# Assessment of genotoxic effects of copper on Cucumber Plant (*Cucumis sativus* L.) using Random Amplified Polymorphic DNA (RAPD-PCR) markers

# تقييم تأثيرات السمية الوراثية للنحاس على نبات الخيار باستخدام مؤشرات التضاعف العشوائي متعدد الأشكال لسلسلة الدنا

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**Abstract**

**The aim of this study was to evaluate the genotoxic effects of copper on *Cucumis sativus* L., fife levels of copper sulfate as aqueous solutions were added as a total concentrate of the soil using the concentrations (100, 200, 300, 400, 500) milligrams per one kilogram of soil, the control soil were using without additional copper sulfate. Random amplified polymorphic DNA (RAPD) technique was used for detection of genotoxic produced by this metal. Twenty one primers were used, of which three did not amplify, three gave extremely faint and ambiguous bands and the rest of fifteen primers generated single and polymorphic bands. All genomic DNA of *Cucumis sativus* L. exposed to copper sulfate solutions displayed polymorphic fragments which were not detectable in DNA of unexposed plants, but high concentration (500 mg/kg soil) of copper sulfate had the strongest effect on genomic DNA of *Cucumis sativus* L. when compared with other concentrations. Thus, this study confirmed that RAPD markers, as a fast, and simple technique can be used for detection of genotoxic effects of copper on *Cucumis sativus* L. plants which are growing in heavy metal polluted soils.**

**Key words: Genotoxic, *Cucumis sativus* L., RAPD-PCR,**

**المستخلص**

**هدف هذه الدراسة تقييم الآثار السمية الوراثية للنحاس على نبات الخيار *Cucumis sativus* L. . أضيفت خمس مستويات 100 ، 200، 300، 400، 500 ملليغرام لكل كيلوغرام واحد من التربة، من كبريتات النحاس بشكل محاليل مائية باعتبارها التركيز الكلي للتربة من دون اضافة كبريتات النحاس الى التربة التي استخدمت كسيطرة.استخدمت تقنية التضاعف العشوائي متعدد الأشكال لسلسة الدنا للكشف عن السمية الوراثية الناتجة من هذا المعدن. استخدم واحد وعشرين بادئ ثلاث منها لم يعطي أي تضخيم وثلاثة اخرى اعطت حزم باهت للغاية وغامق اما البقية فقد اعطت حزم مفردة ومتعددة الاشكال. كل الحمض النووي المجيني لنبات الخيار *Cucumis sativus* L. الذي تعرض لمحلول كبريتات النحاس اظهر قطع متعددة الأشكال التي كانت غير موجودة في الحمض النووي المجيني للنباتات غير ألمتعرضة لكن التركيز العالي 500 ملغ التربة/ كغم من كبريتات النحاس كان له التأثير الأقوى على تسلسل الحمض النووي المجيني لنبات الخيار *Cucumis sativus* L بالمقارنة مع التراكيز أخرى. بذلك اثبتت هذه الدراسة أن مؤشرات التضاعف العشوائي متعدد الأشكال كتقنية سريعة، بسيطة يمكن استخدامها لكشف التأثيرات السمية الوراثية للنحاس على نبات *Cucumis sativus* L . النامي في التربة الملوثة بالمعادن الثقيلة.**

**الكلمات المفتاحية: السمية الوراثية، نبات الخيار، التضاعف العشوائي**

## Introduction

Copper is an essential element, because it is involved in a number of physiological processes such as the photosynthetic and respiratory electron transport chains [1] and as a cofactor or as a part of the prosthetic group of many key enzymes involved in different metabolic pathways, including ATP synthesis [2]. Higher plants take up copper from the soil solution mainly as $Cu^{+2}$ [3]. The progressive increase of $Cu^{+2}$ in aquatic ecosystems arises from various anthropogenic sources including copper mine drainage, copper-based pesticides, industrial and domestic wastes, and antifouling paints [4,5]. Excess in Copper is strongly phytotoxic and may alter membrane permeability, chromatin structure, protein synthesis, enzyme activities, photosynthetic and respiratory processes, and may activate senescence [6,7].

