proceedings

MDPI

Proceedings

Extracts of non-microcystin-producing cyanobacteria affect the plant cytoskeleton and cell cycle

Dimitris Pappas [1,*], Ioannis-Dimosthenis S. Adamakis [2], Spyros Gkelis [1] and Emmanuel Panteris [1,*]

[1] Department of Botany, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, GR-54124, Greece; dtpappas@bio.auth.gr, sgkelis@bio.auth.gr

[2] Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens, Athens, GR-15784, Greece; iadamaki@biol.uoa.gr

* Correspondence: dtpappas@bio.auth.gr; epanter@bio.auth.gr; Tel.: +30 2310 998908

Published: 1 December 2020

Abstract: Studies on the toxicity of cyanobacterial products on plant cytoskeleton have so far focused on the effects of microcystins (MCs), cyanobacterial toxins that inhibit protein phosphatases 1 and 2A, enzymes which are involved in plant cytoskeleton (microtubules and F-actin) organization and cell cycle progression. In this study, we investigated the effects of extracts from two non-microcystin-producing (NMP) cyanobacterial strains, *Microcystis viridis* TAU-MAC 1810 and *Planktothrix agardhii* TAU-MAC 0514, on the cytoskeleton and cell cycle of *Oryza sativa* (rice) root cells. Rice seedling roots were exposed for various time periods (1, 12 and 24h) to aqueous extracts of the aforementioned strains. Treated root tips underwent either immunostaining for α-tubulin or staining of F-actin with fluorescent phalloidin, and DAPI staining of DNA. Fluorescent specimens were observed by confocal laser scanning microscopy (CLSM). Corrected total cell fluorescence (CTCF) was measured to quantify F-actin disorder. To assess cell cycle alterations, cell cycle stage frequencies were calculated. In addition, Evans Blue staining was applied to determine dead cells. Treatment with the extracts affected both microtubules and F-actin, as well as the cell cycle. These findings suggest that bioactive cyanobacterial compounds, apart from MCs, can disrupt the cytoskeleton and cell cycle progression in plant cells.

Keywords: cell cycle; cyanobacteria; cytoskeleton; F-actin; microtubules

1. Introduction

Microcystins (MCs) are monocyclic heptapeptides, produced by several species of cyanobacteria [1]. They are potent hepatotoxins [2], but are also known to negatively affect plants [3,4]. In particular, plant cytoskeleton (F-actin and microtubules) is an established target of MC variants [5,6], the toxicity of which lies on their ability to inhibit protein phosphatases 1 (PP1) and 2A (PP2A) [7], that participate in cytoskeleton organization [8] and cell cycle progression [9].

To date, extracts from cyanobacterial strains that produce MCs have been extensively used to study cytoskeletal and other physiological defects [3,5,10]. In this study, we investigated the effects of extracts from non-microcystin-producing (NMP) cyanobacterial strains on *Oryza sativa* (rice) meristematic root cells, focusing on the plant cytoskeleton and cell cycle.

2. Experiments

2.1. Culture of Cyanobacterial Strains and Biomass Extraction

Two NMP cyanobacterial strains of the TAU-MAC culture collection, *Microcystis viridis* TAU-MAC 1810 and *Planktothrix aghardii* TAU-MAC 0514 [11,12], were cultured using BG-11 medium in a 12:12 h light:dark cycle under white fluorescent lamps. Biomass was collected at the exponential

growth phase, frozen and lyophilized. Dry biomass (150 mg for each strain) was dissolved in 75% (v/v) methanol, sonicated and eventually evaporated, according to [13]. The pellet was resuspended in 5 mL of double-distilled water (ddH_2O). Extracts were filtered and stored at -20°C.

2.2. Plant Material, Cyanobacterial Biomass Extraction and Treatments

Rice (*Oryza sativa* cv Axios) seeds were kindly provided by the National Cereal Institute (Thessaloniki, Greece) and were germinated on soaked filter paper at room temperature (24±1°C) in the dark. Four- to five-day-old seedlings were treated with aqueous cyanobacterial extracts for 1, 12 or 24h, their roots being submerged in tubes containing the extract. For control, seedlings were similarly treated with ddH_2O.

