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Abstract
Microcystins are cyclic heptapeptides hepatotoxins produced by aquatic cyanobacteria such as *Microcystis aeruginosa*. The wide occurrence of toxic microcystins in freshwater is a threat to water quality and health of living organisms. Here, we irrigated an agricultural soil daily with a cyanobacterial extract diluted at environmental concentrations of microcystin–leucine–arginine, from 0.005 to 0.1 mg equivalent MC-LR L^{-1}, for 90 days. We analyzed the impact on the growth and physiology of tomato, *Solanum lycopersicum* cultivar MicroTom. Our results show a stimulation of the tomato plant development, in terms of inflorescence and blooming, after exposure to the lowest concentration, of 0.005 mg eq. MC-LR L^{-1}, during the 40 first days post-germination. That effect was not apparently associated with physiological disturbances of the tomato plants.

Keywords
Microcystins, Irrigation, Soil, Plant development

Introduction

In light of climate global change, the development of cyanobacteria in freshwater, as bloom, will go on world- wide. One main associated concern is the production and release in the environment of different cyanotoxins that are proved toxic for humans. They affect the nervous system (neurotoxins), the skin (dermatotoxins) and the liver (hepatotoxins). Among them, the hepatotoxic microcystins are commonly found in 40–75 % of the cyanobacterial blooms (Sivonen and Jones 1999). Microcystins are cyclic heptapeptides which exhibit high structural variability because of: (1) the identity of amino acids, mainly in positions 2 and
4 of the ring, (2) side-chain modifications, such as desmethylation, and (3) variations of the Adda moiety (3- amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). The MC-LR (microcystin with leucine and arginine in positions 2 and 4, respectively) is the most studied due to its high frequent detection in freshwater, but at least 90

congeners have been identified to date (Puddick et al. 2013). After the Caruaru accident in 1996 (Brazil) where 60 patients died in a hemodialysis center (Pouria et al. 1998), the World Health Organization established a con- centration limit of 1 µg MC-LR L^{-1} in drinking water. In the aquatic ecosystems, the concentrations of microcystins often exceed this limit and induce deleterious effects on aquatic plants and animals (Corbel et al. 2014b). Irrigation is necessary for agricultural production and may use waters contaminated by microcystins and without a prior treatment. Consequently, recent studies focused on the effects of microcystins on terrestrial plants (Chen et al. 2004; Saqrane et al. 2008; El Khalloufi et al. 2012). Phytotoxic impacts of microcystins were observed on growth and plant development, photosynthetic activity and oxidative stress. Nevertheless, in these studies, the exposure time did not generally exceed 30 days and high concentrations of microcystins were used. Recently, we showed the high persistence of microcystins in soils (Corbel et al. 2014a).

We previously highlighted the modification of tomato seedlings growth during 14 days of soil irrigation (Corbel et al. 2015) with water containing realistic environmental concentrations of microcystins. In the present study, we aim at determining the effects of a 90-day irrigation on tomato (*Solanum lycopersicum* cultivar MicroTom) plant growth, development and physiology.

Materials and methods

Culture of *Microcystis aeruginosa* strain PCC7820

The *Microcystis aeruginosa* strain PCC7820 was supplied from the culture collection of the Pasteur Institute (Paris, France). This monoclonal strain was axenically grown in batch culture in 6-L flasks containing 3 L of BG11 liquid medium supplemented with $NaNO_3$ (2 mM) and $NaHCO_3$ (10 mM), at 25 °C, with an incident light intensity of 5-10 µmol of photons m^{-2} s^{-1} provided by cool-white fluorescent tubes (16 h:8 h light/dark cycle), as previously reported by Gupta et al. (2001) and Robillot and Hennion (2001). The cultures were vigorously bubbled with filtered (0.22 µm Millipore, France) air at a flow rate of 0.23 L min^{-1}. After 3 weeks of culture, samples of biomass were collected by centrifugation (25,000g, 5 min) and lyophilized for further extraction of microcystins.

Extraction and identification of microcystin variants of cyanobacterial extract

The method of extraction was described by Corbel et al. (2015). Briefly, freeze-dried cyanobacterial cells were homogenized with aqueous methanol and introduced for 3 min in an ultrasonic bath (Ultrasonic 300), followed by centrifugation at 3000g at 4 °C for 10 min. The supernatant was retained and the residue re-extracted two times as before. Combined supernatants were stored at -20 °C prior to analysis. Microcystins concentrations in the extract were determined using the protein phosphatase type 2A inhibition assay according to Bouaïcha et al. (2001). For confirming the identities of some microcystin–leucine–arginine (MC-LR) variants in the cyanobacterial cells extract, the extract was analyzed by liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) using a Waters Acquity ultra-high-pressure liquid chromatography–electrospray–triple quadrupole system as described by Corbel et al. (2015).

