ASSESSMENT OF DNA DAMAGE USING RANDOM AMPLIFIED POLYMORPHIC DNA IN VEGETATIVE-STAGE BEAN (*PHASEOLUS VULGARIS* L.*)* GROWN UNDER A LOW FREQUENCY ELECTROMAGNETIC FIELD

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Abstract. Electromagnetic field produced by a moving electrically charged object, can be measured everywhere in the environment and it acts as an important effective stress factor on growth and development of plants. Plants are organisms exposed to different levels of biotic and abiotic environmental impacts and are able to distinguish and respond to their surrounding environment with a high specificity. This study aimed to determine the genotoxic effect of a low frequency electromagnetic field (EMF) on the genomic material of plants. Two groups of bean seeds (wet and dry) were subjected to an electromagnetic field of 4 mT. RAPD profiles of EMF-treated seeds showed differences in banding patterns compared to those of the control. There is no significant different between dry and wet treatments. This result forcefully indicated that genomic template stability and band sharing index was significantly affected by the EMF-induced stress in treated seeds. It was concluded that DNA polymorphism detected by RAPD fingerprinting thus could be used as a potential molecular marker for the assessment of electromagnetic field-induced genotoxicity effects in plants and different treatments including chemical and physical motivators.

Keywords: *EMF exposure, genomic template stability, genotoxicity, RAPD, Fabaceae*

Abbreviations: *BSI – band sharing index; GTS – genomic template stability*

Introduction

In recent decades, physical methods based on the usage of electromagnetic fields are developed in the industry, agriculture, etc., and finally their waves distributed in the environment (Racuciu and Creangia, 2005). Levels of electromagnetic fields (EMF) from artificial man-made sources have increased progressively in the past decades and human societies are not aware that magnetic fields and plant development are related to each other. This relationship can be used in agriculture by treating the seeds, water, soil and nutrients in the soil (Rio and Rio, 2013; Cucurachi et al., 2013).

There is a rich literature describing the biological influence of low frequency electromagnetic fields upon vegetal organisms. But the responses elicited in various plant species appear to depend in a complex way on both the physical parameters of the irradiation source (frequency, power density, pulses or continuous waves, duration of exposure, etc.) and the conditions of the biological materials (vegetation stage, pretreatment, environment, etc.), nevertheless different biological parameters may lead to the revealing of different aspects of the interaction between the electromagnetic waves and the irradiated material (Ursache et al., 2009).

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Electromagnetic fields induce several cellular stress responses and change different cellular components such as membranes, proteins, enzymes and genome (Aksoy et al., 2010). Higher plants have been used as efficient bio-indicators of genetic toxicity of environmental pollutants. Several researches have used the chromosome aberration, micronucleus, or comet assays to estimate the effects of genotoxicity on plants (Steinkellner et al., 1999; Angelis et al., 2000; Yildizet al., 2009). Recently, advances in molecular biology have led to the development of a number of selective and sensitive assays for genomic material analysis in eco- genotoxicology. DNA based techniques (RFLP, QTL, RAPD, AFLP, SSR and VNTR) are used to examine the variation at the DNA sequence level (Liu et al., 2007; Enan, 2007). Random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR)-based technique that gives clearance for swift and efficient comparisons of genomic DNA. This technique is used extensively for species classification, genetic mapping, phylogeny and also it has been successfully used to study genotoxicity (e.g. DNA change, point mutation, insertions or deletions and ploidy variation) in different plants (Kumari et al., 2009; Liu et al., 2009; Cenkci et al., 2009).

The common bean [*Phaseolus vulgaris* L. (cv. D81083)], was used as plant material in this study that it is the major source of protein and minerals for example iron, magnesium and etc. in the world. Bean is a diploid (2n = 22), in fact it is important genetic model that has been widely used in physiological, biochemical and molecular analyses of toxicology (Enan, 2007).

The aim of the present study was to evaluate the DNA changes induced by low frequency electromagnetic field (4 mT) using RAPD technique in the vegetative stage.