The heavy metal damage in plants has been detected with chromosome aberration assays, mutation assays, cytogenetic tests and specific locus mutation assays [8], and heavy-metal stress leads to disturbances in the structure and functioning of the Plasma Membrane and accumulation of toxic metals in the cytoplasm [9], Advances and developments in molecular biology have provided new ways of detecting DNA damage on plants [10,11,12]. The detection of genotoxicity with DNA marker has many advantages over other markers. The explorations of random amplified polymorphic DNA (RAPD) as genetic markers have

improved the detection of DNA alterations after the influence of many genotoxic agents [13]. This technique using a single primer able to anneal and prime at multiple location throughout the genome can produce a spectrum of amplification products that are characteristics of the template DNA [14, 15]. RAPD-PCR assay is one of the most reliably used techniques for detecting DNA damage as the amplification stops at the site of the damage. The changes occurring in RAPD profiles following genotoxic treatments include variation in band intensity as well as gain or loss of bands. This has been done through the analysis of band intensities and/or band gain/loss variation between exposed and non-exposed individuals [16].

There are a few studies have been conducted to evaluate the genotoxic effects of elevated levels of heavy metals on *Cucumis sativus* L., but there is no study to investigate the genotoxic effects of cupper on *Cucumis sativus* L. Therefore, and for the important of *Cucumis sativus* L. as a vegetable crop was selected for this study to evaluate the application of RAPD markers as a molecular technique to detect DNA damage in *Cucumis sativus* L., and the genotoxic effects of copper that chosen because it is play a relevant role in aquatic pollution and are potentially toxic.

## Materials and Methods

### Plant material

The experiments was carry out in plastic houses in College of Agriculture-Tikrit University during summer season of 2010, by using plastic containers capacity three kilogram of mixture sand soil. For assessment genotoxicity effects produced from copper factor in *Cucumis sativus* L. plant, six different concentrations of copper sulfate CuSO4, ZhengXin Chemical Co., China, reagent grade, 99.0% purity were used. The copper sulfate were added as a total concentrate of the soil with arithmetic well-known by using the levels 100, 200, 300, 400, 500 milligrams per one Kg of soil, the control soil were using without additional copper sulfate. The chemical analysis and physicals characteristics of the soil that used carry out in laboratory of department of soil sciences, College of Agriculture/ University of Tikrit Table (1). Stock solution of metal were prepared in distilled water and diluted with distilled water to obtain the desired concentrations Table (2).

The seeds were immersed in 3%v/v formaldehyde solution for five minutes to remove fungal contamination thereafter the seeds were washed with deionized water for three times to remove excess formaldehyde. Approximately, 4 seeds of *Cucumis sativus* L. were planted in each plastic container that represents one concentration after one week from added aqueous solutions of copper sulfate to the soil. Tap water was used for irrigation, the analysis of the water was achieved Table (2) in the soil department. After 45 day of cultivate, fresh leaves were collected and transported to the laboratory for molecular analysis biology department, college of science, Tikrit University.

**Table (1): Soil Chemical and physicals characteristics**

| No. | Parameters | Value | No. | Parameters | Value | | |
|---|---|---|---|---|---|---|---|
| 1 | Electrical connecting E.C ds.m$^{-1}$ | 2.31 | 9 | $CaCO_3$ gm/kgm$^{-1}$ | 180 | | |
| 2 | Ph | 7.24 | 10 | $CaCO_4$ gm/kgm$^{-1}$ | 60 | | |
| 3 | Ca C.mole/Liter $^{-1}$ | 7.15 | 11 | Organic Material gm/kgm$^{-1}$ | 14 | | |
| 4 | Mg C.mole/Liter $^{-1}$ | 2.8 | 12 | Cu gm/kgm$^{-1}$ | 63 | | |
| 5 | Sodium C.mole/Liter $^{-1}$ | 1.4 | 13 | Mechanical | .00000000000000000000000000 | Greene | clay |
| 6 | Chloride C.mole/Liter $^{-1}$ | 3.6 | | Analysis of the soil gm/kgm$^{-1}$ | 520 | 300 | 180 |
| 7 | Sulfate C.mole/Liter $^{-1}$ | 5.48 | 14 | Text | Mixture | | |
| 8 | C.E.C C.mole/100mg/soil | 16 | | | | | |

