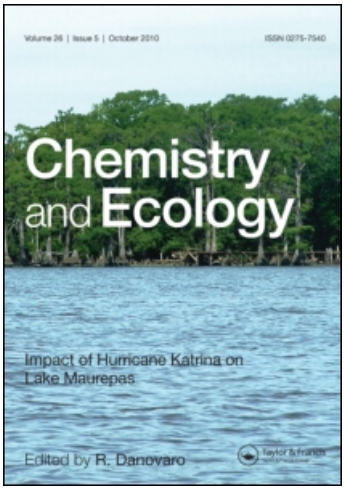

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Allelopathic effect of *Cylindrospermopsis raciborskii* extracts on the germination and growth of several plant species

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Cylindrospermopsis raciborskii is an invasive cyanobacterium and a potential producer of the alkaloid toxin cylindrospermopsin (CYN). Extracts of two strains of *C. raciborskii* were tested for their effects on the germination and growth of *Lactuca sativa*, *Phaseolus vulgaris*, *Pisum sativum* and *Solanum lycopersicum*. Germination was not significantly inhibited for any of the plant species tested, but growth was affected, depending on the species. Root and stem growth in *L. sativa* was generally stimulated by both strains. *Ph. vulgaris* root growth was stimulated by both strains but no effect was visible in stem growth. *S. lycopersicum* root growth was inhibited by both strains and stem growth was inhibited only by the CYN strain. *P. sativum* root growth was also inhibited by both strains but stem growth was stimulated. CYN accumulation was also differential with toxin transfer to the stem. *Ph. vulgaris* accumulated the highest CYN concentration. This study suggests that plants behave differently in their response to this toxin and that roots and stems also show different abilities to react and accumulate the toxin. Knowledge of the impact of CYN- and non-CYN-producing cyanobacteria in different plant species and translocation of the toxin to different plant parts is essential for the avoidance of human as well as environmental health hazards.

Keywords: *Cylindrospermopsis raciborskii*; cylindrospermopsin; allelopathy; plants; germination; growth; bioconcentration

1. Introduction

Cylindrospermopsis (Seenayya and Raju, 1972) is a filamentous, heterocystous freshwater cyanobacterium which is causing increasing concern because of its potential toxicity [1–3] and widespread distribution [4]. Another concern about *Cylindrospermopsis* is based on reports which show that the toxin cylindrospermopsin (CYN) (Figure 1) accumulates in several organisms [5–7], suggesting that it may be bioaccumulated. The first report of human poisoning by *Cylindrospermopsis raciborskii* occurred in Palm Island, Australia [8], where a severe cyanobacteria bloom was treated with copper sulphate which released the toxin and all toxic cellular components into the water. Since then, *C. raciborskii* has been reported upon because of its severe hepatotoxicity [1,9]. However, injuries to other organs such as the kidneys and heart have also been reported [10–12].

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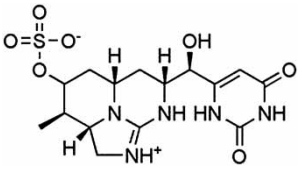

Figure 1. Structure of the cytotoxic alkaloid cylindrospermopsin (CYN), produced by cyanobacteria.

Bloom-forming cyanobacteria produce a variety of toxins, increasing the possibility of toxin uptake and accumulation by plants. Most reports regarding the effects and accumulation of cyanotoxinin plants are on *Microcystis aeruginosa*. Uptake by plants has been reported and rhizomes and stems have been described as the main uptake routes [13–15]. Secondary effects have also been reported, with plant growth being obviously affected [13,14,16]. This has been reported mainly as a reduction in the size of the roots and a decrease in the number of fronds and roots [13–17]. These effects are probably related to a decrease in phosphatase activity and a reduction in chlorophyll (a + b) concentration [15,16,18,19]. Other secondary effects on plants have been reported, namely aerenchyma obturation, altered lignification of cell walls in the axial organs, root and leaf necrosis, frond chlorosis, premature development of lateral roots, root coalescence and early aerenchyma formation [16,18].

