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Nodularin uptake and induction of oxidative stress in spinach (*Spinachia oleracea*)

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ABSTRACT

The bloom-forming cyanobacterium *Nodularia spumigena* produces toxic compounds, including nodularin, which is known to have adverse effects on various organisms. We monitored the primary effects of nodularin exposure on physiological parameters in *Spinachia oleracea*. We present the first evidence for the uptake of nodularin by a terrestrial plant, and show that the exposure of spinach to cyanobacterial crude water extract from nodularin-producing strain AV1 results in inhibition of growth and bleaching of the leaves. Despite drastic effects on phenotype and survival, nodularin did not disturb the photosynthetic performance of plants or the structure of the photosynthetic machinery in the chloroplast thylakoid membrane. Nevertheless, the nodularin-exposed plants suffered from oxidative stress, as evidenced by a high level of oxidative modifications targeted to various proteins, altered levels of enzymes involved in scavenging of reactive oxygen species (ROS), and increased levels of α-tocopherol, which is an important antioxidant. Moreover, the high level of cytochrome oxidase (COX II), a typical marker for mitochondrial respiratory protein complexes, suggests that the respiratory capacity is increased in the leaves of nodularin-exposed plants. Actively respiring plant mitochondria, in turn, may produce ROS at high rates. Although the accumulation of ROS and induction of the ROS scavenging network enable the survival of the plant upon toxin exposure, the upregulation of the enzymatic defense system is likely to increase energetic costs, reducing growth and the ultimate fitness of the plants.

Introduction

Cyanobacteria are a large group of oxygenic photosynthetic bacteria that are present in a wide range of habitats. The occurrence of cyanobacteria in fresh and brackish water often results in surface blooms, which may lead to a release of nutrients accelerating eutrophication as well as to a release of wide range of toxic or bioactive compounds having adverse effects on the faunae (Codd, 1995). Recent studies have demonstrated that certain cyanotoxins may disturb the function of plants as well; for example, microcystin retards the growth of seedlings of a variety of crop plants by inhibiting protein phosphatases and photosynthesis (MacKintosh et al., 1990; Babica et al., 2006). In many organisms, cyanotoxins induce oxidative stress and accumulation of reactive oxygen species (ROS) (Pflugmacher et al., 2007a,b), which may damage cellular components such as DNA, membranes and proteins. The harmful effects of oxidative stress are prevented or reduced by the action of an elaborate ROS scavenging network, which consists of enzymatic components (such as superoxide dismutase (SOD), catalase, glutathione and ascorbate peroxidases and glutathione reductase) and various antioxidants (glutathione, ascorbate and tocopherol) (Apel and Hirt, 2004; Møller et al., 2007).

Cyanotoxins are classified into hepatotoxins (microcystin, nodularin and cylindrospermopsin), neurotoxins (anatoxin-a, anatoxin-a(S) and saxitoxins) and irritant-dermal toxins according to the symptoms they induce in humans and vertebrates. Among cyanotoxins, microcystin produced by *Microcystis* sp. is a common and well studied toxin. It has been shown that, in addition to its toxic effects on animals, microcystin exposure may also result in oxidative stress in plants, which leads to necrosis of the leaves and stunted growth of the plants (Järvenpää et al., 2007; Peuthert et al., 2007). Another important but less studied cyanotoxin is nodularin, produced by nitrogen-fixing *Nodularia spumigena*, which is the most common cyanobacterium species found in the Baltic Sea (Sivonen et al., 1989). Additionally, *Nodularia* sp. has been found in various freshwater lakes (Beattie et al., 2000). Nodularin is a cyclic pentapeptide, hepatotoxin and tumor promoter (Ohta et al., 1994). Exposure to nodularin induces oxidative stress in mussels (Davies et al., 2005; Kankaanpää et al., 2007), fish (Vuorinen et al.,

Abbreviations: APX, ascorbate peroxidase; COX II, cytochrome oxidase II; FW, fresh weight; PRXQ, peroxiredoxin Q; PS, photosystem; PTOX, plastid terminal oxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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2009) and marine macroalga *Fucus vesiculosus* (Pflugmacher et al., 2007b).

Plants growing in the shore zone of the Baltic Sea, or by the lakes occupied by *Nodularia* sp., are occasionally exposed to cyanobacteria-contaminated water, which may disturb the growth of plants, while agricultural crop plants may be particularly contaminated with cyanotoxins when irrigated with surface waters containing cyanobacterial blooms. It is conceivable that the plants can accumulate toxins in the leaves, roots and other organs, which may cause deleterious effects not only to the plants, but also to the heterotrophs fed by contaminated plants. In the present study, we explored the primary physiological target(s) of nodularin on the metabolic pathways of *Spinachia oleracea*, which is an important model organism in plant biochemistry and photosynthesis research. Understanding the impact of nodularin on plant metabolism at the molecular level will allow further studies to determine ecological effects of nodularin exposure to the surrounding ecosystem.

Material and methods

Cyanobacterial and plant material

Nodularin-producing (AV1) and non-nodularin-producing (HKVV) *Nodularia* sp. cells (University of Helsinki Culture Collection (UHCC), Division of Microbiology, Department of Food and Environmental Sciences) were grown under continuous light of 20 μmol photons m^{-2} s^{-1} in Z8 medium with salt and without added nitrogen at 25 °C (Lehtimäki et al., 1997). The cells were harvested after 4 weeks growth in their stationary phase, pelleted and washed with tap water. Thereafter, the cell pellets were weighed, frozen and thawed several times to break the cells and release the toxins. Just before use, the pellets were suspended in tap water.

