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INVESTIGATION OF TOXIN CONTENT IN *CYLINDROSPERMOPSIS RACIBORSKII* (WOŁOSZYŃSKA) SEENAYA AND SUBBA RAJU AND *APHANIZOMENON OVALISPORUM* (FORTI) STRAINS ISOLATED FROM SHALLOW LAKES OF HUNGARY

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Cylindrospermopsin (CYN) is an alkaloid type cytotoxic metabolite produced by several cyanobacterial species, which caused human illnesses. The occurrence of CYN has been mostly associated with tropical and subtropical cyanobacteria, but recently it is appearing in several countries, all over the world. We analyzed CYN concentration and polyketide synthase/peptide synthetase (PKS/PS) genes, important parts of the gene cluster responsible for the CYN biosynthesis, in 14 isolated/collected *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* strains originated mostly from Hungary. CYN and PKS/PS genes were detected in *Aphanizomenon ovalisporum* strains isolated from Spain (of our isolation) and isolated in Israel (ILC-164), but the Hungarian isolate from the hyposaline Lake Szelidi had a lack of production capacity. In the Hungarian samples of *C. raciborskii*, we found no CYN and PKS/PS genes content comparing to CYN producer *C. raciborskii* AQS originated from Australia.

Keywords: *Aphanizomenon ovalsiporum* – Cylindrospermopsin – *Cylindrospermopsis raciborskii* – *PKS/PS gene*

INTRODUCTION

Cyanobacterial blooms in eutrophic lakes cause several problems due to the production of toxins and a number of organoleptic compounds [2, 4]. The filamentous cyanobacterium *Cylindrospermopsis raciborskii* is one of the most notorious cylindrospermopsin (CYN) producers and can be found in freshwater habitats in the temperate, tropical, subtropical regions of the world [5, 9]. This species is of major concern from a water quality and public health perspective, due to its known ability to produce different type of toxins, including the potent hepatotoxic alkaloid CYN, highly toxic paralytic shellfish poisons (PSP) and an unidentified toxin with atypical toxicity [12].

One of the causative toxins is the alkaloid CYN, a water-soluble cyclic guanidine alkaloid hepatotoxin. The chemical structure of CYN was elucidated in 1992 (Fig. 1).

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Abbreviations: CYN – cylindrospermopsin; CE-MEKC – capillary electrophoresis-micellar electrokinetic chromatography; n.d. – not detected; PKS/PS – polyketide synthase/peptide synthetase.

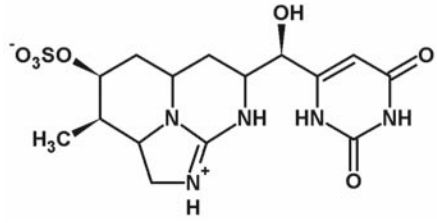

Fig. 1. Chemical structure of the cyanobacterial toxin cylindrospermopsin

It consists of a tricyclic guanidine moiety combined with hydroxymethyluracil having a molecular formula of $C_{15}H_{21}N_5O_7S$ and a molecular weight of 415.43 [8].

Suspected causation of human illnesses by cylindrospermopsin and cattle mortality associated with *C. raciborskii* were reported from Australia. CYN is described as potent hepatotoxin with additional effect on kidneys, heart, thymus, spleen and intestine, whose damage is chiefly caused by inhibition of protein synthesis. Furthermore, CYN-induced mutagenicity by DNA strand breaking and chromosome loss during cell division was proven *in vitro* [11].

The recent reports of *C. raciborskii* from many temperate countries have highlighted the invasive nature of this species, spreading worldwide [9].

Cylindrospermopsin is produced by various genera of cyanobacteria. Besides *C. raciborskii*, CYN was detected in *Umezakia natans*, *Aphanizomenon ovalisporum*, *Raphidiopsis curvata*, *Anabaena bergii* and in *Aphanizomenon gracile* as well [6].

