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Original Research

Effects of Lead Sulfate on Genetic and Epigenetic Changes, and Endogenous Hormone Levels in Corn (*Zea mays* L.)

Filiz Aygun Erturk[1], Guleray Agar[2]*, Esra Arslan[2], Gokce Nardemir[3], Murat Aydin[4], M. Sinan Taspinar[5]

[1]Department of Molecular Biology, Faculty of Science, Avrasya University, Trabzon, Turkey
[2]Department of Biology, Faculty of Science, Ataturk University, Erzurum 25240, Turkey
[3]Department of Molecular Biology and Genetics, Faculty of Science, Agri İbrahim Cecen University, Agri, Turkey
[4]Department of Field Crops, Faculty of Agriculture, Ataturk University, Erzurum, Turkey
[5]Department of Agricultural Biotechnology, Faculty of Agriculture, Ataturk University, Erzurum, Turkey

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Abstract

In this research we aimed to evaluate DNA damage levels, DNA methylation, and protein and pytohormone level changes in corn seedlings (*Zea mays* L.) exposed to 5, 10, 20, and 40 mM concentrations of lead sulfate solution ($PbSO_4$). The results showed that all concentrations of the test material caused a decreasing mitotic index, genomic template stability (GTS), and soluble protein levels, but increased RAPDs profile changes (DNA damage) and DNA hypermethylation. Moreover, in the HPLC (high-pressure liquid chromatography) analyses, it was also observed that Pb contamination caused a decrease in the growth-promoting hormones including gibberellic acid (GA), zeatin (ZA), and indole acetic acid (IAA) levels, in contrast to increasing the absisic acid (ABA) level. The results of this experiment have clearly shown that Pb has a significant impact on the epigenetic mechanisms as well as its genotoxic effects. Some of phytohormone decreases (GA, ZA and IAA) and especially increasing ABA levels under Pb stress may be a part of the defense system against stress.

Keywords: lead sulfate, DNA damage, DNA methylation, total soluble protein level, phytohormones

Introduction

Heavy metal contamination is considered one of the most important problems for the environment and human health because heavy metals are pollutants that cannot be degraded to nontoxic forms [1]. Among the toxic metal contaminants, lead (Pb) pollution can occur from mining and smelting activities, Pb-containing paints, gasoline, and explosives, as well as from the disposal of municipal sewage sludges enriched with Pb [2, 3]. When the soil is lead-contaminated, plants take up lead via their root systems [4]. Pb is known as one of the nonessential metals for plants, and there are a number of reports on its toxic effects such as decreasing on germination rate, growth, and dry mass of roots and shoots [5] reduction in cell division [6], and inhibition of photosynthetic activity besides its genotoxic [3] and epigenetic effects [7, 8]. The genotoxic effect of Pb on plants has been shown by using various genotoxic tests such as comet, micronucleus, or chromosome aberration assays in several studies [6, 9]. However, little is known about the effects of lead on DNA methylation changes in plants. Few papers have shown that DNA hyper-

*e-mail: gagar@atauni.edu.tr

metylation in wheat can occur via Pb treatment [7, 8]. Pb effect on DNA methylation still needs more research. As an epigenetic mechanism, DNA methylation plays an important role in regulating gene expression in plants and in regulating the activity of transposable elements [10] and in paramutation [11-13]. DNA methylation in specific gene expression patterns under epigenetic control are reversible and may show transgenerational inheritance [14, 15]. Epigenetic control for plant adaptation under stress is vital and necessary. DNA methylation exists in virtually all organisms. In eukaryotes (especially methylation) it occurs predominantly on the cytosine residues in symmetrical sequences, such as CpG and CpNpG. Under normal conditions, the ratio of methylated to total cytosines varies from 20% to 30% in plants [16]. Cytosine methylation is numerous polymorphisms and DNA sequence polymorphisms between different plant genotypes [17, 18]. Additionally, plant growth regulators response protect plants against DNA methylation and oxidative stress generated by different stress. Several recent studies have shown that their levels change significantly in plants under a variety of stress conditions including water, salt, temperature, heavy metal, and viral infection [4, 19, 20]. Thus, in the current study we aimed to determine the genotoxic potential of lead and its epigenetic effects on maize seedlings by using the RAPD, mitotic index, and CRED-RA techniques. Furthermore, the relations among genetic-epigenetic and physiological parameters were also determined by measuring the changes in total soluble protein content and phytohormones levels.

