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Freshwater toxic cyanobacteria induced DNA damage in apple (*Malus pumila*), rape (*Brassica napus*) and rice (*Oryza sativa*)

J.Z. Chen [a,b,*], J.Y. Ye [a], H.Y. Zhang [a], X.J. Jiang [a], Y.X. Zhang [a], Z.L. Liu [b]

[a] School of Life Science, Huzhou University, Huzhou 313000, PR China
[b] School of Life Science, Nanjing University, Nanjing 210093, PR China

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ABSTRACT

Cyanobacteria in freshwater ecosystems can present a harmful effect on growth and development of plants through irrigation with contaminated water. In this study, the effects of microcystins (MCs)-containing cyanobacteria extract (CE) on DNA damage of apple, rape and rice were investigated to explore the phytotoxic mechanism of MCs through DNA fragmentation and RAPD analysis. Determination of DNA fragmentation by fluorescent dye DAPI showed that significant DNA damage was observed in rice seedlings after exposure to CE while DNA fragmentation in rape seedlings and apple cultures did not differ significantly between treatment and control groups. Qualitative characterization of genomic DNA fragmentation by agarose gel electrophoresis supported the quantitative determination using DAPI. The main changes in RAPD profiles of rape seedlings following exposure of lower doses of CE were variation in band intensity for the primers F03 and S01, while higher doses of CE caused loss of normal bands and appearance of new bands except band intensity changes. The data presented here demonstrate that DNA damage in plants occurs following exposure of microcystins, and the polymorphic RAPDs may be used as an investigation tool for environmental toxicology and as a useful biomarker for the detection of genotoxic effects of microcystins on plants.

1. Introduction

Toxic cyanobacteria in freshwater ecosystems are of increasing health and environmental concern throughout the world as a result of their ability to produce hepatotoxic heptapeptides, microcystins (MCs) [1]. These toxic compounds have been implicated in wildlife, livestock, and pet fatalities as well as human poisonings worldwide [2–5]. Microcystins exert toxicity by inhibition of the protein phosphatases 1 and 2A that is related to a complete collapse of the liver function with subsequent death or tumor-promoting activity at subacute levels [6,7].

Recent evidence suggests that oxidative stress also plays a role in the pathogenesis of microcystin toxicity in animals and humans [8–10]. MC-LR can induce the generation and accumulation of reactive oxygen species (ROS) in murine liver after exposure [10]. ROS are capable of damaging DNA and other biomolecules; increased formation of ROS can promote the development of malignancy, and increased rates of ROS generation may account for the increased risk of cancer development [9]. In the last few years, DNA damage induced by MCs in animals and humans was well documented [11–15]. Rao et al. [11] demonstrated that both cell-free extract of *Microcystis aeruginosa* and purified toxin microcystin-LR (MC-LR) induced significant DNA fragmentation in mouse cells.

The phytotoxic effects of MCs on both terrestrial and aquatic plants have been investigated and most of the investigations demonstrated that exposure to MCs presented a harmful effect on growth and development of plants [16–20]. However, the toxic mechanism of MCs in plants is less known and, to our knowledge, there is no report on whether MCs cause DNA damage of plants.

Dianchi Lake is situated in Yunnan province of China. In the last 20 years, the occurrence of toxic freshwater blooms of cyanobacteria has been frequently reported in this lake. We have isolated the bloom-forming cyanobacteria *M. aeruginosa* from the eutrophic lake of Dianchi, and by mouse bioassay and HPLC it has been confirmed that the cyanobacteria can produce MCs. Intraperitoneal injection of the bloom cyanobacteria extract (CE) to test mice caused death of the animals with a median LD_{50} of 83 mg/kg body weight, and the mice exposed to the CE showed almost the same symptoms as the mice exposed to standard MC-LR. Reversed-phase HPLC analysis showed that the CE had at least three microcystin variants: microcystin-RR, -LR and -YR, in the proportion of 54.9%, 31.0% and 2.7%, respectively. By ELISA the total microcystins level of the toxic bloom sample was 0.4 μg of microcystin-LR equivalents per mg dry weight of bloom [21]. By using the crude aqueous extract of the toxic bloom, we found that MCs inhibited the growth

* Corresponding author at: School of Life Science, Huzhou University, Huzhou 313000, PR China. Tel.: +86 572 2322003; fax: +86 572 2321166.
E-mail addresses: jzchen@hutc.zj.cn, jzchen@sina.com (J.Z. Chen).

