QUALITATIVE ASSESSMENT OF CADMIUM STRESS USING GENOME TEMPLATE STABILITY IN *Hordeum vulgare*

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Cadmium, a non essential heavy metal, does not play any metabolic role and is considered as toxic element even at very low concentration. It is mainly generated through anthropogenic activities, such as mining deposits, aerial fallout from smelters and from industrial, agricultural, energy and municipal sources (McGrath *et al*., 2001; McBride, 2003; Entezari *et al*., 2006). Cadmium (Cd) contamination has become a severe global issue and is considered a dangerous environmental pollutant not only for its neurotoxic, mutagenic and carcinogenic effects, but also for the high water solubility and thereby easier entry into human body via food (Koizumi and Yamada, 2003). Phytotoxicity of heavy metals such as Cd, Cu and Zn is long known and well documented (Prasad, 2004; Panda and Choudhury, 2005). Metal ions may directly interfere with the metabolic activities by altering the conformation of proteins, for example enzymes, transporters or regular proteins, owing to their strong affinities as ligands to sulfhydryl and carboxylic groups (Sharma *et al*., 2004).

Genotoxicity of Cd is directly related to its effect on structure and function of DNA, which may be determined using a number of laboratory methods (Angelis *et al*., 2000; Unyayar *et al*., 2006). However, there have been few direct experimental demonstrations of the wider relationships between DNA effects and their subsequent consequences at higher levels of biological organization. To address this issue, it is necessary to develop reliable and reproducible genotoxicity assays that can then be used in conjunction with traditional assays for detecting any impairment of population parameters (e.g. growth, reproduction and viability of offspring).

Recently, advances in molecular biology have led to the development of new tools for detection of genetic alteration in response to toxic chemicals tolerance at the level of DNA sequence and structure. DNA based techniques (RFLP, QTL, RAPD, AFLP, SSR and VNTR) are used to evaluate the variation at the DNA sequence level (Cenkci *et al.,* 2009). Random amplified polymorphic DNA (RAPD), developed by Williams *et al*. (1990) and Welsh and McClelland (1990), is a PCR-based technique that amplifies random DNA fragments of genomic DNA with single short primers of arbitrary nucleotide sequence under low annealing conditions. This technique is used extensively for species classification, genetic mapping and phylogeny etc. in addition, their use in surveying genomic DNA for evidence of various types of DNA damage

and mutational events (e.g., rearrangements, point mutation, small insert or deletions of DNA changes) in cells of bacteria, plants and animals (Atienzar *et al.,* 2000; Cenkci *et al*., 2010; Cambier *et al*., 2010). It is suggested that alterations in RAPD profiles due to genotoxic exposure can be regarded as changes in genomic template stability (GTS, a qualitative measure of genotoxic effects) (Atienzar *et al*., 1999).

It is known that, heavy metal hyperaccumulation potential of some plant species is very high (Ozturk *et al*., 2010). This feature makes hyperaccumulators highly suitable for phytoremediation, for clean-up of soil and water. On the other hand, an excess of toxic heavy metal ions induces several cellular stress responses and damage to different cellular components such as membranes, proteins and DNA (Patra *et al*., 1998; Waisberg *et al*., 2003; Jimi *et al*., 2004). Therefore, several plant species, such as *Allium cepa, Hordeum vulgare, Arabiodopsis thaliana, Vicia faba* and *Zea mays*. etc., have been used as good bioindicators of genetic toxicity of environmental pollutants in recent years. There are many studies on heavy metal uptake and accumulation by crop plants (Chlopecka, 1996; Otabbong *et al*., 1997; Ali *et al*., 2004; Al-Qurainy, 2009). On the other hand, tolerant food crops may be dangerous as carriers of toxic metals in the food chain leading to food toxicity.

Barley (*Hordeum vulgare*) has been identified as a plant for efficient uptake and accumulation of Cd with a phytoremediation potential equal to that of mustard plants (Ebbs and Kochain, 1998; Sridhar *et al*., 2007). To be efficient metal accumulator, plants require efficient detoxification and tolerance mechanisms at both the cellular and plant level. Hence it is important to study how the different plant and cellular characteristics are affected when Cd are accumulated in barley plants.

