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RESEARCH ARTICLE

Evaluation of RAPD Technique to Induced DNA Damage by Heavy Metal to Detect the Genotoxic Effect on Mung Bean (*Vigna Radiata* (L.) Wilczek) Seedlings

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ABSTRACT

The toxic heavy metals cause several cellular stress responses such as seedling growth, membrane proteins and DNA damage. In this study, the most important effect of copper, nickel, lead, copper, cadmium and mercury of Mung Bean were investigated the phytotoxicity and genotoxicity based on morphological such as seed germination, seedling growth and DNA damage. An RAPD fingerprinting technique was used to detect the DNA damage in mung bean (*Vigna Radiata*(L.) Wilczek)on seed germination and root growth exposed with different concentration of heavy metals such as 50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l, for one week showed changes in morphological such as radical, coleoptiles, and protein content and DNA profile. A total 12 primers of 60-70 % GC content were amplified by RAPD primers produced 156 bands between 110-1148 base pair in agarose gel electrophoresis .The Changes in DNA Profile include variation in band intensity, presence or absence of some bands profile showed significant effect at 150 mg/l to 200mg/l concentration of heavy metals. The results concluded that DNA damage evaluated by RAPD analysis could be a useful tool for detection of genotoxic effects of different heavy metals on plants.

Keywords: *RAPD, Vigna Radiata, Seedling growth, DNA Damage.*

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INTRODUCTION

The heavy metals are one of the most important dangerous environmental pollutants which usually originates from different industry and agricultural activities such as mining waste disposal and application of pesticides or fertilizers (Shahtash et al, 2010; Agar and Taspinar, 2003). The toxic chemical induced genotoxicity by RAPD in bean (*phaseolus vulgaris* L.) seedling (Cenkci et al, 2009). The heavy metals contain one of the major groups of genotoxic environmental pollutants posing serious threat to human as well as environmental well being (Cenkci et al, 2009). Many researchers investigate to the toxic effect of heavy metal have been demonstrated in plants and animals (Caudra et al., 2004; Shahtash et al, 2010). The similar study such as effect different heavy metal salt solutions on the seed germination and growth of Spinach plants (Lawal et al., 2011). The cadmium was not so much inhibits on seed germination such as shoot growth and root growth and mitotic index of cells, but also induces damage to different cellular components such as membrane, proteins and DNA (Zhang et al., 1994; Liu et al., 1992; Patra and Panda, 1998; Weisberg et al., 2003; Jimi et al., 2003; Shahtash et al., 2010). Cadmium is a toxic metal with a long biological half life and represents serious environmental pollutants for both animals and plants as compare to other heavy metals cadmium is known to interact with proteins influencing protein-proteins and protein-DNA interactions (Enan et al, (2006). Excess amount of heavy metal causes oxidative damage, but some reactive oxygen species (ROS) can participate in signal transduction pathways. Recently, advances in molecular biology have led to using PCR based techniques such as RAPD, AFLP, SSR and VNTR) for analysis of DNA damage in eco-genotoxicity (Savva, 1996, 1998; Cenkci et al., 2009). Random amplified polymorphic DNA (RAPD) is a PCR based techniques 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 (Wolf et al., 2004; Atienzar et al., 1999; Gupta et al., 2009). Lead acetate and copper salts have been reported to high inhibitory tendencies on the germination and growth of tomato verities and (Jaja and Odeoemena. 2004). Irradiation of DNA itself with UV radiation produces multiple DNA photoproducts (Caudra et al., 2004; Sancar et al., 1988) which may alter the nucleotide sequence and cause severe mutations during replication (Jiang et al., 1993). The aim of this study was to detect the genotoxicity such as protein profile and DNA profiling on seed germination induced by different concentration of heavy metals using RAPD techniques. Assessments of the genotoxicity involve comparison of morphological changes, RAPD profiles, and protein profile on seedling growth.