Material and Method

Phaseolus vulgaris L. (cv. D81083) seeds were prepared from khomain research center, Arak, Iran. The seeds were surface- sterilized using sodium hypochlorite at 5% for 10 min, washed two times with distilled water and dried at room temperature. The seeds were divided into two groups, one group was immersed in distilled water for 12 h (wet seeds) while the second group remained dry (dry seeds). Each group was further divided into three subgroups: two sub groups were exposed to EMF (once treated and twice treated subgroups), while the third, non-exposed seeds were used as control (not treated subgroup).The seeds of once treated sub group were exposed to a field of 4mT intensity for 45 min, while those of twice treated subgroup were exposed twice to a 4mT field with a two-hour interval, each time for 45 min. Three replicates were used in the experiment with 30 seeds in each treatment. The electromagnetic field was performed by a locally designed generator which its electrical power was provided by a 220 V and 0.1 A, AC power supply. This system consists of a PVC cylinder with 20 × 20 cm (diameter and length) and 300 turn coil of copper wire. The samples were placed in the middle of a horizontally fixed coil and for exposure. The temperature was measured with a thermometer to be 22±1°C.Then treated and untreated seeds were grown in petri dishes in a growth room with temperature controlled at 28±1° C and a 16 h light/8h dark cycle.

The leaves of plants in vegetative stage were used for genomic DNA extraction. Silica gel was used for the dehydration of fresh leaves and were stored at -20°C. DNA was extracted following the cetyltri methyl ammonium bromide (CTAB) method as described by Saghai-Maroof (Saghai-Maroof et al., 1984). Briefly, fresh plant sample was ground to a fine powder in liquid nitrogen, mixed with 1 mL of CTAB extraction

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buffer [2% hexa decyl trimethyl- ammonium bromide, 100 m M Tris–HCl, (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% PVP-40 and 0.4% 2-mercaptoethanol]. Nineteen 10-mer oligo nucleotide primers (macrogen, Tehran) were used for characterization of genotypes (*Table 1*). *In vitro* amplification using polymerase chain reaction (PCR) was performed using 50 ng of genomic DNA of each genotype, 1.7 µM primer, 200 µM dNTPs (50 µM of each), 10× reaction buffer (100 mM Tris–HCl, pH 8.8, 500 mM KCl, 15 mM $MgCl_2$, 0.8% Nonidet P40) and 1 U Taq DNA polymerasein a final volume of 20 µl per reaction.

Amplifications were performed in a DNA thermocycler (Uvigene, Uvitech Ltd., UK) programmed for 4 min at 94 °C (initial denaturing step), 40 consecutive cycles each consisting of 45 s at 94 °C (denaturing), 45 s at 36 °C (annealing), 60 s at 72 °C (extension), followed by 1 cycle for 8 min at 72 °C (final extension step).The PCR products were analyzed by electrophoresis on 1.5% agarose gels in 0.5× TBE (90 mMTris base, 90 mM boric acid and 2 mM EDTA) buffer. The gel was run at 10 V, visualized under UV light and photographed using ultracam digital imaging.

Table 1. *Sequences of primers used in this study*

Primer name	Primer sequence	Primer name	Primer sequence
OPA-10	5′-GTGATCGCAG-3′	OPA-17	5′-CACCGCTTGT-3′
OPE-11	5′-CAATCGCCGT-3′	OPB-10	5′-CTGCTGGGAC-3′
OPE-01	5′-CCCAAGGTCC-3′	OPD-07	5′-TTGGCACGGG-3′
OPE-12	5′-TTATCGCCCC-3′	OPG-14	5′-GGATGAGACC-3′
OPA-05	5′-AGGGGTCCTG-3′	OPC-08	5′-TGGACCGGTG-3′
OPA-04	5′-AATCGGGCTG-3′	OPB-07	5′-GGTGACGCAG-3′
OPA-01	5′-CAGGCCCTTC-3′	OPC-02	5′-GTGAGGCGTC-3′
OPG-06	5′-GTGCCTAACC-3′	OPA-09	5′-GGGTAACGCC-3′
OPA-07	5′-GAAACGGGTG-3′	OPA-03	5′-AGTCAGCCAC-3′
OPE-06	5′-AAGACCCCTC-3′		