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Table 1
RAPD primers used in this study.

Primer	Nucleotide sequence (5′–3′)	Primer	Nucleotide sequence (5′–3′)
A-01	CAGGCCCTTC	E-20	AACGGTGACC
A-02	TGCCGAGCTG	F-03	CCTGATCACC
B-01	GTTTCGCTCC	F-04	GGTGATCAGG
B-02	TGATCCCTGG	F-05	CCGAATTCCC
C-02	GTGAGGCGTC	G-02	GGCACTGAGG
C-04	CCGCATCTAC	I-04	CCGCCTAGTC
D-02	GGACCCAACC	S-01	CTACTGCGCT
E-17	CTACTGCCGT	S-03	CAGAGGTCCC
E-18	GGACTGCAGA	S-04	CACCCCCTTG
E-19	ACGGCGTATG	S-05	TTTGGGGCCT

and development of rice (*Oryza sativa*) and rape (*Brassica napus*) seedlings; the growth and proliferation of *Malus pumila* shoots *in vitro* was also inhibited. Furthermore, oxidative stress may contribute to the deleterious effects in exposed rice, rape and apple [19,20].

In this paper, we expanded our previous study [19,20] and investigated the effect of aqueous MCs-containing extract of toxic cyanobacteria from Dianchi Lake on DNA damage of apple, rape and rice through determination of DNA fragmentation by DAPI and random amplified polymorphic DNA (RAPD) analysis. The aim of the present study was to explore the genotoxicity of MCs in plants and to explain the deleterious effects of MCs on plants.

2. Materials and methods

2.1. Materials

In vitro propagated apple shoots (*Malus pumila*, 'Chan Fu II' variety) were provided by our laboratory at Nanjing University, and maintained on the media described below until required for assays. Rice seeds (*O. sativa*, 'Liangyoupeijiu' variety) and rape seeds (*B. napus*, 'Suyou No. 1′ variety) used in this study were kindly provided by Jiangsu Tomorrow seeds Ltd of China.

M. aeruginosa bloom material was collected from Dianchi Lake, Kunming in southwestern China, in August 2001, and was lyophilized and stored at −20 °C prior to use.

4′,6-Diamidino-2-phenylindole (DAPI ≥95% purity) was purchased from Fluka Chemie (Buchs, Switzerland). RAPD primers were obtained from SunShineBIO (Nanjing, China) and reported in Table 1. All other chemicals, from Sangon (Shanghai, China) and SunShineBIO, were of analytical or higher grades.

2.2. Preparation of crude aqueous extract of toxic cyanobacteria

Freeze-dried bloom cyanobacteria were suspended in distilled water, freeze-thawed eight times and then centrifuged at 20,000 × *g* for 30 min. The supernatants were pooled and kept at −20 °C until further use.

The toxicity of the extract was assessed by intraperitoneal injection mouse bioassay. The toxin composition of the extract was detected by high-performance liquid chromatography with diode array detection (HPLC-DAD) after the extract was purified by passing through Sep-pak C18 cartridges (Waters), and ELISA was carried out for the determination of total microcystins and the results were expressed as microcystin-LR equivalents (MC-LR equiv) [19,20].