MATERIALS AND METHOD

Plant materials and treatments:

Seeds of mung been (*Vigna Radiata,* L*)* were first surface sterilized by using 15 min incubation in 3 % (w/v) sodium hypochlorite to avoid the fungal contamination, followed by three times washing with distilled water (Shahrtash et al., 2010). Sterilized Petri dishes of approximately 9 cm diameter each containing spread cotton wool were used as sowing container and media. The heavy metals containing such as cadmium was applied as cadmium chloride, mercury was applied as mercuras chloride, lead was applied as lead acetate, nickel was applied as nickel ammonium sulphate and copper was applied as copper sulphate. The different concentration treated with 50, 100, 150 and 200 mg/l selected for experiments of all heavy metals such as cadmium, mercury, lead, nickel and copper is fall within the normal levels recommended for metals in plants (Awofolu et al., 2005). The seeds were then germinated at 24 ºC and growth

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conditions of 16 h light and 8 h dark, an average minimum temperature of 18 ºC and humidity of 65 %.The plant seedlings were treated with heavy metals for 7 days. Control seed germination experiments were similarly carried out in petri dishes containing neatly spread cotton wool, soaked in only distilled water without any of the metal studied. After treatment plants were utilize for protein estimation, DNA extraction and RAPD analysis.

Seed germination studies:

Ten seeds were placed into 20 mm × 120 mm Petri dishes containing one piece of filter paper (125 mm in diameter, Whatman No.1) followed thin layer of cotton. The dishes were filled with 20 ml of water or test solutions, and incubated at 28°C in dark conditions. After 3 days of incubation, the numbers of seed grown were recorded. The seed germination experiment was done in triplicate.

Morphological analysis:

The morphological study such as radical and coleoptiles lengths was shown Table 2 a, b, c, d and e also the number of seed germination was count. The higher concentration of these effluents shows higher inhibition as compare to low concentration. There was no inhibition take place in control plants. The percentage of seed germination was also shown in 2 a, b, c, d and e,

Genomic DNA isolation:

Genomic DNA was isolated from root and first leaves, approximately 300 mg frozen and powdered in liquid nitrogen using Qiagen DNA isolation Kit (Germany). The extracted DNA was stored at -20^{o}C for future use. The quality and quantity of genomic DNA was determined by running the DNA samples on 0.8% agarose gel and taking the absorbance ratio 260nm/280 nm by spectrophotometer. (Gupta et al., 2009) and all the DNA samples were standardized to 50 ng/µl. The intact DNA showing no smearing was selected for further analysis.

RAPD-PCR amplification:
A total of twenty decamer primers of arbitrary sequences, having GC content 60 to 70% (Operon Biotechnologies GmbH, Germany) from the set OPA, OPG and OPO were initially screened for their study. Only 15 primers were selected for further analysis based on their ability of reproducibility and to detect polymorphic amplified products across the pooled sample. To ensure reproducibility, the primers showing no amplification, no consistent banding pattern and generating weak products were discarded. The primers information along with amplified band size range is provided in Table 1.
All the RAPD-PCR reactions were carried out in a mixture of 25µl volume containing approximately 20-30 ng of genomic DNA, 200 µm of each dNTPs, 2.0 mM of MgCl2, 1.0 U of Taq DNA polymerase (Biotool)), 25 pmol of each primer. All the RAPD-PCR reactions were carried out in a thermal cycler (mycycler, BioRad^{TM}, USA) programmed with the following cycling conditions: initial denaturation was carried out at 94 °C for 5 minute followed by 45 cycles programmed for denaturation step at 94 °C for 1 minute, primer annealing step at 36 ^{o}C for 1 minute, and extension step at 72 °C for 2 minutes. The final extension was carried out at 72 °C for 10 minutes. The reactions were carried out as described by Enan et al., (2006).
Agarose Gel Electrophoresis of RAPD Product:

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The PCR amplified products were resolved on 1.8 % agarose gel containing ethidium bromide in 1X TBE buffer [45 mM Tris-borate, 1.0mM EDTA (pH 8.0)] at 70 V for 4 hours along with 100 bp ladder and 500 bp ladder as reference for molecular weight estimation. The amplified DNA was mixed with a one-fifth volume of gel loading buffer with 20µl of this solution loaded on the gel. The amplified band pattern were visualized and documented in a gel documentation system using Genesnap software (Syngene, U K).