The first reports of the observation of *C. raciborskii* in Hungarian waters were published at the end of the 1970s. Later this heterocystous cyanobacterial species appeared in several lakes and ponds, moreover it caused mass production. In addition it has become the dominant organism in the waters which have important recreational utilization, e.g. Lake Balaton, Lake Szelidi, Reservoir at Levelek [9, 10]. The other suspected toxin producer is *Aphanizomenon ovalisporum*. This cyanobacterial species is not common in Hungary, but dominant in a soda lake called Lake Szelidi [10].

The main goal of this study was to determine the CYN producing ability and CYN content of *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* strains and field samples originating mainly from Hungarian shallow waters.

MATERIALS AND METHODS

Sampling and preparation of extracts

The cyanobacterial bloom samples were collected from lakes, reservoirs and ponds of Hungary throughout the summer of 1995–2009. The sampling stations in Hungary are shown in Fig. 2. Moreover we isolated an *A. ovalisporum* strain from Spain

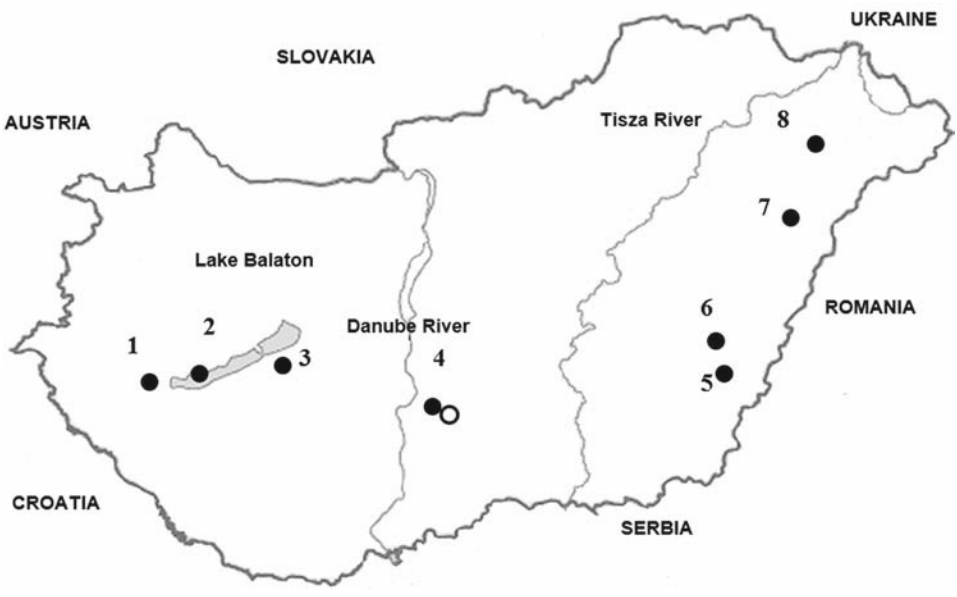

Fig. 2. Map of Hungary with the sampling sites. Symbols ● indicate localities where *C. raciborskii* strains were isolated/collected (1: Kis-Balaton reservoir, 2: Lake Balaton, 3: fish pond, Zamárdi, 4: Lake Szelidi, 5: fish pond, Doboz, 6: backwater, Endrőd, 7: ornamental pond, Debrecen, 8: reservoir Levelek). Symbol ○ indicates the locality where *A. ovalisporum* strain was isolated/collected (Lake Szelidi)

(Blanes, pond of Botanic Garden) in 2005. The samples were stored in brown glass bottles at –7 °C. Prior to cyanotoxin analyses, all the cyanobacterial samples were analyzed by light microscope (Olympus BX-50). The cyanobacteria were identified according to taxonomic keys based on cell or colony morphology [1, 7]. The isolated *C. raciborskii* and *A. ovalisporum* strains were monocultures grown under laboratory conditions. The *C. raciborskii* (AQS) and *A. ovalisporum* (ILC-164) references and isolated strains were grown in combined nitrogen-supplemented Allen medium under constant bubbling with sterile air at 28 °C. Cultures were illuminated with warm white fluorescence tubes for 10 days in 4 L vessels. After harvesting by centrifugation (Beckman Avanti J-25), repetitive freezing-thawing (–7 °C and 1–4 °C, respectively) and filtering, the extracts of cells of reference and bloom samples were ready for CE analysis.

