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Assessment of protein and DNA polymorphisms in corn (*Zea mays*) under the effect of non-ionizing electromagnetic radiation

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Abstract. Many reports highlight biological responses of crop plants after non-ionizing electromagnetic radiation (EMR) exposure based on the phenotypic and physiological levels. So, this study aimed to estimate genetic alterations in proteins, isozymes, and DNA banding patterns as well as the extent of nuclear DNA damage of economic corn (*Zea mays*) under the stress of EMR using accurate and reliable bio-assays like sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isozymes (Leucine- aminopeptidase, Esterases, Peroxidase, and Catalases), random amplified polymorphic DNA- polymerase chain reaction (RAPD-PCR), and Comet Assay, respectively. SDS-PAGE analysis showed distinct polymorphisms (96.66%) between EMR exposed and non-exposed corn seedlings depending on the number and type of bands, their intensities as well as molecular weight which ranged from (60.27 to 192.35 kDa), gain, and loss of bands. The four isozymes generated varies isozymatic polymorphisms based on relative front, zymogram number, and optical intensities. RAPD analysis generated 85 amplified DNA products with high polymorphism values ranged from 90.91 to 100% based on primers, band type, DNA sizes which ranged from 153 to 1008-bp, lose, gain, and intensity of DNA bands. Comet Assay scored highest extent of loosed DNA from nuclei (DNA damage) reached the value of (tailed ratio 20%) at EMR exposed corn nuclei for 5 days compared to non-exposed nuclei which reached the value of (tailed ratio 3%). This study concluded that each EMR exposure time had unique interaction with proteins, isozymes, and DNA of corn cells exhibiting wide range of genotoxic stress and subsequently, adversely effect on growth and yield of this sensitive crop plants.

Keywords: Electromagnetic radiation, *Zea mays*, SDS-PAGE, isozymes, RAPD-PCR, single cell gel electrophoresis.

INTRODUCTION

Nowadays, non-ionizing electromagnetic radiation (EMR) arise from both natural and wide human made sources such as variety of electronic devices (Rio & Rio, 2013) can influence the growth, yield, and quality of

plants based on flux of EMR and exposure time (Nyakane et al., 2019). Important components of cells are proteins that classified into varies classes according to their functions. The stress proteins generated from abiotic stressor like EMR consider a new class from these proteins with functions related to protection of cell (Iderawumi and Friday 2020). When EMR interact with the DNA can stimulate the synthesis of this stress protein, causing DNA strand breaks which increase by increasing of EMR energy (Blank & Goodman, 2012,

Shabrangi et al., 2011). Field parameters and characteristics (frequency, intensity, and wave-shape), cell type, and exposure duration all influence EMR genetic effects. The gene expression changes (for example, genes implicated in cell cycle arrest, apoptosis and stress responses, and heat-shock proteins) are consistent with the results that EMR causes genetic damage (Lai, H 2021).

The study of Ruiz-Gómez and Martínez-Morillo (2009) reported that a major concern of the genotoxic effects of non-ionizing electromagnetic field (EMF) is overproduction of ROS in cells and inducing oxidative stress on protein and DNA because of EMF exposure can induce DNA strand breaks and acts as a co-inductor of DNA damages rather than as a genotoxic agent. The genetic mechanisms by which EMR interact with protein and DNA are radical pair recombination led to increasing the concentration, activity, and lifetime of reactive oxygen species (ROS), which might cause changes in cell cycle, genetic mutation, damage to DNA which can lead to changes in cellular functions and cell death, modification of protein expression and oxidation of proteins, inhibition of enzymes (Kıvrak *et al*., 2017).

Higher plant species differ in their sensitivity and response to environmental stresses because they have a variety of stress perception, signaling, and response skills (Ahanger *et al*., 2017). Corn (*Zea mays*) is one of the world's major cereals and food crops for humans, it belonging to family *Gramineae* and genus *Zea*. Several physical abiotic stresses affect the total production of maize due to damage in its DNA, like EMF (Yan, *et al.,* 2011). It provides a promising genetic bio-monitors model to detect genotoxicity of environmental stress and DNA lesions induced by abiotic stress (Grant & Owens, 2006; Erturk, *et al*., 2014).

Many studies focused on the effect of EMF on plant growth and its development (Ortiz, *et al*., 2015) but rarely concerned with their effects on banding patterns and genetic polymorphisms of proteins, isozymes and DNA. This attracted the attention of this study to explore the interaction of low frequency EMFs (60 Hz^{+}) with proteins (enzymatic and non- enzymatic) using SDS-PAGE and isozymatic technique, respectively and DNA using RAPD- PCR and Single cell gel electrophoresis bioassays.

Identification of the biochemical and molecular mechanisms for plant tolerance like maize to environmental stress is important. Detection of proteins and isozymes alterations at the gene product level is carried out by biochemical markers which measuring allele frequencies for specific genes (Hailu&, Alatawi 2014). Meanwhile, molecular markers monitored differences (polymorphisms) within nucleotide sequences to indicate alterations at the DNA level, such as nucleotide changes: deletion, duplication, inversion, and/or insertion (Qi *et al*., 2014).

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) is a biochemical bioassay that is used to detect genetic differences in polypeptide banding patterns and to profile proteins induced by abiotic stress due to changes in the DNA coding sequences and active structural genes leading to modifications of structure protein, protein interaction, and stressed oxidative proteins (Karaca, 2013). On the other hand, isozyme analysis is a sophisticated biochemical approach that has a wide range of applications in detecting genetic alterations in plant cells (Hailu et al., 2014). Isozymes are enzymatic proteins, arise from multiple gene loci coding for distinct structural polypeptide chains, and their electric charge depends on the amino acids they contain (Hailu et al., 2014). They have different molecular forms showing the same substrate specificity due to changes in the nucleotide sequence of the DNA that codes for the protein, they differ in size, molecular weight, electrophoretic mobility, electric charge, and amino acid content (Karaca, 2013). Native polyacrylamide gel electrophoresis (Native PAGE) is used to differentiate protein variants in isozyme analysis, and an enzyme-specific staining combination, which contains a substrate, co-factor, and oxidized salt, is used to visualize them. (Karaca, 2013).

Recently mutations induced by genotoxic stress, damage and fragmentation of DNA can be estimated using molecular cytogenetic techniques such as RAPD-PCR and the single cell gel electrophoresis (Comet assay) which can determine the direct genotoxic effect of exogenous factors on plant genotypes at the nuclear DNA level (Cenkci *et al*., 2009; Santos, Pourrut, Ferreira de Oliveira, 2015). RAPD (random amplified polymorphic DNA) is a PCR-based technology that uses short randomized nucleotide sequence primers to amplify random DNA segments of genomic DNA without the need for prior genomic DNA knowledge. It can be used to detect genotoxicity, nucleotide sequence polymorphism, and alteration in RAPD profiles induced by environmental genotoxic stresses which lead to changes in the structure

of DNA in living organisms, such as point mutations, tiny insertions and deletions of DNA, and rearrangement of nucleotide sequences, all of which cause DNA damage and lesions (Atienzar & Jha, 2006).

Comet assay, also known as single cell gel electrophoresis, is one of the most modern procedures for analyzing DNA damages brought to agricultural sciences and genetic toxicology in recent years, reflected as single and double-strand breaks, oxidative-induced base damage and DNA-DNA/DNA protein cross linking induced by oxidative and genotoxic environmental factors (Nandhakumar *et al*., 2011). Comet-like shape of nuclei (with a head, the nuclear region and a tail which contains DNA fragments) is formed after electrophoresis and staining with a fluorescent dye and observed by a fluorescence microscopy (Dikilitas *et al*., 2009).

The aim of this study is to investigate biochemical and molecular mechanisms of non- ionizing electromagnetic radiation (EMR) on proteins and DNA of corn (*Zea mays*) based on SDS-PAGE, isozymatic analyses, RAPD-PCR, and comet assay to estimate genetic polymorphism in economic corn crop under the stress of EMR as well as understand how this plant was adapted.