**Table (2): Water characteristics**

| No. | Parameters | Value | No. | Parameters | Value |
|---|---|---|---|---|---|
| 1 | Electrical connecting E.C ds.m$^{-1}$ | 0.46 | 5 | Sodium C.mole/Liter $^{-1}$ | 0.4 |
| 2 | pH | 7.05 | 6 | Chloride C.mole/Liter $^{-1}$ | 1.48 |
| 3 | Ca C.mole/Liter $^{-1}$ | 1.84 | 7 | Sulfate C.mole/Liter $^{-1}$ | 1.32 |
| 4 | Mg C.mole/Liter $^{-1}$ | 0.64 | 8 | Cu mg/lier$^{-1}$ | 0.008 |

**Genomic DNA isolation**

A modified CTAB (hexadecyl trimethyl ammonium bromide) procedure [17] based on the protocol of [18] was used to obtain good quality of DNA. Fresh young leaves 3 gm were ground with liquid nitrogen by mortar and pestle until be powder then mix with 5 mL of preheated 65°C extraction buffer 2% CTAB, 1.4 M NaCl, 20 mM $Na_2EDTA$, 100 mM Tris-HCl, pH 8.0. Mixtures were incubated at 65°C for 60 min. then left at room temperature then extracted with 4 mL chloroform-isoamyl alcohol 24:1. Aqueous phases containing DNA were separated by centrifugation for 15min., 4000rpm at room temperature, then transferred to 2 mL clean tubes. Nucleic acids were precipitated by adding 2/3 of cold isopropanol. Nucleic acids were then washed with 70% ethanol, 10 mM ammonium acetate. After brief air drying, DNA pellets were resuspended in 300 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 and 280 nm using (Nano drop Thermo scientific, Germany) and detected the optimum DNA concentration for RAPD-PCR analysis. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide. Samples were stored at -20°C until further use.

**RAPD-PCR analysis:**

PCR amplification was performed with 21 arbitrary decamer primers Table (3) obtained from Operon Technologies, USA.

**Table (3): Sequence of primers employed**

| Primer | Sequence | Primer | Sequence |
|---|---|---|---|
| OPC-05 | 5'-GATGACCGCC-3' | OPR-12 | 5'-ACAGGTGCGT-3' |
| OPD-20 | 5'-ACCCGGTCAC-3' | OPY-03 | 5'-ACAGCCTGCT-3' |
| OPE-16 | 5'-GGTGACTGTG-3' | OPT-19 | 5'-GTCCGTATGG-3' |
| OPH-14 | 5'-ACCAGGTTGG-3' | OPV-19 | 5'-GGGTGTGCAG-3' |
| OPI-06 | 5'-AAGGCGGCAG-3' | OPX-01 | 5'-CTGGGCACGA-3' |
| OPJ-13 | 5'-CCACACTACC-3' | OPA-11 | 5'-CAATCGCCGT-3' |
| OPL-05 | 5'-ACGCAGGCAC-3' | OPB-17 | 5'-AGGGAACGAG-3' |
| OPM-20 | 5'-ACCAACCAGG-3' | OPE-20 | 5'-AACGGTGACC-3' |
| OPN-07 | 5'-TCGCTGCGGA-3' | OPF-16 | 5'-GGAGTACTGG-3' |
| OPQ-01 | 5'-GGCAGGTGGA-3' | OPG-13 | 5'-CTCTCCGCCA-3' |
| OPQ-17 | 5'-TTCGCCTGTC-3' | | |

