Early physiological and biochemical responses of rice seedlings to low concentration of microcystin-LR

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Abstract Microcystin-leucine and arginine (microcystin-LR) is a cyanotoxin produced by cyanobacteria like Microcystis aeruginosa, and it's considered a threat to water quality, agriculture, and human health. Rice (Oryzasativa) is a plant of great importance in human food consumption and economy, with extensive use around the world. It is therefore important to assess the possible effects of using water contaminated with microcystin-LR to irrigate rice crops, in order to ensure a safe, high quality product to consumers. In this study, 12 and 20-day-old plants were exposed during 2 or 7 days to a M. aeruginosa extract containing environmentally relevant microcystin-LR concentrations, 0.26–78 **1**g/L. Fresh and dry weight of roots and leaves, chlorophyll fluorescence, glutathione S-transferase and glutathione peroxidase activities, and protein identification by mass spectrometry through two-dimensional gel electrophoresis from root and leaf tissues, were evaluated in order to gauge the plant's physiological condition and biochemical response after toxin exposure. Results obtained from plant biomass, chlorophyll fluorescence, and enzyme activity assays showed no significant differences between control and treatment groups. How- ever, proteomics data indicates that plants respond to M. aeruginosa extract containing environmentally relevant microcystin-LR concentrations by changing their metabolism, responding differently to different toxin concentrations. Biological processes most affected were related to protein folding and stress response, protein biosynthesis, cell signalling and gene expression regulation, and energy and carbohydrate metabolism which may denote a toxic effect induced by M. aeruginosa extract and microcystin- LR. The implications of the metabolic alterations in plant physiology and growth require further elucidation.

Keywords Rice · Oryza sativa · Microcystin-LR · GPx · GST · Proteomics

Introduction

Cyanobacteria (blue-green algae) are photosynthetic prokaryotes. Some species are known to produce potent bio-active compounds. Cyanobacteria outbreaks are a frequent phenomenon, often associated with the deterioration of water quality (e.g. increased eutrophication) (Bibo et al. 2008; Saqrane et al. 2008). The release of cyanobacterial bioactive compounds and toxins leads to water contamination and increases the risk of animal and human expo- sure, thus constituting a major environmental hazard. The utilization of contaminated water in agriculture may pose additional risks concerning crop growth and production, and food safety

Microcystins (MCs) are the most frequent cyanobacte-rial toxins in freshwaters (Hitzfeld et al. 2000; Tyler et al. 2009; WHO 2003; Zhang et al. 2007). MCs are produced by species of the genera Microcystis, Anabaena, Planktothrix, Nostoc, Oscillatoria and Anabaenopsis (Dawson1998; Hitzfeld et al. 2000; Ibelings and Chorus 2007; Saqrane et al. 2008). These cyclic heptapeptides are specific inhibitors of the catalytic subunit of protein serine/ threonine phosphatases 1 and 2A (PP1, PP2A) (Hitzfeld et al. 2000; WHO 2003). The side chain of the unusual amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phe- nyldeca-4,6-diene acid (ADDA), and possibly the planar ring portion of the peptide, might be responsible for recognizing as well as inhibiting protein phosphatases, which makes these parts of the molecule essential for its biological activity. MCs have been also referred to as inducing of oxidative stress in animals and plants (Bibo et al. 2008; Crush et al. 2008; Dawson 1998; Saqrane et al. 2007, 2008; WHO 2003). Moreover, MCs possess tumour-promoting activity and may be important liver carcinogens (Dawson 1998; Hitzfeld et al. 2000). More than 80 MC chemical congeners have been identified, being MC-LR one of the predominant variants, containing leucine and arginine as variable amino acids at positions 2 and 4, respectively (Ibelings and Chorus 2007; WHO 2003).

The toxic effects of MCs in plants have also been characterized. MCs have been associated with the inhibition of germination and growth of several agricultural and forage plants (Crush et al. 2008; El Khalloufi et al. 2011; Saqrane et al. 2008). MCs were shown to inhibit the nodulation of fava bean (Vicia faba) and alfalfa (Medicago sativa) (El Khalloufi et al. 2011; Lahrouni et al. 2012). This condition may have implications in nitrogen uptake and development of leguminous plants. Alterations in root architecture and development have been reported for tis-sue-cultured common reed (Phragmites australis) challenged with 0.5–40 **1**g/mL MC-LR. These effects may be attributed to the activity of MC-LR on microtubule organization (Máthé et al. 2009). This work underlines the dose and time effects of exposure to the toxin. For instance, 2 day exposure to 10–40 **1**g/ml MC-LR caused inhibition of mitosis in roots of common reed (Phragmites australis). However, prolonged exposure (20 days)

stimulated or inhibited mitosis in plants according to the toxin concentration, 0.1–5 or 20–40 1g/ml MC-LR, respectively (Máthé et al. 2009).

Research has also highlighted that plants may take up and accumulate MCs. For instance, application of 2.1 mg/L MCs for 15 days to ryegrass, clover, rapeseed, and lettuce plants led to the accumulation of 0.2–1.45 mg/kg toxin (dry weight, DW) in the root system (Crush et al. 2008). Moreover, Peuthert et al. (2007) reported average bioconcentration factors (BCFs) for MCs of 3.6 and 8.4, in shoots and roots respectively, from 11 agricultural species. The presence of MCs in the leaves of crop plants is consistent with the hypothesis of the presence of a root-to-shoot transport mechanism for MCs (Peuthert et al. 2007). The results also suggest that MCs accumulation in shoots, roots and leaves is time and concentration dependent (Peuthert et al. 2007; Saqrane et al. 2007).

Furthermore, data gathered regarding chlorophyll content, PSII activity, lipid peroxidation and the antioxidant-response enzymes glutathione-S-transferase, glutathione peroxidase, and glutathione reductase provided evidence that photosynthesis and oxidative stress play a primary role in MC mediated plant toxicity and growth impairment (Saqrane et al. 2009; Peuthert et al. 2007; Pflugmacher et al. 2007). In addition to these, other molecular effects have been reported. Total activity of protein phosphatase was reduced in alfalfa plants after exposure to 5 1g/L MC-LR, MC-LW or cyanobacterial cell extracts containing equal concentration of MC-LR. This enzyme activity was followed by a MC-LW specific induction of MsPP1c and the MsPP1e gene transcripts (Peuthert et al. 2008). Isoenzyme specific single-stranded DNA (ssDNase) and double- stranded DNA (dsDNase) cleaving activities were reported in root tip meristematic cells of Phragmites australis exposed for up to 20 days to MC-LR (0.5–40 1g/mL). Enzyme activity was consistent with the early induction of chromatin condensation and the subsequent occurrence of necrotic cell death providing evidence for the role of these enzymes in MC induced programmed cell death (Jámbrik et al. 2011).

