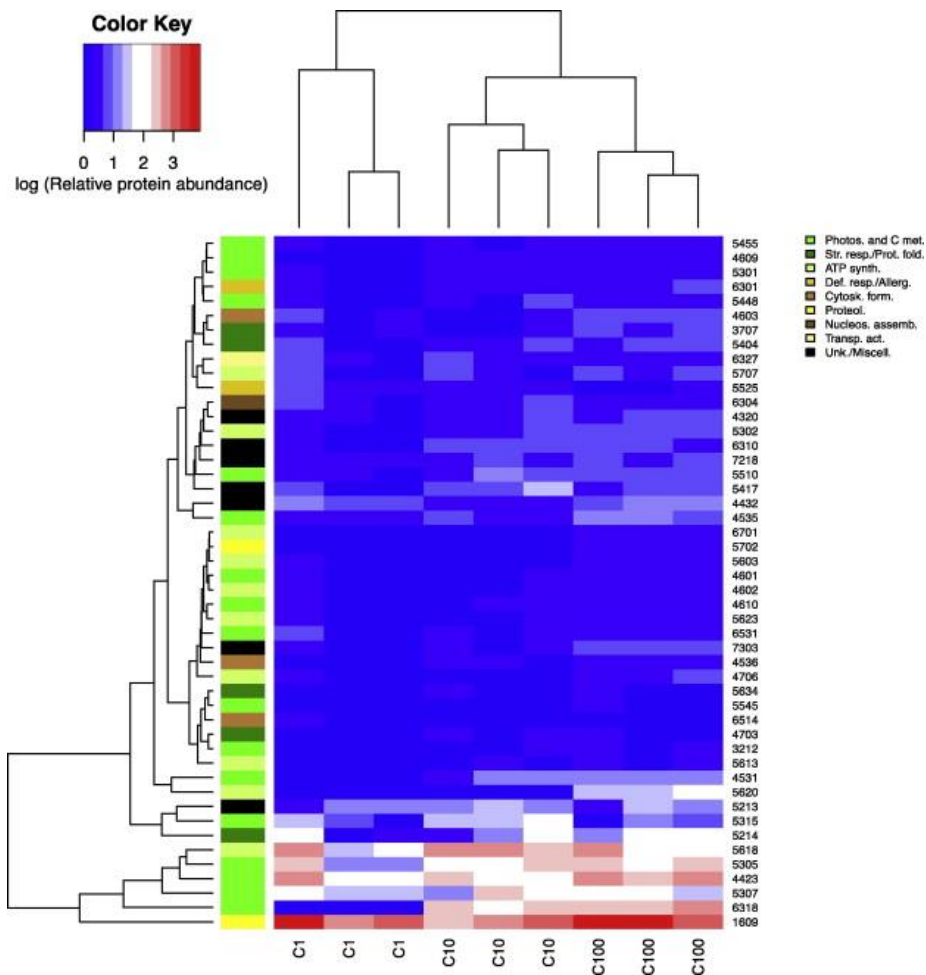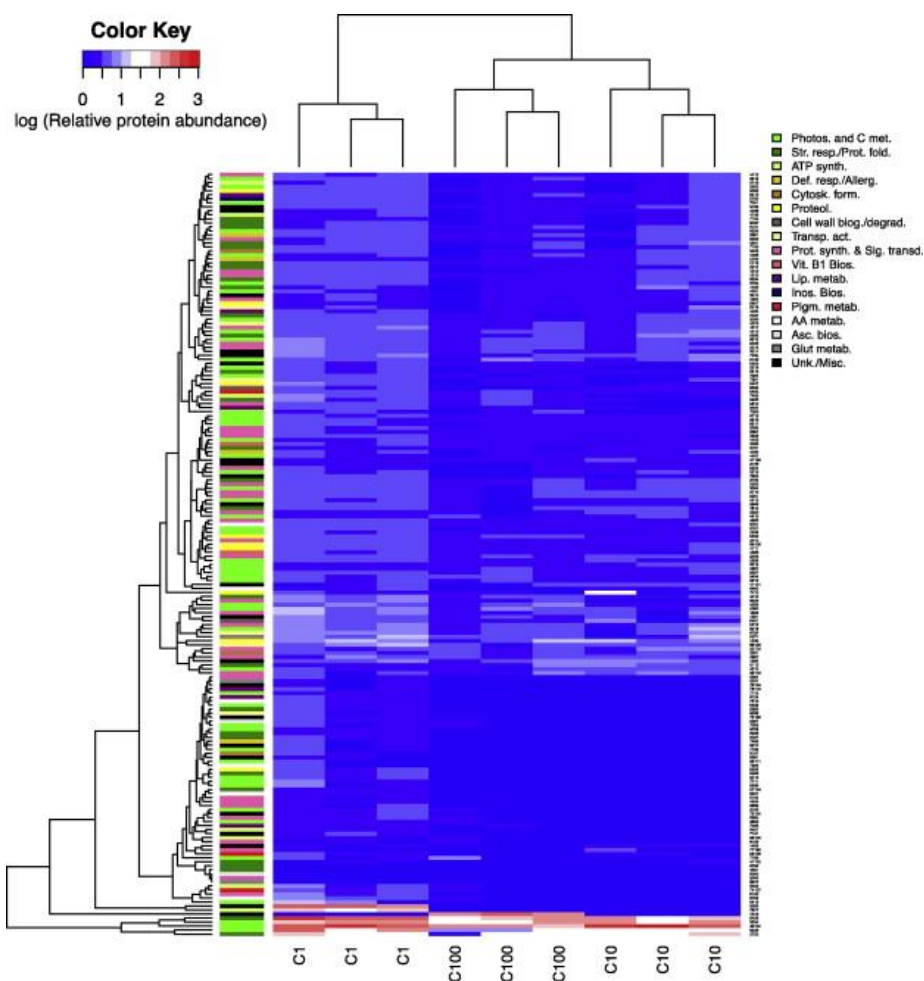
Fig 6. Heat map of the proteins identified from the differentially abundant protein spots of leaf-lettuce plants exposed to MC-LR/CYN. The values shown were normalized and standardized using the control group as the reference. Two hierarchical clusterings wer

