*ISPEC Tarım Bilimleri Dergisi*
*5(3): 529-543, 2021*

***Araştırma Makalesi***

**E-ISSN:2717-7238**
**www.ispecjournal.com**

*ISPEC Journal of Agr. Sciences*
*5(3): 529-543, 2021*

***Research Article***

**Filiz AYGÜN ERTÜRK**[1a*]
**Serap SUNAR**[2a]
**Güleray AĞAR**[3a]

[1]Bayburt University, Health Sciences Faculty, Nutrition and Dietetics Department, Bayburt

[2]Erzincan Binali Yildirim University, Pharmacy Faculty, Department of Pharmacy Professional Sciences, Erzincan

[3]Ataturk University, Faculty of Science, Department of Biology, Erzurum

[1a]**ORCID**: 0000-0002-9973-5669
[2a]**ORCID**: 0000-0002-2011-1117
[3a]**ORCID**: 0000-0002-8445-5082

[*]Corresponding author:
filizayerturk@bayburt.edu.tr

DOI
https://doi.org/10.46291/ISPECJASvol5iss3pp529-543

**Alınış (Received):** 25/04/2021
**Kabul Tarihi (Accepted):** 28/05/2021

**Keywords**
Heavy metal, genomik instability, ABA, CRED-RA, RAPD

# Determination of Cytogenetic and Epigenetic Effects of Manganese and Copper on *Zea mays* L.

**Abstract**
Heavy metal accumulation and its possible effects are prominent problem for not only human health but also for the environment and plant systems due to that heavy metals are non-biodegradable. In this research, it was aimed to examine the impacts of heavy metals on toxicity and genotoxicity in maize. Seeds of corn were subjected to various concentrations of $MnSO_4$ and $CuSO_4$ for determining their effects on DNA methylation, DNA damage levels, protein and phytohormone alterations. The results revealed that an increase in copper and manganese concentrations causes decrease in soluble protein levels, genomic template stability (GTS) and mitotic index but causes an increase in RAPD profile alterations and DNA hypermethylation. Additionally, HPLC analyses show that $CuSO_4$ and $MnSO_4$ contamination reduces growth-promoting hormones, like gibberellic acid (GA), zeatin (ZA) and indole acetic acid (IAA), and increases the abscisic acid (ABA). This study obviously indicated that $CuSO_4$ and $MnSO_4$ have epigenetic and genotoxic effects. A decrease in the phytohormone level (ZA, GA, and IAA) and an increase in the ABA level under $CuSO_4$ and $MnSO_4$ are thought to be a part of the defense system of maize to struggle with stress.

## INTRODUCTION

$Cu^{2+}$ is one of the major elements for plants due to its very important missions in biological activities. $Cu^{2+}$ is used as a mineral nutrient for plants, and it supports the vital processes like mitochondrial respiration, photosynthesis, hormone-signaling pathways, and cell-wall metabolism. Besides these aspects, it is a constructional ingredient of proteins and enzymes (Muccifora, 2007). On the other hand, one of the striking features of $Cu^{2+}$ is its ability to show cytotoxic effects especially in high concentrations (Mediouni et al., 2006). The toxicity mechanism of $Cu^{2+}$ is directly associated with the generation of toxic oxygen species, including hydroxyl radical, hydrogen peroxide and superoxide radical. These oxygen species may lead to some damage to the structural mechanism of some macromolecules, such as DNA, proteins, and lipids (Girotti, 2001). It has been reported that the high density of $Cu^{2+}$ provokes the total chromosomal aberration, DNA mutation, mitotic index, and creates negative impacts on enzymes and protein metabolism in various plant and animal species (Korpe and Aras, 2011). Besides morphological changes, it has been reported that lipid peroxidation resulting in decreased chlorophyll content, increased hydrogen peroxide, and excess production of reactive oxygen species (ROS) $Cu^{2+}$ can also affect biochemical content and induce DNA damage (Atha et al., 2012; Mosa et al., 2018). On the other hand, one of the genotoxic effects of $Cu^{2+}$ was explored on the polytene chromosomes of larvae of *Chironomus riparius* by Michailova et al., (2004).

Mn is also a vital nutrient like Cu and takes part in some physiological activities of plants. Some of these activities are respiratory processes, regulation of reproduction, lipid and carbohydrate metabolism, generating bone marrow and connective tissues. Additionally, it contributes to the care of the brain in animals (Pittman, 2005). Inter alia, Mn is a crucial co-factor element for many enzymes used in DNA biosynthesis, neurotransmitters and in transduction signals (Pittman, 2005; Roth and Garrick, 2003;). Besides these beneficial aspects, conversely, high concentrations of Mn may cause mutagenic impacts on bacteria by affecting DNA replication (Gerber et al., 2002). Moreover $MnCl_2$ affects the Drosophila melanogaster wing spot test. In the study of Brega et al. (1998), the high level of chromosomal aberration was observed in the farm workers who were exposed to pesticides with Mn content. Additionally manganese chloride has a mutagenicity effect against TA1537 strain of *S. typhimurium* (Lima et al. 2007). There are many studies explaining that manganese causes DNA damage in plants (Enan, 2006; Zhou et al., 2013; Yigider et al., 2016). In accordance with the WHO (World Health Organization) data, All Mn forms have mutagenic impact in vivo and in vitro and further research should be performed to clarify the mutagenicity potential of Mn. Hence, the studies carried out in this regard have recorded that high concentrations of Cu and Mn have the potential to bring out mutagenic activation.

Cu and Mn may affect the epigenome, in addition to having genotoxic effects. The high concentration of $Cu^{2+}$ (100-200 mM) causes a high reduction of total histone acetylation in human hepatocyte Hep3B cells. It is determined that, by in vivo and in vitro assays that Cu decreases the histone acetyltransferases (HATs) activity in the study of Kang et al., 2005. At the same time, a number of studies indicate that the modulation of the activity of histone deacetylases (HDACs) by Cu is also possible.

In addition, in response to Cu stress, it has been reported that increases in mRNA level of metallothionein-like genes were observed (Tan et al., 2014). And it has been reported that transcriptional upregulation of autophagy-related (ATG) genes is observed in grapevine under copper stress (Shangguan et al., 2018).

In the light of the events mentioned here, this study aims to detect the genotoxic ability of Cu and Mn and their epigenetic impacts on the seedlings of maize by employing the RAPD and CRED-RA methods and mitotic index (MI). Furthermore, some physiological parameters were determined as a result of the measurement of the alterations in phytohormones and total soluble protein.

## MATERIAL and METHODS

### Plant, chemicals and growth conditions

Copper sulfate ($CuSO_4$) and manganese sulfate ($MnSO_4$) were supplied from Sigma-Aldrich and the seeds from the Agriculture Faculty. Care was taken to select and use the *Zea mays* L. seeds of equal size, and then 2.5% sodium hypochlorite was used for sterilizing the seeds' surfaces for 3 min and they were washed using distilled water five times at minimum. Then, fifteen seeds of equal size were elected and placed into sterile Petri dishes. The selected samples were put in another plates. Two of them are solution-free (control) plates for $CuSO_4$ and $MnSO_4$ and others are 5, 10, 20, 40 mM of $CuSO_4$ and $MnSO_4$. Germination of the seeds consisted of at the 25°C and 50% humidity. The roots were harvested when their length reached at 1.5-3.0 cm. They put in acetic alcohol (1:3) for cytological analysis. One week later, the seedlings were preserved at -80ºC.