Semi-aquatic cultures may be among the most susceptible to cyanotoxin exposure. In this regard, MCs were detected in water chestnuts and rice grains produced at Lake Tai in China, frequently contaminated with heavy cyanobacterial blooms (Chen et al. 2012; Xiao et al. 2009). Rice is the most important staple crop in the world; however, little data has been reported regarding the effects of cyanotoxins in this culture. Chen et al. (2012) have recently reported that high MC-LR concentration (C2.0 1g/mL) affects rice root morphogenesis by inhibiting root elongation, crown root formation, and lateral root development from primordia. Treatment with high concentrations of MC-LR stimulated the production of reactive oxygen species (ROS) and inhibited the production of nitric oxide (NO) in rice roots. In contrast, lower toxin concentrations (0–200.0 1g/L) did not affect rice root growth (Chen et al. 2004). Prieto et al. (2011) reported that the exposure of rice plants to a mixture of Aphanizomenon ovalisporum and Microcystis aeruginosa cell extracts containing the cyanotoxins cylindrospermopsin and MC-LR resulted in a significant increase in glutathione S-transferase and glutathione peroxidase activities, suggesting a synergistic effect of both extracts.

The objective of the present work was to examine the effects of M. aeruginosa extracts with MC-LR concentrations between 0 and 78 1g/L, which cover the concentrations more commonly found in the environment (van Apeldoorn et al. 2007; Campos et al. 2013). This approach will contribute to an enhancement of our understanding of the impact of cyanobacterial toxins on this important crop. Since most studies focus on the toxicological effects produced by high concentrations of MC-LR, this work attempted to determine whether low, environmentally relevant toxin concentrations are deleterious to rice plants or not. Plants were exposed to M. aeruginosa extracts during 2 or 7 days, to mimic short and semi-chronic exposure. 12 and 20 day-old plants were considered to study the effects of MC-LR in early stage of development of this crop. Rice plants were analysed regarding biomass, chlorophyll fluorescence, GST and GPx activity, and protein expression analysis by two-dimensional gel electrophoresis (2DE) and protein identification by matrix-assisted laser desorption/ionization time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry. Biochemical data was gathered in order to register effects that may not be observed by standard physiological parameters. Significant changes in rice proteome are reported, highlighting the cellular stress induced by toxic M. aeruginosa extracts in rice seedlings.

Materials and methods

Cyanobacteria culture and toxic extract preparation

Microcystis aeruginosa (LEGE 91094) cultures were established as described by Saker et al. (2005) under sterile conditions in 6 L glass containers with 4L of Z8 medium (Kótai 1972), at 25 °C, under 22 1Em^{-2} s^{-1} light intensity, and a light/dark period of 14/10 h.

After 4 weeks of growth, biomass was collected by centrifugation at 4 °C, during 15 min, at 4,4959g and thereafter lyophilized (Pinheiro et al. 2013). Freeze-dried M. aeruginosa cells were extracted in aqueous methanol 50 % (v/v) through continuous stirring for 20 min at room temperature. The sample was then sonicated in a water bath for 15 min at room temperature and subsequently ultrasonicated in an ice bath at 60 Hz (VibraCell 50-sonics & Material Inc. Danbury, CT, USA) with five cycles of 1 min. The resulting slurry was centrifuged at 10,0009g for 15 min to remove cell debris. The supernatant was then dried by

rotary evaporation at
35 °C. The resulting residue was then resuspended in 40 mL of distilled water and the extract was stored at −20 °C prior to use. MC-LR concentration was determined by HPLC–DAD as described in section "Detection and quantification of microcystin-LR".

Detection and quantification of microcystin-LR

MC-LR from M. aeruginosa toxic extracts was quantified by high performance liquid chromatography (HPLC-DAD) based on the procedure described by Ramanan et al. (2000). The linear gradient elution consisted of (A) MeOH ? 0.1 % trifluoroacetic acid and (B) H_2O ? 0.1 % TFA (55 % A and 45 % at 0 min, 65 % A and 35 % B at 5 min, 80 % A and 20 % B at 10 min, 100 % A at 15 min, 55 % A and 45 % B at 15.1 and 20 min) with a flow rate of 0.9 mL/min. The injected volume was 40 1L. The DAD range was 210–400 nm, with a fixed wavelength at 238 nm. The MC-LR was then separated on a Merck Lichrospher RP-18 endcapped column (250 mm 9 4.6 mm i.d., 5 1m) equipped with a guard column (4 9 4 mm, 5 1m) both maintained at 45 °C. Calculations were made using the external standard method ($y = 53456 \cdot x - 22{,}587$; $R^2 = 0.99701$).

The cyclic peptide was also analysed by the analytical method of MALDI-TOF/TOF using the procedure described for protein identification ("Protein identification" section). MC-LR was detected by searching the peak signal at 995.0 m/z.

Rice cultures

Peeled Albatros variety rice seeds were sterilized and washed with hydrogen peroxide 10 % (v/v) during 10 min to prevent fungal and bacterial growth. Seeds were then washed three times with sterile distilled water during 10 min, treated with ethanol 70 % (v/v) for 5 min, rinsed with distilled water, and subsequently hydrated for 1 h in sterile distilled water under agitation (Miché and Balandreau 2001).

Seeds were germinated for 12 days in glass trays with wet filter paper and sealed with plastic wrap, at 25 °C and under 22 1Em^{-2} s^{-1} light intensity with a light/dark period of 14/10 h. Water was used on the first 3 days of incubation and then changed to Yoshida medium (Yoshida et al. 1976). The medium was replaced every 3 days from then on. Rice seedlings with 12-days and 2–3 young leaves were subsequently grown in hydroponic cultures at 23 °C and 133 1Em^{-2} s^{-1} light intensity with a light/dark period of 14/10 h. Rice was grown in 750 mL Yoshida medium renewed every 2–3 days. Plant growth and oxidative stress studies were thereafter undertaken with 12- and 20-day-old rice plants.

Plant exposure to toxic M. Aeruginosa extract

The following plant exposure experiments to toxic M. aeruginosa extracts were undertaken:

1. 20-day-old rice plants were exposed to 0.26; 13 and 78 1g/L MC-LR crude extracts, applied to the nutrient solution, for 2 days.
2. 20-day-old rice plants were exposed to 0.26; 13 and 78 1g/L MC-LR crude extracts for 7 days. The nutrient solution and the toxic extracts were replaced twice.
3. 12-day-old rice seedlings were exposed to 13 and 78 1g/L MC-LR crude extracts, applied to the nutrient solution, for 2 days.

Experiments were performed in triplicate (n = 3) and control groups were maintained with nutrient solution without toxic extracts (0 1g/L MC-LR). At the end of the experiments, PSII fluorescence was measured and 3 groups of roots and leaves from each experimental replicate collected separately, weighted, frozen in liquid nitrogen, and kept at −80 °C until being processed for enzymatic assays or for proteomic analysis (performed only on the experiment with 12-day-old plants). Although high concentrations of MCs have been reported in cyanobacterial blooms, the range of concentrations selected for this study are more frequently observed in water bodies, facilitating the comparison of the laboratorial studies with real case scenarios (Oh et al. 2001; Rivasseau et al. 1999; Sivonen et al. 1999). Acute (2 days) and sub-chronic (7 days) periods of expo- sure could provide further insight into the tolerance of plants to toxic cyanobacterial compounds and the physiological and biochemical responses.