2.2. Identification and functional classification of differentially abundant leaf-proteins

Six (100%), 12 (75%), and 38 (83%) proteins from the C1, C10, and C100 groups in the CYN experiment, respectively, and 127 (85%), 74 (73%), and 25 (74%) proteins from the C1, C10, and C100 groups of the MC-LR/CYN experiment, respectively, were successfully identified (Table 1). In both experiments, there were multiple spots corresponding to the same protein, such as ATP synthase CF1 β subunit (spots 4706, 5603, and 5707) in the CYN experiment and chloroplastic-like thiamine thiazole synthase chloroplastic-like (spots 3309 and 4308) in the MC-LR/CYN experiment. Furthermore, some of the identified proteins exhibited an unexpected molecular mass relative to its position in the 2-DE gels (Supporting information Figs. 4 and 8). Similar results have been reported in other proteomics studies, and this variability may be related to the identification of proteins from other plant species, the identification of multiple isoforms of the same protein, and post-translational modifications (Sheoran et al., 2007 and Pinheiro et al., 2013).

The identified proteins were classified based on their putative designated functions, which were mainly gathered from Gene Ontology (UniProt/Swiss-Prot) (details of the identification and changes in abundance

are presented in the Supplementary data; Tables 1 and 2). The identified proteins from the CYN and MC-LR/CYN experiments were classified into 10 (Fig. 7) and 18 functional categories (Fig. 8), respectively. A large number of identified proteins in both experiments are mostly involved in photosynthesis and carbon metabolism, ATP synthesis, and stress/defense response and protein folding (Fig. 7 and Fig. 8). Proteins related to protein synthesis and signal transduction were also well represented in the MC-LR/CYN experiment. To summarize, groups of more comprehensive functions were created to discuss the potential effects of environmentally relevant concentrations of CYN and MC-LR/CYN on leaf-lettuce plants.

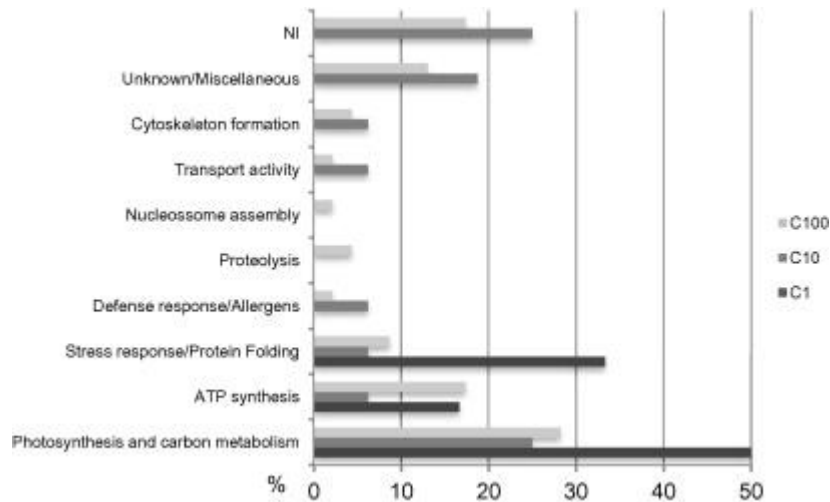
Fig 7. Functional categorization of the proteins identified from the differentially abundant protein spots of leaf-lettuce plants exposed to CYN. C1: 1 μg/l; C10: 10 μg/l; and C100: 100 μg/l.
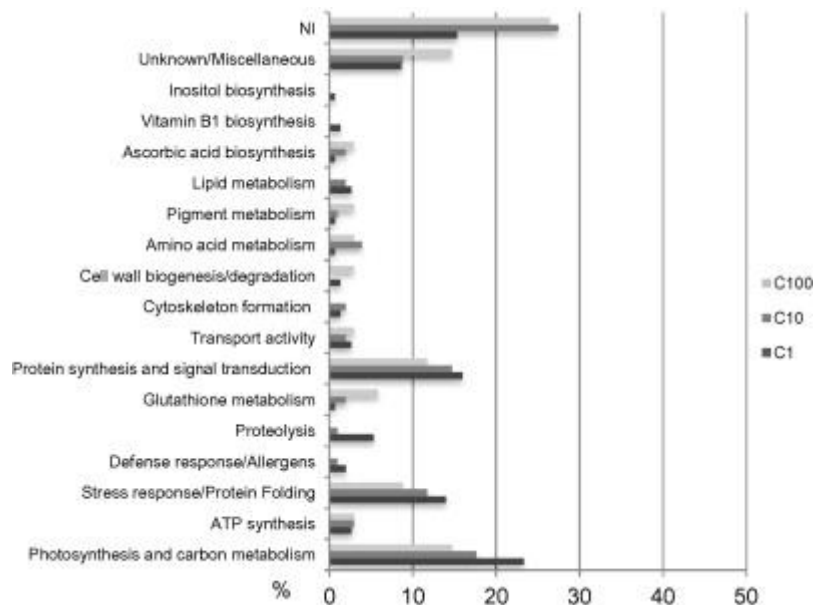

Fig 8. Functional categorization of the proteins identified from the differentially abundant protein spots of leaf-lettuce plants exposed to MC-LR/CYN. C1: 1 μg/l; C10: 10 μg/l; and C100: 100 μg/l.

2.2.1. Energy-related metabolism (photosynthesis/carbon metabolism; ATP synthesis)

The impact of the cyanotoxins on photosynthesis is of major interest because plant productivity and hence crop yield depend strongly of the efficiency of this process.

