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Original article

# Accumulation and Inhibitory Effects of Microcystin on the Growth of Rice and Broccoli

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**Abstract** Microcystins (MCs) produced by cyanobacteria are severe hepatotoxins for mammalian and protein phosphatase inhibitors. Irrigation water for grain and vegetables is often contaminated with cyanobacteria and microcystin during warm seasons. We assessed the effects of various concentrations (0, 0.01 to 10 μg $mL^{-1}$) of microcystin-LR (MC-LR) and microcystin-RR (MC-RR) exposure on *Oryza sativa* (rice) and *Brassica oleraces* var. *italica* (broccoli). The $EC_{50}$ of leaves and roots of rice was 0.9 and 1.1 μg MC-LR $mL^{-1}$, respectively. The no observed effect level (NOEL) of rice was less than 0.1 μg $mL^{-1}$ (100 μg $L^{-1}$). The $EC_{50}$ of the stems and roots of broccoli was 8.7 and 7.2 μg MC-RR $mL^{-1}$, respectively. There was no difference in the germination rate of broccoli among microcystin-RR concentrations. After exposure to 0, 0.01 to 10 μg $mL^{-1}$ MC-RR for seven days, 14, 89 and 154 ng $mg^{-1}$ (dry weight) MC-RR accumulated in *B. oleracea*. These $EC_{50}$ values showed that microcystin-LR and -RR affected the growth of rice and broccoli. These findings suggest that MC is carried into terrestrial ecosystems via irrigation, and that the biota of higher ecological niches can be influenced by MC through bioaccumulation. Therefore, a guideline for MC concentrations in irrigation water should be set using the NOEL.

**Key words:** microcystin, accumulation, *Oryza sativa*, *Brassica oleracea* var. *italica*

## INTRODUCTION

Severe blooms of cyanobacteria have been observed in systems that have undergone eutrophication during warm seasons. Francis (1878) reported the lethal toxicity of cyanobacteria (*Nodularia spumigena*) to animals. Since then, damage to animals such as a death of cattle that drank lake water contaminated with cyanobacteria (Galey *et al.,* 1987), a fatal accident involving more than 50 renal dialysis patients (Jochimsen *et al.,* 1998), damage to fish (Zimba *et al.,* 2001; Liu *et al.,* 2002; Xie *et al.,* 2004) and damage to zooplankton (Rohrlack *et al.,* 2001) have been confirmed worldwide, and the existence of various toxic species (*Microcystis*, *Anabaena*, *Planktothrix*, *Nodularia*, and *Aphanizomenon*) has been recognized (Watanabe *et al.,* 1994; Zurawell *et al.,* 2005). Since toxic cyanobacterial blooms causes health problems for humans and animals, it is important to control blooms of blue-green algae in water resources, including lakes.

In Japan, *Microcystis* is known as a major species that causes blooms of cyanobacteria (Watanabe *et al.,* 1994), and diverse effects of microcystins produced by *Microcystis* against various organisms have been reported (Casanova *et al.,* 1999). Microcystins are cyclic heptapeptides that contain β-amino acid Adda (3-amino-9-methoxy-2,8-trimethyl-10-phenyldeca-4,6-dienoic acid), *N*-methyl hydroalanine (Mdha), *D*-amino acid and two L-amino acids (Botes *et al.,* 1984). Microcystins are a group of over 60 structurally related monocyclic

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heptapeptide hepatotoxins (Sivonen and Jones, 1999), with toxicity that varies based on structural modifications (Rinehart *et al.,* 1994). The 50% lethal doses ($LD_{50}$, mouse toxicity assay) of microcystin-LR, RR and YR have been shown to be 43, 235 and 111 μg $kg^{-1}$, respectively (Gupta *et al.,* 2003). Microcystins are stable and not destroyed by 15 minutes of boiling. Therefore, it is possible to keep microcystins in freezers for over 20 years (Park, personal communication).

Microcystins are produced within the cells of cyanobacteria and usually exist in the cells. Microcystins detected in lakes are often actually the microcystins present in the cells of cyanobacteria. These compounds are generally reported as the amount of intracellular toxin per 1L of environmental water. Dissolved microcystins discharge to environmental water when cyanobacteria cells die or are preyed upon (Harada *et al.,* 1998). Dissolved microcystins are usually diluted in a large amount of environmental water or decomposed by bacteria. Therefore, microcystin concentrations are generally low in lake water (Park *et al.,* 1998; Maruyama *et al.,* 2003). In Lake Suwa, cyanobacteria blooms are observed every year, and the maximum concentration of dissolved microcystin is 3.6 μg $L^{-1}$ (Park *et al.,* 1998). Microcystins dissolve into the water when the cyanobacteria blooms decline and decay. For example, when cyanobacterial blooms are lysed by the addition of copper sulfate solution, the concentrations of dissolved microcystin in the water increase to 1,300-1,800 μg $L^{-1}$ (Jones and Orr, 1994), which is much higher than the guideline value of microcystin-LR for drinking water (1 μg $L^{-1}$; WHO, 1998); therefore, these organisms pose a great risk to human health.