Chlorophyll fluorescence

(10,0009g, 4 °C, 20 min) and the supernatant kept at −80 °C. Protein concentration was determined by the Bradford method (Bradford 1976).

GPx activity was determined through the methods described by Flohé and Gunzler (1984) and Lawrence and Burk (1976) by measuring the consumption of NADPH. The samples were diluted to 0.4 mg/mL of protein and 100 1L of diluted sample were added to each well of a 96 well microplate. The reaction solution with GSH (1 mM), NADPH (0.1 mM), GR (0.08 %, v/v) in phosphate buffer (0.08 M) pH 7.5 was prepared and 80 1L added to each well. To initiate the reaction, 20 1L of 0.0075 % (v/v) H_2O_2 were added to the samples. The activity was monitored by registering the absorbance at 340 nm every 20 s during 5 min in a microplate reader (BioTek, Synergy ST, Winooski VT, USA).

GST activity was determined through the method described by Habig et al. (1974), by measuring the formation of the complex between glutathione and the enzyme substrate 1-chloro-2,4-dinitrobenzene (CDNB). For this assay, the samples were diluted to 0.3 mg/mL of protein, and 100 1L of diluted sample were added to each well. The reaction

solution with CDNB (10 mM), GSH (60 mM) in phosphate buffer (0.3 M) pH 6.5 was prepared and 200 1L were added to each well to start the reaction. GST activity was monitored as described for GPx assay.

GPx and GST activities were determined using the following expression

$$\text{Activity}\left(\text{nkat.mg}^{-1}_{\text{protein}}\right) = \frac{\Delta\text{Abs}/\Delta t(\text{min}^{-1}) \times V_{\text{reaction}}(\text{mL})}{\varepsilon(\mu\text{mol}^{-1}.\text{mL.cm}^{-1}) \times L(\text{cm})} \times \frac{1}{C_{\text{sample}}(\text{mg.mL}^{-1}) \times V_{\text{sample}}(\text{mL})} \times \frac{1000}{60}$$

ΔAbs—Absorbance variation at 340 nm;
Δt—Time variation in min;
ε—Molar extinction coefficient (GPx assay: 6.22 μmol^{-1} mL cm^{-1}; GST assay: 9.6 μmol^{-1} mL cm^{-1});
L—Path length;
$V_{reaction}$—Total volume of the reaction solution;
C_{sample} – Concentration of the sample added to the reaction solution;
V_{sample}—Volume of the sample added to the reaction solution.

Chlorophyll fluorescence in 20-day old plants was determined through pulse amplitude modulation (PAM) fluorometry with PAM 2000 (Walz, Effeltrich, Germany). Plants were dark adapted for at least 30 min, and leavessubsequently illuminated with a pulse of saturating light. The fluorescence emitted was immediately recorded by the instrument. This procedure allows measuring the maximum fluorescence yield of photosystem II (Fv/Fm) that is directly related with the functional state of the PSII protein complex and the photosynthetic efficiency of the plants (Maxwell and Johnson 2000). This procedure was repeated for each replicate of each treatment, and for the control.

GPx and GST activity determination

To assess oxidative stress in plants, activities of glutathione S-transferase (GST) and glutathione peroxidase (GPx) were determined. For protein extraction, plant tissues were disrupted in a mortar with the aid of liquid nitrogen, and homogenized with 1 mL of phosphate buffer 0.08 mol L^{-1} (pH 7.5) per 0.2 g of tissue. The mixture was centrifugedDAbs—Absorbance variation at 340 nm;
Dt—Time variation in min;
e—Molar extinction coefficient (GPx assay: 6.22 1mol^{-1} mL cm^{-1}; GST assay: 9.6 1mol^{-1} mL cm^{-1});
L—Path length;
$V_{reaction}$—Total volume of the reaction solution;
C_{sample} – Concentration of the sample added to the reaction solution;
V_{sample}—Volume of the sample added to the reaction solution.

Protein extraction from rice tissues for two-dimensional gel electrophoresis

Plant tissues were macerated in liquid nitrogen and proteins precipitated using 2 mL of cold acetone precipitation solution with TCA (10 %, v/v) and b-mercaptoethanol (0.07 %, v/v) per 0.1 g of tissue (Campos et al. 2012). The mixture was kept at −20 °C for 1 h and then centrifuged at 4,4959g for 25 min at 0 °C. The protein pellet was resuspended in equal volume of acetone washing solution with b-mercaptoethanol (0.07 %, v/v). The mixture was stored during 1 h at −20 °C and centrifuged in the same conditions. The supernatant was discarded and the protein pellet dried in a rotating evaporator (Acid Resistant Centrivap Concentrator and Centrivap Cold Trap, LAB-CONCO, Kansas City, MO, USA) for 30 min. The protein pellet was solubilized in 2 mL of resolubilisation buffer with urea (7 M), thiourea (2 M), CHAPS (4 %, w/v), DTT (65 mM), ampholytes pH 3-10 (0.8 %, v/v) per 0.1 g of pellet (Campos et al. 2012). Protein solubilisation was undertaken for 1 h and then centrifuged at 10,0009g during 30 min and 4 °C. Protein supernatants were stored at −80 °C. Total protein content was determined through a Bradford assay.

First dimension gel electrophoresis: isoelectric focusing (IEF)

Protein samples (150 1g) were diluted to 125 1L resolubilisation buffer. Samples were centrifuged at 10,0009g during 10 min and loaded in 7 cm and 4–7 pH range IEF gel strips (Bio-Rad, Hercules, CA,USA). Proteins were there- after separated according to their isoelectric point (pI) in a Protean IEF cell (Bio-Rad) with the following program: 16 h at 50 V (active strip rehydration); step 1, 15 min at 250 V; step 2, 2 h voltage gradient to 4,000 V (linear ramp); step 3,
4,000 V until achieving 10,000 V/h (linear ramp). After the first dimension, IEF gel strips were stored at −20 °C until performing the second dimension, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Campos et al. 2012).

Second dimension gel electrophoresis: SDS-PAGE

After IEF, the gel strips were equilibrated using 10 mg/mL dithiothreitol followed by 25 mg/mL iodoacetamide in urea (6 M), glycerol (30 %, v/v), SDS (2 %, w/v) as described in Campos et al. (2009). This procedure reduces and alkylates the proteins. The IEF gel strips were quickly inserted on top of 12 % (w/v) acrylamide SDS-PAGE slab gels (size 8.3 cm 9 7 cm). A solution of melted agarose 0.5 % (w/v) was used to seal the gel

strip in the second dimension gel. Proteins were thereafter separated in a miniProtean II cell (Bio-Rad) at 150 V. After electrophoresis, gels were stained with Colloidal Coomassie Blue (Heinemeyer et al. 2007; Neuhoff et al. 1988).

Gel analysis

2DE Gel images were acquired with a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) and protein spots were detected automatically with PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA, USA). Sensitivity parameters were reproduced for each gel image, and spot detection and matching were done manually. Protein spot intensities were normalized in terms of the total den- sity in the gel image. For protein expression analysis, a master gel was obtained in the software, detecting all the spots present in the 2DE gel images. The presence or absence of spots and quantitative variations in spot intensities were analysed, comparing each spot's intensity in each of all three experimental groups with each other. Only spots that were detected in at least two replicate gels were taken into consideration. Quantitative variations in spot intensity were statistically validated using t Student test (P B 0.05). Three gels were done regarding each experimental condition ($n = 3$).