### Cytological analysis

Feulgen's squash technique was performed to prepare cytological preparations. Various preparations were used for every application for exploring the chemical impacts on the MI. The MI was determined by rating the divided cells to the total.Tthe data was analyzed in new multiple range test of Duncan.

### Analysis of the total protein content

The total protein level of the ground tissue of the *Z. mays* seedling roots was determined quantitively in accordance with the study carried out by Bradford (1976). The data were presented as mean ± SD. A significant difference ($P \leq 0.01$) between the control and treatment specimens was found as a result of one-way ANOVA by conducting Duncan's test.

### Phytohormone analysis

Cytokinins and indole-3-acetic acid were analyzed in accordance with the studies of Kuraishi et al. 1991 and Turker et al. 2008. The gibberellic acid and abscisic acid analysis was performed according to the studies of Unyayar et al. 2006 and Cakmak et al. 1989 with some modification. HPLC analysis was conducted by utilizing the isocratic system. Variance analysis was performed by with one-way ANOVA by using SPSS. Comparison of the averages was performed by Duncan's test (the reliability level is 0.01).

### DNA isolation and RAPD and CRED-RA procedures

Extraction of genomic DNA from seedlings in the form of powder was performed by employing a modified method presented by Li and Quiros (2001). *Z. mays* control groups were scanned for RAPD alteration by utilizing standard 10-base primers. The reaction mixtures (30 ml) were fixed in the way described below: 3.0 ml of 10X buffer, 1.2 ml of dNTPs (10 mM), 1.2 ml of magnesium chloride (25 mM), 2.0 ml of primer (5 mM), 0.4 ml of Taq polymerase (5 units), 19.2 ml of water and 3.0 ml of sample DNA (100 ng/ml). Screening of 40 oligonucleotide primers in total was performed, among which 16 primers (OPA-13, OPH-17, OPA-2, OPA-1, OPA-6, OPH-14, OPH-18, OPY- 1, OPY-6, OPY-8, OPY-15, OPY-16, OPW-1, OPB-8, OPW-7 and OPW-5).

Screening of the control groups of CRED-RA alteration was carried out by utilizing standard 10-base primers. Genome methylation was performed for determining the features of the enzymes of *Msp I* and *Hpa II*. 16.3 µl of water, 2 µl of RE 10X buffer, 0.2 µl of BSA, 1 µl of DNA and 0.5 µl of restriction enzyme were added to 0.5ml tubes for methylation. Incubation was carried out in an oven with 37°C for 4 hours to ensure that the DNA was crop by

enzymes. The PCR program for the RAPD and CRED-RA was performed. 5 min at 94 °C; 4 cycles for 1 min and 30 s at 94 °C, 1 min and 30s at 37 °C and 3 min 72 °C. 41 cycles for 1 min at 94 °C, 1 min at 36 °C, 1 min at 42°C and 3 min at 72°C. The final extension is 7 min at 72 °C and a reduction to 4 °C in thermal cycler.

**Electrophoresis**

PAGE was utilized for protein analysis (Laemmli, 1970). RAPD and CRED-RA PCR products were distinguished with 1.5% agarose gel in electrophoresis using in 0.5xTBE buffer in 70 V for 150 min. UV imaging and gel visualiazation systems were utilized for determining and photographing the amplified DNA products. Research patterns were assessed by means of the Total Lab TL120.

**Data analysis**

GTS was computed by using the following equation. *GTS*=100-(100 x a/n). In the CRED–RA analysis, the mean polymorphism rates were determined for every dose. For the purpose of computing the polymorphism rate, the 100 x a/n formula was performed (Nardemir et al., 2015).

## RESULTS and DISCUSSION

**Mitotic index**

The mitotic index in the plant roots treated by Cu and Mn decreased significantly ($P<0.01$), and this result matches the control. There is an inverse relationship between heavy metal concentration and the mitotic index. In the seeds affected by Cu, the mitotic index declines with a growing dose. Table 1 contains information on the mitotic index change of maize seedlings. As seen from the table, the minimum dose (5 mM) of Cu caused a significant decrease in the mitotic index compared to the control. A further increase in dose causes a differential reduction in the mitotic index which was also substantially lower compared to the control at all doses. Similarly, the mitotic index decreases with the effect of 5-40mM concentration of Mn in comparison with the control (Table 1).

**Table 1.** MI of the root tips of seedlings according to the Cu and Mn concentration.

| Mitotic index (MI) | | |
|---|---|---|
| **Dose (mM)** | **Copper (Cu)** | **Manganese (Mn)** |
| Control | 9.0[a] | 9.0[a**] |
| 5 | 5.7[b] | 5.8[b] |
| 10 | 2.6[b] | 4.5[bc] |
| 20 | 2.1[c] | 2.5[c] |
| 40 | 1.0[c] | 2.1[c] |
| **Average** | **4.1** | **4.8** |

Means of each column followed by the same letter do not differ considerably at the level of $p < 0.01$

The decrease of MI depending on the high dosages of heavy metals shows us the effects of Cu and Mn on the mitotic cell division of somatic cells. In addition, mitotic abnormalities were determined in root tip cells, including multipolar anaphase, anaphase bridges, sticky chromosomes, and micronucleus.

**Total soluble protein**

Table 2 presents information on the alterations in the total soluble protein of maize seedlings. Soluble protein decrease considerably ($P\leq0.01$) with the increase in Cu and Mn concentrations according to the control plantlets. It was also observed that the total protein rate decreases depending on the concentration of Mn in maize

seedlings. SDS-PAGE was used to evaluate the proteins that were acquired from the maize seedlings affected by Mn and Cu. The acquired findings demonstrated that small alterations occurred in total protein band. The mentioned alterations are characterized only by changing the band density (Figure 1-2).

**Table 2.** Total soluble protein of root tips according to the Cu and Mn concentration.

| Total soluble protein (mg/ml) | | |
|---|---|---|
| **Dose (mM)** | **Copper (Cu)** | **Manganese (Mn)** |
| Control | 3.307$^{a**}$ | 3.307$^{a**}$ |
| 5 | 3.293$^{a}$ | 3.157$^{b}$ |
| 10 | 3.200$^{b}$ | 3.190$^{b}$ |
| 20 | 3.180$^{b}$ | 3.200$^{b}$ |
| 40 | 3.130$^{b}$ | 3.213$^{ab}$ |
| **Average** | **3.222** | **3.213** |

Means of each column followed by the same letter do not differ considerably at the level of p< 0.01

