



Long-term treatments with microcystin-LR and diquat reveal their differences in the induction of oxidative stress responses and mitotic alterations in terms of stress recovery in *Arabidopsis* roots[☆]

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ABSTRACT

Microcystin-LR (MCY-LR), a widespread natural cyanobacterial toxin is a potent inhibitor of type 1 and 2A protein phosphatases (PP1, PP2A), inducing oxidative stress as well. Diquat (DQ) is a herbicide that inhibits electron transport to induce strong oxidative stress. Both compounds are affecting natural environments as well as plant and human health. We conducted a comparative analysis on their long-term (48 h) effects on reactive oxygen species (ROS) production, mitosis and histone H3 phosphorylation in root apical meristems of wild-type and PP2A-related mutants of *Arabidopsis thaliana*. MCY-LR did not increase ROS levels in any of the genotypes, but increased significantly the activity of ROS scavenging peroxidases (PODs) in Col0. DQ decreased ROS levels, but did not affect PODs. The paradoxical effect of DQ indicated the initiation of cell death processes. Mitosis was not affected by MCY-LR, but inhibited by DQ. Interestingly, MCY-LR as a phosphatase inhibitor did not affect histone H3 phosphorylation in mitotic cells, but DQ had an increasing effect. As we have shown previously, most of the above parameters changed differently at shorter (24 h) exposures: both compounds increased ROS levels in the phosphatase mutants, while POD activities were not affected; inhibition of mitosis or arrests in early mitosis, as well as increases of histone H3 phosphorylation levels occurred in the presence of both compounds. To conclude, *Arabidopsis* is able of recover partially and transiently from MCY-LR induced stress at long-term exposure, but this is not true for DQ. The environmental relevance of these findings is discussed.

1. Introduction

Microcystin-LR (MCY-LR) is a well-known member of the microcystin family of peculiar cyclic heptapeptides produced by strains of several cyanobacterial genera (Rastogi et al., 2014). The main biochemical targets of microcystins are type 1 and 2A serine-threonine protein phosphatases (PP1 and PP2A) (Mackintosh and Diplexito, 2010). Moreover, there are several reports that demonstrate their oxidative stress inducing effects, raising the possibility that this latter

effect is also related to protein phosphatase inhibition in plants. These studies concentrated mainly on the alterations of oxidized/reduced glutathione ratios and activities of reactive oxygen species (ROS) scavenging enzymes (see Machado et al., 2017; Máthé et al., 2019 for reviews). Microcystins may accumulate in surface water bodies as a consequence of cyanobacterial overproduction and this may cause significant alterations in aquatic plant communities. Also, they may enter into the food chain by spray irrigation of terrestrial crops with microcystin contaminated water to have possible consequences on human

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health (Corbel et al., 2015). Microcystins can exert their environmental toxicity for prolonged time because of their persistence and high stability (Kós et al., 1995).

The harmful effects of MCY-LR are known for both aquatic and terrestrial model plants under laboratory conditions, including alterations of subcellular organization, featuring chromatin, cytoskeleton, membrane compartments (Wiegand and Pflugmacher, 2005; Szigeti et al., 2010; Máthé et al., 2021). Such alterations were detected directly in natural freshwaters as well for plants like *Ceratophyllum* (Ujvárosi et al., 2019). These statements are valid also for crops (Pappas et al., 2020; Campos et al., 2021). Recently, the model plant *Arabidopsis* has been involved in such studies as well (Yin et al., 2005; Jia et al., 2018; Juhász et al., 2023). *Arabidopsis* is considered a good model to study MCY-LR toxicity. It is a well-known eukaryotic model with the whole genome sequence available, and many genotypes bearing fusion protein constructs were created-these genotypes are regularly used for live cell imaging (Mathur, 2007). Moreover, this plant is a good model for toxicity test for another aspect, too: it is resembling many features of cultivated plants, especially those belonging to the Brassicaceae family as being genetically closely related - a closer relative is e.g. *Lepidium sativum* (Franzke et al., 2009).

Diquat (diquat dibromide, DQ) is a dihydropyridine herbicide, that disturbs electron transport processes e.g. during photosynthesis or at the mitochondrial level to promote the formation of reactive oxygen species (ROS) (Zer et al., 1993; Acar, 2021). Several papers show their phytotoxicity including its adverse effects at the subcellular level (Birmingham and Colman, 1983; Acar, 2021; Kelemen et al., 2024). This herbicide may be an environmental pollutant and several case reports are available on its detrimental effects not only on plants, but on humans as well (Roberts and Reigart, 2013). In *Arabidopsis*, DQ increases the levels of free malondialdehyde, related to lipid peroxidation (Weber et al., 2004). DQ may be degraded to water-soluble compounds within weeks in natural water bodies (Simsman and Chesters, 1976).

Since MCY-LR may have oxidative stress inducing effects and as mentioned before, there are several studies showing protein phosphatase-oxidative stress connections, comparisons for the effects of the cyanotoxin and DQ are worthwhile. Their environmental effects are documented and we have already conducted such comparative studies for shorter (24 h) exposures (Freytag et al., 2023). For the same reason, in the present study, besides wild-type (Col 0) *Arabidopsis* plants, we have also used *c3c4* double mutants for catalytic subunits and *fass* (a type of B'' regulatory subunits). These subunits are involved in the regulation of several subcellular events, e.g. microtubular organization and even the production and scavenging of ROS. Further treatment of relevant mutants with protein phosphatase inhibitors and ROS inducers is contributing to a deeper understanding of protein phosphatase-oxidative stress relationships (Spinner et al., 2013; Freytag et al., 2023).

We expected that MCY-LR as a protein phosphatase inhibitor (reversible protein phosphorylation is a key cell cycle regulator) and DQ as a ROS inducer should influence mitotic activities in eukaryotes and particularly in the model plant *Arabidopsis*. Both compounds are able of interfering in key events of eukaryotic cell division. However, the degree and mode of these alterations is largely dependent on the plant species studied, the nature of meristematic tissue involved and the time/dose of exposures (Acar, 2021; Máthé et al., 2021; Kelemen et al., 2024). One notable parameter in this context is histone H3 phosphorylation. This occurs at Ser10, Ser28 and Thr4 positions and characteristic mainly for dividing cells. It is well detectable at prophase, then the degree of phosphorylation reaches a peak in metaphase. It is required for the regulation of metaphase-anaphase transition and decreases gradually at late mitosis (Houben et al., 2007). MCY-LR induces hyperphosphorylation of histone H3 and this leads to mis-segregation of sister chromatids in root apical meristems of *Vicia faba* probably by counterbalancing the protein phosphorylation-dephosphorylation cycles (Beyer et al., 2012). Even DQ alters histone phosphorylation at short-term

exposures of *Arabidopsis* seedlings. The biochemical background of this alteration is not known, however after short-term (24 h) DQ treatments of *Arabidopsis* seedlings we detected decreased phosphorylation levels in the PP2A subunit mutants *fass* and *c3c4*, but not in the wild-type plants Col0 (all these genotypes are involved in the present study as well). These results indicate that alterations of PP2A functionality induced increased sensitivity of plants to DQ-induced oxidative stress and affect protein dephosphorylation events (Kelemen et al., 2024). Other abiotic stresses like high salinity are also affecting histone phosphorylation in *Arabidopsis* and tobacco cell cultures and leaves (Sokol et al., 2007).

