

Physiological and biochemical defense reactions of *Vicia faba* L.–*Rhizobium* symbiosis face to chronic exposure to cyanobacterial bloom extract containing microcystins

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Abstract The presence of cyanotoxins, mainly microcystins (MCs), in surface freshwater represents a serious health risk to aquatic organisms living in the water body, as well as terrestrial animals and plants that are in contact with contaminated water. Consequently, the use of MCs contaminated water for irrigation represents a hazard for cultivated plants and could induce severe economical losses due to crops' yield reduction. The experimental approach undertaken in this work was exposing *Vicia faba* seedlings (inoculated with a *Rhizobium* strain resistant to MCs), to water supplemented with cyanobacterial crude extract containing total microcystins at a concentration of 50 and 100 µg/L (environmental relevant concentrations of MCs dissolved in the raw irrigation water from Lalla Takerkoust Lake-Marrakesh region). After chronic MCs exposure (2 months), biological and physiological parameters (plant growth, nitrogen uptake, mineral assimilation, and oxidative defense mechanisms) were evaluated. The results obtained showed evidence that chronic exposure to cyanobacterial bloom extract containing MCs strongly affected the physiological and biological plants activities; reduction of dry matter, photosynthetic activity, nodule number, and nitrogen

assimilation. At the same time, an increase of oxidative stress was observed, as deduced from a significant increase of the activities of peroxidase, catalase, polyphenoloxidase, and phenylalanine ammonia lyase in leaves, roots, and nodules of faba bean plants exposed to cyanotoxins, especially at 100 µg/L of MCs. This experimentation constitutes a simulation of the situation related to cyanotoxins chronic exposure of seedlings—plants via the contaminated irrigation water. For this reason, once should take into consideration the possibility of contamination of agricultural crops and the quality of irrigation water should be by the way monitored for cyanotoxins biohazard.

Keywords Faba bean · Cyanotoxins · Microcystins-LR · Oxidative stress · Rhizobia · Symbiosis · Antioxidant defense

Abbreviations

CAT	Catalase
Fv/Fm	Ratio of variable to maximum fluorescence—the quantum efficiency of open photosystem II centers
Fm	Maximum fluorescence
F0	Minimum fluorescence yield
HPLC-PDA	High-performance liquid chromatography coupled to a photodiode array detector
MC	Microcystins
MC-LR	Microcystin-LR
NN	Number of nodules
NDW	Nodule dry weight
PAL	L-phenylalanine ammonia lyase
POD	Peroxidase
PPO	Polyphenoloxidase
PSII	Photosystem II

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RDW	Root dry weight
RL	Root length
TPC	Total polyphenols content

Introduction

In Morocco, as well as in many countries located in arid and semi-arid areas, irrigation with raw water from lake reservoirs is a common agricultural and horticultural practice, and an important tool for farmers to improve crop yields. This kind of surface water bodies can contain potentially toxic bloom-forming cyanobacteria, and its use as a source for irrigation water can enable a transfer of cyanotoxins into crop plants (Peuthert et al. 2007; Saqrane and Oudra 2009). In the Marrakech region, Lalla Takerkoust reservoir is an aquatic ecosystem known by frequent and massif proliferations of the toxic cyanobacterium *Microcystis aeruginosa* during late-summer and autumn. The bloom toxicity, diversity of microcystins (MC) variants, and MCs cell quota are being previously monitored since 1994 (Oudra et al. 2001, 2002; El Ghazali et al. 2011). The most common referenced cyanotoxins are microcystins. These cyclic heptapeptides occur as more than 70 different congeners (Zurawell et al. 2005). Among them, microcystin-LR is known to be a potent inhibitor of the protein phosphatase 1 and 2A (PP1 and PP2A) of mammals and higher plants. It has been shown that cyanotoxins are taken up by seedlings (Peuthert et al. 2007) and their toxicity has already been demonstrated in terrestrial plants (Abe et al. 1996; McElhiney et al. 2001; Pflugmacher et al. 2006; Pichardo and Pflugmacher 2010; El Khalloufi et al. 2011; Lahrouni et al. 2012) and aquatic plants (Vassilakaki and Pflugmacher 2007; Kinnear et al. 2008; Saqrane et al. 2009), causing inhibitory effects on development, root growth, and photosynthesis.

First step in cyanotoxins detoxification is the formation of a glutathione conjugate (Pflugmacher et al. 1998). In this context, the activity of glutathione-S-transferase has been shown to increase upon exposition of plants to cyanotoxins (Mitrovic et al. 2004; Pflugmacher et al. 2007).

Furthermore, as a result of exposure to these microcystins, reactive oxygen species (ROS) can be generated (Pflugmacher et al. 2007), which react with other cellular compounds such as lipids, proteins, and DNA (Esterbauer et al. 1991). Plants have the ability to decrease toxins exposure, a well-elaborated antioxidative defense system of plants' functions relieves the negative effects caused by ROS. The antioxidant defenses comprise enzymatic and nonenzymatic mechanisms. Some of the key enzymes of the antioxidant defense are catalase, peroxidase, and superoxide dismutase (Mitrovic et al. 2004; Pflugmacher et al. 2007; Saqrane et al. 2007). Enzymes of the glutathione–ascorbate cycle, such as glutathione reductase, are also involved in the detoxification of ROS produced upon exposure to cyanotoxins (Pflugmacher 2004; Pflugmacher et

al. 2007). Besides redox enzymes, several compounds also participate in alleviating the oxidative stress; the pool of oxidized/reduced glutathione levels are adjusted (Pflugmacher 2004) and phenolic compounds also play an important role in the detoxification of free radicals (Ksouri et al. 2007).