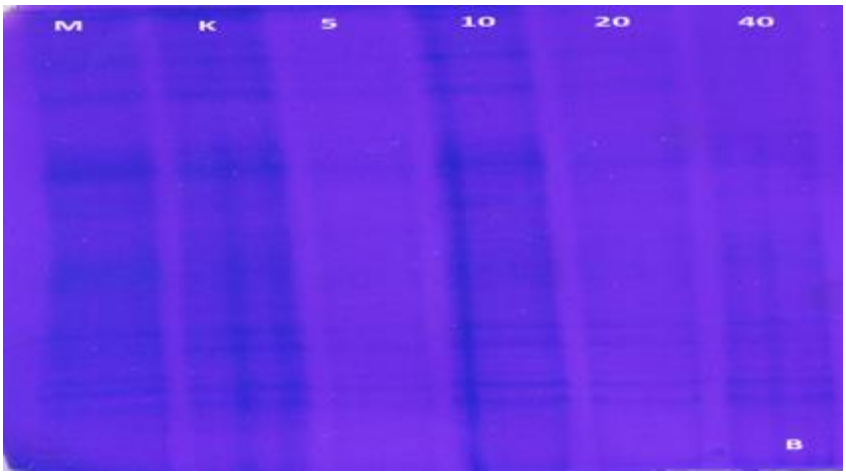


**Figure 1.** Total soluble protein of seedlings via Cu concentration.

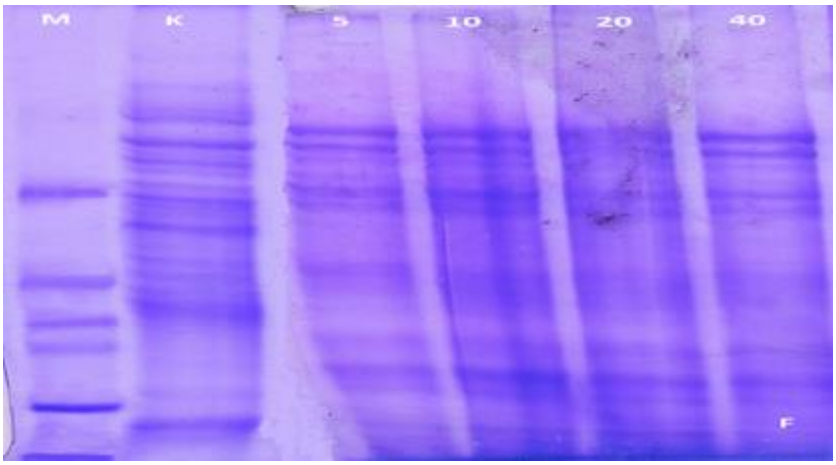


**Figure 2.** Total soluble protein of seedlings via Mn concentration.

**RAPD**

In this study, 40 oligonucleotide primers in total were utilized in the PCR analysis of the products of *Zea mays* genome, and only 16 of them present us specific and stable results. When the samples are compared with the control DNA, effects and changes resulting from Mn and Cu can be explicitly seen on the RAPD patterns (Figure 3-4). The mentioned alterations are characterized by a change in band density, presence and/or absence of DNA fragments (Table 3-4).

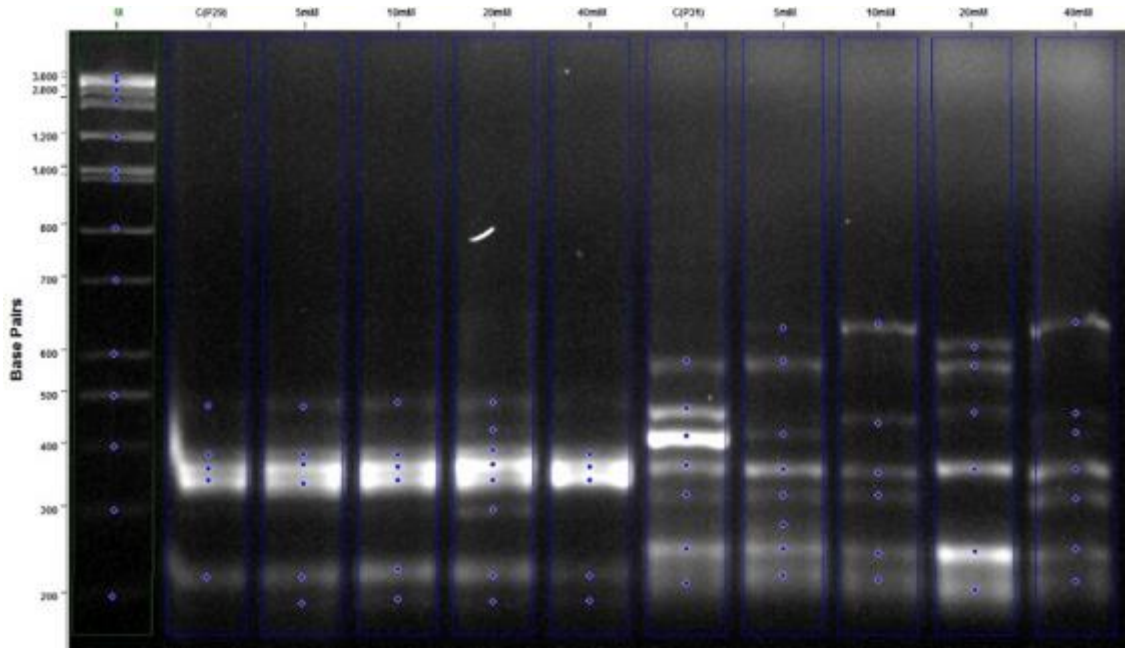


**Figure 3.** RAPD profiles of seedlings subjected and not subjected to Cu with OPW-7 and OPW-5

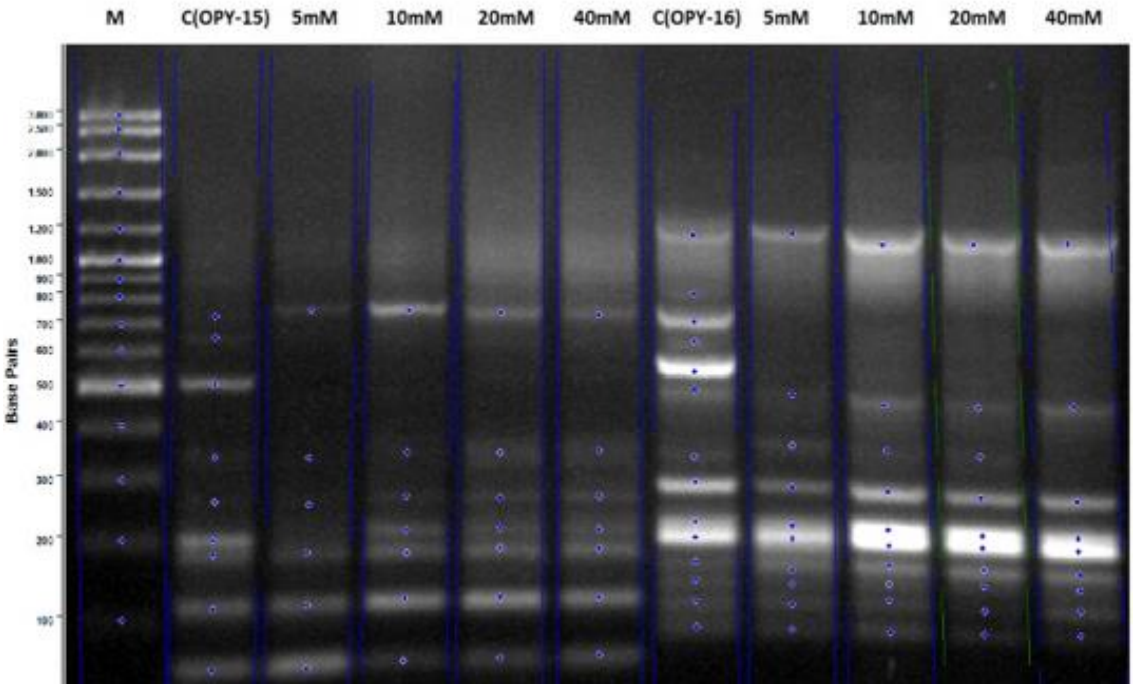


**Figure 4.** RAPD profiles of seedlings subjected and not subjected to Mn with OPY-15 and OPY-16

**Table 3.** Dimension (bp) of disappearance (−) / appearance (+) DNA bands and RAPD profiles of Cu treated seedlings and GTS change