MATERIAL AND METHODS

Plant material

The bio-monitor plant material in this investigation was maize grains (hybrid-323) supplied from the (Agronomy Research Department, Field Crops Institute, Agriculture Research Center, Giza, Egypt). Grains were checked for viability and homogeneity size before being divided into two groups: non-exposed and exposed to electromagnetic radiation (EMR). 30 grains of each group were sterilized and germinated in earthenware pot 60 cm in diameter containing soil obtained from the agriculture field until reached seedlings after thirty-days-old.

EMR Exposure facility

Electromagnetic radiations (EMR) are created when electric current flows: the greater the current, the stronger the magnetic intensity. So, (EMR) have magnetic and electrical properties that surround plant samples within that field. EMR generator system was designed a locally and presented at Biophysics Department, Faculty of science, Zagazig University, Egypt. This system consists of two coils, each formed by 1,000 turns of 1 mm copper wire, with a mean diameter of 260 mm and 25 cm length. EMR were generated by a handmade cylindrical shaped coil that was connected to a 220V AC power supply (ED-345BM, China), to generate electrical current of 60 Hz. EMR intensities were measured through use of magnetic flux meter type 4048 with probe T- 4048, 001, manufactured by USA. To keep the temperature from rising, a standard fan was used. The temperature was measured with a thermometer to be 22+1°C. Thus, a vertical sinusoidal magnetic field of 10 mT was generated in the central zone of the coils system when a 60 Hz sinusoidal electric current passed through the coils.

Corn seedlings were put in a vessel (a glass jar with diameter of 7 cm and height of 12 cm) by placing a glass jar daily in the center zone of the coils system, and then subjected to strengths of EMR (10 mT) for four different durations of exposure 1, 3, and 5 days, termed as (Ex-1, Ex-3, and Ex-5 days) while EMR non-exposed seedlings termed as (Ex-0). Leaves of ten corn seedlings were collected from EMR exposed, and non-exposed, and thoroughly cleaned with distilled water, for removal of any debris and then completely dried in air conditions and then subjected to biochemical and molecular cytogenetic analyses.

Biochemical and molecular cytogenetic bioassays

Dried leaves of EMR exposed, and non-exposed Corn seedlings were defatted and processed into leaf powder according to the methods outlined by Hojilla-Evangelista & Evangelista, (2006) and used for SDS-PAGE, isozymatic, RAPD-PCR, single cell gel electrophoresis analyses.

Biochemical bioassays

Protein extraction and SDS-PAGE analysis

Protein extraction from leaves was carried out as stated in the work of Abdelhaliem and Al-Huqail (2016) with some modifications. 0.2 g of powdered and defatted leaves was added to extraction buffer (0.5 M Tris-HCl, pH 6.8, 2.5% SDS, 5% urea, and 5% 2-merkaptoethanol) in an Eppendorf tube and mixed thoroughly by overtaxing. Extraction buffer was boiled for 5 min before centrifugation at 10,000 *g* for 5 min at 4°C and the supernatant was used. To visualize the mobility of protein on the gel, bromophenol blue was added to the supernatant as a tracking dye. SDS-PAGE was used to examine proteins using 10% SDS-polyacrylamide gels, as described by Laemmli (1970). The protein bands were observed after electrophoresis using Coomassie brilliant blue G-250 staining. Marker proteins (Fermentas) were

used as references.The molecular weights (kDa) of the polypeptide bands formed in the electropherogram were compared to the standard Pharmacia protein marker. To capture the image and determine band

intensities, gels were digitally photographed and analyzed using the Gel Doc Viller Lourmat system.

Data analysis of polypeptide banding patterns

The Bio-Rad video densitometer, Model Gel Doc 2000, was used to determine the number, concentration, and band density of polypeptide bands on each gel lane. Electropherograms of each germplasm of EMR exposed and non-exposed corn plants were evaluated for the presence (1) or absence (0) of protein bands to assess variance in the protein banding pattern. Protein polymorphisms were evaluated based on previous polypeptide banding pattern differences.

Isozymes extraction and Isozymatic analysis

Identification of isozymatic variations induced in EMR exposed, and non-exposed corn seedlings were performed using the sodium dodecyl sulphate-native polyacrylamide gel electrophoresis (PAGE) according to the methods of Majumder, Hassan , Rahim, Kabir (2012). Four isozymes, Leucine-aminopeptidase (LAP. E.C. 3.4.1 1. I), Esterases (EST, E.C. 3.1.1.1), Peroxidase, (PRX E.C. 1.11.1.7), Catalases (CAT, 1.11. 1.6), were used in this study. Isozymes of each sample was extracted according to method of Majumder et al. (2012) that described briefly in the study of Abdelhaliem & Al-Huqail, (2014).

The staining of gel of LAP and CAT isozymes was performed according to protocol of Pasteur, Pasteur, Bouhomme, Catalan, Davidian (1988) while EST and PRX isozymes followed the protocol of Tiwari & Bakshi (2015) for. The Vilber Lourmat gel documentation system was used to photograph the gels. The most common allele at each locus for each isozyme was assigned as relative front mobility (R_f) value. The value R_f was calculated in equation (1).

R_f = Distance of zymograms migrated / Distance of marker dye migrated (Eq. 1)

PAGE and data analyses

Zymograms of each enzyme were observed and studied against an intense fluorescent light. After the staining of LAP, EST, PEX, and CAT isozymes, the isozymatic data were collected and immediately only consistent and clear zymograms were scored. The isozymatic banding patterns were compared among of EMR non-exposed, and exposed corn seedlings based on their relative front (R_f) values on gel electrophoresis, zymogram number, their intensities, and the percentages of polymorphic, unique and non-unique loci. Different isozymatic patterns were scored as discrete variables, the presence "1" or absence "0" of zymogram. Alterations of isozymatic patterns and zymograms at each locus were calculated using the POPGENE 32 version 1.31 software based on the computer program (Labate, 2000).

Molecular cytogenetic bioassays

Genomic DNA isolation and RAPD-PCR analysis

RAPD analysis was performed to analyze the genotoxic effects of EMR exposed, and non- exposed corn DNA. Genomic DNA of powdered and defatted leaves were isolated following a modified Hexadecyl trimethyl ammonium bromide (CTAB) buffer protocol Kit & Chandran (2010) as described briefly in the study of Abdelhaliem & Al-Huqail, (2013). The absorbance of diluted DNA solution at 260 nm and 280 nm was used to measure the purity and concentration of DNA. The DNA quality was evaluated using ethidium bromide-stained agarose gel electrophoresis.

DNA amplification process by PCR

Reactions of DNA amplification by PCR, analysis of amplification products by agarose gel electrophoresis were conducted following the protocol of Williams Williams, Kubelik, Livak, Rafalski, Tingey (1990) with some modifications. The mixture of PCR amplification reaction as described briefly in the study of Abdelhaliem, & Al-Huqail (2016) was consisted of 2.5 µL 10X buffer with 15 mM $MgCl_2$ (Fermentas, Vinius, Lithuania), with 0.25 mM each of dATP, dCTP, dGTP and dTTP (Sigma, St. Louis, MO, USA), 0.5 U Taq DNA polymerase (Sigma), 0.3 mM primer and 50 ng template DNA. The PCR was performed in a Palm thermal Cycler apparatus (Corbett Research) was programmed using the following method: initial denaturation of 4 min at 95°C followed by 45 cycles of 1 min at 95°C, 1 min at 38°C, and 2 min at 72°C with final extension at 72°C for 10 min and a hold temperature of 4°C. Only five primers (P-02, 06, 08, 10, and 14) effectively generated reproducible amplified products after a total of 20 random DNA oligonucleotide primers

(10 mer) were employed in the PCR (University of British Columbia, Canada). Amplification DNA products were analyzed by electrophoresed on 1.5% agarose gel (Sigma) in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8). The run lasted one hour at a constant voltage of 100 volts. For 15 minutes, gels were stained with 0.2 mg/mL ethidium bromide. A UV light transilluminator was used to examine the PCR products. To determine band sizes, a 100-bp DNA ladder (Gibco-BRL, Grand Island, NY, USA) was put into the first lane of each gel. A gel documentation system was used to photograph the gels under UV light (Bio-Rad, Hercules, CA, USA).