Materials and Methods

Plant Material, Inclusion of Lead Sulfate and Germination

Zea mays L. seeds were obtained from the Department of Field Crops, Faculty of Agriculture, Ataturk University (Turkey), and were used in this study as plant material. Seeds were surface-sterilized with 0.5% sodium hypochlorite solution for 10 min and then washed with sterilized water three times. 15 seeds in four replications were placed in 15 cm diameter sterile petri dish with two layers of filter paper and then treated with different lead sulfate ($PbSO_4$) concentrations (5, 10, 20, 40 mM). Tap water was used as control group. The seeds were allowed to germinate at 25±1ºC and 50% humidity in a 16 h photoperiod.

Cytological Analysis

When root length reached approximately 1.5-2 cm, ten root tips (2-3 mm) in each treatment group were excised and transferred to colchicine solution (0.15%) for 2 h and fixed in farmer's solution (3:1; 95% ethanol:glacial acetic acid) for 24 h at 4ºC. Root tips were then hydrolyzed with 1 N HCL for 15 min at 60ºC. For the observation of cell division, root tips were stained with 2% aceto-orcein staining for 2-3 h and then squashed in 45% glacial acetic acid. To determine the effect of lead on mitotic index (MI), 3 slides were examined for each treatment and were counted 1500 cell. MI was calculated as the ratio of the number of dividing cells to the total number of cells.

Total Protein Content and SDS-PAGE

Quantitative estimation of total protein in the ground tissue of roots, shoots, and leaf systems of *Z. mays* L. seedlings were estimated according to [21]. For protein analysis with SDS-PAGE, protein isolation and purification were carried out as described by [22, 23]. Polypeptides of proteins were separated in 12.5% SDS-polyacrylamide gel (PAGE) at 110 V [24]. The gel was stained with Coomassie brilliant blue.

Phytohormones Analysis

Cytokinins (t-Zeatin), indole-3-acetic acide (IAA), gibberellic acid (GA), and abscisic acid were analyzed as described by [21, 25-27], with minor modifications.

Genomic DNA Isolation, RAPD, and CRED-RA Analysis

Leaves were randomly collected from 10 plants for each treatment and were stored at -80ºC. DNA isolation and purification were carried out as described by [29]. 16 primers were used in RAPD PCR reactions. Sequences (5'→3') of primers are CAGCACCCAC (OPA-13), CACTCTCCTC (OPH-17), TGCCGAGCTG (OPA-2), CAGGCCCTTC (OPA-1), GGTCCCTGAC (OPA-6), ACCAGGTTGG (OPH-14), GAATCGGCCA (OPH-18), GTGGCATCTC (OPY-1), AAGGCTCACC (OPY-6), AGGCAGAGCA (OPY-8), AGTCGCCCTT (OPY-15), GGGCCAATGT (OPY-16), CTCAGTGTCC (OPW-1), GTCCACACGG (OPB-8), CTGGACGTCA (OPW-7), and GGCGGATAAG (OPW-5).

RAPD-PCR reaction contained 25 ng genomic DNA, 400 µM dNTP, 10 pmol primer, 2.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase, and 1X PCR buffer (10X) in a total volume of 20 µL. DNA amplification was carried out in a thermocycler programmed as follows: 1 cycle of 5 min at 95ºC, 42 cycles of (1 min at 94ºC, 1 min at 36ºC, and 2 min at 72ºC), 1 cycle of 15 min at 72ºC. PCR products were analyzed using 1% agarose gel electrophoresis and visualized with ethidium bromide staining. The sizes of the fragments were estimated based on a DNA ladder of 100 bp.