Faba bean (*Vicia faba*) is an important legume plant that is widely cultivated, generally in Morocco and particularly in the Marrakech region, and used for human and animal nutrition (MADRP/DERD 2006). It has a high nutritional value as a consequence of its antioxidative capacity, providing high yield of proteins and vitamins (Bond et al. 1985).

The aim of the present study is examining the long-term effects of water contaminated with MCs on the *V. faba*–*Rhizobium leguminosarum* symbiosis. In particular, the principal aimed objectives, are to determine the physiological and biochemical responses of faba bean seedlings, induced by irrigation water containing cyanobacterial crude extract [equivalent to 50 and 100 µg/L MCs]. Moreover, the oxidative stress caused by MCs was evaluated by determining peroxidase (POD), catalase (CAT), polyphenoloxidase (PPO), and phenylalanine ammonia-lyase (PAL) activities in faba bean plants exposed and not exposed to MCs. The originality of the experimental approach consists in using a MCs-tolerant rhizobial strain, *R. leguminosarum* RhOF4 (Lahrouni et al. 2012). According to our knowledge, scarce studies were carried out on the effects of MCs on the oxidative stress and the antioxidant defense of *V. faba* inoculated with a MCs-tolerant symbiotic rhizobial strain.

Material and methods

Extraction of cyanotoxins

The cyanobacterial bloom material (mainly *M. aeruginosa*) was collected in September 2005 from Lalla Takerkoust reservoir. The sample was freeze-dried and the lyophilized material was stored at –20 °C for MCs extraction and HPLC analysis. The methodology of extraction was previously described by El Khalloufi et al. (2011). Basically, the bloom was sonicated for 15 min, stirred for 1 h at room temperature, and centrifuged at 22,000×g for 15 min. The supernatant was collected, aliquoted, and stored at –20 °C until use. This raw bloom extract was used for the experiments. Microcystins quantification by high-performance liquid chromatography coupled to a photodiode array detector (HPLC-PDA) in the raw extract was done in a previous work (El Khalloufi et al. 2011), and corresponded to 22.24 µg MCs/mL. The concentration of MCs in the water used for plant irrigation was adjusted to 50–100 µg/L in order to mimic the natural conditions which occur during bloom event in Lalla Takerkoust lake (cells lysis, cyanotoxins release, MCs dissolved fraction). Natural occurring MCs concentrations in this lake were widely studied for several years (Oudra et al. 2001, 2002; El Ghazali et al. 2011).

Faba bean seedlings and growth conditions

Seeds of commercial cultivar of *V. faba* L. var. *aguadulce* (widely cultivated in Marrakech region) were surface-sterilized with sodium hypochlorite (6 %) for 10 min, then rinsed several times with sterile distilled water and germinated at 26 °C in moist autoclaved sand. After initial germination, seedlings selected for uniformity, were inoculated with a suspension of *R. leguminosarum* strain RhOF4, which has been described as cyanotoxins-tolerant rhizobial strain (Lahrouni et al. 2012) and planted (three per pot) in pots containing sterile sand. The sand was previously washed with distilled water and sterilized at 200 °C for 4 h in a muffle furnace. Chronic and continuous exposure to cyanotoxins was started 1 week after transplanting the germinated faba bean seeds into the pots.

Plants were grown for 5 weeks in a glasshouse under natural conditions during October–November 2010 and irrigated with N-free nutrient solution (Rigaud and Puppo 1975). After germination stage, the seedlings were grown in normal conditions (not in contact with cyanotoxins) and afterwards the pots were separated into three experimental groups. The first group was kept without cyanotoxins (served as a control), while the second and the third groups were irrigated with water containing cyanobacterial bloom extract equivalent to 50 and 100 µg/L MCs, respectively. Cyanotoxins concentrations are in accordance with the environmental realistic concentrations detected in Lalla Takerkoust's water bodies and reflective of possible MCs exposures during the occurrence of natural blooms of *M. aeruginosa* (El Ghazali et al. 2011).

Plant harvest

After exposure to cyanobacterial cell extract, the plants were harvested at the flowering stage, washed, and separated into two groups for each treatment (control, 50 and 100 µg/L). For the first group, nodules were detached from their roots and counted. After desiccation at 70 °C during 72 h, the dry matter of the different vegetative parts of the plants (shoots, roots, and nodules) was quantified. Nitrogen as well as mineral nutrient content in shoots and roots part were dosed. For the other group, nodules root and leaves were separated, the fresh weight determined, and subsequently submerged in liquid nitrogen and stored at –20 °C for biochemical analysis. The activity of antioxidative enzymes POD, CAT, PPO, and PAL was evaluated in different plant organs.

