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Dissolved organic phosphorus use by the invasive freshwater diazotroph cyanobacterium, *Cylindrospermopsis raciborskii*

Fang Bai, Rui Liu, Yanjun Yang, Xiaofei Ran, Junqiong Shi, Zhongxing Wu *

Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education), Chongqing Key Laboratory of Plant Ecology and Resources Research in Three Gorges Reservoir Region, School of Life Science, Southwest University, Chongqing 400715, PR China

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ABSTRACT

This study examines the physiological responses of the diazotrophic cyanobacteria, *Cylindrospermopsis raciborskii*, to different dissolved organic phosphorus (DOP) compounds to explore mechanisms of environmental acclimation in this invasive species. Our results show that the specific growth rates of *C. raciborskii* cells in media treated with β-glycerol phosphate, D-glucose-6-phosphate, and (2-aminoethyl)-phosphinic acid were significantly higher than those of cells grown in phosphorus free media. We observed that maximal net photosynthesis was highest when cells were cultured with D-glucose-6-phosphate and lowest when cells were cultured with glyphosate. Similarly, rates of photosynthetic activity (maximum quantum yield, maximum electron transport rate, and photosynthetic efficiency) were observed to be highest in media treated with D-glucose-6-phosphate. We report that rates of alkaline phosphatase activity to the different organophosphates tested changed markedly in response to the concentration of dissolved inorganic phosphorus (DIP); a result supported by the amount of green fluorescent products revealed by ELF®97 phosphate dye (ELFP) and gene up-regulation for alkaline phosphatase (*phoA*). Our results indicate that *C. raciborskii* is able to use different organic phosphorus to support its growth when phosphorus is limited. In addition, we show that *C. raciborskii* has a higher availability to phosphate (C–O–P) than phosphonate (C–P). The results suggest that the strategic flexibility to environmental phosphorus might play an important role in the domination of *C. raciborskii*.

1. Introduction

Cylindrospermopsis raciborskii (Woloszyńska) (Seenayya and Subba-Raju, 1972) is a freshwater, planktonic, filamentous cyanobacterium that was first recorded in 1978 in Java, Indonesia (Woloszyńska, 1912). Owing to its dependence on high temperatures for growth (optimally at 25 °C) and its adaptability to temperature fluctuations, *C. raciborskii* was originally considered to be restricted to tropical waters (Briand et al., 2004). Indeed, *Cylindrospermopsis* blooms are frequent in the tropics (Branco and Senna, 1994; Fabbro and Duivenvoorden, 1996). In recent decades, however, the species has spread globally into temperate regions. For example, *C. raciborskii* is currently spreading into Germany (Fastner et al., 2003; Stüken et al., 2006), France (Briand et al., 2002), Hungary (Borics et al., 2000), Canada (Hamilton et al., 2005), USA (Hong et al., 2006), Thailand (Chonudomkul et al., 2004), China (Wu et al., 2009), New Zealand (Wood and Stirling, 2003), Austria (Everson et al., 2009), and Brazil (Huszar et al., 2000). In addition, *C. raciborskii* has also invaded a wide variety of water bodies, including ponds, lakes, reservoirs and other drinking and recreational water supplies (Dyble et al., 2006). As *C. raciborskii* can produce toxins, such as hepatotoxic cylindrospermopsin (CYL) and paralytic shellfish-poisoning toxin (PSP), contamination of reservoirs and recreational lakes poses serious health risks to both people and wildlife (Byth, 1980; Bourke et al., 1983; Saker et al., 1999; Lagos et al., 1999). As a result, *C. raciborskii* has garnered increasing attention from both the scientific community and managers of water facilities.

