

## Research Article

# Evaluation of Genetic Variations in Maize Seedlings Exposed to Electric Field Based on Protein and DNA Markers

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The current study analyzed proteins and nuclear DNA of electric fields (ELF) exposed and nonexposed maize seedlings for different exposure periods using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isozymes, random amplified polymorphic DNA (RAPD), and comet assay, respectively. SDS-PAGE analysis revealed total of 46 polypeptides bands with different molecular weights ranging from 186.20 to 36.00 KDa. It generated distinctive polymorphism value of 84.62%. Leucine-aminopeptidase, peroxidase, and catalase isozymes showed the highest values of polymorphism (100%) based on zymograms number, relative front ( $R_f$ ), and optical intensity while esterase isozyme generated polymorphism value of 83.33%. Amino acids were analyzed using high-performance liquid chromatography, which revealed the presence of 17 amino acids of variable contents ranging from 22.65% to 28.09%. RAPD revealed that 78 amplified DNA products had highly polymorphism value (95.08%) based on band numbers, with variable sizes ranging from 120 to 992 base pairs and band intensity. Comet assay recorded the highest extent of nuclear DNA damage as percentage of tailed DNA (2.38%) and tail moment unit (5.36) at ELF exposure of maize nuclei for 5 days. The current study concluded that the longer ELF exposing periods had genotoxic stress on macromolecules of maize cells and biomarkers used should be augmented for reliable estimates of genotoxicity after exposure of economic plants to ELF stressors.

## 1. Introduction

Plants are unique in their ability to serve as in situ monitors for environmental genotoxins that inflict damage to DNA and cause genotoxic stress, which can reduce plant genome stability, growth, and productivity [1]. Maize or corn (*Zea mays*) is a plant belonging to the family of grasses (*Poaceae*) and is one of the most important cereal crops worldwide as human nutrient, a basic element of animal feed and raw material for manufacture of many industrial products [2]. Maize is the oldest plant to have a fully established gene map with the basic genome consisting of 10 chromosomes and is an excellent plant for the detection of genotoxins, mutagenic, and clastogenic substances in the environment [3].

As the result of the development of industrialization and technology, an increasing number of man-made electric fields

(ELFs) have appeared in living plant environment that produce in all places by numerous sources, including nearby high voltage transmission lines, primary and secondary overhead utility distribution lines, and the electrical grounding system [4]. ELF is one kind of stress, which can cause more risk of disorders in the function of biological systems such as morphology, uncoiling immune defense and regulation of the cell division [5]. The final effect of ELF in the number of flowering plants was dependent on voltage and time of exposition of tissues as well as on the electric field polarity [6]. The study of [7] mentioned that the electric field of an extremely low frequency induces electrical potentials and resultant current flows in the aqueous medium that surrounds the living cells leading to various physiological and biochemical responses. The study of [8] evaluated the effect of electric field intensity and exposing time on some

physiological properties of maize grains. The study of [9] concluded that an electric current directed to the plant cells may increase the capacity of some enzymes associated with genes already inside the nucleus. The results of [10] showed that the electrostatic fields with certain intensity can increase the content of free radicals in plant cells.

It is important for detection of genotoxicity and mutagenic potential of various types of environmental stress such as ELF on crop plants, to understand their biological consequences and their molecular action on protein, and DNA of plant cell by introducing biochemical, molecular, and molecular cytogenetic assays. The biochemical assays related to protein-based markers such as nonenzymatic protein (storage proteins), enzymatic proteins (isozymes which are one of the most commonly used protein markers), and amino acids composition [11]; these markers detect the coding regions and the variations at the gene product level.

Proteins are primary gene products of active structural genes; their size and amino acids sequence are the direct results of nucleotide sequences of the genes; hence, any observed variation in protein systems induced by any mutagen is considered a mirror for genetic variations [12]. Variation in the DNA coding sequences frequently causes variation in the primary conformation of the proteins. Determination of protein molecular weight (MW) via polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) is a universally used method in biomedical research; [13] concluded that electrophoresis (SDS-PAGE) of proteins can be economically used to assess genetic variation and relation in germplasm and also to differentiate mutants from their parent genotypes. Some studies used SDS-PAGE for detection of alterations in protein profiles occurring during exposure to electric field [5, 14].

Isozymes are direct gene products that show differences in protein-coding genes and arise from multiple gene loci coding for structurally distinct polypeptide chains. The different proteins that make up a set of isozymes can catalyze the same reaction but differ in molecular weight, their kinetics, substrate affinity, number of subunits, and amino acid sequences, electric charge, or electrophoretic mobility [11, 15]. Protein variants in isozyme analysis are separated chemically by nondenaturing native polyacrylamide gel electrophoresis (Native PAGE) resulting in different banding patterns (zymograms) and electrophoretic mobilities that visualized using an enzyme-specific staining mixture, which includes a substrate, cofactor, and oxidized salt [11]. Isozyme analysis is a powerful biochemical technique with numerous applications in mutagenic potential in plant cell [15, 16].

Recently, advances in molecular biology have led to the development of a number of selective and sensitive assays for detection of the variations at the DNA level, and differences can clearly be shown when comparing DNA fingerprints from individuals exposed and/or nonexposed to genotoxic agents [17]. Molecular assays related to DNA-based molecular techniques, like Random Amplified Polymorphic DNA (RAPD), are a very sensitive method of screening for nucleotide sequence polymorphisms that are randomly distributed throughout the genome, in both coding and

noncoding regions and repeated or single copy (unique) sequences [18]. RAPD-PCR technique has been successfully used to detect DNA damage and mutations in some plant species induced by various types of genotoxic agents [19, 20].

In recent years, a new molecular cytogenetic-based assay, the Comet or single cell gel electrophoresis (SCGE) assay, can theoretically be applied to every type of eukaryotic cell, including plant cells for detecting DNA damage induced by genotoxic agents [1]. The basic principle of this assay is to determine the DNA breaks by measuring the DNA damage which is quantified by the proportion of DNA, which migrates out of the nuclei towards the anode when individual cells or isolated nuclei embedded in single cell agarose gel electrophoresis (comet assay) [21]. After electrophoresis, a damaged nucleus takes on the appearance of “comet-like” shape (with a head, the nuclear region, and a tail which contains DNA fragments that migrate from the nucleus) [21].

The objective of this study to evaluate the possible influence of electric field intensity and exposing time on *Zea mays* seedlings on the levels of biochemical analyses of proteins using SDS-PAGE, isozymes, amino acids composition, and on the levels of DNA damage using RAPD-PCR and comet assay.