The aims of this study were to investigate the DNA changes induced by Cd using RAPD technique, and to compare changes in RAPD profiles with growth parameters such as root and shoot growth of barley seedlings in order to improve the knowledge about the capacity of Graminae species as phytoextractors. *Hordeum vulgare* was chosen as test species because of its worldwide economic importance and extensive cultivation.

MATERIALS AND METHODS

Plant material and growth conditions

The barley seeds used in this study were taken from a commercial variety obtained locally. Prior to starting the experiment, dry mature seeds of barley were soaked at 4°C for 2-3 days in the distilled water, and germinated to primary roots of 2mm long in a Petri dish containing a filter paper of 11 cm diameter at temperature of 15°C in the dark. Uniformly 30 germinated seeds were selected and transferred to plastic pots containing 1 kg of steam sterilized soil at 2.0 cm depth. Pots were incubated in a growth chamber at tem-

perature of 23±1°C and light/dark photoperiod of 15 h light/ 8h dark.

Experimental design

The experiments conducted in the present study were set-up in triplicate, Modified Hoagland's nutrient solution (Hoaglands and Arnon, 1950) was supplied to the plants three times per week. To avoid Cd precipitation, the pH nutrient solutions was adjusted to 6.0. To stimulate heavy metal contamination in soil, barley plant groups were supplied with 0 (control), 10, 20 and 40 ppm Cd, respectively, (in the form of $CdCl_2.2H_2O$, Sigma, grade). After 15 days of sowing, sampling was done to test the toxicity of Cd solutions on plant growth.

The growth inhibition test was performed on the above barley plants, the root and shoot lengths were measured. Inhibitory rate (IR%) of the above indices was calculated by the following formula: IR= 1-(x/y)x100 where x and y are the average values detected in the treated sample and control, respectively.

Genomic DNA isolation

After 15 days of sowing, leaves of 5-8 seedlings were used for genomic DNA extractions. DNA was extracted using the procedure reported by Doyle and Doyle (1990). DNA concentrations of total genomic DNA in each sample were estimated using a spectrophotometer (TU-1880 Double Beam UV-VIS). Electrophoresis of DNA samples was performed on 0.8% agarose gel using λ-*hindIII* DNA as a molecular weight marker.

RAPD procedures

The RAPD-PCR experiments were conducted according to the method of Williams *et al*. (1990) and seven random decanucleotide primers were used in this study (Table 1). Amplification was performed in a DNA thermal cycler (Techne Thermal cycler) using the following conditions: the initial 2 min denaturation at 94°C was followed by 44 cycles of; 1 min at 94°C, 1 min 36°C, 2 min 72°C and final extension at 72°C for 5 min. Negative controls with water, replacing the template DNA, were always included to monitor for contamination. Electrophoresis of RAPD reaction products was preformed on 1.4% agarose gel. 100 bp DNA ladder (Fermentas) was used as molecular size DNA standard. DNA bands were stained with 0.5 mg/ml ethidium bromide, visualized and photographed under UV light. The size of each amplification product was automatically estimated using the UVI soft image analyzer system.

Estimation of genomic template stability

Genomic template stability (GTS%) was calculated as the following: GTS= 1- a/n) x100 Where a is the average number of polymorphic bands detected in each treated sample and n is the number of total bands in the control (Ateinzar *et al*., 1999). Polymorphism in RAPD profiles included disappearance of normal band and appearance of a new band compared to control were measured. The average was calculated for each experimental group exposed to different Cd treatments.

To compare the sensitivity of each parameter, changes in these values were calculated as a percentage of their control.