Mineral nutrients analysis

To determine the inorganic ions Na⁺, K⁺, and Ca²⁺, 0.25 g of dried matter were ashed in a muffle furnace for 6 h at 550 °C, afterwards dissolved in 3 mL of chloridric acid (5 N), and then diluted with distilled water up to 25 mL. These cations were

analyzed in a flame-photometer Jen Woy PFP7 according to the procedure described by Saqrane et al. (2009). Nitrogen content in shoots and roots was dosed by the Kjeldahl procedure as described by Lahrouni et al. (2012).

Photosynthetic parameters

Chlorophyll analysis

Three weeks after exposure to *Microcystis* aqueous extract, chlorophyll (a+b) content was determined to assess the impact of MCs on photosynthetic pigments. Chlorophylls a and b were extracted in the dark, and analyzed following the methodology described by Geider and Osborne (1992). Briefly, 50 mg of leaves were homogenized in 2 mL of aqueous acetone 90 % (v/v). The homogenate was centrifuged (10,000×g for 5 min), and the pellet was extracted twice with 0.5 mL of 90 % acetone. The concentration of chlorophyll a and b was calculated from the absorbance at 664 and 647 nm.

Photosynthetic activity

Photosynthetic activity was assayed by fluorescence in dark-adapted leaves, using a portable fluorometer (Handy Plant Efficiency Analyser, Hansatech Instruments Ltd), after illumination at a light intensity of 3,500 µmolephotons m⁻² s⁻¹. Photosystem II (PSII) was estimated by the ratio (Fm–F0)/Fm (also known as Fv/Fm) (Maxwell and Johnson 2000), where F0 is the minimum fluorescence, corresponding to the open state of PSII reaction centers, and Fm, the maximum fluorescence, corresponding to a closed state of PSII reaction centers. The fluorescence parameters were calculated automatically using Handy PEA v 1.3 software.

Enzymatic assays

Extraction and assay of POD activity

Samples (100 mg) were ground with 5 % polyvinyl pyrrolidone (PVPP), and homogenized with 1.5 mL sodium phosphate buffer (0.1 M, pH 7). The samples were centrifuged at 9,000 rpm at 4 °C for 30 min. The supernatants were used as crude enzyme sources to assay POD enzymatic activity according to the method of Venisse et al. (2001) with some modifications. Guaiacol was used as the substrate. Oxidation of guaiacol to tetraguaiacol was monitored spectrophotometrically at 470 nm for 2 min at 30 °C.

Extraction and assay of CAT activity

The method of extraction as described by Gong et al. (2001) was used in the experiment with some modifications. One hundred milligrams of fresh weight of tissue was

homogenized in 2.5 mL of 50 mM Tris–HCl buffer (pH 8.5) including 2 mM EDTA and 5 % (w/v) PVPP. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatant was used for the activity measurement. Catalase (CAT) activity was determined by following the disappearance of H₂O₂ in the enzyme reaction mixture (Du and Bramlage 1994). The enzyme extract (100 µL) was added to 800 µL assay mixture (50 mM Tris–HCl buffer pH 6.8, containing 5 mM H₂O₂). The reaction was stopped by adding 100 µL 20 % titanium tetrachloride (in concentrated HCl, v/v) after 10 min at 20 °C. A blank was prepared by addition of 100 µL 20 % titanium tetrachloride at zero time to stop the enzyme activity. The absorbance of the reaction solutions was read at 415 nm against water. CAT activity was determined by comparing absorbance against a standard curve of H₂O₂ from 0.25 to 2.5 mM.

Extraction and assay of PPO activity

For PPO assay, samples of 100 mg of different tissues were ground with 5 % polyvinyl polypyrrolidone, and homogenized with 2.5 mL sodium phosphate buffer (0.1 M, pH 6.4). The samples were centrifuged at 9,000 rpm at 4 °C for 30 min. The supernatants were used as crude enzyme sources. The PPO activity was determined according to the method of Jiang et al. (2002), by adding 100 µL of enzyme preparation to 1 mL of 10 mM catechol (dissolved in 100 mM sodium phosphate, pH 6.4) as a substrate. Absorbance at 420 nm was measured. The activity was expressed in units (U) per milligram protein. One unit was defined as ΔA_{420} of 0.01 per minute.

Extraction and assay of PAL activity

Phenylalanine ammonia lyase activity was measured according to the method of Camm and Towers (1973) with some modifications. The reaction mixtures contained 250 µL supernatant with 1 mL 0.1 molL⁻¹ borate buffer (pH 8.8) and 10 mmolL⁻¹ L-phenylalanine. After incubation of the mixtures at 30 °C for 1 h, the reaction was terminated by the addition of 0.2 mL of 5 N HCl. The increase in absorbance at 290 nm, due to the formation of trans-cinnamate, was measured spectrophotometrically.

