Morphological and ultrastructural changes in tobacco BY-2 cells exposed to microcystin-RR

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ABSTRACT

Tobacco BY-2 cells were exposed to microcystin-RR (MC-RR) at two concentrations, 60 μg mL⁻¹ and 120 μg mL⁻¹, to study the changes in morphology and ultrastructure of cells as a result of the exposure. Exposure to the lower concentration for 5 d led to typical apoptotic morphological changes including condensation of nuclear chromatin, creation of a characteristic ‘half moon’ structure, and cytoplasm shrinkage and decreased cell volume, as revealed through light microscopy, fluorescence microscopy, and transmission electron microscopy, respectively. Exposure to the higher concentration, on the other hand, led to morphological and ultrastructural changes typical of necrosis, such as rupture of the plasma membrane and the nuclear membrane and a marked swelling of cells. The presence of many vacuoles containing unusual deposits points to the involvement of vacuoles in detoxifying MC-RR. Results of the present study indicate that exposure of tobacco BY-2 cells to MC-RR at a lower concentration (60 μg mL⁻¹) results in apoptosis and that to a higher concentration (120 μg mL⁻¹), in necrosis.

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1. Introduction

Eutrophication of water bodies may lead to excessive growth of cyanobacterial blooms common in many lakes and rivers all over the world (Skulberg et al., 1984; Carmichael, 1992; Codd, 1995). Many of the cyanobacteria forming the blooms produce different types of cyanotoxins including neurotoxins, hepatotoxins, cytotoxins, and lipo polysaccharide (LPS) endotoxins, which can have a variety of adverse effects on human and animal health and also raise ecological and aesthetic concerns (Carmichael, 1997). Of all the algal toxins, microcystins (mainly microcystin-LR, RR, and YR) are the most toxic and abundant (Carmichael, 1994). Physiological, biochemical, and ultrastructural changes induced by microcystins (MCs) have been studied in animals, such as fish (Liu et al., 2002), rats (Bouaïcha and Maatouk, 2004), freshwater shrimps (Chen and Xie, 2005), mice (Benson et al., 2005), birds (Pašková et al., 2008), and rabbits (Zhao et al., 2008).

Recently, the research on the uptake and effects of MCs on aquatic and terrestrial plants has increased. It has been reported that MCs can accumulate in Ceratophyllum demersum, Elodea canadensis, Vesicularia dubyana, Phragmites australis, Vallisneria natans, Lemna minor, Wolffia arrhiza, and Chlorophora fracta (Pflugmacher et al., 1998, 1999, 2001, 2004; Mitrovic et al., 2005; Yin et al., 2005a). Growth, development and physiological pathway of plants were affected when exposed to MCs (Abe et al., 1996; Kurki-Helasmo and Meriluoto, 1998; McElhiney et al., 2001). Romanowska-Duda and Tarczynska (2002) found that microcystin-LR inhibited the growth of an aquatic macrophyte (Spirodela oligorrhiza) and decreased chlorophyll content. M-Hamvas et al. (2003) found that fresh weight, plant length, the extent of lateral root formation, and anthocyanin content were lower in seedlings of white mustard (Sinapis alba L.) that had been treated with microcystin-LR. The commonly accepted mechanism of action of MCs in plants is inhibition of protein phosphatase type-1 and -2A. Recently, several studies have shown that MCs increase the level of cellular reactive oxygen species generating oxidative stress, and it is generally accepted that MCs toxicity involves oxidative damage (Pflugmacher, 2004)—a hypothesis strengthened by the results of our experiments to study the effects of MC-RR on Arabidopsis thaliana suspension cells and tobacco BY-2 cells (Yin et al., 2005b,c).

Current knowledge on the effect of MCs on plants at the ultrastructural level is scant owing to paucity of data and the limited number of plant species studied. To better understand the toxic effects of MCs on plants, we chose tobacco BY-2 suspension cells and examined the changes in their morphology and ultrastructure following exposure to MCs. We also assessed the possible mechanisms underlying such changes.
2. Materials and methods

2.1. Materials

Microcystin-RR (MC-RR) was extracted and purified using high-performance liquid chromatography with photodiode array detection (HPLC-PDA) (Harada et al., 1988; Lawton et al., 1994). The purity of the extract exceeded 95%, a level regarded as acceptable for general toxicological experiments.