Microcystins specifically act on animal livers (Watanabe *et al.,* 1994), inhibit protein photophatases 1 and 2A (PP1 and PP2A) (Mackintosh *et al.,* 1990; Yoshizawa *et al.,* 1990), and promote carcinogenesis (Nishiwaki-Matsushima *et al.,* 1991, 1992). Microcystins have also been reported to have genotoxic effects (Ding *et al.,* 1999). With regard to higher plants, the inhibition of PP (Mackintosh *et al.,* 1990; Rakwel *et al.,* 2001), the inhibition of growth (Kurki-Helasmo and Meriluto, 1998; McElhiney *et al.,* 2001; M-Hamvas *et al.,* 2002; Wiegand *et al.,* 2002; Gehringer *et al.,* 2003; Jianzhong *et al.,* 2004), the inhibition of photosynthesis (Abe *et al.,* 1996), the inhibition of germination (Casanova *et al.,* 1999), the allelopathic effect (Smith and Doan, 1999; Pflugmacher, 2002), the inhibition of carbon oxide fixation, the inhibition of starch storage, the inhibition of sucrose synthesis (McElhiney *et al.,* 2001) and cell apoptosis (Liyan *et al.,* 2006) have been reported. However, there are fewer reports (Table 2) regarding the effects of microcystins on higher plants than on animals.

**Table 1.** Accumulation of microcystin-RR in broccoli (*Brassica oleracea* var. *italica*) after 14 days of exposed to various concentrations (ng $mg^{-1}$).

| Organism | Exposure concentration (μg $mL^{-1}$) | | | | |
|---|---|---|---|---|---|
| | 0.1 | 0.5 | 1 | 5 | 10 |
| Broccoli (*Brassica oleracea* var. *italica*) | N.D. | 14.0 | 14.5 | 88.9 | 154 |

N.D.: Not Detected

Several studies have been conducted to investigate the bioaccumulation of microcystins through the food chain, and microcystins are found in aquatic organisms (Park *et al.,* 2001; Zurawell *et al.,* 2005). The n-octanol-water partition coefficient of microcystins is about 3 (de Maaged *et al.,* 1999), which is not as high as that of DDT or PCB. Therefore, the accumulation route of microcystins is considered to be absorption via the digestive system. The accumulation of microcystins in aquatic organism has been confirmed in laboratory feeding experiments (Vasconcelos, 1995; Williams *et al.,* 1997; Lirås *et al.,* 1998; Amorim and Vasconcelos, 1999; Vasconcelos *et al.,* 2001; Magalhães *et al.,* 2003; Ozawa *et al.*, 2003; Yokoyama and Park, 2003). Moreover, the accumulation of microcystins in beef cattle has been reported (Orr *et al.,* 2003). It has also been reported that microcystins accumulate in plants, at the bottom of the food chain, as well as in animals (Pflugmacher *et al.,* 1999, 2001). Additionally, microcystins have been found to have effects on metabolism, such as growth and physiologic regulation (Casanova *et al.,* 1999).

Natural lakes are often used as water resources; therefore, it is highly likely that lake water contaminated with blooms of cyanobacteria is utilized for drinking and agricultural purposes, which could lead to microcystin exposure. In this study, we exposed two terrestrial plants, *Oryza sativa* and *Brassica oleraces* var. *italica*, to microcystin-LR, the most toxic microcystin, and to microcystin-RR, which has been observed most often in Asian countries, but studied less intensively than LR.

The purposes of this study were to assess the influence of microcystin-LR on the growth of *Oryza sativa* and the influence of microcystin-RR on *Brassica oleraces* var. *italic* based on growth inhibition. Addition-

