Applied Chemical Engineering (2020) Volume 3 Issue 1
doi:10.24294/ace.v3i1.724

ORIGINAL RESEARCH ARTICLE

Heavy metal-induced oxidative stress and DNA damage as shown by RAPD-PCR in leaves of *Elodea canadensis*

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ABSTRACT

The objective of the study was to evaluate the antioxidant response and DNA damage of heavy metals (Cd, Cu and Cr) in *Elodea canadensis*. Superoxide dismutase (SOD), catalase (CAT), Glutathione reductase (GR) and lipid peroxidation levels of leaves of *Elodea canadensis* which was exposed to different concentrations of heavy metals (Cd: 2, 5, 10, 15, 25 ppb; Cu: 200, 500, 1000, 2500 ppb and Cr: 1, 3, 10, 15, 25 ppb) in a hydroponic culture were determined spectrophotometrically. The highest induction in SOD and CAT activities were determined at highest concentration of heavy metals. The Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) technique was used to investigate the variation of DNA banding patterns of samples that exposed to different concentrations of heavy metals. Changing in band intensity and the gain and loss of bands were demonstrated the genotoxic effect of heavy metals. Bioaccumulation, oxidative responses and DNA damages were shown that *Elodea canadensis* represents a useful bioindicator.

Keywords: *Elodea Canadensis*; Oxidative Stress; DNA Damage; RAPD-PCR

ARTICLE INFO

Received 6 March 2020
Accepted 29 April 2020
Available online 4 May 2020

1. Introduction

Metals form one of the major groups of genotoxic environmental pollutants for soil and aquatic ecosystems. These dangerous pollutants usually originate from industry and agricultural activities like pesticides and fertilizers. The toxic effect of heavy metals in plants and animals has been investigated by many researchers[1–3]. Heavy metal originated phytotoxicity often related to production of reactive oxygen species (ROS) in plants and potentially toxic that compared with molecular oxygen[4].

The metal's molecular damaging action is not clearly known, but studies suggested that the oxidative damage is caused by metal toxicity[5]. There are several cellular stress responses and damages in cellular components like membranes, proteins and DNA. Comet, micronucleus and choromosome aberration assays have been used to measure the genotoxic effect of heavy metals on plants in many studies[6–8].

The advances in molecular biology provide the development of sensitive and useful techniques for DNA analysis in ecotoxicology. Random Amplified Polymorphic DNA (RAPD) is a DNA-based technique and used to detect the differences of DNA fingerprints from control and exposed DNA to a genotoxic effects. DNA damaging agents such as heavy metals and UV radiation in plants induce genomic DNA alterations and

RAPD-PCR can be successfully applied to determine these alterations[9].

The advantage of DNA-based techniques is especially related to sensitivity and short response time of these techniques. RAPD-PCR is also used for species classification, genetic mapping and phylogeny studies. Nowadays, it is known that a novel biomarker assay for the detection of DNA damages and mutations like rearrangements, point mutation, insert or deletions of DNA in many living things has been carried out[10,11]. RAPD fragments are detected by agarose gel electrophoresis and changing in band intensity, and the gain and loss of bands are demonstrated the genotoxic effect of damaging agents.

Heavy metal contamination as a result of natural or anthropogenic activities is a serious problem in the world[12]. The accumulation of these heavy metals in plants causes many physiological and biochemical changes[13]. These pollutants cause production of ROS that can cause important harm to plant cell structures. To overcome the oxidative damage, plants have advanced a common network of antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7)[14].

The objectives of the present study were to investigate DNA damage induced by different heavy metals in *Elodea canadensis* and changes in antioxidants levels.

2. Materials and methods

2.1 Genomic DNA isolation, RAPD amplification method and estimate of genomic template stability

DNA extraction was performed by using CTAB method[15]. The concentration of extracted DNAs was measured at 260 nm and the purity was evaluated by the ratio of OD_{260}/OD_{280} with a spectrophotometer (ACTGene Micro-Spectrophotometer). The A_{260}/A_{280} ratio demonstrates the DNA purity, and 1.8–2.0 values suggest "pure DNA".