The PCR reaction was carried out by using AccuPower PCR premix (BIONEER Company, Korea). Genomic DNA 25 ng and primer 10 picomole were added in each tube, total volume made up to 20µl with distilled water, these used according to the manufacturer's instructions. Tubes were vortex and briefly centrifuged after adding template DNA and primer. PCR amplifications were performed in a Thermal Cycler from Applied Biosystems under amplification conditions were as follows: First denaturation, 1 cycle at 94ºC for 2 minutes; 40 cycles (segment denaturation 92 ºC for 1 minutes; annealing 36 ºC for 1 minutes; extension 72 ºC for 1 minutes) and final extension, 1 cycles at 72 ºC for 7 minutes. The amplification products were separated using 1.5 % agarose gel, stained with ethidium bromide and photographed under UV light, standard molecular weight markers also used in each electrophoresis run [17, 19].

**RAPD Data Scored**

RAPD results were analyzed by scoring the amplified fragment generated as the presence or absence of a fragment that could not amplify, total number of amplified bands across all samples of *Cucumis sativus* L., the unique bands which can be detected horizontally [20].

**Results and Discussion**

A RAPD technique was applied to detect the genetic effects at the DNA level of *Cucumis sativus* L. plant after exposed to copper as heavy metal. This study compared the effects occurring in the genomic DNA of *Cucumis sativus* L. exposed to various levels of copper sulfate 100, 200, 300, 400, 500 mg/one Kg of soil comparison with the control soil were using without addition the copper. Twenty one primers were utilized used for screening genomic DNA of *Cucumis sativus* L. exposed and unexposed to copper. Out of 21 primers, three (OPB-17, OPL-05, OPR-12) could not amplify the genomic DNA and three (OPC-05, OPE-16, OPL-05) gave extremely faint and ambiguous bands. Remaining fifteen primers produced visible and reproducible bands, which further used to amplify genomic DNA from all treated and untreated plant. The results of RAPD profiles generated by the fifteen primers revealed differences between exposed and control plants, with visible changes in the number and size of amplified DNA fragments and loss of normal bands and appearance of new bands. The polymorphic bands of the primers were scored as present (+) and absent (-). An example of RAPD patterns generated by representative primer sets OPM-20, OPA-11, OPG-13, OPY-03 and OPF-16 are shown in Figures (1, 2, 3, 4).

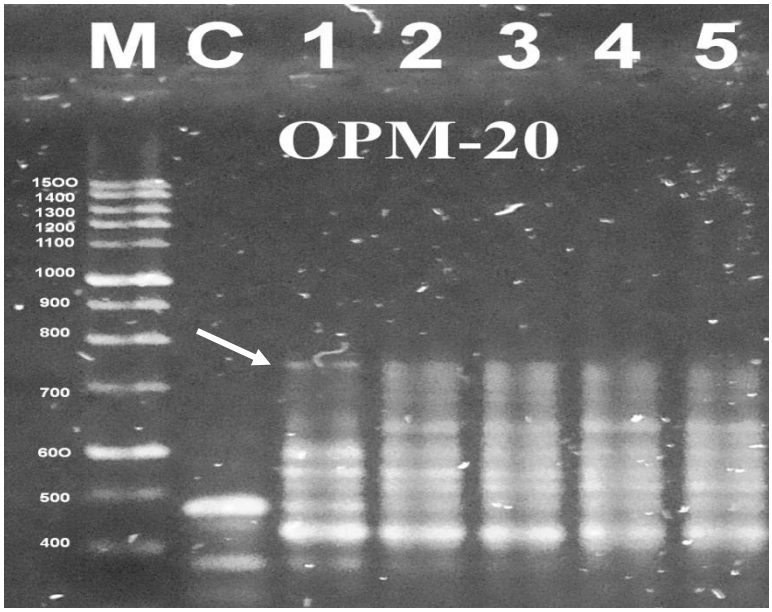


**Fig.(1): RAPD-PCR profiles generated after amplification of genomic DNA of *Cucumis sativus* L. treated with various concentrations of copper using the primer OPM-20 fractionated on a 1.5% agarose gel. Lane M, 1500 bp DNA ladder (Promega); Lane C, plant control without treated; Lane 1, plants treated with concentration 100 mg/kg soil of cupper; Lane 2, treated with concentration 200 mg/kg soil of cupper; Lane 3, treated with concentration 300 mg/kg soil of cupper; Lane 4, treated with concentration 400 mg/kg soil of cupper; Lane 5, treated with concentration 500 mg/kg soil of cupper.**