Statistical analysis

The statistical analyses were carried out using SPSS program (Nie *et al*., 1975). Changes in root and shoot growth were tested statistically by performing one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Evaluation o f plant growth

Phytotoxicity assessment was performed to evaluate the inhibitory effect of Cd-contaminated soil using general toxicological endpoints such as root growth and shoot length. The results showed that root length was significantly inhibited (Table 2 & Fig. 2) in response to increase of 10, 20, 40 ppm Cd concentrations compared to control plantlets, indicating a dose-dependent response. On the other hand, shoot length was non significantly inhibited suggesting that Cd induced a significant reduction in root elongation of barley, whereas stem elongation was not affected. In a previous study on *H. vulgare*, Ali *et al*. (2004) reported that shoots continue their growth after complete inhibition of root growth by several metals suggesting that metals are mainly accumulated in the roots of barley.

The change of RAPD Profile

In eco-genotoxicology, the effective evaluation and proper environmental monitoring of potentially genotoxic pollutants will be improved with development of sensitive and selective methods to detect toxicant-induced alterations in the genome of a wide range of biota (Atienzar *et al*., 1999; Theodorakis *et al*., 2001; Liu *et al*., 2007). The changes in DNA caused by genotoxic chemicals may be monitored using different biomarker assays both at biochemical and molecular level (Savva, 1998).

Aimed at verifying the genetic effect of Cd contamination, the RAPD analysis was performed in the present study on DNA extracted from groups of 5-8 seedlings from each replicate treated with 10, 20, 40 ppm CdCl2 treatments. Seven 10-mer oligonucleotide primers were utilized to discriminate controls from barley plants contaminated with $CdCl_2$. These primers were able to produce reproducible RAPD with template DNA from control and treated barley plantlets. The RAPD analysis yielded 7-21 bands in control (Fig. 1). Polymorphism was evidenced as the presence and/or absence of DNA fragments between the samples. A total of 102 amplification products (loci) from the seven primers were identified in the control seedlings ranging from 142-2599 bp in molecular size (primer C4 and primer C6, respectively (Table 3 and Fig. 1). RAPD patterns generated by the Cd-exposed plantlets were dramatically different from those obtained using control DNA. Different polymorphic bands were detected at each concentration of Cd for different primers. The detected values of polymorphisms P% were 40.1%, 55.8%

and 57.8% for 10, 20 and 40 ppm, respectively.

Previous studies have showed that changes in band patterns observed in DNA fingerprint analyses reflected DNA alterations from single base changes (point mutations) to complex chromosomal rearrangements (Atienzar *et al*., 1999; Atienzar and Jha, 2006; Ozturk *et al*., 2010). Similarly, in the present study, the modifications to genomic DNA were detected by RAPD profiles through the randomly primed PCR reactions. The principal events that were observed in the RAPD patterns to occur subsequent to Cd-exposure were: loss of normal bands, and appearance of new bands in comparison with the normal control plants. Almost, the seven primers detected more than one such alteration in a given sample. In this sense, the obvious disappearance of normal bands and appearance of new bands generated from the exposed seedlings were determined generally as PCR products with high molecular weight. The maximum number of disappearing RAPD bands was found to be at two higher concentrations (20 and 40 ppm) with primers A2 and C4. The bands of molecular size approximately 142-2599 bp disappeared and extra bands (two to four new PCR amplification product) were appeared at all concentrations with molecular size approximately of 152-2240 bp (Fig. 1 & Table 4).

The disappearance of bands may be attributed to the presence of DNA photoproducts (e.g. pyrimidine dimers), which can act to block or reduce the polymerization of DNA in the PCR reaction (Donahue *et al*., 1994; Nelson *et al*., 1996; Atienzar *et al*., 2000). It is suggested that the DNA damage may be serious in the majority of cells of barley seedlings exposed to toxic chemicals. At higher Cd concentrations, it seems that the extent of DNA lesion is so important that the *Taq* DNA polymerase is more often blocked which implies a disappearance of band (depending on the extent of DNA damage). The disappearance of PCR products mainly affected the high molecular weight bands because the odds of obtaining DNA photoproducts increase with the length of the amplified fragment (Ateinzar *et al*., 2000). On the other hand, mutations (new annealing events) can only be responsible for the appearance of new bands if they occur at the same locus in a sufficient number of cells. A minimum of 10% of mutations may be required to get new PCR product visible in agarose gel to be amplified by PCR (Ateinzar *et al*., 2000). Thus, the new bands could be attributed to mutations while the disappeared bands could be attributed to DNA damage.