Amplification of both nuclear DNA that extracted by CTAB method was performed using 2 µl of DNA template in a 15 µl reaction that contains PCR buffer 1.5 µl (10X buffer with $(NH_4)_2$ SO_4, Thermo Fisher), MgCl2 (2.5 mM, Thermo Fisher) 1.2 µl, dNTP (10 mM stock solution) 0.5 µl, BSA (10 mg/mL) 0.6 µl, primers (10µM, Opc) 1.0 µl, Taq Polymerase (5 u/µl, Thermo Fisher) 0.25 µl and filled up with sterile deionized water to the final volume.

The temperature profile for the RAPD-PCR was initially denaturating at 94 ℃ for 2.5 min, followed by 36 cycles at 94 ℃ for 45 s (denaturation), 35 ℃ for 45 s (annealing) and 72 ℃ for 2 min (extension), with final extension at 72 ℃ for 10 min. PCR products were electrophoresed in a Tris-Asedic Acid-EDTA (TAE) buffer by 2% agarose gel for 1.5 h at 80V. DNAs were stained with ethidium bromide and the bands were photographed under UV light. 100 bp DNA ladder was used as a standard marker.

Primer screening for RAPD-PCR was performed using 10 primers. Five out of 10 primers gave polymorphic bands. The sequences of these oligomers were given in **Table 1**. Polymorphism that observed in RAPD profiles contained disappearance of a control band and appearance of a new band. The amplification reactions were repeated at least three times to get reproducible results.

Genomic template stability (GTS; %) was calculated as following:

$$GTS = (1 - a/n) \times 100$$

where "*a*" was the average number of RAPD polymorphic profiles detected in each samples treated and "*n*" the number of total bands found in the control. The polymorphisim that observed in RAPD banding profiles included the disappearance of a normal band and appearances of a new band in comparison with the control. The average polymorphism was calculated for each experimental group exposed to different doses of different heavy metals (Cd, Cu and Cr). For comparing the sensitivity of each parameter, changes occurring in these values were calculated as a percentage of its control (set to 100%).

2.2 Plant material, growth conditions and heavy metal estimation

Fresh samples of *E. canadensis* were obtained from ponds in Kayseri, Turkey. Plants were grown in a growth chamber at 25 ± 1 ℃ during 16/8 light/dark cycle with 350 µmol m^2 s^{-1} of irradiance by cold fluorescent lamps. The plants were growth in Hoagland nutrient solution and the solution was renewed every four days. The solution pH was maintained to 5.8 ± 0.1 by titration with NaOH or HCl solutions (0.1 M) when required. On the 7th day, heavy metal treated plants were harvested from containers. The plants were rinsed twice with distilled water and subsequently, biochemical parameters were determined. Dried samples of plant were digested with 10 mL of concentrated HNO_3, using a CEM microwave digestion system. After digestion, the volume of each sample was adjusted to 25 mL using double deionized water[16]. Determination of the cadmium, chromium and copper concentrations in all samples was carried out by inductively coupled plasma optical emission spectrometry (Varian). The samples were analyzed in triplicate.

2.3 Estimation of lipid peroxidation (MDA)

Lipid peroxidation level was estimated according to the method of Razinger *et al*.[17], with modifications.

2.4 Antioxidative enzymes

2.4.1 Enzyme extraction

Fresh tissue (0.2 g) was homogenized with 5 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone in an ice-cooled mortar. Homogenate was centrifuged at 15000 g for 15 min at 4 ℃[18]. The supernatant was used for enzyme determination.

2.4.2 Superoxide dismutase (EC 1.15.1.11)

The SOD activity (EC 1.15.1.11) was analyzed by measuring the inhibition of the photochemical reduction of nitrobluetetrazolium (NBT)[19]. The assay mixture contained 20 mM phosphate buffer (pH 7.5), 10 mM methionine, 0.1mM NBT, 0.1 mM EDTA, 0.005 riboflavin, 50 µg mL^{-1} of enzyme extract and 0.25 mL of deionized H_2O in a total volume of 3 mL. Riboflavin was added at the end, and the tubes were shaken and then illuminated for 15 min. The absorbance was recorded at 560 nm and the absorbance of the non-irradiated reaction mixture served as a control. Inhibition of 50% of the reaction was defined as one unit of enzyme and the enzyme activity was expressed as nU g^{-1} FW.

2.4.3 Catalase (EC 1.11.1.6)

CAT (EC 1.11.1.6) activity was measured spectrophotometrically by following the consumption of H_2O_2 at 240 nm, according to Aebi[20], in potassium phosphate buffer (150 mM, pH 7) containing 15 mM H_2O_2 and enzyme extract (exactly 50 mg of protein) in a final volume of 1 mL. Addition of H_2O_2 started the reaction.

