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Heavy metal induced DNA changes in aquatic macrophytes: Random amplified polymorphic DNA analysis and identification of sequence characterized amplified region marker

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Abstract

Plants have been used as good bio-indicators and genetic toxicity of environmental pollution in recent years. In this study, aquatic plants *Hydrilla verticillata* and *Ceratophyllum demersum* treated with 10 μmol/L Cd, 5 μmol/L Hg, and 20 μmol/L Cu for 96 h, showed changes in chlorophyll, protein content, and in DNA profiles. The changes in DNA profiles included variation in band intensity, presence or absence of certain bands and even appearance of new bands. Genomic template stability test performed for the qualitative measurement of changes in randomly amplified polymorphic DNA (RAPD) profiles, showed significant effect at the given concentration of metals. Cloning and sequencing of bands suggested that these markers although may not be homologous to any known gene but its conversion as a sequence characterized amplified region (SCAR) marker is useful in detecting the effects of genotoxin agents.

Key words: RAPD; *Hydrilla verticillata*; *Ceratophyllum demersum*; DNA polymorphism, SCAR marker
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Introduction

Higher plants have been reported to produce varied responses to heavy metals in their environment and interfere with the genetic constitution of plants (Conte *et al*., 1998; De Wolf *et al*., 2004). Among the higher plants, aquatic plants possess remarkable ability to concentrate heavy metals in their tissues, known as good biological indicators and play a role in detecting metal toxicity and tolerance (Gupta and Chandra, 1996; Gupta *et al*., 1998). Several plants such as *Allium cepa*, *Hordeum vulgare*, *Arabidopsis thaliana*, *Glycine max*, *Vicia faba*, and *Zea mays* etc. have been used as good bio-indicators of genetic toxicity of environmental pollutants in recent years, and comet assay, micronucleus assay or chromosome aberration assay were used to measure the genotoxic effect of metals on plants (Steinkellner *et al*., 1999; Angelis *et al*., 2000; Liu *et al*., 2005). Advantages of measuring the genotoxicity in plants at DNA level are mainly related to the sensitivity and short response time. Recently, the development of molecular marker technology has provided new tools for detection of genetic alteration in response to heavy metal tolerance by looking directly at the level of DNA sequence and structure. Random amplified polymorphic DNA (RAPD) is a PCR-based technique and extremely efficient for DNA analysis in complex genomes as it is relatively inexpensive and yields information on a large number of loci without having to obtain sequence data for primer design (De Wolf *et al*., 2004; Atienzar *et al*., 1999). RAPD profiles are achieved by PCR with single short primers of arbitrary nucleotide sequence under low annealing conditions. Fragments generated by RAPD are visualized after agarose gel electrophoresis and ethidium bromide staining. The resulting DNA profiles may differ due to band shifts, missing bands or the appearance of new bands. These bands can be scored to evaluate genetic similarities or dissimilarities. Furthermore, its use in surveying genomic DNA to detect various types of DNA damage and mutations (e.g., rearrangements, point mutation, small insert or deletions of DNA and ploidy changes) suggest that they may potentially form the basis of novel biomarker assays for the detection of DNA damage and mutations in cells of bacteria, plants and animals (Savva, 1998; Atienzar *et al*., 2000). However, concerns about reproducibility of DNA fingerprints have limited their wider use in environmental biology. To overcome the reproducibility problem associated with the RAPD analysis, RAPD markers have been converted into sequence characterized amplified region (SCAR; Paran and Michelmore, 1993). Although several reports are available of the development of SCAR marker in many plants (Hernandez *et al*., 1999; Evans and James, 2003), there are no published reports on the development of SCARs in metal treated plants.

The objective of this study was to assess heavy metal

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(Cd, Cu, and Hg) induced DNA changes in two aquatic submerged macrophytes *Hydrilla verticillata* (l.f.) Royle and *Ceratophyllum demersum* L. through RAPD analysis, cloning and sequencing of RAPD band and conversion of RAPD marker into dependable SCAR markers.