There are several studies showing that both MCYs and DQ can accumulate in plants over time, thereby toxic effects may be amplified at long-term exposures both under laboratory conditions and in the environment (Yin et al., 2005; Puri et al., 2008). We have studied previously their toxic effects in *Arabidopsis* in terms of cytological and developmental alterations, oxidative stress responses after relatively short-term (4, 24 h) treatments (Freytag et al., 2021, 2023; Máthé et al., 2021, 2024; Juhász et al., 2023; Kelemen et al., 2024). For the present study, our initial hypothesis was that alterations observed previously are amplified at longer (48 h) exposures. For validating this hypothesis, we aimed to conduct for the first time a comparative investigation of long-term MCY-LR and DQ effects in *Arabidopsis* - mainly alterations of oxidative stress responses, mitotic activities and histone H3 phosphorylation in wild-type and phosphatase mutant *Arabidopsis* seedlings. Treatments were performed with environmentally relevant concentration of MCY-LR and DQ.

2. Materials and methods

2.1. Chemicals used for treatments, plant material and the method of treatments

Microcystin-LR (MCY-LR) was purified at UD, Department of Botany according to a procedure routinely used in our laboratories for decades (Máthé et al., 2024). *Microcystis aeruginosa* BGSD243 was the source of the cyanotoxin and the procedure was according to the method of Kós et al. (1995) as modified by Vasas et al. (2004). The purity of resulting MCY-LR was >95 % as verified by a standard HPLC method, in principle described by Kós et al. (1995) and by LC-MS. This purified compound was used throughout our experiments (for more information, see Máthé et al., 2024).

Diquat (diquat dibromide, DQ) was purchased from Supelco, Bellefonte, PA.

Arabidopsis genotypes used were wild-type (Col0) as well as loss-of-function mutants of catalytic (*c3c4*) and *Fass* (*fass-15* and *fass-5*), a B'' regulatory subunit of PP2A. Mutants were kind gifts of M. Pastuglia and D. Bouchez, INRAE, Versailles, France, who contributed to their description as well (Spinner et al., 2013).

Treatments with MCY-LR and DQ (both used at concentrations of 1 µM) were performed as described previously (Nagy et al., 2018; Freytag et al., 2021). Briefly, seeds were surface sterilized and pre-germinated seeds were used for treatments: for cytological assays (determination of ROS content, of mitotic activities and pH3Ser10 levels) five seedlings were placed per 60-mL petri dish per treatment and each treatment was in at least two replicates per experiment. For enzyme activity (POD, SOD) assays, all treatments were performed in three replicates per treatments, with 10–15 seedlings per petri dish. Chemicals to be tested were dissolved in liquid MS medium with Gamborg's vitamins (Murashige and Skoog, 1962; Gamborg et al., 1968). Three layers of sterile filter papers were soaked with liquid medium, then pre-germinated seeds were placed on them. Treatments lasted for 48 h.

2.2. The assays of reactive oxygen species (ROS) content and scavenging

Total ROS content of living whole primary roots of Col0 as well as

fass and *c3c4* mutants was assayed by labeling them with 10 μM of 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, Mo., USA). The protocol itself and microscopy settings (excitation of the dye was at a wavelength range of 450–480 nm) were described previously (Freytag et al., 2023). DCFH-DA is a dye for labeling all types of ROS (Jambunathan, 2010). Intensity of fluorescence signals of ROS was quantified with the Fiji (ImageJ2/Win64) software and expressed as AIOD (Area Integrated Optical Density, a parameter that shows pixel numbers). Experiments concerning treatments with MCY-LR and DQ were performed as four independent assays.

Total peroxidase (POD) activity of Arabidopsis (Col0) root extracts was assayed with pyrogallol used as co-substrate, by a spectrophotometric method according to M-Hamvas et al. (2010) developed initially for *Sinapis alba*. The oxidation of pyrogallol by PODs was assayed spectrophotometrically at 430 nm with a Hitachi U-2910 (Hitachi HT Sci. Corp., Tokyo, Japan) spectrophotometer and specific activity was expressed as absorbance change $\text{min}^{-1} \text{mg}^{-1}$ protein in the percentage of control (untreated Col0 = 100 %).

Total superoxide dismutase (SOD) activity was assayed from extracts of wild-type (Col0) whole roots on native activity polyacrylamide gels, essentially as described by Freytag et al. (2023). The method itself was based on detection of active SOD isoenzymes by using 2.5 mM nitroblue tetrazolium (NBT, Duchefa, Haarlem, The Netherlands) and riboflavin (Sigma-Aldrich, St. Louis, Mo, USA) as band developers. This method was based on the valid procedures of Giannopolitis and Ries (1977)/Bertrand and Eze (2014). Individual band (Mn-SOD, Fe-SOD, Cu/Zn-SOD) intensities were quantified and their sum was expressed as total SOD activity. Both POD and SOD activity assays were performed in three independent experiments.

2.3. Histochemical/immunohistochemical (IHC) methods, the determination of mitotic activities

Mitotic figures were detected in apical meristems of whole primary root tips with the aid of IHC labeling of microtubules and histone H3 phosphorylated at the Ser10 position (pH3Ser10) and histochemical labeling of chromatin with 4', 6'-diamidino-2-phenylindole (DAPI, at a concentration of 3 $\mu\text{g mL}^{-1}$). Fixation, pre-treatments of whole-mount Arabidopsis samples as well as chromatin and pH3Ser10 labeling were performed the same way and with the same chemicals/antibodies as in the methodology that we described before (Kelemen et al., 2024). Briefly, fixation was performed in paraformaldehyde (PFA) + 0.1 % (v/v) Triton X-100 in 2x microtubule stabilizing buffer (MTSB). Subsequent washing with MTSB was followed with methanol treatments and rehydration, then digestion of cell walls with a Driselase (Sigma-Aldrich, St. Louis, MO., US) - Macerozyme (Serva Electrophoresis GmbH, Heidelberg, Germany) mix, dissolved in 2 mM MES (Sigma-Aldrich, St. Louis, MO., US). Samples were then washed with 1x MTSB and permeabilized with DMSO + IGEPAL (Sigma-Aldrich) in 1x MTSB, washed with MTSB and pre-incubated with (Sigma-Aldrich) in 1x MTSB. Labeling was performed with 50x in BSA + MTSB diluted primary anti-phospho H3 at serine 10 raised in rabbit (Abcam, Cambridge, UK), samples were incubated overnight in 4 °C, then washed with 1x MTSB and labeled with the 100x diluted secondary antibody (Alexa 488 conjugated anti-rabbit IgG generated in goat, Abcam). For the detection of mitotic microtubules by IHC, the procedure was as follows. Simultaneously with the primary antibody labeling for pH3Ser10, a direct IHC employing Cy3-conjugated monoclonal anti- β tubulin antibody (Sigma-Aldrich, generated in mouse, 50-fold diluted) was used. The sequence of labeling was: primary anti-pH3Ser10 antibody + Cy3 conjugated anti- β tubulin antibody, Alexa488 conjugated secondary antibody for pH3Ser10 detection, DAPI. This complex, triple labeling procedure for simultaneous labeling of microtubules, phosphohistone and whole chromatin was necessary for a more precise detection of cells in G2 phase and in different mitotic phases. In Arabidopsis, G2 phase was detected by the presence of peculiar plant microtubule structures,