## 2. Materials and Methods

**2.1. Plant Material and Germination.** Maize grains (hybrid number 323 from Agronomy Research Department, Field Crops Institute, Agriculture Research Center, Giza, Egypt) were used as plant material in this study. Grains were screened for viability and uniformity size and divided into two groups (A and B). 50 grains of each group were sterilized and germinated until reached seven-day-old seedlings in earthenware pot 60 cm in diameter containing soil obtained from the agriculture field. Seedlings of group (A) are exposed to the electric field exposure system while seedling of group (B) was maintained without exposure (unexposure samples).

**2.2. The Electric Field (ELF) Exposure.** In the laboratory, the maize seedlings of group (A) were exposed to an alternating electric field of 50 Hz frequency and 6 kV/m strength generated between two parallel aluminum electrodes of  $60 \times 50 \times 2$  cm dimensions fixed horizontally above and below seedlings for 1, 3, and 5 days. The electric field was derived directly from 50 Hz high voltage setup transformer, manufactured by the “Center of Scientific and Electronic Equipment Maintenance, Faculty of Science, Cairo University.” Maize seedlings exposed to ELF for 1, 3, and 5 days termed as three treatments ( $T_1$ ,  $T_3$ , and  $T_5$ ) while seedlings of group (B) which non-exposed to the electric field termed as zero treatment ( $T_0$ ). Ten seedlings from exposed and nonexposed seedlings were harvested and thoroughly cleaned with fresh water followed by distilled water, for quantitative removal of any foreign particles and then dried in air conditions until completely dried and then subjected to biochemical and molecular analyses.

**2.3. Biochemical Analysis Using SDS-PAGE.** Dried leaves of exposed and nonexposed maize seedlings were used for SDS-PAGE, isozymes, and amino acid analyses.

**2.3.1. Preparation of Leaf Powder and Defatted Preparation.** The dried leaves of ELF exposed and nonexposed maize seedling were milled to leaf powder and defatted according to methods described by [22].

**2.3.2. Extraction of Proteins and SDS-PAGE Analysis.** The protein extraction technique employed was similar to the extraction technique described by [23]. Sample buffer was added to 0.2 g seed flour as extraction liquid and mixed thoroughly in an Eppendorf tube by vortexing. The extraction buffer contained the following components (final concentration): 0.5 M Tris-HCl, pH 6.8, 2.5% SDS, 5% urea, and 5% 2-mercaptoethanol. Before centrifugation at 10,000 g for 5 min at 4°C, the sample buffer was boiled for 5 min. SDS-PAGE was performed using a standard method on a vertical slab gel. Bromophenol blue was added to the supernatant as a tracking dye to watch the movement of proteins on the gel. Protein profiling of samples was performed using SDS-PAGE as described by [24]. Seed proteins were analyzed by SDS-PAGE on 10% polyacrylamide gel. After electrophoresis, the protein bands were visualized by staining with Coomassie brilliant blue G-250. Marker proteins (Fermentas) were used as references. The bands produced in the electropherogram were scored, and their molecular weights were compared to the standard Pharmacia protein marker.

**2.3.3. Protein Imaging and Data Analysis.** Gel photography and documentation were carried out using the Bio-Rad gel documentation system. The number of bands revealed on each gel lane was counted and compared using the Gel Pro-Analyzer software. Quantitative variations in band number and concentration were estimated using the Bio-Rad video densitometer, Model Gel Doc 2000. With regard to variation in protein banding patterns, electropherograms of each exposed and nonexposed sample were scored for the presence or absence of bands.

**2.4. Biochemical Analysis of Amino Acids Composition Using High-Performance Liquid Chromatography (HPLC).** Free amino acids (AAs) were extracted from fine powders of dried leaves for each exposure time as described by [25] with some modifications. Amino acid analyses were performed by HPLC after hydrolysis of samples with 6N HCl at 110°C under vacuum for 24 h on an amino acid analyzer (Applied Biosystems 421 amino acid analyzer, Foster City, CA, USA) as described by [26]. The results of the analysis were expressed as the nitrogen (N) content of the sample: g/100 g of crude protein ( $N \times 6.25$ ). The quality of amino acid composition was tested using the essential amino acid index (EAAI) and the amino acids were quantified by comparing the peak area with corresponding amino acid standard solutions using the Spectra Physics Data System program (Santa Clara, CA, USA).

## 2.5. Biochemical Analysis of Isozymes

**2.5.1. Extraction, PAGE Technique, and Isozyme Staining Methods.** Four isozymes, leucine-aminopeptidase (LAP), esterase (EST), peroxidase (PER), and catalase (CAT), were used in this experiment. The dried leaves of ELF exposed and nonexposed maize seedling were separately milled and defatted according to methods described by [22]. Approximately 0.4 g powdered seed was crushed with acid washed sand and 400 mL extraction buffer. Extraction buffer consisted of 0.1 M Tris-HCl (pH 7.5) containing 20% sucrose as described by [27]. The samples were then centrifuged at 15000 g for 15 min at 4°C; supernatants were collected and used directly for isozyme analyses in a separate vial. Each sample was applied to vertical polyacrylamide gel electrophoresis (4.5% stacking, 9% separating gel) using a mini gel apparatus in Tris-glycine (pH 8.3) buffer as described by [27]. Visualization of enzyme activity after electrophoresis was achieved by histochemically staining the gels. The gels were stained for LAP, EST, PER, and (CAT) separately with specific activity stain solutions as described by [28–31], respectively. Gels were photographed using the Vilber Lourmat gel documentation system.

**2.5.2. Gel Scoring and Identification of Zymogram.** Following [32], isozyme banding patterns were recorded on the basis of number, the relative front ( $R_f$ ) values of zymograms on gel electrophoresis, and their optical intensities. The  $R_f$  value of each respective band on schematic isozyme patterns was determined to allow precise comparisons among the various treatment exposures of maize seedlings to electric field. Data were scored as the presence or absence of zymogram of a unique pattern.

## 2.6. Detection of DNA Damage Based on RAPD-PCR Technique

**2.6.1. Isolation of Genomic DNA.** Genomic DNA from the dried leaves of ELF exposed and nonexposed maize seedling was extracted using the hexadecyltrimethylammonium bromide method as described by [33].

**2.6.2. Qualitative and Quantitative Analyses of Extracted DNA.** DNA yield was measured using a UV-visible spectrophotometer (PerkinElmer, Waltham, MA, USA) at 260 nm. DNA purity was determined by calculating the absorbance ratio at A260/280 nm. Polysaccharide contamination was assessed by calculating the absorbance ratio at A260/230 nm [34]. For quality and yield assessments, electrophoresis was performed for all DNA samples on 0.8% agarose gels that were stained with ethidium bromide; the bands were observed using a gel documentation system (Alpha Innotech, San Leandro, CA, USA) and compared with a known standard lambda DNA marker sample.