1 Materials and methods

1.1 Plant acclimatization and treatment

Plants of *H. verticillata* and *C. demersum* were acclimatized for one week in laboratory conditions using light intensity of 115 μmol/(m^2·s), a 14-h photoperiod at 25 ± 2°C and 10% Hoagland's solution. Experiments were setup in triplicates and each replicate contained 5 plants of equal size (Gupta and Chandra, 1996). Plants were treated with 10 μmol/L Cd, 20 μmol/L Cu, and 5 μmol/L Hg under the above mentioned laboratory conditions for 96 h duration. After harvesting plants were washed, blotted to remove excess water and used for DNA isolation.

1.2 Genomic DNA isolation and PCR amplification

Genomic DNA was isolated from the leaves, frozen and powdered in liquid nitrogen using Qiagen DNA isolation kit (Germany). The quantity and quality of DNA samples were estimated by comparing band intensities on agarose gel and by measuring the 260 nm/280 nm absorbance ratio. Twenty decamers from Kit G and ten from Kit B (Operon Technologies, Alameda, USA) were used as primers. Reactions were performed in 25 μL total volume containing 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 10 pmol primer, 0.5 U Taq Pol and 30 ng template. The conditions of DNA amplification were optimized and followed the procedure by Williams *et al*. (1990) with a few modifications. The PCR protocol consisted of an initial denaturing of 3 min at 94°C, followed by 44 cycles at 94°C for 60 s (denaturation), 36°C for 60 s (annealing) and 72°C for 60 s (extension), with an additional extension period of 10 min at 72°C. A negative control, without genomic DNA, was run with every set of samples to confirm that no contaminating DNA was present in the reactions. Amplification was carried out in a thermocycler (iCycler™, Bio-Rad, USA). The amplified PCR products were separated by electrophoresis on 1.0% agarose gels in 0.5% TBE buffer, visualized and imaged using gel documentation system (Gel Doc Mega, Biosystematics, U.K.) after staining with ethidium bromide. Chloprophyll and protein content were estimated following the method of Arnon (1949) and Bradford (1976), respectively. Fragment sizes of all the amplification products, estimated from the gel by comparison with marker, λDNA double digested with *Hin*dIII and *Eco*RI and scored as presence and absence of bands.

1.3 Genomic template stability test

Each change observed in RAPD profiles such as disappearance or appearance of bands and variation in band intensities in comparison to controls were given the arbitrary score of +1 and the average was calculated for each metal using number of primers used. Primers that did not produce changes in RAPD profiles or which were too difficult to score were not used in calculation. Genomic template stability (GTS, %) was calculated using the equation:

$$\mathrm{GTS} = (100 - (100 \times a/n)) \times 100\% \quad (1)$$

where, a is RAPD changes detected in each sample treated, and n is the number of total bands in the control.

To compare the sensitivity of each parameter (GTS, chlorophyll and protein content), changes in these values were calculated as a percentage of their control (set to 100%).

1.4 Cloning, sequencing, and amplification of SCAR marker

The polymorphic bands were eluted from the gel by QIAGEN DNA gel extraction kit (as detailed by the manufacturer) and cloned in pGEM-T Easy vector (Promega). Sequencing of the cloned fragment was done using Big Dye Terminator kit version 3.0 (Applied Biosystems, Foster City, CA) and analyzed with the 3700 ABI Prizm 96 capillary sequence analyzer. The sequences obtained were analyzed by BLAST program for similarity search. The SCAR primers were designed only from the *Hydrilla* plants sequences. Based on the sequence of the cloned RAPD marker, oligonucleotide primers of 20 nucleotides each, were designed by extending the original ten bases of the RAPD primer with the next ten nucleotides of the DNA sequences at the 3' ends. The SCAR primer sequences were custom synthesized from Sigma, USA. PCR conditions for genomic DNA amplification with SCAR primers were the same as for RAPD, except for the annealing temperatures.