the preprophase bands (PPBs), characteristic for this cell cycle phase and gradually disappearing as the cells progress into prophase. The presence of PPB and the absence of any pH3Ser10 signal altogether confirms that cells are in G2. Prophase cells could be detected with the aid of pH3Ser10 labeling, that is characteristic only during mitosis, while this initial phase of mitosis is difficult to be detected solely by chromatin labeling, due to the small size of chromosomes in Arabidopsis. The distinction of anaphase and telophase/cytokinesis is much easier, when phragmoplast, another plant microtubular structure characteristic for this mitotic phase, appears (see Fig. 3 of the Results section, that confirm these above statements). For microscopic observations, a Nikon Ti-E inverted super-resolution microscope (Nikon Instruments Inc., Melville, NY, USA) was used by employing a Plan Apo VC 60x NA WI DIC N2 objective and NIS-elements Ar software (version number: 5.30.02). Microscopical observations were according to the standard manufacturer settings as follows. For DAPI signal visualization, we used 405 nm laser with 452/45 filter set, emission wavelength was 430/475. For the detection of Alexa 488 signal, we used 488 nm laser and 525/50 filter set, emission wavelength was 500/550 nm. For the detection of Cy3 signal we used 561 nm laser and 593/46 filter set, emission was observed at 570/616 nm in this case. For quantification of pH3Ser10 signal, the same gain settings were employed for all samples (gain value = 1 for this type of CLSM).

Mitotic figures and the occurrence of different mitotic phases were detected by this triple labeling method. For MCY-LR and DQ treatments, assays for the detection of total, early and late mitotic indices as well as investigations of pH3Ser10 signals from primary root apical meristems were performed in four separate sets of experiments. The intensity of pH3Ser10 signals was quantified as for the quantification of ROS levels, employing the pixel number counting facilities (AIOD) of Fiji software: labeling intensities were plotted after subtracting background fluorescence.

2.4. Data analysis

All experiments were performed at least in three independent assays and sample sizes ("n" values) are indicated at each Figure, where appropriate.

Plots illustrating datasets represent the mean $\pm$ SE values and they were created with the aid of Systat Sigma Plot 12.0 ® software (Systat Software, San Jose, CA). "X" symbols on graphs = significant differences between control wild-type (Col0) and control mutants, "*" symbols = significant differences between MCY-LR or DQ treatments within a given genotype. Statistical significances for the differences between controls and treatments were studied by comparing two data groups at once (comparison of genotypes and comparison of single concentration MCY-LR and DQ treatments within a genotype) with t-tests. When Shapiro-Wilk normality test failed, t-tests were performed with Mann-Whitney Rank Sum tests. Symbols for significant differences on the graphs: X, * = significant difference ($P < 0.05$), XX, ** = significant difference ($P < 0.01$), XXX, *** = significant difference ($P < 0.001$). Where it was necessary, for more clarity, important changes were labeled with numbers on graphs.

3. Results

3.1. Exposures to MCY-LR and DQ for 48 h have differential effects on reactive oxygen species (ROS) levels and scavenging in Arabidopsis roots

For the genotypes not treated with MCY-LR/DQ, all protein phosphatase mutants had elevated levels of ROS as compared to wild-type (Col0) and this was valid for root apical meristems (root tips) and differentiated root tissues (Fig. 1a, see labels "X" and "XXX" and numbers "1" and "2" for significance). For differentiated root tissues, *fass-5* could not be examined in this sense, because of its severe developmental alterations: for example in this mutant, root apical meristems