3. Results

3.1 Effects of heavy metals on RAPD profiles

Among the 10 decamer oligonucleotide primers evaluated, only 5 of them presented specific and stable results (**Table 1**). The RAPD fingerprints revealed the occurrence of significant differences between untreated and treated plant materials, with apparent changes in the number, size and the intensity of amplified DNA fragments. The primary observation of RAPD banding patterns generated by the heavy metals exposed plants were the differences in the band intensities, appearance of new bands or absence of normal bands compared with control plants. RAPD banding patterns generated by heavy metal exposed plant materials were different from those obtained control. Different polymorphic bands were determined at each concentration of heavy metals for different primers. GTS did not decrease gradually.

Table 1. Random primers that gave polymorphic bands in RAPD-PCR

RAPD primer	Sequences of primers (5′→3′)	GC%
Opc 2	GTG AGG CGT	70
Opc 5	GAT GAC CGC	70
Opc 8	TGG ACC GGT	70
Opc 9	CTC ACC GTC	70
Opc 15	GAC GGA TCA	60

The number of disappearing RAPD bands was greater at 5 ppb and 25 ppb Cd for Opc 5 and Opc 9, respectively. Maximum number of extra bands appeared with Cu application at 500 and 1000 ppb concentrations in total. The decrease in band intensity was only apparent for *Elodea* exposed to 500 ppb Cu but in Cd treated plant it was observed for different doses. However, the increase in band intensity was clearly obvious for Cd, Cu and Cr treatments especially for Opc 5 primer (**Table 2**, **3**, **4**).

Table 2. Changes of total bands in control, and of polymorphic bands and varied bands in *E. canadensis* exposed to different concentrations of Cd

Primers	**Cd concentration (ppb)**																			
	0	2				5				10				15				25			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Opc 2	5	0	0	0	2	0	0	3	0	0	0	0	2	1	0	0	0	1	0	0	0
Opc 5	7	2	0	0	2	0	3	3	0	0	0	1	0	0	0	0	3	4	0	0	0
Opc 8	3	0	0	0	1	0	2	0	0	0	0	0	1	0	0	0	0	1	1	2	0
Opc 9	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Opc 15	7	0	0	2	2	0	0	0	2	0	0	0	2	0	0	0	1	1	0	2	1
Total bands	28	2	0	2	7	0	5	6	2	0	0	1	5	1	0	0	4	7	4	4	1
a + b		2				5				0				1				11			
a + b + c + d		11				13				6				5				16			

Note: a: indicates appearance of new bands; b: disappearance of normal bands; c: decrease in band intensities; and d: increase in band intensities; a + b: denotes polymorphic bands; and a + b + c + d: varied band**.**

Table 3. Changes of total bands in control, and of polymorphic bands and varied bands in *E. canadensis* exposed to different concentrations of Cu

Primers	**Cu concentration (ppb)**																			
	0	200				500				1000				1500				2500			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Opc 2	5	0	0	0	2	2	0	0	2	2	0	0	2	3	0	0	2	3	0	0	2
Opc 5	7	1	0	0	6	2	0	1	1	2	0	0	0	3	0	0	0	3	0	0	0
Opc 8	3	2	0	0	1	4	0	0	0	4	0	0	0	1	0	0	1	1	0	0	1
Opc 9	6	1	0	0	5	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	4
Opc 15	7	0	0	0	2	0	2	0	2	0	0	0	5	0	0	0	4	0	0	0	3
Total bands	28	4	0	0	16	8	2	1	5	8	0	0	7	7	0	0	11	7	0	0	10
a + b		4				10				8				7				7			
a + b + c + d		20				16				15				18				17			

Note: a: indicates appearance of new bands; b: disappearance of normal bands; c: decrease in band intensities; and d: increase in band intensities; a + b: denotes polymorphic bands; and a + b + c + d: varied band**.**

Table 4. Changes of total bands in control, and of polymorphic bands and varied bands in *E. canadensis* exposed to different concentrations of Cr