Soil and plant exposure experiments

The topsoil (0–20 cm) from a grassland (Pierre Plate, experimental site of INRA Versailles, France) was sampled in August 2012. It was air-dried, sieved (2 mm mesh) and stored at 20 °C for less than 3 months (Corbel et al. 2015). It was a silty sand soil (76 % sand, 13 % silt and 11 % clay), with 37.8 % of organic matter and the pH = 5.6. Soil microcosms consisted in 350 g of dry soil per pot adjusted at 50 % of the maximum water holding capacity with MilliQ water and incubated for stabilization during 2 weeks. Then, one seed of *S. lycopersicum* cultivar MicroTom, from INRA (Avignon, France), was planted in each pot. We carried out daily irrigation, to adjust at 70 % of water holding capacity, with aqueous extract of cyanobacteria prepared from aqueous methanol (75 %, v/v) diluted in nutritive solution of N–P–K (15–10–30), 0.75 g L^{-1}, with final concentrations of 0.005, 0.02, 0.05 and 0.1 mg eq. MC-LR L^{-1}. For controls, samples of nutritive solution containing methanol (3.5 % final concentrations) were used. There were five replicates for each treatment. Microcosms were incubated in greenhouse chamber: 16:8 h light/dark cycle, light intensity of 160 µmol photon m^{-2} s^{-1} provided by cool-white fluorescent tubes (Osram, Cool DayLight 865) and temperature of 26 °C/21 °C light/dark cycle. The exposition held 90 days and the total quantities of microcystins brought after irrigation with 0.005, 0.02, 0.05 and 0.1 mg eq. MC-LR L^{-1}, were, respectively: 0.05, 0.18, 0.46 and 0.92 µg eq. MC-LR g^{-1} of dry soil. Then, plants were harvested, weighed and frozen (-20 °C) before freeze-dried and toxin extraction.

Plant phenology and physiological indicators

Plant growth and development

The ‘‘biologische Bundesanstalt, Bundessortenamt und CHemische industrie’’ scale (BBCH scale), described for Solanaceae by Feller et al. (1995), was used to describe the development of plants, every day, during the 90 days of exposition to cyanobacterial extract. For example, the emergences of leaves and inflorescences or blooming flowers were monitored every day. After exposure, the leaves were harvested and their areas were obtained after scanning (Agfa SnapScan E50) and analyzing with ImageJ 1.46 (2012) software. All organs harvested (roots, shoots, leaves and fruits) were frozen (-20 °C) before to be freeze-dried. After this step, the dry biomasses of organs were established.

Physiological parameters of plants

The chlorophyll fluorescence was measured one week before harvesting. The maximum efficiency of photosystem II was obtained with the ratio Fv/Fm that is used as a sensitive indicator of plant photosynthetic performance with an optimal of around 0.83 measured for most plant species. The values could decrease when plants were exposed to abiotic (Takayama et al. 2011) or biotic stress (Wagner et al. 2006). Measurements were taken according to Murchie and Lawson (2013), in the dark, with a portable photosynthesis system Li-Cor 6400 (Li-Cor Biosciences, Lincoln, NE, USA) on one leaflet of the leave number 3 from the plant collar. Furthermore, the concentration of chlorophylls a and b was obtained on the same leaf according to Geider and Osborne (1992). Briefly, 50 mg of leaf (fresh weight, fw) was homogenized in 0.5 mL of aqueous acetone 80 % (v/v) in a mortar and pestle which was kept in ice. Then, the homogenate was centrifuged (10,000g, 5 min at 4 °C), and the pellet was extracted twice again with 0.5 mL of 80 %

acetone. The three extracts were pooled, and the concentrations of chlorophylls a and b (µg g^{-1} fw) were calculated from the absorbance at 663.6 and 646.6 nm using Eqs. (1, 2) and (3) (Porra 2002):

$$\text{Chl } a = 12.25 \times \text{Abs}663.6 - 2.55 \times \text{Abs}646.6 \quad (1)$$

$$\text{Chl } b = 20.31 \times \text{Abs}646.6 - 4.91 \times \text{Abs}663.6 \quad (2)$$

$$\text{Chl } a + b = 17.76 \times \text{Abs}646.6 + 7.34 \times \text{Abs}663.6 \quad (3)$$