2 Results

Seven primers out of thirty used for amplification resulted in specific and stable DNA profiles in the plants (Table 1). The RAPD profiles showed substantial differences between exposed and unexposed plants, with changes in the number, size and intensity of the bands. The approximate size range of the RAPD products was 200–1000 bp. Out of 243 bands, 110 bands showed the changes as compared to their controls. The RAPD profiles generated by 7 primers showed in Fig. 1a–1e indicate the differences between control and treated plants. Arrows on the gels

Table 1 Different Operon primer sequences resulting in consistent profiles

Sequence number	Primer ID	Sequence of the primer
1	OPG-6	GTG CCT AAC C
2	OPG-7	GAA CCT GCG G
3	OPG-8	TCA CGT CCA C
4	OPG-9	CTG ACG TCA C
5	OPG-10	AGG GCC GTC T
6	OPG-13	CTC TCC GCC A
7	OPB-20	GGA CCC TTA C

OPG denotes primers from Operon technologies, Kit G, while OPB is from Kit B.

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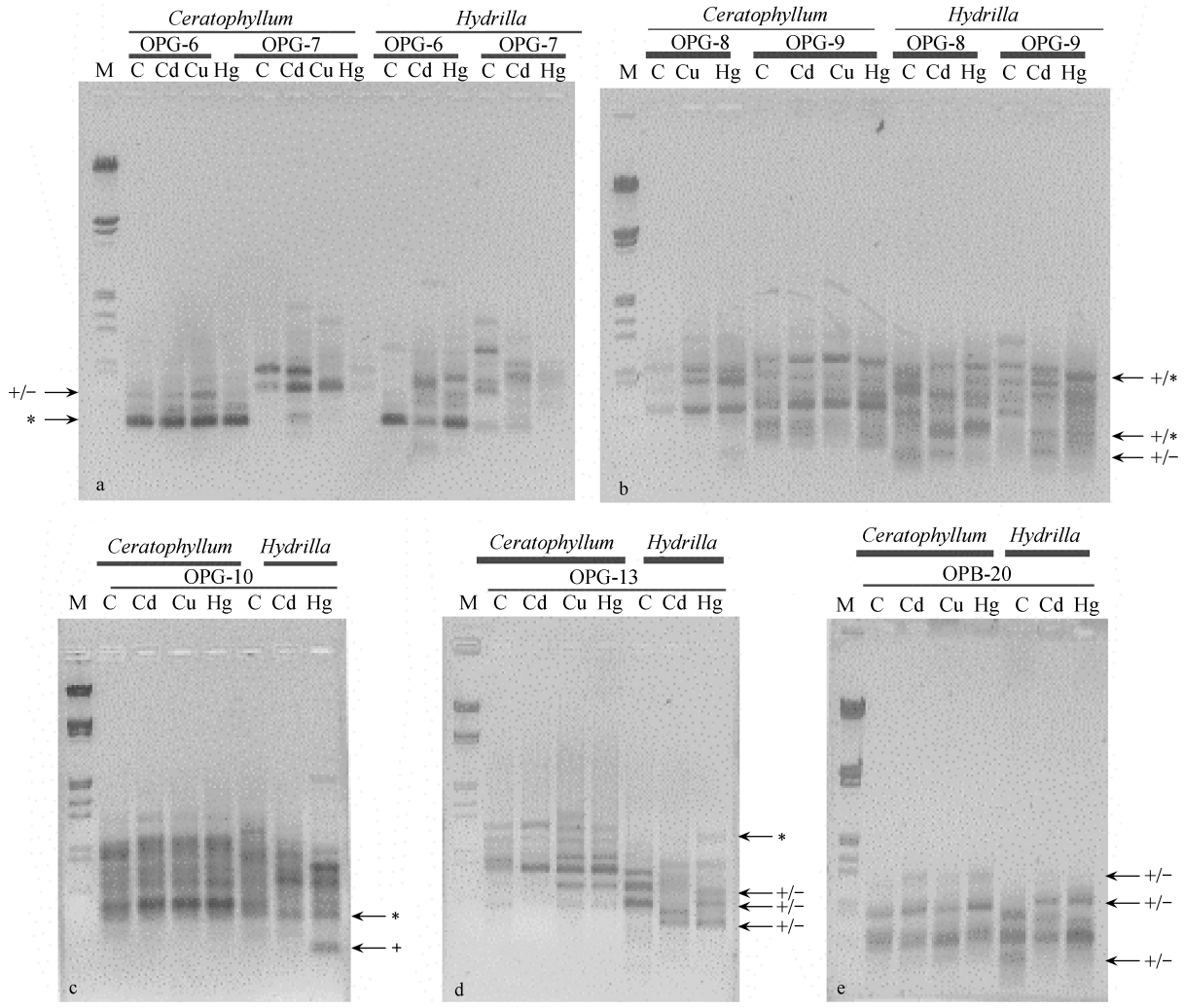