In the C1 group of the CYN experiment, although only six proteins showed differential abundance, three (50%) were related to energy production, and these decreased in abundance (plastocyanin, ATP synthase CF1 β subunit, and NADP-dependent malate dehydrogenase (pyruvate metabolism)). In contrast, energy production appears to be enhanced in the leaves of the plants in groups C10 and C100, the latter of which presented the highest number of proteins involved in this process. The abundance of chlorophyll a-b-binding proteins, which gather and transfer light energy to photosynthetic reaction centers, was increased; in particular, chlorophyll a-b-binding protein 8 exhibited a 50.7-fold increase in abundance in the C10 group. Oxygen-evolving enhancer proteins, which are responsible for water oxidation in photosystem II (PS II), are also increased in abundance in groups C10 and C100; and the same results were obtained with quinone oxidoreductase-like protein At1g23740, ATP synthase CF1 α (C10 and C100) and β subunits (C100), and ATPase epsilon chain (C100). In addition to proteins associated with primary photosynthesis reactions (light reactions), proteins involved in the Calvin cycle (carbon fixation reactions) exhibit an increase in abundance in group C100: ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RuBP activase), ribulose bisphosphate carboxylase/oxygenase activase 1 (RuBisCO activase 1), phosphoribulokinase (PRK), and sedoheptulose-1,7-bisphosphatase (SBPase).

The effects of MC-LR/CYN were much more complex and appear to have been stronger in groups C10 and C100, the gels of which showed several absent proteins. The combined MC-LR/CYN exposure affected not only photosynthesis but also cellular respiration. Interestingly, in group C1, several proteins involved in energy production are increased in abundance. In detail, these proteins are associated with the light reactions of photosynthesis (chlorophyll a-b-binding proteins, oxygen-evolving enhancer proteins, chloroplast PsbO4 precursor, cytochromeb_6/f heme-binding protein 2-like, ATP synthase α subunit, PS I reaction center subunit II, PS II stability/assembly factor HCF136, quinone oxidoreductase-like protein At1g23740 chloroplastic-like, and ferredoxin-NADP reductase), photorespiration (gamma carbonic anhydrase-like 2), the Calvin cycle (RuBP activase and large subunit, RuBisCO activase 1, and PRK), glycolysis and pentose phosphate pathway (β-xylosidase/α-L-arabinofuranosidase 2-like, fructan 1-exohydrolase IIa, triosephosphate isomerase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, phosphoglycerate kinase 3, ribose-5-phosphate transaldolase), tricarboxylic acid (TCA) cycle (mitochondrial dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 2), and oxidative and photo phosphorylation (mitochondrial ATP synthase subunit delta′ and chloroplastic soluble inorganic pyrophosphatase 1). In contrast, few proteins related to photosynthesis and cellular respiration were increased in abundance in groups C10 and C100 (chloroplast light-harvesting chlorophyll a/b-binding protein, RuBP activase (C100), pyruvate dehydrogenase E1 component subunit β-like, transaldolase-like, and chloroplastic soluble inorganic pyrophosphatase 1).

Furthermore, there were proteins that were absent in the gels or exhibited decreases in abundance in groups C10 and C100, and these proteins are associated with the light reactions of photosynthesis (oxygen-evolving enhancer protein 1, ATP synthase α subunit, ATP synthase gamma chain, and chloroplastic-like isoform 1) and the Calvin cycle (RuBP activase, transketolase, putative), which is dependent on the ATP and NADPH generated by the light reactions. Additionally, proteins involved in glycolysis (pyrophosphate--fructose 6-phosphate 1-phosphotransferase β subunit, and glyceraldehye-3-phosphate dehydrogenase (C100)) and the TCA cycle (putative cytosolic NADP-malic enzyme, α-isopropylmalate synthase, succinate dehydrogenase, isocitrate dehydrogenase (NAD+) (C100), and malate dehydrogenase (C100)) were absent in the gels or presented decreases in abundance in groups C10 and C100. Several studies have proposed that photosynthesis-related proteins are differentially regulated under abiotic stress. In general, photosynthesis is impaired in sensitive plants and enhanced in tolerant plants (Kosová et al., 2011 and Abreu et al., 2013). Nothing definite is known regarding how CYN interferes in plant photosynthesis. However, negative effects of MC-LR in the photosynthesis activity of plants have been reported (Abe et al., 1996, Pflugmacher, 2002 and El Khalloufi et al., 2011). Our results show that MC-LR/CYN concentrations of 10 and 100 μg/l may produce harmful effects on the energy production of lettuce leaves. Nevertheless, the highest number of proteins that decreased in abundance was found in group C10, which may suggest that the effects are produced through different mechanisms. The roots exposed to 100 μg/l MC-LR/CYN had a high amount of exudates (data not shown). It could be hypothesized that this accumulation could prevent the uptake of the toxins, thereby mitigating their negative effects, but can also impair the uptake of water and nutrients, which can be related to the significant reduction in the fr. wt of these lettuce leaves ($p < 0.05$) (Fig. 1). In contrast, the fr. wt of the lettuce leaves of group C1 was significantly increased ($p < 0.05$), likely due to the enhancement of photosynthesis and carbon metabolism. As mentioned above, no differences were found in the fr. wt of lettuce leaves in the CYN experiment (Fig. 1); thus, it is likely that the increase in abundance of proteins related to photosynthesis and carbon metabolism in group C100 may contribute to the maintenance of cellular homeostasis and an active adaptation to the stress promoted by CYN. Plant productivity and hence crop yield depend strongly on the prevailing photosynthetic rates. The Calvin cycle is autocatalytic and can thus be enhanced by increases in the concentrations of its biochemical intermediates (Taiz and Zeiger, 2002). Some of the identified proteins belonging to the Calvin cycle, such as SBPase (which exhibits a 11.8-fold increase in abundance in group C100 of the CYN exposure experiment), have been reported to be effective for the improvement of abiotic stress tolerance (Abreu et al., 2013) and have also been studied to determine whether their use may increase crop production (Lefebvre et al., 2005). Lefebvre et al. (2005) reported that an increase in SBPase activity in tobacco plants (*Nicotiana tabacum*) leads to improvements in the photosynthesis rate, levels of sucrose and starch, leaf area, and biomass, which may be associated with an increase in the RuBP regenerative capacity. Because photosynthesis and carbon metabolism seem to have been the main biological processes affected by CYN and MC-LR/CYN, it is hypothesized that some of these identified proteins could be investigated as potential biomarkers of these cyanotoxins.