Analyses of DNA banding patterns

Visualization of amplified DNA products on agarose gel electrophoresis was carried out using Photo Print (Vilber Lourmat, France) imaging system. Banding patterns generated by RAPD were analyzed using one-dimensional software (Advanced American Biotechnology and Imaging, Fullerton CA 92831, USA) while DNA polymorphisms in RAPD profiles included disappearance of a band and appearance of a new band with respect to the non-exposed profile, were calculated using several amplified DNA parameters such as losses of normal bands, appearance of new bands, the number of polymorphic (unique and non-unique DNA bands), and monomorphic DNA bands, and the molecular sizes of DNA bands as well as intensity of bands for each EMR exposed sample compared to non-exposed one. Amplified DNA products were scored based on the presence (1) or absence (0) of DNA bands for each primer and DNA band intensity estimated by using image analysis software. Polymorphic DNA bands (unique and non-unique) and monomorphic bands were also scored.

Estimation of genetic Polymorphisms

Polymorphism (*P*, in %) of protein or isozyme or DNA were estimated according to Gjorgieva *et al.*, (2013) based on the lost bands (non-unique) and the appearance of a new band (unique bands) as well as the monomorphic bands (bands with the same loci at all samples) as in equation (2).

$$\text{Polymorphism } \% = [a+b/c] \times 100 \quad \text{(Eq. 2)}$$

Where *a* is the number Polymorphic bands (a is new bands unique, *b* is the number of lost non- unique bands and *c* is the total number of scored bands (Polymorphic and monomorphic bands).

Isolation of nuclei and Comet Assay (Single cell gel electrophoresis) technique

Isolation of nuclei and slide preparation

The nuclei of EMR exposed, and non-exposed corn leaves were isolated following the protocol of Juchimiuk *et al.*, (2006). Five hundred mg of leaves were rinsed in distilled water twice, dried with a paper towel and then placed in a glass petri dishes containing 200 µL of cold Tris-HCl buffer, pH 7.5 (on ice). Under yellow light, leaves were carefully sliced into a "fringe" with a new razor blade to release nuclei into the buffer. This approach of nuclei isolation found to be the most effective in obtaining low DNA lesions in non-exposed samples. Each slide was covered with a mixture of 55 l nuclear suspension and 55 l LMP agarose (1% generated with phosphate-buffered saline) and cover slipped at 40°C after being coated with 11% NMP agarose and dried. After putting the slide on ice for at least 5 min., the coverslip was removed. The coverslip was then replaced after 110 l of LMP agarose (0.5%) was poured on the slide. The coverslip was removed after 5 minutes on ice.

Single cell gel electrophoresis technique

Comet assay slides were prepared as described by Juchimiuk et al. (2006). The corn nuclei slides were horizontally put in a gel electrophoresis tank with freshly prepared cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) and incubated for 15 min. At 4°C, electrophoresis was carried out at 16 V, 300 mA for 30 min. The gel was then neutralized by washing three times in 400 mM Tris-HCl, pH 7.5, and then stained for five minutes with ethidium bromide (20 g/mL). The gels were immediately dipped in ice-cold distilled water after staining and examined.

Comet imaging and software analysis

The level of DNA damage in 50 randomly chosen nuclei was examined in each slide using a computerized image analysis system or a fluorescence microscope with an excitation filter of 546 nm and a barrier filter of 590 nm (Komet Version 3.1. Kinetic Imaging, Liverpool, UK). Tail DNA (TD percent, relative proportion of DNA in the comet tail) and tail moment (TM, integrated value of density multiplied by DNA migration distance from nuclei) were used as DNA damage parameters to quantify nuclear DNA damages (Juchimiuk et al., 2006). The

percentage of nuclei having tails was also estimated, as well as relative tail length.

RESULTS

Biochemical genetic bioassays

SDS-PAGE bioassay

The electrophoretic profiling of polypeptide banding patterns based on molecular weight (kDa), band number, band intensity, fractionation of bands, appearance of new bands and the loss of some bands as parameters of polypeptide banding showed variations between EMR exposed and non-exposed corn seedlings by SDS-PAGE analysis (Table 1 and Figure 1). There were 39 polypeptide bands with molecular weights ranging from 60.27 to 192.35 kDa, 29 of which were polymorphic with a value of 74.36%, according to the data obtained, (24 unique bands with a value of 61.54%, plus 5 non-unique bands with value of 12.82%) in addition to one monomorphic band value of 2.56%.SDS-PAGE analysis indicated distinctive polymorphism value of 96.66% based previous banding variations. When comparing EMR exposure samples to non-exposed samples, there were noticeable differences in the number of polypeptide bands and molecular weight. The highest number of polypeptides bands was 12, with a value 30.77% scored at corn seedlings exposed to EMF for 5 days while the lowest number was 8, with a value 20.51% for non-exposed samples (control).

Unique polypeptide bands obtained from SDS-PAGE were varied in molecular weight (kDa), and in number and intensity of bands among EMR exposed and non-exposed samples (Table 1). As a result, unique bands can be employed as a tool for the appearance new characteristic polypeptide bands that are specific for each EMR exposure time. The highest number of unique polypeptides bands was 9, with a value of 37.50% for samples exposed to EMR for 5 days while the lowest number (4 unique bands), and with a value of 16.67 scored at samples exposed to EMR for one a day (Table 1).

Isozymatic bioassay

LAP, EST, PRX, and CAT isozymes used in this study revealed clear isozymatic polymorphisms among EMR exposed and non-exposed Zea mays reached the values of 91.66 % for LAP and EST, 88.89% for CAT, and 80.00% for PEX based on zymograms number, loci, R*f* values, and optical densities generated by each isozyme, separately (Tables 2, 3, 4 and 5; Figures 2 and 3 A and B).

A total of 72 different electrophoretic zymograms produced by the four isozymes showed varied relative front (R*f*) values, varying from 0.01 to 1.20 and varied values of optical densities (OD). Of these zymograms, 38 with a value of 52.77% were polymorphic (27 unique zymograms with a value of 37.5% plus 11 non- unique zymograms with a value of 15.28%). The higher number of zymograms was (19) generated by EST and PRX while LAP and CAT isozymes generated 17 zymograms. The four enzymes scored maximum number of zymograms (21) at corn seedling exposed to EMF for 5 days compared with 13 zymograms scored for non-exposed samples.

On the other hand, LAP , EST, PRX, and CAT isozymes generated unique zymograms varied in R*f* values and optical densities. The highest number of unique zymograms produced by four isozymes was 9; with a value of 33.33% recorded for samples exposed to EMR for 1 and 5 days, in comparison to 5 unique zymograms; with a value of 18.51 % for non-exposed samples (Tables 2, 3, 4 and 5).

Molecular cytogenetic bioassays

RAPD-PCR bioassay

Profiles and banding patterns of amplified DNA bands generated by RAPD exhibited clear variations among corn seedlings exposed to EMR compared to non-exposed one (Table 6 and Figure 4). Only five of the 20 random decamer primers examined revealed distinct alterations in the amplified DNA banding patterns and provided specific and reliable results and consistent bands. 85 DNA bands were produced by RAPD analysis, the sizes of these bands ranged from 153 and 1008 bp in length. The RAPD analysis, on the other hand, identified three types of amplified DNA bands (polymorphic, monomorphic, and polymorphic), which differed quantitatively and qualitatively in band number, size (bp), and intensity on an agarose gel (Table 6). There were, 59 bands were polymorphic (unique and non-unique bands) with a value of 69.41% (36 unique bands with a value of 42.35% and 23 non-unique bands with a value of 27.06%) and 5 monomorphic bands with a value of 5.88%. An average of 17 bands per primer was scored. Furthermore, Table 6 shows the total polymorphisms produced by the five primers, which reached a value of 97.01%. The primers differed with respect to the value of polymorphisms detected. The highest level of polymorphism (100%) was revealed by primers (P-14) because of it do not detect any monomorphic bands, followed by primers (P-02 and P-10) which recorded polymorphism value of 90.91%, P-06 recorded polymorphism value of (88.89%)

Table 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins of EMF exposed and non-exposed *Zea mays* seedlings for days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5) using the documentation system Gel Doc Bio Rad system 2000. Lanes 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5), respectively.