2.3. Plant treatment

M. pumila shoots were established on solid MS medium [22] supplemented with 1.0 mg/l 6-benzyladenine (BA) and 0.5 mg/l α-naphthaleneacetic acid (NAA). The crude aqueous extract of toxic cyanobacteria was filter sterilized and added to the sterilized medium to give a range of doses of the extract (equivalent to 0, 0.03, 0.3 and 3 μg MC-LR/ml). *M. pumila* shoot cultures were maintained at 25 ± 2 °C under a 12 h photoperiod provided by cool white fluorescent lamps at 50 μmol/m^2 s as measured at culture level. After 7 d and 14 d of culture, *M. pumila* shoots were sampled for the examination of DNA damage.

Germination tests were performed on a range of doses of the extract (equivalent to 0, 0.024, 0.12, 0.6 and 3 μg MC-LR/ml). Before germination experiments, rice seeds were dipped in various concentrations of the extract for 24 h. Germinations were carried out in 12 cm × 12 cm × 5 cm (length × width × height) plastic boxes, on which three filter papers were placed. The papers were saturated with 10 ml of different concentrations of the extract or distilled water for control, and 100 seeds were placed on each box. Four replicate boxes were used for every concentration of microcystins. The germination boxes were closed and placed at room temperature (15–25 °C) for rape, and for rice the boxes were placed into incubators in a variable-temperature mode (from 8:00 am, 25 °C 2 h, 30 °C 8 h, 25 °C 4 h, 20 °C 10 h). The incubator was illuminated by fluorescent lights, with a daylight photon flux density of 150 μmol/m^2 s in the center, which maintained a 12 h photoperiod. During germination, 4 ml of the extract at identical concentration was added at 3-day intervals to prevent dryness. After 10 d the laboratory germination experiments were terminated, and the rice and rape seedlings (excluding roots) were weighed and placed at −70 °C for examining DNA damage.

2.4. DNA extraction and agarose gel electrophoresis

DNA was extracted from 0.2 or 0.5 g of rice and rape seedlings (excluding roots) or *in vitro* apple cultures by cetyl trimethyl ammonium bromide (CTAB)-based procedure according to the method described in Murry and Thompsom [23] with some modifications. After grinding, the tissue was transferred to centrifuge tubes and immediately added into 4 ml of extraction buffer preheated at 65 °C. The extraction buffer contained 2% CTAB (W/V), 100 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaC1, 3% PVP (polyvinylpyrolidone) (W/V) and 2% β-mercaptoethanol. The mixture was then shaken softly up and down ten times and allowed to incubate for 1 h in a water bath at 65 °C. The sample was then extracted twice with an equal volume of chloroform/isoamyl alcohol (24:1) and once with phenol/chloroform/isoamyl alcohol (25:24:1), and nucleic acids were precipitated from the aqueous phase with 1/5 volume of 3 M NaAc (pH 5.2) and 2 volume of ethanol. RNA was removed by treatment with RNase A at 100 μg/ml for 1 h at 37 °C. After precipitation and pelleting, DNA was rehydrated in TE buffer (1 mM Tris–HCl, pH 8.0, and 0.1 mM EDTA, pH 8.0).

Electrophoresis was carried out using 1.2% agarose gels for 2 h in constant voltage mode (5 V/cm). The gels were stained with ethidium bromide.

2.5. DNA fragmentation examination

DNA fragmentation was examined fluorimetrically using DAPI [11,24]. After centrifugation of the aqueous DNA at 27,000 × *g* for 30 min, the DNA content in the supernatant fraction (DNA fragments) and the pellet (intact chromatin) was determined respectively. Briefly, the fluorescence of 2 ml of buffer (100 mM NaCl, 10 mM EDTA, and 10 mM Tris, pH 7.4) containing 100 ng/ml DAPI was measured after the addition of 20 μl of DNA samples at 360 nm of excitation wave length and 450 nm of emission wave length. The DNA fragmentation was expressed as the ratio of the DNA content of the supernatant to the total DNA.

Table 2
Effect of CE containing MCs on DNA fragmentation.