2.2.2. Stress and defense response (stress response/protein folding; glutathione metabolism; proteolysis; defense response/allergens)

In the stress/defense response, plants need to regulate a variety of processes that require energy. In fact, the profile of abundance of proteins involved in the stress/defense response followed the same pattern observed in the analysis of energy-related proteins: group C100 of the CYN experiment and group C1 of the MC-LR/CYN experiment stand out due to their high number of proteins that exhibited an increase in abundance. Although it is well recognized that ROS are generated during normal plant metabolism (e.g., in chloroplasts), it has also been stated that the cyanotoxins CYN and MC-LR induce oxidative stress in plants (Pflugmacher et al., 2006, Pflugmacher et al., 2007, Stüven and Pflugmacher, 2007, Saqrane et al., 2009 and Prieto et al., 2011). A protein involved in glutathione (GSH) metabolism (S-formylglutathione hydrolase), specifically in GSH synthesis, was only present in the C group of the MC-LR/CYN experiment. However, protein IN2-1 homolog B-like (glutathione-S-transferase (GST) superfamily) increased in abundance in group C100 (3.3-fold). Runnegar et al. (1995) showed that CYN inhibits GSH synthesis in cultured rat hepatocytes. It is interesting to note that in this study this effect was not observed in lettuce leaves exposed to CYN even at a concentration of 100 μg/l. Nevertheless, it is well recognized that MC-LR is detoxified by conjugation with GSH via GST (Pflugmacher et al., 1998). It can be hypothesized that the simultaneous exposure to MC-LR/CYN may lead to a reduction of GSH pool in cells due to MC-LR detoxification (via GST), which may have resulted in a higher requirement of GSH biosynthesis. Because CYN is an inhibitor of GSH synthesis, it can impair the capacity of plants to detoxify MC-LR. On the other hand, the weakened response of lettuce plants to MC-LR may contribute to oxidative stress and may enhance its toxic effects in a concentration-dependent manner, leading to an inefficient response of plants to CYN and consequently the inhibition of a higher number of proteins. In contrast, at low concentrations, lettuce plants appear to be able to cope with oxidative stress by inducing the production of other antioxidant enzymes. Chloroplastic Cu/Zn superoxide dismutase (SOD), ascorbate peroxidase (APX), other peroxidases, and ferritin, which is reported to be responsive to stress, were identified in leaf-lettuce plants exposed to 1 μg/l MC-LR/CYN (chloroplastic 2-Cys peroxiredoxin BAS1, peroxiredoxin 2, thioredoxin-dependent peroxidase, oxidoreductase, chloroplastic SOD [Cu–Zn], aldo-ketoreductase 2-like, and chloroplastic peroxiredoxin-2E (also in the group C10)). The coordinated action of these enzymes prevents the oxidative damage of cells generated by ROS. SOD catalyzes the dismutation of superoxide radical to hydrogen peroxide and oxygen. The resulting hydrogen peroxide is reduced by APX and other peroxidases to yield water (Pflugmacher et al., 2006). However, if the detoxification enzymes produced are not sufficient, proteins damaged by ROS may accumulate in plant cells under stress conditions. Some of the strategies developed by plants to overcome this accumulation are to refold misfolded proteins using helper proteins, such as chaperones, and to remove these by protease activity. Heat shock protein (HSP) 70, which is one of the most important HSPs involved in the plant response to abiotic stresses (Abreu et al., 2013), was decreased in abundance in the C1 group of the CYN exposure experiment. However, in group C100, putative thioredoxin-dependent peroxidase and RuBisCO large subunit-binding protein subunit α, which has chaperone and refolding activity, exhibited increases in abundance. In addition, the chloroplastic protein

peptidyl-prolyl cis–trans isomerase (PPIase) FKBP16-3, which is a folding catalyst, exhibited a 107-fold increase in abundance in group C100 (CYN exposure). With respect to the MC-LR/CYN experiment, several proteins involved in redox homeostasis and some proteins with chaperone functions (chloroplastic PPIase CYP38, RuBisCO subunit binding-protein α subunit precursor, 60-kDa chaperonin α subunit, chloroplastic20-kDa chaperonin, protein disulfide isomerase-like 2-3-like, calreticulin (C10), protein disulfide isomerase (C10), and HSP90 (C10)) were increased in abundance in groups C1 and C10 (exhibited less fold-variation in group C10). The late embryogenesis abundant protein (LEA), Lea14-A, is well recognized for its role in stress tolerance (Abreu et al., 2013); however, this protein was absent in the gels of groups C1 and C10 of the MC-LR/CYN experiment. Additionally, some proteins involved in redox homeostasis and some proteins with chaperone activity were either decreased in abundance or absent in the gels of groups C10 and C100 of the MC-LR/CYN experiment (chloroplastic chaperone protein ClpC (C10), glutaredoxin S16 (C10), thylakoid-bound ascorbate peroxidase (C10), thioredoxin reductase 2-like (C10), PITH domain-containing protein At3g04780 (C100), and chloroplastic chaperone protein ClpB3). The latter protein was also decreased in abundance in the C1 group. Almost all of the chaperones and peroxidases are chloroplastic, which suggests that photosynthetic complexes are the most affected by CYN and MC-LR/CYN. In addition to detoxification and chaperone proteins, an increase in the abundance of proteases and proteasomes was observed in group C1 of the MC-LR/CYN experiment. Probable 26S proteasome non-ATPase regulatory subunit 7, which belongs to the proteolytic complex that regulates cytosolic protein turnover by the ubiquitin pathway (Taiz and Zeiger, 2002) and plays an essential role in removing regulatory proteins and abnormal polypeptides in plants during stress, exhibited a 4-fold increase in abundance. The putative zinc-dependent protease was increased in abundance in group C100 of the CYN exposure experiment.