The cultured strains and bloom samples with known volumes were filtered through GF/C discs (Whatman, Maidstone, UK), and the cell materials were frozen and stored at –7 °C. Prior freezing the samples were divided into two parts: the first destined for analysis of cyanotoxins was frozen and thawn-twice (to break the cells), and then filtered through a glass fiber filter (GF/C, Whatman); the second was lyophilized for measurement of dry mass.

Quantitation of cylindrospermopsin content of plankton samples and cyanobacterial strains

The cyanotoxin (cylindrospermopsin) content was determined with the help of capillary electrophoresis as described by our laboratory earlier [13]. The CE electrolyte contained 25 mM sodium tetraborate (pH 9.1) and 100 mM SDS. Separation of compounds was followed at 270 nm, since the elevated alkaline pH shifted the absorption maximum of CYN toward 270 nm. On several occasions purified CYN was added to crude extracts (to crude cell lysates or to culture supernatants) before CE. When direct biosample injection was performed the use of interanalysis rinses become of particular importance to remove all proteineous components, which have a high tendency to stick to the capillary walls and cause variations in electroosmotic flow rates and migration times. Therefore, after each electrophoretic run the capillary was washed with 0.5 M NaOH (5 min), acetonitrile (2 min), and water (5 min). In the case of MEKC the SDS micelles strongly interact with the sample proteins, causing the proteins to be eluted after the toxin peaks of interest [13].

PS gene and PKS gene amplification

DNA extraction was achieved by the phenol–chloroform method [12]. PCR was used to analyse the presence or absence of the *PKS* and *PS* specific gene region, which are important parts of the gene cluster responsible for CYN biosynthesis. The molecular mass marker 120 was GeneRulerTM 1 kb DNA Ladder (Fermentas), the photo preparation and the analysis of the results was achieved using Cleaver GelDoc system.

For the PS and PKS gene amplification, the PCR reaction was performed in 25 µl containing 12.5 master mix (Fermentas; PCR buffer, 0.05 U/µl Taq Polimerase, 4 mM $MgCl_2$, 0.4 nM dNTP), forward and reverse primer, DNA template (100 ng). Amplification was performed in a T Gradient thermocycler, consisting of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C and 2 min at 72 °C and a final extension step of 7 min at 72 °C. For the PKS gene amplification, the PCR reaction was performed as above, using PKS M4 (5'-GAAGCTCTGGAATCCGGTAA-3') and PKS M5 (5-AATCCTTACGGGATCCGGTGC-3) primers. For the PS gene amplification, the PCR reaction was performed as above, using PS M13 (5'-GAAGCTCTGGAATCCGGTAA-3') and PS M14 (5-AATCCTTACGGGATCCGGTGC- 3) primers [12]. The PCR products were electrophoresed in 1% agarose gel.

RESULTS

Morphology of Aphanizomenon ovalisporum *and* Cylindrospermopsis raciborskii *strains*

C. raciborskii strains were identified according to the morphological features by Komarek [7]. Morphological characteristics of *C. raciborskii* revealed solitary, straight or slightly curved trichomes that had a range in length of 40–185 μm. Heterocysts were ovoid in shape and always terminal. There were either one or two terminal heterocysts on each trichome.

Two strains of our isolates were identified as *A. ovalisporum* in accordance with the morphological features designated earlier [1] with straight or slightly curved trichomes. There were also heterocysts at various locations throughout the trichomes. Akinetes were either spherical or broadly oval.