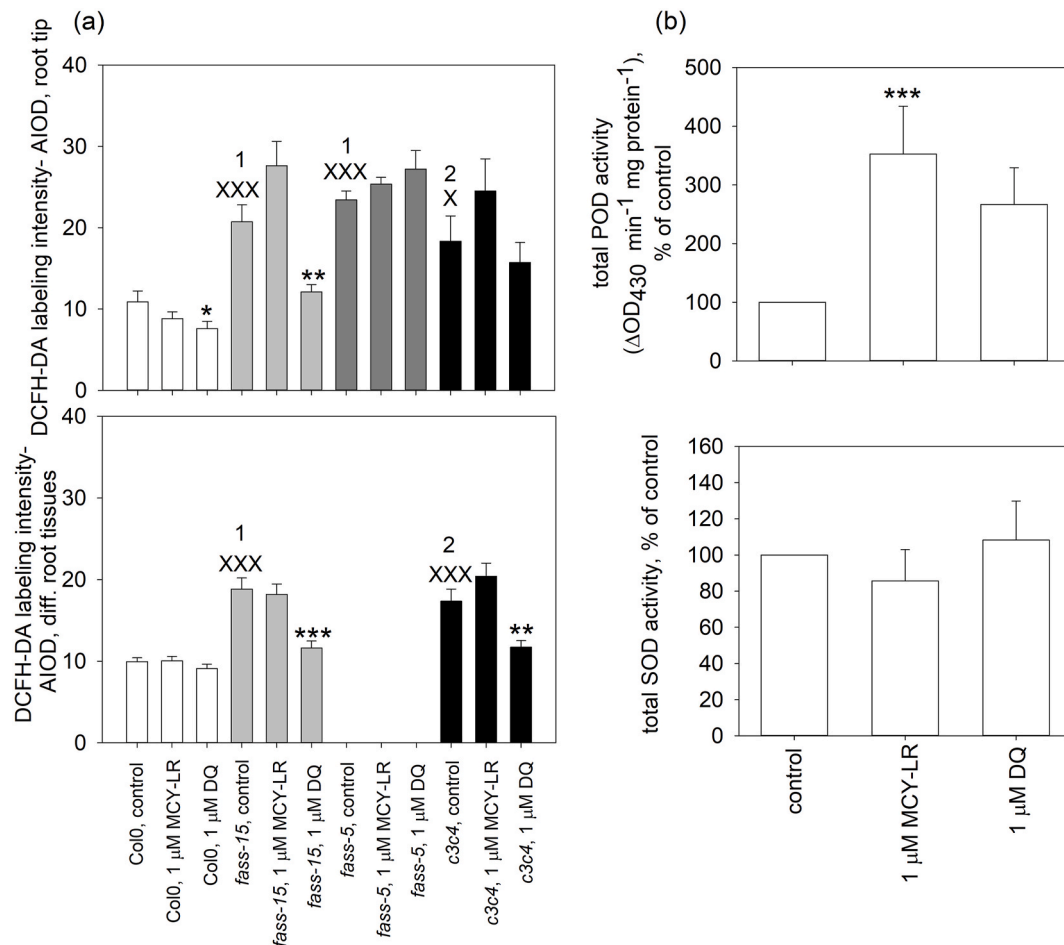


Fig. 1. Differential effects of MCY-LR and DQ on reactive oxygen species (ROS) production and enzymatic scavenging. **(a)** ROS production as revealed by DCFH-DA labeling. Untreated *fass* mutants (number 1) and *c3c4* (number 2) show higher ROS levels, than Col0 both in the root apical meristems (RAMs) and differentiated zones of primary roots. DQ decreases ROS levels in the RAM of Col0 and *fass-15* as well as in differentiated root tissues of *fass-15* and *c3c4*. MCY-LR does not have significant effects. *fass-5* mutants did not develop well differentiated roots, thus there are no data for differentiated root tissues for this genotype. Sample size was $n \geq 6$ for each treatment per each independent experiment. **(b)** The effects of MCY-LR and DQ on total POD and SOD activities of Col0 whole roots. MCY-LR induces a significant increase of POD activity. None of the drugs induce any significant changes in total SOD activities. “AIOD” is the number of pixels to show signal intensity. “X” symbols on graphs = significant differences between control wild-type (Col0) and control mutants, “***” symbols = significant differences between treatments within a given genotype. Symbols for significant differences on the graphs: X, * = significant difference ($P < 0.05$), ** = significant difference ($P < 0.01$), XXX, *** = significant difference ($P < 0.001$). Statistical analysis used the Mann-Whitney rank sum test.

are present, but there is a lack of an intact root system. When we compared MCY-LR treatments of each genotype to their own controls, we could not find significant differences ($P \geq 0.05$) in any root tissue, in any of the genotypes. On the other hand, DQ treatments induced significant decreases of ROS levels in root tips of Col0 and *fass-15* and in differentiated tissues of *fass-15* and *c3c4* (Fig. 1a, see “*, **, ***” labels for significances).

Next, we assayed activities of ROS scavenging enzymes of whole Col0 roots. Total peroxidase (POD) activity was significantly elevated at MCY-LR treatments, but in case of exposures to DQ, this elevation was not significant (Fig. 1b, upper chart). Total superoxide dismutase activities remained practically unchanged at any of the treatments (Fig. 1b, lower chart).

3.2. Exposures to MCY-LR and DQ for 48 h have differential effects on mitotic activities in *Arabidopsis* root apical meristems

For the different genotypes not treated with MCY-LR/DQ, G2 index (the percentage of meristematic cells in this cell cycle phase out of the total number of root apical meristematic cells) was significantly lower in *fass-5*, than in the other three genotypes examined (Fig. 1a, “XX” label).

Total mitotic activities were significantly lower in *fass-5* and *c3c4*, as compared to wild-type (Col0) root tips (Fig. 2b and Suppl. Fig. 2, upper charts, labels “XXX”). The percentage of early mitotic cells was reduced significantly only in *c3c4*, while late mitosis was inhibited both in *fass-5* and *c3c4* (Fig. 2b and Suppl. Fig. 2, middle and lower charts). In case of *fass-5*, this also meant that there was a partial arrest of cells in early mitosis (number “1” on the middle charts, Fig. 2b and Suppl. Fig. 2).

At treatments with MCY-LR, significant decrease of the percentage of cells in G2 phase could be observed only in *fass-5*, where untreated seedlings did also show a dramatically decreased G2 index (Fig. 2a). As concerning cell division activities, the cyanotoxin did not induce any significant changes of total as well as early or late mitotic indices (Fig. 2b). However, in case of *fass-15*, MCY-LR induced a slight arrest of cells in early mitosis (Fig. 2b, middle chart, number “1” for this genotype).

At treatments with DQ, G2 index was decreased dramatically as compared to the controls for each *Arabidopsis* genotype (Fig. 2a, “*, **” labels). This was true for mitotic activities, including both early and late mitotic indices (Fig. 2b, “*, **, ****” labels, all charts; Fig. 3). Moreover, in case of *fass-15*, almost all dividing cells were arrested in early phases (see number 1 on Fig. 2b/middle chart and compare this to late mitotic