**Table 2.** Toxicity of microcystins against various higher plants.

| Organism | Common name | Microcystin | $IC_{50}$ *$EC_{50}$ (mg $L^{-1}$) | Effect level (mg $L^{-1}$) | Time | Response | References |
|---|---|---|---|---|---|---|---|
| *Sinapis alba* L. | Mustard | RR | 0.8 | | 7 days | Growth inhibition | Kurki-Helasmo and Meriluoto, 1998 |
| do. | | RR | 1.6* | | 7 days | Growth inhibition | McElhiney *et al.*, 2001 |
| do. | | LR | 1.9* | | 7 days | Growth inhibition | do. |
| do. | | LF | 7.7* | | 7 days | Growth inhibition | do. |
| *Solanum tuberosum* | Potato | LR | | 0.005 | 16 days | Weight decrease | do. |
| do. | | LR | | 0.5 | 3 weeks | Necrosis | do. |
| *Phaseolus vulgaris* | Podded peas | LR | | 1 | 18 days | Growth inhibition | do. |
| *Lemna minor* L. | Duckweed | RR | > 5 | | 96 hours | Growth inhibition | Weiβ *et al.*, 2000 |
| do. | Duckweed | LR | | 10 | 5 days | Growth inhibition | Mitrovic *et al.*, 2005 |
| *Lemna gibba*. | Duckweed | RR, WR | | 0.15 | 2 days | Growth inhibition | Saqrane *et al.*, 2007 |
| *Spirodela oligorrhiza* | Waterweed | Crude | 0.45 | | 4 days | Growth inhibition | Romanowska-Duda and Tarczyńska, 2002 |
| do. | | Crude | 0.2 | | 7 days | Growth inhibition | do. |
| do. | | LR | 0.3 | | 4 days | Growth inhibition | do. |
| *Ceratophyllum demersum* | Waterweed | LR | | 1 | 6 weeks | Growth inhibition | Pflugmacher, 2002 |
| *Vesicularia dubyana* | Aquatic moss | LR | | 0.05 | 1 day | Photosynthetic oxygen production decrease | Wiegand *et al.*, 2002 |
| *Lepidium sativum* | Watercress | Crude | | 1 | 2 days | Growth inhibition | Gehringer *et al.*, 2003 |
| *Brassica napus* L. | Rape | LR, YR, RR | | 0.12 | 10 days | Growth inhibition | Mathe *et al.*, 2007 |
| *Brassica oleracea* | Cabbage | RR | | 0.1 | 6 days | Growth inhibition | Liu *et al.*, 2008 |
| *Phragmites australis* | Reed | LR | 12 | | 22 days | Growth inhibition | Jianzhong *et al.*, 2004 |
| *Spinacia oleracea* | Spinacia | Crude LR | | 0.0005 | 6 weeks | Photosynthetic oxygen production decrease | Pflugmacher, 2007 |
| *Oryza sativa* L. | Rice | LR | | 0.6 | 10 days | Growth inhibition | Jianzhong *et al.*, 2004 |
| *Oryza sativa* L. | Rice | LR | 3.8* | | 1 week | Shoot extend inhibition | This study |
| do. | | LR | 1.1* | | 1 week | Root extend inhibition | do. |
| do. | | LR | 0.9* | | 2 weeks | Shoot extend inhibition | do. |
| do. | | LR | 1.6* | | 2 weeks | Root extend inhibition | do. |
| *Brassica oleracea* var. *italica* | Broccoli | RR | 8.72* | | 7 days | Shoot extend inhibition | do. |
| do. | | RR | 7.18* | | 7 days | Root extend inhibition | do. |

$IC_{50}$: Half maximal (50%) Inhibitory concentration
$EC_{50}$: Half maximal (50%) effective concentration

ally, the quantities of accumulated microcystin-RR in broccoli were determined.

## MATERIALS AND METHODS

### 1. Materials

Rice for the experiment (*Oryza sativa*) was purchased from JA (Farmers Cooperative of Japan, Asama Onsen, Matsumoto, Japan). As pretreatments, we screened rice with a specific gravity of saltwater of 1.14 (2 kg salt per 10 L water), and sterilized the seeds by hot-water treatment, which is usually used for organic cultivation. Hot water treatment was conducted at 60˚C for 20 minutes, after which the seeds were soaked at 10˚C for ten days (accumulated temperature, 100˚C) for germination. Germination was stimulated at 30˚C so that the length of the shoots would be similar. Plastic vessels (PET, diameter 100 mm × height 44 mm, volume 200 mL) were used as incubation containers, and the plants were incubated in a Biotron LH200-RDS incubator (Osaka, Japan).

The seeds of broccoli (*Brassica oleracea* var. *italica*, Tohoku 06273PK) were used for the exposure experiment. As pretreatments, the seeds were sterilized by hot-water treatment at 40˚C for 2-3 hours. Glass vessels (diameter 100 mm × height 44 mm, volume 200 mL) were used as incubation containers, which were placed in an incubation room.

### 2. Purification of microcystins for growth inhibition experiments

Blue-green algae samples were collected from Chikato Pond (Matsumoto, Japan), freeze-dried, and the microcystins were then purified. Specifically, distilled water (50 mL) was added to the freeze-dried samples, placed in hot water and then heated. The samples were then centrifuged (LCO6-SP, TOMY, Tokyo, Japan) at 1,400 g for 20 minutes, after which the supernatant was extracted. The residues in the centrifuge tubes were subsequently stirred and the process from centrifugation to supernatant extraction was repeated two more times. Next, the supernatant was cleaned up by solid phase extraction using an ODS cartridge (octadecylsilane, 5.0 g). Subsequently, the inside of the cartridge was washed with distilled water (50 mL) and 20% methanol (100 mL). Finally, the microcystins were eluted with 90% methanol (100 mL). After removal of the solvent by rotary evaporator (REN-1VN, Iwaki, Tokyo, Japan), the microcystins were dissolved in 100% methanol (5 mL). The extract was then added to a silica-gel cartridge (2.0 g) that had been pretreated with 100% methanol (10 mL). Finally, the cartridge was washed with methanol (15 mL), and the microcystins were eluted with 70% methanol (20 mL).

The purified microcystin fractions were injected into an HPLC (ChromNAV, Jasco, Tokyo, Japan) using a syringe for qualitative analysis of microcystin-LR and RR. The multi-wavelength detector (MD-2015Plus, Jasco, Tokyo, Japan) was set to 238 nm, and the intelligent column oven (Jasco, Tokyo, Japan) was set to 40˚C. A reverse phase ODS column (Cosmosil 5C18-AR IL, 20 × 250 mm, Nacalai, Japan) was used for microcystin isolation. For preparative isolation, a mixed solution of 0.05 M phosphate buffer (pH=3) and aqueous methanol (100% for HPLC)=42 : 58 was used for the mobile phase, and the flow-rate was 12 mL $min^{-1}$. Qualitative analysis of microcystin-LR and RR was conducted by comparison of the retention times and UV spectrum analysis when necessary. Quantitative analysis of microcystin-LR and RR was conducted based on the peak area output from the detector and the recorder (LC-Net II/ADC, Jasco, Tokyo, Japan). The amount of microcystin-LR and RR in the fractions was determined using calibration curves obtained from the peak area and the amount of microcystins in standard samples (Wako Ltd., Japan). Finally, the solvent in the microcystins was removed using a rotary evaporator (REN-1VN, IWAKI, Tokyo, Japan), and then re-dissolved in dimethylsulfoxide (DMSO) for the growth inhibition experiments.