| Primers | Copper (Cu) Concentrations | | | | |
|---|---|---|---|---|---|
| | **Control** | **5 mM** | **10 mM** | **20 mM** | **40 mM** |
| **OPA-13** | 7 | -454 | -454,-363 | -454 | -454 |
| **OPH-17** | 12 | -- | -- | -- | -168 |
| **OPA-2** | 8 | -618, -300,+113,+28 | -618,+113,+28 | -618,+445, +113,+28 | -618,+445,-300, +113,+28 |
| **OPA-1** | 11 | +312 | +312,-209 | +312,-209,-126 | +312,-209,-126 |
| **OPA-6** | 9 | -- | +251 | +251 | +251 |
| **OPH-14** | 5 | -478,-212 | -478,-212 | -212 | -212 |
| **OPH-18** | 5 | -- | -- | +266,-72 | -72 |
| **OPY-6** | 10 | -133 | -133 | -133 | -432,-133 |
| **OPY-1** | 7 | -559, -316,+262 | -559,+262 | -559,+280 | -559,+385, +262 |
| **OPY-8** | 8 | +419,+308 | +419,+308 | +419,+308 | +419,+308 |
| **OPY-15** | 12 | -650,+413,+373, +298,+251 | -650,+413, +373,+298,+251 | -650,+413, +373,+298,+251 | -650,+413, +373,+298 |
| **OPY-16** | 10 | +187 | +187 | +187 | +457,+187 |
| **OPW-1** | 11 | -507,-449, -261 | -507,-261, -252 | -507,-261, -252 | -507,-261, -252 |
| **OPB-8** | 6 | +507,+452 | +507,+452 | +646,+558,+507,+452 | +646,+558,+507,+452 |
| **OPW-7** | 8 | +192 | +192 | +429,+301,+192 | -478,+192 |
| **OPW-5** | 8 | +634,-472,+281 | +634,-586,-472,-19 | +611,-419,-323 | +634,-586 |
| **Total band** | **137** | **29** | **31** | **36** | **37** |
| **Polymorphism** | | **21.16** | **22.63** | **26.27** | **27.00** |
| **GTS value** | | **78.84** | **77.37** | **73.72** | **63.00** |

Genomic template stability (GTS) was utilized to determine the alterations in RAPD profiles. There was a tendency of GTS values to decrease with an increase in the concentration of heavy metal treatments. The above-mentioned findings may indicate the events like DNA protein cross-links, abasic sites, complex chromosomal rearrangements, single and double-strand breaks, modified bases, oxidized bases, bulky adducts and point mutations (Atienzer et al., 1999; 2000).

**Table 4.** Dimension (bp) of disappearance (−) / appearance (+) DNA bands and RAPD profiles of Mn treated seedlings and GTS change

| Primers | Manganese (Mn) Concentrations | | | | |
|---|---|---|---|---|---|
| | Control | 5 mM | 10 mM | 20 mM | 40 mM |
| **OPA-13** | **7** | +365 | +365 | +365 | -300 |
| **OPH-17** | **12** | +1355,+588, -102 | +1355,+588 ,-242,-102 | +1355,+588,-102 | -242,-102 |
| **OPA-2** | **8** | +390,+164, -85 | +431,+390,+292,-269,+164-144,-39 | +390,-269, +164,-85,-39 | -39 |
| **OPA-1** | **11** | -65 | -65 | -65 | -65 |
| **OPA-6** | **9** | -612,+481 | -612,+481 | -612,+481 | -612,+481,-313 |
| **OPH-14** | **5** | -1132,-789, -488,-348,-322 | -1132,-789,-488, -348,-322 | -1132,-789, -348,-322 | -1132,-789, -488,-348,-322 |
| **OPH-18** | **5** | +563,-389,-83 | +563,-389,-83 | +563,-389,-83 | -389,-83 |
| **OPY-6** | **10** | -313,+154,-83 | +356,-313 | +991,-313,+154 | +356,-313 |
| **OPY-1** | **7** | -149 | -433,-149,+124,+41 | -433,-312,-149,+124 | -433,-312,-149,+124, +41 |
| **OPY-8** | **8** | -1073,+684,-508,+259,+200,-76 | -1073,+829,-508 | -1073,+600,-508,+259,+200,-76 | -1073,+829, -508,+171 |
| **OPY-15** | **12** | -650,-503 | -650,-503 | -650,-503 | -650,-503,-200 |
| **OPY-16** | **10** | -815,-709, -633,-535 | -815,-709,-633,-535 | -815,-709,-633,-535,-339 | -815,-709, -633,-535 |
| **OPW-1** | **11** | -- | -- | -- | -- |
| **OPB-8** | **6** | +604,+414, +346,+295 | +1118,+414,+295,-156 | +604,+414,+346,+295,-127 | -252,-203 ,-188,-156 |
| **OPW-7** | **8** | -819,-772,+600, -337,+263,-138 | -819,-772,+600,-337,+263,-138 | -819,-772,+600,-337,+263,-138 | -819,-772,+600,-337,-138 |
| **OPW-5** | **8** | -893,+455, +406, +328,+302 | -893,+587,+406, +328,+302 | -893,+455,+406 | -893,+587, +455, +406,+328 |
| **Total band** | **137** | **49** | **53** | **53** | **47** |
| **Polymorphism** | | **35.77** | **38.69** | **38.69** | **54.01** |
| **GTS value** | | **64.23** | **61.31** | **61.31** | **45.98** |

## CRED-RA

For the purpose of determining epigenetic alterations on the genome of maize, eight primers were determined to employ the CRED-RA method (Figure 5-6). They are OPY-6, OPY-15, OPB-8, OPA-1, OPA-2, OPW-1, OPH-17 and OPH-18. Table 5-6 present the CRED-RA method results and contain information on the methylation changes of maize seedlings. As it can be understood from the results, heavy metals may alter the methylation situation and cause DNA hypermethylation.

