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Effects of two toxic cyanobacterial crude extracts containing microcystin-LR and cylindrospermopsin on the growth and photosynthetic capacity of the microalga *Parachlorella kessleri*

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ABSTRACT

Secondary metabolites produced by cyanobacteria like microcystins (MC) and cylindrospermopsin (CYN) have been frequently studied because of their toxicity in humans and other animals. However, the function(s) of these metabolites remain largely unknown. The objective of this work was to deepen the knowledge in this research area and characterize the allelopathic effects of the cyanotoxins MC and CYN on the green alga *Parachlorella kessleri*. Several assays were carried out under controlled laboratory conditions with cyanobacterial cell extracts from *Microcystis aeruginosa* and *Chrysosporum ovalisporum*, producers of MC-LR and CYN respectively, at concentrations of 55 and 150 µg/L. The effects of CYN at 150 µg/L was also studied in *P. kessleri* growing in Z8 medium with altered nutrient composition. The data obtained indicate that growth rate and photosynthetic pigments were not affected by the exposure to cyanobacterial toxic extracts compared to control. However, the growth rate and photosynthetic pigments content changed during the 14 days of *P. kessleri* cultivation, in both groups (control and treatment), which may reflect the culture conditions and *P. kessleri* growth curve. It was also possible to verify that nutrients' concentration of the microalga culture medium does not influence the growth rate of the green microalga or the biological activity of the cyanobacterial extracts. In conclusion, under these specific laboratory conditions, there were no toxic or allelopathic effects of *M. aeruginosa* and *C. ovalisporum* crude extracts, which demonstrates the high tolerance of this phytoplanktonic species for the cyanobacterial toxins MC and CYN and other metabolites.

1. Introduction

Cyanobacteria are anaerobic photoautotrophic prokaryotes widely distributed in freshwaters [1]. These organisms have important ecological functions such as the fixation of atmospheric nitrogen and contribute to the primary productivity of the aquatic ecosystems. However, water eutrophication may lead to an overgrowth of those microorganisms with adverse consequences for the environment [2, 3]. This overgrowth is influenced by several climatic factors and water quality, among which temperature and nutrient availability play a primary role, leading sometimes to severe phytoplankton communities' shifting and biodiversity reduction in the aquatic environment [4–6]. These adverse effects may be associated to the excessive consumption of oxygen and/or to the formation of dense mats on the water surface, which blocks the light and reduces the growth of other phytoplankton species. In addition, several genera of cyanobacteria such as *Microcystis* and *Aphanizomenon* synthesize various secondary metabolites which exhibit toxic activity to other organisms and therefore also contribute to the degradation of the aquatic environment and controlling the phytoplankton growth. Some of these compounds, such as microcystins (MC) and cylindrospermopsin (CYN), are harmful to many organisms, including animals and humans, and therefore are classified as toxins [7–9].

Microcystins are cyclic heptapeptides with the general chemical

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structure cyclo-(D-alanine-X-DMeAsp-Z-Adda-D-glutamate-Mdha), in which X and Z are variable L-amino acids, D-MeAsp is D-erythro-β-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid [10]. The study of this cyanotoxin has led to a good knowledge of its mechanisms of action in animals (see review in Valério et al. [11]) and the identification of > 200 MC chemical variants produced by cyanobacteria [12, 13]. The initial event triggering the toxic action of MC is their binding to the phosphatase proteins PP1 and PP2A with the subsequent inhibition of their enzymatic activity [14–16]. Cylindrospermospin, synthesized by polyketide synthase genes, is an alkaloid of low molecular weight (415 Da) with the chemical formula of $C_{15}H_{21}N_5O_7S$ having a tricyclic guanidine moiety and a hydroxymethyluracil group. The zwitterionic nature of this cyanotoxin makes it very soluble in water [9]. The mode of action of CYN is less known, but it was postulated that it inhibits the protein and glutathione syntheses [17–20]. Worldwide environmental concentrations in water bodies of MC can vary between 0.041 and 29,163 μg/L and concentrations of CYN between 0.002 and 2×10^6 μg/L [19, 20].

The action of cyanotoxins in the aquatic environment, and especially on the composition of the phytoplankton communities is not well understood. The decrease in phytoplankton biodiversity is thought to be associated with the allelopathic and toxic effects of the compounds produced by cyanobacteria. However, the response of phytoplankton species to cyanotoxins is very heterogeneous and seems to be species-dependent [21–23]. The effects reported on green microalgae are complex but likely to result from the combination of multiple stress factors, including an array of several bioactive compounds produced by the many cyanobacterial species, light intensity, temperature and nutrient availability [21, 24–26]. Also, the co-culturing of the cyanobacteria and the microalgae [24, 25, 27, 28], or exposing microalgae to a crude cyanobacterial extract [23, 25, 29–31], pure cyanotoxins [22, 23, 31] or to the media where cyanobacteria grown [28, 32] can lead to different research outcomes. For example, the toxicological potential of crude extracts of *Aphanizomenon ovalisporum*, a CYN-producing cyanobacterium, is higher than the pure CYN, which shows that the combined effect of different metabolites may be more adverse than the isolated toxic compound [23, 29]. In addition, cyanotoxins' effects in green microalgae occur in the photosynthetic pigments and photosynthesis rate [24, 25, 28, 33, 34] and on the oxidative stress and detoxification [29, 35].