2.3. Tubulin Immunolabeling

Control and extract-treated root rips were fixed in 4% (w/v) paraformaldehyde (PFA) solution in PEM buffer (50 mM PIPES, 5mM EGTA, 5 mM $MgSO_4$, pH 6.8) + 5% (v/v) dimethyl sulfoxide (DMSO) for 1 h. Cell wall digestion was performed with a 3% (w/v) Macerozyme R-10 + 3% (w/v) cellulase Onozuka R-10 solution in PEM buffer for 90 min. Root tips were squashed on coverslips coated with poly-L-lysine, and the squashes were left to dry. The adherent cells were extracted for 1h with a 5% (v/v) DMSO + 1% (v/v) Triton X-100 solution in phosphate-buffered saline (PBS, pH 7.2). Rat anti-α-tubulin (YOL 1/34) as primary antibody (diluted 1:50 in PBS) and anti-rat IgG AlexaFluor 488 as secondary antibody (diluted 1:300 in PBS) were used. Specimens were incubated with each antibody overnight at room temperature. DNA was counterstained with 0.9 μM 4′, 6-diamidino-2-phenylindole (DAPI) for 5-10 min. Fluorescent specimens were mounted with anti-fade medium [PBS 1:2 glycerol (v/v) + 0.5% (w/v) p-phenylenediamine] and examined under a Zeiss Observer.Z1 microscope, equipped with the LSM780 confocal laser scanning (CLSM) module and the appropriate filters for each fluorophore. Imaging was achieved with ZEN2011 software, according to the manufacturer's instructions.

2.4. F-actin Labeling

F-actin of control and extract-treated root tips was pre-stabilized with 300 μM m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) in PEM + 0.1% (v/v) Triton X-100 for 30 min in the dark. Fixation was performed with 4% (w/v) PFA in PEM + 5% (v/v) DMSO + 0.1% (v/v) Triton X-100 + DyLight 554-phalloidin 1:400 and fixed samples were washed with PEM buffer, extracted with 5% (v/v) DMSO + 1% (v/v) Triton X-100 in PBS for 1h and incubated with DyLight 554-phalloidin (1:40 in PBS) at 37°C for 2h. DAPI was used for DNA counterstaining. Samples were mounted with anti-fade medium and observed with the Zeiss Observer.Z1 microscope, equipped with the LSM780 confocal laser scanning module.

2.5. Fluorescence Intensity Measurements

Single cortical CLSM sections and maximun intensity projections of serial CLSM sections of control and extract-treated roots were measured for fluorescence intensity using ImageJ. The Corrected Total Cell Fluorescence (CTCF) was calculated using the equation:

CTCF = Integrated Density – (Area of Selected Cell x Mean Fluorescence of Background Readings)

Thirty individual cells from three different roots were measured for each treatment and data were statistically analyzed (ANOVA with Dunnett's test).

2.6. Cell Cycle Analysis

Alterations in cell cycle progression of extract-treated meristematic root cells were assessed with CLSM observations of whole-mount specimens, labelled for F-actin and stained with DAPI. Cell cycle stages (interphase, preprophase/prophase, metaphase/anaphase and cytokinesis) were recognized according to F-actin arrangement and/or chromatin state. At least 1000 meristematic cells from three different roots per treatment were counted. Statistical analysis of data (chi-squared test, df = 6) was performed, with a significance of $P < 0.05$.

2.7. Detection of Dead Cells

Control and extract-treated roots were stained with Evans Blue for detection of dead cells, according to [14]. Roots were incubated in 0.25% (w/v) aqueous Evans Blue extract for 15 min, washed twice and incubated overnight in double-distilled water. Samples were then observed with a stereomicroscope.