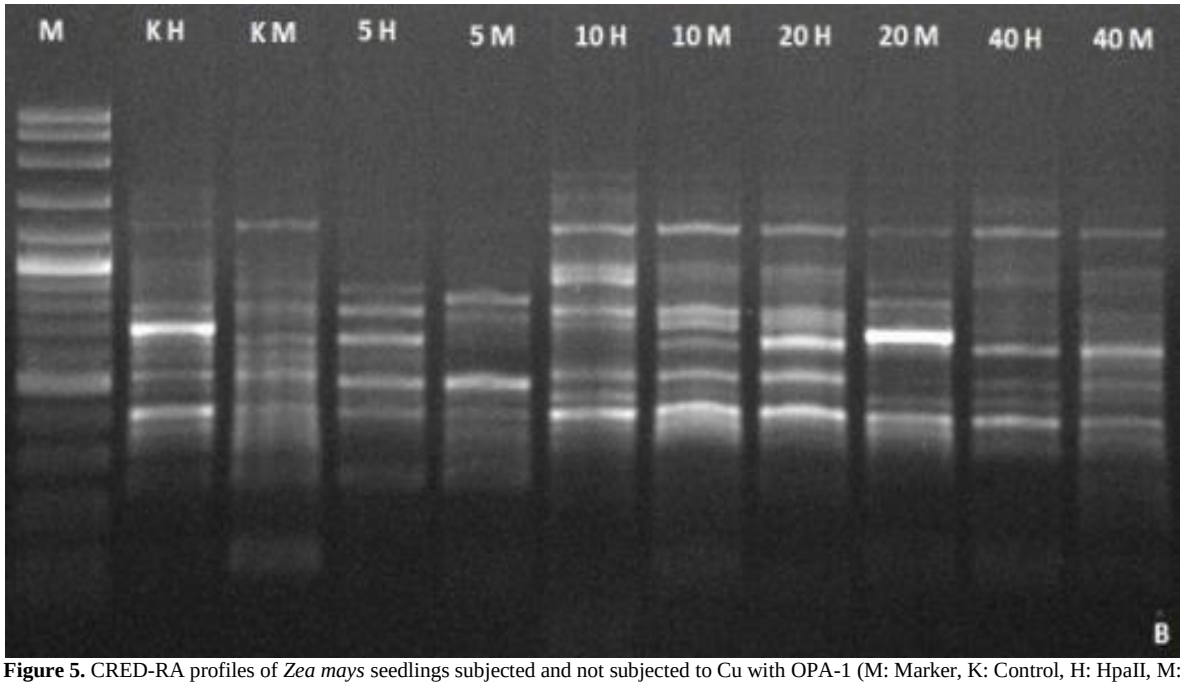


**Figure 5.** CRED-RA profiles of *Zea mays* seedlings subjected and not subjected to Cu with OPA-1 (M: Marker, K: Control, H: HpaII, M: MspI).

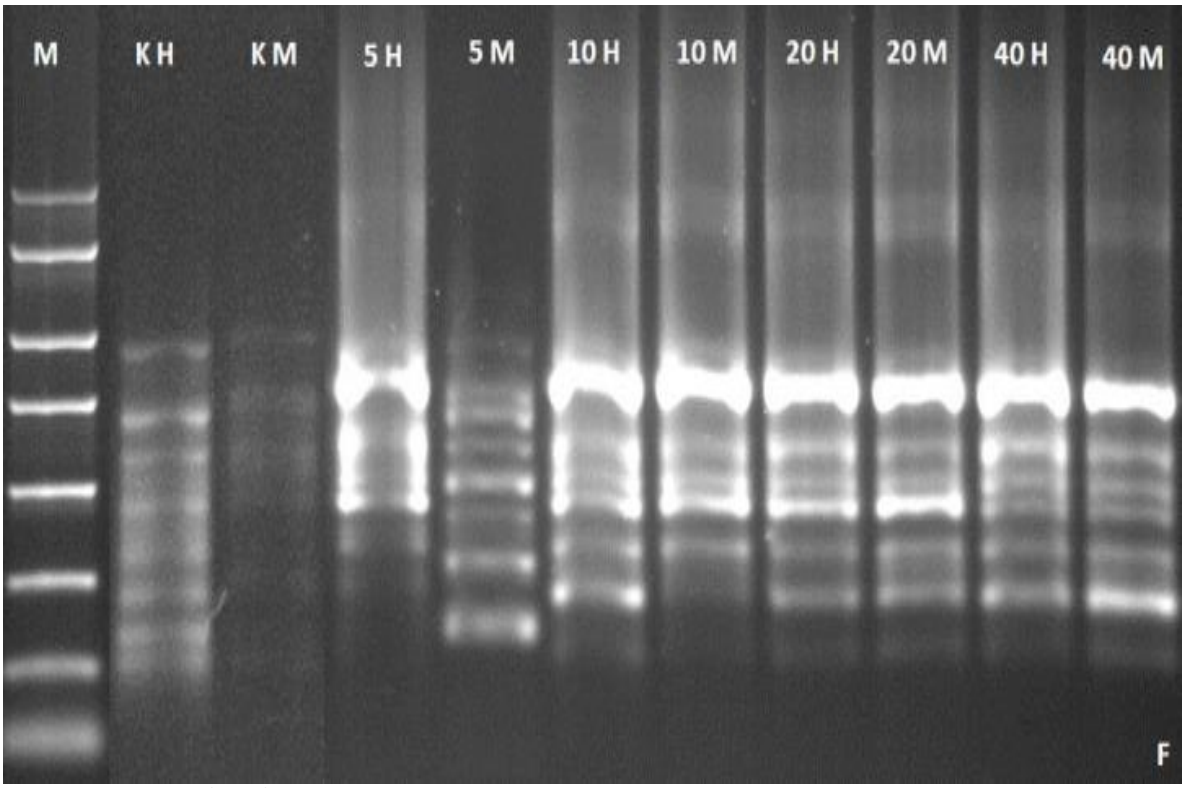


**Figure 6.** CRED-RA profiles of *Zea mays* seedlings subjected and not subjected to Mn with OPY-6 (M: Marker, K: Control, H: HpaII, M: MspI).

**Table 5.** CRED-RA band numbers according to Cu concentration and polymorphism %

| Primers | Control | | Total Band Number | | | | | | | | Total polymorphic band number | | | | | | | | % Polymorphism | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5 mM | | 10 mM | | 20 mM | | 40 mM | | 5 mM | | 10 mM | | 20 mM | | 40 mM | | 5 mM | | 10 mM | | 20 mM | | 40 mM | |
| | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M |
| **OPH-17** | 6 | 7 | 9 | 7 | 9 | 7 | 6 | 4 | 4 | 5 | 5 | 3 | 4 | 3 | 5 | 4 | 3 | 4 | 55.5 | 42.8 | 44.4 | 42.8 | 83.3 | 100 | 75 | 80 |
| **OPA-1** | 5 | 5 | 6 | 8 | 7 | 8 | 6 | 2 | 0 | 9 | 5 | 3 | 6 | 4 | 5 | 6 | 5 | 5 | 83.3 | 37.5 | 85.7 | 50 | 83.3 | 100 | 0 | 55.5 |
| **OPH-18** | 8 | 6 | 6 | 7 | 6 | 8 | 8 | 8 | 8 | 7 | 4 | 2 | 5 | 5 | 4 | 5 | 6 | 4 | 66.6 | 28.5 | 83.3 | 62.5 | 50 | 62.5 | 75 | 57.1 |
| **OPY-6** | 5 | 5 | 5 | 0 | 0 | 1 | 6 | 0 | 1 | 2 | 0 | 5 | 5 | 4 | 1 | 5 | 4 | 3 | 0 | 0 | 0 | 100 | 16.6 | 0 | 100 | 100 |
| **OPY-15** | 11 | 11 | 10 | 12 | 10 | 12 | 12 | 12 | 5 | 5 | 4 | 5 | 4 | 4 | 3 | 6 | 9 | 6 | 40 | 41.6 | 40 | 33.3 | 25 | 50 | 100 | 100 |
| **OPW-1** | 9 | 9 | 8 | 9 | 3 | 9 | 5 | 9 | 5 | 8 | 7 | 3 | 6 | 5 | 9 | 5 | 8 | 5 | 87.5 | 33.3 | 100 | 55.5 | 100 | 55.5 | 100 | 62.5 |
| **OPB-8** | 4 | 4 | 5 | 6 | 6 | 6 | 7 | 5 | 5 | 7 | 1 | 2 | 4 | 4 | 5 | 3 | 5 | 5 | 20 | 33.3 | 66.6 | 66.6 | 71.4 | 60 | 100 | 71.4 |
| **OPA-2** | 8 | 8 | 9 | 9 | 8 | 8 | 9 | 8 | 8 | 7 | 3 | 3 | 2 | 2 | 4 | 3 | 4 | 3 | 33.3 | 33.3 | 25 | 25 | 44.4 | 37.5 | 50 | 42.8 |
| **Average** | **7** | **7** | **7** | **7** | **6** | **7** | **7** | **6** | **5** | **6** | **4** | **3** | **5** | **4** | **5** | **6** | **6** | **4** | **48.2** | **31.2** | **55.6** | **54.4** | **59.2** | **58.1** | **75** | **71.1** |