2.2. Cell culture and MC-RR treatment

The tobacco BY-2 suspension cell line (Nicotiana tabacum L. cv. Bright Yellow 2) was cultured in KCMS liquid medium and maintained as described earlier (Yin et al., 2005c). The cells were treated during their phase of exponential growth (3-d-old cell culture) with 60 μg mL⁻¹ or 120 μg mL⁻¹ MC-RR for 5 d. A stock solution of MC-RR in deionized water was added to the liquid medium to yield the desired final concentration. The concentrations were selected on the basis of preliminary experiments, which had shown marked differences in cellular morphology and growth between tobacco BY-2 cells treated with MC-RR at 40–100 μg mL⁻¹ and those treated with MC-RR at 100–150 μg mL⁻¹. The cells were harvested for morphological and ultrastructural observations at 3 d and 5 d, respectively. All experiments were repeated three times.

2.3. Morphological observation by light microscopy

The samples were examined for cell morphology under a light microscope (Nikon Eclipse E600) equipped with a Nikon 35C camera.

![Fig. 1](image1)

*Fig. 1.* (a–c) Morphological alterations of tobacco BY-2 cells exposed to 60 and 120 μg mL⁻¹ MC-RR, respectively. (a) Control cells for 5 d, scale bar = 45 μm. (b) Cells treated with 60 μg mL⁻¹ MC-RR for 5 d, scale bar = 48 μm. (c) Cells treated with 120 μg mL⁻¹ MC-RR for 5 d, scale bar = 45 μm. (d–f) Morphological alterations of tobacco BY-2 cells stained with Evan’s blue exposed to 60 and 120 μg mL⁻¹ MC-RR, respectively. (d) Control cells for 5 d, scale bar = 30 μm. (e) Cells treated with 60 μg mL⁻¹ MC-RR for 5 d, scale bar = 32 μm. (f) Cells treated with 120 μg mL⁻¹ MC-RR for 5 d, scale bar = 29 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

![Fig. 2](image2)

*Fig. 2.* Nuclear morphology of tobacco BY-2 cells exposed to MC-RR. Cells from control (a) and 60 μg mL⁻¹ MC-RR treated group (b) after 5 d exposure stained with DAPI and observed under a fluorescence microscope. The arrows in (b) indicate the formation of numerous apoptotic nuclei, such as chromatin condensation. Scale bar = 14 μm in (a), scale bar = 15 μm in (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The viability of plant cells can be tested quickly and conveniently by staining them with Evan’s blue (0.5% w/v) as described by Taylor and West (1980). Living cells do not take up the stain and retain their natural colour because the stain cannot penetrate beyond the plasma membrane; cells damaged by MC-RR cannot keep the stain from penetrating and thus stain deep blue, readily distinguishable from viable cells upon microscopic examination.

2.4. Morphological observation by fluorescence microscopy

Morphology of the cell nuclei was studied by staining them with DAPI, a fluorescent dye that binds to DNA. Untreated cells and those exposed to the lower concentration (60 \mu g mL\(^{-1}\)) of MC-RR were stained with DAPI. The final concentration of the stain in a phosphate buffer solution (PB, pH 7.5) was 10 mg L\(^{-1}\). The cells were incubated for 20 min at room temperature in the dark, and images of the nuclei were observed with a fluorescence microscope (Nikon Eclipse E600) with excitation at 330–380 nm and emission at 420 nm (Dhar-Mascareno et al., 2005).

2.5. Ultrastructural observation by transmission electron microscopy

Ultrastructural changes were studied by observing ultrathin sections of cells under a transmission electron microscope (TEM). Untreated cells and those exposed to MC-RR at either of the two concentrations were washed twice with PBS, fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2) at 4°C for 4 h, washed again with PBS, and post-fixed in 1% osmium tetroxide at room temperature for another 2 h. After dehydration under a graded series of ethanol solutions of varying concentrations and embedding with Epon812 resin and acetone, ultrathin sections were cut and stained with uranyl acetate and lead citrate for examination under the TEM (Huang et al., 2008).

3. Results

3.1. Observation by light microscope

The untreated cells were easily identifiable: they appeared clear and intact, with nearly round nuclei. Cells exposed to the lower concentration appeared shrunken, with irregularly shaped nuclei, whereas those exposed to the higher concentration were swollen, with indistinct cellular boundaries following lysis of cell membranes (Fig. 1a–c). Almost all the untreated cells had retained their natural colour despite staining with Evan’s blue; in the group of cells that had been exposed to the lower concentration, a few cells were stained deep blue whereas such cells were widespread in the group exposed to the higher dose (Fig. 1d–f).