Fig. 1 RAPD agarose gel electrophoresis profiles of the plants *C. demersum* and *H. verticillata* using primers OPG-6 and 7 (a) OPG-8 and 9 (b), OPG-10 (c), OPG-13 (d) and OPB-20 (e). Lanes indicated by M contains l DNA double digested with *Hin*dIII and *Eco*RI as molecular marker, C (control), Cd (10 μmol/L), Cu (20 μmol/L), and Hg (5 μmol/L). Selected changes were indicated in comparison to control patterns as: *: variation in band intensities; +: appearance of a new band; –: disappearance of a normal band.

Table 2 Metal induced specific SCAR primer sequences derived from cloned RAPD fragments using *Hydrilla* plants and optimal annealing temperature for each set of reactions

RAPD primer	SCAR primer	Sequence (5'–3')	Annealing temperature (°C)
OPB-20	MG_{500} 1F	**GGA CCC TTA C** ATC AAG TTG G	60
	MG_{500} 1R	**GGA CCC TTA C** GGA ACA GTT T	
OPG-10	MG_{200} 4F	**AGG GCC GTC T** CCG CAA GTG G	65
	MG_{200} 4R	**AGG GCC GTC T** TCT AGT GAT T	

The numbers preceding the R (reverse) and F (forward) refer to the approximate size of the SCAR band (bp). The nucleotides represent in bold is the sequence of the RAPD primers used.

shows some obvious modifications in RAPD profiles. The main observation or changes following the metal treatment were the difference in the intensities of bands, appearance of some new bands or absence of bands. *Hydrilla* and *Ceratophyllum* plants showed 15 and 18 new bands after treatment with Cd and Hg, respectively, with the primers OPG- 6, 7, 9, 11, 13, and OPB-20. However, treatment with Cu showed only 6 new bands in *Ceratophyllum* plant with primer OPG-9 and OPG-13. Extra band of 200 bp appeared with primer OPG-10 in Hg treated *Hydrilla* plants (Fig. 1c). The reproducibility of RAPD profiling confirmed that the variation in band intensities was stable (data not shown).

Changes in RAPD profiles were also measured as GTS (which is a qualitative measurement reflects changes in RAPD patterns) in relation to the pattern showed in the control plants. Results of GTS of the control and treated plants were compared with reduction in chlorophyll and protein contents. Figure 2 shows a reduction in chlorophyll, protein content as well as GTS compared to their controls in *Hydrilla*. The reduction in GTS, chlorophyll and protein contents were more pronounced in *Hydrilla* compared to *Ceratophyllum* (data not shown).

