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Full Length Research Paper

Application of inter simple sequence repeat (ISSR marker) to detect genotoxic effect of heavy metals on *Eruca sativa* (L.)

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As an influence of the Mediterranean diet, *Eruca sativa* (rocket salad) is eaten all over the world in salads and soups. It belongs to plant order Capparales (glucosinolate-containing species) and it is from the family Brassicaceae. Predominantly, the leaves of this species is eaten raw or cooked, although flowers are also consumed. Assessment of environmental contamination on ecology (plant) at molecular and population levels is important in risk quantification and remediation study. Heavy metal toxicity in plants is to induce oxidative stress linked to oxidation of proteins and membrane lipids but also to alterations of DNA damage response. *E. sativa* has been investigated in our study which is of agronomical importance and widely used in European countries. We studied three heavy metals Zn, Pb and Cd which showed a dose-dependent effect on radicle and coleoptile lengths of *E. sativa*. The radicle length was more affected than the coleoptiles length under all concentration tested plant. The ranking of genotoxic potencies in all three heavy metals was in the descending order: $Cd^{2+} > Pb^{2+} \geq Zn^{2+}$. Among these heavy metals, high concentration of Cd (150 mg/l) and Pb (150 mg/l) generated mutations along with changed morphology of seedlings. The radicle and coleoptile lengths (cm) under high concentration of Cd were decreased as compared to low, medium and high concentrations treated seedlings with Pb and Zn. 20 ISSR primers were used, of which four did not amplify, three gave single band and the rest of thirteen primers generated upto six bands (an average of 4 bands per primer). Sixteen primers exhibiting amplified products gave monomorphic; only two primers (OPC-5 and OPC-7) gave unique extra band in seedlings treated with medium and high concentrations of heavy metals Cd and Pb, respectively. The dendrogram was constructed to evaluate the genetic distance generated among the seedling treated with various heavy metals at various concentrations. The similarity matrix values were found from 42.8 to 100% and these values showed the genetic divergence among the seedlings treated with various concentrations of heavy metals.

Key words: Bioindicator, environmental pollutant, genetic diversity, mutation.

INTRODUCTION

Eruca sativa (L.) is an annual herb and is one of the varieties of mustard. The plant originated in the Mediterranean region, but is presently found around the world. It is extensively consumed in some European countries, e.g., Italy and is also used in Indian cooking. The leaves and sprouts of the plant are widely used in salads for their hot pungent taste and can add flavour to any boring salad. The plant also has a wide spread medicinal use. Traditionally, its use as astringent, diuretic, digestive, emollient, tonic, depurative, laxative, rubefacient and stimulant is well documented (Uphof, 1968; Yaniv et al., 1998; Perry, 1978). It is a member of the family Brassicaceae and is a valuable genetic resource for cabbage, rapeseed and other *Brassica* crops. Tender leaves are reported to have stimulant, stomachic, diuretic and anti-scorbutic activity (Bhandari and Chandel, 1996). In recent years, attention has been given to investigating the occurrence of genotoxic agents in the environment. The 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 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. They can be used for assaying arrange of environmental conditions in different habitats. In addition, it has been shown that for some chemical agents, comparable results in terms of genetic abnormalities are obtained in plant or animal systems (Minissi and Lombi, 1997) and that plants are more sensitive to some stressors (that is, herbicides) than animals (Wang and Freemark, 1995). Furthermore, plant-based assays applied to toxicity screening in the environmental field would reduce animal sacrifice and testing costs. The use of plants as bioindicators of genetic toxicity of environmental pollutants has been reported in several studies (Grant, 1994; Knasmuller et al., 1998). Mutagenic activity of chemicals has been analysed 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). Chromosome aberration assays, mutation assays, cytogenetic tests and specific locus mutation assays were performed on plants (Constantin and Nilan, 1982; Tardiff et al., 1994) for detection of heavy metal damage. The advantage of measuring the effect of genotoxic chemical directly on DNA is mainly related to the sensitivity and the short response time. Recently, enormous advances and developments in molecular biology have provided new ways of detecting DNA damage (Conte et al., 1998; Savva, 2000; Citterio et al., 2002). Following the original description of the PCR (Mullis and Faloona, 1987), modifications were described enabling the generation of DNA fingerprints which have been used to screen for biodiversity.