Protein identification

Differentially expressed proteins were excised from the 2DE gels and in gel trypsin digested following the procedure described in Campos et al. (2012). The tryptic pep- tides were desalted and concentrated using reversed phase microcolumns (Gobom et al. 1999). The peptides were eluted directly onto the MALDI plate using the matrix a- cyano-4-hydroxycinnamic acid (5 mg/mL) prepared in acetonitrile (70 %, v/v) and TFA (0.1 %, v/v). Samples were analyzed by MALDI-TOF/TOF Mass spectrometry (4700 Proteomics Analyzer, AB SCIEX, Foster City, CA, USA). Peptide mass spectra data was collected in positive MS reflector mode in the range of 700–4,000 (m/z) and was calibrated internally using trypsin autolysis peptide peaks. Several of the highest intensity and/or relevant non-tryptic peaks were selected for MS/MS analysis. Proteins were identified by combination of the Peptide Mass Fingerprint and MS/MS peptide sequencing approaches following an already published procedure (Gomes et al. 2013) using the software GPS Explorer (Version 3.6; AB SCIEX). Proteins were searched in a locally stored copy of the two sections of the UniProtKB protein sequence knowledgebase (Swiss- Prot and TrEMBL, release 2011_12) using the Mascot search engine (Version 2.1.04) (Perkins et al. 1999). The search included peaks with a signal-to-noise ratio greater than 10 and allowed for up to two missed trypsin cleavage sites. For a match to be considered, a confidence interval (CI) of at least of 99 %, calculated by AB SCIEX GPS Explorer software, was required.

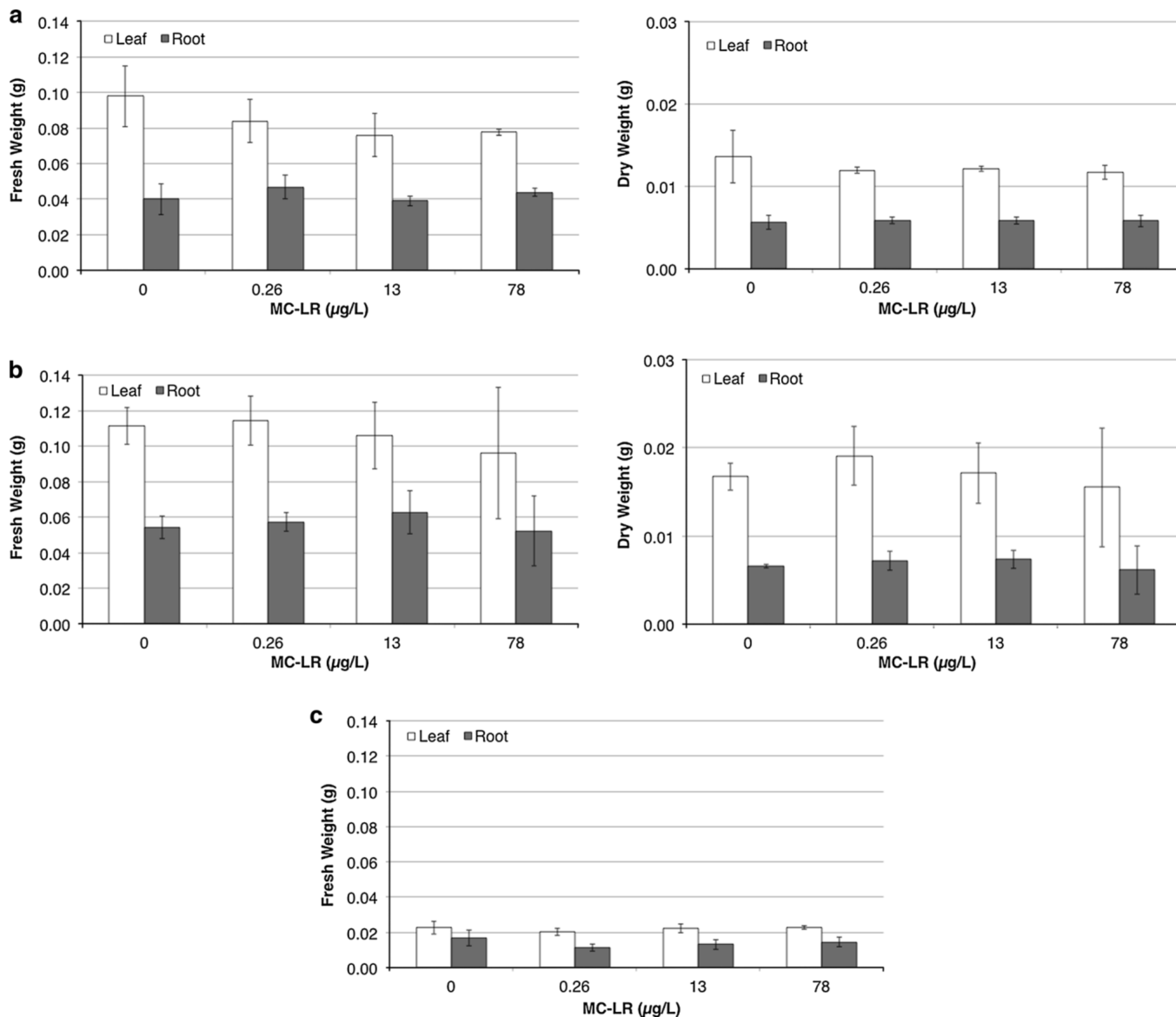

Fig. 1 Fresh weight and dry weight values from 20 days-old rice plants exposed for 2 days (a), and 7 days (b), and 12 days-old plants exposed for 2 days (c) to M. aeruginosa extract with MC-LR at 0.26, 13 and 78 **1**g/L. Results expressed as mean value ± standard deviation

Statistical analysis

For statistical analysis of the results obtained (n = 3) Student's t test was used, with a confidence limit of 95 % ($P < 0.05$).

Results

Rice growth parameters

No significant differences in root and leaf fresh and dry weights from 20-day-old rice plants exposed for 2 days to M. aeruginosa extracts were registered, as displayed in Fig. 1a. Increasing the time of exposure to M. aeruginosa extracts to 7 days did not affect rice growth (Fig. 1b), neither did exposing rice seedlings (12-days-old) to M. aeruginosa extracts with the same MC-LR concentrations for 2 days (Fig. 1c).