Fig. 3. Toxic effects of MC-RR on the plasma membrane of tobacco BY-2 cells after exposure to 60 and 120 \mu g mL\(^{-1}\) MC-RR, respectively. (a) Showing normal plasma membrane of control cells, scale bar = 10 \mu m. (b) Showing the phenomenon of plasmolysis (black arrow) in the low dose group after 3 d exposure, scale bar = 10 \mu m. (c) Showing the gaps in the plasma membrane (black arrows) in the high dose group after 3 d exposure, scale bar = 5 \mu m.

Fig. 4. Toxic effects of MC-RR on the endoplasmic reticulum of tobacco BY-2 cells after exposure to 60 and 120 \mu g mL\(^{-1}\) MC-RR, respectively. (a) Showing normal endoplasmic reticulum (black arrow) of control cells, scale bar = 0.2 \mu m. (b) Showing the whirling of RER (black arrow) in the low dose group after 5 d exposure, scale bar = 0.2 \mu m. (c) Showing the serious swelling and degranulation of RER (white arrows) in the high dose group after 5 d exposure, scale bar = 1 \mu m.
3.2. Observation by fluorescence microscope

In untreated cells, single and round nuclei were clearly visible (Fig. 2a). However, in cells exposed to the lower concentration for 5 d, chromatin became condensed and migrated to the edge of the nucleus, clearly visible as a moon-like circle or a ring inside the nuclear envelope (Fig. 2b).

3.3. Observation by transmission electron microscopy

3.3.1. Plasma membrane

The cell plasma membrane was intact and in close contact with the cell wall in untreated cells. After 3 d exposure, plasmolysis was evident in the low-concentration group whereas the plasma membrane was disrupted in the high-concentration group (Fig. 3a–c).

3.3.2. Endoplasmic reticulum (ER)

In untreated cells, abundant rough endoplasmic reticula (RER) were concentrated around the cell nuclei and borders of the cell membrane (Fig. 4 a) with some zymogen granules and electron-dense material evident inside the cisternae. In the low-concentration group, no changes were evident in RER and smooth-surfaced endoplasmic reticula (SER) until 5 d, after which the whirling of ER were observed (Fig. 4b). The ER was wrapped around other organelles including mitochondria. However, there was widespread swelling of ER in the high-concentration group within 5 d of exposure. At 5 d, the expansion and vacuolation of cisternae of ER were severe and some ribosomes became detached from the surface of the cisternae (Fig. 4c). In places, the swollen ER was transformed into vesicles dispersed throughout the cytoplasm by fragmentation or separation.

3.3.3. Mitochondria

Untreated cells showed many mitochondria at the membrane border (Fig. 5a). The mitochondria had high electronic density with abundant and acerose cristae. In the low-concentration group, the mitochondria were slightly swollen, with lower electronic density and fewer cristae than those in the control group after 5 d of exposure (Fig. 5b). However, in the high-concentration group, the mitochondria began to lose the cristae and their matrix with time (Fig. 5c–e). At 3 d, the mitochondria were slightly swollen, with only a few affected cristae (Fig. 5c), although badly damaged mitochondria with very low electron density and very few cristae were also observed (Fig. 5d). At 5 d, the damage was serious: the affected mitochondria had no cristae and were completely vacuolar (Fig. 5e).

3.3.4. Nucleus

Nuclei in untreated cells were round, with large and round nucleoli of very high and uniform electronic density. There was some rich heterochromatin in the nuclei, located generally just inside the nuclear membrane. Among heterochromatin there was

![Fig. 5. Toxic effects of MC-RR on the mitochondria of tobacco BY-2 cells after exposure to 60 and 120 μg mL⁻¹ MC-RR, respectively. (a) Mitochondria (black arrows) from control cells, scale bar = 0.5 μm. (b) Showing slight dilation of mitochondria (black arrows), with lower electronic density and less cristae in the low dose group after 5 d exposure, scale bar = 0.2 μm. (c) Showing the mitochondria with lower electronic density and less, short cristae in disorder in the high dose group after 3 d exposure, scale bar = 0.2 μm. (d) Showing the mitochondria with very low electron density and few cristae in disorder (black arrows) in the high dose group after 3 d exposure, scale bar = 0.2 μm. (e) Showing the swelling and vacuolation of mitochondria (black arrow) in the high dose group after 5 d exposure, scale bar = 0.5 μm.](image-url)
some euchromatin (Fig. 6a). In the low-concentration group, after 5 d exposure, the cells were shrunk and the chromatin had condensed and concentrated at the margin, although the nuclear membrane was still intact (Fig. 6b). However, in the high-concentration group, the nuclear membrane was distorted after 3 d exposure (Fig. 6c and d); after 5 d exposure, the chromatin in the nucleus became a mass of irregular fragments and the nuclear membrane was cleft in places (Fig. 6e).