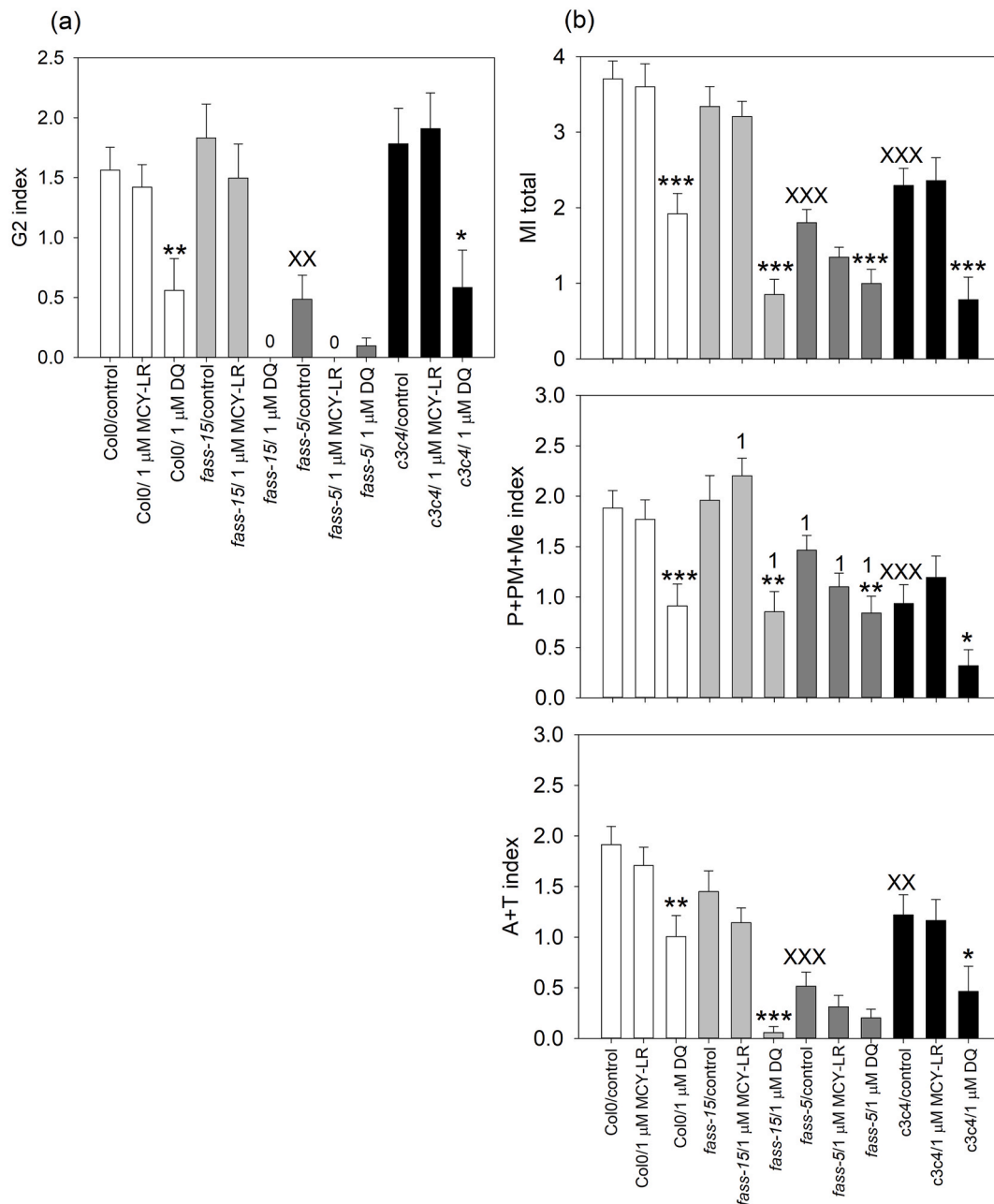


Fig. 2. Differential effects of MCY-LR and DQ on the mitotic activities of primary root apical meristems (RAMs). Sample size was $n \geq 6$ for each treatment per each independent experiment. **(a)** G2 indices show that for untreated genotypes, *fass-5* show a significant decrease as compared to Col0 (see the “XX” label). DQ, but not MCY-LR induces significant decreases in Col0, *fass-15* and *c3c4*, while both drugs induce decreases in *fass-5*. **(b)** total mitotic activities (upper chart) as well as separate early (middle chart) and late (lower chart) mitotic indices. For untreated genotypes, *fass-5* and *c3c4* show significant decreases of total and late mitotic activities as compared to Col0 (see the “XXX/XX” labels on the top and bottom graphs). For early mitotic activities, this decrease is significant only for *c3c4* (see “XXX” label on the middle graph). Numbers 1 on the middle graph indicate blocks in early mitosis. DQ, but not MCY-LR has significant decreasing effects for all mitotic phases in all genotypes. P – prophase, PM-prometaphase, Me-metaphase, A-anaphase, T-telophase. “X” symbols on graphs = significant differences between control wild-type (Col0) and control mutants, “***” symbols = significant differences between treatments within a given genotype. Symbols for significant differences on the graphs: * = significant difference ($P < 0.05$), XX, ** = significant difference ($P < 0.01$), XXX, *** = significant difference ($P < 0.001$). Statistical analysis used *t*-test and the Mann-Whitney rank sum test.

index value for this genotype and this treatment on the lower chart). This means that mitotic arrest observed to untreated root tips of this genotype was further amplified by DQ treatment.

3.3. Exposures to MCY-LR and DQ for 48 h have differential effects on the levels of histone H3 phosphorylation in *Arabidopsis* root apical meristems

The progress of histone H3 phosphorylation during *Arabidopsis* mitosis in root apical meristems untreated with MCY-LR/DQ confirmed the general statement made by Houben et al. (2007) for plants. pH3Ser10 signals were observed only during the M phase of cell cycle.

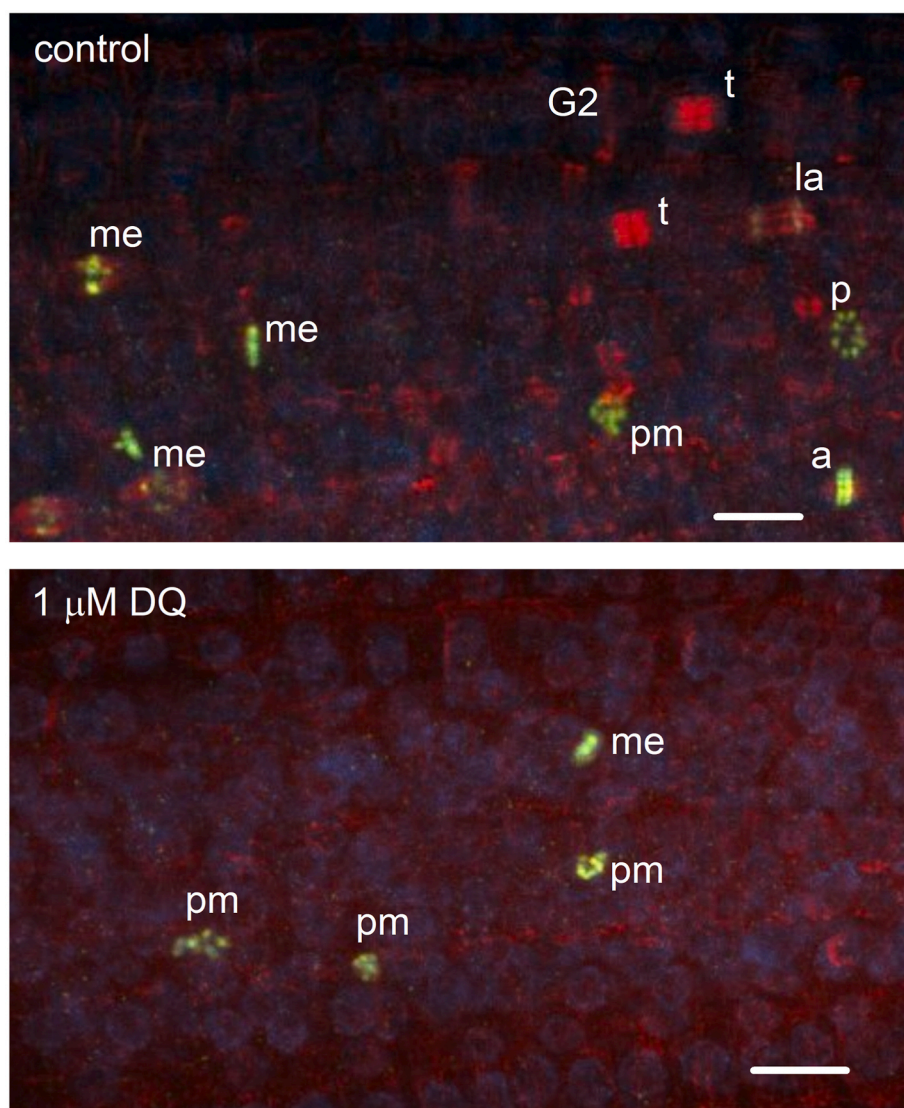


Fig. 3. Representative merged images of 2D projections of confocal Z-stacks for chromatin (background blue signals for chromatin for cells in all cell cycle phases), pH3Ser10 (green signals) and microtubule (red signals) in root apical meristems of Col0: controls top image, treatments with 1 μ M DQ (b). G2-shows the presence of preprophase bands (PPBs) and the lack of phosphohistone signals; p-prophase, shows pH3 signals as dots representing centromeres of chromosomes; pm-prometaphase; me – metaphase; a – anaphase; la-late anaphase showing weakening of pH3Ser10 signal; t-telophase, shows strong labeling for phragmoplast microtubules and the lack of pH3Ser10 signal. DQ treatments bottom image decrease the numbers of mitotic figures. Scalebars:10 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