Primers	**Cr concentration (ppb)**																			
	0	1				3				10				25				50			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Opc 2	5	0	0	0	1	0	0	0	3	0	0	0	2	0	0	0	3	1	2	1	0
Opc 5	7	2	5	0	1	1	0	0	5	1	0	0	2	1	0	0	2	2	4	0	1
Opc 8	3	2	0	1	0	2	0	0	1	4	0	0	2	2	0	1	2	3	1	1	1
Opc 9	6	0	0	0	3	0	0	0	3	0	3	0	2	0	4	0	0	0	6	0	0
Opc 15	7	0	4	0	1	0	4	0	1	0	4	0	1	0	0	1	3	0	4	2	0
Total bands	28	4	9	1	6	3	4	0	13	5	7	0	9	3	4	2	10	6	17	4	2
a + b		13				7				12				7				23			
a + b + c + d		20				20				21				19				29			

Note: a: indicates appearance of new bands; b: disappearance of normal bands; c: decrease in band intensities;and d: increase in band intensities; a + b: denotes polymorphic bands; and a + b + c + d: varied band**.**

Finally, it was defined that maximum number of extra bands appeared with Opc 5 at 25 ppb Cd (four new bands), Opc 8 at 500 and 1000 ppb Cu (four new bands) and Opc 8 at 10 ppb Cr (four new bands). The extra bands that appeared were determined to be of maximum 1100 bp molecular size (**Figure 1**). The occurrence of polymorphism was due to the loss and/or gain of the bands in the treated plant with heavy metals in comparison with the control. The highest

polymorphism was obtained from Cr application with 82% at 50 ppm concentration.

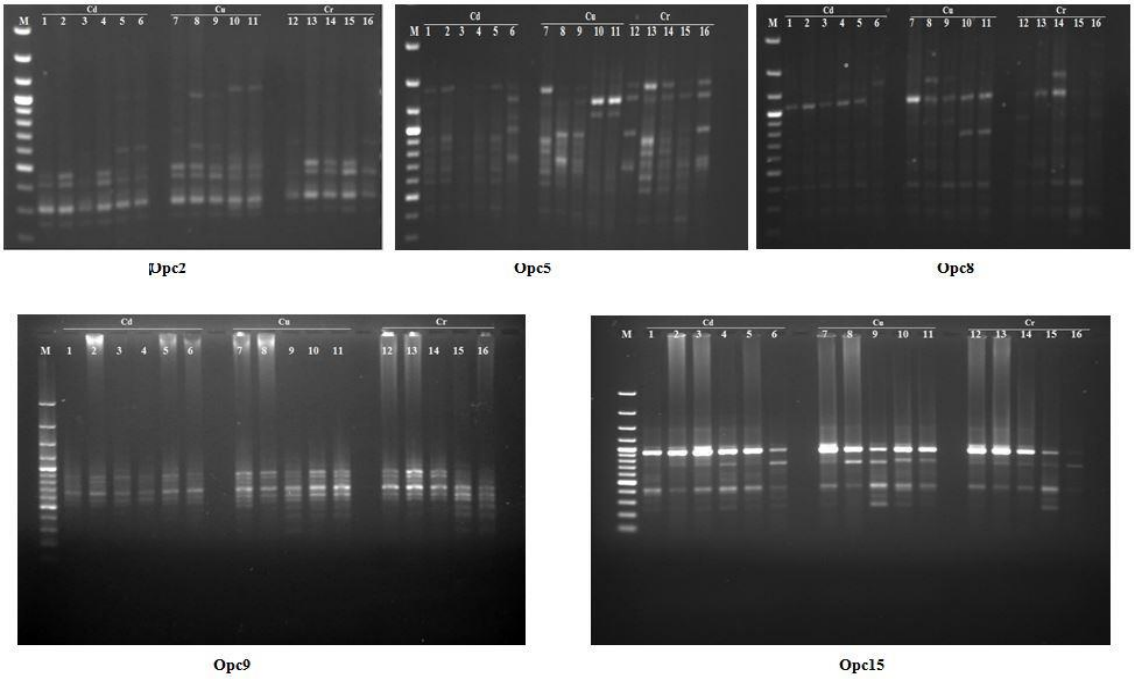
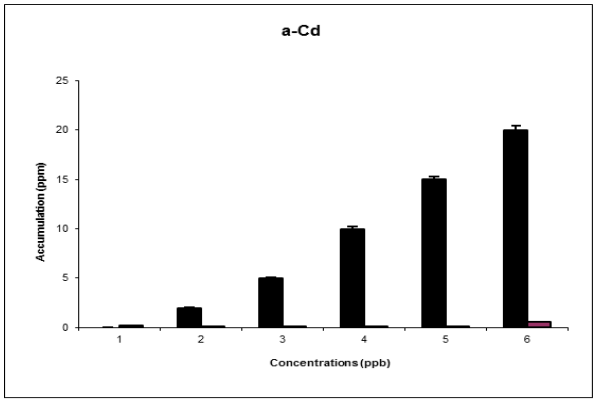