Numerous factors may have facilitated the successful spread of *Cylindrospermopsis raciborskii* into temperate regions, including buoyancy (Ramberg, 1984; Padisák, 1997), superior shade tolerance (Padisák, 1997), a high affinity for ammonium uptake (Padisák, 1997; Présing et al., 2001; Burford et al., 2006), superior N_2-fixation ability (Padisák, 1997; Burford et al., 2006), tolerance to grazing (Padisák, 1997), efficient use of nitrate and iron (de Souza et al., 1998; Bouvy et al., 1999, 2001; McGregor and Fabbro,

* Corresponding author. Tel.: +86 23 68254532; fax: +86 23 68254533.
E-mail address: wuzhx@swu.edu.cn (Z. Wu).

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2000), a rate of high phosphate uptake and phosphate storage capacity (Isványovics et al., 2000), and climate change effects (O'Neil et al., 2012). Despite this knowledge, however, bloom formation by *C. raciborskii* remains poorly studied and difficult to predict; largely because it is dependent on interactions among numerous stochastic factors (Padisák and Reynolds, 1998).

Nutrient availability is thought to be the one of the primary factors governing the growth of phytoplankton. In particular, the quantity of phosphorus in the environment mediates phytoplankton biomass and influences phytoplankton species composition (Dyhrman et al., 2007). Phosphorus loading is thus predicted to play an important role in the growth and production of phytoplankton and benthic algae, as well as the occurrence of cyanobacteria blooms (Wu et al., 2000; Paerl et al., 2001; Young et al., 2010). Similar suggestions are also applied in the dominance of *Cylindrospermopsis raciborskii* (Isványovics et al., 2000; Posselt et al., 2009; O'Neil et al., 2012). However, studies examining the effect of phosphorus on the growth and dominance of *C. raciborskii* reveal several inconsistencies (Posselt et al., 2009). For example, Isványovics et al. (2000) suggested that a capacity for high phosphorus storage might promote the dominance of *C. raciborskii*. However, a later study revealed that while *C. raciborskii* exhibits rapid uptake of dissolved inorganic phosphorus (DIP), its affinity for DIP is weaker than that observed in two other bloom-forming cyanobacteria, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* (Wu et al., 2009). Nevertheless, low surface DIP concentrations (<0.06 μM DIP) may be sufficient to promote blooms of *C. raciborskii* (Padisák and Isványovics, 1997).

Alternatively, *Cylindrospermopsis raciborskii* might display a unique strategy of phosphorus use. Dissolved phosphorus is comprised of non-reactive inorganic compounds, such as polyphosphates (Diaz et al., 2008), and organic compounds (dissolved organic phosphorus, DOP) derived from the degradation of glycolipids, glycoproteins, antibiotics and phosphonolipids (Clark et al., 1998; Kolowith et al., 2001; Dyhrman et al., 2006; White and Metcalf, 2007). Although DOP constitute the majority of dissolved phosphorus, non-reactive inorganic phosphorous compounds are regarded as the most important source of phosphorus for microalgae (Clark et al., 1998; Kolowith et al., 2001). It has been demonstrated that some phytoplankton alter their physiologies so as to utilize DOP compounds for sustained growth (Cembella et al., 1984; Dyhrman et al., 2006; Ilikchyan et al., 2009). In addition, Padisák (1997) showed that *Cylindrospermopsis* blooms occurred in lakes that have high amounts of DOP. Latterly, some genes about to the utilize of inorganic and organic P, such as high affinity phosphate binding proteins (*pstS*, *sphX*), phosphanate uptake (*phnC,D,E*) and metabolism (*phnG-M,X,W*), and phosphorus ester metabolism (*phoA*), have been proved in *C. raciborskii* (Stucken et al., 2010; O'Neil et al., 2012). It is possible that *C. raciborskii* is similarly able to utilize DOP. However, the mechanisms by which *Cylindrospemopsis* responds to different dissolved organic phosphorus are not yet well established.

In this study we analyzed rates of growth, photosynthesis, metabolic enzyme activity and gene expression in *Cylindrospermopsis raciborskii* so as to investigate the ability of *C. raciborskii* to use dissolved organic phosphorus compounds, and to determine the effects of increasing organic P inputs on the occurrence of *C. raciborskii*.