They were detectable in prophase, increased in prometaphase to reach a peak in metaphase and gradually decreased until early telophase. Late telophase and cytokinetic cells did not show the signal any more (Fig. 3). This is true not only for Col0, but to different degrees, for (untreated) phosphatase mutants as well (Fig. 4). *Fass* mutants showed significantly lower degrees of histone H3 phosphorylation as compared to Col0 (Fig. 4e).

MCY-LR did not induce significant changes of pH3Ser10 signals in any of the genotypes examined (Fig. 4a–d). As concerning DQ treatments, these could not be examined in terms of pH3Ser10 levels in phosphatase mutants, due to their very low percentage of mitotic cells (see Fig. 2b, upper chart for mitotic indices). For mutants, the low number of samples did not allow us to perform valid statistical analysis. In case of Col0, this herbicide induced significant increases of pH3Ser10 signals during early mitotic phases, but remained relatively unchanged at late mitosis (Figs. 3 and 4a).

4. Discussion

The concentrations (1 μ M) of the two toxic compounds used in this work are environmentally relevant. As for DQ, laboratory experiments to simulate environmental effects are often using even much higher concentrations, than our study. These is the case of an onion (*Allium cepa*) test system, where a range of 87–348 μ M was used (Acar, 2021). Another dihydropyridine herbicide, paraquat (methyl viologen) was used at 10 μ M to study the mechanisms of herbicide extrusion in *Arabidopsis* (Lv et al., 2021). We can assume that the alterations induced by a relative low DQ concentration used here are pointing out, that the herbicide may be environmentally effective even at higher dilutions. Concerning MCY-LR, environmentally relevant concentrations are considered in general to be in the nM range, however, there are plenty of cases where toxic cyanobacterial overproduction releases this compound into freshwaters even at μ M concentrations (see Máthé et al., 2007; Chia et al., 2022 for examples). Also, experimental design required equimolar use of MCY-LR and DQ to perform a valid

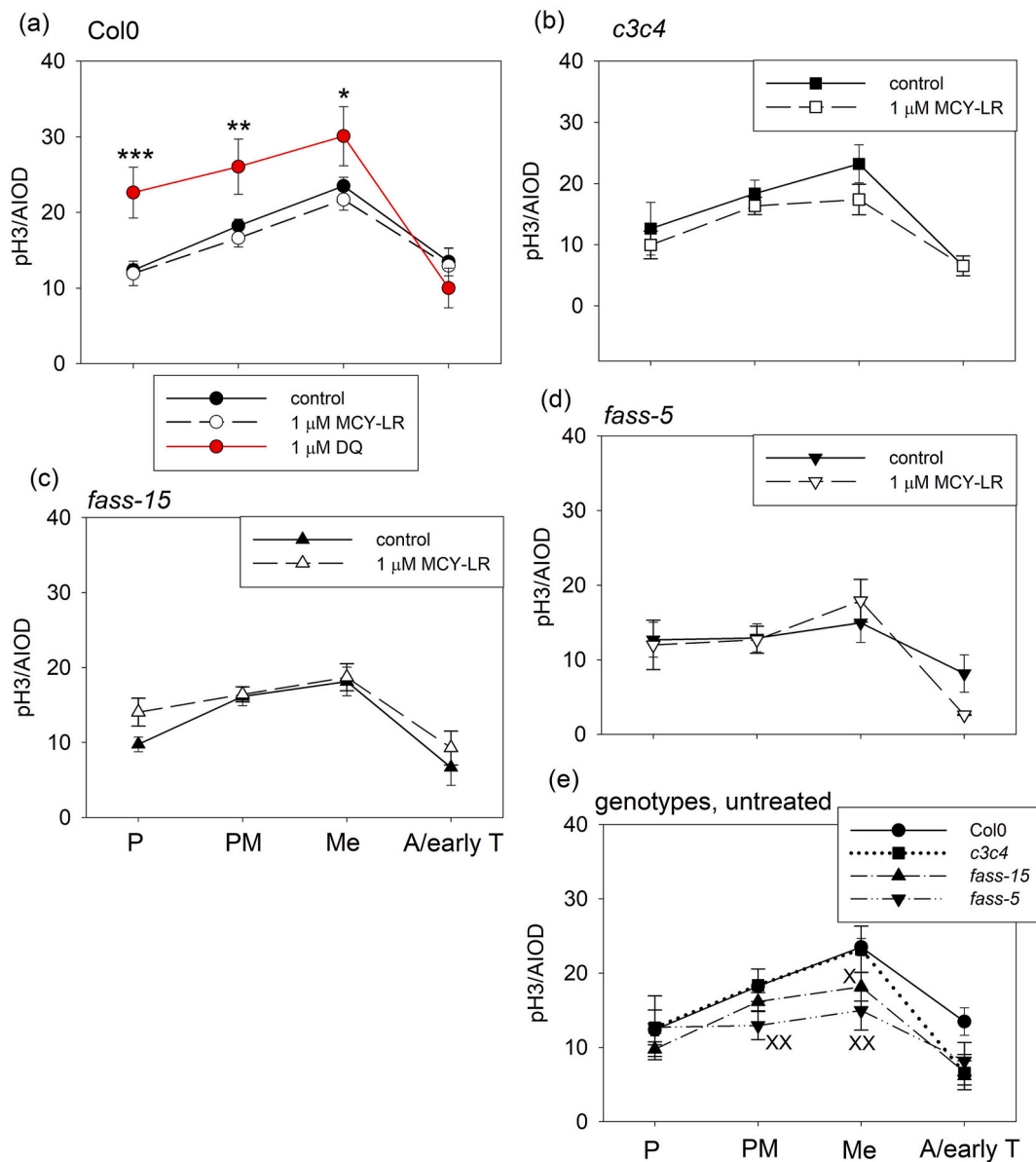


Fig. 4. (a–d) Differential effects of MCY-LR and DQ on the phosphorylation of histone H3 at Ser10. Note that MCY-LR has no significant effects in any of the genotypes studied. Sample size was $n \geq 5$ for each treatment per each independent experiment. (a) For Col0, we had a sufficient number of DQ treated samples (mitotic figures) to reveal significant increases of pH3Ser10 levels in early mitotic phases. (e) A comparison of untreated genotypes show that while we did not detect significant changes for *c3c4*, both *fass-15* and *fass-5* show significant decreases of pH3Ser10 levels. “AIOD” is the number of pixels to show signal intensity. P–prophase, PM–prometaphase, A/early T–anaphase to early telophase. “***” symbols = significant differences between treatments within a given genotype. Symbols for significant differences on the graphs: * = significant difference ($P < 0.05$), ** = significant difference ($P < 0.01$), *** = significant difference ($P < 0.001$). Statistical analysis used *t*-test and the Mann-Whitney rank sum test. For (d), significant differences were labeled, with “X, XX, and XXX”, since here we compared different genotypes untreated with MCY-LR/DQ.

comparison of their effects.