Figure 1. RAPD profiles of genomic DNA from *E. canadensis* exposed to varying heavy metal concentrations with different primers. (M: DNA marker; 1: Control; 2–16: increasing doses of heavy metal treatments)**.**

3.2 Heavy metal contents

The results related to the accumulation of Cd, Cr and Cu in *E. canadensis* are presented in **Figure 2a**, **2b** and **2c**. The maximum Cd accumulation was found to be 0.47 ppm DW at 15 ppb Cd. On prolonged exposure to higher concentrations of Cd (25 ppb), there was a significant decline in the Cd accumulation rate. According to **Figure 2b**, the higher Cr accumulation was obtained from 25 ppb Cr application (0.049 ppm) and the differences in all treatments were significant for *E. canadensis.* Similarly, the maximum Cu accumulation was found to be 6.8 ppm at 2500 ppb. The analysis of one-way ANOVA showed that the differences in all the treatments were significant ($P < 0.05$).



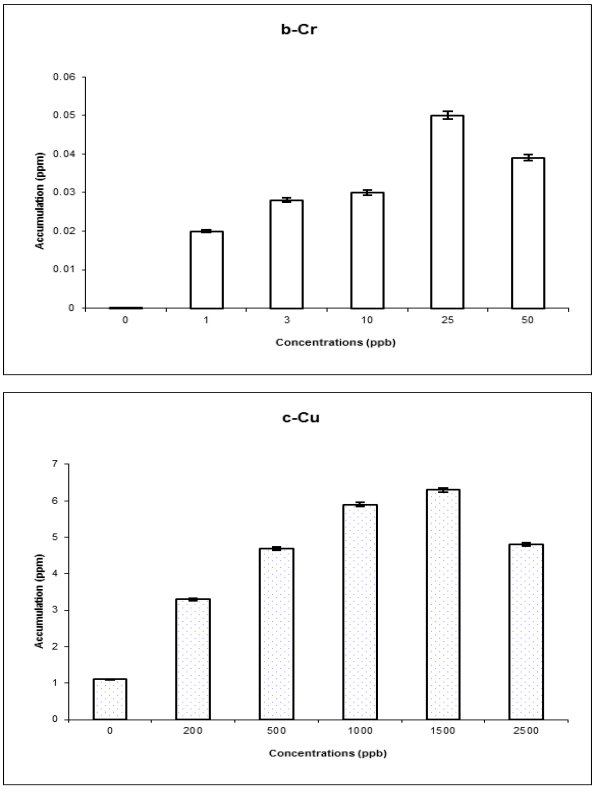


Figure 2. Heavy metal accumulation in *E.canadensis***.**

3.3 Effect of heavy metals on the level of MDA

The effect of Cd, Cu and Cr on MDA concentration is presented in **Figure 3a**, **b** and **c**. In the experiments, increasing in MDA

concentration in *E. canadensis* was observed. MDA concentration was linearly with increased heavy metal levels in the solution. The analysis of one way ANOVA showed that the differences of all treatments were significant ($P < 0.05$) for plant.