A genomic DNA sample from each treatment was separately digested with HpaII and MspI endonucleases (which cut the sequence 5'-C/CGG-3' with different sensitivity to cytosine methylation; MspI cuts if the inner C is methylated, whereas HpaII cannot cleave in the presence of methyl groups). After checking digestion on agarose gel, 1µl of each digestion product were amplified with random primers (OPA-1, OPA-2, OPH-18, OPY-6, OPY-15, OPW-1, OPB-8, and OPH-17). Amplification and visualization conditions for CRED-RA are the same as described for RAPD analysis.

Table 1. Pytohormones content, total soluble protein content, and mitotic index in maize exposed to lead.

Lead (pb) Dose (mM)	Total soluble protein (mg/ml)	Mitotic Index (MI)	Pytohormones (µg/mL)			
			GA	Zeatin	IAA	ABA
0 (control)	3.307^{ab}*	9.0^{a}*	363.00±1.54^{a}*	2.4±0.11^{a}*	20.09±0.57^{a}*	6.5±0.11^{a}*
5	3.370^{a}	4.1^{b}	284.46±0.49^{b}	2.2±0.17^{ab}	19.60±0.05^{a}	8.8±0.17^{b}
10	3.337^{a}	3.6^{bc}	278.70±0.40^{c}	2.0±0.23^{abc}	18.70±0.11^{b}	12.4±0.11^{c}
20	3.240^{bc}	2.6^{bc}	265.73±0.33^{d}	1.7±0.11^{bc}	18.60±0.23^{b}	13.9±0.23^{d}
40	3.213^{c}	2.0^{c}	210.23±0.01^{e}	1.5±0.23^{c}	16.20±0.02^{c}	17.2±0.11^{e}

*The difference between values with the same letter in each column is not significant at the level 0.05

RAPD patterns were evaluated using the Total Lab TL120 computer software. Genomic template stability (GTS, %) was calculated as follows:

$$GTS = 100 - (100 \times a/n)$$

...where *a* in formula is the average number of polymorphic bands detected in each treated sample, and *n* is the number of total bands in the control. Polymorphisms in RAPD profiles included the disappearance of a normal band and appearance of a new band compared with the control. The average was calculated for each experimental group. To compare the sensitivity of each parameter, changes in these values were calculated as a percentage of their control (set to 100%). The average number of polymorphisms (%) was calculated for each dose to realize CRED-RA analysis. To calculate the number of polymorphisms (%), the following formula was used $100 \times a/n$.

Statistical Analysis

Mitotic index, total protein content, and phytohormone content were tested using SPSS statistical according to complete random design, and mean separation was performed by Duncan's multiple test at 0.05 level.

Results

Changes of Mitotic Index (MI) Value in Maize Seedlings Subjected to Various Pb Concentrations

Pb decreased significantly the MI of *Zea mays* root meristems when compared with control as seen in Table 1, and the mitotic index decreased progressively with increasing Pb concentration. Such a decrease was found to be statistically significant ($P<0.05$). All concentrations of Pb used in this study increased the frequencies of abnormalities such as C-mitosis, chromosomal stickiness, and anaphase and telophase bridges involving one or more chromosome micronuclei and fragments.

Total Protein Content

All concentrations of Pb affected the total proteins, and the protein content decreased with increasing Pb concentrations at a statistically significant level ($P<0.05$) (Table 1). Also, the proteins obtained from the seedlings of maize treated with different concentrations of Pb were investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that little changes are available in the total protein band pattern. These changes are only characterized by variation in band intensity (Fig. 1).

