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Full Length Research Paper

RAPD profile for the assessment of genotoxicity on a medicinal plant; *Eruca sativa*

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***Eruca sativa* (family: Brassicaceae) has gained an importance as a vegetable and spice, especially among Europeans. The genotoxicity of three heavy metals, viz., Zn, Pb and Cd was studied on *E. sativa* which showed a dose-dependent effect on radicle and coleoptile lengths. The radicle length was more affected as compared to coleoptiles length under all tested concentrations. The ranking of genotoxic potencies of three heavy metals was as in descending order: Cd^{2+}> Pb^{2+}≥ Zn^{2+}. The high concentration of Cd and Pb at 150 mg/l showed genotoxic effects, thereby length (cm) of radicle and coleoptile were decreased at high concentration of Cd as compared to low, medium and high concentrations of Pb and Zn. Random amplified polymorphic DNA (RAPD) technique was used for detection of genotoxicity produced by these metals. Twenty decamer primers were used, of which four did not amplify, three gave single and polymorphic band and the rest of thirteen primers generated upto 5 bands (an average of 4 bands per primer). Sixteen primers showed amplified products as monomorphic, whereas three primers (OPC-11, OPC-12, and OPC-13) showed unique extra band from seedlings treated with medium and high concentrations of Cd, Pb and Zn respectively. Genetic divergence among the seedlings was evaluated with dendrogram and similarity matrix value was obtained from 47.83 - 95.83%.**

Key words: Environmental pollutant, genetic toxicity, heavy metals, mutation.

INTRODUCTION

Eruca sativa L., commonly known as rocket plant belongs to Brassica plant family, contains more than 350 genera. It is immensely used as vegetable and spice. *E. sativa* originated in the Mediterranean region and now is found around the world. The leaves are immensely consumed as salad in some European countries due to their hot pungent taste. The plant also has a widespread medicinal uses. The essential oil from the leaves was characterized by a high content of sulfur- and nitrogen-containing compounds. The essential oil extracted from the leaves of *E. sativa* contains 67 volatile compounds which represents 96.52% of the oil (Mitsuo et al., 2002). The oil from seed of *E. sativa* has promising pharmacological efficacy and potential bio-active compounds as compared to different aerial and root plant extracts (Khoobchandani et al., 2010). The main active constituents found in leaves are 4-methylthiobutylisothiocyanate and 5-methylthio-pentanonitrile with (60.13%) and (11.25%) respectively. Some species of Brassicaceae strongly inhibit tumorigenesis (Lynn et al., 2006; van Poppel et al., 1999; Verhoeven et al., 1996; Verhoeven et al., 1997). The extract of *E. sativa* has erucin and erysolin which showed antigenotoxicity on human hepatoma (HepG2) cells

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towards benzo(a)pyrene induced toxicity (Lamy et al., 2008) and this property may also due to the non-nutrient compounds produced in the enzymatic cleavage of glucosinolates, named isothiocyanates (ITCs) (Conaway et al., 2002; Hecht, 1999; Keum et al., 2005). These ITCs are formed by a reaction of glycosinolates with endogenous enzyme myrosinase, which is released by chopping or chewing the vegetables (Holst and Williamson, 2004). ITCs showed protective effect against genotoxicants as they modulate the activity of enzymes involved in biotransformation (Steinkellner et al., 2001a). Recent studies have focused on volatile and non-volatile compounds of *E. sativa* which has ten different ITCs (Bennett et al., 2006; Jirovetz et al., 2002). The ethanolic extract of *E. sativa* seeds showed antioxidant activity and exert a protective effect on mercuric chloride induced renal toxicity (Alam et al., 2007). Its extract also possesses anti-secretory, cytoprotective, and anti-ulcer activities against gastric lesions (Alqasoumi et al., 2009).

In recent years, attention has focused for investigating the occurrence of genotoxic agents in the environment. Increasing concern of the general public and of governments for the welfare of humans and natural environments requires the assessment of new sensitive and efficient methods for early detection of environmental genotoxic risk. The difficulties arising from direct chemical measurements of pollutants in the field and the interpretation of such measurements in terms of bioavailability have stimulated a strong interest in bioindicators and biomarkers (Lowry, 1995). Bioindicators of contamination make it possible to detect subtle forms of pollution that are hard to measure in the field.