### 3. Exposure of *Oryza sativa* to microcystin-LR

Dechlorinated water (50 mL) containing microcystin-LR of known concentration and five grains of rice was placed into plastic vessels, which were then placed in an incubator (BIOTRON LH200-RDS, Osaka, Japan). The temperature was set to 25.0 ± 0.5˚C and the light-dark cycle was 16 h/8 h.

The rice was exposed to microcystin-LR at concentrations of 0 (control), 0.01, 0.1, 0.5, 1, 5 and 10 μg $mL^{-1}$ for 14 days so that germination of the rice would be stimulated and the rice could be grown to a 1.5 true leaf. The maximum concentration of ethanol was less than 1%. The experiment was repeated three times. After exposure, the lengths of the true leaves and roots were measured using a micrometer caliper (CD-15, Mitutoyo

Co., Japan), and the results of each concentration were compared (Fig. 1).

### 4. Exposure of *Brassica oleracea* var. *Italia* to microcystin-RR

Cellulose papers were placed inside glass vessels, after which dechlorinated water containing microcystin-RR of known concentration was added and 20 broccoli seeds were placed on the papers. The broccoli was then exposed to microcystin-RR in the incubation room at 22.0 ± 1.0°C under a light-dark cycle of 16 h/8 h. To avoid inhibition of germination by light, from day 0 (the start date of the experiment) until day 3, the experiments were conducted under dark conditions.

Broccoli was exposed to 0 (control), 0.1, 0.5, 1, 5 and 10 μg of microcystin-RR $mL^{-1}$ for 14 days so that the germination of broccoli would be stimulated and it could be grown to a 1.5 true leaf. The maximum DMSO concentration was less than 1% and the experiment was repeated three times. After exposure, the lengths of the true leaves and roots were measured using a micrometer caliper. The plant samples were freeze-dried using a freeze dryer (FDU-2100, Japan) and the dry weight was measured using an automatic balance (Sartrius 1712MPS, Germany). The plant samples were stored in 100% methanol (3 mL) for 24 hours under cool and dark conditions for quantitative analysis of the chlorophyll-*a*. Next, the samples were centrifuged in using an LCO6-SP centrifuge at 1,400 g for 20 minutes, after which the supernatant was extracted and its absorbance was measured (238 nm) using an absorption spectrometer (V-530, Jasco, Tokyo, Japan). The amount of chlorophyll-*a* (μg $mg^{-1}$ dry weight) was calculated using the UNESCO method (1966).

### 5. Analysis of microcystin-RR in *Brassica oleracea* var. *Italia*

Freeze-dried broccoli samples were ground to powder using a mortar and pestle. Microcystin-RR in the freeze-dried broccoli samples was then extracted using the acetic acid extraction method. Briefly, the sample and 5% acetic acid (10 mL) were placed into centrifuge tubes together and then stirred for 30 minutes using a stirrer and a stir bar. The samples were then centrifuged at 1400 g for 20 minutes, after which the supernatant was collected. This process was repeated two more times, after which the supernatant was cleaned by solid phase extraction. Next, the supernatant was loaded into an