	Days after treatment	Concentration of microcystins (μg MCs/ml)	% DNA fragmentation[a]
Rice	10	0 (CK)	16.3 ± 2.1
		0.024	42.3 ± 3.9**
		0.12	25.1 ± 2.7*
		0.6	70.8 ± 8.2***
		3	33.4 ± 4.0*
Rape	10	0 (CK)	17.2 ± 1.8
		0.024	18.5 ± 1.5
		0.12	16.7 ± 2.3
		0.6	17.7 ± 1.6
		3	18.9 ± 2.2
Apple	0	0 (CK)	15.4 ± 1.7
	7	0 (CK)	15.9 ± 1.6
		0.03	14.6 ± 1.8
		0.3	16.9 ± 1.4
		3	14.4 ± 1.5
	14	0 (CK)	17.0 ± 1.9
		0.03	16.1 ± 2.0
		0.3	15.3 ± 2.4
		3	17.5 ± 1.9

[a] Values are the means ± S.D. ($n = 4$).
* Significant difference from the control is shown as: $P < 0.05$.
** Significant difference from the control is shown as: $P < 0.01$.
*** Significant difference from the control is shown as: $P < 0.001$.

2.6. RAPD reactions

RAPD reactions were performed according to the following protocol: initial denaturation at 94 °C for 1 min followed by 35 cycles at 94 °C for 40 s, 38 °C for 90 s, 72 °C for 2 min, and a final single step of 72 °C for 5 min. Reactant concentrations were 200 μM dNTPs, 2.5 ng/μl of DNA template, 1.0 ng/μl of RAPD primer, and 0.75 unit of *Taq* DNA polymerase in a final reaction volume of 20 μl. RAPD products were separated by electrophoresis in 1.2% agarose gels and stained with ethidium bromide.

2.7. Statistical analysis

The data are expressed as mean ± standard deviation (S.D.). Significant differences between data sets were detected by one-way analysis of variance (ANOVA). Results were considered significant at a P level <0.05.

3. Results

3.1. DNA fragmentation analysis

DNA fragmentation was determined in apple shoots and rice and rape seedlings after exposure to cyanobacteria extract (CE) containing MCs (Table 2). Significant increase in DNA fragmentation was observed in rice seedlings exposed to CE containing 0.024, 0.12, 0.6 and 3 μg/ml MCs ($P < 0.01$, $P < 0.05$, $P < 0.001$, and $P < 0.05$), which corresponded to 260%, 154%, 434% and 205% of control, respectively. In contrast, no significant change in DNA fragmentation was observed in rape seedlings after various doses of CE. Similarly, the DNA fragmentation in apple shoots was not significantly altered 7 d and 14 d after CE exposure (Table 2). On electrophoresis, the DNA fragmentation appeared as a smear due to degradation (Figs. 1 and 2), which corresponded with the quantitative results obtained fluorimetrically using DAPI.

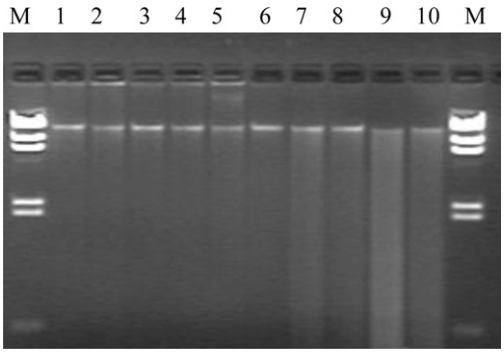

Fig. 1. Genome DNA of rape and rice seedlings following treatment of seeds with different doses of CE containing MCs. Lane M: lambda DNA/*Eco*RI + *Hin*dIII DNA size markers; MCs concentrations for rape seeds from lane 1 to lane 5 are 0, 0.024, 0.12, 0.6 and 3 μg/ml, respectively; MCs concentrations for rice seeds from lane 6 to lane 10 are 0, 0.024, 0.12, 0.6 and 3 μg/ml, respectively.