Extraction and analysis of phenolic compounds

The extraction of leaf, root, and nodules samples for the determination of total phenolics was made and the total phenolic content (TPC) of extracts was assessed by using the Folin-Ciocalteu phenol reagent method (Singleton and Rossi 1965). To 100 µL of the sample extracts (diluted 1:5 (v/v) with methanol), 1,750 µL of bidistilled water, and 250 µL of Folin-Ciocalteu reagent (diluted 1/3 v/v) were

added; after 5 min incubation at room temperature, 500 µL of sodium carbonate (20 % w/v) were added. The extracts were mixed and incubated for 30 min at 30 °C before measuring the absorbance at 760 nm. A mixture of water and reagents was used as a blank. The TPC was expressed as caffeic acid equivalents in milligrams per gram of fresh weight of plant tissue.

Statistical analysis

The experimental design was a randomized complete block. The growth values, the parameters related to nodulation were means of seven replicates per treatment and parameters related to the enzymatic activities were means of five replicates per treatment. Data were analyzed by variance analysis, and the mean separation was achieved by LSD test by the COSTAT software. All numeric differences in the data were considered significantly different at the probability level of $P \leq 0.05$.

Results

Plant growth responses and symbiotic performance

The bloom extract used in this work was previously analyzed (El Khalloufi et al. 2011). HPLC-PDA data obtained from the extract of the *M. aeruginosa* bloom used in this study revealed a mixture of five variants of MCs: DMC-LR (4.20 %); MC-(H4)-YR (4.32 %); MC-LY (8.32 %); MC-FR (9.45 %), and MC-LR (73.71 %). The total MCs content of the bloom extract was 22.24 µg of MC/mL.

After 4 weeks of faba bean exposure to cyanobacterial bloom extract containing 50 and 100 µg/L total MCs, no visual morphological changes were perceptible compared with the controls. At the end of the experiment (5 weeks), the number of leaves and nodes were counted and the length of shoot and root parts were measured. The obtained results showed that there were no significant differences in total nodes and leaves number and shoot length between plants exposed and not exposed to cyanobacterial bloom extract (Table 1). However, the effect of MCs exposure seems to be related with a significant decrease of shoot dry weight (up to 31 % at 100 µg/L total MCs).

The root part appeared to be more sensitive to MCs than the shoot. All the investigated root parameters, root length (RL), root dry weight (RDW), total nodule dry weight (NDW), and the total number of nodules (NN), showed decreased values in the presence of MCs (Table 1). Indeed, a reduction of 19 % in RL was registered after exposure to 100 µg/L cyanotoxins MC, whereas up to 53 % reduction in RDW was observed at this MCs concentration. In addition, remarkable visual changes were noticed on plant roots

Table 1 Effect of cyanotoxin (MC) treatments on growth and nodulation parameters of *Vicia faba* inoculated with the *Rhizobium leguminosarum* strain RhOF4

Parameters	MC treatment (µg/L)		
	0	50	100
Total number of leaves (plant ⁻¹)	22.14±2.19 ^a	20.42±2.52 ^a	21.85±1.88 ^a
% Decrease		7.74	1.29
Total number of nodes (plant ⁻¹)	8.28±0.55 ^a	8.00±0.74 ^a	8.14±0.58 ^a
% Decrease		3.44	1.72
Shoot length (cm)	44.42±3.49 ^a	44.28±3.10 ^a	42.92±2.30 ^a
% Decrease		0.32	3.37
Shoot dry weight (g)	2.04±0.09 ^a	1.69±0.13 ^b	1.40±0.05 ^c
% Decrease		17.15	31.37
Root length (cm)	22.57±1.40 ^a	19.55±1.61 ^a	18.28±2.47 ^b
% Decrease		13.35	18.98
Root dry weight (g)	1.19±0.09 ^a	0.83±0.07 ^b	0.55±0.03 ^c
% Decrease		30.25	53.78
Total number of nodules (plant ⁻¹)	90.14±13.12 ^a	64.57±7.2 ^b	49.85±5.33 ^c
% Decrease		28.36	44.69
Nodule dry weight (gramplant ⁻¹)	0.19±0.003 ^a	0.15±0.001 ^b	0.12±0.001 ^c
% Decrease		21.05	36.84

Values are means of seven replicates. Differences in the data were considered significantly different at the probability level of $P \leq 0.05$ (indicated by different letters)

exposed to MCs. This effect became more evident as the experiment progressed after 18 days; the roots of plants exposed to MCs were dark brown due to necrosis and very short (Fig. 1). Nodulation was very sensitive toward MCs; the reduction of two nodulation parameters, NN and NDW, reached 44 and 36 % at 100 µg/L of MC, respectively (Table 1).

Content of nitrogen and mineral nutrients in plant tissues

Figure 2 illustrates the mineral nutrient (Ca^{2+} , K^+ , and Na^+) contents in *V. faba* plants treated with cyanotoxins (50 and



Fig. 1 Aspect of roots of faba bean plants grown in the absence (A) and in the presence (B, C) of cyanotoxins. Short roots with browning color are observed at 50 µg/L (B) and 100 µg/L (C) total MCs

100 µg/L total MC) in comparison to the controls (unexposed). A significant decreases in K^+ (around 20 %) and Ca^{2+} (around 30 %) contents in shoots, relative to controls, were observed (Fig. 2a). In the roots, only K^+ contents differed significantly between the plants exposed and not exposed to cyanotoxins (up to 20 % reduction in K^+ levels in roots). As for Na^+ , cyanotoxins caused a significant increase of Na^+ contents both in shoots and roots of *V. faba* (between 5 and 15 %). The effect of cyanotoxins on nitrogen content by faba bean plants inoculated with RhOF4 was also established. The total nitrogen contents in both shoots and roots of *V. faba* were significantly decreased ($p < 0.05$) especially at exposure to 100 µg/L total MC (Fig. 2c). Nevertheless, this reduction was up to a 10–15 %. Since no N was added to the watering solution (except for the low amount of N present in the cyanobacterial crude extract added to the watering solution), the N content of the plants reflects differences in N_2 -fixation in root nodules.