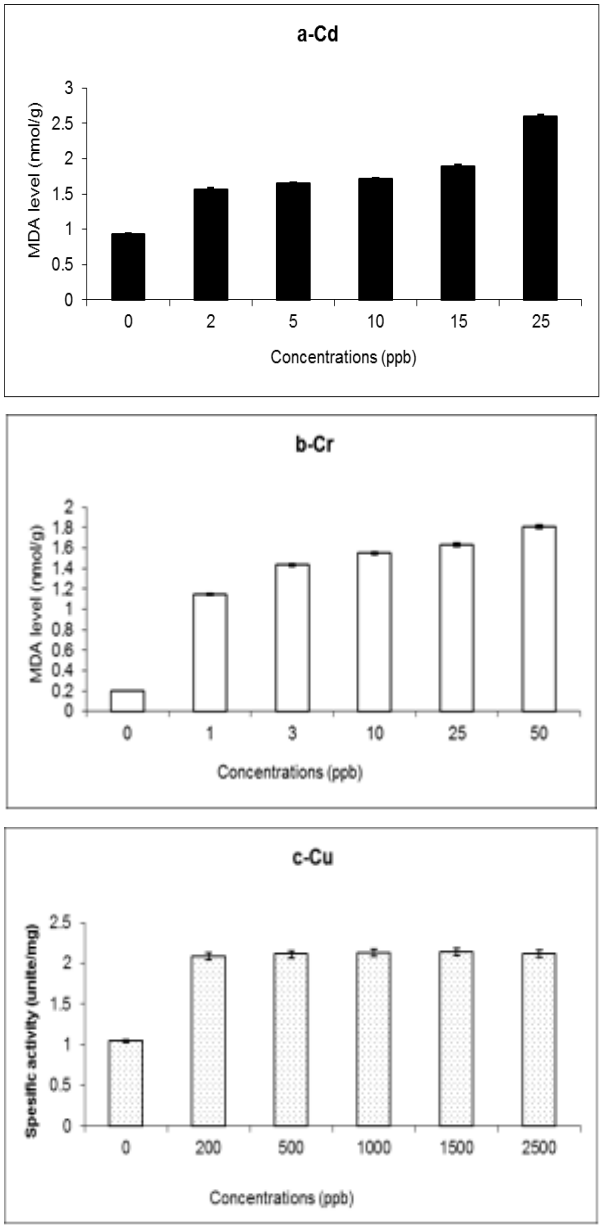


Figure 3. MDA activities in *E.canadensis* upon Cd **(a)**, Cr **(b)** and Cu **(c)** exposures. Values represent mean ± S.E. (n = 3). Asterisks indicate significant differences at $P < 0.05$**.**

3.4 Effects of heavy metals on SOD and CAT activity

In this study, the decline in catalase activity was observed with the increasing concentration of Cd in *E. canadensis*. The highest concentration of Cd (15 ppb) proved to be extremely toxic declining CAT activity (**Figure 4a**). Plants exposed to Cd present significant differences ($P < 0.05$). Similarly, the highest concentration of Cu (1500−2500 ppb) and Cr (25 and 50 pbb) proved to be toxic declining CAT activity (**Figure 4b** and **c**).

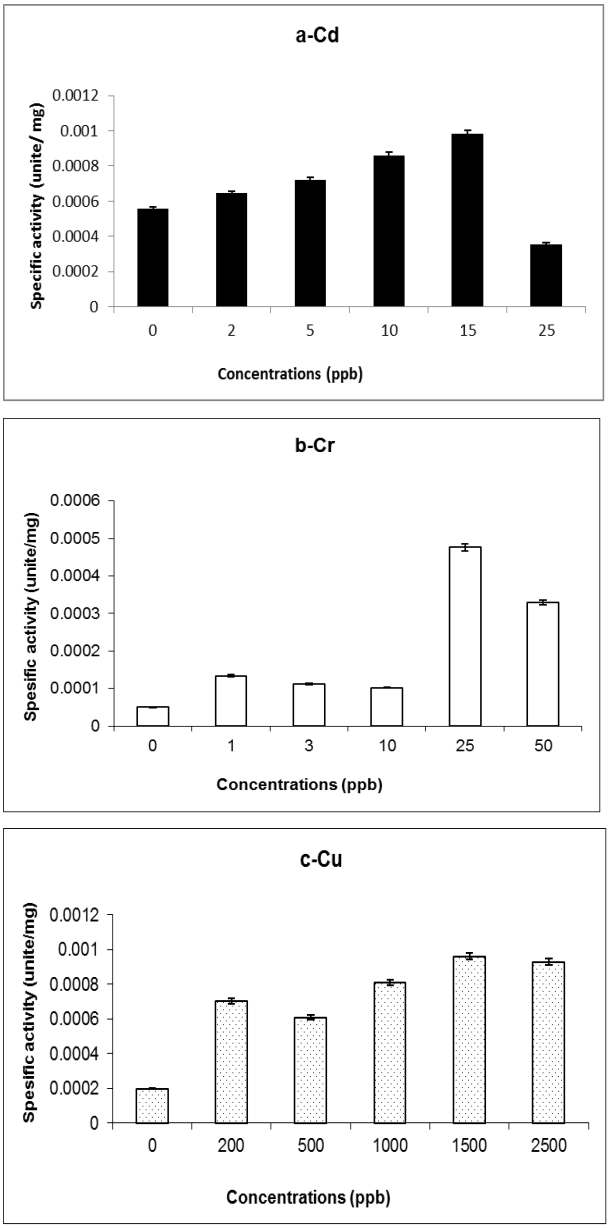


Figure 4. CAT activities in *E.canadensis* upon Cd **(a)**, Cr **(b)** and Cu **(c)** exposures. Values represent mean ± S.E. (n = 3). Asterisks indicate significant differences at $P < 0.05$**.**