Plants are good bioindicators because they play a significant role in food chain transfer and in defining habitat. They are easy to grow and adaptable to environmental stress, and can be used for the assessment of environmental conditions in different habitats. Furthermore, plant-based assays applied for toxicity evaluation in the field would reduce animal sacrifice and testing costs. The use of plants as bioindicator for evaluation of genotoxicity has been reported in several studies (Grant, 1994; Knasmuller et al., 1998).

The mutagenic activities of chemicals has been analyzed with different plant systems such as *Allium cepa* (Fiskesjo, 1997), *Vicia faba* (Koppen and Verschaeve, 1996), *Trifolium repens* (Citterio et al., 2002), and *Tradescantia virginiana* (Fomin et al., 1999). The heavy metal damage in plants has been detected with chromosome aberration assays, mutation assays, cytogenetic tests and specific locus mutation assays (Constantin and Nilan, 1982; Tardiff et al., 1994) respectively. Recently, tremendous advances and developments in molecular biology have provided new ways of detecting DNA damage on plants (Conte et al., 1998; Savva, 2000; Citterio et al., 2002). The detection of genotoxicity with DNA marker has many advantages over other markers.

Some heavy metals at low doses are essential micronutrients for plants, but higher doses of these may cause metabolic disorders and growth inhibition for most of plants species (Claire et al., 1991). Researchers have observed that some plants species are endemic to metalliferous soils and can tolerate greater than usual amounts of heavy metals or other toxic compounds. The heavy metals mainly Pb, Cu, Mn and Cd affect DNA integrity in plant cells.

The effect of different concentra-tions of Pb was examined on seed germination, seedling growth and some metabolites of *E. sativa* (Faheed, 2005). A considerable reduction in fresh and dry matter as well as shoot and root length was obtained as a result of increasing Pb concentrations. Approximately 400 plants that accumulate high content of metals have been reported in recent years and these belong to family, Brassicaceae, Euphorbiaceae, Asteraceae, Lamiaceae or Scrophulariaceae (Macnair and Tansley, 1993). The largest group of these plant called 'metal hyperaccu-mulators' and found in the genus *Alyssum* (Brassicaceae), in which Ni concentrations can reach upto 3% of leaf dry biomass (Kramer et al., 1996). In few studies, the seeds have been exposed to the contaminants (Claire et al., 1996; Vojtechova, 1991; Xiong, 1998) and genotoxic effects were observed.

Randomly amplified polymorphic DNA (RAPD) is a simple technique and require little amount of DNA for PCR amplification and can be used for genotoxicity assessment. The resulting DNA profiles may differ due to band shifts, missing bands or the appearance of new bands. These bands are 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 the cells of bacteria, plants and animals (Savva, 1998; Atienzar et al., 2000). Since, *Eruca sativa* is a heavy metal accumulator, but high concentration of these metals damage to plant cells and cause genotoxicity. In this paper we evaluated the use of RAPD to detect DNA damage on *E. sativa* caused by heavy metals.

MATERIALS AND METHODS

Eruca sativa seeds were obtained from the local market of Riyadh and the experiment was conducted at Department of Botany and Microbiology, College of Science, King Saud University, Kingdom of Saudi Arabia. The solution of Zn, Pb and Cd were prepared in autoclaved deionized distilled water. The low, medium and high concentrations of these heavy metals were selected after screening of various concentrations on seed germination in petriplate. The low, medium and high concentrations of metals used for seed treatment

were as: 50, 100, and 150 mg/l, respectively.

The seeds were immersed in 3% v/v formaldehyde solution for five minutes to remove fungal contamination thereafter the seeds were washed with deionized water for three times to remove excess formaldehyde. Approximately, 20 seeds were put on Whatman filter paper and covered it with another filter paper, labeled and added 5 ml solution of each concentration. The top of the plates was closed, kept in a germinator at 25°C. Each treatment was replicated three times for statistical purposes. The radicle and coleoptile lengths were measured in 8 days old seedlings and compared with untreated seedlings. The seedlings were harvested after two weeks for the assessment of genotoxicity produced from different heavy metals.