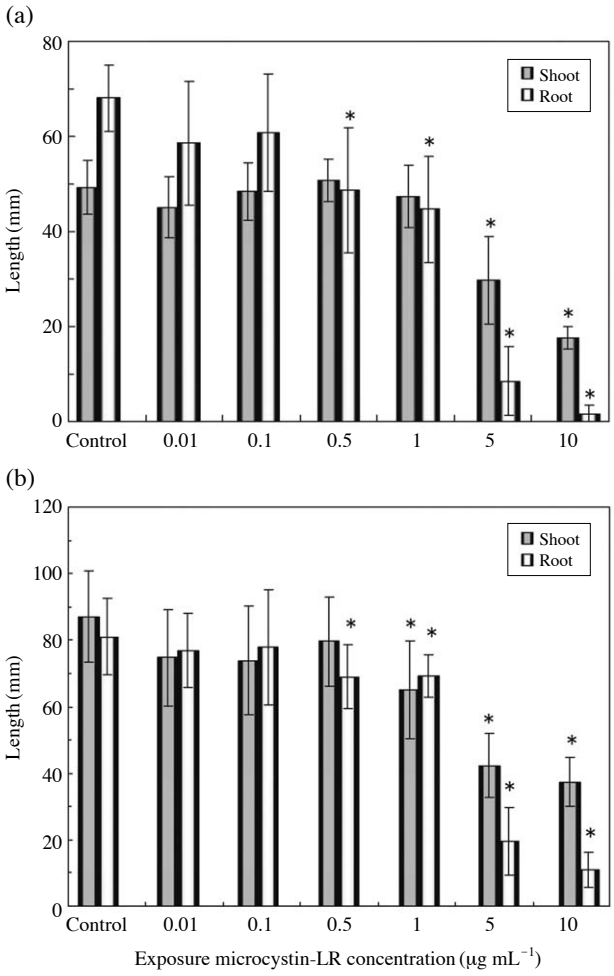


**Fig. 1.** Inhibitory effects of microcystin-LR on the growth of rice (*Oryza sativa*) shoots and seminal roots exposed to various concentrations. (a) Effects of microcystins on the lengths (during 7 days of exposure), (b) Effects of microcystins on the lengths (during 14 days of exposure). Each bar represents the average of 10 plants. Error bars indicate the standard deviation. Asterisks indicate significant differences from control plants ($P < 0.01$, one way ANOVA, Fisher's PLSD).

HLB cartridge (500 mg, Milford, USA) that had been pretreated with 100% methanol (6 mL) and distilled water (6 mL) to absorb the microcystins. Subsequently, the inside of the cartridge was washed with 5% methanol (15 mL), after which the microcystin-RR was eluted using 100% methanol (20 mL).

After condensation of the solvent using a rotary evaporator, the extract was added to a Silica-gel cartridge (2.0 g) that had been pretreated with 100% methanol (10 mL). Methanol (15 mL) was then added to the cartridge, after which the microcystins were eluted with

70% methanol (20 mL). After removal of the solvent by evaporation, the microcystin-RR was dissolved in 100% methanol.

The cleaned up microcystin fractions (5 μL) were injected into an HPLC (ChromNAV, Jasco, Tokyo, Japan) using a syringe for qualitative and quantitative analysis of microcystin-RR.

### 6. Statistics

Significant differences in rice (true leaves and seminal roots) were identified by one-way ANOVA (analysis of variance), which was conducted using Excel 2000 (SSRI, social information service). Results were considered significant at a $P<0.01$. The individual means of each treatment were compared using Fisher's Protected Least Significant Difference when necessary.

Differences among groups of broccoli (the lengths of true leaves, shoots and roots, dry weight, and chlorophyll-*a*) were determined by one-way ANOVA. Results were considered significant at $P<0.05$.

The probit method (EcoTox-Statistics Ver. 2.5, The Society of Environmental Toxicology of Japan) was used for calculation of the 50% effective concentration ($EC_{50}$).

## RESULTS

### 1. Effects of microcystin-LR exposure on *Oryza stiva*

The lengths of true leaves and seminal roots of rice (*Oryza sativa*) decreased with increasing exposure concentrations after seven days (Fig. 1). The lengths of true leaves and seminal roots of the rice did not differ significantly between the 5 μg $mL^{-1}$ and 0.5 μg $mL^{-1}$ treatments. Additionally, the no observed effect level (NOEL) of the rice was less than 0.1 μg $mL^{-1}$ (100 μg $L^{-1}$). The number of secondary roots in plants exposed to microcystin-RR was lower than in the controls, and some true leaves exposed to toxin levels of 10 μg $mL^{-1}$ showed significantly lower chlorophyll-*a* content (necrosis) and became decolorized when compared to those in other groups. Many seminal roots snapped in plants treated with more than 1 μg $mL^{-1}$ of toxin. The snapped seminal roots were thinner and their root densities were lower when compared to the control. The $EC_{50}$ values of true leaves and seminal roots were 3.8 μg $mL^{-1}$ and 1.1 μg $mL^{-1}$, respectively.

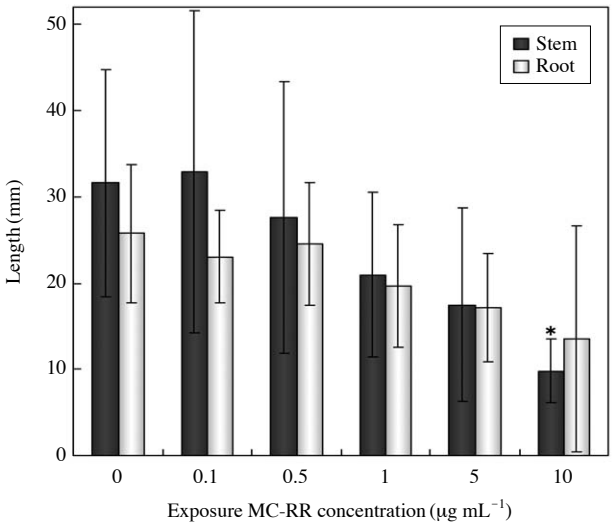


**Fig. 2.** Inhibitory effects of microcystin-RR on the growth of broccoli (*Brassica oleracea* var. *italic* stems and roots) exposed to various concentrations. Average lengths, MC-RR exposure concentrations (μg $mL^{-1}$). Asterisks indicate significant differences from control plants ($P<0.05$, one way ANOVA, Fisher's PLSD). Error bars indicate the standard deviation.