RAPD profile and data analysis:

Scoring and matching of bands in different gels were done by Genesnap and Genetool softwares (Syngene, U. K.) along with the standard size ladders (100 bp and 500 bp). For the inter gel analysis and comparison of the patterns, a distinct and well-separated bands were selected. The presence and absence of RAPD band was recorded as '1' and '0' respectively. The binary coded characters (1, 0) were used for the genetic analysis. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix for each sample. The binary data generated were used to estimate the levels of polymorphism by dividing the polymorphic bands by the total number of scored bands.

Statistical Analysis:

All the experiments were conducted in triplicates and data are presented as mean ±SD.

RESULTS

The RAPD profile showed the genotoxic effect of different concentration of heavy metals changes in the number, size and intensity of the bands on different light conditions. Twelve primers out of twenty used for amplification resulted in specific and stable DNA profile in the plants (Table 1). Treatment of Seven days old seedling with different concentration of heavy metals condition showed the maximum inhibition seed germination and RAPD Profile such as the germination growth was more affected in descending order: Hg,>Cd>Pb>Ni>Cu in normal light conditions. The main observation or changes following the heavy metal treatment were the differences in the intensities of bands, appearance of some bands or absence of bands.

Effect of heavy metals on seed germination and shoot length growth:

The result (Table 2) shows the inhibitory effect of the heavy metal on the shoot length of mung bean seedling at four concentrations (50, 100, 150 and 200 mg/l). The shoot length decreased with the increased the concentration of heavy metals, the high concentration of heavy metals (50 and 100 mg/l) showed that there is no growth was observed. The morphological data was supported by some other researchers such as in plants cadmium has more toxic effect on seed germination and root growth. The shoot length was decreased under treatment of both heavy metals, and it was severely affected by Hg as compared to Cd at same concentration (Table 2d & e). The shoot length was observed at 50 and 100 mg/L of Hg and Cd treatment was 6.60 ± 0.29 cm and 2.62 ± 0.46 cm, whiles at 100 mg/L of Hg and Cd, the shoot length was 5.36 ± 0.35 cm and 5.27 ± 0.20 cm respectively. The less toxic effect was found when treated with $Cu^{2+,}$ Ni^{2+} and Pb^{2+} (Table 2a, b&c). The toxicity of these heavy metals after being performed in petriplate, further it was performed in pot in green house.

The plant growth such as height, leaf area per plant, fresh and dry weight per plant in pot experiment was observed after a time interval of 25, plants, and plant height was more affected by Hg than Cd, while of same concentrations of both metals applied.

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Table 1. Primer name, sequence, GC content, Tm value ºC

No.	Primers	Sequence (5' 3')	G+C (%) content	Tm valueºc
1.	OPA 03	AGTCAGCCAC	60%	32.0
2.	OPA 12	TCGGCGATAG	70%	34.0
3.	OPA 13	CAGCACCCAC	70%	34.0
4.	OPA 20	GTTGCGATCC	60%	32.0
5.	OPG 01	CTACGGAGGA	60%	32.0
6.	OPG 03	CTAGTGCTAC	50%	30.0
7.	OPG 06	GTGCCTAACC	60%	32.0
8.	OPG 07	GAACCTGCGG	70%	34.0
9.	OPG 08	TCACGTCCAC	60%	32.0
10.	OPG 11	TGCCCGTCGT	70%	34.0
11.	OPG 12	CAGCTCACGA	60%	32.0
12.	OPG 13	CTCTCCGCCA	70%	34.0
13.	OPG 16	AGCGTCCTCC	70%	34.0
14.	OPO 01	GGCACGTAAG	60%	32.0
15.	OPO 02	ACGTAGCGTC	60%	32.0
16.	OPO 08	CCTCCAGTGT	60%	32.0
17.	OPO 10	TCAGAGCGCC	70%	34.0
18.	OPO 12	CAGTGCTGTG	60%	32.0
19.	OPO 14	AGCATGGCTC	60%	32.0
20.	OPO 20	ACACACGCTG	60%	32.0

Table. 2 (a). Different concentration of copper, number of seed germination, % of seed germination, root length and shoot length.