3.2. RAPD analysis

In order to explore the possible DNA damage in exposed rape seedlings, RAPD technique was used although DNA damage was not examined fluorimetrically through DAPI after exposure of rape seeds to CE. In RAPD analysis, a total of 20 ten-mer primers (Table 1) were screened for polymorphism between rape seedlings exposed to CE containing 3 μg/ml MCs and the control (Fig. 3). The results demonstrated that 18 or 90% primers detected one or more RAPD while 2 gave weak amplification. The generated polymorphic RAPDs were reflected by variation in band intensity, disappearance of bands and appearance of new PCR products.

Following primer screening, we further assessed genetic variation among rape seedlings exposed to various doses of CE containing MCs with primers F03 and S01. Band intensity changes occurred mainly for rape seedlings exposed to CE containing 0.024 μg/ml MCs for the primer F03, while for two higher CE concentration (0.12 and 0.6 μg/ml MCs) two new faint RAPD bands of about 1300 and 300 base pairs (bp) in size appeared except band intensity changes (Fig. 4). After exposure of CE containing 3 μg/ml MCs, three new bands including two bold bands (approximately 1200 and 600 bp) were found, and an increase in band intensity with molecular size of about 930 bp occurred for rape seedlings (Fig. 4). Primer S01 produced more RAPD fragments than primer F03 that generated only three unambiguous bands for control rape seedlings (Figs. 4 and 5). The decrease in band intensity was particularly obvious for two lower CE concentration (0.024 and

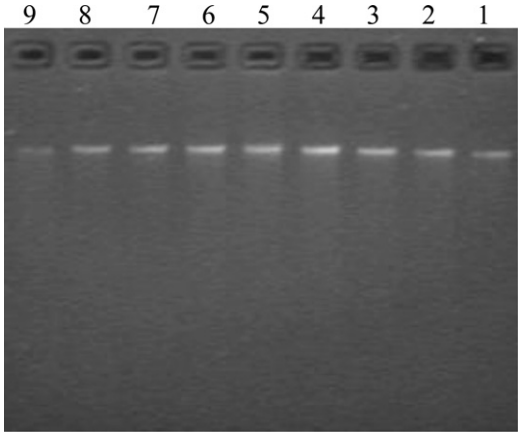

Fig. 2. Genomic DNA of *in vitro* apple shoots on media supplemented with different doses of CE containing MCs. Lane 1: control; lane 2 to lane 5: 0, 0.03, 0.3 and 3 μg/ml MCs for 7 d; lane 6 to lane 9: 0, 0.03, 0.3 and 3 μg/ml MCs for 14 d, respectively.

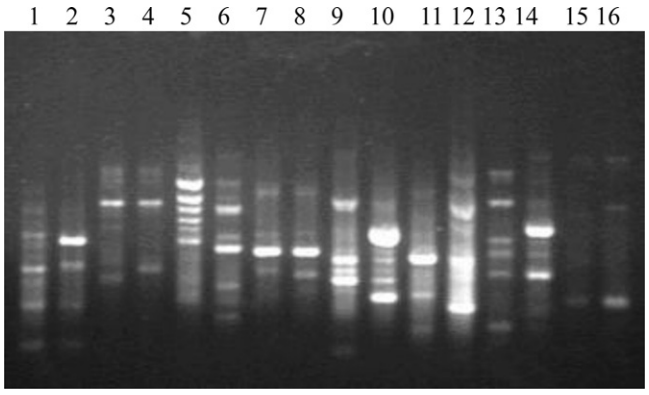

Fig. 3. Screening of primers for rape seedling genomic DNA. Lane 1: primer S05, 3 μg/ml MCs; lane 2: primer S05, 0 μg/ml MCs; lane 3: primer S04, 3 μg/ml MCs; lane 4: primer S04, 0 μg/ml MCs; lane 5: primer S01, 3 μg/ml MCs; lane 6: primer S01, 0 μg/ml MCs; lane 7: primer F05, 3 μg/ml MCs; lane 8: primer F05, 0 μg/ml MCs; lane 9: primer F03, 3 μg/ml MCs; lane 10: primer F03, 0 μg/ml MCs; lane 11: primer E20, 3 μg/ml MCs; lane 12: primer E20, 0 μg/ml MCs; lane 13: primer E17, 3 μg/ml MCs; lane 14: primer E17, 0 μg/ml MCs; lane 15: primer B02, 3 μg/ml MCs; lane 16: primer B02, 0 μg/ml MCs.