Endpoint sensitivity

Qualitative analysis has shown considerable variation among the treated and control plantlets. In this experiment, the genomic template stability was used to reflect changes in RAPD profiles and to compare the changes of RAPD profiles with modifications in traditional growth parameters (root and shoot length) in barley plantlets. Following exposure to as-

cending Cd concentration, both root growth and GTS of exposed plants decreased gradually according to control group (Fig. 2). However, shoot length was less sensitive in comparison to the two parameters. Inhibition of root growth is regarded as the first obvious effect of heavy metals in plants (Ali *et al*., 2004).

Genomic template stability stabilized after 20 ppm Cd of exposure. According to Liu *et al*. (2007), this result could be attributed to multiple changes in RAPD profiles (e.g. appearance of new bands, disappearance of normal bands). The disappeared band was compensated by the low frequency of newly appearing bands at 20 or 40 ppm Cd concentration. On the other hand, GTS is related to the level of DNA damage, the efficiency of DNA repair and replication. The results revealed that for Cd concentrations used, DNA replication was dramatically reduced due to higher level of DNA damage and it can be assumed that DNA damages were repaired to some extent and that DNA replication was not totally inhibited.

DNA fingerprinting offers a useful biomarker assay in toxicology study, changes in RAPD profiles induced by pollutants can be regarded as changes in genomic DNA template stability and this genotoxic effects can be directly compared with alterations in other parameters. RAPD analysis have been reported as more sensitive test than classic genotoxic tests since it was capable of detecting temporary DNA changes that may not finally manifest themselves as mutations (Labra *et al*., 2003; Liu *et al*., 2007). The present results of Cd exposure in barley seedlings are consistent with the earlier studies on other organisms (Enan, 2006; Liu *et al*., 2007; Cambier *et al*., 2010).

Finally, a comparison between the untreated and treated genomes revealed that the RAPD analysis can be employed to evaluate the manner in which the environmental pollutants modify the structure of the DNA present in *H. vulgare*. The effects of each category of DNA damage (e.g., strand breakage, modified bases, and bulky adducts) on the RAPD profiles can only be speculated when the amplicons are analysed (e.g., sequencing) and more specific methods, such as the comet assay and 32P-postlabelling assay are needed to obtain a quantitative data (De Wolfe *et al*., 2004).

The conclusions of the research conducted in the present study are: 1) the RAPD analysis is a highly sensitive method for the detection of DNA damage induced by environmental pollutants like toxic Cd, 2) the growth parameters most sensitive to Cd exposure is root growth, 3) increasing the concentration of Cd exposure affects the genomic template stability and may induce a gain or loss in the number of bands and 4) the genome template stability is a highly sensitive parameter compared with the traditional indices such as root growth and shoot length. Thus, changes at the DNA level may be the precursors of some of the numerous effects reported at higher levels of biological organization. This study suggests that the

RAPD analysis used in conjunction with other biomarkers from higher levels of biological organization such as growth parameters can be a powerful tool for identifying DNA damage induced by Cd contamination, which may be useful for risk assessment of environmental contamination and remediation.

SUMMARY

Assessment of genotoxins-induced DNA damage and mutations at molecular level is important in eco-genotoxicology. In this research, RAPD was used to detect DNA damage in Barley (*Hordeum vulgare*) seedling exposed to toxic ascending cadmium (Cd) at concentrations of 10, 20, 40 ppm for 15 days. Substantial inhibition of root growth was observed with an increase in the Cd concentration, whereas shoot growth was non significantly inhibited compared to the unexposed plantlets. For the RAPD analyses, seven RAPD primers were found to produce a total of 102 bands with molecular size of 142-2599 bp of control seedlings. Results produced from RAPD analysis indicated that the evident changes occurred in DNA of exposed barley seedlings included gain or loss of bands compared with the control seedlings. New amplified fragments at molecular size from approximately 152-2240 bp appeared almost for 10, 20, and 40 ppm Cd with seven primers, and the number of missing bands was dose- dependent. The results suggested that genomic template stability (GTS) reflecting changes in RAPD profiles was the most sensitive endpoint compared with the traditional indices such as root and shoot growth. The DNA polymorphisms detected by RAPD profiles can be applied as a tool in risk assessment of Cd stress on plants.