3. Results

3.1. Cytoskeletal Alterations

3.1.1. Effects on F-actin Organization

The extracts of both NMP strains induced time-dependent alterations on F-actin. The fine actin filaments observed in control root tips (Figure 1A) were significantly affected, starting from 1h of treatment. Roots treated with the 1810 extract exhibited more severe alterations, ranging from bundling after 1h of treatment (Figure 1B) to the formation of thick F-actin aggregates after 12h (Figure 1C) and, eventually, total collapse of F-actin network after 24h (Figure 1D). Roots treated with the 0514 extract exhibited milder alterations, mainly disorientation, which was detectable at all time points and intensified progressively (Figure 1E-G).

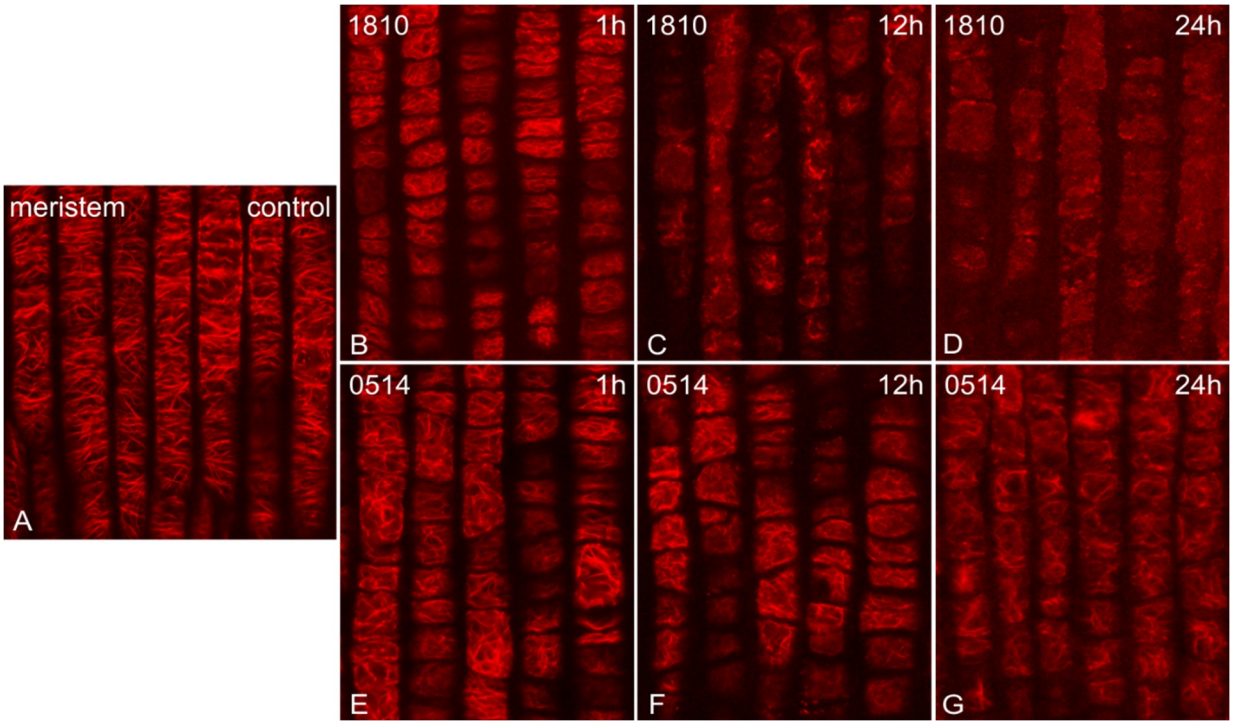

Figure 1. Single cortical CLSM sections of *Oryza sativa* root protodermal cells, stained for F-actin, either control (A) or treated with extracts from non-microcystin-producing (NMP) cyanobacterial strains: *Microcystis viridis* TAU-MAC 1810 (B-D) and *Planktothrix aghardii* TAU-MAC 0514 (E-G). Control meristematic cells exhibit a dense network of fine actin filaments, with a dominant transverse orientation (A). After 1h of treatment, bundling (B) and disorientation of F-actin (E *cf.* A) is observed. After 12h, roots

treated with the 1810 extract exhibited actin aggregates and an overall actin network disorganization (C), while in roots treated with the 0514 extract F-actin bundling intensified (F). After 24h, the F-actin network either collapsed (D) or exhibited heavy bundling and disorientation (G).