Some heavy metals at low doses are essential micronutrients for plants, but in higher doses they may cause metabolic disorders and growth inhibition for most of the 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 on DNA integrity in plant cells. The effect of different concentrations 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 hyperaccumulate metals have been reported in recent years. Hyperaccumulator plants are found in Brassicaceae, Euphorbiaceae, Asteraceae, Lamiaceae or Scrophulariaceae families (Macnair and Tansley, 1993). The largest group of these so-called 'metal hyperaccumulators' is found in the genus *Alyssum* (Brassicaceae), in which Ni concentrations can reach 3% of leaf dry biomass (Kramer et al., 1996). In a few studies, the seeds have been exposed to the contaminants (Claire et al., 1996; Vojtechova and Leblova, 1991; Xiong, 1998) and genotoxic effects were observed. Since, *Eruca sativa* is a heavy metal accumulator but high concentration of these metals damage to the plant cells and generate mutation. In this paper the application of ISSR as molecular marker to detect DNA damage in *E. sativa* (L.) caused by environmental pollutants was evaluated.

MATERIALS AND METHODS

E. sativa seeds were obtained from the local market of Riyadh and the experiments were conducted at Department of Botany and Microbiology, College of Science, King Saud University, Kingdom of Saudi Arabia. The solution of Zn, Cd and Pb were prepared in autoclaved deionized distilled water. The low, medium and high concentrations of these heavy metals were selected after application of various concentrations on seeds in Petri plate experiments. The low, medium and high concentrations of heavy metals used for seed treatment were (50, 50, 50 mg/l), (100, 100, 100 mg/l) and (150, 150, 150 mg/l) for Cd, Pb and Zn respectively. The seeds were immersed in 3% (v/v) formaldehyde solution for five minutes to avoid fungal contamination. After that, the seeds were washed with deionized water three times to remove excess formaldehyde. About 20 seeds were placed on Whatman filter paper and covered with another filter paper and each plate labeled. Then 5 ml of different concentrations of Zn, Cd and Pb were added. The top of the plates were closed and left in a germinator with temperature set at 25°C. The radicle and coleoptiles lengths were measured in 8 days old seedlings. Each treatment was replicated three times for statistical purposes. Further, morphological variants of seedlings produced under various concentrations was investigated. The radicle and coleoptile lengths were measured in various heavy metals treated seedlings and compared with untreated seedlings. The seedlings were harvested after two weeks for detection of mutation produced under various concentrations of 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

Table 1. List of ISSR Primers used in the study.

S/N	Primer sequence
1	5'-CACACACACACAGG-3'
2	5'-CTCTCTCTCTCTCTCTAC-3'
3	5'-CTCTCTCTCTCTCTCTTG
4	5'-CACACACACACAAC
5	5'-CTCTCTCTCTCTCTCTGC
6	5'-CACACACACACAAG
7	5'-CACACACACACAGT
8	5'-GAGAGAGAGAGAGG
9	5'-GTGTGTGTGTGTGG
10	5'-GAGAGAGAGAGACC
11	5'-GTGTGTGTGTGTCC-3'
12	5'-CACCACCACGC-3'
13	5'-GAGGAGGAGGC-3'
14	5'-CTCCTCCTCGC-3'
15	5'-GTGGTGGTGGC-3'
16	5'-CAGCAGCAGCAGCAG-3'
17	5'-CAACAACAACAACAA-3'
18	5'-GACAGACAGACAGAC-3'
19	5'-GATAGATAGATAGAT-3'
20	5'-CACACACACACACACAGT-3'