**Table 6.** CRED-RA band numbers in different Mn concentrations and polymorphism %.

| Primers | Control | | Total Band Number | | | | | | | | Total polymorphic band number | | | | | | | | % Polymorphism | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5 mM | | 10 mM | | 20 mM | | 40 mM | | 5 mM | | 10 mM | | 20 mM | | 40 mM | | 5 mM | | 10 mM | | 20 mM | | 40 mM | |
| | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M | H | M |
| **OPH-17** | 5 | 7 | 7 | 7 | 8 | 8 | 10 | 10 | 7 | 7 | 2 | 0 | 7 | 2 | 7 | 7 | 2 | 3 | 28.5 | 0 | 87.5 | 25 | 70 | 70 | 28.5 | 42.8 |
| **OPA-2** | 4 | 4 | 1 | 1 | 3 | 3 | 3 | 3 | 1 | 1 | 3 | 3 | 5 | 5 | 3 | 5 | 3 | 3 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **OPA-1** | 5 | 4 | 2 | 0 | 0 | 0 | 2 | 0 | 2 | 2 | 5 | 4 | 5 | 4 | 5 | 4 | 6 | 6 | 100 | 0 | 0 | 0 | 100 | 0 | 100 | 100 |
| **OPH-18** | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **OPY-15** | 10 | 10 | 8 | 11 | 9 | 10 | 10 | 10 | 11 | 11 | 8 | 8 | 7 | 7 | 10 | 9 | 9 | 10 | 100 | 72.7 | 77.7 | 70 | 100 | 90 | 81.8 | 90.9 |
| **OPW-1** | 12 | 11 | 0 | 0 | 8 | 8 | 8 | 8 | 2 | 2 | 12 | 11 | 8 | 7 | 10 | 7 | 10 | 9 | 0 | 0 | 100 | 87.5 | 100 | 87.5 | 100 | 100 |
| **OPB-8** | 11 | 11 | 6 | 6 | 6 | 4 | 8 | 5 | 9 | 7 | 8 | 9 | 6 | 7 | 7 | 6 | 8 | 8 | 100 | 100 | 100 | 100 | 87.5 | 100 | 88.8 | 100 |
| **OPY-6** | 5 | 5 | 2 | 2 | 6 | 6 | 2 | 6 | 2 | 6 | 3 | 4 | 1 | 6 | 3 | 7 | 3 | 7 | 100 | 100 | 16.6 | 100 | 100 | 100 | 100 | 100 |
| **Average** | **7** | **7** | **4** | **4** | **5** | **5** | **6** | **7** | **5** | **5** | **5** | **4** | **5** | **5** | **6** | **7** | **5** | **6** | **66** | **46.5** | **60.2** | **60.3** | **82.1** | **68.4** | **74.8** | **79.2** |

**Phytohormone levels**

The phytohormone level changes of maize seedlings by the effect of copper and manganese were given in Table 7-8. The ABA content of the seedlings increases with the increasing heavy metal concentration. On the contrary, a decrease in the Z, GA, IAA content is observed with an increase in the Mn and Cu concentrations in comparison with control ($P \leq 0.01$). The above-mentioned findings are also clearly dose-dependent.

**Table 7.** Phytohormone content of seedlings at variable Cu concentration

| | Phytohormones (µg/mL) | | | |
|---|---|---|---|---|
| | **Gibberellic acid** | **Zeatin** | **Indole acetic acid** | **Abscisic acid** |
| **Control** | 363.00±1.15$^{a}$ | 2.4±0.11$^{a.b}$ | 20.09±0.57$^{a}$ | 6.5±0.11$^{a}$ |
| **5** | 359.83±0.02$^{b}$ | 2.6±0.05$^{a}$ | 19.80±0.02$^{a}$ | 10.39±0.02$^{b}$ |
| **10** | 356.33±0.82$^{c}$ | 2.4±0.11$^{a.b}$ | 19.50±0.28$^{a.b}$ | 14.50±0.11$^{d}$ |
| **20** | 349.03±0.01$^{d}$ | 2.4±0.05$^{a.b}$ | 19.20±0.28$^{a.b}$ | 10.69±0.51$^{c}$ |
| **40** | 348.26±0.02$^{d}$ | 2.1±0.17$^{b}$ | 18.50±0.23$^{b}$ | 16.90±0.57$^{e}$ |

Means of each column followed by the same letter do not differ considerably at the level of $p < 0.01$.

**Table 8.** Phytohormone content of seedlings at variable Mn concentration

| | Phytohormones (µg/mL) | | | |
|---|---|---|---|---|
| | **Gibberellic acid** | **Zeatin** | **İndole acetic acid** | **Abscisic acid** |
| **Control** | 363.00±1.54$^{a}$ | 2.4±0.11$^{a}$ | 20.09±0.57$^{a}$ | 6.5±0.11$^{a}$ |
| **5** | 341.13±0.05$^{b}$ | 2.2±0.11$^{a}$ | 19.40±0.02$^{a.b}$ | 9.4±0.23$^{b}$ |
| **10** | 324.46±0.02$^{c}$ | 2.2±0.11$^{a}$ | 18.80±0.05$^{b.c}$ | 13.69±0.05$^{c}$ |
| **20** | 324.40±0.23$^{c}$ | 2.0±0.17$^{a.b}$ | 18.10±0.01$^{c}$ | 14.10±0.57$^{c}$ |
| **40** | 316.10±0.02$^{d}$ | 1.6±0.17$^{b}$ | 18.07±0.04$^{c}$ | 17.10±0.05$^{d}$ |

Means of each column followed by the same letter do not differ considerably at the level of $p < 0.01$

## DISCUSSION

The environmental contamination of heavy metals affects plants adversely. Particularly the high concentrations of heavy metal ions such as Mn and $Cu^{2+}$, which are beneficial elements for plants at normal levels, inhibit the growth of plants and compromise many biochemical processes (Dimitrova and Ivanova, 2003). In this study, the effects of $Cu^{2+}$and Mn on the mitotic index, RAPD band profile changes, amount of total protein and phytohormones levels were investigated based on dose. In previous studies, $Cu^{2+}$ and Mn originated changes of the mitotic index and mitotic abnormalities (chromosomal stickiness, C-mitosis, anaphase and telophase bridges including one or more chromosome micronuclei and fragmentation) in different plants were investigated (Yıldız et al., 2009). The underlying reason for the restriction of the mitotic activity and the creation of a number of aberrations induced by Mn and $Cu^{2+}$ is the effect of Mn and $Cu^{2+}$ on DNA synthesis and enzymatic inhibitors of the enzyme system needed for the chain reaction of DNA synthesis. Hence, RAPD was used to evaluate the genotoxicity effects of Mn and $Cu^{2+}$ in this study.