3.1.2. Effects on F-actin Fluorescence Intensity

CTCF measurements (Figure 2) showed that fluorescence intensity in extract-treated meristematic root cells decreased progressively. Even after short treatments (1h) the intensity was significantly lower compared to control cells. The lowest levels were recorded after 24h.

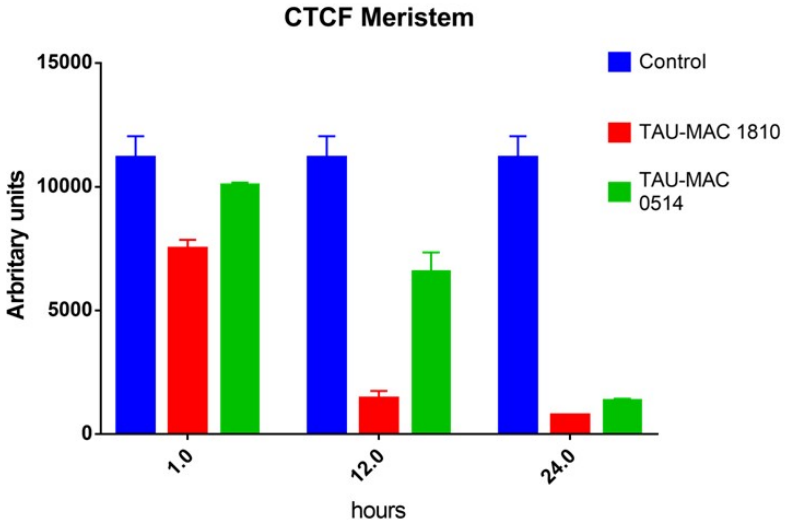

Figure 2. Graph exhibiting F-actin fluorescence intensity measurements in *Oryza sativa* root meristematic cells, stained for F-actin, either control (blue) or treated with extracts from NMP cyanobacterial strains: *Microcystis viridis* TAU-MAC 1810 (orange) and *Planktothrix aghardii* TAU-MAC 0514 (green). Fluorescence intensity decreased significantly, in a time-dependent manner, in root cells treated with the extracts. Error bars represent standard error. All data show a statistical significant difference compared to control (ANOVA with Dunnett's test), $P < 0.05$. n=30.

3.2. Effects on Microtubules and Chromatin

Both cyanobacterial extracts induced alterations on the microtubule network (Figure 3). Meristematic cells treated with the 1810 extract exhibited major defects after 1h of treatment (Figure 3G-J), which evetually led to the disappearance of microtubules after 12h, in parallel with abnromal chromatin condensation (Figure 3K). The effect of 0514 extract was not so acute, as only minor defects were observed after 1h of treatment (L-R). No significant alterations were detected after longer treatments.