Spinach seeds (*Spinachia oleracea* L. cv. Nores) were purchased from Nelsons OY (Finland). The seeds were sown on vermiculite, and the seedlings were transferred after germination to the pots filled with commercial soil (Kekkilä, Finland). The spinach plants were grown under 250 μmol photons m^{-2} s^{-1}, an 8 h photoperiod and a temperature of 23 °C in three distinct groups. Each group was composed of eight plants grown in two separate pots. During the growth period of five weeks, one of the groups was watered daily with tap water, and for the second group, the tap water was supplemented twice a week with 0.25 g fresh weight (FW) of *Nodularia* HKVV cell extract (dissolved in 30 mL of tap water) per plant. The third group was watered with tap water supplemented twice a week with 0.25 g fresh weight (FW) of *Nodularia* AV1 cell extract (dissolved in 30 mL of tap water) per plant (0.34 μM nodularin), which was the dose resulting in severe visible symptoms during the growth period of five weeks. Cyanobacterial extracts were provided by watering the bottom of the soil and care was taken not to contaminate the surface of the plants.

Because the focus was on the primary effects of nodularin, the metabolism of young, healthy-looking leaves of the nodularin-treated plants was studied, and the small, dark green leaves were harvested for the experiments. The metabolism of the old, but still vital, leaves with clear visible symptoms (chlorotic spots) was studied in order to detect later steps of intoxication, while the completely bleached leaves were excluded from the analyses. For comparison, leaves of corresponding age were studied in the control and HKVV-treated plants.

Quantitation of nodularin

Nodularin content of the roots and leaves of spinach and soil as well as cyanobacterial water extracts was quantified. Weighted (wet weight) roots, leaves and soil were treated for 60 min without shaking in 15 mL plastic tubes with 70% methanol in 80 °C. Prior to HPLC injection, methanol extracts were filtered through 0.2 μm filters. LC–MS analyses of extracts were performed with an Agilent 1100 Series LC/MSD Ion Trap XCT Plus System (Agilent Technologies, Palo Alto, CA) using a Phenomenex Luna C18(2) (150 × 2.0 mm, 5 μm, Phenomenex, Torrance, CA) LC-column. The mobile phase was composed of 0.1% formic acid in water (A) and 2-propanol (B). The gradient run was from 20% B to 70% (B) over 20 min and then a 9 min wash with 100% (B) at a flow rate of 0.15 mL min^{-1} at 40 °C using 5 or 10 μl injections. Nodularin was detected with a diode array detector (238 nm) and with MS using electrospray ionization (ESI) set in positive ion mode. The nebulizer gas (N_2) pressure was 35 psi (240 kPa), desolvation gas flow rate 8 L min^{-1} and the desolvation temperature 350 °C. The capillary voltage was set to 5000 V, the capillary exit offset was 300 V, the skimmer potential was 66 V and the trap drive value was 73. Spectra were recorded at a scanning range of 100–850 m/z and rate of 26,000 m/z s^{-1}. Product ion spectra using auto MS mode with precursor ion m/z 825.5 was recorded and nodularin quantification was based to the integration of the nodularin-specific product ion peak m/z 389.3 from the extracted ion chromatogram. Eight reference nodularin (gift from Zbigniew Grzonka, Faculty of Chemistry, University of Gdańsk, Poland) solutions from 0.5 to 9000 ng/mL 70% methanol were prepared. The linear response curve (R^2 = 0.99996) for calculating nodularin concentrations in extracts and further nodularin amounts in solid samples was obtained.

Germination test

For germination tests, 100 spinach seeds were sown on vermiculite and watering of plants with cyanobacterial extracts (0.5 g FW) was started immediately. After two weeks of treatment, the number of germinated seeds from each treatment was counted.

Pigment and α-tocopherol analysis

Pigments (chlorophyll a and b, neoxanthin, violaxanthin, lutein and β-carotene) and α-tocopherol were extracted from leaf discs (diameter 5 mm) with 300 μL of pure methanol. After centrifugation and filtration of the extracts, photosynthetic pigments were separated by HPLC according to Gilmore and Yamamoto (1991) with a reverse phase C18 column (LiChroCART 125-4, Hewlet Packard), series 1100 HPLC device with diode array and fluorescence detector (Agilent Technologies, Palo Alto, CA). Buffer A consisted of acetonitrile–methanol–Tris–HCl buffer 0.1 M pH 8.0 (72:8:3, v/v) and buffer B consisted of methanol–hexane (4:1, v/v). A constant flow rate 0.5 L min^{-1} was used. The program started with an isocratic run with buffer A for 4 min followed by a linear gradient for 15 min from 0% buffer B to 100% buffer B. The isocratic run of buffer B lasted 26 min. The column was re-equilibrated between samples for a minimum of 10 min with buffer A. Pigments were detected at 440 nm, and α-tocopherol by fluorescence (λ_{ex} = 295 nm, λ_{em} = 340 nm). Pigment standards were supplied from DHI Lab Products and the α-tocopherol was supplied from Sigma–Aldrich.

Determination of chlorophyll content

The chlorophyll content from isolated thylakoid membranes was determined as described in Porra et al. (1989).

Photosynthetic activity

Oxygen evolution

To measure the Photosystem (PS) II oxygen-evolving capacity, the thylakoid membranes were isolated and electron transfer