**2.6.3. PCR Amplification Using Random Primers of RAPD.** The PCR reaction mixture contained 2.5  $\mu$ L 10x buffer with 15 mM MgCl<sub>2</sub> (Fermentas, Vinius, Lithuania), with 0.25 mM each dNTP (Sigma, St. Louis, MO, USA), 0.3  $\mu$ M primer,



0.5 U *Taq* DNA polymerase (Sigma), and 50 ng template DNA. The PCR reaction was performed in a Palm Cycler apparatus (Corbett Research) using the following method: initial denaturation of 4 min at 95°C followed by 40 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. A total of 20 random DNA oligonucleotide primers (10-mer) were independently used in the PCR reactions (University of British Columbia, Canada) according to [35] with some modifications. Only 5 primers (P-02, 06, 08, 10, and 14) successfully generated reproducible DNA amplification products. For DNA amplification, the PCR was run for 35 cycles, which consisted of a denaturation step (1 min at 95°C), annealing step (1 min at 35°C), and elongation step (2 min at 72°C). After 34 cycles, a final extension period was added (5 min at 72°C). Amplification products were electrophoresed on 1.5% agarose gel (Sigma) in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8). The gel was run at 100 V constant voltage for 1 h. Gels were stained with 0.2 µg/mL ethidium bromide for 15 min. The PCR products were visualized under a UV light transilluminator. The 100-base pair DNA ladder (Gibco-BRL, Grand Island, NY, USA) was loaded into the first lane of each gel to evaluate band sizes. The gels were photographed under UV light using a gel documentation system (Bio-Rad, Hercules, CA, USA).

**2.6.4. Scoring and Data Analyses.** After separating PCR products by agarose gel electrophoresis, the gels were visualized using a Photo Print (Vilber Lourmat, France) imaging system. Quantitative variations in numbers and sizes of bands as well as their optical intensities were analyzed using Bio-One D++ software (Vilber Lourmat, France). The RAPD bands were scored as the presence or absence of DNA bands.

## 2.7. Detection of DNA Damage Based on Single Cell Gel Electrophoresis (Comet Assay) Technique

**2.7.1. Isolation of Nuclei.** The nuclei suspension was used in the alkaline single cell gel electrophoresis assay, as described by [36]. Dried leaves of ELF exposed and nonexposed of maize seedlings were placed in a small Petri dish containing 200 µL of cold 400 mM Tris-HCl buffer, pH 7.5 (on ice). Using a razor blade, the leaf was gently sliced into a “fringe” to release nuclei into the buffer under yellow light. Each slide previously coated with 1% normal melting point (NMP) agarose and dried was covered with a mixture of 55 µL of nuclear suspension and 55 µL of low melting point (LMP) agarose (1% prepared with phosphate-buffered saline) at 40°C and cover slipped. The slide was placed on ice for at least 5 min, and coverslip was removed. Then 110 µL of LMP agarose (0.5%) was placed on the slide and coverslip was mounted again. After 5 min on ice, the coverslip was removed.

**2.7.2. Single Cell Gel Electrophoresis (SCGE).** Slides of SCGE were prepared as described by [36]. The slides with the plant cell nuclei were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer

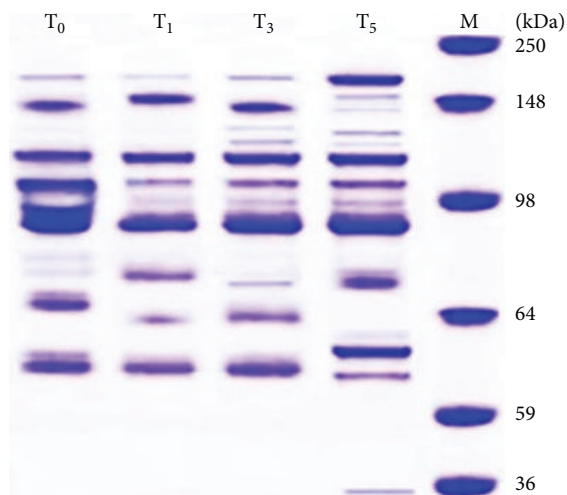


FIGURE 1: SDS-PAGE banding patterns of proteins of ELF exposed and nonexposed maize seedlings.

(300 mM NaOH, 1 mM EDTA, pH > 13) and incubated for 15 min. Electrophoresis was performed at 16 V, 300 mA for 30 min at 4°C. Then the gels were neutralized by washing three times in 400 mM Tris-HCl, pH 7.5 and stained with ethidium bromide (20 µg/mL) for 5 min. After staining, the gels were dipped in ice-cold distilled water and immediately analyzed.

**2.7.3. Imaging and Analysis Software.** In each slide, DNA-damage extent of 50 randomly chosen cells was analyzed and assessed quantitatively by visual scoring or using the fluorescence microscope with an excitation filter of 546 nm and a barrier filter of 590 nm using computerized image analysis system (Komet Version 3.1. Kinetic Imaging, Liverpool, UK). Percentage of nuclei with tails, the relative tail length, tail DNA (TD%), relative percentage of DNA in the comet tail, and tail moment (TM, integrated value of density multiplied by migration distance) were used as parameters of DNA damage.

## 3. Results

**3.1. SDS-PAGE Analysis.** The proteins electrophoretic banding patterns of ELF exposed and non-exposed maize leaves using SDS-PAGE exhibited distinctive quantitative and qualitative alterations compared to nonexposed one ( $T_0$ ) as shown in Table 1 and Figure 1. These protein alterations based on changes in polypeptides molecular weights (MWs), bands intensities, fractionation of some bands, appearance of new bands (unique bands), and disappearance of some bands (polymorphic bands). SDS-PAGE analysis revealed total of 46 polypeptides bands with different bands intensities and MWs that ranged from 186.20 to 36.00 KDa. Out of which, 22 bands were polymorphic with 84.62% polymorphism (16 bands were unique with value of 34.04%; 6 bands were nonunique with value of 12.77%) and 4 bands were monomorphic bands

TABLE 1: Electrophoretic banding analysis of proteins of ELF exposed and nonexposed maize seedlings.