All amplifications were repeated twice in order to confirm the reproducible amplification of scored fragments and only reproducible bands obtained in repeated experiments were taken into account. The marked changes observed in RAPD profiles (disappearance or appearance of bands, increase or decrease in band intensity in comparison with untreated control treatments) were evaluated (*Table 2*).
Genomic template stability (GTS, %) was calculated as follows:

$$GTS\ (\%) = (1 - \alpha/n) \times 100 \qquad \text{(Eq. 1)}$$

Where α is the average number of polymorphic bands detected in each treated sample and n is the number of total bands in the control. (Savva, 2000)
In order to evaluate the band sharing index (BSI) that is considered as a measure of similarity between two samples, following equation was used:

$$BSI = 2s/(a+b) \qquad \text{(Eq. 2)}$$

where *s* is the number of bands shared between two samples, *a* is the number of bands in the first sample and *b*is the number of bands in the second sample. A BSI value of 1

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indicates that the two samples are identical, while a BSI value of zero indicates that the two samples are totally different (Savva, 2000).

Results

In order to verify the geneotoxic effect of low frequency EMF, the RAPD analysis was performed on DNA extracted from the 30-d-old plants. In total, nineteen 10-mer oligo nucleotide primers of 60-70% GC content were utilized for screening the bean genome for modification in response to EMF. Seventeen primers generated specific and stable results. The total number of bands was 174, 175, 171, 179, 177, 172 in untreated dry seed, once treated dry seed, twice treated dry seed, untreated wet seed, once treated wet seed and twice treated wet seed, respectively, ranged from 100 to 1500 bp. The maximum and minimum of bands observed in untreated wet seed and twice treated dry seed RAPD profiles respectively. On the other hand, seeds in wet condition had more bands than those in dry condition. As observed in *Table 2*, RAPD profiles of the controls have more bands in comparison to treatments.

Table 2.*Changes in total bands in control, and of polymorphic bands and varied bands in EMF-treated seedlings. a: appearance of new bands, b: disappearance of normal bands, c: decrease in band intensity, and d: increase in band intensity. a+b denotes polymorphic bands, and a+b+c+ d denotes varied bands*

No. of primers treatments																
	Dry seed								**Wet seed**							
	No EMF	**Once EMF**				**Twice EMF**				**No EMF**	**Once EMF**				**Twice EMF**			
		a	b	c	d	a	b	c	d		a	b	c	d	a	b	c	d
OPA-10	6	0	0	1	0	1	1	1	1	6	0	0	0	0	1	1	1	1
OPE-11	8	0	0	0	0	2	3	1	1	8	2	0	0	1	3	4	0	0
OPE-01	8	0	0	0	0	1	2	1	0	8	0	0	0	0	1	2	0	0
OPE-12	12	0	0	0	0	0	2	0	0	12	0	0	0	0	0	2	0	1
OPA-05	10	0	0	0	0	2	1	0	0	10	1	0	0	0	1	1	0	0
OPA-04	13	0	0	0	0	0	3	0	0	12	0	0	0	0	0	1	0	0
OPA-01	13	0	0	0	0	4	3	0	0	13	0	0	0	0	3	3	0	0
OPG-06	10	1	0	0	0	2	1	0	0	10	0	0	0	0	2	1	1	1
OPA-07	9	2	2	0	0	1	3	0	0	11	0	0	0	0	0	3	0	0
OPE-06	11	0	0	0	0	4	1	0	2	11	0	0	0	0	3	2	0	2
OPA-17	8	1	2	0	0	1	1	0	0	8	0	0	0	0	2	0	0	0
OPB-10	5	0	0	0	0	0	2	0	0	7	0	2	1	0	0	5	2	0
OPD-07	8	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0
OPG-14	12	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0
OPC-08	7	0	0	0	0	0	1	0	1	7	0	0	0	0	0	1	0	1
OPB-07	12	2	0	0	0	1	0	0	0	12	0	0	0	0	2	1	0	0
OPC-02	8	0	1	0	0	1	1	0	0	8	0	0	0	0	1	1	0	0
OPA-09	12	1	1	2	0	1	2	2	0	13	0	1	0	0	1	3	0	0
OPA-03	2	0	0	0	0	3	0	0	0	3	0	2	0	0	4	1	0	0
Total bands	174	7	6	3	0	24	27	5	5	179	3	5	1	1	24	31	4	6
a + b		13				51					8				55			
a + b + c + d		16				61					10				65			