Genomic DNA isolation for PCR analysis

Reagents and chemicals

The stock solution concentration were: cetyl trimethyl ammonium bromide (CTAB) 3% (w/v), 1 M Tris-Cl (pH 8), 0.5 M EDTA (pH 8), 5 M NaCl, absolute ethanol (AR grade), chloroform: Isoamylalcohol (24:1 [v/v]), polyvinylpyrrolidone (PVP) (40 000 mol wt) (Sigma), β-mercaptoethanol. All the chemicals used in the experiments were of analytical grade. The extraction buffer consisted of CTAB 3% (w/v), 100 mM Tris-Cl (pH 8), 25 mM EDTA (pH 8), and 2 M NaCl respectively. The PVP and β-mercaptoethanol were freshly prepared and added in the extraction buffer.

DNA extraction

DNA was isolated from seedlings using a modified CTAB method (Khan et al., 2007). The young seedlings were ground into extraction buffer (100 mM Tris buffer pH 8, 25 mM EDTA, 2 M NaCl, 3% CTAB, 3% PVP). The suspension was gently mixed and incubated at 65°C for 20 min with occasional mixing. The suspension was then cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged at 12,000 rpm for 5 min. The clear upper aqueous phase was then transferred to a new tube and added 2/3 volume of ice-cooled isopropanol and incubated at -20°C for 30 min. The nucleic acid was collected by centrifugation at 10,000 rpm for 10 min. The resulting pellet was washed twice with 80% ethanol. The pellet was air-dried under a sterile laminar hood and the nucleic acid was dissolved in TE (10 mM Tris buffer pH 8, 1 mM EDTA) at room temperature and stored at 4°C until used. The RNA from crude DNA was eliminated by treating the sample with RNase A (10 mg/ml) for 30 min at 37°C. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 and 280 nm. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide.

PCR amplification (McClelland et al., 1995) was performed with arbitrary decamer primers obtained from Sigma Company Technology, USA. The PCR reaction was carried out in 20 µl volume of master mixture purchased from Amerson Company (UK). In master mixture, 30 ng of template DNA and 30 ng of primer were added in each tube. Tubes were vertexed and briefly centrifuged after adding template DNA and primer in master mixture. The amplification was done on 96 well plates on a Primus PCR machine as per the programme: First denaturation at 94°C for 3 min, segment denaturation at 94°C for 1 min, annealing at 35.5°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 3 min was performed for amplification.

RESULTS AND DISCUSSION

DNA fingerprinting allows precise, objective and rapid identification of plant populations. The changes in DNA caused by genotoxic chemicals may be monitored using different molecular markers at biochemical as well as molecular level (Savva, 1998). RAPD detects alterations in the genomic DNA and clearly shows the detection of DNA damage induced by pollutants. However, it is only a qualitative method through which nature and amount of DNA can be speculated. RAPD is a reliable, sensitive and reproducible and has the potential to detect a wide range of DNA damage (e.g. DNA adducts, DNA breakage) as well as mutations (point mutations and large rearrangements), therefore can be applied to genotoxicity and carcinogenesis studies (Atienzar and Jha, 2006). The disappearance of a normal RAPD product may be related to the events such as DNA damage (e.g. single and double-strand breaks, modified bases, a basic sites, oxidized bases, bulky adducts, DNA–protein cross-links), point mutations and/or complex chromosomal rearrangements induced by genotoxins (Atienzar et al., 1999, 2000).