(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 ⅔ volume of ice-cooled isopropanol was added 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 was done with ISSR primers according to the protocol developed by Zeitkiewicz et al. (1994). The 20 ISSR primers synthesized from Sigma Company were used to amplify the genomic DNA extracted from untreated and treated seedlings (Table 1). 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 38°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

E. sativa (rocket salad) is widely used in Europe and in many countries. It is regarded as a special food or even a delicacy. It is also used as a vegetable (and not just as a condiment), in the sense that cooked leaves are used for the preparation of special dishes like 'pasta e rucola' or 'bresaola' a sort of dry meat seasoned with cheese, rocket leaves and olive oil (Bianco, 1995). In the Indian subcontinent and in Pakistan in particular, special ecotypes of *E. sativa* are cultivated for seed production. The seeds are used to extract oil often named 'jamba oil' which has many interesting uses such as for illumination or in the production of pickles (Padulosi, 1995). Studies on inducible adaptive response in plants to metals or genotoxic stress are arousing interest as that would augment our understanding the basis of evolution of metal tolerance in plants. Tolerant plants are attracting attention owing to the promise they offer in crop production as well as in phytoremediation (Kochian, 1995; Salt et al., 1995). While evolution of metal tolerance has been generally attributed to the tolerant genes (Macnair and Tansley, 1993) and genecological studies suggested that metal stress might induce adaptive changes involving transient expression of certain genes, commonly called as 'stress genes'. The several plant species have been used as bioindicators and several tests have been developed to evaluate the toxicity of environmental contaminants on vegetal organisms. We studied genotoxicity of three heavy metals Cd, Pb and Zn on *E. sativa* (L.). In our study all three heavy metal concentrations affected the radicle and coleoptile lengths and it was found dose dependent. All treated seedlings with heavy metals were compared with untreated seedlings. Among the three heavy metals, Cd has been found strong inhibitory effect on morphological as well as DNA markers. At high concentrations of Zn, Pb and Cd, the radicle lengths (cm) was found 1.56 ± 0.16, 0.73 ± 0.12 and 1.0 ± 0.080, respectively, as compared with untreated seedling which was found 4.5 ± 0.13 (cm) (Table 2). Similarly, at high concentrations of Cd, Pb and Zn the coleoptile lengths (cm) was found 3.05 ± 0.17, 2.93 ± 0.04 and 0.43 ± 0.04, respectively, as compared with untreated seedlings which was found 5.2 ± 0.21 cm (Table 3). The radicle length was more affected than the coleoptiles length (Figure 1). The root length was more sensitive parameter than shoot length at every 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 Cd was more accumulated in the radicle and its length was decreased as 1 cm as compared to control radicle length which was found 4.5 cm (Figure 1j). The tobacco genotoxicity was caused by Cd and DNA damages as measured by Comet assay (Gichner et al., 2004). The cadmium was more accumulated in the root and it was analysed by inductively coupled plasma optical emission spectrometry that roots accumulate almost 50-fold more cadmium than

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

Zn treatment		Pb treatment		Cd treatment	
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

Each values are mean ± SD for three replicates in each group.
l: low concentration; m: medium concentration; and h: high concentration.

Table 3. Effects of various concentrations of heavy metals on radicle 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

Each values are mean ± SD for three replicates in each group.
l: low concentration; m: medium concentration; and h: high concentration.

above-ground parts of the tobacco seedlings.