Polymorphic bands (ranging from 200–1000 bp with

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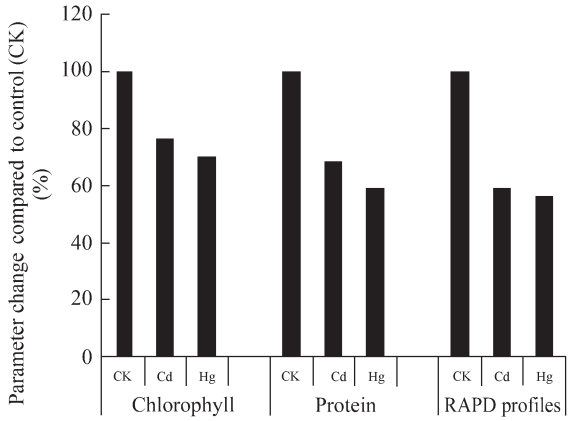

Fig. 2 Genomic DNA template stability as RAPD profiles was compared with chlorophyll and protein content in *H. verticillata* exposed to different metals for 96 h. CK: control; Cd: 10 μmol/L; Hg: 5 μmol/L.

OPB-20 and OPG-10) were eluted from *Hydrilla* plants treated with 10 μmol/L Cd and 5 μmol/L Hg were eluted and cloned in pGEM^T Easy vector. These polymorphic bands were sequenced and the one having 200 bp (Fig. 2) was submitted to NCBI database (Acc. No DQ136313). The size of the inserted DNA fragments was confirmed by both PCR (using operon primers and M13 universe and reverse primer) and restriction digestion analysis. However, the analysis of sequence did not reveal any similarity with any known sequences present in the genebank database.

The designed metal induced specific SCAR primer pairs (Table 2) were used to amplify genomic DNAs of *Hydrilla* plants. A single, distinct and brightly resolved band of the same size as the original RAPD fragment was obtained in control (500 bp) and Hg treated (200 bp) *Hydrilla* samples using MG1 and MG4 SCAR primers, respectively. Reduction of the annealing temperatures did not generate an alternate or extra allele other than the SCAR, confirming the specificity of the SCAR primers (Figs. 3a and 3b).

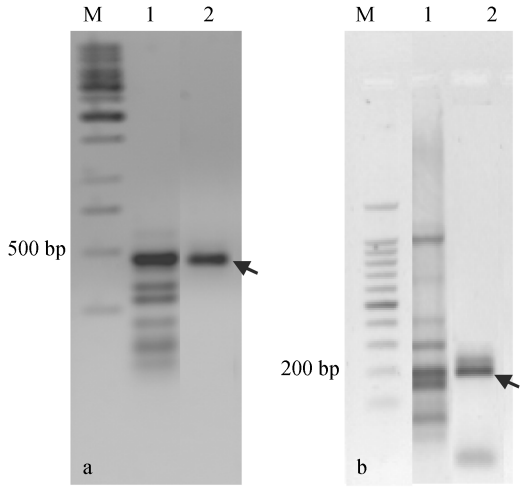

Fig. 3 Agarose gel profiles showing the amplification product from the genomic DNA of *Hydrilla*. (a) control plants, lane M: 1 kb DNA ladder; lane 1: operon primer; lane 2: SCAR primer. (b) Hg treated plants, lane M: 100 bp DNA ladder; lane 1: operon primer; lane 2: SCAR primer.