The results show that all of the Mn and Cu concentrations cause changes in the RAPD profile. These changes may indicate a variety of DNA damages, including single- and double-strand breaks, point and deletion mutations, DNA-protein cross-links, 8-hydroxyguanine, even bulky adducts, homologous recombination, and complex chromosomal rearrangements. Metal binding mostly takes place on N7 and O6 of guanine, the N7 and N1 of adenine bases and the N3 of pyrimidines (Anastassopoulou, 2003). Alterations in the RAPD profiles are possibly induced by the alterations in oligonucleotide priming-sites and/or interactions between DNA polymerase and damaged DNA (Nelson *et al*., 1996). It has been indicated that conformational B-to-Z conversion in some DNA fragments can be caused by the

transition from weak complex DNA Cu (II) to strong complex DNA Cu (I) in naked double-stranded DNA (Prützs et al., 1990). Thus, the above-mentioned structural modifications possibly have a considerable impact on the kinetics PCR events. Similar results were stated in studies conducted previously (Atienzar et al. 2001; Aydin et al., 2012; Korpe and Aras, 2011).

The GTS value, which is considered as an indicator of RAPD profile alterations, represents a qualitative method that is utilized to determine the genotoxic impacts of heavy metals and is regarded to have higher sensitivity compared to growth parameters, including dry weight, root length, and total soluble protein content. In the current study, a decrease has been observed in genomic template stability with an increase in Mn and $Cu^{2+}$ concentrations. Previous studies have shown that reduced GTS values induce the effects of genotoxins Mn and $Cu^{2+}$ (Aydin et al., 2012; Vyas et al., 2009). Besides genetic damage through oxidative as well as nonoxidative (DNA adducts) mechanisms, metals may also lead to considerable alterations in DNA methylation and histone modifications which provokes gene expression reactivation or epigenetic silencing. A number of studies conducted recently have demonstrated that some heavy metals, including Pb, Cd, Ni, Co, and Zn, lead to alterations in DNA methylation. Kang *et al*. (2005) demonstrated that the high concentration of $Cu^{2+}$ caused a decrease in global histone acetylation and HAT activity. Mylonas et al. (2005) reported that $Cu^{2+}$ directly binds to histones by utilizing short sequence models from H2A, H3, and H4. Cu that binds to the C-terminal peptide of H2B (H2B94-125) may step in the ubiquitination of Lys120 that has been related to gene silencing (Zavitsanos et al., 2011).

Although $Cu^{2+}$ is known as a molecule responsible for histone modification, to date no study has demonstrated the impacts of Mn and Cu on DNA methylation and histone modifications. In this study, it is shown for the first time that Mn and $Cu^{2+}$ cause DNA hypermethylation. Besides, the adverse effects of Mn and $Cu^{2+}$ on corn seedlings are investigated by using protein analysis. The results showed that Mn and $Cu^{2+}$ decreases total protein and change the profiles protein electrophoresis. The genetic and chromosomal rearrangements, point mutations, deletions, DNA insertions or methylation may bring about the mentioned changes. These findings are consistent with DNA methylation, mitotic index, and RAPD results. In this research, the effects of Mn and $Cu^{2+}$ on phytohormone levels were observed. The results showed that Mn and $Cu^{2+}$ cause a reduction in GA, IAA and Z and an increase in ABA in seedlings. GA, Z, and IAA are synthesized under unstressed conditions and their impacts induce growth events in plants. On the contrary, ABA represents a growth inhibitor, which is an important "stress hormone" in plants. Thus, an increase is observed in the synthesis of ABA in leaves, particularly under stressful conditions, and ABA moves to every part of the plant for the purpose of inhibiting growth and protecting the plant against biotic and abiotic stress. It has been stated in the studies conducted that ABA stimulates the activities of alternative respiration pathways that are related to the prevention of the creation of reactive oxygen species in plants (Ozfidan et al., 2013). Additionally, it is a known fact that ABA induces the synthesis of certain types of new proteins (Chinnusamy et al., 2008; Taspinar et al., 2009). These results indicate that ABA can take part in changing the gene expression mechanism by the epigenetic control in plants.

The above-mentioned results are consistent with DNA methylation and protein analysis results. However, future research is required in order to enlighten the possible genotoxic and epigenetic mechanism of $Cu^{2+}$, Mn and how ABA is affected by DNA methylation in plants.

## CONCLUSIONS

The results indicated that an increase in the $MnSO_4$ and $CuSO_4$ concentrations decreases the mitotic soluble protein levels, index, GTS, while increases the CRED-RA changes (DNA hypermethylation) and RAPD profile alterations (DNA damage). Additionally, HPLC analyses revealed that $CuSO_4$ and $MnSO_4$ contamination reduced the levels of growth-promoting hormones, such as zeatin, gibberellic acid and indole acetic acid, whereas it increased the abscisic acid level. Finally, it was concluded that an increase in heavy metals (copper and manganese) causes an increase in epigenetic and genotoxic effects.

## ACKNOWLEDGEMENTS

The current study was supported by BAP (Ataturk University). We thanks all of the participants in the study.

## REFERENCES

Anastassopoulou, J. 2003. Metal–DNA interactions. J. Mol. Struct., 651: 19-26.

Atha, D.H., Wang, H., Petersen, E.J., Cleveland, D., Holbrook, R.D., Jaruga, P., Dizdaroglu, M., Xing, B., Nelson, B.C. 2012. Copper oxide nanoparticle mediated DNA damage in terrestrial plant models. Environ. Sci. Technol., 46: 1819–1827.

Aydin, S.S., Gokce, E., Buyuk, I., Aras, S. 2012. Characterization of stress induced by copper and zinc on cucumber (*Cucumis sativus L.*) seedlings by means of molecular and population parameters. Mutat. Res., 746: 49-55.

Brega, S.M., Vassilieff, I., Almeida, A., Mercadante, A., Bissacot, D., Cury, P.R., Freire-Maia, D.V. 1998. Clinical cytogenetic and toxicological studies in rural workers exposed to pesticides in Botucatu, Sao Paulo, Brazil. Public Health Rep., 14: 109-115.

Cakmak, I., Marschner, H., Bangert, F. 1989. Effect of zinc nutritional status on growth, protein metabolism and levels of indole-3-acetic acid and other phytohormones in bean (*Phaseolus vulgaris* L.). J. Exp. Bot., 40: 404-412.