RAPD Analysis

In total, 40 10-mer priming oligonucleotides were used in RAPD and 16 of them were selected for their stable results and produced the total number of 512 bands. Table 2 represents the summary of all polymorphic bands in RAPD profile and Fig. 2 presents all RAPD bands of the select 4 primers. Compared with the PCR products obtained from the control DNA, Pb treatments resulted in apparent changes in RAPD patterns. These changes are characterized by variation in band intensity, loss of normal bands, or appearance of new bands (Table 2 and Fig. 2).

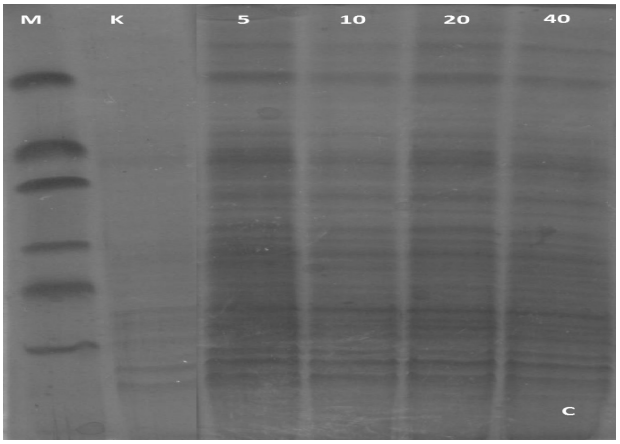

Fig. 1. Comparison of total soluble protein levels in maize seedlings exposed to different concentrations of Pb.

Table 2. The number of bands in control and disappearance (−), and/or appearance (+) of DNA bands with molecular sizes (base pair, bp) for all the primers in the roots of Pb treated maize seedlings.

Primers	Control	+/-	Lead (Pb) (mM)			
			5	10	20	40
OPA-13	7	+	263	629, 584, 516, 266	516, 263	629, 516, 266
		-	424			228
OPH-17	12	+	--	--	--	--
		-	--	--	--	--
OPA-2	8	+	646	1063, 646, 178	646, 178	1063, 646, 178
		-	718, 392, 314	718	718, 392	718, 392
OPA-1	11	+	--	--	--	--
		-	382	--	--	--
OPA-6	9	+	765, 457	765, 747, 666, 457	765, 747, 666, 457	765, 747, 666, 457
		-	710, 639, 419	639, 419	639, 419	639, 419
OPH-14	5	+	367, 231	231	231	231
		-	427, 190	427, 400, 263, 190	190, 263, 427, 400	190, 263, 427, 400
OPH-18	5	+	794, 342	342	794, 342	794, 342
		-	765, 732, 506, 279, 188	765, 732, 506, 279, 188	765, 732, 506, 279, 188	765, 732, 506, 279, 188
OPY-6	10	+	797, 622, 548	797, 622, 548	797, 622, 548	797, 622, 548
		-	750	750	750	750
OPY-1	7	+	329		329	
		-	643	643	643	643
OPY-8	8	+	603	--	603	603, 434
		-	770, 720	932, 832, 770, 720, 543, 334	932, 832, 770, 543, 334	932, 832, 770, 543, 334
OPY-15	12	+	302	302	302	617, 325,
		-	--	--	260	260
OPY-16	10	+	--	--	--	--
		-	276, 255	255	504, 276, 255	297, 255
OPW-1	11	+	432, 328	551	--	--
		-	--	--	--	--
OPB-8	6	+	--	--	--	--
		-	--	--	--	--
OPW-7	8	+	409	--	409	542
		-	372	372	372	372
OPW-5	8	+	521, 333	521, 407, 386, 333	521, 386, 333	521, 386, 333
		-	--	--	--	--
Total band	137		41	44	46	49
Polymorphism			29.92	32.11	33.58	35.77
GTS value			70.08	67.89	66.42	64.23

Table 3. CRED-RA band amounts in various Pb concentrations and polymorphism %.