Amount of Copper (mg/l)	**No of Seed germination (20)**	**% of seed germination**	**Root Length Radical (cm)**	**Shoot Length Coleoptiles (cm)**
Control (0)	20	100%	3.1+0.9	9.1+0.8
50	19	95%	2.1+0.7	5.6+0.8
100	14	70%	1.8+0.5	4.2+0.2
150	15	75%	1.7+0.3	3.2+0.1
200	5	25%	1.0+0.3	2.0+0.1

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Table 2 (b). Different concentration of Nickel, number of seed germination, % of seed germination, root length and shoot length.

Amount of Nickel (mg/l)	**No of Seed germination**	**% of seed germination**	**Root Length Radical (cm)**	**Shoot Length Coleoptiles (cm)**
Control (0)	19	95%	3.4+0.8	8.5+0.8
50	17	85%	2.2+0.7	5.8+0.9
100	11	55%	1.7+0.6	4.3+0.3
150	14	70%	0.7+0.2	1.2+0.1
200	6	30%	0.5+0.1	0.6+0.1

Table 2 (c). Different concentration of Lead, number of seed germination, % of seed germination, root length and shoot length.

Amount of Lead (mg/l)	**No of Seed germination**	**% of seed germination**	**Root Length Radical (cm)**	**Shoot Length Coleoptiles (cm)**
Control (0)	20	100%	3.1+0.9	9.1+0.8
50	17	85%	2.1+0.7	5.6+0.8
100	10	50%	1.8+0.5	4.2+0.2
150	6	30%	0.7+0.2	1.3+0.1
200	0	10%	0.1+0.1	0.6+0.1

Table 2 (d). Different concentration of Cadmium, number of seed germination, % of seed germination, root length and shoot length.

Amount of Cadmium(mg/l)	**No of Seed germination**	**% of seed germination**	**Root Length Radical (cm)**	**Shoot Length Coleoptiles (cm)**
Control (0)	19	95%	3.3+0.9	8.1+0.8
50	17	85%	2.1+0.7	5.6+0.8
100	10	50%	1.8+0.5	4.2+0.2
150	6	30%	0.7+0.2	1.2+0.1
200	0	0%	0.0+0.0	0.6+0.1

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Table 2 (e). Different concentration of mercury, number of seed germination, % of seed germination, root length and shoot length.

Amount of Mercury (mg/l)	No of Seed germination	% of seed germination	Root Length Radical (cm)	Shoot Length Coleoptiles (cm)
Control (0)	18	90%	3.1+0.9	9.1+0.8
50	16	80%	2.1+0.7	5.6+0.8
100	11	55%	1.8+0.5	4.2+0.2
150	8	40%	0.7+0.2	1.2+0.1
200	1	5%	0.0+0.0	0.2+0.1

Table 3. Genetic Profile of different concentration of these heavy metals amplified by OPG -02 primers. Ten most appropriate bands size were observed with the indicated size .The Plus sign indicate the presence of bands and negative sign indicates absence of bands.

Heavy Metals	Nickel					Copper				Lead				Cadmium				Mercury			
Size (bp)	Control	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	15 0	200
1128	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
1080	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
950		-	+	+	+	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-
770	+	-	+	+	+	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-
610	-	-	+	+	+	-	+	-	+	-		-	-	+	-	-	-	-	+	-	-
518	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	
420	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
340	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
303	+		+	-	-	-	-	-	-	-	-	-		+	+	-	-	-	-	-	-
Total bands	4	0	6	5	4	0	4	1	5	0	0	0	0	4	1	0	0	2	1	0	0

Table 4. Genetic Profile of different concentration of these heavy metals amplified by OPG -12 primers. Ten most appropriate bands size were observed with the indicated size .The Plus sign indicate the presence of bands and negative sign indicates absence of bands.

Heavy Metals	Nickel					Copper				Lead				Cadmium				Mercury			
Size (bp)	Contro l	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200
1148	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1080	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
960	+	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
790	-	+	+		+	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
580	-	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	+	-	-	-
518	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-	
410	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-
290	-	-	+	+	+	+	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-
220	+	+	+	+	-	+	+	-	+	+	-	-	+	-	+	+	-	-	-	-	-
110	+	+	+	+	-	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-
Total bands	5	6	6	5	6	7	5	4	1	2	4	4	3	0	4	3	0	3	0	0	0

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Figure 1: RAPD PCR product generated by OPG-12 from mung bean plant treated with different heavy metals Lane 1: control and Lane M: low range DNA rulers (100–3000 bp).