Proteins related to the bacterium defense response also presented changes in abundance in the CYN and MC-LR/CYN experiments. Eugenol synthase 1, which catalyzes the synthesis of the phenylpropene eugenol, a defense compound with antimicrobial properties, exhibited an increase in abundance in group C10 of the CYN experiment and in group C1 of the MC-LR/CYN experiment. In the latter experiment, harpin binding protein 1 also presented increases in abundance. The increase in abundance of pathogenesis-related (PR) proteins may be promising for the improvement of the response to abiotic stress; however, it may also constitute a threat to food safety because most of these proteins have allergenic potential. Thaumatin-like protein-like, which appears to have allergenic properties (Palacín et al., 2010), was increased in abundance in group C100 of the CYN experiment (2.7-fold) and in group C1 of the MC-LR/CYN experiment (2.2-fold).

2.2.3. Protein synthesis and signal transduction (transcription, RNA processing and translocation, and translation)

Several proteins involved in protein synthesis and signal transduction were found in almost all of the groups of the MC-LR/CYN experiment. The C1 group presented a high number of these proteins that were

increased in abundance. Although to a lower extent, groups C10 and C100 also showed proteins associated with: mRNA processing (chloroplast putative ribonucleoprotein (C1, C10, and C100), chloroplast 31-kDa ribonucleoprotein (C1 and C100)), transcription factors containing DNA-binding motifs and regulators of transcription (transcription factor Pur-alpha 1-like, U2 small nuclear ribonucleoprotein A, zinc finger protein, proliferating cell nuclear antigen, minor allergen Alt *a*, nascent polypeptide-associated complex subunit alpha-like), and mRNA transport and translation (eukaryotic translation initiation factor 3 subunit D-like, elongation factor 1-beta, eukaryotic translation initiation factor 3 subunit F-like, eukaryotic translation initiation factor 3 subunit J-like (C10), chloroplast 30S ribosomal protein S1 (C1 and C10), 60S acidic ribosomal protein P0, and chloroplast 50S ribosomal protein L12 (C10)) that presented increases in abundance. The mRNA processing and translational apparatus may indicate a rapid protein synthesis, which could be related to the increase and maintenance of the fr. wt of lettuce leaves in groups C1 and C10, respectively. Nevertheless, some proteins associated with RNA recognition motifs and regulators of ribonuclease activity (poly (A)-binding protein (C10), and regulator of ribonuclease activity A (C10)) and translation (eukaryotic translation initiation factor 5A (C10), 40S ribosomal protein (C10), eukaryotic translation initiation factor 3 subunit K-like (C10), and elongation factor 2 (C100)) decreased in abundance or were absent in the gels of groups C10 and C100. In this study, the changes in abundance of proteins related to protein synthesis suggest that this activity may be of particular importance in the lettuce response to MC-LR/CYN. The little that is known regarding how CYN inhibits protein synthesis in plants is that the soluble proteins associated with the eukaryotic translation system appear to be the target of the toxin (Froscio et al., 2008). Additionally, this toxin appears to interfere with the elongation step, which indicates that elongation factors may also be a target (Froscio et al., 2008). In fact, in group C100 of the MC-LR/CYN experiment, elongation factor 2 was the unique protein that exhibited a decrease in abundance. The potential decrease of energy-related enzymes (e.g., Calvin cycle enzymes), likely as a result of protein synthesis inhibition, may lead to reduced synthesis of carbohydrates, which are essential for the support of cell division and elongation (Taiz and Zeiger, 2002). This decrease may also have contributed to the reduction in the fr. wt of lettuce leaves; however, this hypothesis should be further studied. Exposure to CYN results only in an increased abundance of histone H4 in the C100 group. This protein is a core component of the nucleosome and plays a central role in transcription regulation, DNA repair, DNA replication, and chromosomal stability. It could be hypothesized that CYN at a concentration up to 100 μg/l is not sufficient to cause cessation of protein synthesis; however, MC-LR may potentiate this effect. Furthermore, phosphorylation and dephosphorylation play important roles in signal transduction, and in the MC-LR/CYN experiment, proteins involved in signal transduction pathways (14-3-3-like protein D-like (C1), 14-3-3 protein 1-like, and 14-3-3 protein (C10)) present increases in abundance in groups C1 and C10. The plant 14-3-3 isoforms regulate a diverse range of proteins, including kinases, transcription factors, structural proteins, ion channels, and pathogen defense-related proteins (Denison et al., 2011). The differential accumulation of these proteins appears to be implicated in the response to abiotic stress (Abreu et al., 2013).

2.2.4. Transport activity

Proteins and lipoproteins involved in the transport of macromolecules, small molecules, and ions, mainly in the chloroplast, exhibited changes in abundance in both experiments. In groups C10 and C100 of the CYN experiment, temperature-induced lipocalin exhibited 4.4- and 2.5-fold increases in abundance, respectively. This protein as well as chloroplast processing peptidase-like, protein TIC 62 chloroplastic-like, and apolipoprotein d, also increased in abundance in group C1 of the MC-LR/CYN experiment. However, in groups C10 and C100, chloroplastic protein TIC 62 was decreased in abundance, and GTP-binding nuclear protein Ran1A was absent in the gels of the C10 group.