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Table (1): The sequences of the primers used for RAPD-PCR amplification.

List of primers	Primer	Sequences of primers (5'-3')
1	C6	CTG CGGGACT
2	A2	TGCCATGCTG
3	C4	GTCCGCTGGA
4	D1	ATGAGGTCTG
5	G4	ATGGTGCTGA
6	B1	TCATGCCAGG
7	D5	TCTCGCCCAG

Table (2**):** Effects of Cd stress on root length and shoot length.

Cd concentration (ppm)	Root in barley seedling		Shoot in barley seedlings	
	Root length (cm)	Inhibitory rate%	Shoot length (cm)	Inhibitory rate%
0	11.3 ± 0.5	0.0	4.90 ±1.03	0.0
10	9.6± 0.3*	15.04	4.77± 0.61^{ns}	2.65
20	8.1± 0.3*	28.30	4.50± 0.39^{ns}	8.10
40	7.2± 0.4**	36.20	4.4±0.15^{ns}	10.20

*, ** indicate values that differ significantly from the control at $p<0.05$, $p<0.01$ respectively, and ns (not significant).

Table (3): The number of bands in control with all primers and polymorphic bands in Cd-exposed barley plants.

Primers	Cd concentration (ppm)						
	0	10		20		40	
		a	*b*	*a*	*b*	*a*	*b*
B1	7	3	2	3	2	2	2
G4	9	3	3	3	3	4	3
A2	15	2	4	1	13	1	14
D5	17	2	4	4	4	3	4
D1	17	2	1	1	5	1	6
C6	16	3	3	2	5	2	6
C4	21	4	5	4	7	3	8
Total bands	102	19	22	18	39	16	43
a+b		41		57		59	

a: indicates appearance of new bands, b: disappearance of normal bands, a + b denote polymorphic bands.

Table (4): Molecular sizes (base pair, bp) of disappearance (-) and appearance (+) of DNA bands with all primers in Cd- treated barely plants.

Primers		Cd concentrations (ppm)		
		10	20	40
B1	+	310; 540; 900 bp	310; 540; 900 bp	310; 540 bp
	-	280; 650 bp	280; 650 bp	280; 650 bp
G4	+	652; 899; 2240 bp	652; 899; 2240 bp	495; 652; 899; 2240bp
	-	799; 1000; 1200 bp	799; 1000; 1200 bp	799; 1000; 1200 bp
A2	+	152; 1340 bp	152 bp	152 bp
	-	197; 232; 1526; 1646 bp	197; 232;472; 480; 556; 612; 703; 756; 940; 1021; 1251; 1526; 1646 bp	197; 232; 428; 472; 480; 556; 612; 703; 756; 940; 1021; 1251; 1526; 1646 bp
D5	+	571; 944 bp	428; 571; 944; 1243 bp	428; 571; 944 bp
	-	538; 550; 604; 900 bp	538; 550; 604; 900 bp	538; 550; 604; 900 bp
D1	+	285; 723 bp	723 bp	723 bp
	-	1155 bp	800; 1155; 1248; 1861; 1984 bp	800; 1155; 1248; 1861; 1984 bp
C6	+	501; 610; 700 bp	610; 700 bp	610; 700 bp
	-	2100; 2488; 2599 bp	1105; 1702; 2100; 2488; 2599 bp	1105; 1702; 1876; 2100; 2488; 2599 bp
C4	+	801; 1074; 1399; 1532 bp	801; 1074; 1399; 1551 bp	801; 1074; 1551 bp
	-	142; 306; 500; 833; 1149 bp	142; 306; 430; 492; 703; 905; 1302 bp	142; 306; 430; 492; 703; 905; 1155; 1302 bp

(+) = appearance of new band; (-) = disappearance of a normal band.