After a 14-day exposure to microcystin-LR, the lengths of the true leaves and seminal roots were longer than after 7-days of exposure, but the growth of the true leaves and seminal roots was more inhibited as the concentrations of microcystins increased (Fig. 1). Significant differences in the lengths of true leaves and seminal roots were observed in response to treatment with concentrations of more than 1 μg $mL^{-1}$ and 0.5 μg $mL^{-1}$, respectively. The photosynthetic pigment of the bleached true leaves (the 7-day exposure to microcystin-LR) recovered. The treated groups had smaller numbers of secondary roots than the control, and the number of their roots did not increase. Some seminal roots snapped in the treatment area, as observed in the 7-day exposure experiment. The $EC_{50}$ of the true leaves and seminal roots was 0.9 μg $mL^{-1}$ and 1.6 μg $mL^{-1}$, respectively (Fig. 1).

### 2. Effects of microcystin-RR exposure on *Brassica oleracea* var. *italica*

The results of the 7-day exposure experiment showed that the lengths of stems/petioles and seminal roots of broccoli decreased as exposure concentrations of microcystin-RR increased when compared with the control (Fig. 1). Specifically, the results indicated that the growth

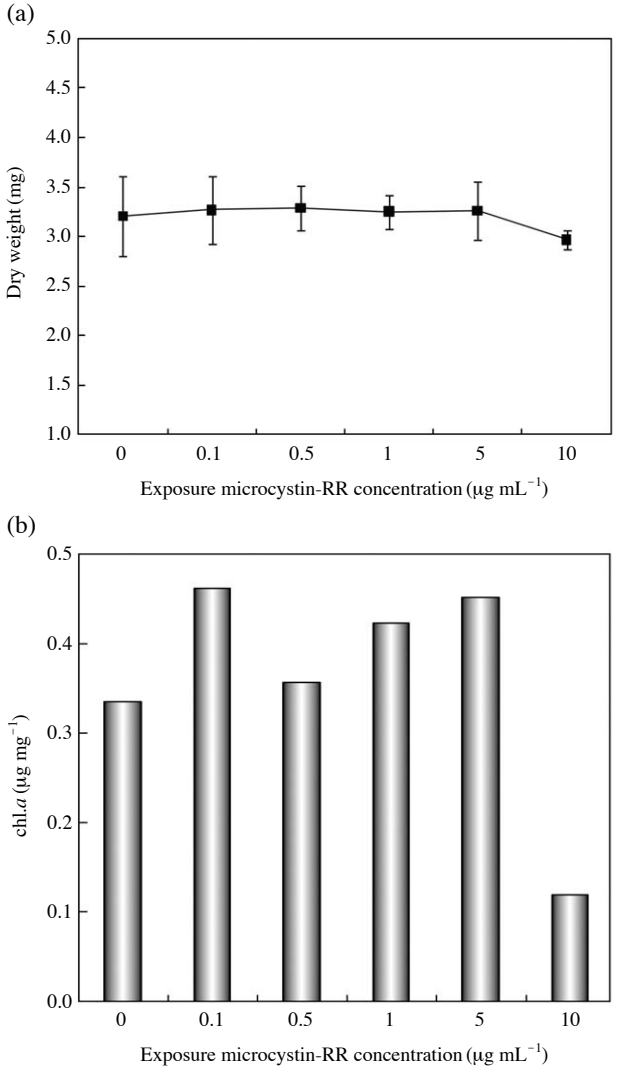


**Fig. 3.** Effects of microcystin-RR on Chlorophyll-*a* content (μg $mg^{-1}$ dry weight) in broccoli (*Brassica oleracea* var. *italica*) after 14 days of exposure to various concentrations. Error bars indicate the standard deviation. There were no significant differences observed ($P > 0.05$, one way ANOVA, Fisher's PLSD).

of broccoli was inhibited by exposure to microcystin-RR. The lengths of the stems and petioles differed significantly between plants treated with 10 μg $mL^{-1}$ and the controls (Fig. 2). However, the length of seminal roots did not differ significantly among groups. When compared with the length of seminal roots, stems and petioles showed different growth rates and were more heavily influenced by microcystin-RR. The $EC_{50}$ of the stems and petioles was 8.7 μg $mL^{-1}$, while that of the seminal roots was 7.2 μg $mL^{-1}$. In proportion to the test duration, difference in growth rate between the control and the high concentration exposure area (5, 10 μg $mL^{-1}$) became bigger. Additionally, the dry weight of the plants decreased somewhat as the concentration of microcystin-RR increased, but no significant difference was observed among groups (data not shown). There was also no difference in the germination rate of broccoli among microcystin-RR concentrations. Furthermore, there was no difference in chlorophyll-*a* in plants exposed to 5 μg $mL^{-1}$ of toxin or lower, but the chlorophyll levels greatly decreased in plants exposed to 10 μg $mL^{-1}$ (Fig. 3). In plants exposed to microcystin-RR at 1 μg $mL^{-1}$ or higher, the leaves of broccoli were bleached (chlorosis), and this effect increased with exposure concentrations; however, the pigment was not recovered (data not shown). Furthermore, necrosis was observed in response to exposure to 10 μg $mL^{-1}$ of microcystin, and the leaves and seminal roots became thin and black.

### 3. Accumulation of microcystin-RR in broccoli

Analysis of microcystin-RR in broccoli (dry weight) indicated that the toxin was not present in plants exposed to 0.1 μg $mL^{-1}$ or less. However, microcystin-RR was detected in plants exposed to 0.5 to 10 μg $mL^{-1}$ microcystin-RR. In plants exposed to 1, 5 and 10 μg $mL^{-1}$ of the toxin, 14.5, 88.9 and 145 ng $mg^{-1}$ of microcystin-RR were accumulated, respectively, indicating that the accumulation was dose dependent (Table 1).