In our study all three heavy metals Cd, Pb and Zn showed a serious damage on radicle and coleoptile lengths and it was dose dependent. All treated seedlings with metals were compared with untreated seedlings. Among three heavy metals Cd showed strong inhibitory effect on morphological as well as DNA markers. At high concentrations of Zn, Pb and Cd, the radicle lengths (cm) was 1.56 ± 0.16, 0.73 ± 0.12 and 0.43 ± 0.080, as compared with untreated seedlings which was found 4.5 ± 0.13 (cm) (Table 1). Likewise, at high concentrations of Zn, Pb and Cd, the coleoptile lengths (cm) was 3.05 ± 0.17, 2.93 ± 0.04 and 1.00 ± 0.04, as compared with untreated seedlings which was found 5.2 ± 0.21(cm) (Table 2). The radicle length was more affected than the coleoptiles length (Figure 1). The root length was more sensitive parameter than the shoot length at each Pb concentration in *E. sativa* (Faheed, 2005). The high concentration of Cd (150 mg/l) had strong inhibitory effect on radicle as well as coleoptile lengths and its concentration was more accumulated in the radicle (Figure 1j). In initial experiment a total of 20 primers were screened on genomic DNA of treated and untreated rocket samples. Out of 20 primers, four could not amplify the genomic DNA and three gave extremely faint and ambiguous bands. Remaining sixteen primers produced visible and reproducible bands, which further used to amplify genomic DNA from all treated and untreated seedlings. Three primers produced only single band, whereas thirteen primers produced up to 5 bands with an average of 4 bands per primer. Different size band obtained in RAPD profile was from 250 - 2000 bp. An example of RAPD patterns generated by representative primer sets OPC-11, OPC-12 and OPC-13 are shown in (Figures 2, 3 and 4). Changes

Table 1. Effects of various concentrations of heavy metals on radicle length (cm) of *Eruca sativa* (L.).

Zn treatment		Pb treatment		Cd	
Control	4.50 ± 0.13	Control	4.50 ± 0.13	Control	4.50 ± 0.13
a_l	3.20 ± 0.16	b_1	3.43 ± 0.09	c_1	2.53 ± 0.24
d_g	2.20 ± 0.21	e_m	1.50 ± 0.16	f_m	1.83 ± 0.12
g_h	1.56 ± 0.16	h_h	0.73 ± 0.12	i_h	0.43 ± 0.080

Note: Each value is mean ± SD for three replicates in each group
l: low concentration
m: medium concentration
h: high concentration.

Table 2. Effects of various concentrations of heavy metals on coleoptile length (cm) of *Eruca sativa* (L.).

Zn treatment		Pb treatment		Cd treatment	
Control	5.20±0.21	Control	5.20±0.21	Control	5.20±0.21
a_l	3.56±0.16	b_1	4.16±0.12	c_1	3.76±0.20
d_g	3.53±0.04	e_m	3.20±0.16	f_m	3.00±0.16
g_h	3.05±0.17	h_h	2.93±0.04	i_h	1.00±0.04

Note: Each value is mean ± SD for three replicates in each group
l: low concentration
m: medium concentration
h: high concentration.

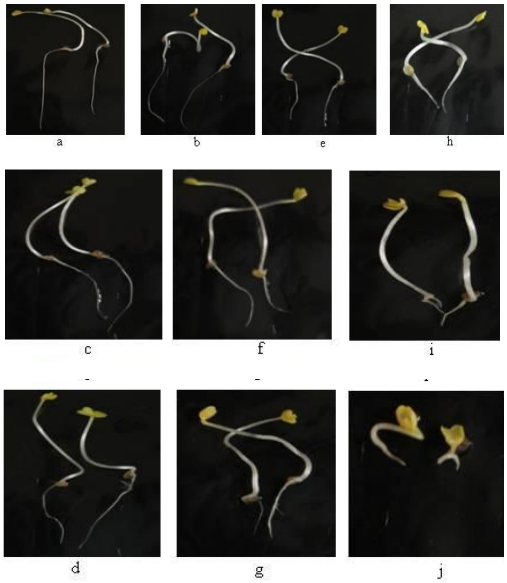

Figure 1. 8 days old seedlings grown in glass petriplate treated with various concentrations of heavy metals. a- control; b- 50 mg/l (Zn); e- 100 mg/l(Zn); h- 150 mg/l (Zn); c- 50 mg/l (Pb); f- 100 mg/l (Pb); i- 150 mg/l (Pb); d- 50 mg/l (Cd); g- 100 mg/l (Cd); j- 150 mg/l (Cd).