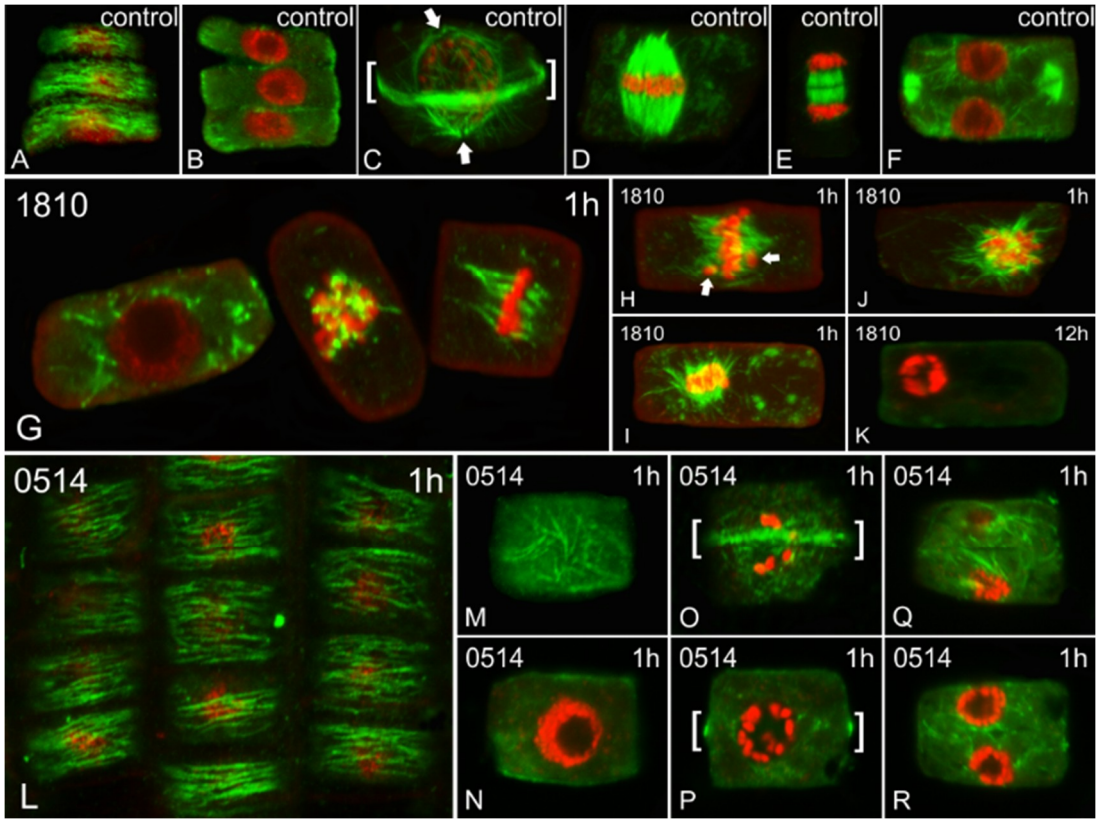

Figure 3. Single cortical (A, L, M, O, Q), single central (B, F, G, N, P, R) and maximum intensity projections (C-E, H-K) of serial CLSM sections of *Oryza sativa* root meristematic cells, after *α*-tubulin immunostaining (green) and DNA staining with DAPI (pseudo-coloration in red). Cells depicted are either control (A-F) or treated for various time periods (indicated on each image) with extracts from non-microcystin-producing (NMP) cyanobacterial strains: *Microcystis viridis* TAU-MAC 1810 (G-K) and *Plankothrix aghardii* TAU-MAC 0514 (L-R). The images A&B, M&N, O&P and Q&R depict the same cells at cortical and central sections. A–F: Control cells at interphase exhibit dense, transverse cortical (A) and scarce endoplasmic (B) microtubules. Preprophase/prophase cells exhibit the typical preprophase band of microtubules (brackets in C), as well as perinuclear microtubules converging on two distinctive poles (arrows in C). Mitotic spindles with aligned chromosomes at the equator can be observed in control metaphase cells (D) and control cytokinetic cells exhibit typical phragmoplasts (early stage in E and later stage in F). G-K: Cells treated with the 1810 for 1h exhibited numerous endoplasmic microtubules (left cell in G *cf.* B), abnormally short microtubules attached to chromosomes (middle cell in G) and malformed mitotic spindles (right cell in G *cf.* D). Misaligned chromosomes, outside the equator plate (arrows in H *cf.* D) can also be observed, as well as masses of chromosomes attached to abberant spindle-like microtubules (I, J). After 12h, no tubulin polymers could be detected, while chromatin was abnormally condensed (K). L-R: Cells treated with the 0514 extract exbihited typical cortical microtubules at interphase after 1h (L *cf.* A). Occasionally, interphase cells with disoriented cortical microtubules could be spotted (M *cf.* A), but exhibited no endoplasmic microtubules (N). Certain affected preprophase cells exhibited almost typical preprophase bands of microtubules (brackets in O), but lacked perinuclear microtubules (P *cf.* C). Some cytokinetic cells exhibited abnormal phragmoplasts (Q, R *cf.* F).