There is little information concerning the effects of CYN on plants. Exposure of tobacco (*Nicotiniana tabacum*) pollen to CYN led to the inhibition of germination with an IC_{50} of 300 $\mu g\,CYN \cdot mL^{-1}$ [20]. The action of CYN was compared with that of cycloheximide, a known protein synthesis inhibitor, although CYN was less potent, with toxic effects detected at 5 $\mu g\,CYN \cdot mL^{-1}$. The growth of *Sinapsis alba* seedlings was also affected, with an IC_{50} value of 18.2 $\mu g\,CYN \cdot mL^{-1}$ when exposed to CYN [21]. CYN also seems to affect root growth in the common reed (*Phragmites australis*) [22]. In fact, plants exposed to CYN concentrations ranging from 0.5 to 40 $\mu g \cdot mL^{-1}$ showed root growth inhibition with an IC_{50} value of 0.5 $\mu g\,CYN \cdot L^{-1}$. Most of these studies used very high CYN concentrations, not found in nature, but Kinnear et al. [23] used an environmentally relevant concentration and studied the exposure of the duckweed *Spirodela oligorrhiza* to concentrations up to 120 $\mu g\,CYN \cdot L^{-1}$. They showed that *Spirodela oligorrhiza* is able to respond physiologically to concentrations up to 50 $\mu g\,CYN \cdot L^{-1}$, and significant toxic effects were only registered for concentrations $> 100\,\mu g\,CYN \cdot L^{-1}$.

To date, several cyanobacterial species have been shown to produce CYN, including *Aphanizomenon flos-aquae* in Germany [24], *Anabaena lapponica* in Finland [25], *Aphanizomenon ovalisporum* in Israel [26], *Umezakia natans* in Japan [27], *Anabaena bergii* in Australia [28] and *Raphidiopsis curvata* in China [29]. Ecological and economical effects of water contaminated with cyanobacteria and with CYN on plants have to be assessed to estimate the risks of watering plants with eutrophic water.

This article reports on the effects of water contaminated with CYN-producing and non-CYN-producing cyanobacteria on the growth of four edible plant species: *Lactuca sativa*, *Phaseolus vulgaris*, *Pisum sativum* and *Solanum lycopersicum*. Effects of extracts on germination, root and stem growth, as well as CYN accumulation are reported.

2. Material and methods

2.1. *Culture preparation*

To obtain a biomass of *C. raciborskii*, CYN-producing and non-CYN-producing strains [7] were cultured in 4 L of Z8 medium [30]. They were grown in the laboratory at 25 °C, under a light

intensity of 20.8–27.4 $\times 10^{-6}$ E $\cdot$ m^{-2} $\cdot$ s^{-1} with a light/dark cycle of 14/10 h until they reached the end of the exponential phase. When the cultures reached a cell density of 10^6 cells $\cdot$ mL^{-1}, three subcultures were prepared for each toxic and non-toxic strain of *C. raciborskii* with 10^4, 10^5 and 10^6 cells $\cdot$ mL^{-1}, corresponding to a total of 0.57, 5.7 and 57.0 μg CYN $\cdot$ L^{-1}, respectively.

2.2. *Germination assay*

Seeds of four plant species commonly used in agriculture, *L. sativa*, *Ph. vulgaris*, *P. sativum* and *S. lycopersicum* were soaked in a 10% bleach solution for $\sim$ 20 min to eliminate the possibility of fungal and bacterial growth. After that, they were rinsed with ultrapure water to remove the bleach. Germination assays were performed in Petri dishes lined with a disc of paper. All seeds were placed an equal distance from each other in the Petri dishes to avoid interference in development. For *L. sativa* and *S. lycopersicum*, 10 seeds were placed in each Petri dish moistened with 3 mL of *C. raciborskii* cultures (of the three concentrations, both toxic and nontoxic) or with 3 mL of distilled water (control sample). The same procedure was used for *P. sativum* and *Ph. vulgaris*, with the exception that only five seeds were placed per each Petri dish. For each sample, three replicates were carried out. Petri dishes were then sealed with Parafilm®, to prevent evaporation, and placed in the dark at 24 $\pm$ 1 °C. One week later, the number of germinated seeds (those with root growth of 2 mm or more) was recorded for each plant.