Photosynthetic activity

In order to evaluate the effect of cyanotoxins on the photosynthetic activity, two parameters were evaluated, chlorophyll content and chlorophyll fluorescence (Fig. 3). The obtained results showed that exposure of *Medicago sativa* seedlings to different concentrations of MCs led to photosynthetic disorders. Indeed, 100 µg/L total MC induced significantly lower total chlorophyll “a” and “b” contents than the control plants (Fig. 3a; maximum reductions of 10–15 % were observed). The photosynthesis performance was determined after dark adaptation by analyzing Fv/Fm, which

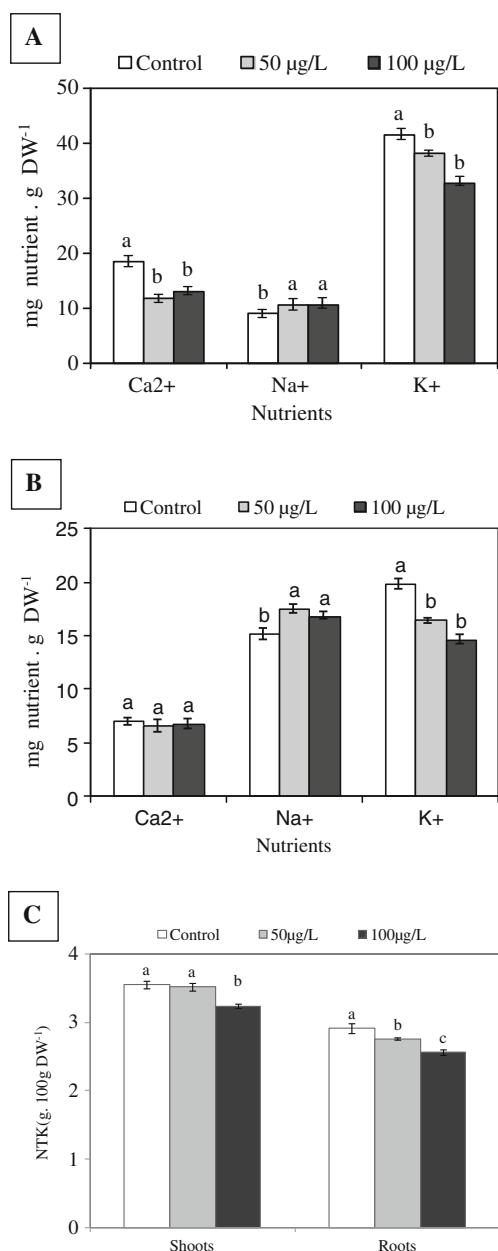


Fig. 2 Mineral nutrient accumulation in shoot (a) and root (b), and nitrogen assimilation (c) by *V. faba* L. inoculated with the MCs-tolerant strain RhOF4 exposed to cyanobacterial bloom extract containing 50 and 100 µg/L total MCs, compared to unexposed control plants. Data are means of seven replicates; differences in the data were considered significantly different at the probability level of $P \leq 0.05$

represents the maximum quantum yield of PSII (Maxwell and Johnson 2000). The Fv/Fm value decreased by 10–15 % as the concentration of toxin increased (Fig. 3b). This value was around 0.8 for the control plants; whereas for exposed plants this value was significantly lower (0.740 and 0.658 when faba bean plants were exposed respectively to 50 and 100 µg/L total MC). Values of Fv/Fm lower than 0.8 will reveal the plant stress condition, indicating a particular photo-inhibition phenomenon (Johnson et al. 1993).