2.2.5. Structural activity (cytoskeleton formation; cell wall biogenesis/degradation)

It is well recognized that MC-LR alters the cytoskeletal structure of animal cells (Toivola and Eriksson, 1999). Phosphorylation/dephosphorylation and the synthesis of certain regulatory proteins are key mechanisms in cytoskeletal regulation (Máthé et al., 2013). Similarly, CYN induces the reorganization of the cytoskeleton in animal cells (CHO K1 cells) (Fessard and Bernard, 2003). In this study, exposure to CYN promoted a decrease in the abundance of actin in the C10 group. In contrast, plastid-dividing ring protein, which is related to microtubule-based process and protein polymerization, increased in abundance in group C100 of the CYN experiment and in group C1 of the MC-LR/CYN experiment. Additionally, fibrillin, a glycoprotein that contributes to the structural integrity and assembly within or outside cells, increased in abundance in groups C1 and C10 of the MC-LR/CYN experiment. UDP-arabinopyranose mutase 1, which is involved in the biosynthesis of the cell wall, exhibited an increase in abundance in group C1 of the MC-LR/CYN exposure experiment. However, xyloglucan endotransglucosylase/hydrolase, an enzyme involved in wall assembly, decreased in abundance in groups C1 and C100 of the MC-LR/CYN exposure experiment. Exposure to these cyanotoxins appears to lead to the reorganization of cytoskeletal components in lettuce leaves, and it could be hypothesized that the differential abundance of proteins involved in cytoskeleton assembly and cell wall biosynthesis played a role in the changes of the observed fr. wt of lettuce leaves.

2.2.6. Other metabolisms (amino acid metabolism; pigment metabolism; lipid metabolism; ascorbic acid biosynthesis; vitamin B1 biosynthesis; inositol biosynthesis; hormone regulator)

Overall, proteins involved in the metabolism of amino acids, pigments, lipids, ascorbic acid, vitamin B1, inositol, and hormone regulation exhibited increases in abundance in group C1 and decreases in abundance in groups C10 and C100 of the MC-LR/CYN experiment.

Ketol-acid reductoisomerase, which promotes the formation of amino acids containing a branched carbon skeleton, increased in abundance in the C1 group. In contrast, other proteins that have been identified to be involved in amino acid metabolism, such as acetohydroxyacid synthase 1, diaminopimelate decarboxylase 2 chloroplastic isoform 1, vitamin-b12 independent methionine synthase 5-methyltetrahydropteroyltriglutamate-homocysteine, and putative thiosulfate sulfurtransferase (C100)

decreased in abundance or were absent in the gels of groups C10 and C100. Chloroplastic coproporphyrinogen-III oxidase, which is involved in porphyrin and chlorophyll metabolism and biosynthesis, increased in abundance in the C1 group, and chloroplastic uroporphyrinogen decarboxylase was absent in the gels of the C10 group. The polyphenol oxidase precursor, which is involved in the pigment biosynthetic process, was absent in the gels of the group C100. A diverse family of lipases and esterases (GDSL esterase/lipase At5g45670, GDSL esterase/lipase LTL1-like, and bifunctional epoxide hydrolase 2-like) and a key enzyme of the type II fatty acid synthesis (FAS) system (enoyl-ACP reductase 1) presented increases in abundance in the C1 group. However, the protein 2-hydroxyacyl-CoA lyase-like, which plays a key role in redox signaling and lipid homeostasis, and the protein acetyl-CoA C-acetyltransferase, which is involved in the beta oxidation pathway of fatty acid degradation, were absent in the gels of the C10 group. Phosphomannomutase, which is involved in ascorbic acid biosynthesis, was absent in all gels of the groups of the MC-LR/CYN experiment. Chloroplastic-like thiamine thiazole synthase and L-myo-inositol-1-phosphate synthase, which are involved in vitamin B1 and inositol biosynthesis, respectively, increased in abundance in the C1 group. Auxin-binding protein ABP20-like, a probable receptor for the plant growth-promoting hormone auxin, exhibited an increase in abundance in groups C1 and C10 (to a higher extent in group C1), whereas abscisic acid receptor PYR1-like, a plant hormone associated with signal transduction, was absent in the gels of the group C10. The simultaneous exposure to low concentrations of MC-LR and CYN (1 μg/l) appears to stimulate the synthesis of different constituents in lettuce leaves that are concomitantly involved in plant growth and development and also in the response to stress, such as hormones, amino acids, lipids, important membrane components, and vitamins. Exposure to environmental stress induces several physiological changes in plants that can alter the chemical composition and thus the quality of crops (Wang and Frei, 2011). Dependent on numerous factors, such as the time of exposure and the crop species, the overall plant stress response appears to result in increasing the concentrations of some constituents, such as proteins and antioxidants (Wang and Frei, 2011). At low concentrations of MC-LR/CYN exposure, positive changes in the nutritional quality of lettuce leaves may be attained. However, this issue should be further studied through the quantification of the respective constituents in plants exposed to these cyanotoxins.

2.2.7. Unknown/miscellaneous

Proteins with no well-defined function (several functions or unknown function) were identified as unknown/miscellaneous.

3. Conclusions

In this study, we applied a differential-expression proteomics approach to understand the mechanisms underlying the response of leaf-lettuce plants to environmentally relevant concentrations of CYN and MC-LR/CYN. The abundance of proteins was affected in a concentration-dependent manner by the simultaneous activation of several metabolic pathways, which are mainly related to photosynthesis,

response/defense to stress, and protein synthesis and transduction. The activation of these pathways appears to confer tolerance to lettuce plants against CYN at concentrations up to 100 μg/l. The simultaneous exposure to MC-LR and CYN resulted in a 'dualistic response', and exposure to a concentration of 1 μg/l promoted an increase in the abundance of proteins associated with general biological processes and a significant increase in the fr. wt of leaves. In contrast, the concentrations of 10 and 100 μg/l appeared to be deleterious to lettuce because a high number of proteins associated with general biological processes exhibited decreases in abundance or were absent in the gels. The latter concentration also promoted a reduction in the fr. wt of the leaves. This effect is of major concern because the occurrence of mixtures of cyanotoxins is expected to become increasingly recurrent. This study also provides new insights into potential protein markers of exposure to cyanotoxins and of novel proteins that may confer tolerance to CYN and MC-LR/CYN, although these need to be functionally characterized and validated. Furthermore, the proteomics analysis was found to be suitable for the discovery of some traits associated with the quality and safety of edible tissues of lettuce exposed to environmentally relevant concentrations of CYN and MC-LR/CYN, such as the presence of allergenic proteins.