Primers	H/M	Lead (Pb) (mM)			
		5	10	20	40
OPA-1	H	0	33.3	33.3	44.4
	M	50	25	0	50
OPA-2	H	37.5	62.5	25	42.8
	M	22.2	42.8	40	50
OPH-18	H	42.8	40	40	44.4
	M	37.5	44.4	40	44.4
OPY-6	H	40	60	100	100
	M	50	25	100	75
OPY-15	H	28.5	33.3	42.8	37.5
	M	25	33.3	33.3	55.5
OPW-1	H	11.1	25	100	100
	M	42.8	100	37.5	37.5
OPB-8	H	40	55.5	60	42.8
	M	33.3	50	62.5	83.3
OPH-17	H	50	57.1	100	37.5
	M	66.6	14.2	66.6	37.5
Avarage	H	31.2	45.8	62.6	56.1
	M	40.9	41.8	47.4	54.1

Some primers resulted in alteration of a few amplification products (OPW-5), while others (OPY-8) gave more complicated patterns of gains or losses. After Pb treatment, in total 137 normal RAPD bands from the control disappeared. In addition, 86 new bands appeared in all concentrations of Pb treatment. Polymorphism value was calculated as 29.92, 32.11, 33.58, and 35.77% for 5, 10, 20, and 40mM doses, respectively. Based on these alterations, GTS values were calculated as 70.08, 67.89, 66.42, and 64.23%, respectively (Table 2). It was observed that GTS values decreased with increased Pb concentrations.

CRED-RA Analysis

For CRED-RA analysis only eight oligonucleotide (OPA-1, OPA-2, OPW-1, OPY-6, OPB-8, OPY-15, OPH-17, OPH-18) products used for RAPD analyzing gave specific and stable bands (Fig. 3). Compared to the PCR prod-

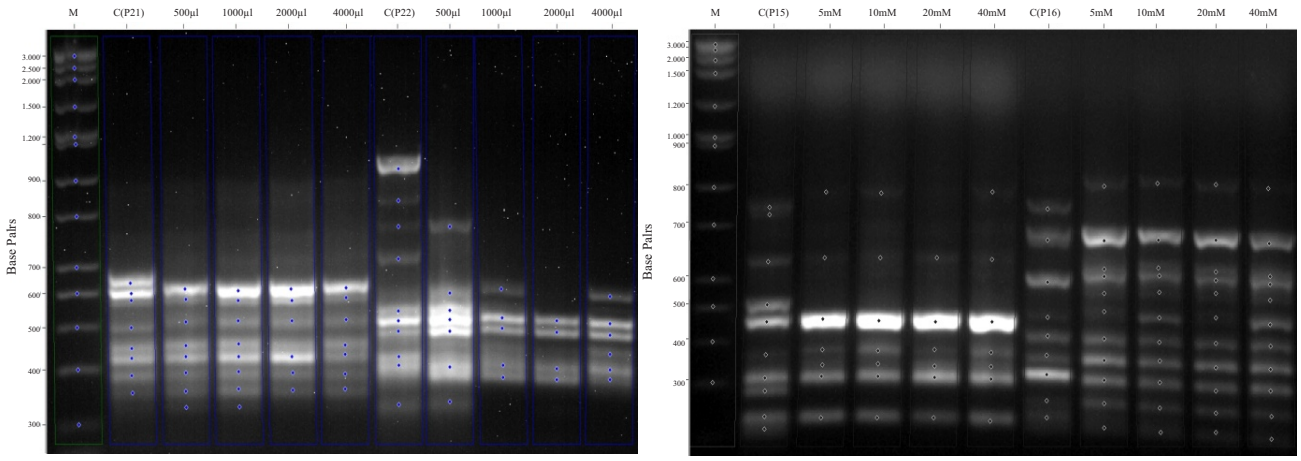

Fig. 2. Randomly amplified polymorphic DNA profiles of Pb-exposed and nonexposed *Zea mays* seedlings with primers of OPY-6, OPY-8, OPH-18, and OPY-1.

ucts obtained from the control DNA, dose-related DNA hypermethylation was detected. This ranged from a net hypermethylation of 40.9% in plants grown in the presence of the 5mM of $PbSO_4$ to 54.1% in plants grown with the highest concentration methylation increase (Table 3).