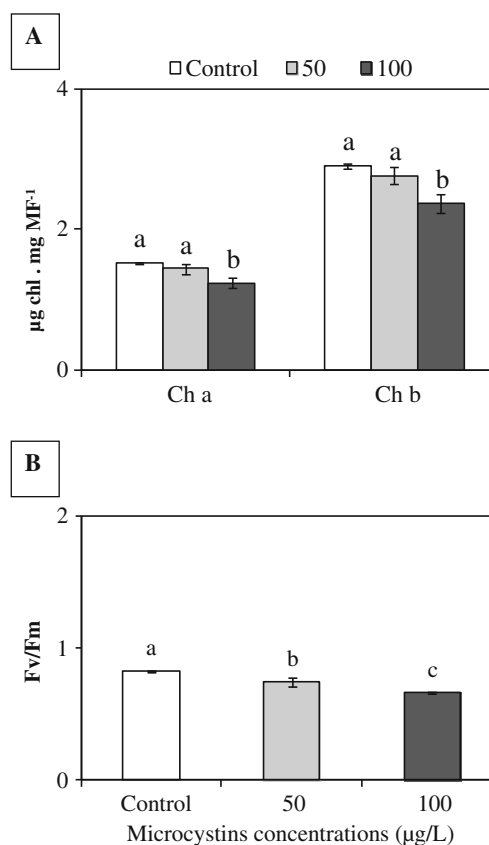


Fig. 3 Chlorophyll (a+b) content (a) and effect on photosystem II (b) of plants exposed to *Microcystis* extract for 4 weeks. Photosynthetic activity was assayed as Fv/Fm fluorescence. Significant differences between control and treated plants are indicated by letters a, b, and c. $P < 0.05$

Antioxidant defense reaction of rhizobia–*V. faba* symbiosis under MCs exposure

To provide further evidence for the influence of cyanobacterial secondary metabolites in plant stress, several ROS scavenging enzymes have been determined. The activities of POD and CAT showed marked increases, especially when the plants were exposed to cyanobacterial bloom extract containing 100 µg/L total MCs, indicating a strong increase of the oxidative damage (Fig. 4). The activities of POD and CAT were induced in a dose-dependent manner. At 50 µg/L total MCs, increases of 200–300 % in enzyme activities were found, depending on the enzyme and the specific tissue (Fig. 4). In general, at dose 100 µM total MCs/L, induction of POD and CAT reached 300–400 % relative to control unexposed plants. Moreover, for POD, the highest activity levels were found in nodules, as compared to leaves and roots, whereas CAT showed similar activity in all the tissues.

In order to evaluate the effect of cyanotoxins on the detoxification process mediated by phenolic compounds, three parameters were measured; quantification of PPO and PAL

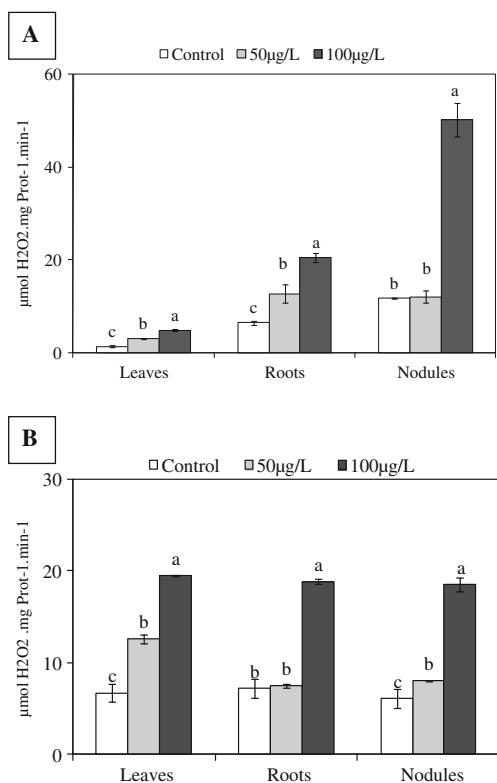


Fig. 4 Activity of peroxidase (a) and catalase (b) in faba bean plants after 4 weeks exposure to 50 and 100 µg/l MC-LR compared to control plants cultivated without cyanotoxins. Significant differences between control and treated plants are indicated by letters *a*, *b*, and *c*. $P < 0.05$

activities and phenolics content. In leaves of plants exposed to MCs, PAL activity was strongly induced in a dose-dependent manner (Fig. 5a). Maximum induction was recovered at 100 µg/L (3-fold increased activity). However, in roots and nodules changes were minor or no significantly different. By contrast, the PPO activity showed the maximum induction in nodules (up to 10-fold increased level of activity), whereas the increases of activity in roots and leaves were of three to four times (Fig. 5b). Regarding the concentration of phenolic compounds, there was an increase in all the tissues, which was related to microcystins concentration (Fig. 5c). In leaves and roots, the increase of phenolic compounds reached 1.5–2-fold, whereas in nodules a 2.5-fold increase in phenolic compounds was observed at the maximum MCs concentration.

Discussion

This study shows the long-term effects of cyanobacterial crude extract containing cyanotoxins on the physiological and biochemical defense reactions of faba bean–rhizobia symbiosis. Two important approaches were undertaken in this work: on the one hand, the experimental design used mimics the real MCs concentration present in raw irrigation water used in the Marrakech region. On the other hand, the