In the initial experiments, a total of 20 primers were screened with treated and untreated rocket samples. Out of 20 primers, four could not amplify the rocket genomic DNA and three gave extremely faint and ambiguous bands. Sixteen responding primers which produced clear cut and reproducible bands were further used to amplify genomic DNA from all treated and untreated seedlings. Three primers produced only single band, while the rest thirteen primers produced 2 - 5 bands with an average of 4 bands per primer. The bands generated were primer dependant and were in the size range of 250 to 2000 bp. An example of the ISSR patterns generated by representtative primer sets OPC-5 and OPC-7 are shown in (Figures 2 and 3). The high concentration of Cd (150 mg/ L) created mutation and produced more number of unique fragments in PCR amplification. DNA from plants exposed to heavy metals solution displayed polymorphic fragments which were not detectable in DNA of unexposed plants. The unique fragments produced with primer OPC-5 of sizes 1.7 kb and 250 bp (Figure 2) and 1.4 kb and 900 bp (Figure 3) were found with primer OPC-7 at high concentration of Cd. The high concentration of Cd showed to be a genotoxic dose in our study on *E. sativa* seedlings. The genetic diversity of heavy metal-tolerant populations of *Silene paradoxa* (L.) (Caryophyllaceae) was determined by using a chloroplast microsatellite analysis (Mengoni et al., 2001). All sixteen responding primers, except two, revealed monomorphic banding patterns indicating a high degree of homogeneity in the rocket seedling variants. Out of 20 primers screened, only two primer (OPC-5 and OPC-7) revealed polymorphic bands. It was repeated at least three-times with the same results, indicating its reproducibility. Thus, ISSR fingerprints appear to be stable in detection of mutation of those plants growing in heavy metal polluted soils.

Some fragments were absent at low, medium and high concentrations of Cd while these were present under the treatment of Zn and Pb, respectively. The high concentration treated seedlings with Cd and Pb produced more bands as compared to low concentration treated and control seedlings. The RAPD technique was used in Cd treated barley and the changes occurring in RAPD profiles of the root tips following Cd treatment included alterations in band intensity as well as gain or loss of bands compared with the control seedlings (Liu et al., 2009). New amplified fragments in treated barley at molecular size from approximately 154 to 2245 bp appeared almost for 10, 20 and 40 mg/l Cd with 9 primers and the number of missing bands enhanced with the increasing Cd concentration for nine primers (Liu et al., 2009). The PCR fragment of size 1.3 kb was missing in seedlings treated with 100 and 150 mg/l of Cd and 150 mg/l of Pb while same band was found at lower concentrations of heavy metals including untreated seedling. The PCR fragment of size 1kb was found present in untreated and low concentration of Cd and Pb (50 mg/l) treated seedlings but was found absent in seedlings treated with higher concentrations. The low concentration of Cd (50 mg/l), Pb (50 mg/l) and Zn (50 mg/l) did not damage the cells and no mutation to be detected at these concentrations. The medium concentration of Cd (100 mg/l), Pb (100 mg/l) and Zn (100 mg/l) strongly affected morphological as well