Lanes Rows	Molecular weight (kDa)	Polypeptide bands in each lane								
		Lane1		Lane 2		Lane 3		Lane 4		Band types
		kDa	%	kDa	%	kDa	%	kDa	%	
1	192.35	0	-	0	-	1	10.90	0	-	U
2	184.13	1	2.27	0		0	-	0	-	U
3	180.15	0	-	1	5.67	0	-	0	-	U
4	158.02	0	-	0	-	0	-	1	6.67	U
5	154.61	0	-	0	-	1	5.98	0	-	U
6	148.00	0	-	1	4.08	0	-	0	-	U
7	145.31	1	10.40	0	-	0	-	0	-	U
8	138.81	0	-	0	-	1	5.31	1	1.55	Non-U
9	130.19	0	-	1	1.17	0	-	0	-	U
10	126.66	0	-	0	-	1	2.23	0	-	U
11	118.79	1	11.10	0	-	0	-	0	-	U
12	115.57	0	-	1	17.40	1	13.00	0	-	Non- U
13	112.44	0	-	0		0		1	17.60	U
14	105.45	1	10.40	1	5.51	0	-	0	-	Non-U
15	104.49	0	-	0	-	1	6.53	0	-	U
16	89.84	1	28.30	1	27.60	1	21.50	1	24.60	M
17	80.24	0	-	0	-	0	-	1	8.28	U
18	76.16	1	2.70	0	-	0	-	0	-	U
19	75.50	0	-	0	-	0	-	1	1.43	U
20	71.66	0	-	0	-	0	-	1	4.24	U
21	71.04	0	-	1	15.20	0	-	0	-	U
22	70.42	0		0		1	13.40	0	-	U
23	67.43	0	-	0	-	0	-	1	2.07	U
24	66.27	1	13.40	0		0	-	0	-	U
25	63.66	0	-	0	-	0	-	1	2.07	U
26	62.00	0	-	1	16.90	1	13.90	1	1.00	Non-U
27	62.22	0	-	0	-	0	-	1	10.30	U
28	61.45	1	21.50	0	-	0	-	0	-	U
29	60.91	0	-	1	6.46	1	7.12	0	-	Non-U
30	60.27	0	-	0	-	0	-	1	21.20	U
No. of polypeptide bands			8		9		10		12	
Total polypeptide bands						39				
% of total bands			20.51		23.07		25.64		30.77	

	Unique (U) bands		(Non-U) bands		Polymorphic bands		Monomorphic bands		Polymorphisms %
	No	%	No	%	No	%	No	%	
Frequency of polypeptide bands and Polymorphisms	24	61.54	5	12.82	29	74.36	1	2.56	96.66

	Lane1		Lane2		Lane 3		Lane 4	
	No.	kDa	No.	kDa	No.	kDa	No.	kDa
No.and MW(kDa) of unique polypeptide bands	6	184.13–145.31-76.16–66.27–61.45	4	180.1501–48.00–130.19–71.04	5	192.35–154.61 126.66–104.49–70.42	9	158.02–112.44–80.24–75.50–71.66–67.43–63.66–62.22-60.27
Total unique bands					24			
% of unique bands		25		16.67		20.83		37.50

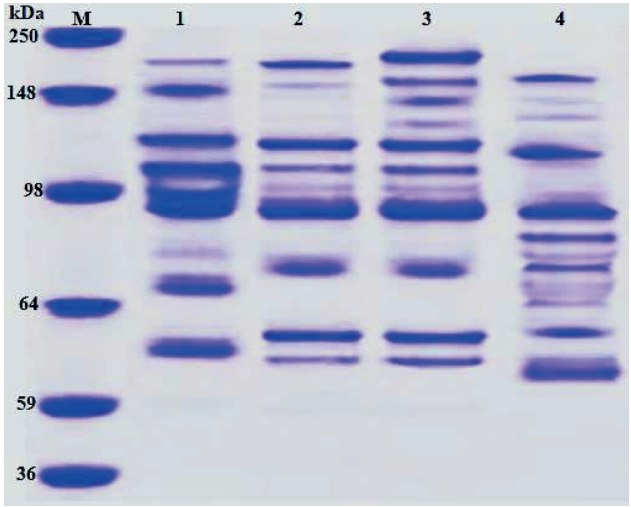

Figure 1. Polypeptide banding patterns analyzed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique of non-exposed and EMR exposed seedlings of *Zea mays* for days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5) based on relative front (R*f*) Values and optical densities (OD). Lanes 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex- 3,and Ex-5), respectively.

while primer (P-08) scored the lowest level of polymorphism value of (81.83%). These genetic DNA polymorphisms based on the gain and/or loss of DNA bands in EMR exposed samples compared to the non-exposed one (control). Besides, the number of RAPD amplified DNA bands varied among EMR exposed corn seedlings compared to control and correlated positively with increasing exposure time of EMR. The highest number of amplified DNA bands was 26, with a value of 30.59%, which was produced by five primers and was detected in EMR exposed sample for 5 days exposure time compared to 21 DNA bands, with a value of 24.71% which recorded at non-exposed samples. Unique amplified DNA bands created by RAPD analysis were distinctive loci specific for one exposure time based on their number, their molecular sizes, and optical intensities. The highest number of unique DNA bands produced by five primers was 13, with a value of 36.11% recorded in EMR exposed samples for 5 days exposure time compared to 10 DNA unique bands, with a value of 27.78% which recorded at non-exposed samples, while the lowest number of unique DNA bands was six, with a value of 16.67% for EMR exposed samples for one days exposure time (Table 6).

Single Cell Gel Electrophoresis Technique bioassay

The Comet Assay or single cell gel electrophoresis assay (SCGE) is one of the very widely used assays to microscopically detect DNA damage at the level of a single cell. Cells containing damaged DNA have the appearance of a comet with a bright head and tail. The SCGE or comet test was employed in this investigation to identify nuclear DNA (nDNA) damage caused by an electromagnetic radiation stressor in corn seedlings for different exposure times (Table 7 and Figure 5). The extent of DNA migration from nuclei (tailed ratio), tail length μm, % of tailed DNA (TD percent), and tail moments(TM) were utilized to evaluate the level of DNA damage caused by the comet assay. The recent findings revealed that each EMR exposure time led to inconsistent differences in the level of DNA damage in corn nuclei. EMR exposed samples for 5 days exposure time (Ex-5) detected the highest DNA migration from corn nuclei (tailed ratio 20%) with tail length (2.88 μm), TD% (2.79%), and TM (8.04); this demonstrated that this EMR exposure time had clastogenic and genotoxic stress increased nDNA damage of corn cells in comparison to non-exposed nuclei (Ex-0) which detected the lowest DNA migration (tailed ratio 3%) with tail length (0.99 μm), TD% (1.05%), and TM (1.73).