2.3. *Growth assay*

Germinated plants were placed on Petri dishes with 3 mL of cyanobacterial extract or with water (control) and placed at 24 $\pm$ 1 °C, under a light intensity of 20.8–27.4 $\times 10^{-6}$ E $\cdot$ m^{-2} $\cdot$ s^{-1} with a light/dark cycle of 14/10 h. The growth of the different plants was measured after 15 days, when root and stem lengths were measured.

2.4. *CYN quantification in the roots and stem*

Roots and stems were weighed separately and extracted with 50% methanol (5 mL methanol $\cdot$ g plant^{-1}). Plant extracts were prepared by crushing, followed by ultrasonication on ice for 15 min to release CYN from the cells; the methanol was then fully evaporated. In order to remove cell fragments, extracts were centrifuged at 4600 rpm for 5 min, allowing the supernatant to be recovered. The supernatant was filtered through a 0.45 μm filter prior to analysis. CYN detection in extracts of both lower (root) and upper (stem) parts of the plants was carried out using an immunological assay (Cylindrospermopsin ELISA – Microtiter Plate®; ABRAXIS), with a detection limit of 0.040 ppb (μg $\cdot$ L^{-1}).

2.5. *Statistical analysis*

One-way analysis of variance (ANOVA) and post-hoc Tukey tests were used to analyse significance of the differences on germination, root and stem growth (SPSS version 16.0).

3. Results

3.1. *Germination assay*

After one week of incubation in the presence of cyanobacterial extracts, all plant species registered a germination rate > 70%. Controls had a germination rate of 90–100%. The presence of CYN did

not interfere significantly with germination rate except for *S. lycopersicum* ($p < 0.05$). The lowest observed germination rate, 73.3%, was obtained for *L. sativa* after exposure to 10^6 cells $\cdot$ mL^{-1} of the non-CYN extract, being significantly different from all other concentrations and the control ($p < 0.05$). For *P. sativum*, all treatments produced a 100% germination rate.

3.2. *Growth assay*

During the growth assay, some Petri dishes showed plant mortality and a high standard deviation. For *S. lycopersicum*, most of the plants that had germinated died during CYN treatment. In fact, only one valid replica for each concentration (10^6, 10^5 and 10^4) survived. Of the seeds that had germinated, few were able to develop during exposure. The number of plants exposed to the toxin was insufficient to allow us to quantify CYN in this species.

Analysis of the variation in root length relative to that of the control for all species and treatments shows that *S. lycopersicum* and *P. sativum* are affected by both CYN and non-CYN producers (Figure 2). *S. lycopersicum* is affected equally by both strains, showing that CYN seems not to be the main toxic substance affecting root development. In fact, significant differences in root length were found only when considering cell density ($p < 0.05$) and not the presence or absence of toxin. *P. sativum* is affected more by the CYN producer, although no significant differences were found ($p = 0.189$). By contrast, *Ph. vulgaris* root length is stimulated by exposure to CYN and non-CYN cyanobacteria. Both extracts produced a stimulatory effect that, in the case of the non-CYN producer, was concentration dependent. *L. sativa* showed stimulation of root length for the two lowest cyanobacteria concentrations and both strains, although at 10^6 cells $\cdot$ mL there was significant inhibition by the CYN-producing strain ($p < 0.05$).

The variation in stem length relative to controls for plants exposed to the two cyanobacteria strains showed different behaviour (Figure 3). *L. sativa* and *P. sativum* growth was stimulated by the two strains, particularly the non-CYN producer. *Ph. vulgaris* was affected slightly by both