Rows	Mol. weights	Electric field treatments								Band types
		Lanes								
		Lane T <sub>0</sub>		Lane T <sub>1</sub>		Lane T <sub>3</sub>		Lane T <sub>5</sub>		
		KDa	%	KDa	%	KDa	%	KDa	%	
1	186.20	1	2.27	1	4.40	1	9.21	1	10.54	M
2	180.15	0	—	0	—	0	—	1	11.80	U
3	155.00	0	—	0	—	0	—	1	1.10	U
4	151.27	0	—	1	12.00	0	—	0	—	U
5	146.31	1	10.40	0	—	1	11.20	1	1.90	Non-U
6	131.38	0	—	0	—	0	—	1	1.66	U
7	130.19	1	1.00	1	1.09	1	1.62	0	—	Non-U
8	118.79	0	—	0	—	0	—	1	1.11	U
9	116.71	1	22.10	1	19.60	1	21.60	1	24.21	M
10	106.42	1	10.40	1	14.60	1	16.90	1	17.90	M
11	96.84	0	—	0	—	1	5.14	1	6.18	Non-U
12	89.06	1	28.30	0	—	0	—	0	—	U
13	85.29	1	32.30	1	22.50	1	26.60	1	28.00	M
14	80.16	1	2.70	0	—	0	—	0	—	U
15	78.84	0	—	1	12.66	1	5.14	1	2.62	Non-U
16	75.55	0	—	0	—	0	—	1	15.90	U
17	69.27	1	4.66	0	—	0	—	0	—	U
18	66.66	1	13.40	0	—	0	—	0	—	U
19	64.77	0	—	0	—	1	21.50	0	—	U
20	63.45	0	—	1	12.60	0	—	0	—	U
21	62.91	0	—	0	—	0	—	1	21.30	U
22	62.11	1	9.39	0	—	0	—	0	—	Non-U
23	61.45	1	22.50	1	20.10	0	—	0	—	Non-U
24	61.23	0	—	0	—	1	21.50	0	—	U
25	60.91	0	—	0	—	0	—	1	17.30	U
26	36.00	0	—	0	—	0	—	1	5.97	U
Number of polypeptide bands of each lane		<b>12</b>		<b>9</b>		<b>11</b>		<b>15</b>		
Total polypeptide bands		<b>47</b>								
% of total bands of each lane		<b>25.53</b>		<b>19.15</b>		<b>23.40</b>		<b>31.92</b>		
Types and frequency of polypeptide bands		Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		Polymorphism %
		Number	%	Number	%	Number	%	Number	%	
		16	34.04	6	12.77	46.81	22	4	8.51	84.62

with value of 34.04%. It also generated considerable value of polymorphism reaching 84.62%. SDS-PAGE generated 8 unique bands at the electric field treatment after 5 days (T<sub>5</sub>) with MWs (180.15, 155.00, 131.38, 118.79, 75.55, 62.91, 60.91, and 36.00 KDa) compared to nonexposed treatment (T<sub>0</sub>) which recorded 5 unique bands with MWs (146.31, 89.06, 80.16, 69.27, and 66.66 KDa). It also generated 2 unique bands at the electric field treatments (T<sub>1</sub> and T<sub>3</sub>) with MWs (151.27, 63.45 KDa for T<sub>1</sub> and 64.77, 61.23 KDa for T<sub>3</sub>). These unique bands can be used as markers for the protein alterations at these treatments. The maximum and minimum number of

bands (15 bands with value 31.92% and 9 bands with value 19.15%, resp.) was found at ELF treatment (T<sub>5</sub> and T<sub>1</sub>, resp.) compared to 12 bands with value of 25.53% at nonexposed treatment (T<sub>0</sub>).

3.2. *Isozymes Analyses.* LAP, EST, PER, and CAT isozymes used in the current study showed clear polymorphisms between exposed maize seedlings to ELF treatment (T<sub>1</sub>, T<sub>3</sub>, and T<sub>5</sub>) compared to nonexposed one as shown in Tables 2–5 and Figure 2. These polymorphisms are based on the number of zymograms, the R<sub>f</sub> values, and their optical

TABLE 2: Distribution of leucine-aminopeptidase (LAP) zymograms of ELF exposed and nonexposed maize seedlings.

Rows	$R_f$ value	Electric field treatments								Zymogram types
		Lane T <sub>0</sub>		Lane T <sub>1</sub>		Lane T <sub>3</sub>		Lane T <sub>5</sub>		
		$R_f$	OD	$R_f$	OD	$R_f$	OD	$R_f$	OD	
1	0.20					√	17.5			U
2	0.25	√	34.9							U
3	0.33					√	38.7			U
4	0.34							√	54.8	U
5	0.35			√	29.5					U
6	0.65					√	32.9			U
7	0.66	√	35.1	√	34.3			√	24.4	Non-U
8	0.87							√	20.8	U
9	0.88			√	36.3	√	28.5			Non-U
10	0.89	√	29.9							U
Number of zymograms in each lane		3		3		4		3		
Total zymograms										13
% of zymograms in each lane		23.08		23.08		30.77		23.08		
Frequency of isozymetic bands and Polymorphism		Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		% of polymorphism
		Number	%	Number	%	Number	%	Number	%	
		8	61.54	2	15.38	10	76.92	0	0	100

TABLE 3: Distribution of esterase (EST) zymograms of ELF exposed and nonexposed maize seedlings.

Rows	$R_f$ value	Electric field treatments								Zymogram types
		Lane T <sub>0</sub>		Lane T <sub>1</sub>		Lane T <sub>3</sub>		Lane T <sub>5</sub>		
		$R_f$	OD	$R_f$	OD	$R_f$	OD	$R_f$	OD	
1	0.13			√	43.60					U
2	0.14					√	55.60	√	52.20	Non-U
3	0.16	√	34.30							U
4	0.34			√	10.80					U
5	0.36					√	11.40			U
6	0.38							√	11.70	U
7	0.39	√	13.50							U
8	0.57	√	25.80	√	33.00	√	19.20	√	17.10	M
9	0.77	√	26.30	√	12.70	√	13.80	√	19.00	M
10	0.85			√	30.40					U
11	0.88					√	26.30			U
12	0.91	√	32.40							U
Number of zymograms in each lane		5		5		5		4		
Number of total zymograms										19
% of zymograms in each lane		26.32		26.32		26.32		21.05		
Frequency of isozymetic bands and polymorphism		Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		% of polymorphism
		Number	%	Number	%	Number	%	Number	%	
		9	47.37	1	5.26	10	52.63	2	10.53	83.33

TABLE 4: Distribution of peroxidase (PER) zymograms of ELF exposed and nonexposed maize seedlings.

Rows	$R_f$ value	Electric field treatments								Zymogram types	
		Lane $T_0$		Lane $T_1$		Lane $T_3$		Lane $T_5$			
		$R_f$	OD	$R_f$	OD	$R_f$	OD	$R_f$	OD		
1	0.07	√	24.6							U	
2	0.08			√	36.30					U	
3	0.09					√	23.7	√	30.00	Non-U	
4	0.34	√	57.9							U	
5	0.35			√	35.7	√	25.5			Non-U	
6	0.36							√	36.3	U	
7	0.60							√	33.7	U	
8	0.67			√	28					U	
9	0.73	√	17.5			√	50.8			Non-U	
10	0.78							√	11.8	U	
11	0.80			√	10.5					U	
12	0.88							√	9.66	U	
13	1.05							√	8.34	U	
Number of zymograms in each lane		3		4		3		6			
Number of total zymograms											16
% of zymograms in each lane		18.75		25.00		18.75		37.5			
Frequency of isozymetic bands and polymorphism		Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		% of polymorphism	
		Number	%	Number	%	Number	%	Number	%		
		10	52.50	3	18.75	13	81.25	00	00	100	