Another leaflet of this leaf (number 3 from the collar) is used for analyzing the fatty acids [phospholipidic fatty acids (PLFA) trans-methylated to yield analyzable fatty acid methyl esters (FAME)] according to the norm Afnor X31- 233 on the determination of the effects of microcystins on the foliar fatty acid content of *Lactuta sativa* (AFNOR 2012; Le Guedard et al. (2008) and suitable for tomatoes (Verdoni et al. 2001). The fatty acid composition was used as bio-marker of contaminated soil with metallic or organic pollutants. For the experiment, 50 mg fw was immersed with 1 mL of MeOH/H_2SO_4 solution (40/1, v/v), in a closing tube, during 1 h at 80 °C. After cooling, 750 µL hexane and 1.5 mL of MilliQ water were added. The tubes were shaken manually during 20 s before centrifugation (300g, 5 min). The organic phase was then sampled, and quantification of FAME was performed by gas chromatography–mass spectrometry (GC–MS) on a BPX column (60 m, SGE) according to Shahzad et al. (2012). External standards of FAME (C16:0; C16:1, C18:0, C18:1; C18:2 and C18:3) were used for compounds identification and calibration. The ratios (4) and (5) were calculated:

$$\text{FAME } 1 = \frac{\%\text{C18:3}}{\%\text{C18:0} + \%\text{C18:1} + \%\text{C18:2}} \quad (4)$$

$$\text{FAME } 2 = \frac{\%\text{C16:0}}{\%\text{C16:1}} \quad (5)$$

Statistical analysis

Data were represented by the mean ± standard deviation. The normality and homogeneity of variance were checked with Kolmogorov-Smirnov and Bartlett tests (p \ 0.05). If these conditions were checked, an ANOVA and a Tukey post hoc comparison were performed. In contrast, data were compared with a nonparametric Kruskal-Wallis test and ‘‘nparcomp’’ post hoc comparisons. All statistical analyses were performed using ‘‘R’’ software, and significance level was 0.05.

Results and discussion

The objective of the present work was to determine potential effects of a 90-day irrigation with water containing realistic environmental concentrations of microcystins on tomato (*S. lycopersicum* cultivar MicroTom) plant growth, development and physiology.

Quantification and identification of microcystins in cyanobacterial extract

The total concentration of microcystins in the extract calculated with the protein phosphatase assay and expressed

according to microcystin–leucine–arginine was 21.6 mg eq. MC-LR L^{-1}. It corresponded to 6.8 mg eq. MC-LR g^{-1} cyanobacteria's cells dry weight. The cyanobacterial extract of M. aeruginosa (PCC 7820) was characterized, and the different congeners identified were described in Corbel et al. (2015). The microcystins congener the most abundant in this extract was the MC-LR (68.4 % of the total microcystins). This extract diluted at concentrations from 0 to 0.1 mg eq. MC-LR L^{-1} was used daily for soil irrigation.

Effects of microcystins on plant growth, development and physiological parameters

Irrigation of the soil–tomato systems with water containing microcystins for 90 days did not disturb the global growth of tomatoes, with total plant biomasses similar to the control group (Table 1). Results of biomasses obtained regarding each organ were also similar in treated and non- treated plants. Considering the stages of phenological development, the kinetics showed no difference in the delay necessary for the emergence of leaves, regardless of the concentration of microcystins (Fig. 1A). Conversely, the kinetics concerning the emergence of the inflorescence and the blooming of the first flower differed for the treatment with the lowest concentration, 0.005 mg eq. MC-LR L^{-1} (Fig. 1B, C). The emergence of the first inflorescence appeared nearly 24 days after planting for the treatment with 0.005 mg eq. MC-LR L^{-1}, whereas the inflorescence appeared after 28 and 29 days for the tomato plants untreated and treated with 0.1 mg eq. MC-LR L^{-1}, respectively. There was a significant difference between the delays of inflorescence emergence for treatment with 0.005 and 0.1 mg eq. MC-LR L^{-1}, until the emergence of the inflorescence number 6. The blooming of the first flower of the inflorescence number 1 confirmed this gap. It appeared the 33^{th} day with the treatment with 0.005 mg eq. MC-LR L^{-1}, and the 38^{th} day for tomatoes treated with 0.1 mg eq. MC-LR L^{-1}. Treatments with cyanobacterial extract did not modify the production of infructescences and fruits (data not shown). The number of aborted flowers from the treated plant with water containing microcystins and untreated plants was unchanged. Conversely, results differed in the literature, partially explained by the strong concentrations used. El Khalloufi et al. (2012) reported a decrease in tomato plants (*Lycopersicum esculatum*) biomass and height after 30 days of exposure with a concentration of 2 mg eq. MC-LR L^{-1}, 20-fold higher than our higher concentration. In the same way, other crops species: *Zea mays*, *Lens esculenta*, *Triticum durum* and *Pisum sativum*, showed an increase in growth after exposure with 0.5 mg eq. MC-LR L^{-1} during 30 days. The results obtained seemed species and concentration dependent. A recent study showed an increase in tomato seedling growth after 14 days of irrigation with environmental concentrations of this cyanobacterial extract comprised between 0.005 and 0.1 mg eq. MC-LR L^{-1} (Corbel et al. 2015). As observed in this work, exposure to 0.005 mg eq. MC-LR L^{-1} of cyanobacteria extract advanced by 4 days the tomato development, by comparison with plants treated with 0.1 mg eq. MC-LR L^{-1} or untreated plants. Table 1 shows values of several parameters measured on the tomato leaves after 90 days of the various treatments. Firstly, the areas of leaves were similar, between 17 and 21 cm2 despite the concentration of cyanobacterial extract input. The concentrations of chlorophyll a and b, represented by the a/b ratio, were also similar to the control group with values comprised between 2.3 and 2.4. Furthermore, the chlorophyll fluorescence, indicated by the Fv/Fm ratio values, near 0.8, was not significantly modified by microcystins exposure. The composition of leaf fatty acids was unmodified by treatments with cyanobacterial extract. Furthermore, the physiological parameters considered in this study did not show a stress period for the tomato plant. Other studies indicated effect on chlorophyll concentration in *Z. mays* or *L. esculenta* when the concentrations of exposure were, respectively, higher than 4.2 and 2.1 mg eq. MC-LR L^{-1} (Saqrane et al. 2009) and the chlorophyll fluorescence