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 (Nelson et al., 1996). Appearance of new 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). The Low concentration of Cd (50 mg/l) also created mutation and produced more number of unique fragments in PCR amplification. DNA from seedlings of *E. sativa* exposed to heavy metals solutions displayed polymorphic fragments which were not detectable in DNA of unexposed plants. The unique fragments produced with primer OPC-11 of sizes 650 bp (Figure 2), and 1800 and 500 bp(Figure 4) were found with primer OPC-13 at low concentration of Cd (50 mg/l) respectively. The high concentration of Cd produced more unique band at genotoxic dose of these heavy metals but high concentration of Zn (150 mg/l) did not created a unique band. All sixteen responding primers, except three

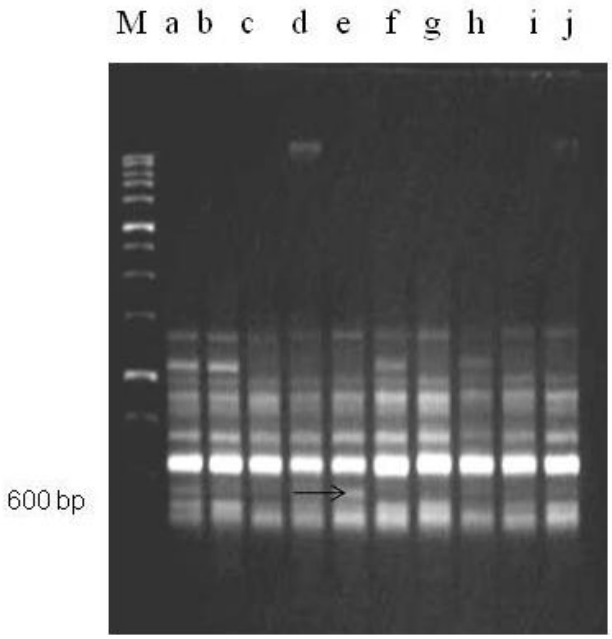

Figure 2. RAPD fingerprints for *Eruca sativa* treated with various concentrations of heavy metals with primer OPC-11. Lane M- 1 kb DNA ladder; Lane a- control; Lane- b, c, d (seeds treated with Zn, Pb and Cd with concentration 50, 50 and 50 mg/l,). Lane- e, f, g (seeds treated with Zn, Pb and Cd with concentration 100, 100 and 100 mg/l). Lane- h, i, j (seeds treated with Zn, Pb and Cd with concentration 150, 150 and 150 mg/l).

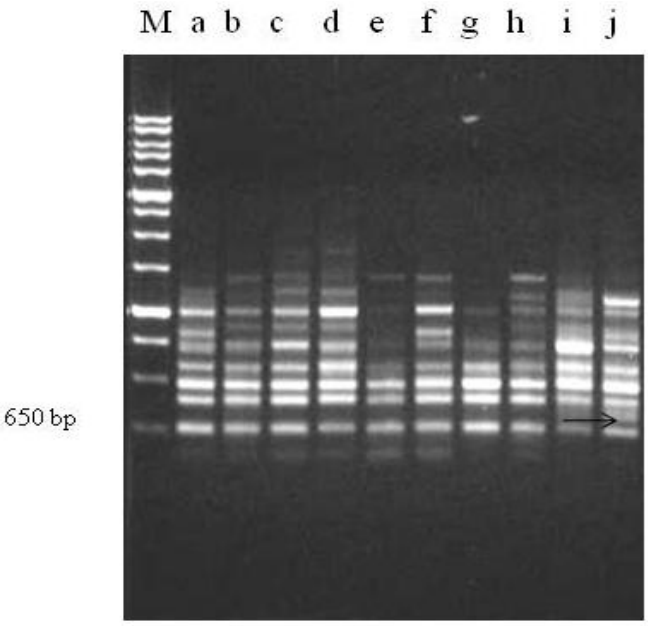

Figure 3. RAPD fingerprints for *Eruca sativa* treated with various concentrations of heavy metals with primer OPC-12. Lane M-1 kb DNA ladder; Lane a; control; Lane-b, c, d (seeds treated with Zn, Pb and Cd with concentration 50, 50 and 50 mg/l). Lane- e, f, g (seeds treated with Zn, Pb and Cd with concentration 100, 100 and 100 mg/l). Lane- h, i, j (seeds treated with Zn, Pb and Cd with concentration 150, 150 and 150 mg/l).

three, produced monomorphic banding patterns indicating a high degree of homogeneity in the rocket seedlings which differed from each other morphologically. The three primers (OPC-11, OPC-12 and OPC-13) revealed polymorphic and unique band which repeated three-times for their reproducibility and same results was found in each experiment.