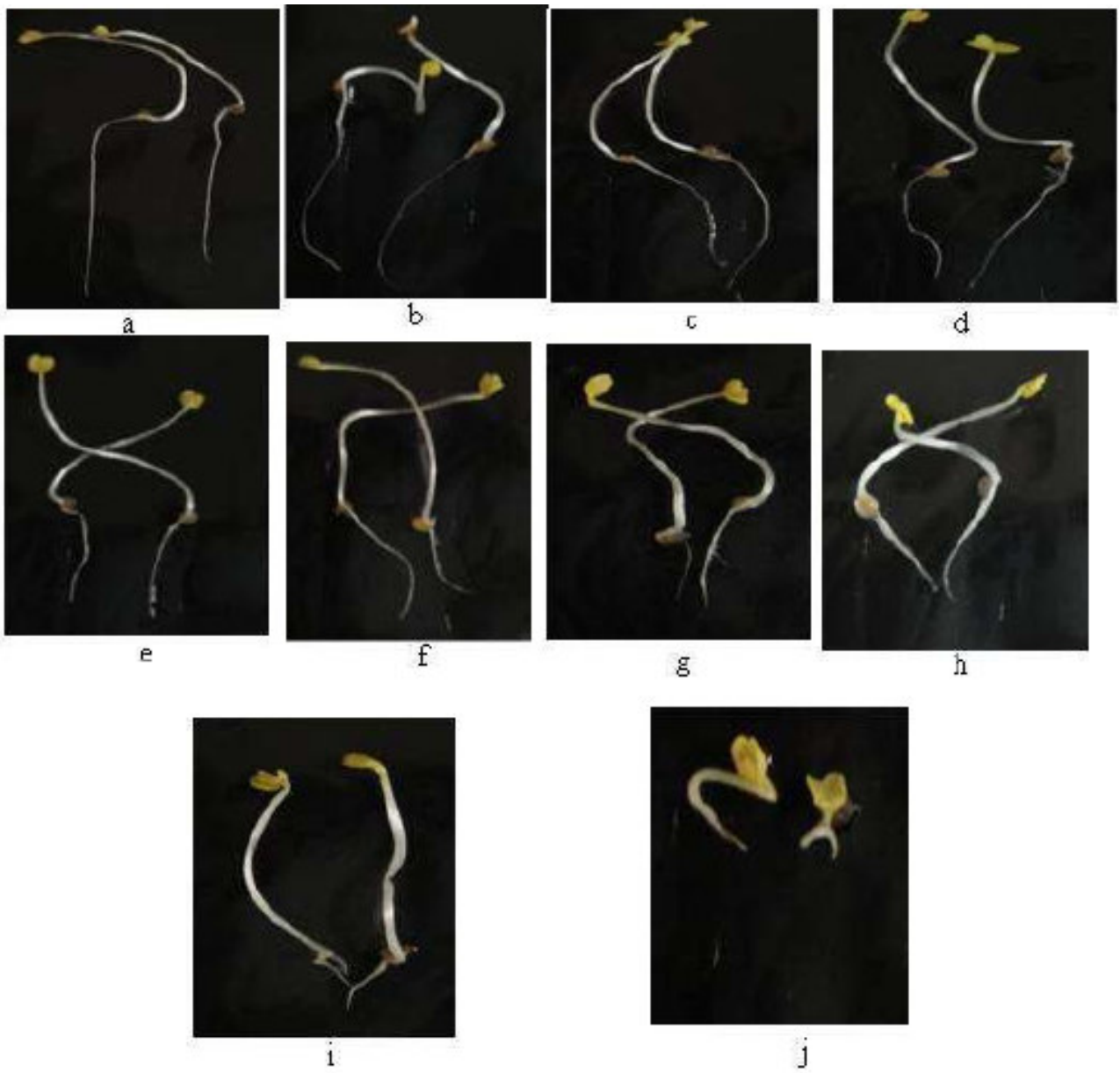

Figure 1. Eight days old seedlings grown in glass petriplate treated with various concentrations of heavy metals. a- control; b- 50 mg/l (Zn); e- 100 mg/l(Zn); h- 150 mg/l (Zn); c- 50 mg/l (Pb); f- 100 mg/l (Pb); i- 150 mg/l (Pb); d- 50 mg/l (Cd); g- 100 mg/l (Cd); j- 150 mg/l (Cd).

as DNA markers and produced mutations as compared to low concentrations treated seedlings. The barley seedlings treated with Cd (30 - 120 mg/l) caused DNA damage and polymorphism was detected by RAPD method (Liu et al. 2005). The changes occurring in random amplified polymorphic DNA (RAPD) profiles of root tips following Cd treatment included variation in band intensity, loss of normal bands and appearance of new bands compared with the normal seedlings (Liu et al. 2005). The genotoxicity of heavy metals in kidney-bean (*Phaseolus vulgaris*) seedlings was studied with RAPD (random amplified polymorphic DNA) analysis and polymorphisms became evident as the presence and/or absence of DNA fragments in treated samples compared with the untreated one at150 and 350mgl^{-1} respectively (Enan, 2006). At 350mgl^{-1}, a high number of both missing bands and new amplified fragment were observed and thus, this concentration showed the mutagenic effect on *Phaseolus vulgaris* (Enan, 2006).