Changes of Pytohormone levels in Maize Seedling Subjected to Various Pb Concentrations

The alterations of pytohormone level maize seedlings are presented in Table 1. ABA content in seedlings increased noticeably ($P \leq 0.05$) compared with the control plant, let alone the increase of Pb concentrations following 7 days as seen in Table 1. On the other hand, Z, GA, and IAA content decreased with the increased concentrations of Pb compared with the control seedlings ($P \leq 0.05$). These results were also in a clear dose-dependent manner.

Discussion

Some research has determined that contamination with Pb reduces MI activity and causes mitosis abnormalities such as C-mitosis, chromosomal stickiness, anaphase, and telophase bridges involving one or more chromosome micronuclei and fragments in plant species [6, 9]. The inhibition of mitotic activity and production of several aberrations caused by Pb treatment have been explained as being the effect of the treatment on DNA synthesis and its action as an enzymatic inhibitor of the enzyme system required for the chain reaction of DNA synthesis. Our results revealed that Pb treatment causes an increase in chromosomal aberration frequencies and a decrease in MI levels. Besides, our study used RAPD for evaluation of genotoxic effects of Pb. Recently, DNA fingerprinting has offered a useful biomarker assay in genotoxicity [30]. It is suggested that after proper optimization, especially the RAPD as a reliable, sensitive, and reproducible assay, has the potential to detect a wide range of DNA damage (e.g. DNA adducts, DNA breakage) as well as mutations (point mutations and large rearrangements) and therefore can be applied to genotoxicity and carcinogenesis studies [31]. Our results showed that all concentrations of Pb caused RAPD profile changes. RAPD profile changes can induce a range of DNA damage such as single and double-strand breaks, DNA-protein cross-links, point and deletion mutations, 8-hydroxyguanine, even bulky adducts, complex chromosomal rearrangements and homologous recombinations [32]. In a previous study, similar results have also been obtained [3]. A decreasing GTS value, regarded as an indication of the RAPD profile changes, has been observed with increasing Pb concentrations. Previous studies have shown GTS value decreasing the induced effect of genotoxins [3, 33-35].

In addition to genetic damage via both oxidative and nonoxidative (DNA adducts) mechanisms, metals can also cause significant changes in DNA methylation and histone modifications, leading to epigenetic silencing or reactivation of gene expression. Recently, several studies have shown that the same heavy metals such as Cd, Pb, Co, Ni, and Zn cause changes in DNA methylation. Ge et al. have shown that Pb contamination caused DNA hypermethylation in rice and wheat [7]. These findings are in accordance with our results. Besides, higher concentrations of Pb have demonstrated that it causes DNA hypomethylation by decreasing DNA methyltransferases, DNMT1, and DNMT3A, reduction in post translationally modified histones such as H3K9ac, H4K8ac, H4K12ac, and H3K4me2 in adult primates [36].

In addition, we investigated the adverse effects of Pb on maize seedlings using protein analysis. The results demonstrated that all concentrations of Pb have significantly changed the protein patterns of the seedlings when compared with control as seen in Table 1. Such a decrease was found to be statistically significant ($p < 0.05$). Results of