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RAPD profiles showed remarkable differences between untreated control and treated plants with apparent changes (disappearance and/or appearance, increase and decrease of band intensity) in the number and size of amplified DNA bands for different primers. The changes in RAPD profiles were summarized for treated seedlings in comparison to their controls (*Table 2*). RAPD pattern screated by OPD-07, OPG- 14 and OPC-08 primers were identical for all treatments and the controls (*Fig. 1*). For OPD-07, OPG-14 primers, there was no change in RAPD profiles in all samples.

RAPD pattern in plants exposed to EMF showed variations in the appearance and disappearance of bands, as well as changes in band intensity in both dry and wet conditions. The number of polymorphic bands (appearance of new bands+ disappearance of normal bands) and varied bands (appearance of new band + disappearance of normal band + decrease in band intensity + increasein band intensity) were 13, 16 and 51, 61 bands in the once EMF treatment and twice EMF treatment in dry condition, respectively. Similarly in the once EMF and twice EMF treatments in wet condition the number of polymorphic bands and varied bands were 8, 10 and 55, 65 bands, respectively.

In the once EMF treatments, the number of new bands was low (7 and 3 new bands in dry and wet, once EMF treatments, respectively), while the number of new bands was high (24 and 24 bands in dry and wet twice EMF treated, respectively) in the twice EMF treatments. The number of disappeared RAPD bands was more at twice EMF treatments (27 and 31 bands) than once EMF treatments (6 and 5 bands). No significant difference was observed between once EMF treatments and their controls. Furthermore, the decrease and increase in band intensity were observed for plants exposed to all EMF treatments (once and twice) used in this experiment for nine of the primers. Changes in band intensity were greater in twice EMF than in once EMF treatments (*Table 2*).

For each treatment group, the value of Band Sharing Index (BSI) and Genomic Template Stability (GTS) compared to their controls were calculated (*Table 3*). Results showed that with increase the time of exposed to the EMF, BSI and GTS values decreased in all treatments that showed the existence of variation among of RAPD profiles in all treatments compared to their controls. The estimated GTS and BSI characters were found to be lower (<1.00) in all treatments compared to the controls. Similar results were detected in twice EMF treatments compared to once EMF treatments. According to these characters, the seeds treated with once EMF showed the nearest distance (BSI: 0.92, 0.96. GTS: 92.52%, 95.53%) to their controls, but twice EMF treatments showed the furthest distance (BSI: 0.71, 0.70. GTS: 70.68%, 69.27%).

Table 3. *Effect of EMF on Band Sharing Index (BSI) and Genomic Template Stability (GTS) of treated bean seeds compared to the control*

	Treatments			
	Dry seed		**Wet seed**	
Character	**Once EMF**	**Twice EMF**	**Once EMF**	**Twice EMF**
BSI	0.92	0.71	0.96	0.70
GST	92.52 %	70.68 %	95.53 %	69.27 %

As indicated in *Table 3*, quantity of GTS and BSI characters are similar. As seen in our general results, wet seeds exposed to an EMF were much more affected than the dry seeds.