The genotoxic effect of the three heavy metals was quantified using cluster analysis by comparing DNA from treated seedlings with the control seedlings. The dendrogram was constructed using NTSYS pc programme version 2.2 for all 10 untreated and treated seedlings. At

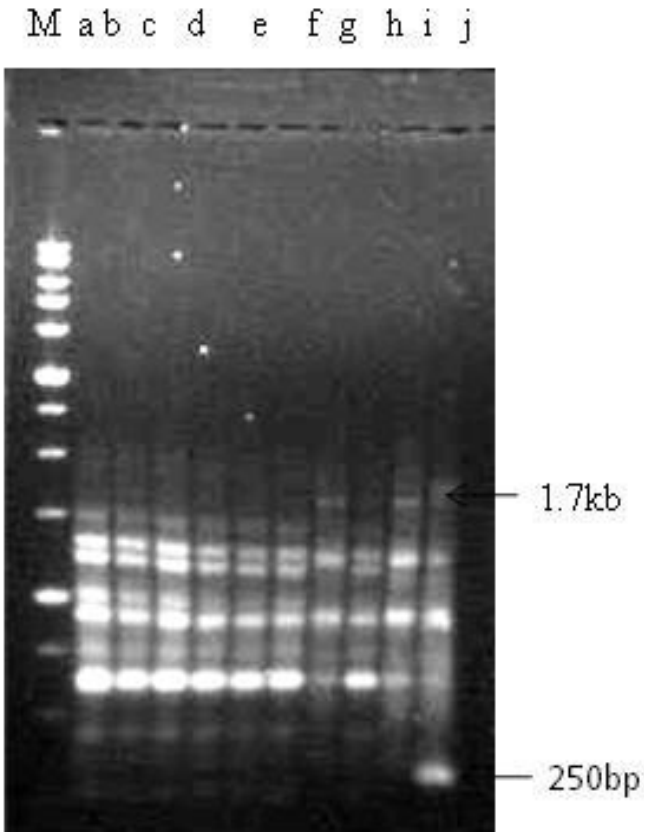

Figure 2. ISSR fingerprints for *Eruca sativa* (L.) treated with various concentrations of heavy metals with primer OPC-5. Lane M: 1 kb DNA ladder; Lane a: control; Lanes b, c, d: seeds treated with Zn, Pb and Cd with concentration 50, 50 and 50 mg/l; lanes e, f, g: seeds treated with Zn, Pb and Cd with concentration 100, 100 and 100 mg/l; lanes h, i, j: seeds treated with Zn, Pb and Cd with concentration 150, 150 and 150 mg/l.