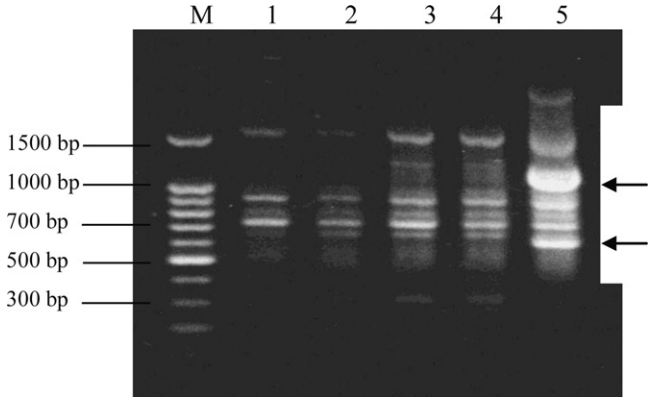

Fig. 4. RAPD products of genomic DNA from rape seedlings using primer F03 following treatment of rape seeds with different doses of CE containing MCs. Lane M: DNA size marker; lane 1: control; lane 2: 0.024 μg/ml MCs; lane 3: 0.12 μg/ml MCs; lane 4: 0.6 μg/ml MCs; lane 5: 3 μg/ml MCs. Arrow indicates polymorphic bands generated.

0.12 μg/ml MCs) for the primer S01 (Fig. 5). There was also a slight band intensity decrease for 0.6 μg/ml MCs-containing treatment. In comparison with lower CE treatment, CE containing 3 μg/ml MCs generated at least three new RAPD fragments including two bold

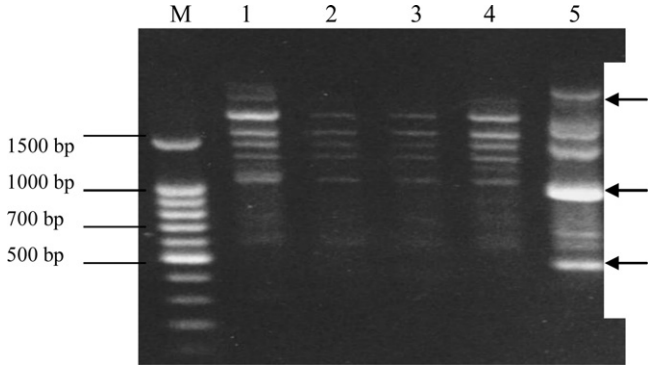

Fig. 5. RAPD products of genomic DNA from rape seedlings using primer S01 following treatment of rape seeds with different doses of CE containing MCs. Lane M: DNA size marker; lane 1: control; lane 2: 0.024 μg/ml MCs; lane 3: 0.12 μg/ml MCs; lane 4: 0.6 μg/ml MCs; lane 5: 3 μg/ml MCs. Arrow indicates polymorphic bands generated.

bands (approximately 1000 and 450 bp) with more than two disappearing RAPD bands (Fig. 5). These detected polymorphic RAPDs indicated substantial differences between unexposed and exposed rape seedlings with apparent changes in the number, size and the intensity of amplified DNA fragments.