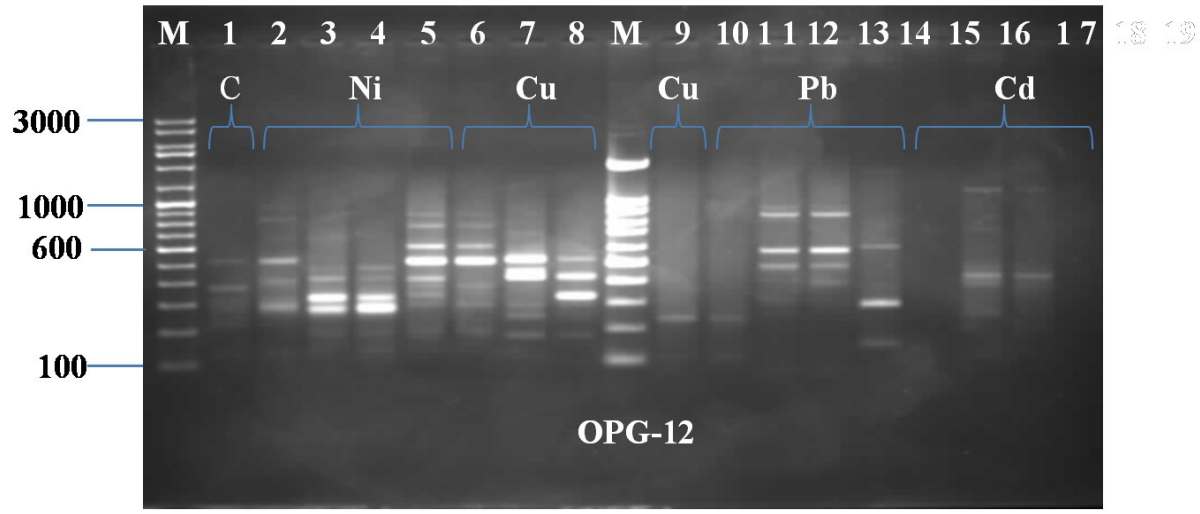
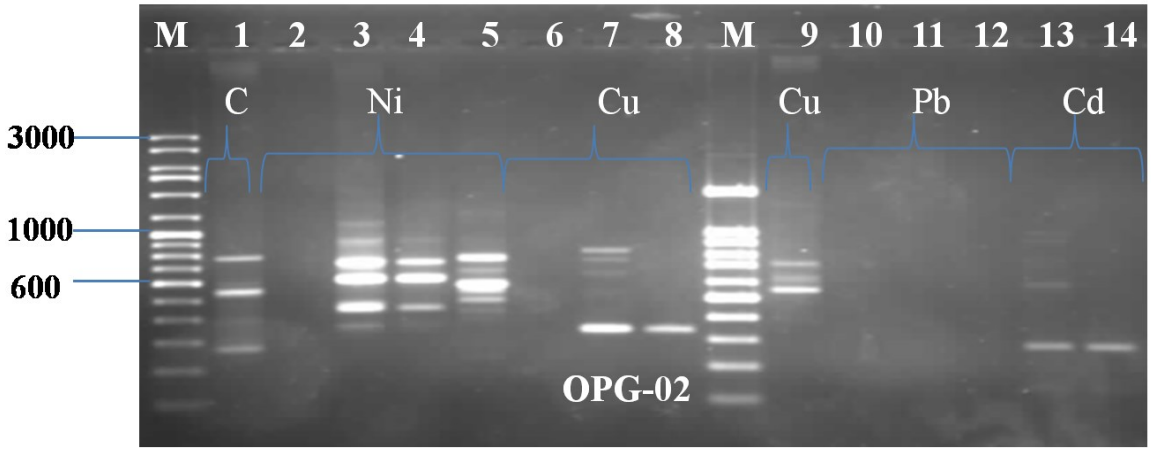


Figure 2: RAPD PCR product generated by OPG-02 from mung bean plant treated with different heavy metals Lane 1: control and Lane M: low range DNA rulers (100–3000 bp).