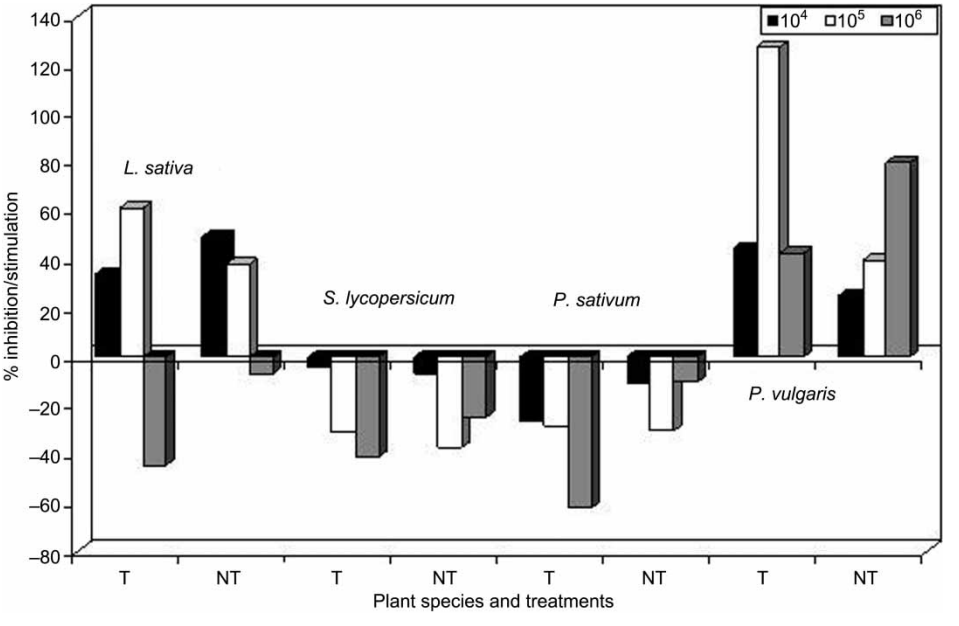

Figure 2. Effects (% stimulation/inhibition compared with controls) of two-week exposure to CYN (T) and non-CYN (NT) cyanobacteria on root growth in *Lactuca sativa*, *Phaseolus vulgaris*, *Pisum sativum* and *Solanum lycopersicum.*

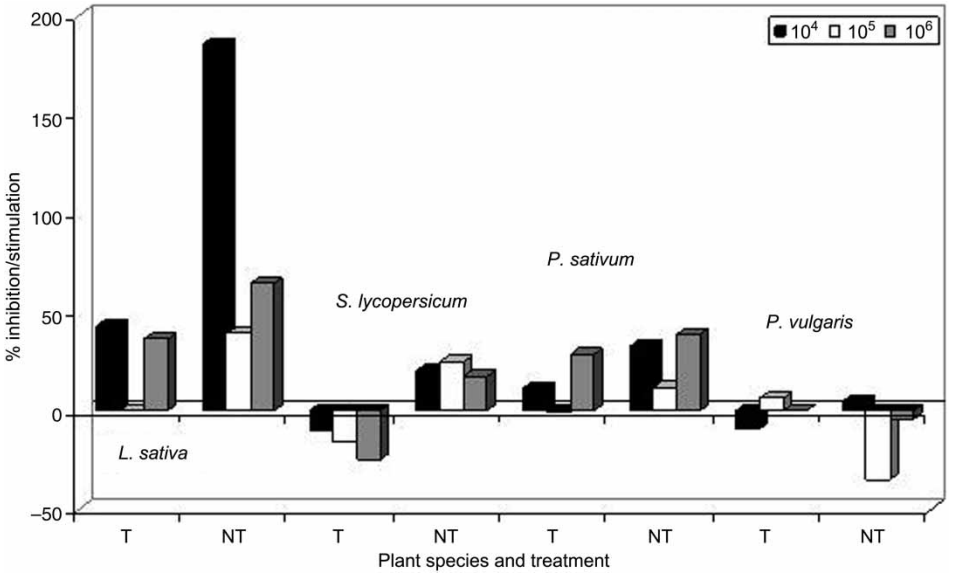

Figure 3. Effects (% stimulation/inhibition compared with controls) of two-week exposure to CYN (T) and non-CYN (NT) cyanobacteria on stem growth in *Lactuca sativa*, *Phaseolus vulgaris*, *Pisum sativum* and *Solanum lycopersicum.*

Table 1. Stimulatory (+) or inhibitory (−) effects and cylindrospermopsin (CYN) bioconcentration in the stems of plants exposed to *Cylindrospermopsis raciborskii* extracts with and without CYN.

Plant species	Root	Stem	CYN in stem
Lactuca sativa	+	+	++
Solanum lycopersicum	+	CYN, + no CYN	*
Pisum sativum	−	+	+
Phaseolus vulgaris	+	=	+++

Note: =, no effect; *, no data.

strains with no significant differences, and *S. lycopersicum* was inhibited by the CYN strain and stimulated by the non-CYN strain ($p < 0.05$). As mentioned above, most of the *S. lycopersicum* plants died at the end of the exposure period, showing that this species is the most sensitive to CYN (Table 1).