Rice photosynthetic efficiency

Maximum quantum yield of PSII is a reliable measurement of photosynthetic efficiency. It was registered here to complement the characterization of the physiological status of rice plants. No variations were reported in the maximum quantum yield of plants exposed to M. aeruginosa extracts during 2 days (Fig. 2a), suggesting that the presence of MC-LR in the nutrient solution, in an interval of concentrations between 0.26 and 78 **1**g/L, may not affect

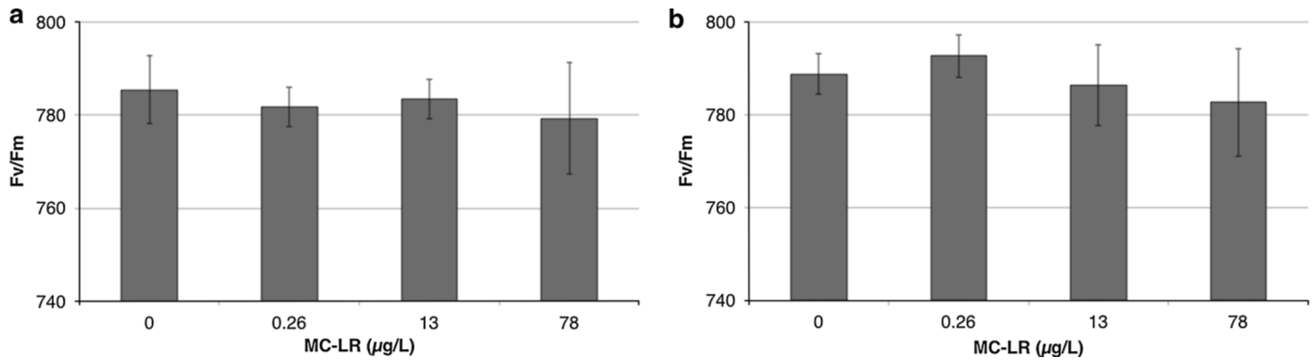

Fig. 2 Maximum fluorescence values (Fv/Fm) from 20 day-old rice plants exposed for 2 days (a), and 7 days (b) to M. aeruginosa extract, with MC-LR at 0.26, 13 and 78 **1**g/L. Results expressed as mean value ± standard deviation

Fig. 3 GPx and GST activities from 20-day-old rice plants exposed for 2 days (a), and 7 days (b), and 12-day-old rice plants exposed for 2 days to M. aeruginosa extract, with MC-LR at 0.26, 13 and 78 **1**g/L (c). Results are expressed as mean value ± standard deviation. Data significantly different from control on GPx activity assay (a) on roots (*) (Student's test, P ∖ 0.05)

photosynthesis. Moreover, increasing the time of plant exposure to M. aeruginosa extracts to 7 days did not alter the maximum quantum yield in rice plants (Fig. 2b).

GPx and GST enzyme activity

No significant differences in root and leaf GST activities were registered in 20-day-old rice plants exposed for 2 or 7 days to M. aeruginosa extracts (Fig 3a, b). However, it was possible to detect an increase in root-GPx activity during the acute exposure (2 days) to 0.26 and 13 μg/L MC-LR M. aeruginosa extracts (Fig. 3a). Regarding the other experimental conditions tested, GPx activity remained close to control levels (Figs. 3b, c). These results suggests that exposure to MC-LR in this range of concentrations have minimal impact on these enzymes' activity.

Rice proteomic analysis

2DE and MALDI-TOF/TOF mass spectrometry approaches were applied to study rice root and leaf proteomes. In order to increase the number of proteins covered by the study, a large fraction of the proteome was first separated over a broad pI interval (pI 4–7), and subsequently according to the molecular mass (MM) (from ~20 to 100 kDa). Only 12 day-old plants were considered for proteomics analysis since the results accomplished on GPx and GST activity and biomass (fresh weight data) suggest that there are no major differences in the response of plants in the two growth stages to M. aeruginosa extracts and MC-LR. Moreover, we expect this analysis to be sufficient to infer on whether MC-LR affects rice proteome or not. High resolution of protein spots was achieved in 2DE gels over the range of pI and MM intervals chosen (Fig. 4). Differential protein expression was analysed taking into consideration the quantitative variations in protein abundance. A summary of the proteome variations detected by this method is displayed in Fig. 5. 51 and 42 proteins were differentially expressed in roots and leaves, respectively, from exposed rice groups, in comparison to control. Moreover, variations between exposed groups comprised 116 root and 6 leaf proteins. Only 2 and 4 detected proteins from roots and leaves respectively, overlapped at the variations detected in 13 and 78 μg/L MC-LR exposed groups. The high number of differentially expressed proteins (DEP) associated to the rice root system might be related to the multiple metabolic processes putatively involved in the organ response to MC-LR and other M. aeruginosa bioactive compounds.

Protein identification and assignment of functional groups

In Table 1, the functions and expression levels of each identified protein are shown. 28 proteins were identified combining MS and MS/MS analysis; 9 in roots and 19 in leaves. The most representative protein groups in roots are involved in protein folding and stress response, and cell signalling and gene expression regulation, while in leaves protein folding and stress response, and energy and carbohydrate metabolism are the most representative protein functional groups (Table 1).

Functional groups specifically assigned to roots are cell signalling and gene expression regulation (14-3-3 protein and G-box binding factor) and cell structure (tubulin alpha-1). Instead, methylmalonate semi-aldehyde dehydrogenase, involved in inositol and propanoate metabolism, glutamine synthetase, a key enzyme from the nitrogen metabolism, and flavone 3′-O-methyltransferase, acting in flavonoid biosynthesis, were identified specifically in leaves. All these proteins were down-regulated in 13 μg/L MC-LR treated plants with the exception of glutamine synthetase, which was down-regulated in 78 μg/L MC-LR treated plants.

In roots from 13 μg/L MC-LR treated plants, all proteins were down-regulated in respect to control plants. Nevertheless, in roots from 78 μg/L MC-LR treated plants, all proteins were up-regulated in regard to 13 μg/L MC-LR and control plants. In leaves, the majority of proteins were down-regulated.

Six and three folding and stress response proteins were down-regulated in leaves from plants exposed to 13 and 78 μg/L MC-LR, respectively, in comparison with the control group. One cell death associated protein is up-regulated in leaves when comparing 78 μg/L MC-LR treated plants to the 13 μg/L MC-LR plants. In roots, chaperonin was down-regulated in 13 μg/L MC-LR treated plants in regard to control plants, and heat shock protein 83 was up-regulated when comparing the 78 μg/L MC-LR treatment with the control group.

Alpha tubulin, a cytoskeleton component, was down-regulated in roots of 13 μg/L MC-LR treated plants, contrasting the expression in 78 μg/L MC-LR treated plants.

14-3-3 protein, g-box binding factor, cell signalling and gene expression regulation proteins, were down-regulated in roots from 13 μg/L MC-LR plants in comparison to control and 78 μg/L MC-LR plants.

Pyruvate decarboxylase isozyme 2, sucrose synthase 1, sucrose synthase, fructokinase-2 and triosephosphate isomerase, are proteins acting in energy and carbohydrate metabolism. In leaves, sucrose synthetase 1 was down-regulated in both MC-LR groups, while the second isoform was up-regulated in the 13 μg/L MC-LR group only. Fructokinase and triosephosphate isomerase were down-regulated in 13 and 78 μg/L MC-LR groups, respectively. Root pyruvate decarboxylase was up-regulated in 78 μg/L MC-LR treated plants in regard to control and to 13 μg/L MC-LR plants.