Chinnusamy, V., Gong, Z., Zhu, J.K. 2008. Abscisic acid-mediated epigenetic processes in plant development and stress responses. J. Integr. Plant Biol., 50: 1187-1195.

Dimitrova, I., Ivanova, E. 2003. Effect of heavy metal soil pollution on some morphological and cytogenetical characteristics of flax (*Linum usitatissum* L.). J. Balkan Ecol., 4: 212-218.

Enan, M.R. 2006. Application of random amplified polymorphic DNA (RAPD) to detect the genotoxic effect of heavy metals. Biotechnol. Appl. Biochem., 43: 147–154.

Gerber, G.B., Leonar, A., Hantson, P., 2002. Carcinogenicity, mutagenicity and teratogenicity of manganese compounds. Crit. Rev. Oncol. Hematol. 42: 25-34.

Girotti, A.W. 2001. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J. Photochem. Photobiol. Biol., 63: 103-113.

Kang, J., Chen, J., Shi, Y., Jia, J., Wang, Z. 2005. Histone hypoacetylation is involved in 1, 10-phenanthroline-$Cu^{2+}$-induced human hepatoma cell apoptosis. J. Biol. Inorg. Chem., 10: 190-198.

Korpe, D.A., Aras, S. 2011. Evaluation of copper-induced stress on eggplant (*Solanum melongena* L.) seedlings at the molecular and population levels by use of various biomarkers. Mutat. Res. 719: 29–34.

Kuraishi, S,, Tasaki, K., Sakurai, N., Sadatoku, K. 1991. Changes in levels of cytokinins in etiolated squash seedlings after illumination. Plant Cell Physiol., 32: 585-591.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of head of Bacteriophage-T4. Nature, 227: 680-685.

Li, G., Quiros, C.F. 2001. Sequence-Related Amplified Polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in Brassica. Theor. Appl. Genet., 103: 455-461.

Lima, P.D., Leite, D.S., Vasconcellos, M.C., Cavalcanti, B.C., Santos, R.A., Costa-Lotufo, L.V., Pessoa, C., Moraes, M.O., Burbano, R.R. 2007. Genotoxic effects of aluminum chloride in cultured human lymphocytes treated in different phases of cell cycle. Food Chem. Toxicol. 45: 1154-1159.

Mediouni, C., Benzarti, O., Tray, B., Mohamed, H.G., Jemal, F. 2006. Cadmium and copper toxicity for tomato seedlings. Agron. Sustain. Dev., 26: 227–232.

Michailova, P., Petrova, N., Ilkova, J., Bovero, S., Brunetti, S., White, K., Sella, G. 2006. Genotoxic effect of copper on salivary gland polytene chromosomes of *Chironomus riparius* Meigen 1804 (*Diptera, Chironomidae*). Environ. Pollut., 144: 647-654.

Mosa, K.A., El-Naggar, M., Ramamoorthy, K., Alawadhi, H., Elnaggar, A., Wartanian, S., Ibrahim, E., Hani, H. 2018. Copper nanoparticles ınduced genotoxicity, oxidative stress, and changes in superoxide dismutase (SOD) gene expression in cucumber (Cucumis sativus) plants. Front. Plant. Sci., 9: 872.

Muccifora, S. 2007. Effects of copper on spore germination, growth and ultrastructure of *Polypodium cambricum* L. gametophytes. Environ. Pollut., 153: 369–375.

Nelson, J.R., Lawrence, C.W., Hinkle, D.C. 1996. Thymine–thymine dimer bypass by yeast DNA-polymerase-zeta. Science, 272: 1646-1649.

Nepravishta, R., Bellomaria, A., Polizio, F., Paci, M., Melino, S. 2010. Reticulon RTN1-C(CT) peptide: a potential nuclease and inhibitor of histone deacetylase enzymes. Biochemistry, 49: 252-258.

Ozfidan, C., Turkan, I., Sekmen, A.H., Seckin, B. 2013. Time course analysis of ABA and non-ionic osmotic stress-induced changes in water status, chlorophyll flourescens and osmotic adjustment in *Arabidopsis thaliana* wild-type (Colombia) and ABA deficient mutant (aba2). Environ. Exp. Bot., 86: 44-51.

Pittman, J.K. 2005. Managing the manganese: molecular mechanisms of manganese transport and homeostasis. New Phytol. 167: 733-742.

Prütz, W.A., Butler, J., Land, E.J. 1990. Interaction of copper (I) with nucleic acids. Int. J. Radiat. Biol., 58: 215-234.

Roth, J.A., Garrick, M.D. 2003. Iron interactions and other biological reactions mediating the physiological and toxic actions of manganese. Biochem. Pharmacol., 66: 1-13.

Shangguan, L., Fang, X., Chen, L., Cui, L., Fang, J., 2018. Genome-wide analysis of autophagy-related genes (ARGs) in grapevine and plant tolerance to copper stress. Planta, 247:1449-1463.

Song, M.O., Li, J., Freedman, J.H. 2009. Physiological and toxicological transcriptome changes in HepG2 cells exposed to copper. Physiol. Genom., 38: 386-401.

Tan, J., He, S., Yan, S., Li, S., Li, H., Zhang, H., Zhao, L., Li, L. 2014. Exogenous EDDS modifies copper-induced various toxic responses in rice. Protoplasma, 251:1213-1221.

Taspinar, M.S., Agar, G., Alpsoy, L., Yildirim, N., Bozari, S. 2011. The protective role of zinc and calcium in *Vicia faba* seedlings subjected to cadmium stress. Toxicol. Ind. Health, 27: 73-80.

Turker, M., Battal, P., Agar, G., Gulluce, M., Sahin, F., Erez, M.E, Yildirim, N. 2008. Allelopathic effects of plants extracts on physiological and cytological processes during maize seed germination. Allelopathy J., 21: 273-286.

Unyayar, S., Çelik, A., Çekiç, F.Ö., Gözel, A. 2006. Cadmium-induced genotoxicity, cytotoxicity and lipid peroxidation in *Allium sativum* and *Vicia faba*. Mutagenesis, 21: 77-81.

Yıldız, M., Cigerci, I.H., Konuk, M., Fidan, A.F., Terzi, H. 2009. Determination of genotoxic effects of copper sulphate and cobalt chloride in *Allium cepa* root cells by chromosome aberration and comet assays. Chemosphere, 75: 934-938.

Yigider, E., Taspinar, M.S., Sigmaz, B., Aydin, M., Agar, G. 2016. Humic acids protective activity against manganese induced LTR (long terminal repeat) retrotransposon polymorphism and genomic instability effects in Zea mays. Plant Gene, 6:13-17.

Zavitsanos, K., Nunes, A.M., Malandrinos, G., Hadjiliadis, N. 2011. Copper effective binding with 32–62 and 94–125 peptide fragments of histone H2B. J. Inorg. Biochem., 105: 102-110.

Zhou, C.P., Qi, Y.P., You, X., Yang, LT., Guo, P., Ye, X., Zhou, X.X., Ke, F.J., Chen, L.S. 2013. Leaf cDNA-AFLP analysis of two citrus species differing in manganese tolerance in response to long-term manganese-toxicity. BMC Genomics, 14:621.