## DISCUSSION

### 1. Toxic effects of microcystin-LR

The results of this study confirmed that exposure to microcystin-LR and -RR affected the growth of rice and pigmentation of broccoli. The findings presented here also revealed that microcystin-RR accumulated in exposed broccoli.

Gehringer *et al.* (2003) suggested the bioassay of *Lepidium sativum* for microcystin-LR toxicity tests as a simple, inexpensive and sensitive evaluation method. Since bioassay tests using plants including *Lepidium sativum* are easy to conduct, it would be ideal to use these tests to evaluate the influence of microcystins before conducting chemical analyses such as HPLC.

Table 2 shows the effects of microcystin concentrations in higher plants. The $EC_{50}$ of rice shoots after the two weeks of exposure to microcystin-LR was found to

be 0.9 μg $mL^{-1}$ in the present study, which was similar to the results of a study conducted Jianzhong *et al.* (2004), who reported an $EC_{50}$ of 0.6 μg $mL^{-1}$. The $EC_{50}$ obtained for other higher plants such as mustard (*Sinapis alba* L.) were also similar (0.8 μg $mL^{-1}$). The $EC_{50}$ of the stems of broccoli and petioles exposed to microcystin-RR was 8.7 μg $mL^{-1}$, which was similar to the results ($>5$ μg $mL^{-1}$) obtained by Weiβ *et al.* (2000).

The results of microcystin-RR exposure experiments of broccoli revealed that the length of stems and petioles varied in response to different exposure concentrations. The length of seminal roots also varied in response to different exposure concentrations, and growth inhibition was confirmed in response to exposure to more than 1 μg $mL^{-1}$ of microcystin-RR. Irrigation using water that had blooms of cyanobacteria for ten days was found to lead to the adhesion and residue of *M. aeruginosa* and microcystin-LR to lettuce (Codd *et al.,* 1999). It is highly likely that the surface of plants and grains grown in contaminated irrigation water could be exposed to high concentrations of cyanobacteria and its toxins, because of cell breakdown of residual cyanobacteria and evaporation of water. Since the broccoli used for this research was grown in a greenhouse, it may also have been affected by cyanobacteria. Furthermore, the results of this study suggest that irrigation water containing microcystin-RR can influence the productivity of grains.

A bioassay experiment ($LD_{50}$) of rats showed that the toxicity of microcystin-LR was 12 times higher than that of microcystin-RR (Botes *et al.,* 1982; Kusumi *et al.,* 1987). However, there was no significant difference in the toxic effects of microcystin-RR and LR on plants. Other studies have shown that the toxic effects of microcystin-RR are more severe than those of LR (McElhiney *et al.,* 2001; Mitrovic *et al.,* 2005). Microcystin-RR is more hydrophilic than microcystin-LR, which is considered to lead to the difference in toxicity between microcystin-RR and LR. However, few studies have been conducted to compare the toxicities of microcystin-RR and LR toward plants. Since microcystin-RR is the most common toxin produced by cyanobacteria in Asia, further research regarding the toxicity of microcystin-RR is necessary.

The effects of microcystin on chlorophyll in plant cells indicated that the amounts of chlorophyll in potato shoots decreased as the microcystin concentrations increased (McElhiney *et al.,* 2001). The amounts of chlorophyll in broccoli showed the greatest decrease in response to microcystin-RR exposure at 10 μg $mL^{-1}$. The level of chlorophyll in potato shoots was found to depend on the concentration of microcystin-LR; however, the level of chlorophyll in broccoli was only affected by the highest concentration of microcystin-RR exposure. These findings indicate that the effects of microcystin-RR would be smaller than those of microcystin-LR toward photosynthesis pigments such as chlorophyll in plants.

It has been reported that the chlorophyll-*b* content in plants exceeded the chlorophyll-*a* content in connection with the increase of exposure concentrations of microcystins (Pflugmacher, 2002; Wiegand *et al.,* 2002). A lack of chlorophyll was confirmed when microcystins were found to be present in high concentrations (Kurki-Helasmo and Mariluoto, 1998). A similar phenomenon occurred in rice exposed to microcystin-LR. Subsequent recovery of the pigment likely occurred due to the detoxification process (Pflugmacher, 2002). Pflugmacher (2002) reported the recovery of the amounts of photosynthetic oxygen evolved, and also the detoxifying processes of microcystin in aquatic macrophytes. In their study, *Ceratophyllum demersum* was exposed to microcystins for 24 hours, and the photosynthetic oxygen evolution was recovered after five days. They suggested that this recovery of photosynthetic oxygen-evolving production was caused by 1) detoxification through GST (glutathione S-transferases) and 2) promotion of protein transformation by fixation of D1 protein of photosynthetic organs and MC-LR. In our study, recovery of the pigment was observed after exposure to 10 μg $mL^{-1}$ of toxin for seven days following chlorosis, which was similar to the results of previous studies (Pflugmacher, 2002). However, recovery of pigment was not observed following the exposure of broccoli to microcystin-RR. These findings were likely due to the short duration of the experiment.