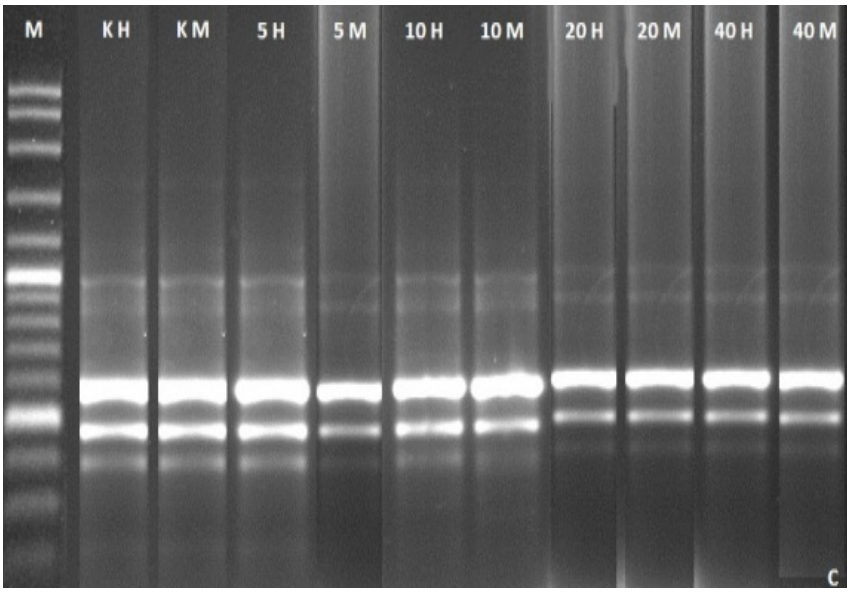

Fig. 3. Coupled restriction enzyme digestion-random amplification (CRED-RA) profiles of Pb-exposed and nonexposed *Zea mays* seedlings with primers of OPY-15 (M – Marker, K – Control, H – HpaII, M – MspI).

protein electrophoresis showed that there was a change in the profiles of protein bands. Changes in protein band profiles may cause point mutations, genetic and chromosomal rearrangements, deletion, insertions, and methylation in DNA. Also, a decrease in total protein levels can be suggested as one of the mechanisms for their genotoxic effects in a cell. On the other hand, the total soluble protein content of root tips of maize seedlings (as an important indicator of reversible or irreversible changes in the epigenetic mechanism), exhibited a positive correlation with Pb concentrations.

In this study, the effects of Pb on phytohormones levels also has been reported. Our results showed that Pb caused a decrease in GA, Z, and IAA levels and an increase in ABA in maize seedlings. The degree of these effects was mostly dependent on the concentration of Pb. GAs, Z, and IAA stimulate growth in plants. These phytohormones are synthesized in unstressed conditions and their effects cause growth events in plants. In contrast, ABA is growth inhibitory and it has been well known as a "stress hormone" in plants. This means that the synthesis of ABA increases especially during stress conditions in the leaves, and it moves to all plant parts to inhibit growth and protect the plant against biotic and abiotic stresses. That is why we think that the levels of phytohormones stimulating growth (GA, Z, and IAA) decreased while ABA synthesis increased in the seedlings. It has been reported that ABA encourages activity of the alternative respiration pathway, which is related to preventing the formation of reactive oxygen species (ROS) in plants [37]. The ROS levels increase in any stress conditions and cause oxidative stress. In this situation, it is possible to say that ABA synthesis increases to reduce oxidative damage in plants. In addition it has been well known that ABA causes synthesis of some new protein types, inhibits some proteins, and also increases and decreases the synthesis of some proteins that synthesize under stress conditions in plant cells [33, 38, 39]. This means that ABA may play a role in the change of the gene expression model by epigenetic mechanisms in plants. These findings are in accordance with DNA methylation results, but more information on molecular mechanisms of the role of DNA methylation of ABA in plants is needed.

In summary, when we put together the all results, we may say that Pb has a genotoxic effect and causes DNA hypermethylation in corn seedlings. RAPD and CRED-RA methods can be used as a useful investigational tool for genetic and epigenetic changes inducing genomic alterations. Because of the stress, the synthesis of ABA increases while GA, Z, and IAA levels decrease to protect against damages to Pb stress in corn seedlings.

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