Figure 5 a–**c** indicated that SOD activity increased linearly with increasing heavy metal levels. The maximum SOD activity was recorded at 25 ppb Cd (1.19 ± 0.0085 U $mg^{-1)}$. Results showed that, at the highest concentration of Cd, the SOD accumulation increases. Additionally, the significant differences were found in SOD activity among the treatments. At 1500 ppb Cu (2.261 ± 0.0075 U mg^{-1}), the maximum SOD

activity was recorded. At the highest concentration of Cu, the SOD accumulation increases. Additionally, the significant differences were found in SOD activity among the treatments. Similarly, SOD activity increased linearly with increasing Cr levels in *E. canadensis.*

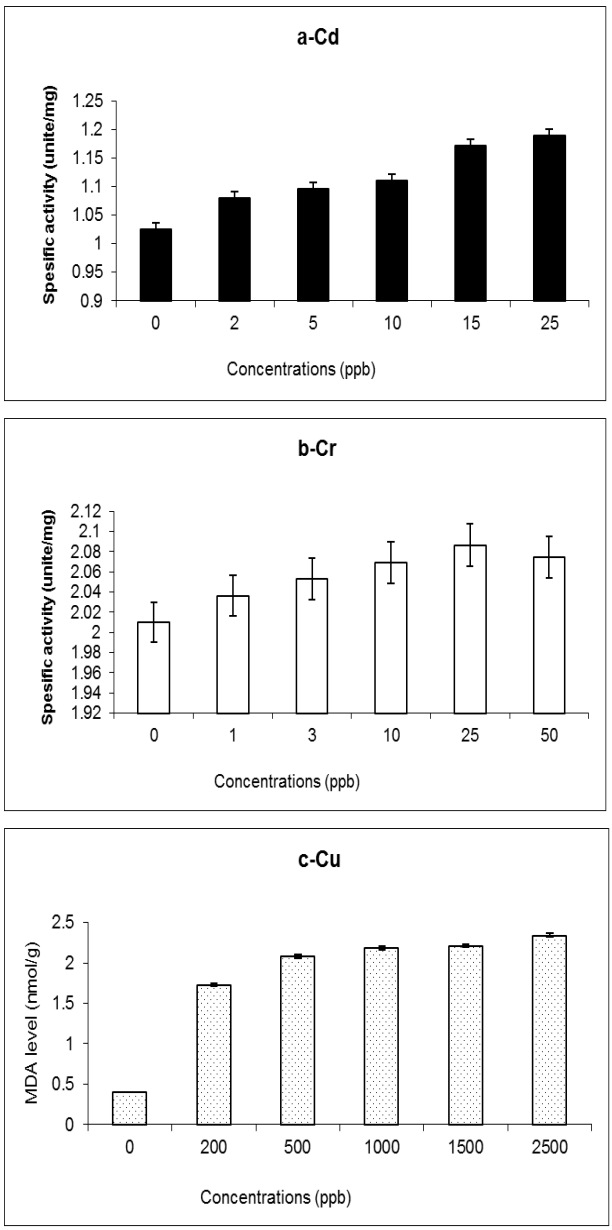


Figure 5. SOD activities in *E. canadensis* upon Cd **(a)**, Cr **(b)** and Cu **(c)** exposures. Values represent mean ± S.E. (n = 3). Asterisks indicate significant differences at $P < 0.05$.

4. Discussion

Heavy metals induce some cellular stress responses and damages of cellular components like DNA, proteins and membranes. RAPD-PCR is a selective and sensitive technique for DNA analysis in ecogenotoxicological studies. DNA fingerprints can be used to show the differences between samples that exposed and nonexposed to genotoxic agents[21]. This technique gives evidence about DNA mutation that treated to any genotoxic agents like heavy metals.