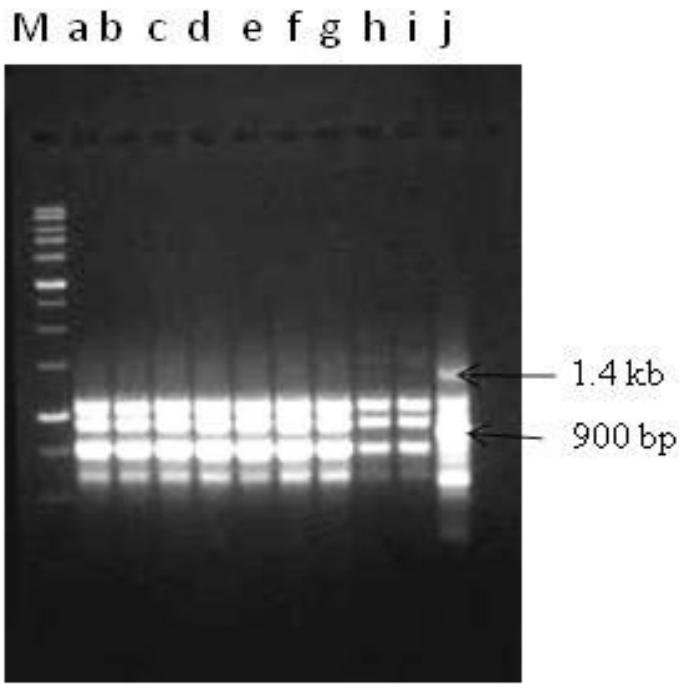

Figure 3. ISSR fingerprints for *Eruca sativa* (L.) treated with various concentrations of heavy metals with primer OPC-7. Lane M: 1 kb DNA ladder; Lane a: control; Lanes b, c, d: seeds treated with Zn, Pb and Cd with concentration 50, 50 and 50 mg/l; Lanes e, f, g: seeds treated with Zn, Pb and Cd with concentration 100, 100 and 100 mg/l; lanes h, i, j; seeds treated with Zn, Pb and Cd with concentration 150, 150 and 150 mg/l.

72% of similarity level, all 10 seedlings were grouped into three clusters (Figure 4). There was a distinct distance found between the band patterns of treated and the untreated seedlings. The first cluster had seedlings treated with low and medium concentrations of heavy metals including untreated seedling. First cluster has seedlings a, b, c, d, e, f and h except g (Cd treated) seedling which showed low percent similarity to low and medium concentrations treated seedlings. Second cluster consisted seedlings g and i treated with medium and high concentrations of Cd (100 mg/l) and Pb (150 mg/l). Third cluster consisted only of one seedling i, which was treated with Cd with concentration of 150 mg/l. The Cd treated seedling with high concentration showed very low similarity (42.8%) with the seedlings 'a' (untreated), 'b' (Cd, 50 mg/l), c (Pb, 50 mg/l) and d (Zn, 50 mg/l) (Table 4). Seedling j showed high similarity to the g seedling and both were treated with Cd with high and medium concentrations and 50% similarity was found between j and g. ISSR analysis indicated that all the three heavy metals showed genotoxicity and Cd and Pb at high concentrations induced DNA changes in different target sequences. Similarly, AFLP analysis was used to determine the genotoxic effect of potassium dichromate and dihydrophenanthrene and it resulted that both pollutant showed genotoxicity in *Arabidopsis thaliana* (L.) Heynh. (ecotype Wassilewskija) (Labra et al., 2003).

In conclusion, the heavy metal Cd, at medium and high concentrations damaged the seedlings of *E. sativa* (L.) and creates mutation. The comparison between 'untreated' and 'treated' genomes showed that ISSR analysis can be used to evaluate how the environmental pollutants modify the structure of DNA in living organisms. On the basis of these considerations we suggest that ISSR method is a powerful tool for measuring qualitative and quantitative genotoxic activity due to 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. This could open up possibilities for designing specifically targeted assays and new approaches to risk assessment.