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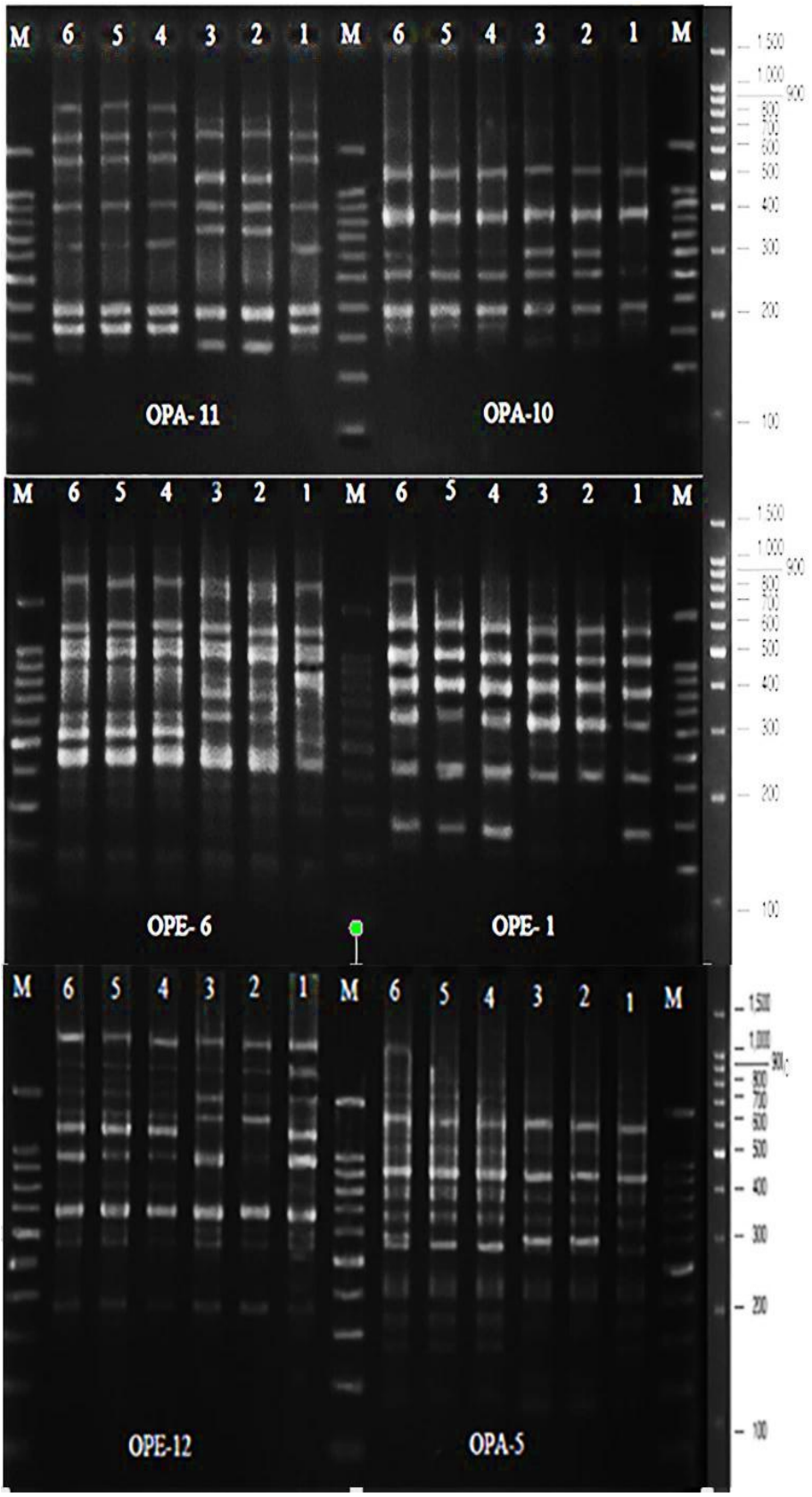