intensities. A total of 63 different electrophoretic zymograms were observed for the 4 isozymes. LAP, EST, PER, and CAT analyses generated 13, 19, 16, and 15 zymograms, respectively, with different  $R_f$  values ranging from 0.20 to 0.89 for LAP, 0.13–0.91 for EST, 0.07–1.05 for PER, and 0.04–0.99 for CAT. LAP, EST, PER, and CAT analyses generated higher number of zymograms (4, 5, 6, and 5 with values 30.77%, 26.32%, 37.5%, and 33.33%, resp.) at treatments ( $T_3$ ), ( $T_0$ ,  $T_1$ , and  $T_3$ ), ( $T_5$ ), and ( $T_0$ ), respectively, while lower number of zymograms (3, 4, 3, and 3 with values 23.08%, 21.05%, 18.75%, and 20.00%, resp.) was scored at treatments ( $T_0$ ,  $T_1$ , and  $T_3$ ), ( $T_5$ ), ( $T_0$  and  $T_3$ ), and ( $T_3$  and  $T_5$ ), respectively. On the other hand, LAP analysis revealed 10 polymorphic zymograms (8 unique and 2 nonunique zymograms). These zymograms generated a high value of polymorphism of 100% while EST analysis revealed 10 polymorphic zymograms (9 unique and 1 nonunique zymograms) and 2 monomorphic zymograms with a considerable polymorphism value of 83.33%. Moreover, PER analysis revealed 13 polymorphic zymograms (10 unique and 3 nonunique zymograms) while CAT analysis revealed 12 polymorphic zymograms (10 unique and 2 nonunique zymograms). These two isozymes generated a high value of polymorphism of 100%.

3.3. *Amino Acid Analysis.* The results of EAAs and NEAAs compositions of dried leaves of exposed and nonexposed maize seedlings are shown in Table 6. HPLC analysis revealed

the presence of 17 amino acids, 9 of which are essential in humans. The free amino acids contents were ELF exposure time, dependent as evident from their reduction by ELF treatments ( $T_1$ ,  $T_3$ , and  $T_5$ ) compared to zero treatment ( $T_0$ ). The highest content of total free amino acids (FAA) was scored at  $T_0$  with value of 28.09%, while the lowest content of FAA was at ELF treatment  $T_5$ , with value of 22.65%, respectively. Total FEAA content (62.73%) was higher than FNEAA (38.27%). The current data showed that the contents of all types of FAAs were prevalent in control ( $T_0$ ) except the NEAA proline was prevalent in ELF treatment  $T_5$ . Furthermore, the EAA Arginine scored the highest content among all types of FAAs.

3.4. *Detection of DNA Damage Based on RAPD-PCR Technique.* RAPD analysis was employed in the present study to evaluate the extent of the DNA alterations in ELF exposed and nonexposed maize leaves as shown in Table 7 and Figure 3. Twenty random primers were used for the RAPD analysis, in which only five primers of them (P-02, 06, 08, 10, and 14) succeeded to produce clear reproducible DNA bands and gave satisfactory results with many alterations in the RAPD profiles. In total, Seventy-eight (78) amplified DNA products were scored; these bands varied in number of specific random sequences, band intensity, and sizes, ranging from 120 to 992 bp. An average of 15.60 bands per primer was scored. RAPD analysis generated highly polymorphism values of

TABLE 5: Distribution of catalase (CAT) zymograms of ELF exposed and nonexposed maize seedlings.

Rows	$R_f$ value	Electric field treatments								Zymogram types
		Lane $T_0$		Lane $T_1$		Lane $T_3$		Lane $T_5$		
		$R_f$	OD	$R_f$	OD	$R_f$	OD	$R_f$	OD	
1	0.04	√	24.90							U
2	0.06			√	29.90					U
3	0.08			√	24.00					U
4	0.09	√	32.40					√	40.70	Non-U
5	0.10					√	33.70			U
6	0.33			√	24.30					U
7	0.34	√	32.9			√	66.30	√	59.30	Non-U
8	0.71	√	34.70							U
9	0.75							√	58.9	U
10	0.88					√	25.5			U
11	0.91			√	51.7					U
12	0.99	√	11.8							U
Number of zymograms in each lane		5		4		3		3		
Number of total zymograms		15								
% of zymograms in each lane		33.33		26.67		20.00		20.00		
Frequency of isozymetic bands and polymorphism		Unique (U)		(Non-U)		Polymorphic		Monomorphic (M)		% of polymorphism
		Number	%	Number	%	Number	%	Number	%	
		10	66.67	2	13.33	12	80.00	00	00	100

95.16% and 75.64% (59 in total) DNA polymorphic bands, of which 51 bands were unique with value 65.35% and 7 bands were nonunique with value 8.97% in addition to 3 bands which were monomorphic with value 3.85%. The maximum number of gene products (23 bands) with value 29.49% was observed at ELF treatment  $T_5$ , whereas the minimum number of bands (16) with value 20.51% was at ELF treatment  $T_1$  compared to zero treatment  $T_0$  at which RAPD analysis scored 21 bands with value 26.92%. On the other hand, the highest number of gene products (21 bands) was generated by primer P-14, whereas the lowest number (13 bands) was generated by the primers 02 and 06. Furthermore, the highest value of polymorphism (100%) was revealed by the primer -10 and 14, whereas the lowest one (87.50%) is revealed by primer 02.

**3.5. Detection of DNA Damage Based on Single Cell Gel Electrophoresis (Comet Assay) Technique.** The present study used the comet assay to evaluate DNA damage induced in maize seedlings under stress of electric field exposure as shown in Table 8 and Figure 4. The higher percentage of tail DNA (TD %) and tail moments (TM) (fraction of migrated DNA multiplied by some measure of tail length) was used as parameters of DNA damage. The current data showed remarkable variations in the extent of DNA damage in *Zea mays* nuclei. ELF treatment  $T_5$  showed the highest DNA migration reached (tailed 15%) with tail length 2.25  $\mu\text{m}$ , % of

tailed DNA (2.38%), and tail moment unit 5.36; this indicated that this ELF treatment had genotoxic stress increased damage of nDNA of maize cells compared to zero treatment  $T_0$  which showed the lowest DNA migration reached (tailed 4%) with tail length 1.31  $\mu\text{m}$ , tailed DNA % 1.40% and tail moment unit 2.30.

## 4. Discussion

**4.1. SDS-PAGE Analysis.** The present study observed that SDS-PAGE analysis exhibited distinctive qualitative and quantitative alterations in electrophoretic SDS-proteins of exposed and nonexposed maize seedlings. These alterations are based on variations in number of polypeptide bands, molecular weights, and intensities of polypeptides bands as well as gain or loss of protein bands that led to high levels of protein polymorphisms. Electrophoretic analysis of protein provides information concerning the structural genes and their regulatory systems that control the biosynthetic pathways of that protein. Each polypeptide band represents the final products of transcriptional and translational events occurring due to active structural genes [37]. The changed protein products caused by ELF exposure time may result from base changes in DNA or altering protein sites or changes in amino acid sequences or frame shift mutations. Additionally, they may serve as genetic markers because they can be quite polymorphic and their variability is generally highly heritable [38].