DISCUSSION

The current study used SDS-PAGE and isozymatic, RAPD-PCR, and SCGE as accurate, reliable, to detect genetic effect of 60 Hz EMR on proteins, isozymes, and DNA, respectively. SDS PAGE and isozymatic analyses are biochemical bioassays generated genetic polymorphisms at the level of gene product such as alterations in non-enzymatic and enzymatic proteins (storage proteins and isozymes, respectively) and amino acids. SDS-PAGE analysis revealed varied polypeptide banding patterns and high level of protein polymorphisms among EMR exposed corn seedlings and non-exposed samples depend on number of bands, their molecular weights, and band intensity, the gain of new protein bands (unique bands) and the loss of normal bands (non-unique bands). The banding pattern of electrophoretic polypeptide may be due to interaction of EMR with the transcriptional events occurring during the expression of genes under EMR stressor leading to different mutations in sequencing of mRNA and changes in amino acids of proteins as end products and consequently, polypeptide banding patterns of proteins on electrophoretic gel of SDS-PAGE (Sadia *et al.*, 2009).

On the other hands, the high levels of polymorphisms based on the gain of polypeptide bands or loss of others which generated by SDS-PAGE analysis may be resulted from conformational changes in the amino acid

Table 2. Distribution of leucine-aminopeptidase (LAP) zymograms of EMF non-exposed and exposed *Zea mays* seedlings for days of exposure times (Ex-0, Ex-1, Ex-3, and Ex-5) based on relative front (R*f*) Values and optical densities (OD). Lanes 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex- 3, and Ex-5), respectively.

Rows	R*f* Values	Leucine-aminopeptidase (LAP) zymograms								Zymogram types
		Lane 1		Lane 2		Lane 3		Lane 4		
		R_f	OD	R_f	OD	R_f	OD	R_f	OD	
1	0.19							√	38.4	U
2	0.25	√	34.9							U
3	0.35			√	5.15					U
4	0.40							√	10.8	U
5	0.43			√	28.3					U
6	0.46					√	32.4			U
7	0.66	√	35.1	√	33.6	√	25.9	√	31.3	M
8	0.85							√	30.4	U
9	0.88	√	29.9							U
10	0.90					√	41.7			U
11	0.95			√	38.1					U
12	1.20			√	7.23	√	4.34	√	3.05	Non-U
No. zymograms		3		5		4		5		
Total zymograms		17								
% of zymograms		17.64		29.41		23.53		29.41		

	Lane 1		Lane 2		Lane 3		Lane 4	
	No.	R*f*	No.	R*f*	No.	R*f*	No.	R*f*
No. and R*f* of unique loci	2	0.25–0.88	3	0.35–0.43–0.95	2	0.46–0.90	3	0.19–0.40–0.85
% of unique loci	20.00		30.00		20.00		30.00	

	Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		Polymorphisms
	No.	%	No.	%	No.	%	No.	%	%
Frequency of isozymetic bands and Polymorphisms	10	58.82	1	5.88	11	64.71	1	5.88	91.67

sequences of proteins, or may result from gene duplication followed by a point mutation (insertion or addition of nitrogenous base sequences) that encodes the fractionated polypeptide bands led to appearing (gain) of new bands (unique bands) or may be result from deletion of sequences or loss of genes and consequently, deficiency of amino acids between mutated sites of polypeptide chain of EMR-exposed samples led to loss of protein bands (non-unique) (Galani *et al.*, 2011). Moreover, variation in the number of polypeptide bands and band intensities observed in EMR-exposed samples in comparison to the control may be resulted from changes in nitrogenous bases of DNA, in protein sites or amino acid sequences or frameshift mutations led to changes in bands number while band intensity may be resulted from duplication of gen or point mutation which led to manufacturing of longer and shorter of polypeptide chains (Shikazono *et al.*, 2005). Additionally, EMR-exposed corn seedlings for 5 days caused alteration in profile and banding patterns of proteins as evident in increasing bands number more than non-exposed ones.

On native-PAGE, LAP, EST, PEX, and CAT isozymes employed in this work displayed numerous zymograms at various loci. These variations in electrophoretic zymogramatic patterns and isozymatic polymorphisms based on R_f values and zymograms intensi-

Table 3. Distribution of esterase (EST) zymograms of EMF non-exposed and exposed *Zea mays* seedlings for days of exposure times (Ex-0, Ex-1, Ex-3, and Ex-5) based on relative front (R*f*) Values and optical densities (OD). Lanes 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex-3, and Ex-5), respectively.

Rows	R*f* Values	Esterase (EST) zymograms								Zymogram types
		Lane 1		Lane 2		Lane 3		Lane 4		
		R_f	OD	R_f	OD	R_f	OD	R_f	OD	
1	0.15			√	33.8	√	42.2			Non-U
2	0.16	√	34.3							U
3	0.29							√	57.6	U
4	0.36					√	15.6			U
5	0.38			√	10.2					U
6	0.39	√	13.5							U
7	0.57	√	25.8			√	22.9			Non-U
8	0.58			√	19.7			√	20.0	Non-U
9	0.69							√	3.62	U
10	0.78	√	26.3	√	36.3	√	19.4	√	8.54	M
11	0.97	√	1.50					√	21.40	Non-U
12	1.10			√	2.52					U
No. zymograms		5		5		4		5		
Total zymograms		19								
% of zymograms		26.31		26.31		21.05		26.31		

	Lane 1		Lane 2		Lane 3		Lane 4	
	No.	R*f*	No.	R*f*	No.	R*f*	No.	R*f*
No. and R*f* of unique loci	3	0.16–0.39–0.77	3	0.38–0.79–1.10	1	0.36	2	0.29–0.69
% of unique loci	42.86		42.86		14.29		28.57	

	Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		Polymorphisms
	No.	%	No.	%	No.	%	No.	%	%
Frequency of zymograms and Polymorphisms	7	36.84	4	21.05	11	57.90	1	5.26	91.66

ties among EMR- exposed corn seedlings for different exposure times compared to non-exposed ones. The alterations in zymogramatic patterns may be due to mutation or changes in the DNA nucleotide sequence that codes for the protein leading to the substitution of one to several amino acids or changes in amino acids compositions that result in a change in the net charge of a proteins consisted isozyme (Karaca, 2013). They might also be attributable to a gene's interaction with oxidative stress caused by EMR exposure times or to changes in enzyme conformation which altering the rate of proteins migration on PAGE and their relative front mobility as well as efficiency and stability of the isozyme (Kumar, Gupta, Misra, Modi, Pandey, 2009). On the other hand, alteration in the electrophoretic zymograms mobility may be due to changes in the sequences of encoding DNA or from the shapes and different sizes of the affected isozyme (Karaca, 2013).

Recently, RAPD-PCR and single-cell gel electrophoresis (SCGE) are molecular and cytogenetic bioassays used in this study at DNA level to detect reliable and accurate genetic polymorphisms in banding patterns and DNA damages induced by EMR-oxidative stress on nuclear DNA of corn seedlings. RAPD bioassay is used in this study to provide information about nucleotide sequence polymorphisms that might have occurred across coding and non- coding regions of the entire genome (Elsh & McClelland, 1991). The data obtained

Table 4. Distribution of peroxidase (PEX) zymograms of EMF non-exposed and exposed *Zea mays* seedlings for days of exposure times (Ex-0, Ex-1, Ex-3, and Ex-5) based on relative front (R*f*) Values and optical densities (OD). Lanes 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex-3, and Ex-5), respectively.

Rows	Rf Values	Peroxidase (PER) zymograms								
		Lane 1		Lane 2		Lane 3		Lane 4		Zymogram
		R_f	OD	R_f	OD	R_f	OD	R_f	OD	types
1	0.01			√	2.5	√	1.5	√	1.0	Non-U
2	0.10	√	24.6	√	35.7	√	32.4	√	44.6	M
3	0.38	√	57.9	√	37.0	√	24.9	√	43.4	M
4	0.59					√	23.4			U
5	0.62			√	2.00					U
6	0.73	√	17.5					√	2.50	Non-U
7	0.88							√	12.00	U
8	0.89			√	27.4					U
9	0.92					√	19.2			U
10	1.20							√	2.00	U
No. zymograms		3		5		5		6		
Total zymograms		19								
% of zymograms		15.78		26.31		26.31		31.57		

	Lane 1		Lane 2		Lane 3		Lane 4	
	No.	Rf	No.	Rf	No.	Rf	No.	Rf
No. and Rf of unique loci	0	–	2	0.62–0.89	2	0.59–0.92	2	0.88–1.20
% of unique loci	0.00		33.33		33.33		33.33	

	Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		Polymorphisms
	No.	%	No.	%	No.	%	No.	%	%
Frequency of zymograms and Polymorphism	6	31.57	2	10.52	8	42.10	2	10.52	80.00

scored variations in DNA polymorphisms and banding patterns among EMR-exposed *Zea mays* compared to the non-exposed ones based on primers used, alterations in the bands number of DNA, their sizes, intensities, the gain of new amplified DNA bands (unique), and disappearance of normal bands (non-unique). Additionally, this study found that these variations was dependent on EMR exposure times.