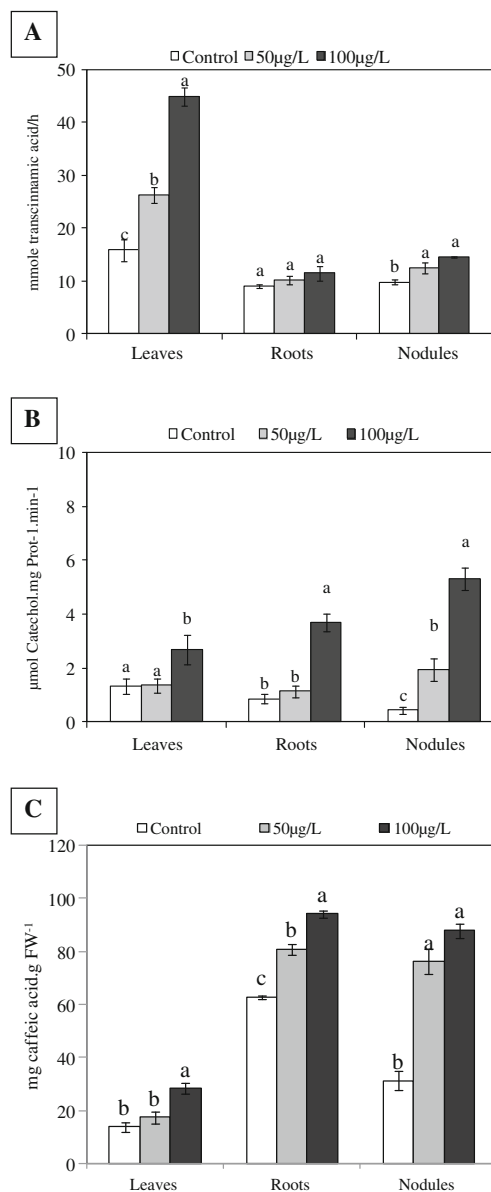


Fig. 5 Activity of phenylalanine ammonia lyase (a), polyphenoloxidase (b), and phenolic compounds content (c) in faba bean plants after 4 weeks exposure to 50 and 100 µg/L total MCs compared to control plants. Significant differences between control and treated plants are indicated by letters *a*, *b*, and *c* ($P < 0.05$)

Rhizobium strain used for plant inoculation is a MC-tolerant strain. The approach followed in this work is trying to reproduce the natural conditions occurring after cyanobacterial bloom (cell lysis and cyanotoxin release in the water). In our experiments, the raw crude extract is used, instead of pure MCs. In this case, all other substances present in the cell extract are also present in the water, and can affect the plants upon irrigation. Thus, we must also consider that cyanotoxins are only around 1 % of all the substances of the bloom extract (Bláha et al. 2009), so the influence of other substances, besides cyanotoxins, cannot be ruled out.

However, this is the “real” situation occurring in water reservoirs.

Several studies have shown that the effect of cyanotoxins on crop plants depends on plant sensitivity, the concentration of cyanotoxins, and the time of exposure. In general, the effect was more apparent on roots than in shoots. In our case, no morphological alterations in the shoot were observed, whereas a marked effect on roots, which looked brown, shorter, and thinner. Similar results were shown in other legumes, such as *Phaseolus vulgaris* (McElhiney et al. 2001) and in *M. sativa* (El Khalloufi et al. 2011). Exposure time is a key factor in the extent of the damage. Whereas short exposure times did not affect dry matter of several varieties of bean or rice plants (Pichardo and Plugmacher 2010; Prieto et al. 2011), long-term exposure produced necrosis in leaves of *Phaseolus vulgaris* (McElhiney et al. 2001) and spinach (Pflugmacher et al. 2007). Plant sensitivity is also critical; while stem malformations, plant browning, and root inhibition occurred in white mustard (*Sinapis alba* L.) at 5 µg/L MC-LR (Kós et al. 1995), no significant differences were reported in broccoli and mustard exposed up to 10 µg/L MCs (Järvenpää et al. 2007).

Few studies have focused on the effects of the cyanotoxins to rhizobia–legume symbiosis. Nodulation is one of the plant processes most affected by cyanotoxins (El Khalloufi et al. 2011). In a previous work, several *Rhizobium* strains were isolated, with different tolerance against MCs (Lahrouni et al. 2012). In this work, a MC-tolerant strain of *R. leguminosarum* was used for plant inoculation in order to minimize the toxic effects of MCs on the plant. The use of resistant *Rhizobium* strains has been reported to alleviate plant stress caused by different abiotic factors. For instance, heavy-metal-resistant *Rhizobium* strains help legume plants cultivated in heavy-metals-polluted soils by protecting plant roots (Dary et al. 2010; Reichman 2007; Wani et al. 2008). Analogously, salt-tolerant *Rhizobium* strains also improve the yield of legumes under salt or drought stress (Ali et al. 2009; Bianco and Defez 2009; Singla and Garg 2006). From a practical point of view, application of a MC-tolerant *Rhizobium* may improve plant yield and nodulation when plants are irrigated with MCs-containing water. Our results indicate the successful root infection with this particular rhizobial strain (RhOF4), even at the highest MCs concentration, although nodulation was decreased. However, the reduction of 45 % in the NN led to only 15 % reduction in N content. These results suggest that nodules were effective in nitrogen fixation. Other positive effect of inoculation is related to the accumulation of toxic compounds in plants. The use of metal-resistant *Rhizobium* decreased the amount of metals taken up by plants (Dary et al. 2010; Wani et al. 2008). It could be interesting to test whether the use of a MCs-resistant *Rhizobium* could decrease the amount of toxins present in plant tissues, thus decreasing the entrance of the toxins into the food chain.