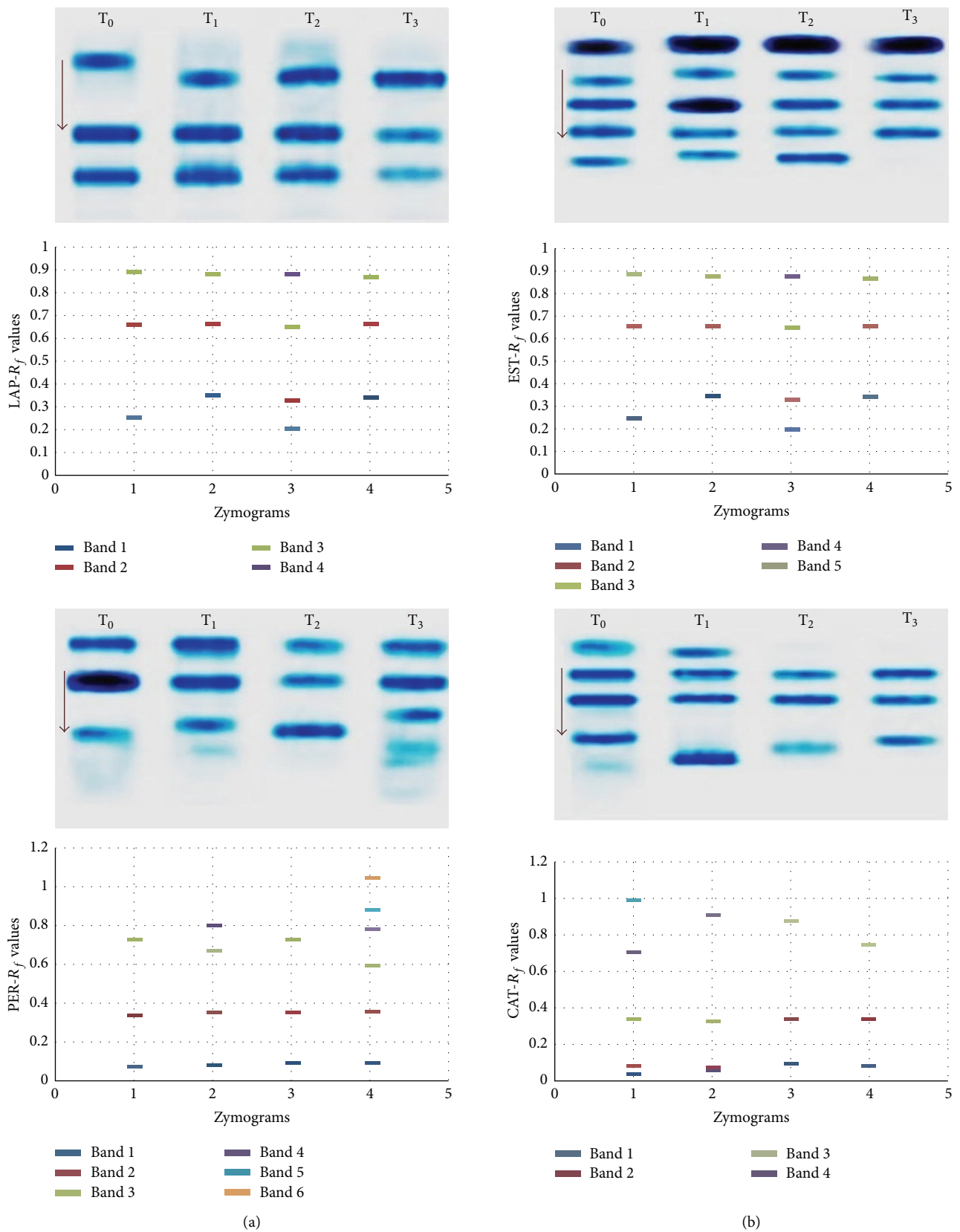


FIGURE 2: Banding pattern (a) and schematic distribution (b) of LAP, EST, PER, and CAT zymograms ( $R_f$  values) in ELF expose and nonexposed maize seedlings.

TABLE 6: Essential and nonessential amino acid composition of ELF exposed and nonexposed maize seedlings.

Free amino acids ( $\mu\text{mol}/100\text{ mg dry weight}$ )	Electric field treatments			
	T <sub>0</sub>	T <sub>1</sub>	T <sub>3</sub>	T <sub>5</sub>
	Essential amino acids (EAA)			
Arginine	8.29	7.63	7.21	7.19
Histidine	2.92	2.77	2.15	1.13
Isoleucine	1.74	1.68	1.35	1.30
Lysine	2.13	1.88	1.65	2.02
Methionine	2.47	2.25	1.97	2.03
Leucine	0.84	0.83	0.80	0.74
Phenylalanine	1.34	1.22	1.13	1.19
Threonine	2.06	1.89	1.59	1.42
Valine	1.33	1.21	1.21	1.24
Total of EAA at each treatment	23.12	21.36	19.06	18.26
Sum			81.80 = 62.73%	
% total of EAA at each treatment	28.26	26.11	23.30	22.32
	Nonessential amino acids (NEAA)			
Alanine	2.68	2.46	1.85	1.78
Aspartic acid	1.68	1.52	1.44	1.39
Glutamic acid	1.48	1.25	1.05	1.01
Cysteine	2.33	0.45	0.46	0.39
Proline	0.49	2.27	2.05	2.76
Tyrosine	1.41	1.28	1.28	0.98
Asparagine	2.38	2.21	2.14	2.12
Glutamine	1.66	1.63	1.51	1.33
Total of NEAA at each treatment	14.11	13.07	11.78	11.76
Sum			50.72 = 38.27%	
% total of NEAA at each treatment	27.82	25.77	23.23	23.19
Total number of EAAs and non-EAAs	37.23	34.43	30.84	30.02
Sum			132.52	
%	28.09	25.98	23.27	22.65

The appearance of new bands (unique bands) may be explained on the basis of mutational events at the regulatory system of unexpected gene(s) or on the basis of band subfractionation which could be attributed to duplication of gene followed by the occurrence of point mutation that encoded the fractionated band [16] or result from different DNA structural changes (breaks, transpositions, deletion, etc.) which led to change in amino acids and consequently protein formed [16]. On the other hand, the disappearance of some protein bands which led to formation of polymorphic bands could be attributed to the loss of genetic material which may be due to the breaking of a small number of peptide bonds to form polypeptides of shorter length than the original protein [16].