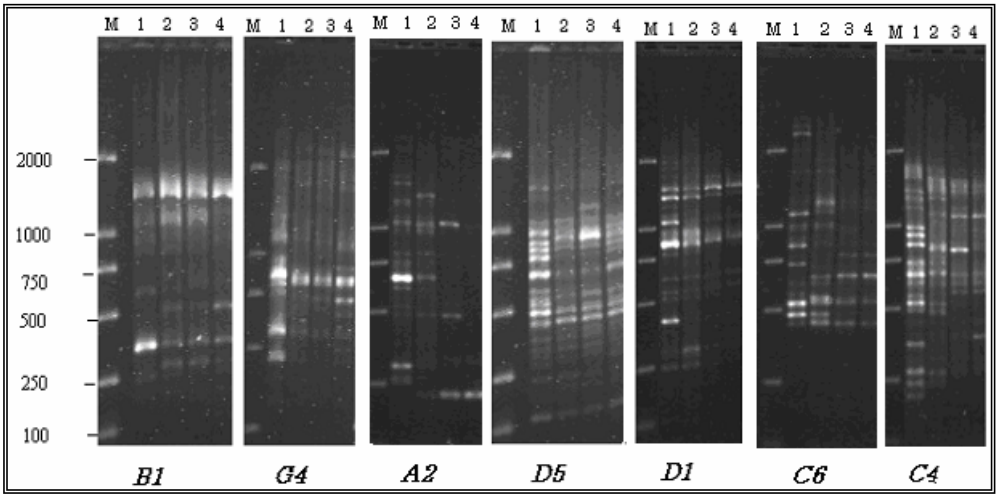

Fig. (1): RAPD profiles of genomic DNA from leaves of barley seedlings exposed to Cd. Lane 1= control; 2 = Cd (10 ppm); 3= Cd (20 ppm); and 4 = Cd (40 ppm), respectively. M= DNA ladder (100 bp).

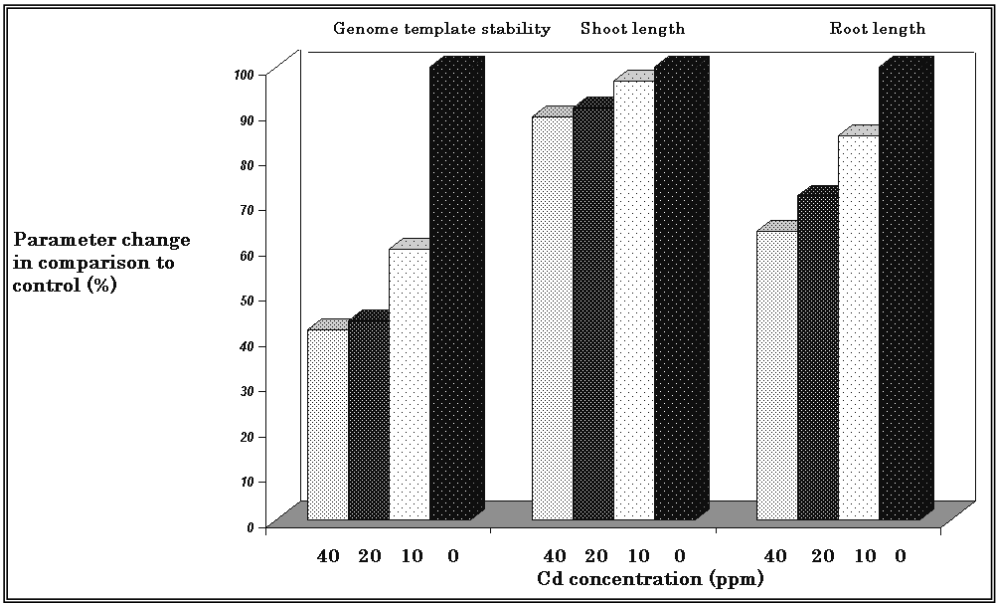

Fig. (2): Comparison between root growth, shoot length and genomic DNA template stability in barley seedlings exposed to various Cd levels.