4. Discussion

It is well known that oxidative stress plays an important role in the toxicity of MCs in animals [8–10] and plants [19,20,25]. Microcystin-induced oxidative DNA damage has been observed in mouse liver *in vivo* and in baby hamster kidney cells and mouse embryo fibroblast primary cells *in vitro* [11], in human colon adenocarcinoma CaCo-2 cells [14], and in whitefish liver cells [15]. However, to our knowledge, no investigation into DNA damage after exposure of plants to MCs is found to date. In this study, MCs-containing cyanobacteria extract induced significant increase in DNA fragmentation determined fluorimetrically using fluorescent dye DAPI in rice seedlings, but no significant changes were observed in exposed rape seedlings and *in vitro* apple shoots. Our previous study showed that the total MCs concentration determined by ELISA in the extracts of rape and rice seedlings after 10 d exposure to CE containing 3 μg/ml MCs and apple shoots after 7 d and 14 d exposure to the same concentration of CE was 651.00 ± 78.71, 5.40 ± 0.85, 225.00 ± 25.62, and 510.23 ± 141.10 ng MC-LR equiv/g FW, respectively; higher MCs levels accumulated by rape seedlings and *in vitro* apple shoots accounted for more powerful inhibition effect on the growth and development than rice seedlings [19,20]. It seems that these previous results are not in agreement with the present study in which we found obvious DNA fragmentation qualitatively and quantitatively in exposed rice seedlings rather than in rape seedlings and apple shoots. This inconsistency suggests that DNA fragmentation is likely to be a signal or an indicator, and due to up-regulation of the expression of DNA repair genes, lower harmful effect of CE containing MCs was observed in rice seedlings. Similarly, higher expression of DNA repair genes in rice seedlings after 10 d exposure of CE containing 3 μg/ml MCs probably accounted for lower DNA fragmentation when compared to 0.024 and 0.6 μg/ml MCs treatments. In addition, it is probable that there are different MCs-resisting mechanisms between terrestrial and aquatic plants, or aquatic plants may have more powerful MCs degradation mechanism. And it is likely that other DNA damage forms occur in exposed rape seedlings and apple shoots, which has been confirmed by RAPD in this study.

RAPD is a PCR-based technique that has been widely used to detect DNA damage caused by various environmental pollutants and mutagens such as heavy metal ions [26,27], pesticide [28] and radiation [29,30]. This is the first report that we know of that employs RAPD assay for evaluating genotoxic effects of microcystins. Although RAPD assay provides just qualitative results, it has proved useful to detect genomic instability manifested such as point mutation, genetic and chromosomal rearrangement, deletion and insertion, and to allow detection of low doses of pollutants [27]. Therefore, this RAPD method can complement other well-established techniques in genotoxicity [31]. In the field of ecotoxicology, most RAPD studies describe the RAPD changes such as differences in band intensity as well as gain/loss of stable RAPD bands [31]. Similarly, in the present study, DNA damage in MCs-containing CE treated rape seedlings was reflected by changes in RAPD profiles: alteration in band intensity, disappearance of amplified bands, and appearance of polymorphic bands occurred in the profiles (Figs. 3–5). However, no DNA damage in MCs-containing CE exposed rape seedlings was detected quantitatively using fluorescent dye DAPI and qualitatively by agarose gel electrophoresis while significant DNA fragmentation in exposed rice seedlings

was found. These findings suggest that there are different classes of oxidative damage to DNA in rape and rice after exposure to MCs, and RAPD is an effective and sensitive technique for examining the genotoxicity in plants induced by cyanobacterial toxins. Aquatic plants can come into direct contact with microcystins after the senescence and lysis of cyanobacterial blooms, and terrestrial plants can also be exposed to microcystins via contaminated irrigation water. Clearly, more work is needed to determine the possible genotoxicity defense systems and molecular mechanisms of microcystin-induced DNA damage in terrestrial and aquatic plants.

5. Conclusions

The present study has demonstrated that different types of DNA oxidative damage exist in microcystins-exposed rice, rape and apple. This work also provides suggestive evidence that RAPD fingerprinting can be a powerful strategy for assessing the genotoxic effects of microcystins on plants, and the RAPD fingerprints of plants in natural freshwater bodies may be used as an investigation tool for freshwater toxicology.

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