Jianzhong *et al.* (2004) reported that the germination rate of *Oryza sativa* L. was not affected by the microcystin concentrations. In this study, the germination rate of broccoli was the same as rice (*Oryza sativa* L), which confirms their findings. The decline in the number of crown roots of rice was affected by exposure to microcystin-LR, but five stems of rice did not appear in the control group (stems) as well. Therefore, it is difficult to determine if the decline of crown roots was due to microcystin exposure. However, the snapped seminal roots were thinner and had lower density when compared to those of the control group. These findings indi-

**Table 3.** Bioaccumulation of microcystins exposed various concentrations in aquatic and terrestrial plants (ng $mg^{-1}$ fresh weight).

| Organism | Microcystin | Exposure concentration (μg $mL^{-1}$) | | | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.02 | 0.1 | 0.5 | 0.6 | 1 | 2.5 | 3 | 5 | 6 | 10 | 20 | |
| Aquatic plant | | | | | | | | | | | | | |
| Duckweed (*Lemna minor*) | MC-LR | | | | | | | 0.046 | | | 0.107 | 0.288 | Mitrovic *et al.* (2005) |
| Cladophorales (*Chladophora fracta*) | MC-LR | | | | | | | 0.041 | | 0.042 | 0.041 | | do. |
| Tape grass leaf (*Vallisneria natans*) | MC-RR | | | | | | | | | | *ca.* 0.4 | | Liyan *et al.* (2005) |
| Tape grass root (*Vallisneria natans*) | MC-RR | | | | | | | | | | *ca.* 1.4 | | do. |
| Terrestrial plant | | | | | | | | | | | | | |
| Mustard stem (*Sinapis alba* L.) | MC-LR | | | | | | *ca.* 35 | | *ca.* 60 | | | | Kurki-Helasmo *et al.* (1998) |
| Mustard leaf (*Sinapis alba* L.) | MC-LR | | | | | | *ca.* 80 | | *ca.* 80 | | | | do. |
| Rice (*Oryza sativa* L.) | MC-LR | N.D. | 0.002 | | 0.005 | | | 0.005 | | | | | Jianzhong *et al.* (2004) |
| Rape (*Brassica napus* L.) | MC-LR | 0.002 | 0.008 | | 0.12 | | | 0.651 | | | | | do. |
| Broccoli (*Brassica oleracea* var. *italica*) | MC-RR | | N.D. | 14.0* | | 14.5* | | | 88.9* | | 154* | | This study |

N.D.: Not detected
*ng $mg^{-1}$ dry weight

cate that growth inhibition due to microcystin-LR affected both the length and thickness of rice roots.

## 2. Bioaccumulation

The intake of microcystin-RR into broccoli was verified by quantitative analysis of microcystin-RR in broccoli using HPLC. The bioaccumulation of microcystins in aquatic and terrestrial plants in response to exposure to various concentrations of these toxins is shown in Table 3. Following exposure to 0.5 μg $mL^{-1}$ exposure, the level of accumulated microcystin-LR was below the detection limit or very low. However, exposure to microcystin-RR and -LR at levels of 1 μg $mL^{-1}$ and above resulted in their accumulation at levels proportional to their exposure concentrations. It is conceivable that the accumulation observed in response to exposure to 5 and 10 μg $mL^{-1}$ microcystin was dependent on concentration, but that the accumulation in response to exposure to 0.5 and 1 μg $mL^{-1}$ did not. Kurki-Helasmo *et al.* (1998) reported that the amount of microcystin-LR accumulated in the leaves of mustard (*Sinapis alba* L.) after 7 days of exposure was about 80 μg $mg^{-1}$, which was similar to the results observed in the present study. Based on these results, it is not possible to determine if the measured microcystin-RR was absorbed onto the epidermal cells of broccoli or absorbed into broccoli. However, it is possible that microcystin-RR absorbed to epidermal cells of broccoli following exposure to less than 1 μg $mL^{-1}$ without concentration dependence, and that it was absorbed in broccoli exposed to 5-10 μg $mL^{-1}$ in a concentration dependent manner. The amount of microcystins found in terrestrial plants was greater than that observed in aquatic plants (Table 3). It is reasonable to assume that defense mechanisms and metabolic systems of aquatic plants against microcystins have developed since they inhabit an environment that is susceptible to cyanobacteria. In contrast, terrestrial plants such as broccoli are less likely to have evolved defenses to cyanobacteria; therefore, they are not resistant to microcystin-RR, leading to greater accumulation of these compounds. Plants are primary producers and humans regularly eat vegetables such as broccoli. Accordingly, microcystins can be brought into the food web of the terrestrial ecosystem, after which they can affect organisms in higher ecological niches through bioaccumulation. McElhiney *et al.* (2001) also suggested that when microcystins accumulate in the cells of vegetables or grains and humans eat them, this ex-

posure route can have greater effects on human bodies than the exposure route of drinking water, which has a guideline of microcystin-LR concentrations set to 1 μg $mL^{-1}$ (WHO, 1988). Therefore, a guideline for microcystin concentrations in irrigation water should be set as soon as possible using the NOEL. Further studies investigating the mechanism of absorption, accumulation and metabolism of microcystins by vegetables and grains are also warranted.

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