4. Experimental

4.1. General experimental procedures

The lettuce plants (*L.sativa* L. var. 'Susybel') were exposed to environmentally relevant concentrations (1, 10, and 100 μg/l) of a CYN and MC-LR/CYN mixture in a hydroponic system. The CYN and MC-LR were purified and quantified by high-performance liquid chromatography (HPLC) and then diluted in the culture medium to the desired concentrations of exposure. All of the HPLC solvents were of high-purity chromatography grade and were filtered (Pall GH Polypro 47 mm, 0.2 μm) and degassed with an ultrasound bath. After exposure, to investigate the leaf-proteome profiles, the proteins of lettuce leaves were separated by 2-DE, and those that were differentially abundant were then identified by MALDI-TOF/TOF MS.

4.2. Cyanobacterial culture and toxin purification and quantification

4.2.1. Cultures of *M. aeruginosa* and *C. 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, 4495g), frozen at −80 °C, and then freeze-dried. Due to the high hydrophilicity of CYN, the culture medium from *C. raciborskii* was also frozen at −80 °C and 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 address the specific effects of CYN and MC-LR/CYN on the lettuce leaf-proteome and to avoid interference from other potentially toxic metabolites of cyanobacteria crude extracts (e.g., lipopolysaccharides) (Pietsch et al., 2001).

4.2.2. 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 cells and medium (0.7 g) were first sonicated in a bath for 15 min in 5 ml of 0.1% (v/v) trifluoroacetic acid (TFA) (spectrophotometric grade) and then subjected to five cycles ultrasonication with Vibra-Cell at 60 Hz for 1 min. The homogenate was stirred for 1 h at room temperature and centrifuged ($20,000g$, 4 °C, 20 min), and the supernatant was collected. The cell pellet was subjected to a second extraction. The supernatants were pooled and stored at −20 °C. CYN was thereafter purified using a Waters Alliance e2695 HPLC system coupled with a photoelectric diode array (PDA) 2998 on a semi-preparative Gemini C18 column (250 mm x 10 mm i.d., 5 μm) from Phenomenex that was maintained at 40 °C. The isocratic elution utilized a 5% methanol (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 i.d., 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 series of ten samples included a blank and two different standard concentrations. The chromatographic purity of CYN was of 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).

4.2.3. 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) 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 & Material Inc. Danbury, CT, USA). The homogenate was centrifuged at $10,000g$ for 15 min to remove the cell debris. The resulting supernatant was then collected and stored at 4 °C. The pellet was re-extracted with an equal volume of solvent, and the pooled supernatants were subjected at a flow rate of at 1 ml/min to solid-phase extraction with a Water Sep-Pak® Vac 6-ml C18 cartridge preconditioned with 100% MeOH and distilled water. The loaded column was washed with 20% (v/v) MeOH, and the MC-LR was then eluted using 80% (v/v) MeOH. The MC-LR fraction was evaporated by rotary evaporation at 35 °C to remove the entire MeOH portion. The concentrated MC-LR extract was thereafter purified and quantified by HPLC–PDA. 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 of which were acidified with 0.1% (v/v) 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 nm and 238 nm. The fraction with purified MC-LR was then evaporated with nitrogen air for 1 day until all of the solvent was removed. The residue was resuspended in distilled water to the desired concentration. The chromatographic purity of MC-LR was of 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 i.d., 5 μm) equipped with a guard column (4 × 4 mm, 5 μm), both of which were 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 no. 018K1209, 10.025 μg/ml in MeOH, 98% purity, Cyano Biotech GmbH, Berlin, and Germany). The system was calibrated using a set of seven dilutions of the MC-LR standard (0.5–20 μg/ml) in 50% (v/v) MeOH. Each vial was injected in duplicate, and every HPLC run series of ten 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).

4.3. Plant exposure to CYN and MC-LR/CYN mixture

Lettuce is an important leafy vegetable worldwide that contains substantial amounts of health-promoting phytochemicals (including polyphenols, carotenoids, and vitamin C). Lettuce has been shown to be an excellent experimental system for the assessment of the effects of MC-LR (Crush et al., 2008 and Pereira et al., 2009), and although its genome has not been sequenced, lettuce has been the object of genomics and proteomics studies (Choi et al., 2008 and Cho et al., 2009). The lettuce plants used in the experiments were purchased at four to five weeks' maturity after sowing in a commercial soil substrate. The roots were carefully washed with tap water until complete soil removal was achieved, and twenty lettuce plants were then transferred to the holes of plastic boards (PVC), which were placed on black glass trays (35 × 25 × 5 cm deep) in a hydroponic system that was continuously aerated. The roots were completely immersed in 3 l of culture medium (Jensen and Malter, 1995), pH 6.5. The lettuce plants were acclimated for 1 week with fluorescent white light (light/dark cycle of 14/10 h) and a temperature of 21 ± 1 °C. After the acclimation period, the culture medium was renewed, and the plants were exposed to CYN and the MC-LR/CYN mixture at ecologically relevant concentrations of 1, 10, and 100 μg/l for five days. After the exposure time, the plants were harvested. The roots and leaf tissues were separated, weighed (fr. wt) and stored at −80 °C for proteomic analysis. Three biological replicates for each experimental group (control (C), C1, C10, and C100) were prepared in a total of 12 trays.

4.4. Proteomics analysis

A proteomics analysis of the lettuce roots was attempted; however, likely due to salt interference, a slow progression of the first-dimensional isoelectric focusing (IEF) and the heat produced on the immobilized pH gradient (IPG) gel strips may the analysis impracticable.