PS gene and PKS gene amplification

Eleven strains of *C. raciborskii* were included in this study, 10 from Hungarian waters and a CYN producing strain of *C. raciborskii* AQS from Australia. All the Hungarian samples tested negative for PKS and PS genes (Table 1). AQS was the only strain giving positive amplification products for both of the genes (Table 1).

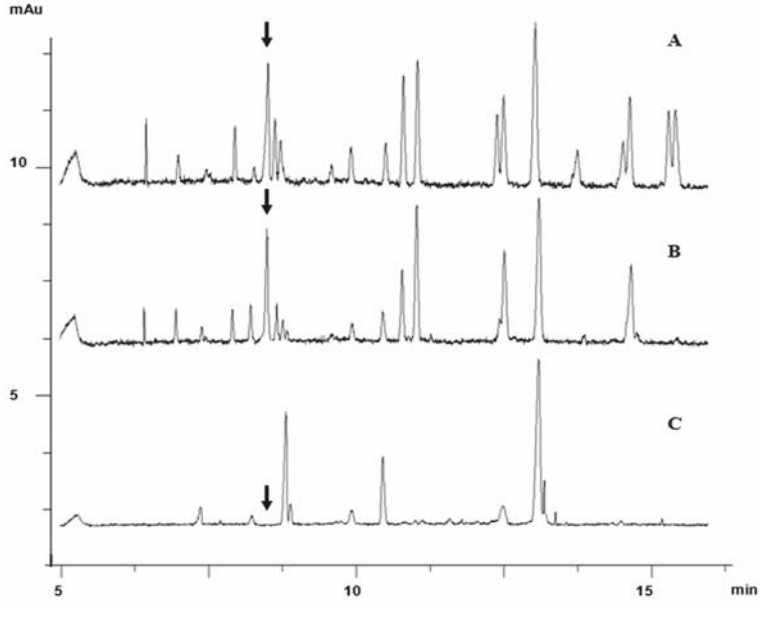

Fig. 3. Electropherograms of *A. ovalsiporum* strains **A**: Israel (ILC-164) arrow shows the peak of CYN; **B**: Spain, Blanes (BGSD-300) arrow shows the peak of CYN; **C**: Hungary, Lake Szelidi (BGSD-301) arrow shows the lack of CYN; MEKC: 25 mM sodium tetraborate, 100 mM SDS, pH 9.3

Three strains of *A. ovalisporum,* a well-known CYN producing strain from Israel (ILC-164) and two of our isolates were tested for PKS and PS genes. While the (ILC-164) strain from Israel and the *A. ovalisporum* isolated from Spain were positive, the Hungarian samples isolated from Lake Szelidi tested negative for PKS and PS genes (Table 1).

Quantitation of cylindrospermopsin content of plankton samples and cyanobacterial strains

CYN was not detected in the cultures of *C. raciborskii* strains isolated from Hungary, and also not detected in the *C. raciborskii* dominated field samples. CYN was detected only in *C. raciborskii* AQS from Australia (Table 1).

Two of the three *A. ovalisporum* isolates contained CYN. The (ILC-164) strain from Israel and *A. ovalisporum* isolated from Spain was positive for the alkaloid hepatotoxin. Neither isolated *A. ovalisporum*, nor the collected *A. ovalisporum* containing plankton sample from Lake Szelidi contained CYN (Fig. 3 and Table 1).