3.3. *Quantification of CYN in the roots and stem*

Analysis of the CYN content in the roots of the exposure plants showed that there is an uptake which is concentration dependent (Figure 4). The measured values show that the accumulation rate for the species increases in the order *P. sativum* > *Ph. vulgaris* > *L. sativa*. The inverse relationship between root development and CYN uptake may indicate that the toxin is more readily absorbed by *P. sativum*, leading to higher toxicity and inhibiting root growth. Transfer of the CYN to the upper part of the plant (stem) was observed in the three analysed species (Figure 5). As expected, the highest accumulation was observed at the highest exposure, but the species that accumulated the highest concentration was *Ph. vulgaris*. In fact, this species accumulated up to 6.8 μg CYN · g^{-1} plant, more than one order of magnitude higher than in *L. sativa*. It is interesting to note that the stem of *Ph. vulgaris* was not significantly affected by exposure to CYN at any

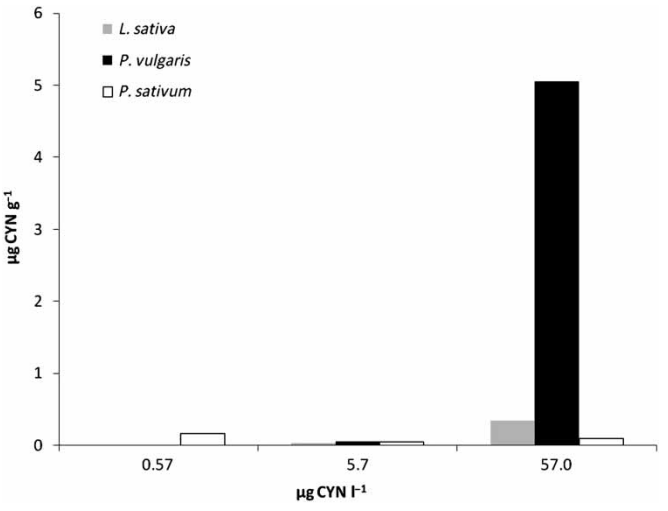

Figure 4. Cylindrospermopsin accumulation ($\mu g\,CYN \cdot g^{-1}$ plant) in the roots of *Lactuca sativa*, *Phaseolus vulgaris* and *Pisum sativum* after a two-week exposure.

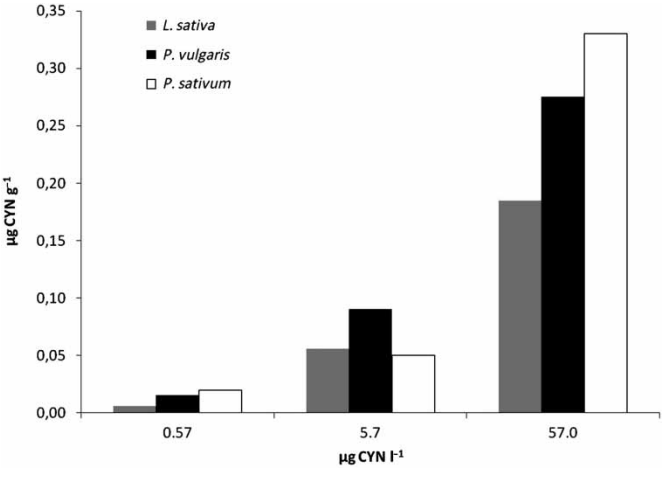

Figure 5. Cylindrospermopsin accumulation ($\mu g\,CYN \cdot g^{-1}$ plant) in the stems of *Lactuca sativa*, *Phaseolus vulgaris* and *Pisum sativum* after a two-week exposure.

of the tested concentrations, compared with controls, even at the high accumulation rate of the toxin. *L. sativa* accumulated a maximum of $0.4\,\mu g\,CYN \cdot g^{-1}$ plant in the stem and its growth was stimulated by the presence of the extract, showing that the toxin seems not to affect the growth of the plant.