3.3.5. Cytoplasm

The untreated cells showed abundant endoplasmic reticulum, mitochondria, and other organelles, all with normal ultrastructures in the cytoplasm (Fig. 7a). In the low-concentration group, the cells had shrunk and the cytoplasm was denser. Some lysosomes and vacuoles were seen in the cytoplasm and some mitochondria-like fragments were clearly visible in the lysosomes after 5 d exposure (Fig. 7d). In the high-concentration group, the cytoplasm was far less dense and vacuoles were frequent after 3 d exposure (Fig. 7b). At 5 d, many starch grains and lysosomes were present in the cytoplasm (Fig. 7c and e) but normal ER and mitochondria were very few.

4. Discussion

Microcystins are known to induce cell apoptosis or necrosis in fish and mammals (Tencalla and Dietrich, 1997; McDermott et al., 1998; Fischer et al., 2000; Mankiewicz et al., 2001; Li et al., 2004; Pichardo et al., 2005). In the present work, the mode of plant cell death following exposure to MC-RR was studied in tobacco BY-2 cells. Staining with Evan’s blue showed that deeply stained cells were present in both the low-concentration and the high-concentration groups (Fig. 1e and f), indicating that both the doses are lethal to cells, while cannot distinguish the form of cell death.

Light microscopy revealed that death of cells from the lower concentration of MC-RR was accompanied by cell shrinkage, but membrane blebbing was not observed (Fig. 1b). Simultaneously, distinct changes were observed in chromatin morphology, such as chromatin condensation at the nuclear envelope and/or broken chromatin (Fig. 2b). Ultrastructural studies revealed the details of extensive damage following exposure to a low concentration of MC-RR: plasmolysis, nuclear and cytoplasmic condensation (Figs. 3b and 6b). The protoplast shrank away from the cell wall and the plasmolysis of plasma membrane was significant; the nuclear envelope although nearly in good shape, was much smaller, and nuclear chromatin underwent marked condensation and became attached to the nuclear membrane. These typical ultrastructural changes were evidently compatible with cell apoptosis (Noodén et al., 2004). Moreover, the changes were compatible with cell apoptosis (Noodén, 2004) and similar to the changes observed in cells treated with known inducers of plant cell apoptosis such as Cd2+ (Kuthanová et al., 2004) and hydroxyl radicals (Lei et al., 2003). Taken together, these data strongly suggest that BY-2 cells exposed to MC-RR at low concentrations die through apoptosis. However, one important characteristic of apoptosis, namely fragmentation of cell contents into distinct, multiple, membrane-bound apoptotic bodies by membrane blebbing, was seen less frequently in this study. Since plant cells are constrained and supported by cell walls, cell fragmentation and phagocytosis may occur obscurely. However, annexin binding indicates that the migration of phosphatidyl serine to the outer surface of the plasma membrane, which triggers phagocytosis in animals, also occurs in plants (O’Brien et al., 1997).

Furthermore, we also found that the ER was wrapped around other organelles, including mitochondria (Fig. 4b). In animals, it is known that mitochondria and ER are often closely associated, thereby providing suitable conditions for communication between the two organelles (Rutter and Rizzuto, 2000; Walter and Hajnóczky, 2005). Indeed, mitochondria and the ER maintain a local Ca2+ communication conduit, allowing highly efficient and effective transfer of Ca2+ between the two organelles. It is also known that ER-targeted stress can activate Ca2+ efflux from the ER; this Ca2+ is then redistributed to the mitochondria and leads to programmed cell death (PCD) (Walter and Hajnóczky, 2005). Ding and Ong (2003) maintain that the activation of calpain and Ca2+/calmodulin-dependent protein kinase is critical to apoptosis induced by MC. Our most recent study found that the opening of mitochondrial permeability transition pore (mPTP) was involved in the process of MC-induced apoptosis in tobacco BY-2 cells (Huang et al., 2008). Arpagaus et al. (2002) report that opening of mPTP can be mediated by many factors including the build-up of Ca2+ in the mitochondrial matrix: perhaps it is the close proximity of ER to mitochondria in tobacco BY-2 cells that results in the ER inducing the opening of mPTP via Ca2+ efflux.