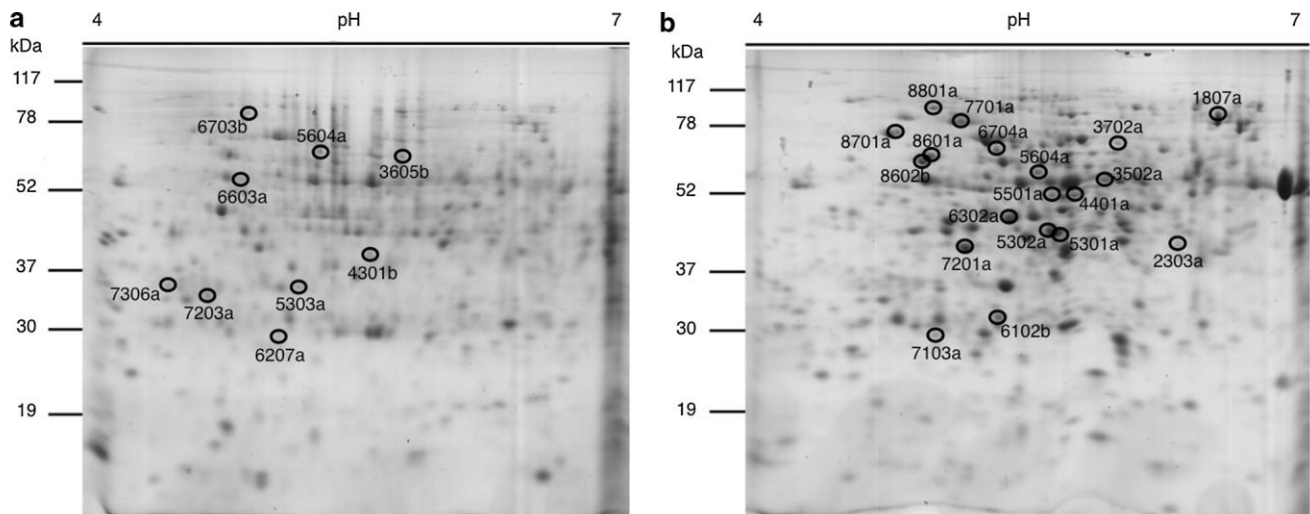

Fig. 4 Two-dimensional maps of rice root (a) and leaf (b) proteins from 12-day-old control plants. Total protein loaded was 150 1g and gels were stained with Colloidal Coomassie Blue. Proteins differentially expressed in 12-day-old plants exposed during 2 days to 0.0, 13 and 78 1g/L MC-LR (Table 1) are marked in the maps with a circle

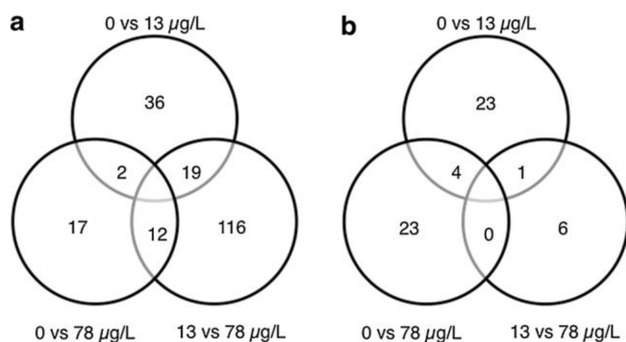

Fig. 5 Venn diagram representation of the number of differentially expressed proteins (DEP) detected after comparison of root (a) and leaf (b) proteomes from 12-day-old rice plants exposed for 2 days to 0.0, 13 and 78 1g/L MC-LR

60S acidic ribosomal protein, asparagine-tRNA ligase and protein disulfide-isomerase are involved in protein biosynthesis and folding. Asparagine-tRNA ligase and protein disulfide-isomerase were down-regulated in leaves from 13 1g/L MC-LR group in regard to the control. In roots, 60S acidic ribosomal protein was up-regulated in treatment 78 1g/L MC-LR in relation to control and to 13 1g/L MC-LR treated plants.

One uncharacterized protein was up-regulated in roots from 78 1g/L MC-LR group in regard to control and to 13 1g/L MC-LR groups. Other two uncharacterized proteins were up-regulated in leaves from 13 1g/L MC-LR group in regard to control.

Chloroplast PsbO2 and ATP-dependent zinc metalloprotease FTSH 2 chloroplastic proteins intervene in photosynthesis. Both proteins were down-regulated in roots and leaves from 13 1g/L MC-LR treated plants in regard to control plants.

Discussion

Physiological effects of toxic M. aeruginosa extract in rice plants

In none of the assays a significant difference in plant weight or photosynthetic efficiency between any of the treatments in comparison to their respective control groups was detected. It appears that exposure to a M. aeruginosa toxic extract in concentrations corresponding to MC-LR in a range of 0.26–78 1g/L, for 2 or 7 days, doesn't impair plant growth or inhibits photosynthetic efficiency.

A study was made in which rice and rapeseed plants were exposed to an extract in doses equivalent to 0, 24,000, 120,000, 600,000 and 3,000,000 1g/L MC-LR. In this study, seedling growth was inhibited, as well as the elongation of primary roots (Chen et al. 2004). However, the concentrations used were much higher than the ones in the present study, and the exposure was done on younger plants, which may have caused an increase in toxicity. Another study reports that rice plants exposed for 10 days to MC-LR in a range of 10–200 1g/L were not affected in regard to the number of crown roots and lateral roots (Chen et al. 2012). These results may be comparable to the absence of root growth inhibition obtained in the present work. In the same study, plant growth was significantly inhibited after 5 days of exposure to high concentrations, 500–4,000 1g/L MC-LR.

A study comparing the effects of exposure of rice and rapeseed plants to a mixture of MC variants found indications that rice plants may have evolved a mechanism that makes them more tolerant to MC toxicity (Chen et al. 2004). Although the growth of rice plants was impaired

due to

Table 1 Differential expression of identified proteins from root and leaf of 12-day-old rice plants exposed for 48 h to M. aeruginosa extracts containing MC-LR at 13 and 78 **1**g/L, in different functional groups