Number of bands, product yield and clarity of the banding profiles are used for detection of polymorphism. Appearance or disappearance of bands is probably to be due to the changes in primers sites. This is likely caused by rearrangements, point mutations and DNA damages in the primer binding sites[22].

Five primers, Opc 2, Opc 5, Opc 8, Opc 9 and Opc 15, produced unique bands in *E. canadensis* that treated with different heavy metals (Cd, Cu, and Cr) in the present study. These bands can be used as a marker for detection of genotoxic effect of heavy metals on *E. canadensis*. The low doses of heavy metals, especially Cd, were tolerable and polymorphism rate was comparatively low. Many researchers have used RAPD-PCR to evaluate metal toxicity changes in genetic patterns between treated and non-treated samples[21,23,24].

Our results showed that maximum number of polymorphic bands obtained from Cr with the highest concentration of heavy metal. In Cd application, the largest number of new bands was obtained with the highest dose of heavy metal similarly with Cr application. Cd has been reported by many researchers to cause DNA damages, such as single and double-strand breaks, modified bases, a basic site, DNA-protein cross-links, oxidized bases etc., in many organisms[3,25–27]. These structural changes after heavy metal treatment can significantly affect the PCR events and new PCR products can be occurred because of changing in priming sites.

RAPD technique can be successfully used to determine the DNA damages in living things. Although this technique is a qualitative method, RAPD analysis can be used as a sensitive method for environmental toxicology and useful biomarker system for an early warning. The present results suggest that RAPD-PCR in conjunction with antioxidant enzyme analysis can prove a powerful ecotoxicological tool.

In the present study, *E. canadensis* were grown in hydroponic culture in the presence of increasing Cd, Cu and Cr concentrations to evaluate its possible defense mechanisms. The results obtained indicated that at highest levels Cd, Cu and Cr were toxic to *E. canadensis.* The level of malondialdehyde (MDA) content has been considered as an indicator of oxidative stress that shows that plants are under high-level antioxidant stress.

In the experiments, MDA concentration increased linearly with increased heavy metal levels in the solution. Similar results were obtained with duckweed species by Uruç Parlak and Demirezen Yılmaz[16]. Gupta *et al.*[28] stated that higher MDA content shows the oxidative stress and this maybe one of the potential mechanisms by which toxicity due to heavy metals is manifested in plant tissues. Plants under environmental stresses, such as droughtand heavy metals, their production of ROS production will increase[29].

The increased activity of antioxidative enzymes in a plant indicated the formation of ROS and the present study indicated the generation of oxidative stress in *L. gibba* since all studied enzymes activity increased at high heavy metal levels[30]. In other words, *E. canadensis* analyzed in this work contains high Cd, Cr and Cu concentrations sufficient to activate ROS production and then oxidative stress.

Superoxide dismutase, the first enzyme in the detoxifying process, converts superoxide radicals to H_2O_2 at a very fast rate. The enhanced SOD activity observed in the present study is consistent with studies in which other plant species were treated with heavy metals[31]. The effect of heavy metal stress on SOD expression is likely to be governed by the tissue and sub cellular sites, at which oxidative stress is generated as supported by the higher activity of SOD in roots than in leaves of metal stressed plants[18].

Our data showed significant increases in CAT activity were observed between the treatments. The results obtained indicated that CAT activity decreased linearly with increasing Cd, Cu and Cr levels. Contrary to our results, a decline in the specific activity of catalase with increase in Cr concentration (20–80 ppm, 0.5 mM) has been reported by Shankar *et al.*[32]. According to Willekens *et al.*[33], it is likely that excess production of ROS by heavy metals can inactivate CAT probably by inactivating enzyme-bound heme group. These inconsistent results of differences in the plant organs were studied, and the durations and concentrations of metals were utilized.

5. Conclusion

Considering these results, we strongly suggest that higher heavy metal levels may cause oxidative stress in *E. canadensis* cells and may cause membrane damage through production of ROS and interferes with chlorophyll metabolism. Therefore, the data shown here can be used to illustrate how *E. canadensis* responds to its stressful environment. Among the antioxidative enzymes, SOD and CAT appear to play key roles in the plant's antioxidative defense mechanism under heavy metal toxicity. The ability of *E. canadensis* to both accumulate and tolerate moderate heavy metal level used in this study could be partly derived from ROS detoxification through an efficient antioxidant system.

Conflict of interest

The authors declare that they have no conflict of interest.

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