The relatively long-term exposure (48 h) of all genotypes of Arabidopsis seedlings to MCY-LR did not induce significant changes of root ROS levels. Untreated PP2A mutants did show relevant increased oxidative stress response, showing that alterations in the functioning of Foss and C3/C4 subunits render plants to be more sensitive to ROS induction. However, even in these more sensitive mutants, MCY-LR did not induce further increase of ROS. Meanwhile to our surprise, DQ decreased, rather than increased ROS levels, with the exception of *fass-5* (Fig. 1a). This may indicate that DQ could initiate cell death processes in roots, thereby lowering stress responses as well. However, chromatin analysis of non-mitotic cells did not detect any symptoms of cell death like nuclear blebbing, chromatin condensation or fragmentation. DAPI

staining of DNA (same method as used for labeling of mitotic chromatin, see Section 2.3. of Materials and methods) showed intact nuclei with normal degree of chromatin condensation, as for controls. Meanwhile, POD and SOD activities were well measurable, indicating that cell viability was not completely altered (Fig. 1b). Interestingly, the activities of these ROS scavenging enzymes were not altered significantly by DQ, while MCY-LR induced notable increases of POD activities in Col0. This may explain why the cyanotoxin did not affect ROS levels: these were probably scavenged effectively to confer toxin resistance of seedlings at long-term exposure.

The above long-term effects of the two compounds were markedly different to shorter exposure time (24 h). Phosphatase mutants showed increased ROS levels both at MCY-LR and DQ exposure and neither POD,

nor SOD activity was increased in MCY-LR treated Col0 (Freytag et al., 2023 and Suppl. Fig. 1), meaning that they could not cope with this stress at short-term. Meanwhile, we detected increases of phosphorylated histone H2AX levels in Col0 root tips treated with MCY-LR or DQ (24 h treatments). This suggests that oxidative stress did occur in Col0 during treatments, because higher phosphorylation levels of H2AX indicate that repair mechanisms of ROS-induced DNA damage were initiated (Freytag et al., 2023). Overall, at 24 h of exposure, both MCY-LR and DQ induced oxidative stress in Arabidopsis, while at 48 h treatments, seedlings seem to be resistant to MCY-LR, but not to DQ. It is worth mentioning however, that at 24 h exposure, DQ did induce nuclear blebbing (but no chromatin condensation or fragmentation) in Col0 (Kelemen et al., 2024). In Arabidopsis, MCY-LR acts primarily as a protein phosphatase inhibitor and less as a ROS inducer, as confirmed by previous studies as well (e.g. Freytag et al., 2021; Máthé et al., 2024), while DQ acts primarily as an oxidative stress inducer as confirmed by this study and Kelemen et al. (2024). Interestingly, another study showed that prolonged exposures amplified the effects of another microcystin congener, MCY-RR in Arabidopsis Col0: it increased the activities of ROS scavenging enzyme families SOD and catalase (CAT) along with amplifying of oxidative stress symptoms like lipid peroxidation and glutathione oxidation (Yin et al., 2005). N.b. this latter study used a completely different experimental system: cell suspension cultures and not young seedlings used in the present work.

There are other studies showing that oxidative stress is amplified with the increase of microcystin exposure time. For example, in the aquatic plant *Ceratophyllum demersum* the amount of oxidized glutathione increases continuously over time, hydrogen peroxide production is increased during the first 24 h of exposure and remains constantly elevated thereafter, in the 72 h exposure period. During this period, antioxidant enzyme activities increase continuously (Pflugmacher, 2004).

Concerning cell division activities at 48 h, mitotic indices were significantly decreased in the untreated *fass-5* and *c3c4* mutants as compared to Col0 (and for *fass-5*, this is valid for G2 index as well) - (Fig. 2, and Suppl. Fig. 2). However, except for the G2 index of *fass-5*, 48 h exposures to MCY-LR did not affect significantly any mitotic parameters in any of the studied genotypes. Exception was *fass-15*, a mutant more sensitive to the cyanotoxin, where MCY-LR induced arrest in early mitosis. In contrast, DQ decreased significantly both the G2 and mitotic indices in all genotypes and induced severe arrest at early mitosis in *fass-15* (Fig. 2). It is worth mentioning that in contrast to *fass-5*, untreated *fass-15* does not show this mitotic arrest symptom (Fig. 2b and Suppl. Fig. 2).

As for oxidative stress, mitotic activities in plants exposed for 48 h to MCY-LR were markedly different to 24 h exposures. At 24 h, MCY-LR inhibited mitosis in Col0, but did not induce mitotic arrest in *fass-15* (under publication elsewhere). 24 h DQ treatment had effects similar to 48 h exposure (see Kelemen et al., 2024 for 24 h exposures). Overall, as for oxidative stress, several aspects of mitotic effects indicate that at longer exposures, Arabidopsis seedlings became more resistant to MCY-LR, but not to DQ.

Since we know that regulation of entry into mitosis is regulated by protein phosphorylation, MCY-LR as a protein phosphatase inhibitor is likely to be used as a tool in plant cell cycle regulation research. There is a number of publications showing the effects of MCY-LR on plant cell division. These previous papers show that at longer exposure times, mitotic indices are partially restored, but the frequency of mitotic anomalies increases (in *Phragmites australis*, Máthé et al., 2009, concentration of MCY-LR was at the μM range), or mitosis is delayed at short-term exposures, and at long-term exposures, mitosis is blocked (in *V. faba*, Beyer et al., 2012, concentration of MCY-LR was at the μM range). These studies indicate that mitotic anomalies are amplified over time. On the other hand, onion root meristems show no significant changes of mitotic indices at long-term (7d) exposures to low (nM range) MCY-LR concentrations (Pamplona-Silva et al., 2018), but there are no

data for short-term exposures or for higher MCY-LR concentrations. These studies suggest that the relevant effects of the cyanotoxin are largely dependent on the plant species/genotypes as well as the employed concentration and exposure time. Concerning DQ, we mention the publication of Acar (2021), who reported inhibitions of mitotic activities together with cytogenetic alterations, micronucleus formation and nuclear blebbing in root tips of onion treated for 72 h with far higher DQ concentrations, than used in the present study.