RAPD profiles of OPM-20 primer produced polymorphic bands with genomic DNA of *Cucumis sativus* L. after treated with various levels of copper sulfate compared with the control, such as the presence of new DNA bands after exposed to copper sulfate solutions displayed polymorphic fragments which were not detectable in DNA of unexposed plants. For example, the low concentration of cupper 100 mg/kg soil created mutation and produced more number of new unique fragments in PCR amplification for most primers. The unique fragment produced with primer OPM-20 of size 750 bp was found Figure (1).

The results of RAPD analysis using OPA-11 primer generated polymorphic bands are illustrated in (Figure 2). RAPD profiles showed substantial differences between control and exposed samples to copper sulfate solutions. High concentrations 500 mg/kg soil of copper sulfate had the strongest effect on genomic DNA of *Cucumis sativus* L. when compared with other concentrations because of give bands with molecular weight 800 and 850 bp were absent in the control sample and in all exposed samples.

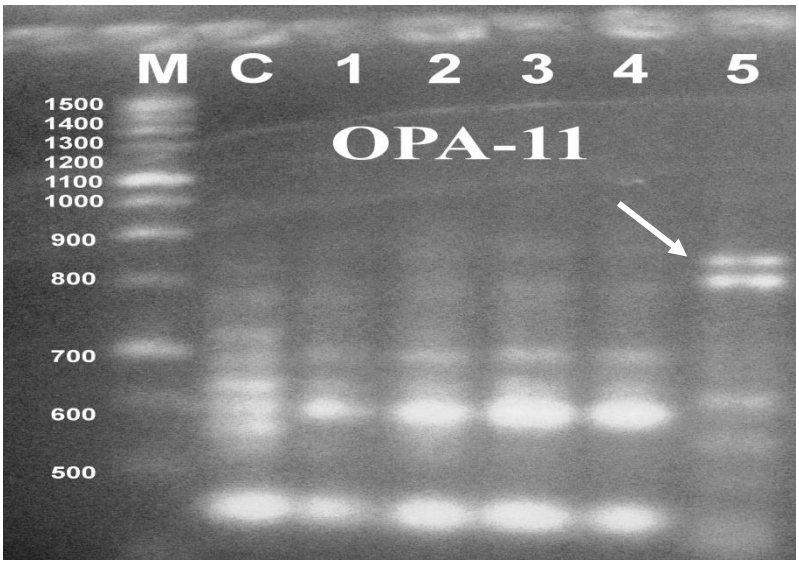


**Fig.(2): RAPD-PCR profiles generated from genomic DNA of *Cucumis sativus* L. treated with various concentrations of copper using the primer OPA-11 fractionated on a 1.5% agarose gel. Lane M, 1500 bp DNA ladder (Promega); Lane C, plant control without treated; Lane 1, plants treated with concentration 100 mg/kg soil of cupper; Lane 2, treated with concentration 200 mg/kg soil of cupper; Lane 3, treated with concentration 300 mg/kg soil of cupper; Lane 4, treated with concentration 400 mg/kg soil of cupper; Lane 5, treated with concentration 500 mg/kg soil of cupper.**