significantly decreased, for an exposure of 0.5 mg eq. MC- LR L^{-1}, traducing a stress period. Conversely, the concentration that significantly altered the chlorophyll fluorescence in tomato plants was approximately 22 mg eq. MC-LR L^{-1} (El Khalloufi et al. 2012). The tomatoes were less affected than other crop plants such as *Z. mays* and *L. esculenta*. With realistic ‘‘environmental’’ concentration of 0.1 mg eq. MC-LR L^{-1}, used in this study, 20-fold lower than the concentration used by El Khalloufi et al. (2012), we can conclude that tomato growth and physiology are not impacted after a 90-day irrigation with water containing microcystins.

Conclusion

We showed for the first time that long-term irrigation, during 90 days, of a soil–plant system with water containing realistic environmental concentrations of microcystins accelerated the development of tomato plants. It allowed the emergence of the first inflorescences 4 days earlier for tomato plants treated with 0.005 mg eq. MC-LR L^{-1} by comparison with the unexposed control and overexposed plants. The biomass and physiological parameters of tomato plants were unmodified during exposure to microcystin exposure, revealing for a lack of disturbance on growth. We conclude that a long exposition of plants to low amounts of microcystins can accelerate their development and that microcystins are not always associated with toxic effects. The mechanisms involved should be determined as a way for future research.

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Table 1. Growth and physiological parameters monitored on tomato *Solanum lycopersicum* cultivar MicroTom exposed for 90 days to water containing various concentrations of microcystins

	Concentrations (mg eq. MC-LR L^{-1})				
	Control	0.005	0.02	0.05	0.1
Plant dry biomasses (g)	21.2 ± 1.2	20.2 ± 1.6	21.5 ± 1.2	22.0 ± 0.6	21.4 ± 0.4
Leaves area (cm^2)	17.6 ± 1.6	17.3 ± 4.6	16.7 ± 2.95	18.9 ± 2.1	21.0 ± 4.5
Chlorophyll ratio (*a*/*b*)	2.29 ± 0.23	2.34 ± 0.06	2.41 ± 0.08	2.41 ± 0.03	2.40 ± 0.07
Chlorophyll fluorescence (*Fv*/*Fm*)	0.792 ± 0.009	0.788 ± 0.009	0.792 ± 0.013	0.791 ± 0.004	0.794 ± 0.006
Leaf fatty acid composition					
FAME 1	7.2 ± 0.4	7.5 ± 0.7	7.3 ± 0.7	7.7 ± 1.1	7.6 ± 1.0
FAME 2	144.9 ± 16.6	138.4 ± 11.0	132.6 ± 13.2	154.4 ± 17.0	150.4 ± 20.1

The absence of effects of microcystins on these parameters, suggesting the lack of physiological disturbances of the plants. Fatty acid methyl esters (FAME 1 and FAME 2) ratios are calculated in ‘‘Physiological parameters of plants’’ section. Results are expressed as mean ± standard deviation (n = 5)

Fig. 1. Development parameters monitored on tomato plant (*Solanum lycopersicum* cultivar MicroTom) exposed during 90 days to water containing various concentrations of microcystins. A Emergence of the leaf number; B emergence of the inflorescence number; C blooming of the first flower of the inflorescence number, according to the ‘‘biologische Bundesanstalt, Bundessortenamt und CHemische industrie’’ scale (BBCH scale). The gray backgrounds show the zone where an advance by several days is noticed for the development of plants exposed to 0.005 mg eq. MC-LR L^{-1} at $P < 0.05$, suggesting a simulation due to low concentrations of microcystins. Results data are expressed as the mean (n = 5)