4. Discussion

The absence of significant effects on the germination of the different plant species exposed to extracts of *C. raciborskii* was not expected because previous studies have shown that CYN affected the germination of *N. tabacum* pollen [20], and growth of *Sinapsis alba* seedlings was also inhibited by the toxin [21]. Nevertheless, in both studies, the IC_{50} values were 300 and

18.5 μg CYN · mL^{-1}, which are three to four orders of magnitude higher than used here. Work carried out with another cyanobacteria hepatotoxin, microcystin (MC), caused differential germination success depending on the plant species [31]. *Lens esculenta* was the most resistant species, whereas *P. sativum* was the most sensitive. By contrast, Pereira et al. [32] showed that exposure to extracts of cyanobacteria with and without MC did not affect the germination of two grass species (*Festuca rubra* L. and *Lolium perenne* L.) or lettuce (*Lactuca sativa* L.) seeds. It seems that sensitivity or resistance to cyanotoxins is concentration and species dependent. Saqrane et al. [31]. used μg MC-LR · mL^{-1}, whereas Pereira et al. [32] used μg MC-LR · L^{-1}, as in this study. Ecologically relevant cell density and toxin concentrations seem not to have any significant effects on seed germination. Toxin uptake may be easier in some species and detoxication may also be more efficient in others, enhancing resistance.

The effects of CYN on plant growth were shown by Vasas et al. [21]. An EC_{50} value of 18.2 μg CYN · mL^{-1} was found by measuring growth reduction in mustard (*Sinapsis alba*) seedlings. Metcalf et al. [20] found that exposure to CYN caused inhibition of tobacco (*Nicotiniana tabacum*) pollen germination at concentrations in the range 5–1000 μg CYN · mL^{-1}. Kinnear et al. [33] showed that exposure of water thyme (*Hydrilla verticilata*) to extracts containing 25–400 μg CYN · L^{-1} caused concentration-dependent effects. They recorded that the greatest increase in root growth was recorded at the maximum toxin concentration – 400 μg CYN · mL^{-1}. This showed stimulation of the root by exposure to the extract containing CYN. In this study, this was also true for *Ph. vulgaris*. *L. sativa* root growth was also stimulated, except at the highest CYN concentration (Table 1). Exposure to increasing CYN concentrations was negatively correlated with chlorophyll a, chlorophyll b and total chlorophyll. Exposure to *C. raciborskii* whole-cell extracts containing CYN appears to promote the redistribution of plant resources in *H. verticillata*. Increasing root production and decreasing main stem elongation might reflect a strategy of the plant during a bloom to optimise survival. A decrease in chlorophyll along with an increase in CYN might indicate that the toxin interferes with chlorophyll synthesis.

Beyer et al. [22] showed that exposure of *Phragmites australis* to CYN (0.5–40 μg CYN · mL^{-1}) led to decreased root elongation and increased root number. But these authors used very high CYN concentrations not expected to normally occur in the environment, compared with our range of 0.57–57 μg CYN · L^{-1}. It is interesting to note that in our study the plants that showed a stimulatory effect on root growth following exposure to CYN were those that had the highest CYN bioconcentration in the stem (Table 1). Increased root area may have increased bioconcentration. These two species, *L. sativa* and *Ph. vulgaris*, had the lowest root CYN concentration, which might indicate rapid translocation to the stem and/or more efficient detoxication of CYN in the roots. In *Phragmites australis*, the maximum detoxication enzyme activities occur in the roots [34].

Exposure of *H. verticillata* to extracts of *C. raciborskii* producing CYN (25–400 μg CYN · L^{-1}) did not lead to bioconcentration of the toxin in the plant, although there was some bioaccumulation at the highest concentrations. Exposure to 50 μg CYN · L^{-1} or lower resulted in little or no free CYN in the plant tissues [35]. Nevertheless, the maximum values were < 1 μg CYN · g^{-1} dry plant weight [33]. There was a positive correlation between CYN concentration and plant biomass. In our study, toxin bioconcentration was plant and concentration dependent. Plants that showed stimulatory effects on root growth and stimulation or no effect on stems were those that bioconcentrate the highest CYN values. This may indicate physiological mechanisms of resistance that are lacking in the other species.

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