3.3. Effects on Cell Cycle Progression

In root tips treated with the extracts, cell cycle stage frequencies were significantly altered, starting from 1h of treatment (Table 1). Treatment with the 1810 extract dramatically affected chromatin state after 12h, as no cell cycle stages could be distincted. On the other hand, treatment with the 0514 extract hindered the cell cycle stage progression after 12h and, after 24h, almost all cells observed were halted at interphase.

Table 1. Percentages (%) of occurrence of cell cycle stages in *Oryza sativa* root tips, either control or treated for various time periods (1, 2 and 24h) with extracts from the NMP cyanobacterial strains,

Microcystis viridis TAU-MAC 1810 and *Planktothrix aghardii* TAU-MAC 0514. Kruskal–Wallis analysis of variance showed a significant time-dependent variation in the percentage of cells in different cell cycle phases (chi-squared; χ^2: 15,8, n = 35, df = 6, $P < 0.05$).

Cell Cycle Stage	Control [1]	TAU-MAC 1810			TAU-MAC 0514		
		1h	12h	24h	1h	12h	24h
Interphase	91.6	90.16	0	0	91.2	91.53	96.7
Preprophase/Prophase	3.73	4.11	0	0	3.4	3.09	2.5
Metaphase/Anaphase	1.02	0.08	0	0	0.58	0.3	0
Cytokinesis	3.65	3.49	0	0	2.66	2.59	0
Abnormal Chromatin	0	2.17	100	100	2.16	2.49	0.8

[1] Data from [5].

3.4. Induction of Cell Death

The occurrence of dead cells in extract-treated roots was confirmed by Evans Blue staining (Figure 4). Dead cells were detected at all time points and the effects were particularly harsh after 24h of treatment with the 1810 extract (Figure 4B).

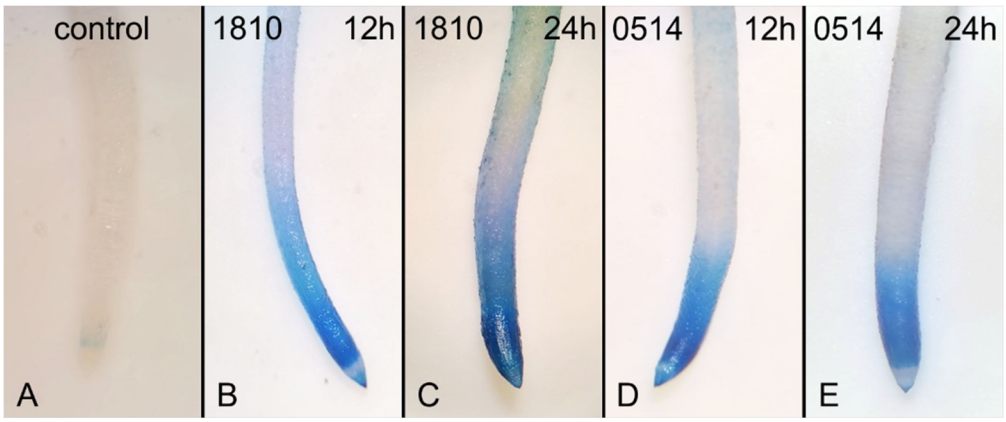

Figure 4. Images of *Oryza sativa* roots, control (A) or treated with extracts from non-microcystin-producing (NMP) cyanobacterial strains, *Microcystis viridis* TAU-MAC 1810 (B, C) and *Planktothirx aghardii* TAU-MAC 0514 (D, E), after staining with Evans Blue for detection of dead cells. After 12h, both extracts induced cell death in rice root tips (B, D *cf.* A). After 24h, enhancement of the staining was visible in 1810-treated roots (C *cf.* B). No significant increase of staining was observed in roots treated with the 0514 extract for 24h (E *cf.* D).