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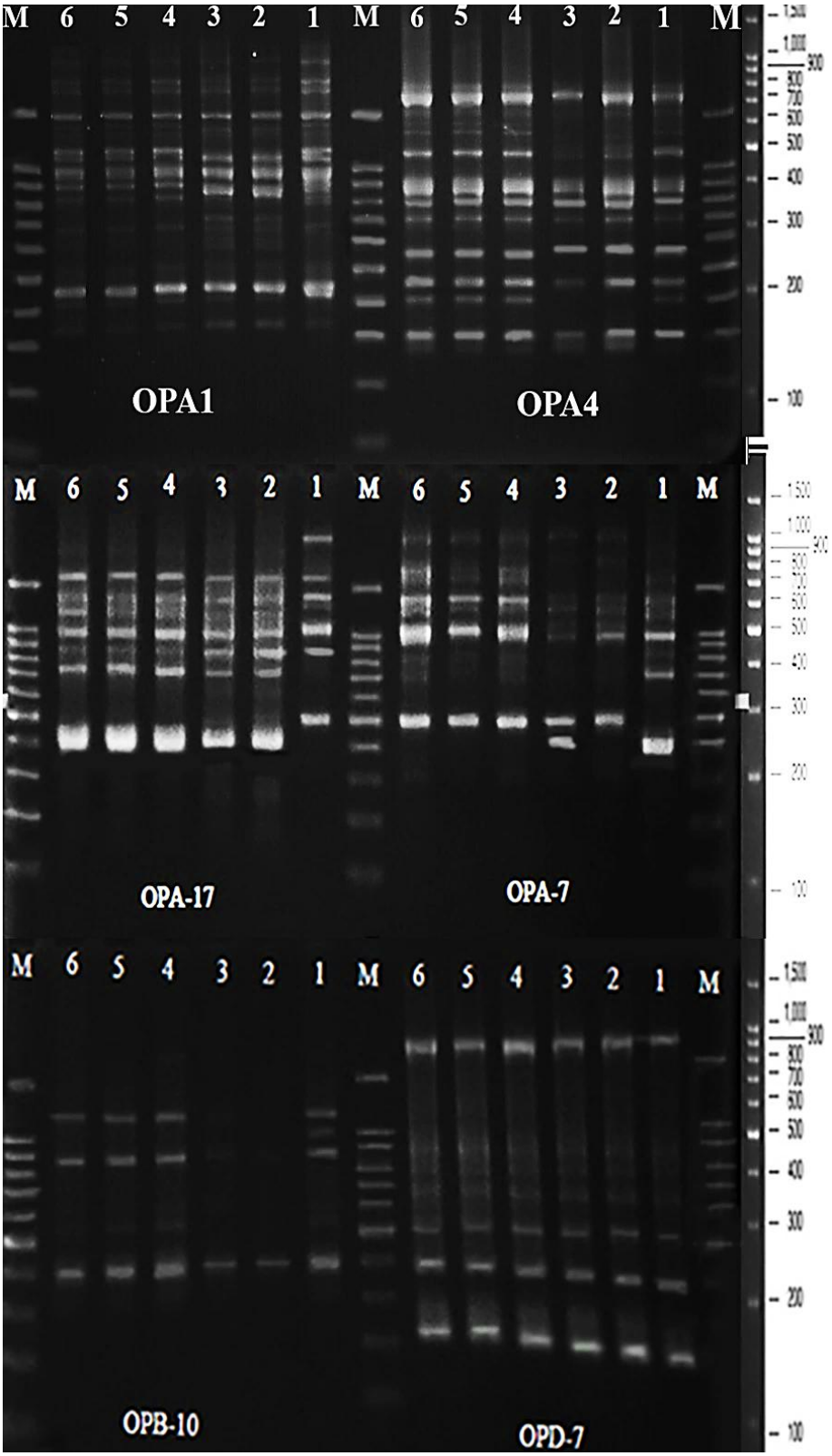