The number of amplified DNA bands may be related to the number of nucleotides and their directions within genomic DNA sequences that are complementary to the sequence of the related primer (Abdelhaliem & Al-Huqail, 2016). DNA polymorphisms generated by RAPD analysis may be due to alterations in DNA nucleotide sequences during duplication of DNA or gene expression under the EMR stressor such as additions of the amplified DNA bands, insertions of new nitrogenous bases, and transpositions of genes within genomic DNA that led to appearance of new DNA bands (unique DNA bands) (Atienzar & Jha, 2006).or due to the deletion of existing genes present on genomic DNA or transpositions of genes from their own DNA to another DNA or due to the hybridization site of a primer in one gene that is altered at a single nucleotide in second amplified gene that can eliminate of a specific amplified nucleotide sequences from second gene amplified resulting of disappearance of amplified DNA genes (non-unique bands) (Welsh & McClelland, 1991). Therefore, the unique bands can be assumed as a characteristic bioassay specific for each corn germplasm affected by EMR.

The comet assay (single-cell gel electrophoresis) is a simple method for measuring DNA strand breaks (DNA damage) in eukaryotic nuclei. Cells containing damaged DNA have the appearance of a comet with a bright

Table 5. Distribution of catalase (CAT) zymograms of EMF non-exposed and exposed *Zea mays* seedlings for days of exposure times (Ex-0, Ex-1, Ex-3, and Ex-5) based on relative front (R*f*) Values and optical densities (OD). Lanes 1–4 represented the days of exposure times (Ex-0, Ex- 1, Ex-3, and Ex-5), respectively.

Rows	R*f* Values	Esterase (EST) zymograms								Zymogram types
		Lane 1		Lane 2		Lane 3		Lane 4		
		R_f	OD	R_f	OD	R_f	OD	R_f	OD	
1	0.02	√	2.00	√	2.34			√	1.0	Non-U
2	0.09	√	32.4	√	32.9	√	25.9	√	21.1	M
3	0.22							√	15.4	U
4	0.33			√	17.2	√	27.9			Non-U
5	0.34	√	32.9					√	21.5	Non-U
6	0.69							√	57.6	U
7	0.71	√	34.7			√	46.2			Non-U
8	0.85			√	49.9					U
9	0.94					√	3.34			U
No. zymograms		4		4		4		5		
Total zymograms		17								
% of zymograms		23.53		23.53		23.53		29.41		

	Lane 1		Lane 2		Lane 3		Lane 4	
	No.	R*f*	No.	R*f*	No.	R*f*	No.	R*f*
No. and R*f* of unique loci	0	–	1	0.85	1	0.36	2	0.29–0.69
% of unique loci	0.00		25.00		25.00		50.00	

	Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		Polymorphisms
	No.	%	No.	%	No.	%	No.	%	%
Frequency of zymograms and Polymorphism	4	23.53	4	23.53	8	47.06	1	5.88	88.89

head and tail. In contrast, undamaged DNA appears as an intact nucleus with no tail. In this study, It used to determine the degree of DNA damage induced by EMR in corn seedlings. SCGE data illustrated notable alterations in the degree of DNA damage in corn nuclei exposed to EMR for one, three-, and five-days dependent on exposing time. This may be due to interaction of EMR with the DNA can stimulate the synthesis of this stress protein, causing DNA strand breaks which increase by increasing of EMR energy (Blank & Goodman, 2012). This nuclear DNA (nDNA) damages led to increase in migration of DNA fragments (tail) from the nucleus (head). This revealed that the increased EMR-exposure of corn seedlings induced DNA lesions in their cells. The DNA lesions induced by EMR may be directly due to energy deposition in cells or indirectly due to reactive oxygen species (ROS) and oxidative DNA damage (Kıvrak et al., 2017). They showed that a major concern of the genotoxic effects of non-ionizing electromagnetic radiation (EMR) is overproduction of ROS in cells and inducing oxidative stress on protein and DNA because of EMR exposure can induce DNA strand breaks and acts as a co-inductor of DNA damages rather than as a genotoxic agent. They also interpreted the genetic mechanisms by which EMR interact with protein and DNA are radical pair recombination led to increasing the concentration, activity, and lifetime of reactive oxygen species (ROS), which might cause changes in cell cycle, genetic mutation, damage to DNA leading to changes in cellular functions and cell death, modification of protein expression and oxidation of proteins, and inhibition of enzymes.