2. Materials and methods

2.1. Culture conditions and experimental design

Cylindrospermopsis raciborskii FACHB 1096 was obtained from the Culture Collections of the freshwater algae section of the Institute Hydrobiology, Chinese Academy of Sciences (FACHB-Collection; Wuhan, China). The strain was purified by lysozyme and antibiotic treatment according to methods described in Sarchizian and Ardelean (2010). Regular fluorescence microscopic inspection after acridine orange and 4′, 6-diamidino-2-phenylindole staining revealed that the biomass of the contaminating bacteria never exceeded 1% of the cyanobacterial biomass in the course of our experiment. This purified strain was grown in MA medium (Ichimura, 1979) at 25 ± 1 °C, under a white light with an intensity of 25 μmol photons m^{-2} s^{-1} and a 12 h light/12 h dark cycle. Cells in the logarithmic growth phase were collected by centrifugation at 4000 rpm for 8 min, then washed three times with a P-free medium and then inoculated in a P-free medium for three days to remove excess phosphate. This process was undertaken for all samples in batches.

Experimental treatments consisted of the application of one of five different forms of phosphorus (β-glycerol phosphate, D-glucose-6-phosphate, (2-aminoethyl)-phosphinic acid, glyphosate and the dipotassium phosphate (K_2HPO_4)) to the P-free MA medium, with a control treatment (no phosphorus added) also tested. The organic phosphorus were purchased from Sigma–Aldrich (USA) (>99%, analysis-grade or chromatography analysis-grade) and differed in bonding patterns (β-glycerol phosphate, D-glucose-6-phosphate include a carbon-oxygen-phosphorus bond (C–O–P), while (2-aminoethyl)-phosphinic acid, glyphosate have a carbon-phosphorus bond (C–P)). The initial concentration of *Cylindrospermopsis* in each treatment was OD_{680} = 0.2, which equals to the concentration of Chl *a*, about 0.652 mg l^{-1}. All treatments were carried out in triplicate.

2.2. Analysis of chlorophyll a and the specific growth rate

Chlorophyll *a* and cell density was measured in cultures every two days. Chlorophyll *a* was extracted with 90% acetone and measured according the methods of Nusch (1980). The specific growth rate (μ) was calculated according to the following equation: $\mu = (\ln C_{t_2} - \ln C_{t_1})/(t_2 - t_1)$, where C_{t_2} and C_{t_1} are cell density at times t_2 and t_1, respectively. The cell density was determined using a hemocytometer chamber under a Nikon CE-I microscope (Nikon, Japan), The cell number of filaments was obtained through the method: firstly, measuring the size and cell number of different individual in order to count the average cell number per unit; secondly, determining the size of each filament; lastly, converting individual size to cell number.

2.3. Analysis of photosynthetic oxygen evolution

Following the exponential growth phase, filaments were sampled from each experimental phosphorous treatment and centrifuged for 8 min at 4000 rpm. The pelleted cells were then washed and suspended in the corresponding medium. Photosynthetic oxygen evolution was measured with a Clark-type oxygen electrode (Chlorolab 2, Hansatech, UK) at 25 °C. Illumination was set from 0 to 1200 μmol photons m^{-2} s^{-1}, with oxygen evolution measured for at least 10 min at each irradiance value. Parameters for the photosynthetic responses to irradiance curves (*P–I* curves) were analyzed according to the equation (Henley, 1993):

$$P = P_m \times \tanh\left(\frac{\alpha I}{P_m}\right) + R_d,$$
$$I_k = \frac{P_m}{\alpha}, I_c = -\frac{R_d}{\alpha}$$

where P represents photosynthetic rate at irradiance I; P_m is the maximum photosynthesis rate; α is the slope of light-limited part of *P–I* curve; I is the irradiance; R_d is the dark respiration rate; I_k is the saturating irradiance for photosynthesis; and I_c is the light compensation point.