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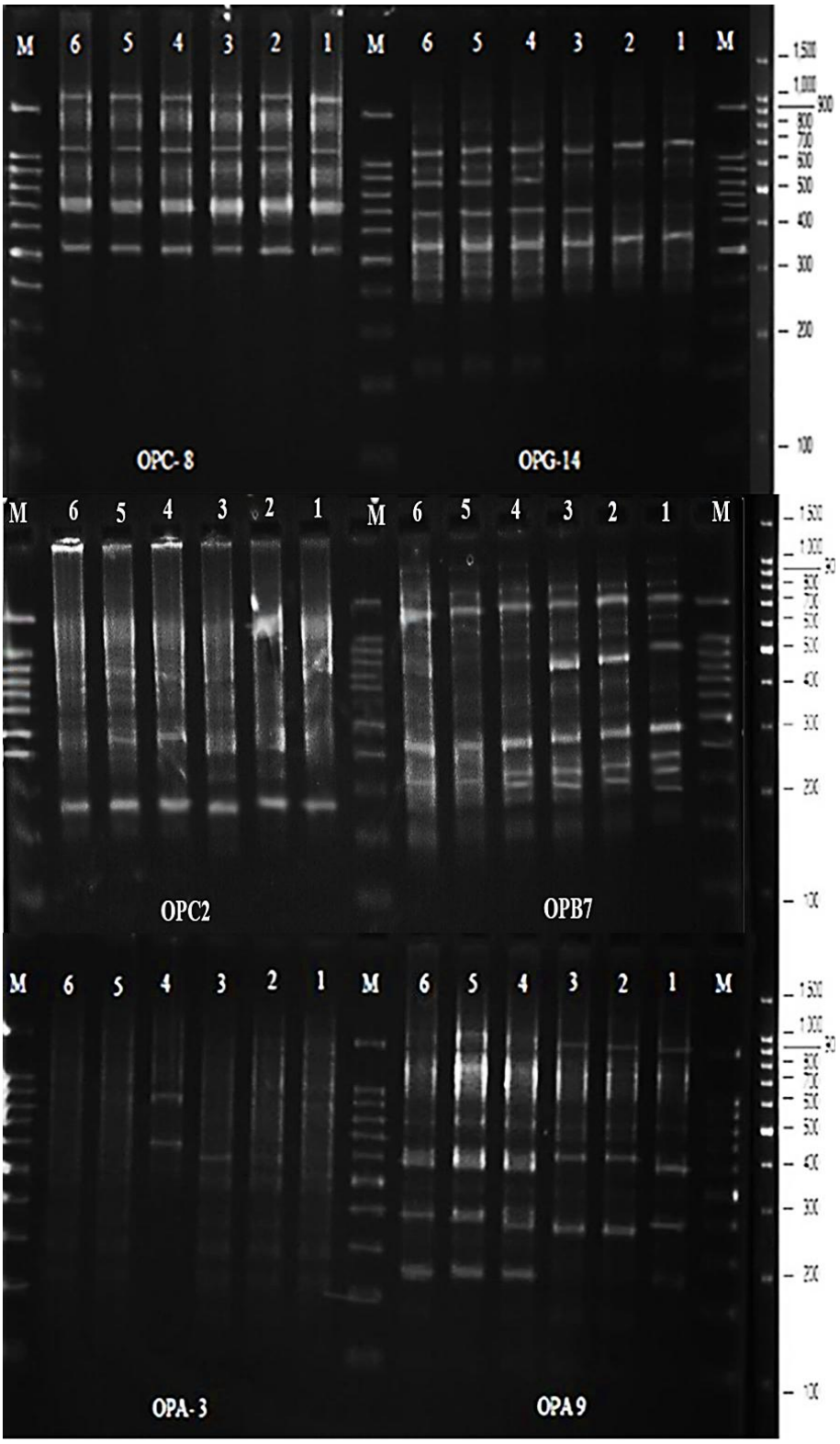

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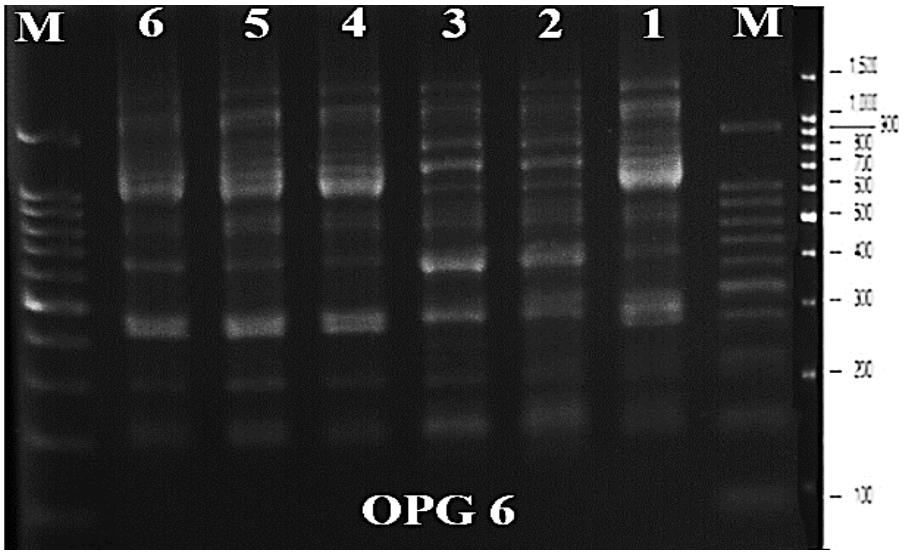