4. Discussion

Extracts from two NMP cyanobacterial strains were shown to negatively affect meristematic root cells of *Oryza sativa*. Both components of the plant cytoskeleton (F-actin and microtubules) were affected, in a time-dependent manner but in a different way, as the 1810 extract exhibited more adverse effects, leading to eventual collapse of the plant cytoskeletal system after longer treatments (12 and 24h). In plant cells, MCs are known to disrupt both microtubules [15] and F-actin [5]. Althought *Microcystis* is a genus notorious for producing MCs, *Microcystis viridis* TAU-MAC 1810 strain was not found to produce any, by LC-MS/MS analysis [12]. However, apart from MCs, *Microcystis* species have been found to produce a wide range of bioactive compounds, such as microginins, microviridins and cyanopeptolins [1]. Similarly, *Plankothrix* produces a variety of secondary metabolites, many of which are yet to be characterized [1]. Investigations on the toxicity of such compounds against the plant cytoskeleton could be a focal point of future research.

F-actin appeared to be more sensitive than microtubules. This is especially highlighted by treatment with the 0514 extract. These results imply a possible independent mechanism of toxicity

for each cytoskeletal element in plant cells. The cytoskeletal alterations, along with the abnormal condensation of chromatin observed in treated cells (especially with the 1810 extract), justify the observed decrease in dividing cells. Interestingly, the vast majority of root meristematic cells treated with the 0514 extract for 24h were arrested at interphase. MCs are able to not only cause cytoskeletal abnormalities in mitotic cells but also to alter the mitotic index of treated plant tissues [15,16]. Our findings suggest that similar effects on plant cell division may be exerted by cyanobacterial bioactive compounds other than MCs.

These cytoskeletal alterations and the disruption of the cell cycle progression could be associated to the cell death effects observed in roots treated with the extracts [17]. Despite the fact that the exact nature of these cell death phenomena has not been determined yet, this is an important indication that the presence of certain NMP cyanobacterial strains in the aquatic environment could pose a threat to plant species exposed to their compounds, which are present in water and are often accessible to crop species (such as rice) through irrigation.

5. Conclusions

- Extracts from NMP cyanobacterial strains are able to negatively affect both F-actin and microtubules in root meristematic cells of *Oryza sativa*, as well as the cell cycle. Therefore, bioactive cyanobacterial compounds, other than MCs, could be able to disrupt the cytoskeleton and cell cycle progression in plant cells.
- Treatment with the extracts induced cell death in root tips.
- Not only strains that produce MCs, but also NMP strains may be toxic for plants.

Acknowledgments: This research was co-financed by Greece and the European Union (European Social Fund-ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" (MIS-5000432), implemented by the State Scholarships Foundation (ΙΚΥ). D.P. is a State Scholarships Foundation scholar. I.-D.S.A. was funded by the National and Kapodistrian University of Athens. E.P. was funded by the AUTh Research Committee, grant number 91913.

Author Contributions: D.P., I.-D.S.A., S.G. and E.P. conceived and designed the experiments; D.P. performed the experiments; I.-D.S.A. and D.P. analyzed the data; S.G. and E.P. contributed reagents/materials/analysis tools; D.P., I.-D.S.A., S.G. and E.P. wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CLSM: Confocal Laser Scanning Microscope