Furthermore, the changes in band intensity could be interpreted on the basis of gene duplication or point mutation that leads to production of shorter and longer polypeptide chains and alteration in the structural genes which may be due to the changes in regulator gene(s) expression [16].

The distinction protein polymorphisms shown between ELF exposed and nonexposed samples in the present study may be resulting from insertions or deletions between mutated sites of protein bands and could be used as biomarkers for identification of ELF stressed plants [38]. Additionally, the increase in ELF exposure time causes alterations in the relative mobility of bands, intensities, expression of new proteins, and suppression of some proteins.

*4.2. Isozymes Analyses.* Because isozymes, being proteins, are direct products of gene expression that show differences in protein-coding genes and produced by different alleles and gene loci [11]. Electrophoretic banding patterns (zymograms) generated can be effectively correlated to change in gene sequence, mutation, and gene interaction with ELF [11]. This may be explained by the presence of multilocus isozyme forms from gene duplication through mutation, polyploidization, and chromosomal aberrations [11].



TABLE 8: The extent of nuclear DNA damage of ELF exposed and nonexposed maize nuclei.

Electric field treatments	Tailed %	Untailed %	Tail length ( $\mu\text{m}$ )	Tail DNA %	Tail moment unit
T <sub>0</sub>	4	96	1.31	1.40	2.30
T <sub>1</sub>	6	94	1.72	1.97	3.39
T <sub>3</sub>	10	90	1.93	2.19	4.23
T <sub>5</sub>	15	85	2.25	2.38	5.36

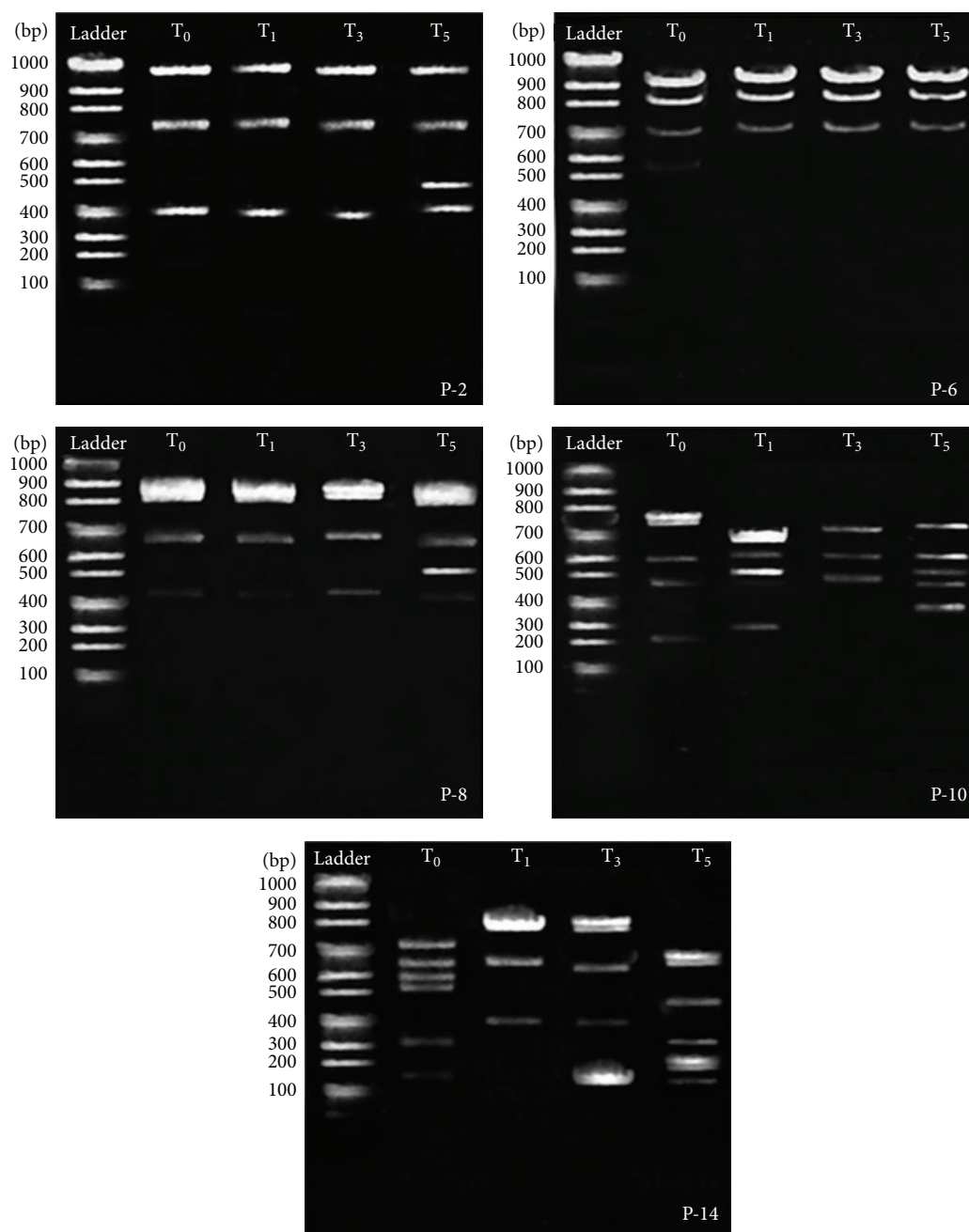


FIGURE 3: RAPD products of DNA fragments extracted from ELF exposed and nonexposed maize seedlings using 5 random primers.