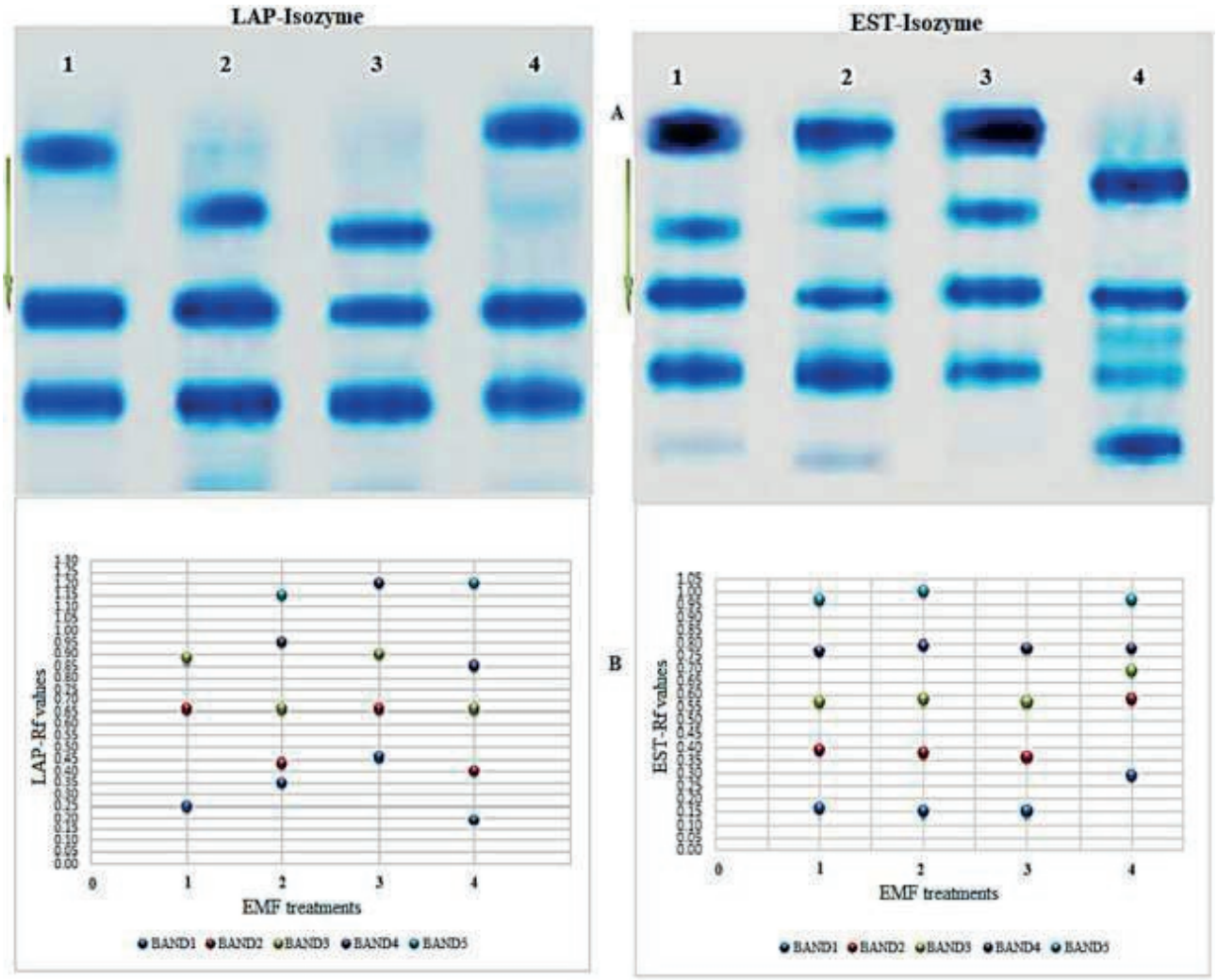

Figure 2. (A) Isozymatic patterns and (B) schematic distribution of Leucine-aminopeptidase (LAP) and Esterase (EST) zymograms (R_f values) of non-exposed and EMR exposed seedlings of *Zea mays* for days of exposure times (Ex-0, Ex-1, Ex-3, and Ex-5) based on relative front (R*f*) Values and optical densities (OD). Lanes 1–4 represented the days of exposure times (Ex-0, Ex- 1, Ex-3, and Ex-5), respectively.

CONCLUSION

The data obtained in current study observed that the longer EMR exposing time could induce notable alterations in banding patterns profile generated by SDS-PAGE, isozymatic, and RAPD bioassays in addition to distinct extent of DNA damages as estimated by comet assay. Therefore, this study concluded that each EMR exposing time had unique interaction with proteins, isozymes, and DNA in corn cells exhibiting wide range of genetic and oxidative stress on these macromolecules. Due to these distinct alteration, it might be asserted that the exposure of economic crop plants to EMR may change gene expression and subsequently, will affect their growth and grain yield. Also, it concluded that bio-assays used should be augmented for accurate and precise estimation of alterations of protein and DNA profiles after EMR exposure of crop plants and for providing excellent results and understanding how this plant was adapted.

AUTHOR CONTRIBUTION

E. M. A. and H. M.A. designed and performed the experiments. E. M. A. analyzed the data and discussed the results. E. M. A. and R. S. S. wrote the manuscript in consultation with A. A. B., and H. M.A. All of the authors read and approved the manuscript.

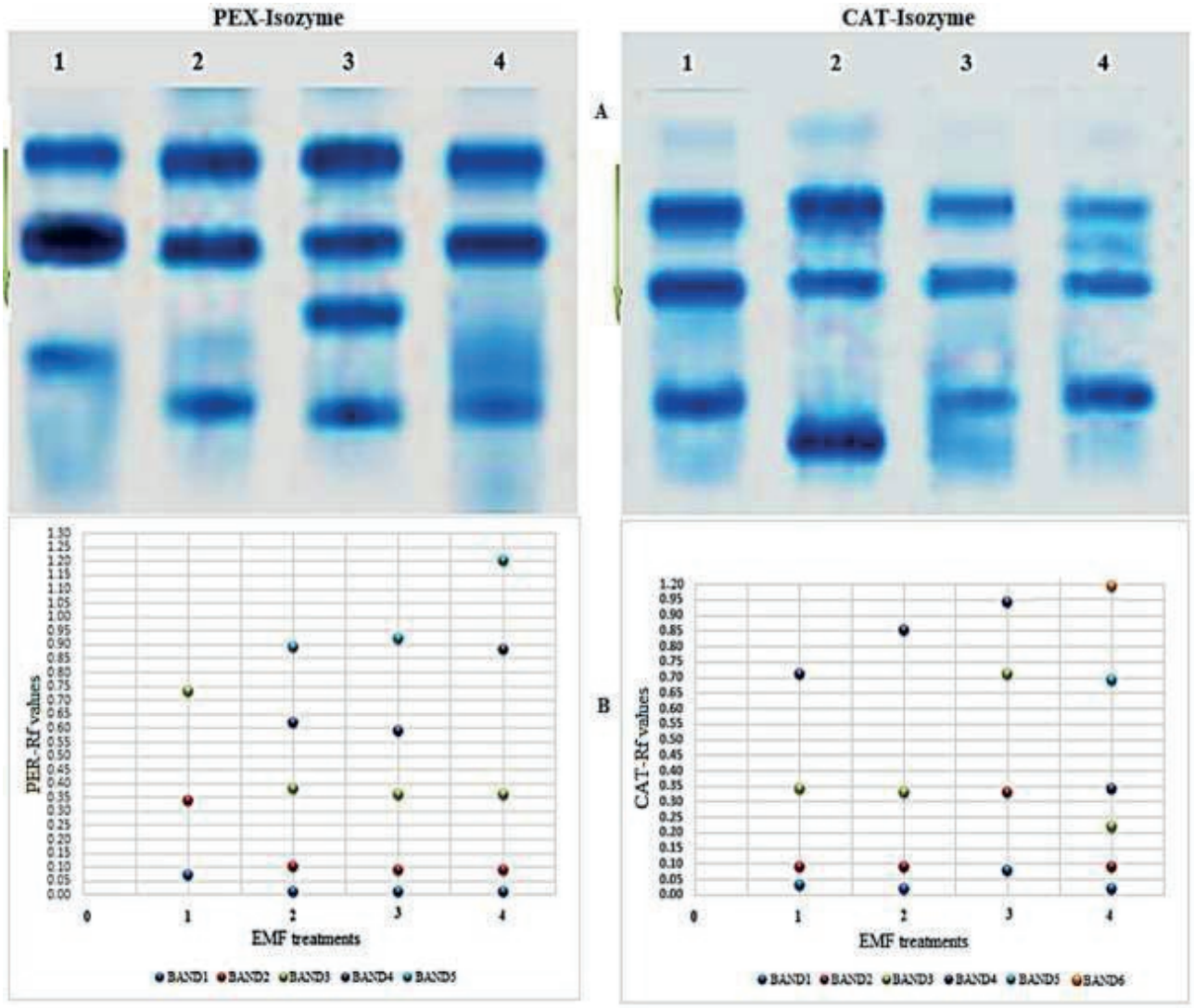

Figure 3. (A) Isozymatic patterns and (B) schematic distribution of Peroxidase (PEX) and Catalases (CAT) zymograms (R_f values) of non-exposed and EMR exposed seedlings of *Zea mays* for days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5) based on relative front (R*f*) Values and optical densities (OD). Lanes 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex- 3,and Ex-5), respectively.

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Table 6. Amplification banding patterns and genomic template stability (GTS) of nuclear DNA analyzed by RAPD-PCR isolated from EMF non-exposed and exposed *Zea mays* seedlings for days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5) and analyzed by Bio-One D++ software (Vilber Lourmat, France). Lanes 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5), respectively.