4.4.1. Protein extraction from lettuce leaves

The leaf tissues from one lettuce plant exposed for five days to the above-mentioned concentrations of CYN and MC-LR/CYN were ground in liquid nitrogen to a powder with a pestle and mortar. The protein extraction was performed immediately with acetone containing 10% trichloroacetic acid and 0.07% β-mercaptoethanol for 1 h at −20 °C. After centrifugation at $4495g$ and 0 °C for 45 min, the pellet was washed with acetone containing 0.07% β-mercaptoethanol for 1 h at −20 °C and centrifuged at $4495g$ and 0 °C for 50 min. The pellet was dried with nitrogen gas, and the proteins were then solubilized in solubilization buffer (SB) composed of urea (7 M), thiourea (2 M), 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate (CHAPS) (4%, w/v), dithiothreitol (65 mM), ampholytes, pH 4–7 (0.8%, v/v), and polyvinylpolypyrrolidone (0.025 g/mL) for 1 h. The homogenate was centrifuged at $16,000g$ and 4 °C for 20 min. The supernatant was collected, and the proteins were quantified according to the method described by Bradford (1976) using bovine serum albumin as the standard. The protein samples were stored at −80 °C until further analysis.

4.4.2. IEF and 2-DE

IEF and 2-DE were performed according to Puerto et al. (2011). Briefly, 300 µl of SB with 400 µg of proteins were loaded in 17-cm pH 4–7 IPG gel strips (Bio-Rad, Hercules, CA, USA). The proteins were separated by IEF in a Protean IEF Cell (Bio-Rad, Hercules, CA, USA) using the following program: 16 h at 50 V (strip rehydration); step 1, 15 min at 250 V; step 2, linear voltage increase to 10,000 V over 3 h; step 3, linear increase from 10,000 V to 90,000 V/h; and step 4: 500 V/h. After rehydration, wet paper wicks were placed between the IPG gel strip and electrode to remove the excess salts from the samples. The IPG gel strips were frozen at −20 °C prior to 2-DE. The IPG gel strips were equilibrated with 10 mg/ml dithiothreitol in buffer containing urea (6 M), SDS (2%, w/v), glycerol (30%, v/v), and Tris (50 mM), pH 8.8, for 15 min and then with 25 mg/ml iodoacetamide in the same buffer for 15 min. The equilibrated IPG gel strips were placed on top of 12% (w/v) acrylamide SDS-PAGE slab gels (20 cm × 20 cm) and sealed with 0.5% agarose. The proteins were separated by SDS-PAGE in a Protean Xi Cell (Bio-Rad, Hercules, CA, USA) at 18 mA per gel for 30 min and then at 24 mA per gel until the dye reached the bottom of the gel. One 2-DE gel was run for each experimental replicate for a total of 24 gels. The gels were stained overnight at room temperature with Colloidal Coomassie Blue as described by Neuhoff et al. (1988).

4.4.3. Gel image acquisition and protein abundance variation analysis

The image acquisition and analysis of the protein abundance from the 2-DE gels were performed as described previously by Puerto et al. (2011). Briefly, the gel images were acquired using a calibrated

scanner (GS-800, Bio-Rad, Hercules, CA, USA), and the protein spots were detected automatically with the PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA, USA). The spot intensities were normalized based on the total density in the gel image, and manual spot corrections, including re-matching, were also made using the software. The protein spots were considered differentially abundant when the intensity levels exhibited at least a two-fold difference that was statistically significant at a level of $p < 0.05$ (univariate approach, t-test).

4.4.4. Protein identification by MALDI-TOF/TOF MS and peptide mass fingerprinting

A selection list of the spots of interest was generated based on the differentially abundant protein spots ($p < 0.05$) between the C group and the C1, C10, and C100 groups of the CYN and MC-LR/CYN exposure experiments. The spots were excised from the gels and automatically digested with trypsin and spotted on MALDI targets as previously described by Printz et al. (2013). The identification of proteins was performed through mass spectrometry (MALDI-TOF/TOF). For each spot 1 MS spectrum was acquired and the 10 highest peaks selected for MS/MS analysis, excluding known contaminants and trypsin autocleavage products. All of the searches were conducted allowing for a peptide mass tolerance of 100 ppm and a fragment mass tolerance of 0.5 Da within NCBInr Viridiplantae (Green Plants) (downloaded 2013.01.18; 1,162,105 sequences) and EST_Lettuce databases (downloaded on 2013.04.19; 1,763,496 sequences; 357736570 residues) using MASCOT (Matrix Science, www.matrixscience.com, London, UK). Variable modifications [Dioxidation (W), Oxidation (HW), Oxidation (M), Trp → Kynurenine (W)] and fixed modifications [Carbamidomethyl (C)] have been allowed for the database search. In general, a protein was considered identified when the protein scores were greater than 73 and were significant ($p < 0.05$). When a protein or peptide was identified based on an EST sequence or a protein with a trivial name, the protein sequence was used in a Blast alignment. Proteins were considered as being significantly identified when two individual non-identical peptides surpassed the threshold for identification or when one peptide resulted in a protein e-value of <0.005. All identifications were manually validated and extra data was acquired when insignificant identifications were obtained. The manual validations were done as previously described by Printz et al. (2013). For some spots, manual sequence determinations were performed and the sequences found used for cross-species identification with FASTS and MSBlast.

4.5. Statistical analysis

The statistical analysis of the fr. wt of the lettuce plants ($n = 10$) was conducted using the Mann-Whitney U test ($p < 0.05$) (*IBM® SPSS® Statistics version 21.0 for Mac OS X*). The remaining statistical analyses and plots were performed using *R software 2.15.1* (*R Core Team*, 2012). A multivariate analysis using PCA was performed after the data were pre-processed through missing value imputation (*sequential KNN* method, *R Environment*) and logarithm transformation. Significant differences between the groups in each PCA dataset were tested by ANOSIM test ($p < 0.05$). The *heatmap.2* function in the *gplots package* was used to generate the heat map. The clustering method that was used with this function was the default

method, which consists of hierarchical clustering using the complete linkage method operated with a matrix of dissimilarities calculated as *Euclidean* distances.

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