Table 1
Cyanobacterial strains/phytoplankton samples in this study

Species	Strain no.	Origin	PSgene/ PKSgene	CYN mg·g^{-1}
Cylindrospermopsis raciborskii	BGSD-266*	Lake Balaton, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	BGSD-2000	Lake Balaton, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	BGSD-2001	Lake Balaton, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	BGSD-410	Kis-Balaton reservoir, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	BGSD-280	Lake Szelidi, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	AQS**	Australia	+/+	3.94
Cylindrospermopsis raciborskii	phytoplankton samples	fish pond Doboz, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	phytoplankton samples	backwater, Endrőd, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	phytoplankton samples	ornamental pond Debrecen, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	phytoplankton samples	fish pond Zamardi, Hungary	–/–	n.d.
Cylindrospermopsis raciborskii	phytoplankton samples	reservoir Levelek, Hungary	–/–	n.d.
Aphanizomenon ovalisporum	ILC-164***	Israel	+/+	4.78
Aphanizomenon ovalisporum	BGSD-300	Blanes, Spain	+/+	4.52
Aphanizomenon ovalisporum	BGSD-301	Lake Szelidi, Hungary	–/–	n.d.

* We thank dr. Attila Kovács for kindly helping with *Cylindrospermopsis rciborskii* strain.
** We thank dr. Törökné Kozma Andrea for kindly helping with *Cylindrospermopsis rciborskii* AQS strain.
*** We thank dr. Ora Hadas for kindly helping with *Aphanizomenon ovalisporum* ILC164.

DISCUSSION

Since cyanobacterial toxin CYN was isolated in 1992 from an Australian strain of *C. raciborskii* [8], the occurrence of this species has been documented in every continent. For the last few years, several studies have included information about its occurrence in European lakes, e.g. Spain, Germany, Italy, Czech Republic, Poland and Finland. Despite several indications of *C. raciborskii* occurrence in European lakes, these strains were not found to be producers of CYN [3, 6, 12].

In the cases when CYN producer was found in European lakes, cyanobacterial species were from the *Aphanizomenon* and *Anabaena* genera, were suspected for this ability [6, 12].

Because of the inconclusive results of the CYN production ability of Hungarian *C. raciborskii,* we tested several strains collected and isolated by us. In addition an *A. ovalisporum* strain was tested, which was the dominant species in Lake Szelidi (Table 1).

Lake Szelidi is situated in the Kalocsa Plain, in the lowlands of the Danube basin. It is a typical mortlake, that had been cut off from the Danube after the Ice Age. It is 5 kilometers long, 120–150 meters wide and 3–4 meters deep. It is a hyposaline lake with natrium-, hydrocarbonate- and chloride ion dominance. Because the lake is very popular as a recreational water, the toxicity of the dominant *A. ovalisporum* in summer could be crucial [10].

The PS and PKS genes are encoding enzymes involved in secondary metabolite biosynthesis in several cyanobacterial species. Simultaneous presence of both genes seems to be associated with the ability to produce the alkaloid CYN [12]. According to our CE data in the analysed set of Hungarian *C. raciborskii* strains no CYN production was observed. When PCR detection of PS and PKS genes was compared with CYN content, the expected amplicons were only found in the CYN positive strains: the Australian *C. raciborskii* strain (AQS), *A. ovalisporum* (ILC-164) from Israel and an *A. ovalisporum* strain from Spain isolated by us (BGSD-300; Table 1). The data related to the global distribution of CYN, are still not complete. Although we did not detect CYN in any of the investigated strains and collected samples from Hungary, we did it in a European *A. ovalisporum* strain isolated from Spain. Beyond giving support to the described non-toxic nature of European *C. raciborskii* strains, our results confirm that the two-gene directed PCR method could be a rapid approach to evaluate the cylindrospermopsin-related toxicity of cyanobacterial strains.

Considering the variety of CYN producers observed around the world, *A. ovalisporum* may be one of the several species of cyanobacteria that are producing CYN in the freshwater of Hungary, but not in Lake Szelidi. More species, especially from the genus *Aphanizomenon* and *Anabaena* should be collected, isolated and tested from Hungary. On the other hand, this study demonstrates that *C. raciborskii* is very unlikely that it could be a CYN producer in Hungary.

Although our study does not demonstrate the occurrence of cylindrospermopsin in Hungary, we suggest that not only the presence of the frequently occurring micro-

cystins should be monitored in waters used for drinking and recreation, but CYN must be studied in this respect as well.

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