Some fragments in *E. sativa* were absent at low, medium and high concentrations of Cd while these were present in seedlings treated with Zn and Pb respectively. The seedlings obtained at high and medium concentrations of Cd and Pb showed more bands as compared with low concentrations and control. Further, RAPD has been used in many plants for detection of genotoxic doses. It was used in Cd treated barley and changes occurred in RAPD profiles of the root tips seen as with alterations in band intensity and loss of bands compared with the control seedlings (Liu et al., 2009). DNA damage and polymorphism was detected by RAPD in barley seedlings treated with Cd (30 - 120 mg/l) (Liu et al., 2005) and variation in band intensity, loss of normal bands and appearance of new bands compared with the normal seedlings was observed (Liu et al., 2005). The genotoxicity of heavy metals in kidney-bean (*Phaseolus vulgaris*) seedlings was studied with RAPD (random amplified polymorphic DNA) and polymorphisms became evident as the presence and/or absence of DNA fragments in treated samples compared with the untreated one at150 and 350 mgl^{-1} (Enan, 2006). The high number of both missing and new amplified fragment was observed at 350 $mgl^{-1}$and showed the mutagenic effect on *P. vulgaris* (Enan, 2006). Similarly, *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, DNA profiles and DNA changes was investigated with RAPD marker (Gupta et al., 2009). Since, excess heavy metals cause disturbance in physiological reaction of a cell as Pb contamination inhibited chlorophyll biosynthesis and its toxicity was assessed by RAPD marker (Cenkci et al., 2010). Furthermore, similar result was obtained from *Arabidopsis thaliana* exposed with Pb, Mn, Cd etc. (Conte et al., 1998) using RAPD technique. Changes in DNA profile was also assessed under heavy metal stress in plant *Daphnia magna* (Atienzar et al., 2001). Heavy metals, viz. Pb, Cu and Cd affect DNA of *Silene paradoxa*, kidney bean and barley plants which showed similar type of response (Mengoni et al., 2000;

Table 3. Jaccard's coefficient of similarity matrix for RAPD data for 10 untreated and treated variants of *Eruca sativa*.

	a	b	c	d	e	f	g	h	i	j
a	1.0000									
b	0.9583	1.0000								
c	0.9167	0.9565	1.0000							
d	0.8333	0.8696	0.9091	1.0000						
e	0.7083	0.7391	0.6957	0.7619	1.0000					
f	0.8750	0.9130	0.8696	0.7826	0.7273	1.0000				
g	0.6250	0.6522	0.6818	0.6667	0.6000	0.7143	1.0000			
h	0.8333	0.8696	0.8261	0.7391	0.6818	0.8636	0.6667	1.0000		
i	0.8333	0.8696	0.9091	0.8182	0.6087	0.8636	0.7500	0.8182	1.0000	
j	0.7083	0.6667	0.6957	0.6087	0.4783	0.6522	0.600	0.6818	0.7619	1.000