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The reduction in plant height was found more at 100mg of Hg per 300 gram of soil, and its effect was continued upto seven days of sowing. At this concentration, the plant height was 8.92 ± 0.43 cm, 7.50 ± 0.51cm and 9.78 ± 0.86 cm after 2, 4 and 7days after s owing, and result showed the highly significant differences as compared to untreated plants.

Effect of heavy metals on RAPD bands pattern:

The RAPD fingerprints showed the apparent changes in the number and size of the amplified DNA fragments. The RAPD profiles generated by these primers revealed differences between control and exposed plants, with visible changes in the number and size of amplified DNA fragments. The RAPD patterns generated by heavy metals using RAPD primers were the differences in the intensities of bands appearance of new bands or absence of bands. Primer OPG-12 and OPG-02 showed good results in all primers (Fig1 & 2). The unique band pattern was showed by primer OPG-02 and OPG-07 to differentiate the number of bands in control and treated samples.

The RAPD profile obtained with the RAPD primers produced 156 bands between 205-3048 base pair in agarose gel electrophoresis. In a total of 156 bands only 74 bands were polymorphic (47.4%) in all primers (Table 3 & 4). The higher concentration of all heavy metals such as 50 and 100% showed the loss of bands more than 50% as compare to control samples.

DISCUSSION

The mung bean is the one of the most widely used in the study of genetics and molecular biology. The changes in DNA damage by heavy metals may be monitored using different biomarker assay both at biochemical and molecular level (Savva, 1998; Gupta et al 2009). The effect of heavy metal on morphological data such as shoot growth and root growth affected to the higher toxic effect syndrome due to high accumulation of heavy metals within the plant body biomass (Jansen et al., 1996). The RAPD assay performs well in terms of number of bands, product yield and clarity of the profile. Although the generation of RAPD profiles has often been criticized as unreliable (Ellsworth et al., 1993). Disappearing bands are likely to be due to changes in oligonucletide primers sites, originated from rearrangements and less likely from point mutations and DNA damage in the primer binding sites (Nelson et al., 1996; Liu et al., 2005; Enan.2006; Liu et al., 2009).

The RAPD was used to evaluate the genotoxicity created by heavy metals on different light conditions as presence or absence of band in PCR amplification. Seven primers OPG 2, OPG-7, OPG-8, OPG-13, OPO-8, OPO-12 and OPA-13 produced unique bands in *Vigna Radiata* at 100 mg/300g of soil for all heavy metals. These unique bands clearly differentiated the seedlings treated with Hg and Cd, and would be act as marker for assessment of genotoxic effect. The low concentration of these heavy metals (25mg/300 g of soil) didn't create any mutation in the genome and these were tolerable. In this study the number of bands patterns in treated plant seedling was evaluated to appearance or disappearance of bands. The 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 *Phaselous vulgaris* (Atienzar et al., 2000; Liu et al., 2005; Enan et al, 2006). Lead ,copper and cadmium affect DNA of Silene paradoxa, Kideny and barley plants showed the similar type of response as it was

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observed in the present study (Mengoni et al.,2000; Enan,2006; Liu 2005) Similar effect on GTS was reported due to UV in a marine alga *palnaria palnata* (Atienzar et al., 2000).

Our finding results support that DNA polymorphisms detected by RAPD can be considered as a powerful biomarker assay for detection of the genotoxic effects of environmental pollutants like heavy metals. These markers would be widely applicable to study the effect of genetic contamination on plants. The RAPD-PCR based assay is fast, reliable and easy to conduct in any laboratory for assessment of environmental hazardous metals on plants.

CONCLUSION

The present result showed that mercury and cadmium inhibit the seed germination at higher concentration as compare to low concentration in normal light. The heavy metal such as Lead, nickel and copper did not so much inhibit on seed germination. Genetic characterization of these oligonucleotide markers would be able to indicate that such primer could amplify heavy metal induced changes in DNA damage and toxicological studies. Finally but not least to required more study for better understanding the detection of genotoxicity.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest in this publication.

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