CTCF: Corrected Total Cell Fluorescence

DAPI: 4′, 6-diamidino-2-phenylindole

DMSO: dimethyl sulfoxide

LC-MS/MS: Liquid Chromatography with tandem Mass Spectrometry

MBS: m-maleimidobenzoyl-*N*-hydroxysuccinimide ester

MCs: Microcystins

NMP: Non-microcystin-producing

PBS: Phosphate Buffer Saline

PEM: PIPES EGTA $MgSO_4$

PFA: Paraformaldehyde

PP1: Protein Phosphatase 1

PP2A: Protein Phosphatase 2A

References

1. Meriluoto, J.; Spoof, L.; Codd, G.A. *Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis*; 2017; ISBN 9781119068686.
2. Runnegar, M.T.; Kong, S.; Berndt, N. Protein phosphatase inhibition hepatotoxicity of microcystins. *Am. J. Physiol.* **1993**, *265*, 224–230.
3. Pflugmacher, S.; Jung, K.; Lundvall, L.; Neumann, S.; Peuthert, A. Effects of cyanobacterial toxins and cyanobacterial cell-free crude extract on germination of alfalfa (*Medicago sativa*) and induction of oxidative stress. *Environ. Toxicol. Chem.* **2006**, *25*, 2381–2387.
4. Chen, J.; Han, F.X.; Wang, F.; Zhang, H.; Shi, Z. Accumulation and phytotoxicity of microcystin-LR in rice (*Oryza sativa*). *Ecotoxicol. Environ. Saf.* **2012**, *76*, 193–199.
5. Pappas, D.; Gkelis, S.; Panteris, E. The effects of microcystin-LR in *Oryza sativa* root cells: F-actin as a new target of cyanobacterial toxicity . *Plant Biol.* **2020**, *22*, 839–849.
6. Máthé, C.; M-Hamvas, M.; Vasas, G. Microcystin-LR and cylindrospermopsin induced alterations in chromatin organization of plant cells. *Mar. Drugs* **2013**, *11*, 3689–3717.
7. MacKintosh, C.; Beattie, K.A.; Klumpp, S.; Cohen, P.; Codd, G.A. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **1990**, *264*, 187–192.
8. Hoffman, A.; Taleski, G.; Sontag, E. The protein serine/threonine phosphatases PP2A, PP1 and calcineurin: A triple threat in the regulation of the neuronal cytoskeleton. *Mol. Cell. Neurosci.* **2017**, *84*, 119–131.
9. Brautigan, D.L.; Shenolikar, S. Protein Serine/Threonine Phosphatases: Keys to Unlocking Regulators and Substrates. *Annu. Rev. Biochem.* **2018**, *87*, 921–964.
10. Prieto, A.; Campos, A.; Cameán, A.; Vasconcelos, V. Effects on growth and oxidative stress status of rice plants (*Oryza sativa*) exposed to two extracts of toxin-producing cyanobacteria (*Aphanizomenon ovalisporum* and *Microcystis aeruginosa*). *Ecotoxicol. Environ. Saf.* **2011**, *74*, 1973–1980.
11. Gkelis, S.; Panou, M. Capturing biodiversity: linking a cyanobacteria culture collection to the "scratchpads" virtual research environment enhances biodiversity knowledge. *Biodivers. Data J.* **2016**, *4*, e7965-1.
12. Gkelis, S.; Panou, M.; Konstantinou, D.; Apostolidis, P.; Kasampali, A.; Papadimitriou, S.; Kati, D.; Di Lorenzo, G.M.; Ioakeim, S.; Zervou, S.-K.; et al. Diversity, Cyanotoxin Production, and Bioactivities of Cyanobacteria Isolated from Freshwaters of Greece. *Toxins (Basel).* **2019**, *11*, 436.
13. Gkelis, S.; Lanaras, T.; Sivonen, K. The presence of microcystins and other cyanobacterial bioactive peptides in aquatic fauna collected from Greek freshwaters. *Aquat. Toxicol.* **2006**, *78*, 32–41.
14. Eleftheriou, E.P.; Adamakis, I.D.S.; Panteris, E.; Fatsiou, M. Chromium-induced ultrastructural changes and oxidative stress in roots of *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **2015**, *16*, 15852–15871.
15. Beyer, D.; Tándor, I.; Kónya, Z.; Bátori, R.; Roszik, J.; Vereb, G.; Erddi, F.; Vasas, G.; M-Hamvas, M.; Jambrovics, K.; et al. Microcystin-LR, a protein phosphatase inhibitor, induces alterations in mitotic chromatin and microtubule organization leading to the formation of micronuclei in *Vicia faba*. *Ann. Bot.* **2012**, *110*, 797–808.
16. Máthé, C.; Beyer, D.; Erdodi, F.; Serfozo, Z.; Székvölgyi, L.; Vasas, G.; M-Hamvas, M.; Jámbrik, K.; Gonda, S.; Kiss, A.; et al. Microcystin-LR induces abnormal root development by altering microtubule organization in tissue-cultured common reed (*Phragmites australis*) plantlets. *Aquat. Toxicol.* **2009**, *92*, 122–130.
17. Smertenko, A.; Franklin-Tong, V.E. Organisation and regulation of the cytoskeleton in plant programmed

cell death. *Cell Death Differ.* **2011**, *18*, 1263–1270.