Figure 1. *RAPD profiles in plants exposed to different EMF treatments; the numbers represented the groups. 1: once EMF in dry seed, 2: Twice EMF in wet seed, 3: Twice magnetic field in dry seed, 4: control in wet seed, 5: control in wet seed, 6: once EMF in wet seed, M: DNA ladder (100–1500 bp)*

Discussion

In this study, we focused on the potential of RAPD assay to evaluate genotoxic effects in plants exposed to low frequency EMF for two different durations (a EMFof4mT intensity for 45 min, and a 4mT EMF with two-hour interval, each time for 45 min) and two conditions (dry and wet).

To detect different types of DNA change and genotoxic effect induced by chemical motivators in plants, RAPD method has been utilized extensively (Aksoy et al., 2010; Liu et al., 2007; Enan, 2007). The changes in DNA quality and quantity were detected by RAPD patterns created by randomly primed PCR reactions. These modifications may include changes in oligo nucleotide priming sites and variations in the activity of the *Taq* DNA polymerase. In this study, according to the RAPD profile, the obvious disappearance and appearance of bands produced by EMF in comparison to the untreated controls were considered to detect distinct changes between treatments. Our results showed a greater number of disappearance bands (69 bands) than appearance bands (58 bands) suggesting that the disappearance of normal bands and band intensity may be related to DNA change, point mutations and/or complex chromosomal rearrangements (Wolf et al., 2004). Also the appearance of new bands may unfold a modification in some oligo nucleotide priming sites due to mutations, large deletions, and/or homologous recombinations (Atienzar et al., 1999). The presence of new bands in this experiment may also be the result of genomic template instability related to the level of DNA change, the efficiency of DNA repair and replication (Atienzar et al., 1999).Therefore appearance of new bands could be related to mutations while the lost bands could be due to DNA change (Atienzar and Jha, 2006).

The RAPD patterns acquired with 19 primers were different for controls. Treatment of bean seeds with EMF induced genomics changes in plants. The number of disappeared and appeared bands in the twice EMF was higher than that of the once EMF treatments. Our results (*Figure 1* and *Table 2*) indicated that polymorphism

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increased with increasing EMF exposure time. On the other hand, the genomic template stability and Band Sharing Index in the treatments decreased with increasing EMF exposure time. Therefore, this study revealed the existence of a time exposing- effect relationship. Based on this, a correlation between EMF exposure period and level in EMF-induced DNA change in plants was shown by using the comet assay. Previous studies have also shown that changes in RAPD profiles can be regarded as changes in genomic DNA template stability, and this genotoxic effect can be directly compared with alterations in other parameters (Atienzar et al., 2000). Genotoxicity studies can be elicited qualitatively and quantitatively via the comparison of the control and treated groups. The diagnostic analysis contains band intensity differences or disappearance and/or appearance of RAPD bands are important parameters in RAPD method (Wolf et al., 2004).Although great progress has been achieved in the elucidation of molecular mechanisms of EMF, how plants sense and transduce the signal of EMF stress is still an important to picto be addressed.

Conclusion

In conclusion, our data support the view that RAPD analysis is a highly sensitive method for the detection of DNA change induced by environmental pollutants such as the magnetic fields. Also our results confirmed that low frequency electromagnetic fields have genotoxic effects on DNA structure. Moreover, as the purpose in this research was to establish the existence of DNA change for hazard identification in risk evaluation of genotoxicity studies, the presence in the DNA of any of these varied RAPD profiles can provide adequate evidence for identification of the genotoxic effect.

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