Histone H3 phosphorylation at Ser10 shows interesting changes at 48 h DQ exposures in Col0: it increases phosphorylation signals at early mitosis in a significant manner (Fig. 4a). Currently we do not know the mechanism of histone hyperphosphorylation in the presence of the herbicide. Unfortunately, due to the low sample number (histone H3 phosphorylation is detectable only during mitosis and DQ severely lowered mitotic indices), we could not study histone phosphorylation in the phosphatase mutants for a relatively high (1 μM) DQ concentration for Arabidopsis. 24 h of DQ (1 μM) exposure is increasing histone H3 phosphorylation in prophase cells of Col0 and *fass-5*, but has a decreasing effect in *fass-15* and *c3c4* (Kelemen et al., 2024). As concerning MCY-LR, 48 h exposures do not change significantly the levels of pH3Ser10 in any of the genotypes studied (Fig. 4). This is surprising again, since MCY-LR is a phosphatase inhibitor, thus it is expectable that it will induce hyperphosphorylation. Indeed, 24 h exposure induces increases H3 phosphorylation levels in all genotypes (Kelemen et al., 2025). Short-term (12–16 h) MCY-LR exposures of *Vicia faba* lateral roots induce dramatic increases of pH3 levels, and these effects are maintained even after six days of exposure (Beyer et al., 2012). For Arabidopsis seedlings, it seems again that longer (48 h) toxin exposures do not show any more alterations that are regularly detected at shorter exposures, but we do not know whether longer (72 h or more) cyanotoxin exposures affect histone H3 phosphorylation.

There are other subcellular alterations that can be detected at short-term but they are “repaired” at longer exposures in Arabidopsis. At 4 h exposure, MCY-LR induces the fragmentation of vacuoles (Nagy et al., 2018), but at 24 or 48 h of exposure, the vacuolar system seems to be normal (Suppl. Fig. 3). Also, the cyanotoxin increases plastid stromule frequency, that is decreased back to control level at as soon as 24 h of exposure. Similar recovery is detected after short-term alteration of the mitochondrial fission-fusion cycle (Juhász et al., 2023).

Data on (MCY-LR/DQ) untreated phosphatase mutants reveal that as compared to wild-type seedlings, they are more sensitive to oxidative stress and their mitotic activity is impaired, which highlights their importance in the study of subcellular regulatory events in plants.

Overall, we can conclude that Arabidopsis seedlings can partially recover their alterations induced by MCY-LR at long-term (48h) exposures. However, this recovery is only transient, because after two or more days of exposure to MCY-LR – which is a long period for a rapidly growing Arabidopsis seedling-plantlets are still growing, but development of lateral and even primary roots is altered (Freytag et al., 2021). It is worth mentioning, that Ma et al. (2023) showed increases of transcript levels for different PP2A subunits after MCY-LR treatments of rice and cucumber seedlings. Although they did not measure changes of expression at the protein level, they suggested that increase of PP2A expression may be a compensatory mechanism to MCY-LR toxicity. This may be a plausible mechanism, however in the study of Ma et al. growth and physiological alterations were in reality amplified over longer exposure times and they were partially recovered only after the removal of the cyanotoxin and not at more prolonged exposures to it (they performed MCY-LR treatments for a 7 days period that was followed by removal of the toxin and growing and monitoring plants for further 7 days). Although bacterial degradation of microcystins in freshwaters has been demonstrated (Bourne et al., 1996), plants are not known to possess similar mechanisms. On the other hand, there is evidence that many plants are able of detoxifying this cyanobacterial compound by conjugation e.g. with glutathione (Pflugmacher et al., 1998), however this mechanism is documented only for a limited plant species to date.

For example, it seems not to function in *Arabidopsis* (Col0), since glutathione-S-transferase (GST) activity, the main enzyme system required for the formation of glutathione conjugates, remains unchanged even after longer exposures to microcystin (MCY-RR) (Yin et al., 2005). According to the present study, the *Arabidopsis* system does not refute our original hypothesis concerning the amplification of deleterious effects of MCY-LR over time, but as stated above, seedlings are able of transient defence against cyanotoxin-induced stress. Concerning DQ, we can assume that the latter maintains or amplifies its altering effects even at long-term exposure. Previous studies demonstrate that long-term exposure to DQ may have growth inhibitory effects under field conditions, and this is dose- and plant species-dependent (Puri et al., 2008). Meanwhile, as mentioned in the Introduction section, there are several studies on other plants confirming that the accumulation of microcystins in plants over a prolonged exposure will decrease gradually plant vigor. Such phenomena were observed for example for rice and partially for cucumber (Ma et al., 2023). Even our *Arabidopsis* system showed that seven days of MCY-LR exposure induced growth inhibition in Col0 plants, however this is characteristic mainly for MCY-LR concentrations higher than 1 μM . The consequences of such very long long-term exposures are not subject of the present paper and will be included in a future publication.

Our main conclusions regarding toxicities of MCY-LR and DQ are:

1. At 48 h of exposures, *Arabidopsis* plants are able of transient recovery from the short-term effects of 1 μM MCY-LR, but this is not true for DQ, a compound with different mechanism of action. Our study contributes to the understanding of differential reactions of plants to different pollutants. In *Arabidopsis*, MCY-LR acts primarily as a protein phosphatase inhibitor, while DQ acts primarily as a ROS inducer. However, we point out that physiological and developmental parameters like altered auxin distribution and root development in the presence of MCY-LR (Freitag et al., 2021), are not recovered over time, pointing out that *Arabidopsis* plants are not able of complete defence against MCY-LR stress.
2. Since DQ increases phosphohistone levels both at short- and long-term, we suggest further evidence for the regulatory effects of protein phosphorylation on oxidative stress responses in plants.
3. Our studies concerning the effects of MCY-LR make more evident that at long-term, the model plant *Arabidopsis* can cope transiently with microcystin induced stresses that alter cellular functioning at short-term exposure. This issue should be taken into account, when we consider the environmental toxicity of this compound, since it is evident, that its long-term effects are largely plant species/genotype dependent.
4. DQ as a herbicide may have long-term detrimental and persistent environmental effects on plants.

CRedit authorship contribution statement

Tamás Garda: Methodology, Investigation, Conceptualization. **Gabriella Petra Juhász:** Methodology, Investigation, Conceptualization. **Adrienn Kelemen:** Methodology, Investigation. **Jaideep Mathur:** Methodology, Investigation. **László Ujlaky-Nagy:** Methodology, Investigation. **Csongor Freitag:** Methodology, Investigation. **Márta M-Hamvas:** Methodology, Investigation. **Milán Riba:** Methodology, Investigation. **Csaba Máthé:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2025.126748>.

Data availability

Data will be made available on request.

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