Another important point to note is the tolerance observed of laboratory-grown microalgae to cyanotoxins and cyanobacterial extracts, leading to the hypothesis that these bioassays are poorly representative of the natural systems and therefore of limited use in risk assessment. In a binary mixture, the cyanotoxins MC-LR and CYN acting together demonstrated a synergistic effect on the growth of *Chlorella vulgaris*, but the toxic potential of CYN only increases in the presence of low concentrations of MC-LR. However, the cyanotoxin MC-LR alone is not toxic to the microalga *C. vulgaris* even at high concentrations (37 mg/L) [30]. In addition, the photosynthetic activity seems to be more affected by the presence of cyanotoxins than the growth rate since ecological relevant concentrations of MC-LR and cyanobacterial crude extracts stimulate or inhibit this process in several *Rhodophyta* and *Bacillariophyta* microalgae species [31]. Moreover, the co-culture of living cells of the toxic *Microcystis aeruginosa* and of the non-toxic *M. panniformis* with *Monoraphidium convolutum* and *Scenedesmus acuminatus* inhibit the algae growth, but the cyanobacterial crude extracts had no effects on the growth of these two green algae [27].

The main objective of the present work was to further understanding the toxicity and the allelopathic effects of the cyanobacteria *M. aeruginosa* and *Chrysosporum ovalisporum* crude extracts in the green microalga *Parachlorella kessleri*. For that purpose, it was established two sets of experiments: one to assess the effects of cyanobacterial crude extracts containing MC-LR and CYN at two environmentally relevant concentrations (55 and 150 μg/L) on *P. kessleri*, and a second experiment to assess the influence of the Z8 medium composition on the toxicity/allelopathy of a cyanobacterial crude extract containing CYN at 150 μg/L on the *P. kessleri* performance. In all the experiments, the time and group dependent data (growth rate, photosynthetic pigments, cyanotoxins' uptake by *P. kessleri* and stability of the cyanotoxins MC-LR and CYN) was analyzed.

2. Material and methods

2.1. Cyanobacteria and microalgae strains

The cyanobacterial strains *M. aeruginosa* LEGE 91094 and *C. ovalisporum* LEGE X-001 were obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) at CIIMAR (Matosinhos, Portugal) (http://lege.ciimar.up.pt/) [36]. *M. aeruginosa* LEGE 91094 was isolated from a pond (Lagoa da Vela) at Mira, Portugal [37], while *C. ovalisporum* LEGE X-001 was originally isolated from Lake Kinneret, Israel during a bloom [38].

The cyanobacterium *C. ovalisporum* LEGE X-001, formerly known as *A. ovalisporum* LEGE X-001, was re-classified using molecular-based methods by Ramos et al. [36]. The green microalga strain used in this study was originally obtained from the Coimbra Collection of Algae (ACOI) as *C. vulgaris* ACOI 879 and their identification was re-evaluated in this work according to molecular-based methods and it is now maintained at LEGE-CC under the code name LEGE Z-001. The genomic DNA of strain ACOI 879 (LEGE Z-001) was extracted and the genes nuclear small-subunit ribosomal (SSU) 18S rRNA gene, nuclear ITS rRNA and chloroplast SSU 16S rRNA were amplified, cloned and sequenced at GATC Biotech (Constance, Germany). The gene sequences were deposited in the GenBank database under the accession numbers MG835608 (nuclear 18S rRNA gene and ITS region) and MG835609 (chloroplast 16S rRNA gene) (Supplementary S1, Methods). The phylogenetic analysis was performed using the MEGA7 package [39], the Maximum Likelihood method, 1000 bootstrap, the Tamura-Nei (TN93) model of evolution, an estimated gamma shape parameter (+G) of 0.1000 and the proportion of invariant sites (+I) was 44.82%.

2.2. Growth of microalgae and cyanobacteria

The two cyanobacteria, *C. ovalisporum* LEGE X-001 and *M. aeruginosa* LEGE 91094, were cultured in the Z8 medium at 24 °C, photoperiod 16 h/8 h (light/darkness) and light intensity of 10 μmol/m^2 s [23, 29] in 15 L vessels for 3 months and aerated with filtered air (0.22 μm membrane). Biomass was collected by centrifugation, lyophilized and stored at −80 °C [40, 41]. Green microalga *P. kessleri* (LEGE Z-001) was grown in Z8 medium in the same conditions. The two main cyanotoxins present in the crude extracts of *M. aeruginosa* and *C. ovalisporum* are MC-LR [23, 40] and CYN, respectively [23, 29, 41]. So, only those two cyanotoxins were quantified by HPLC (see Section 2.3).

2.3. Extraction and quantification of cyanotoxins

To extract microcystins, lyophilized biomass of *M. aeruginosa* was suspended in ultrapure water (Millipore, Madrid, Spain) and lysed by sonication in a water bath (RK 100H, Bandelin Sonorex™, Berlin, Germany) for 15 min followed by probe sonication in ice, 5 cycles of 1 min at 60 HZ (Vibracell VC50, Sonic & Materials Inc., Newtown, CT, USA). The homogenate was centrifuged (60 min, 4 °C, and 70,737 xg) and the supernatant stored at −20 °C [42]. Cylindrospermopsin was extracted from lyophilized *C. ovalisporum* with 0.1% trifluoroacetic acid (TFA) in ultrapure water, homogenized for 30 min, lysed by ultrasound for 15 min at 35 Hz in a water bath followed by probe sonication in ice at 20 Hz for 5 min. Then, the homogenate was centrifuged at 4542 ×*g*, 4 °C, 10 min, and the supernatant fraction were stored at −20 °C [41]. The cyanobacterial crude extracts were used as a source of the toxins CYN and MC-LR for the subsequent exposure experiments.