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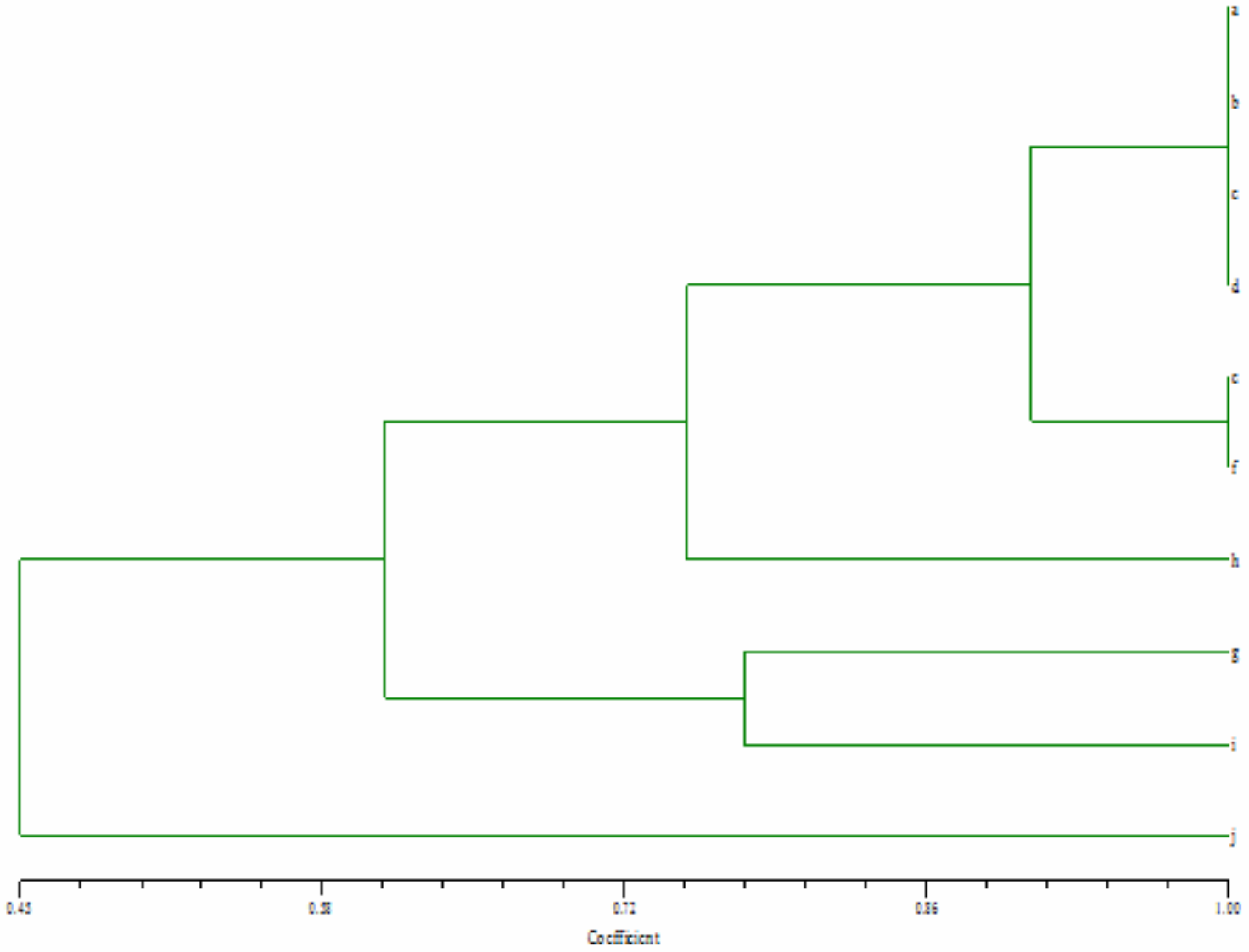

Figure 4. UPGMA dendrogram showing clustering of 10 untreated and treated seedlings of *Eruca sativa* L.

Table 4. Jaccard's coefficient of similarity matrix for ISSR data for 10 untreated and treated variants of *Eruca sativa* L.

	a	b	c	d	e	f	g	h	i	j
a	1.000									
b	1.000	1.000								
c	1.000	1.000	1.000							
d	1.000	1.000	1.000	1.000						
e	0.909	0.909	0.909	0.909	1.000					
f	0.909	0.909	0.909	0.909	1.000	1.000				
g	0.666	0.666	0.666	0.666	0.727	0.727	1.000			
h	0.727	0.727	0.727	0.727	0.800	0.800	0.700	1.000		
i	0.500	0.500	0.500	0.500	0.545	0.545	0.777	0.666	1.000	
j	0.428	0.428	0.428	0.428	0.461	0.461	0.500	0.416	0.454	1.000

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