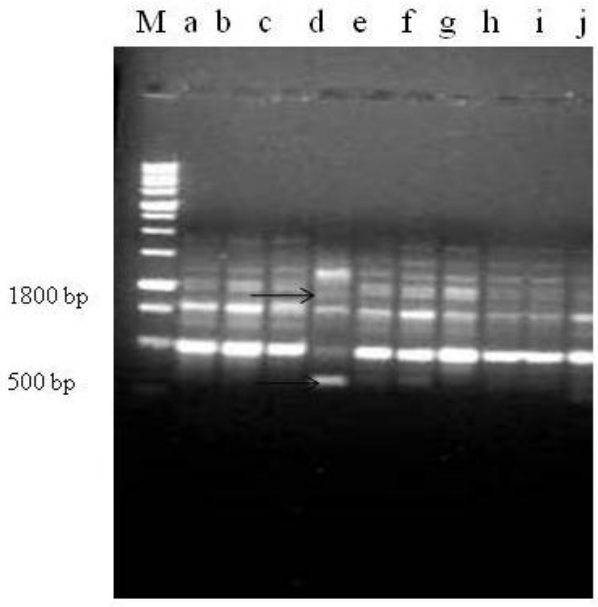

Figure 4. RAPD fingerprints for *Eruca sativa* treated with various concentrations of heavy metals with primer OPC-13. Lane M-1 kb DNA ladder. Lane a; control; Lane-b, c, d (seeds treated with Zn, Pb and Cd with concentration 50, 50 and 50 mg/l). Lane- e, f, g (seeds treated with Zn, Pb and Cd with concentration 100, 100 and 100 mg/l). Lane- h, i, j (seeds treated with Zn, Pb and Cd with concentration 150, 150 and 150 mg/l).

Enan, 2006; Liu et al., 2005) as observed in the present study. The genotoxic effects of three heavy metals on *E. sativa* were evaluated using cluster analysis and comparison was made among treated seedlings and control. The dendrogram was constructed using NTSYS pc programme version 2.2 for all treated and untreated seedlings. All seedlings were grouped into four clusters at 73% of similarity level. The first cluster has treated and untreated seedlings with low, medium and high concentrations of metals. First cluster has seedlings (Figure 5) a, b, c, d, f, i and h except e (Zn treated) which showed low percent of similarity to low and medium concentrations treated seedlings. Second cluster consist only one seedling 'e' which was obtained at medium concentrations of Zn (100 mg/l). Third and fourth clusters also have one seedling 'g' and 'j' which were obtained at 100 and 150 mg/l of Cd. The Zn treated seedlings at medium concentration showed very low similarity (47.83%) with seedlings 'j' (Cd, 150 mg/l) (Table 3). The seedling 'b' treated with Zn showed high similarity to 'a' (untreated). The comparison among 'untreated' and 'treated' genomes showed that RAPD analysis can be used for evaluation of toxicity on plants caused by environmental pollutants. On the basis of these considerations we could suggest that RAPD technique is a powerful tool for measuring quailtative and quantitative genotoxic activities produced by environmental pollutants. This method can be applied to a wide range of bioindicator organisms and may become a universal methodology to identify target genes for specific genotoxic agents. 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. In conclusion, the heavy metal Cd, at medium and high concentrations damaged the seedlings of *E. sativa* and cause mutations. Further, sequence characterized amplified regions (SCAR) marker would be developed for detection of individualized heavy metals on *E. sativa* and it would also helpful in detection of genotoxic doses. Since, the leaf of *E. sativa* is immensely used for salad purpose in European countries; therefore, biomarkers are necessary for detection of high concentration of heavy metals in this species where it is growing, and keep human health free from hazardous materials. Thus, RAPD fingerprints

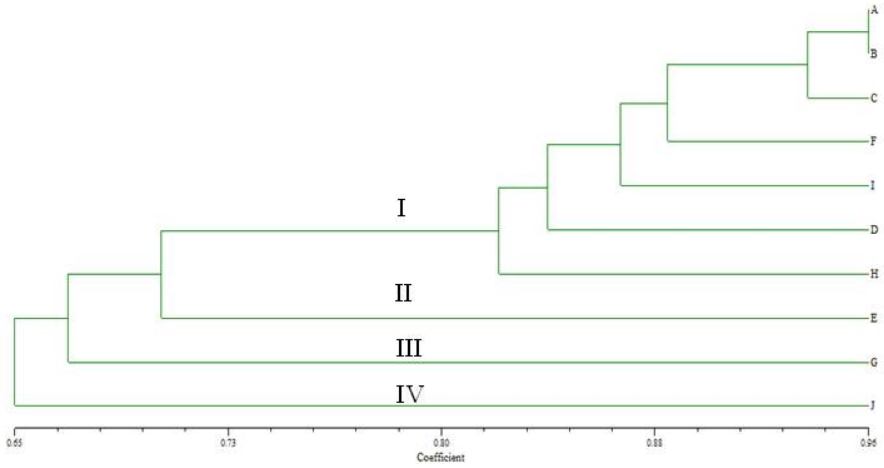

Figure 5. UPGMA dendrogram showing clustering of 10 untreated and treated seedlings of *Eruca sativa*.

appear to be stable in detection of genotoxic doses of those plants which are growing in heavy metal polluted soils.

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