3 Discussion

The changes in DNA caused by genotoxic chemicals may be monitored using different biomarker assays both at biochemical and molecular level (Savva, 1998). RAPD profiles detect alterations in genomic DNA with the use of arbitrarily primed PCR reactions and clearly show promise in the detection of pollutant-induced DNA effects. However, it is only a qualitative method through which nature and amount of DNA can only be speculated. The present study shows the first report on metal induced RAPD profiling in aquatic plants. Changes observed in the DNA profiles such as modifications in band intensity and loss of bands may be due to the changes in oligonucleotide priming sites mainly due to genomic rearrangements, and less likely to point mutations or DNA damage in the primer binding sites or the presence of DNA photoproducts which can block or reduce the polymerization of DNA in the PCR reaction (Nelson *et al*., 1996). Appearance of new PCR products or appearance of bands could be attributed to the presence of oligonucleotide priming sites which become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations (resulting in new annealing events) or large deletions (bringing two pre-existing annealing sites closer) or homologous recombination (Atienzar *et al*., 1999). Presence of some extra band in this experiment (e.g., 200 bp in Hg treated *Hydrilla* plants) with OPG-10 primer showed that the effect can be involved in DNA repair and replication mechanism or it may also be the result of genomic template instability related to the level of DNA damage and the efficiency of DNA repair and replication (Atienzar *et al*., 1999). Furthermore, similar results with respect to metal toxicity changes in genetic pattern were also reported in *Arabidopsis thaliana* when exposed to Pb, Mn, Cd etc. (Conte *et al*., 1998) using the RAPD technique. Changes in DNA profiles also assessed under heavy metal stress in *Daphnia magna*, *Hordeum vulgare* and *Phaseolus vulgaris* (Atienzar *et al*., 2001; Liu *et al*., 2005; Enan, 2006). Lead, copper, and cadmium affect DNA of *Silene paradoxa*, kidney and barley plants showed the similar type of response as it was observed in the present study (Mengoni *et al*., 2000; Enan, 2006; Liu *et al*., 2005). Molecular characterization of contaminant-indicative RAPD markers has also been done in mosquitofish under radionuclide stress by Theodorakis and Bickham (2004). The results also indicated that the genomic template stability test was significantly affected by the metal stress which was further correlated with the decrease in chlorophyll and protein content. Similar effect on GTS was reported due to UV in a marine alga *Palnaria palnata* (Atienzar *et al*., 2000) and heavy metals in barley (Liu *et al*., 2005).

Presence and absence of certain bands gives limited information. More definitive analysis could be obtained after cloning and sequencing of RAPD bands. Therefore, the polymorphic bands obtained were cloned and sequenced. The gene sequences did not show similarity to any other known sequences present in the database. This is not

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surprising as the genome sequence information available from the aquatic plants is very limited. Secondly, the chances of operon primers used in the study to anneal at the intron regions of the genes cannot be ruled out. Finally, it will be interesting to use these cloned fragments as a probe to analyze the transcripts in *Hydrilla* by Northern blot technique before we can come to any concrete conclusion.

The present study showed that the RAPD primers are able to amplify PCR products from aquatic plants, but the knowledge towards the mutation being caused in these plants by heavy metal pollution is very limited. The sensitivity of the RAPD reaction to a number of reaction parameters at a low annealing temperature has failed to generate consistent profiles even under laboratory conditions, which necessitates designing SCAR primers from polymorphic RAPD bands. Identification of SCAR marker from aquatic plants under heavy metal exposure may have significant application in combating metal pollution in water bodies. Although the markers are not homologous to any known sequences but these may be novel markers for metal induced selection. These markers would be widely applicable to study the effect of contaminants on population genetics and its adaptation to different stresses.

4 Conclusions

The presented results showed that the DNA polymorphisms detected by RAPD analysis can be applied as a suitable biomarker assay for the detection of genotoxic effects of heavy metal contamination on plants. Molecular characterization of these markers would be able to indicate that such primers could amplify heavy metal induced changes in DNA and thus have wide applicability in toxicological study. Finally, further research is required to better understand the potential and limitations of the RAPD assay for the detection of DNA damage and mutations.

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References

Angelis K J, Mcguffie M, Menke M, Schubert I, 2000. Adaption to alkylation damage in DNA measured by the comet assay. *Environmental and Molecular Mutagenesis*, 36: 146–150.

Arnon D E, 1949. Copper enzymes in isolated chloroplast, polyphenol oxidase in Beta vulgaris. *Plant Physiology*, 24: 1–15.