Intoxication with MCs (at 100 µg L⁻¹) also had an adverse effect on photosynthetic activity of faba bean plants, as can be deduced from chlorophyll and Fv/Fm values. Impaired photosynthesis of terrestrial plants by cyanobacterial toxins was detected in many species, such as potato, maize, lens, wheat, pea, spinach, and alfalfa (El Khalloufi et al. 2011; Pflugmacher et al. 2007; McElhiney et al. 2001; Saqrane et al. 2009). This effect was related to the content of chlorophyll (Lichtenthaler 1987; Saqrane et al. 2009) or to impaired carboxylation efficiency (Abe et al. 1996). At the concentrations used in this work, these changes in photosynthesis led to a decrease of 30 % shoot dry matter, although other morphological changes were not seen. It could be possible that the defense mechanisms induced allow the plant to stand these MC concentrations, without being affected.

The presence of cyanotoxins has also effects on plant nutrition. K⁺ and Ca²⁺ content decreased whereas Na⁺ increased. Saqrane et al. (2009) have reported that MCs led to an increase in mineral nutrients content (K⁺ and Ca²⁺) in the roots of *Lens esculenta*, *Pisum sativum*, *Zea mays*, and *Triticum durum* plants. These results indicate that MCs may cause a disruption of the membrane permeability.

The promotion of oxidative stress in plants caused by exposure to cyanobacterial crude extract has been previously shown in different plants. In this work, several enzymes were determined aimed to test whether chronic exposure to bloom extract induced an oxidative stress in faba bean. In order to avoid damaging consequences of hydrogen peroxide, the most stable ROS, plants have evolved various enzymes able to detoxify this compound. Increased activity of antioxidative enzymes such as POD and CAT in all organs of faba bean clearly indicated that oxidative stress is promoted in plants exposed to cyanobacterial crude extract containing MCs. Analogous results were reported spinach (Pflugmacher et al. 2007), bean (Pichardo and Pflugmacher 2010), rape (*Brassica napus*) and rice (*Oryza sativa*) (Chen et al. 2004), coontail (*Ceratophyllum demersum*) and alfalfa (*M. sativa*) (Pflugmacher 2004; Pflugmacher et al. 2006; El Khalloufi et al. 2011), and in the cyanobacteria *Synechocystis* sp. (Vassilakaki and Pflugmacher 2007).

PAL and PPO are induced by a variety of stimuli, particularly under conditions of stress, including pathogen attack, wounding, and disease. PAL catalyzes the conversion of phenylalanine to cinnamic acid, which has an antioxidant effect in stress conditions (Archana et al. 2011; Boo et al. 2011). In our experiment, PAL activity in leaves and nodules was significantly altered when plants were watered with the cyanobacterial crude extract, while no changes were distinguished in this enzyme in roots. PPO may be involved in the browning reaction, or alternatively the browning reaction could be a secondary result of other metabolic events (Zhou et al. 2003).

Besides these enzymatic activities, others could be also involved in ROS scavenging, such as SOD, enzymes of the glutathione–ascorbate cycle, or the glutathione-S-transferase, involved in the formation of a glutathione–MC complex (Mitrovic et al. 2004; Pflugmacher et al. 1998, Pflugmacher 2004; Pflugmacher et al. 2007).

Phenolic compounds play an important role in the detoxification of free radicals including $O_2^{\cdot-}$, H_2O_2 , $\cdot OH$, and 1O_2 (Ksouri et al. 2007). At the gateway from primary metabolism, L-phenylalanine ammonia lyase (PAL; EC 4.3.1.5) plays a pivotal role in phenolic synthesis and it has been amply demonstrated that there is a correlation between increased activity of this key enzyme and increases in phenolic compounds in response to different stimuli in plants (Boudet 2007; Sakihama et al. 2002). This could explain the increase of phenol contents in *V. faba* plants irrigated with cyanobacterial crude extract containing cyanotoxins, similar results to those found in *Lemna gibba* and alfalfa (Saqrane et al. 2007; El Khalloufi et al. 2011). Besides phenolic compounds, some other substances, such as ascorbate, dehydroascorbate, glutathione, glutathione disulfide, and tocopherol were reported to act as antioxidant scavengers in plants treated with cyanotoxins (Pflugmacher et al. 2007).

Conclusion

The present work clearly shows the oxidative stress induction in *V. faba* L. var. *aguadulce* during its symbiosis with a MC-tolerant *Rhizobium* strain exposed to cyanobacterial crude extract containing MC-LR. The activities of POD, CAT, PPO, and PAL were significantly increased in leaves, roots, and nodules of faba bean plants exposed to cyanobacterial bloom extract containing MCs at 100 $\mu g/L$ of MCs. Survival of plants under stress conditions is possible if several antioxidants cooperate together with antioxidant enzymes, providing a good defense system and a quick and balanced regeneration of the active reduced forms of the antioxidants.

The cyanotoxins had also a negative effect on growth, nodulation process, nitrogen uptake, and mineral elements assimilation by *V. faba*–*rhizobia* symbiosis. The role of selected strains of *rhizobia* highly tolerant to cyanotoxins may be of great value and a friendly biotechnological pathway in order to improve faba bean productivity and tolerance towards cyanotoxins stress.

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