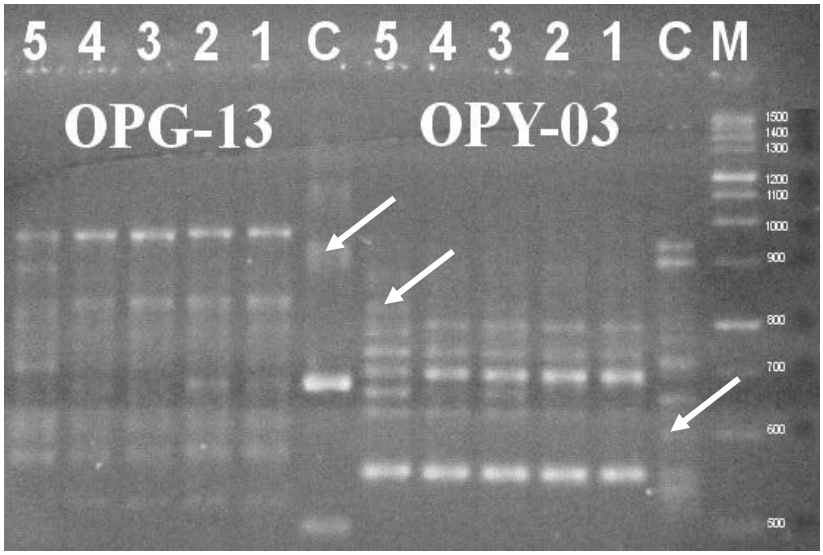


**Fig.(3): RAPD-PCR profiles generated from genomic DNA of *Cucumis sativus* L. treated with various concentrations of copper using the primers OPG-13and OPY-03 fractionated on a 1.5% agarose gel. Lane M, 1500 bp DNA ladder; Lane C, plant control without treated; Lane 1, plants treated with concentration 100 mg/kg soil of cupper; Lane 2, treated with concentration 200 mg/kg soil of cupper; Lane 3, treated with concentration 300 mg/kg soil of cupper; Lane 4, treated with concentration 400 mg/kg soil of cupper; Lane 5, treated with concentration 500 mg/kg soil of cupper.**

Interestingly, as show in figure (3) when used OPY-03 primer gives many bands were present in exposed samples but absent in control and vice versa. Band with molecular weight 600 bp was present in control sample and absent in all exposed samples, whereas the band with molecular weight 830 bp was present in high concentrations 500 mg/kg soil of copper sulfate. OPG-13 primer give bands with molecular weight 1000 bp were present the control sample while was absent in all exposed samples.

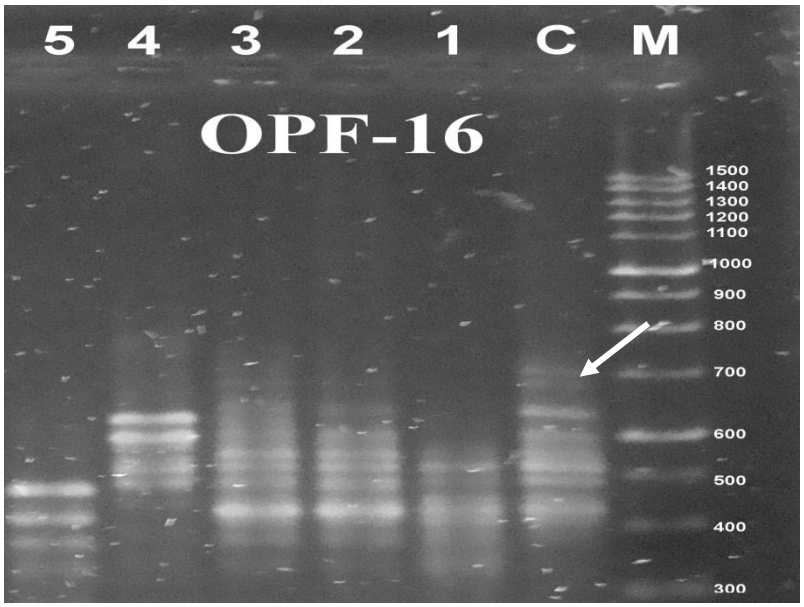


**Fig.(4): RAPD-PCR profiles generated from genomic DNA of *Cucumis sativus* L. treated with various concentrations of copper using the primer OPF-16 fractionated on a 1.5% agarose gel. Lane M, 1500 bp DNA ladder (Promega); Lane C, plant control without treated; Lane 1, plants treated with concentration 100 mg/kg soil of cupper; Lane 2, treated with concentration 200 mg/kg soil of cupper; Lane 3, treated with concentration 300 mg/kg soil of cupper; Lane 4, treated with concentration 400 mg/kg soil of cupper; Lane 5, treated with concentration 500 mg/kg soil of cupper.**