Atienzar F A, Conradi M, Evenden A J, Jha A N, Depledge M H, 1999. Qualitative assessment of genotoxicity using random amplified polymorphic DNA: comparison of genomic template stability with key fitness parameters in *Daphnia magna* to benzo[a]pyrene. *Environmental Toxicology and Chemistry*, 18: 2275–2282.

Atienzar F A, Cordi B, Donkin M B, Evenden A J, Jha A N, Depledge M H, 2000. Comparison of ultraviolet-induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmate*. *Aquatic Toxicology*, 50: 1–12.

Atienzar F A, Cheung V V, Jha A N, Depledge M H, 2001. Fitness parameters and DNA effects are sensitive indicators of copper-induced toxicity in *Daphnia magna*. *Environmental Toxicology*, 59: 241–250.

Bradford A, 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Analytical Biochemistry*, 72: 248–254.

Conte C, Mutti I, Puglisi P, Ferrarini A, Regina G, Maestri E, Marmiroli N, 1998. DNA fingerprinting analysis by a PCR based method for monitoring the genotoxic effects of heavy metals pollution. *Chemosphere*, 37: 2739–2749.

De Wolf H, Blust R, Backeljiau T, 2004. The use of RAPD in ecotoxicology: A review. *Mutation Research*, 566: 249–262.

Enan M R, 2006. Application of random amplified polymorphic DNA to detect genotoxic effect of heavy metals. *Biotechnology and Applied Biochemistry*, 43: 147–154

Evans K M, James C M, 2003. Identification of SCAR markers linked to powdery mildew resistance in apple. *Theoritical and Applied Genetics*, 106: 1178–1183.

Gupta M, Chandra P, 1996. Bioaccumulation and physiological changes in *Hydrilla verticillata* (l.f.) Royle in response to mercury. *Bulletin of Environmental Contamination and Toxicology*, 56: 319–326

Gupta M, Tripathi R D, Rai U N, Chandra P, 1998. Role of glutathione and phytochelatin in *Hydrilla verticillata* (l.f.) Royle and *Vallisneria spiralis* L. under mercury stress. *Chemosphere*, 37(4): 785–800

Hernandez P, Martin A, Dorado G, 1999. Development of SCARs by direct sequencing of RAPD products: A practical tool for the introgression and marker assisted selection of wheat. *Molecular Breeding*, 5: 245–253.

Liu W, Li P J, Qi X M, Zhou Q X, Zheng L, Sun T H, Yang Y S, 2005. DNA changes in barley (*Hordeum vulgare*) seedlings induced by cadmium pollution using RAPD analysis. *Chemosphere*, 61: 158–167

Mengoni A, Gonelli C, Galardi F, Gabbrielli R, Bazzicalupo M, 2000. Genetic diversity and heavy metal tolerance in populations of *Silene paradoxa* L. (*Caryophyllaceae*): A random amplified polymorphic DNA analysis. *Molecular Ecology*, 9(9): 1319–1324.

Nelson J R, Lawerence C W, Hinkle D C, 1996. Thymine-thymine dimmer bypass by yeast DNA-polymerase-zeta. *Science*, 272: 1646–1649.

Paran I, Michelmore R W, 1993. Development of reliable PCR based markers linked to doeny mildew resistance genes in lettuce. *Theoritical and Applied Genetics*, 85: 985–993.

Savva D, 1998. Use of DNA fingerprinting to detect genotoxic effects. *Ecotoxicology and Environmental Safety*, 41: 103–106.

Steinkellner H, Kassie F, Knasmuller S, 1999. Tradescantia-micronucleus assay for the assessment of the clastogenicity of Austrian water. *Mutation Research*, 426: 113–116.

Theodorakis C W, Bickham J W, 2004. Molecular characterization of contaminant-indicative RAPD markers. *Ecotoxicology*, 13: 303–309.

Williams J, Kubelik A R, Livak K J, 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research*, 18: 6531–6535.

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