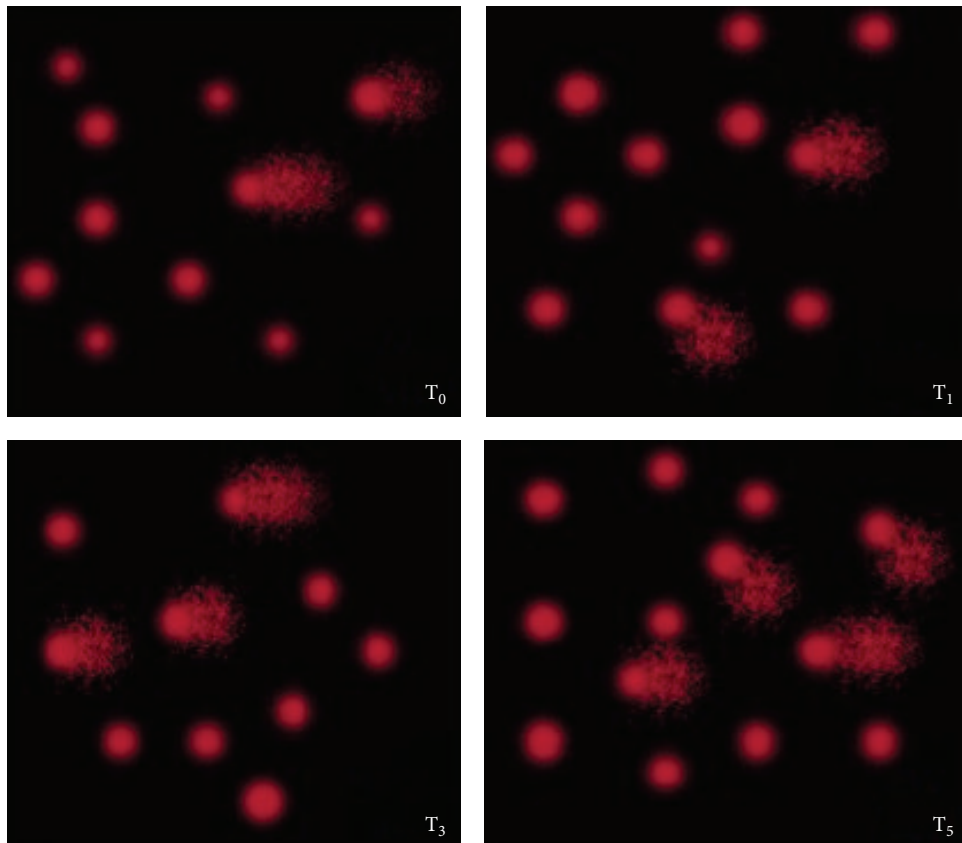


FIGURE 4: Comets images showed varying extent of DNA damage of ELF exposed and nonexposed maize nuclei.

The electrophoretic banding profiles generated by LAP, EST, PER, and CAT isozymes revealed multiple bands on different loci with different  $R_f$  values and unequal intensities. The  $R_f$  value of each respective band for schematic isozyme patterns was determined to compare various ELF treatments. The different electrophoretic mobilities of zymograms resulted from the different sizes and shapes of enzyme molecules; therefore, their variations indicate genetic variation induced due to ELF stress [27].

If an amino acid substitution occurs in a protein molecule due to ELF stress, net charge may be altered or conformational changes in isozymes may occur, consequently altering the migration rate of proteins in an electric field, as well as their electrophoretic mobilities as well as catalytic efficiency and stability [11]. Each band on a gel electrophoresis revealed in the current study can be assigned a descriptive value based on either the net migration of the band from the origin (an  $R_f$  value) or its position relative to that of the band coded by the most common allele. The changes in the mobility of enzymes in an electrical field may be reflecting changes in the encoding DNA sequences. On the other hand, differences in isozymetic patterns generated between different ELF treatments have been attributed to one or more of the following causes: (i) changes in the level of isozyme expression; (ii) genetic transcription of different isozyme loci; and (iii) posttranslational modifications changing the electrophoretic mobility; these were found in agreement with [11].

**4.3. Amino Acid Analysis.** Amino acids analysis by HPLC showed variations in the amount of free amino acids between ELF exposed and nonexposed maize seedlings; these variations may reflect the oxidative stress induced by intensity of the electric fields treatments at certain exposing time [10] which may lead to biosynthesis or degradation of protein by increasing the free radical oxygen content inside maize cells. These oxidative proteins may undergo loss of activity or be denatured leading to oxidation of amino acid chains and generating stable and reactive products such as protein hydroperoxides, which result in fragmentation of proteins. The side chains of all the amino acid residues, particularly cysteine and methionine, are susceptible to oxidation by the action of ROS [39]. On the other hand, these quantitative variations of amino acids will affect DNA gene expression, synthesis of proteins, activity of isozymes, and redox-homeostasis. Free amino acids, especially proline, can accumulate in plant cells due to stress and serve as osmotically active solute, a ROS scavenger, and a molecular chaperone stabilizing the structure of proteins, thereby protecting cells from damage caused by stress [40].

**4.4. Detection of DNA Damage Based on RAPD-PCR Technique.** RAPD assay used in the current study showed that various ELF treatments and nonexposed one exhibited distinctive qualitative and quantitative alterations in the RAPD profiles based on number of gene products, the amplified DNA sizes, their intensities, and appearance or disappearance



of DNA bands that led to generation high levels of DNA polymorphism. The number of amplification products may be related to the number and direction of genome sequences complementary to the primer. High levels of polymorphism generated by RAPD in the present study are considered to be a better parameter for measuring genetic variation patterns and reflect heritable changes in the nucleotide sequences, both in coding and noncoding regions [41].

These DNA polymorphisms may result from DNA structural changes within base-pair sequences of DNA between oligonucleotide primer binding sites in the genome during DNA replication or gene expression under genotoxic stresses of electric field, such as a nucleotide substitution within a target site, base pair insertion or deletion of a DNA-fragment within the amplified regions, rearrangements of genomic DNA, or inversions, translocations, and transpositions of genes which result in the loss or gain of DNA bands resulting in different DNA lengths and consequently highly level polymorphisms [18].

On the other hand, appearance of new DNA bands (unique bands) is usually resulting from different DNA structural changes (insertions of the amplified DNA bands, breaks, transpositions, additions, etc.), while disappearance of some bands (polymorphic bands) and band intensity may correlate with the level of photoproducts in DNA template after ELF exposure which can reduce the number of binding sites for Taq polymerase and the starting copy number of a particular DNA sequence within genome [17] or may be due to the deletion of DNA segments. The present finding supports this claim that DNA polymorphisms detected by RAPD can be considered as a powerful biomarker assay for detection of the genotoxic environmental stress like ELF.

**4.5. Detection of DNA Damage Based on Single Cell Gel Electrophoresis (Comet Assay) Technique.** Comet assay data showed remarkable variations in the extent of DNA damage in maize nuclei exposed to ELF treatments which reflect specific action on nDNA damage producing increased migration of DNA fragments (comet tail) from the nucleus (comet head). This confirmed that the increased exposure of maize seedlings to ELF led to produce DNA lesions in their cells. This DNA damage may be directly as a results of deposition of energy in cells or indirectly as a result of free radical formations as ROS and oxidative damage that they are capable of inducing several major types of DNA lesions such as DNA strand breaks, deletion or insertion of base pairs, pyrimidine dimers, cross-links, and base modification, such as alkylation and oxidation leading to DNA damage [42]. These lesions cause increased DNA migration losing higher amount of DNA as comets from ELF maize nuclei. The DNA damage results in various molecular and physiological effects, such as reduced protein synthesis, cell membrane destruction, and damage to mitochondrial proteins, which affects growth and development of the whole organism [43].

## 5. Conclusions

The current study concluded that each ELF exposing time had special interference with maize cells exhibiting wide

range of genetic and oxidative action on protein and DNA macromolecules. Also, it concluded that the biomarkers used should be combined and augmented for reliable estimates of genetic variability after exposure of economic plants to ELF stressors.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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