RAPD profiles showed substantial differences between control and exposed samples to copper sulfate. When the OPB-19 primer was used with genomic DNA of *Cucumis sativus* L. it caused disappearance of DNA band with size of 700 bp in all treatments compared with the control Figure (4).

The results refer to changes in RAPD profiles that reflect DNA effects were compared to all genomic DNA of *Cucumis sativus* L. exposed to copper sulfate solutions compared with unexposed plants displayed polymorphic fragments which were not detectable in DNA of the unexposed plants. Changes observed in the DNA profiles such as modifications in band intensity and loss of bands may be due to the changes in oligonucleotide priming sites mainly due to genomic rearrangements and less likely to point mutations or DNA damage in the primer binding sites [21]. Appearance of new bands could be attributed to the presence of oligonucleotide priming sites which become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations (resulting in new annealing events) or large deletions (bringing two preexisting annealing sites closer) or homologous recombination [22].

In this study, RAPD analysis showed that there were detectable genetic changes when the genomic DNA of *Cucumis sativus* L. was exposed to copper. Appearing bands may be the result of structural changes induced by copper adducts and/or by nongenotoxic events such as transposition, DNA amplification, and so on. Other changes in DNA patterns such as the variation in band intensities can be attributed principally to the presence of bulky adduct that potential block the PCR enzyme. Finally, the changes observed in RAPD patterns are likely to be due to the sum of all DNA alterations (e. g., adducts, mutations, rearrangements, structural changes) induced by copper. In addition, dimmers can alter the structure of the DNA. If so, such structural changes are likely to have a significant effect on the kinetics of PCR events. New PCR products can be amplified because some sites become accessible to the primers after structural change or because the same mutations have occurred in the genome [23]. In *vivo* copper exposure involves complex interactions. The amount of ingested copper in food and water is usually relatively low, and most humans and animals are able to control excessive amounts of copper in the body by either decreased absorption or increased excretion. Usually, copper is linked to prosthetic groups or tightly bound in storage or transport proteins (like ceruloplasmin) and is, therefore, not available for oxidation reactions. DNA copper damage might be limited or not evident at low copper concentrations but at high concentrations free copper may exert a profound genotoxic effect, as observed in this work. Free copper induces ROS production and several types

of DNA damage, such as base alteration and DNA strand breaks, which may cause extensive cell death [24]. The genotoxicity of copper could appear via the action of induced reactive oxygen species, while the inhibition of DNA repair enzymes could be caused by a non-specific binding of Cu2+cations to essential sites in the enzyme molecule [25]. Copper can induce a range of DNA damage such as single- and double-strand breaks, modified bases, a basic sites, DNA-protein cross-links and even bulky adducts representing in trastr and dimerization of adjacent purine bases (dimmers) [26,27]. Copper, as with other transition metals, catalyses the Fenton type and reduction of hydrogen peroxide to form hydroxyl radical, one of the most reactive radical oxygen species [28]. The clear correlation between copper treatment and percentage of DNA polymorphism support the effectiveness of RAPD for investigating environmental toxicity [29, 30,31].

In conclusion, the heavy metal copper at low, medium and high concentrations caused DNA damage to *Cucumis sativus* L. and mutations. Since, the *Cucumis sativus* L. is immensely used for salad purpose in most countries; therefore, biomarkers are necessary for detection of high concentration of heavy metals in this plant where it is growing, and keep human health free from hazardous materials. Moreover, RAPD were fast and simple technique for genotoxicity.

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