Tobacco BY-2 cells treated with MC-RR at a high concentration showed obvious morphological changes different from those observed in the untreated control group and in the low-concentration group, including the disruption and lysis of cell membrane, as well as sporadic congregation (Fig. 1c). Ultrastructural alterations in the high-concentration group were mainly concentrated in the membrane and marked swelling of organelles (Figs. 3c, 4c, 5c–e and 6c–e). With time, swelling of cells and rupture of the plasma membrane and the nuclear membrane became prominent, discharging the cell contents into the surrounding space. MCs have been shown to induce the formation of reactive oxygen species (ROS) that might cause serious cellular damage such as peroxidation of lipid membranes (Ding and Ong, 2003), which, in turn, can cause structural damage to membranes (Popova and Popov, 2004). Therefore, changes in the integrity of membranes may be the result of oxidative stress induced by MC-RR. These ultrastructural changes in cells in the high-concentration group were consistent with the symptoms of cell necrosis (Noodén, 2004). Mustard seedlings also showed necrosis of cotyledons exposed to MC-RR (M–Hamvas et al., 2003).

Other toxic effects observed in the high-concentration group included structureless cytoplasm and the development of lysosomes and vacuoles (Fig. 7b, c, and e). Similarly, in the low-concentration group there were increased lysosomes, which were seen to contain still recognizable intracellular material under degradation, pointing to autophagy, which is a conserved mechanism for the degradations of cellular contents in order to recycle nutrients or break down damaged or toxic material (Bassham, 2007). Autophagy is activated as an adaptive catabolic process in response to different forms of metabolic stress (Levine and Kroemer, 2008) and may protect tobacco cells from stress induced by MC-RR. The number of vacuoles increased in both low- and high-concentration groups (Fig. 7b and d) and some deposits accumulated in the vacuoles, a response that may play a significant role in either detoxifying MC-RR or increasing tolerance to it to prevent free circulation of MC in the cytosol by confining the toxin to a limited area. Wiegand and Pflugmacher (2005) describe how MC, by binding to glutathione, makes the toxin more soluble in water, supports the excretion of toxin from animal cells, and, in the case of plants, deposits it in the vacuole or binds it to cell walls.

The present study indicates that MC-RR at low and high concentrations has different effects on the morphology and ultrastructure of tobacco BY-2 cells (Figs. 1–7), describing for the first time ultrastructural modifications in plant cells exposed to MCs, and shows that death from MC-RR toxicity is by apoptosis when the toxin is administered at a lower concentration (60 µg mL−1) and by necrosis when at a higher concentration (120 µg mL−1). Moreover, the storage of toxin as electron-dense deposits in vacuoles appears to play an important role in maintaining the toxin in the cytoplasm and nucleus of the cells at comparatively low levels. Autophagy,
Fig. 6. Toxic effects of MC-RR on the nucleus of tobacco BY-2 cells after exposure to 60 and 120 µg mL\(^{-1}\) MC-RR, respectively. (a) Showing the nucleus of control cells, scale bar = 2 µm. (b) Showing serious apoptotic cells with cell shrinking and chromatin condensation in the low dose group after 5 d exposure, scale bar = 2 µm. (c) Showing the distortion of nuclear membrane in the high dose group after 3 d exposure, scale bar = 2 µm. (d) Showing the gap (white rectangle) in the nuclear membrane and condensation of chromatin in the high dose group after 3 d exposure, scale bar = 2 µm. (e) Showing the necrosis of cell in the high dose group after 5 d exposure, scale bar = 2 µm.

Fig. 7. Toxic effects of MC-RR on the cytoplasm of tobacco BY-2 cells after exposure to 60 and 120 µg mL\(^{-1}\) MC-RR, respectively. (a) The control cells, scale bar = 0.5 µm. (b) Showing the vacuoles (black arrows) in the high dose group after 3 d exposure, scale bar = 1 µm. (c) Showing the starch grains (black arrows) in the high dose group after 5 d exposure, scale bar = 0.5 µm. (d) and (e) Showing the numerous lysosomes (big arrows) and vacuoles (small arrows) in the low and high dose groups after 5 d exposure, scale bar = 1 µm and 0.5 µm, respectively.
when activated, might also constitute a form of adaptation to stress to ensure the survival of the cell.

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