			Spot intensity							
			MC-LR (**1**g/L)							
Oryza sativa L.	Spot #	Protein identification	0.0	13		78			Accesion Number	Biological function
Root	6207	Chaperonin	287 ± 78	109 ± 64	;	249 ± 119			Q69Y99_ORYSJ	Protein folding, stress response
	6703	Heat shock protein 83	178 ± 112	217 ± 51		1121 ± 183	:	*	B6UCZ5_MAIZE	
	7203	14-3-3 protein	1107 ± 316	426 ± 106	;	1213 ± 210			Q10E23_ORYSJ	Cell signalling and gene expression regulation
	7306	G-box binding factor	609 ± 222	200 ± 58	;	612 ± 60		*	Q0JCM0_ORYSJ	
	3605	Pyruvate decarboxylase isozyme 2	315 ± 75	208 ± 37		523 ± 56	:	*	B7ERZ5_ORYSJ	Energy and carbohydrate metabolism
	4301	60S acidic ribosomal protein	886 ± 333	633 ± 260		1808 ± 217	:	*	J3MQ37_ORYBR	Protein biosynthesis and folding
	5604	Putative uncharacterized protein	558 ± 271	441 ± 129		1101 ± 170	:	*	B8BD19_ORYSI	Uncharacterized proteins
	5303	Chloroplast PsbO2	512 ± 145	141 ± 19	;	418 ± 104		*	I0B7J2_NICBE	Photosynthesis
	6603	Tubulin alpha-1 chain	776 ± 142	346 ± 146	;	1884 ± 846		*	Q10DN1_ORYSJ	Structural
Leaf	5301	Cell death associated protein	6329 ± 347	4063 ± 411	;	2665 ± 1926	;		Q6J657_ORYSJ	Protein folding, stress response
	5501	Cell death associated protein	850 ± 107	463 ± 17	;	751 ± 121		*	Q6J657	
	5604	TCP-1/cpn60 chaperonin	1135 ± 5	727 ± 50	;	884 ± 187			B8ALS4	
	7701	Heat shock protein 81-2	2286 ± 126	970 ± 375	;	880 ± 311	;		Q69QQ6	
	8701	70 kDa heat shock protein	1092 ± 102	836 ± 12	;	1070 ± 251			I1R581	
	8801	90 kDa heat shock protein	1156 ± 190	583 ± 223	;	845 ± 259			Q5Z9N8	
	8602	60 kDa chaperonin alpha subunit	2964 ± 246	2528 ± 948		2380 ± 191	;		Q2QU06	
	1807	Sucrose synthase 1	3812 ± 298	1479 ± 611	;	1392 ± 84	;		SUS1_ORYSJ	Energy and carbohydrate metabolism
	2303	Sucrose synthase	919 ± 125	1278 ± 144	:	1168 ± 112			A2XHR1_ORYSI	
	7201	Fructokinase-2	5047 ± 160	4388 ± 282	;	4367 ± 766			A2YQL4	
	6102	Triosephosphate isomerase	2269 ± 188	1864 ± 350		1789 ± 192	;		A2Z3G7_ORYSI	
	3702	Asparagine-tRNA ligase	624 ± 74	274 ± 108	;	548 ± 182			Q93WM3_ORYSJ	Protein biosynthesis and folding
	8601	Protein disulfide-isomerase	2653 ± 304	2136 ± 103	;	2386 ± 819			F4MG95	
	4401	Putative uncharacterized protein	908 ± 254	470 ± 83	;	679 ± 415			A5BI23_VITVI	Uncharacterized proteins
	7103	Putative uncharacterized protein	1060 ± 294	470 ± 151	;	705 ± 232			A2YMN1_ORYSI	
	3502	Methylmalonate semi-aldehyde dehydrogenase	1081 ± 9	583 ± 160	;	813 ± 230			Q6Z4E4_ORYSJ	Inositol, propanoate and nitrogen
	6302	Glutamine synthetase	4453 ± 401	3560 ± 753		3056 ± 345	;		Q0J9E0	metabolism
	6704	ATP-dependent zinc metalloprotease FTSH 2, chloroplastic	955 ± 242	505 ± 131	;	748 ± 241			Q655S1	Photosynthesis
	5302	Flavone 3^{0}-O-methyltransferase 1	2289 ± 600	952 ± 427	;	2333 ± 1991			Q6ZD89	Flavonoid biosynthesis

:: up-regulation in respect to control; ;: down-regulation in respect to control; *: up-regulation in respect to 13 1g/L (P \ 0.05)

exposure to MC at 120–3,000 1g/mL and the plants had their primary root growth inhibited, this effect can be partially overcome by developing more powerful lateral roots enabling the plant to take up nutrients and maintain the metabolism and growth (Chen et al. 2004). Fv/Fm values measured in this study lay between 780 and 817. This indicates the plants were not at their optimal state of growth (Baker and Rosenqvist 2004; Maxwell and Johnson 2000) and parameters such as temperature and light intensity need to be established for this rice variety. Despite this condition, rice plants showed a constant growth, and no significant differences in Fv/Fm were registered between control and exposure groups indicating that exposure to M. aeruginosa cell extract containing MC-LR does not cause photosystem II damage, or inhibition of plant's photosynthetic capacity.

Although the results obtained point to the tolerance of rice to M. aeruginosa extracts and MC-LR, this hypothesis should be taken with caution when considering field growing rice. One must bear in mind that field plants may be challenged by a combination of stress factors (e.g. pathogen attack, water contaminants, temperature stress, nutritional shortage, drought, etc.) reducing their ability to respond and cope with cyanotoxin's toxic activity. On the other hand, the hypothesis that growth inhibition effects induced by low toxin doses may arise with long term or chronic exposures cannot be discarded, and therefore this condition needs to be evaluated. In this aspect, 7-day exposure of rice up to 78 1g/L MC-LR could be insufficient to report toxicity based on plant biomass (FW and DW) and PS II fluorescence. Licopersicum esculentum growth inhibition (e.g. stem length and plant biomass, FW, variations) was reported by El Khalloufi et al. (2012) in 30-day exposure experiments to 2.22–22.24 1g/mL MC M. aeruginosa extracts. PS II fluorescence was less sensitive than plant biomass, varying only in plants exposed to the highest toxin dose (El Khalloufi et al. 2012). Lahrouni et al. (2012) reported differences in growth of rhizobia inoculated Medicago sativa (e.g. shoot and root DW variations) when exposed for 6 weeks to 100 1g/L MC M. aeruginosa extracts. Concomitantly, Saqrane et al. (2009) registered variations in FW and DW for Triticum durum, Zea mays, Pisum sativum and Lens esculenta plants exposed for 30-days to 0.5, 1.05, 2.1 and 4.2 mg equivalent MC-LR/mL. Moreover, in all plants tested, and at the various MC concentrations, treatment with cyanobacterial extract produced a decrease in the Fv/Fm in regard to control. Fv/Fm decline as well as growth, was dose and plant species-dependent (Saqrane et al. 2009).

Biochemical response of rice plants exposed to toxic M. aeruginosa extract

The enzyme GPx scavenges peroxides, which can arise in an organism exposed to environmental stress due to the presence of ROS, using reducing agents, such as the tripeptide glutathione (Miao et al. 2006; Navrot et al. 2006). When a plant is under oxidative stress, phospholipid hydroperoxide glutathione peroxidase (PHGPx), an enzyme in the GPx family that has been reported to be present in plants, is up-regulated (Eshdat et al. 1997; Faltin et al. 2010). PHGPx is a member of the GPx family, and it constitutes a defence against lipid peroxidation, since it can reduce phospholipid hydroperoxides and complex hydroperoxy lipids from biomembrane lipid layers, as well as H_2O_2 and other organic hydroperoxides (Faltin et al. 2010; Navrot et al. 2006). A study in which tobacco BY-2 cells were exposed to 50,000 1g/L of MC-RR showed the gradual increase of GPx activity in a time dependent manner (Yin et al. 2005). Again, the exposure concentration used is very high, unlike the range used in the present study. In this work the only instance in which exposure to MC-LR producing M. aeruginosa cell extracts seemed to have an effect in GPx activity was when 20-day-old rice plants were exposed for 2 days to M. aeruginosa extract with 0.26 and 13 1g/L MC-LR. No significant differences in regard to control groups were found in other treatment groups, which can be attributed to the putative activation of defense mechanisms (e.g. systemic acquired resistance, SAR) in 20 day-old-plants exposed for 7 days to MC-LR, or to an elevated activity of GPx in 12-day-old plants.