Primer code	Primers sequences (5′→3′)	Amplicon sizes (bp)	Number of scorable bands in each lane				Types of DNA bands										Polymorphisms
							Polymorphic								Monomorphic		
							Total bands		Unique (U)		Non -U		Total				
			Lane1	Lane2	Lane3	Lane4	No	%	No	%	No	%	No	%	No	%	%
P-02	GGA CCC AAC C	1008-170	3	3	4	5	15	17.65	5	5.88	3	3.53	8	9.41	1	1.18	90.91
P-06	ACC TGA ACG G	938-709	4	3	3	5	15	17.65	4	4.71	6	7.06	10	11.76	1	1.18	88.89
P-08	GTG TGC CCC A	877-438	3	3	4	5	15	17.65	6	7.06	3	3.53	9	10.59	2	2.35	81.83
P-10	GGT CTA CAC C	774-214	5	5	3	7	20	23.53	9	10.59	5	5.88	14	16.47	1	1.18	90.91
P-14	CTT CCC CAA G	846-153	5	5	5	4	20	23.53	12	14.12	6	7.06	18	21.18	0	–	100.00
Overall total bands in each lane			21	19	19	26	85	100	36	42.35	23	27.06	59	69.41	5	5.88	92.19
Sum			85														
% of total bands in each lane			24.71	22.35	22.35	30.59											

Primer code	No. and Sizes (bp) of unique amplified DNA bands							
	Lane 1		Lane 2		Lane 3		Lane 4	
	No.	Sizes	No.	Sizes	No.	Sizes	No.	Sizes
P-02	1	985	1	979	1	1000	2	1008–450
P-06	2	688–595	0	–	0	–	2	780–640
P-08	0	–	1	706	2	650–500	3	780–520
P-10	2	600–200	2	550–418	1	366	4	690–640–493–297
P-14	5	721–652–594–528–153	2	371–153	3	797–483–406	2	846–700
Total unique bands	10		6		7		13	
Sum of unique bands	36							
% of unique bands	27.78		16.67		19.44		36.11	

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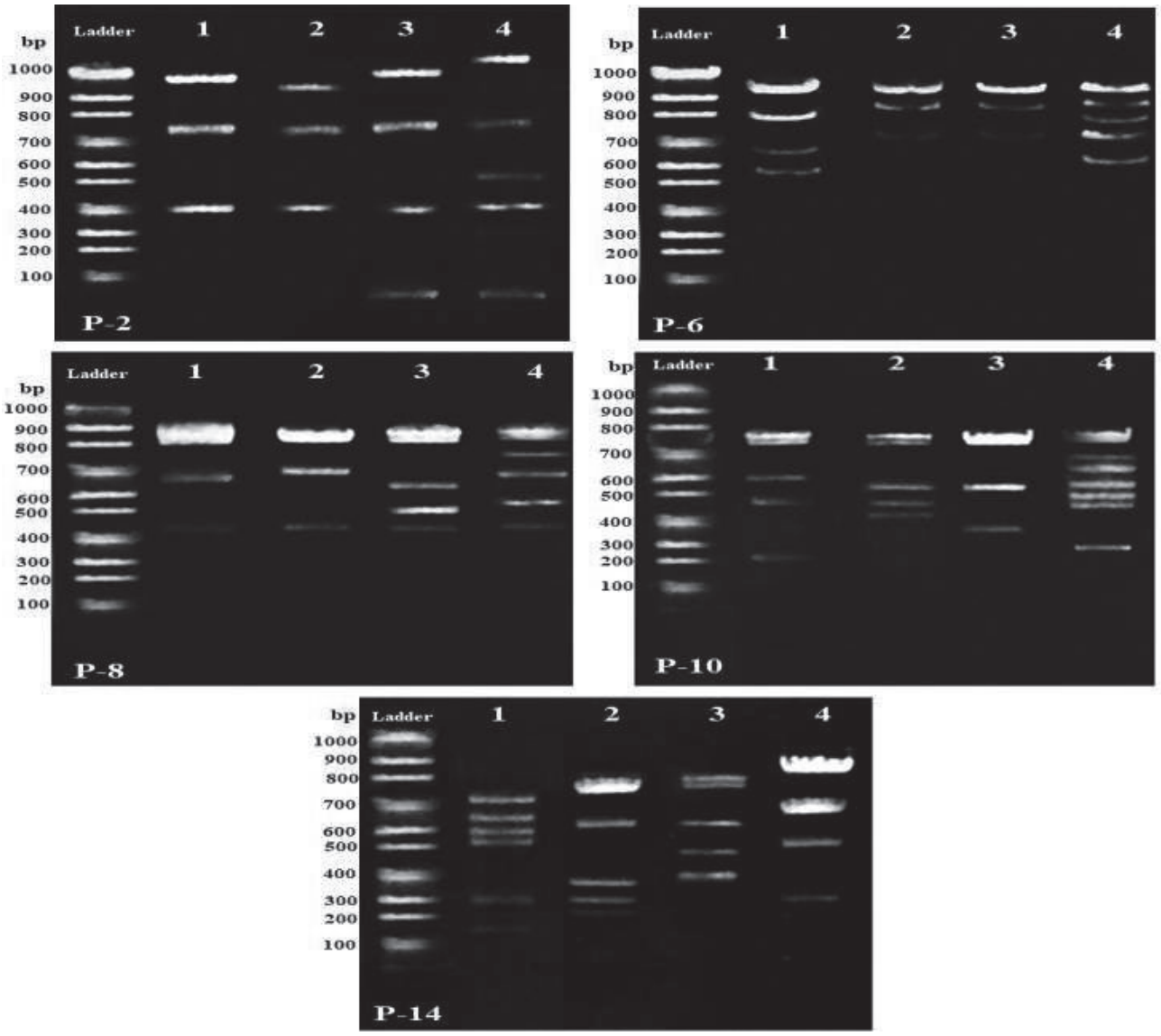

Figure 4. Amplified DNA banding patterns of genomic DNA isolated from non-exposed and EMR exposed seedlings of *Zea mays* for days of exposure times (Ex-1, Ex-3,and Ex-5) and analyzed by RAPD-PCR DNA using five random primers (P-02, 06, 08, 10, and 14). Lanes 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5), respectively.

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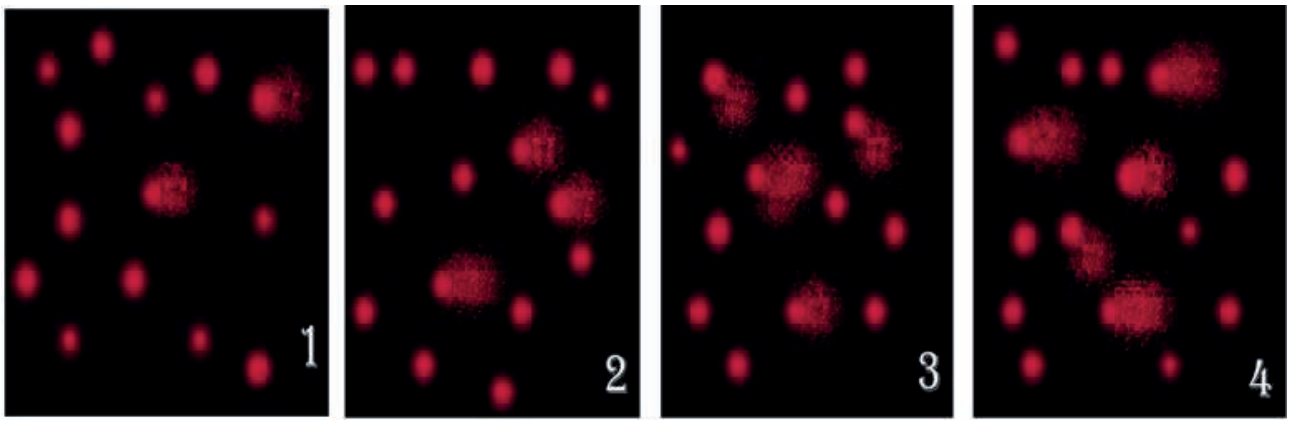

Figure 5. Comet nuclei prepared by Comet assay show the variable extent of nuclear DNA damage in the nuclei isolated from non-exposed and EMR exposed *Zea mays* for days of exposure times (Ex-1, Ex- 3,and Ex-5). Images 1–4 represented the days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5), respectively.

Table 7. Damage extent of nuclear DNA generated by Comet assays in EMF non-exposed and exposed *Zea mays* nuclei for days of exposure times (Ex-0, Ex-1, Ex-3,and Ex-5).

EME exposure time (days)	Tailed %	Un-tailed%	Tail length (μm)	Tail DNA %	Tail Moment Unit
Ex-0	3	97	0.99	1.05	1.73
Ex-1	7	93	2.05	2.19	4.49
Ex-3	12	88	2.46	2.52	6.20
Ex-5	20	80	2.88	2.79	8.04

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