GST is used as an indicator of physiological stress in an organism because it plays an important role, for example, in the oxidative stress defence and in detoxification of a variety of xenobiotic compounds, by binding glutathione to substrates, making them more polar and less reactive (Lee et al. 2011; Marrs 1996). In plants, it's involved in the detoxification of herbicides and pesticides, organic pollutants and naturally occurring toxins (Lee et al. 2011). GST protects cells from a wide range of stresses, including oxidative stress (Lee et al. 2011; Marrs 1996; Pflugmacher 2004). In the specific case of an organism exposed to MCs, GST seems to be involved in the toxin detoxification (Bibo et al. 2008; Crush et al. 2008; Saqrane et al. 2007). A study in which Arabidopsis thaliana suspension cells were exposed to 1,000 and 5,000 1g/L MC-RR for 2 days showed no GST activity variation in regard to control (Yin et al. 2005). It's of notice that this range of concentrations is much higher than the range used in this work (0.26–78 1g/L MC-LR). However, results from a study in which several spinach variants were exposed to low concentrations of MCs (0.5 1g/L) from a crude cell extract, both microsomal GST and soluble GST activities were elevated in all variants except for one, the increase being by 6–146 % in comparison to the control groups (Pflugmacher et al. 2007). Values for GST activity in control groups were 0.20–0.30 nkat/mg protein in microsomes, and between 1.00 and 2.00 nkat/mg protein in the cytosol, and in treatments they lay in a range from 0.30 to 0.80 nkat/mg protein in microsomes, and from 1.00 to 3.5

nkat/mg protein in the cytosol (Pflugmacher et al. 2007). Taking this into account, it would be expected to observe an increase in GST activity in rice plants exposed to M. aeruginosa cell extracts. On the other hand, in the present study, exposure of rice plants to 0.26–78 **1**g/L of MC-LR to a M. aeruginosa extract did not seem to influence GST's activity. Values of GST activity in leaves, both in control and treatments, fall in a range of 0.2–3.2 nkat/mg protein, with no significant differences between control and exposure groups which points to this toxin not having a detrimental effect on rice plant's oxidative stress level, nor on its need for increasing its detoxification mechanisms.

Taking into account the results obtained in GPx and GST enzymatic assays, we may consider that the used concentrations of MC-LR in M. aeruginosa extract did not induce oxidative stress in rice plants. It's possible that GPx and GST might not be the best biomarkers of physiological stress in rice. GST may lack sensitivity towards MC (Bibo et al. 2008; Lee et al. 2011; Marrs 1996; Saqrane et al. 2007), while GPx activity is low and its function poorly characterized in plants (Faltin et al. 2010; Navrot et al. 2006). Measurement of catalase, superoxide dismutase, glutathione, or products of lipid peroxidation, or protein phosphatase bioassays could be used in further assays to study the extension of oxidative stress in rice plants caused by exposure to MC-LR (Asensi et al. 1999; Chen et al. 2004; Lambert et al. 1994; Oh et al. 2001; Vinagre et al. 2012). In this regard, mass-spectrometry based sensitive methods have been suggested for the identification and quantitation of oxidative stress parameters and oxidation products (Lee and Britz-McKibbin 2009; Levison et al. 2013). On the other hand, the lack of response of the two biochemical markers may have been due to a poor capacity of the plant to take up and accumulate the toxin. In such a hypothesis, the toxicity induced inside the cells would be supressed with the basal GPx and GST levels. The research has been also emphasizing the genetic factors that contribute to the differences in GST and GPx activities between species and varieties. Since the enzymatic activities showed no response to the treatments, a more sensitive proteomics approach was employed to investigate further effects on plant metabolism.

Results obtained from proteomic analysis show that there were proteins with altered expression in plants exposed to toxic M. aeruginosa extract containing MC-LR (13 and 78 **1**g/L) in comparison to the control groups. Another important point of this study, revealed by proteomics, is that different concentrations of the toxin in the medium may trigger different biochemical responses in plants.

Chaperonins and heat shock proteins are known to act in protein folding and to respond to a variety of stresses, being, therefore, recognized as important biomarkers of cellular stress. Several members of this class and two cell death associated proteins were down-regulated in roots and leaves of MC-LR treated plants, which may indicate that processes related with protein stability and function are affected and may impair plants' homeostasis (Wang et al. 2004). HSP83, which was an exception, was markedly over-expressed in roots of plants exposed to the highest toxin concentration. The down-regulation of a tubulin isoform in the lower MC-LR exposure concentration might indicate that structural alterations occur in root tissues under this exposure condition. The results also show that different MC-LR concentrations might induce different biochemical responses and particularly in the roots with HSP83 and proteins with functions in cell signalling and gene expression regulation, carbohydrate metabolism and protein biosynthesis displaying significant variations between treatments.

In rice leaves, processes related with protein folding and stress response, energy and carbohydrate metabolism are likely to be affected to a different extent in MC-LR treated plants, as assessed by the down-regulation of several protein markers. Variations in FTSH 2 and PsbO2 proteins would presuppose an alteration in the photosynthesis reactions and thereby in the levels of chlorophyll fluorescence. Nevertheless, this parameter was not altered in treated plants, which may evidence the low impact of M. aeruginosa extract and MC-LR in photosynthesis or, instead, the low sensitivity of this parameter to evaluate more subtle biochemical alterations. Other putative down-regulated processes assigned specifically to 13 **1**g/L MC-LR treated plants are protein flavonoid biosynthesis, inositol, propanoate and nitrogen metabolism. Together, the results point to a decrease in the metabolism of rice, which may denote a putative toxic effect induced by M. aeruginosa extract and MC-LR.

Conclusions

In this work it was observed that rice plants exposed to 0–78 **1**g/L MC-LR, during 2 and 7 days did not suffer major alterations in biomass and chlorophyll fluorescence. Moreover, the lack of variations in GST and GPx activities in plants challenged with M. aeruginosa extracts, suggests no major alterations in rice antioxidant mechanisms and the putative absence of oxidative stress. However, this assumption should be supported by additional data provided by alternative techniques to measure oxidative stress. The possible lack of sensitivity of the conventional parameters to measure toxicity was then covered with an investigation of the rice proteome. Proteomics analysis on 12-day-old plants exposed for 2 days to M. aeruginosa extracts (13–78 **1**g/L MC-LR) showed significant alterations in protein expression, with emphasis on protein folding and stress response members, energy, carbohydrate and nitrogen metabolism, and protein biosynthesis. The physiological implications associated to this metabolic outcome require further elucidation. Physiological and growth responses may be only registered by performing

longer exposure experiments (chronic exposures).

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