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THE CYTOTOXIC EFFECTS OF ZINC AND CADMIUM METAL IONS ON ROOT TIP CELLS OF *PHASEOLUS VULGARIS* L. (FABACEAE)

Kültiğin ÇAVUŞOĞLU[*], Emine YALÇIN[*], Aysun ERGENE[**]

[*]Department of Biology, Faculty of Science and Art, Giresun University, 28049 Debboy Location, Giresun-TURKEY. E-mail: kultigincavusoglu@mynet.com
[**]Department of Biology, Faculty of Science and Art, Kirikkale University, 71450 Yahsihan, Kırıkkale-TURKEY

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Abstract: The present study was designed to evaluate the cytotoxic effects of different concentrations of zinc (Zn) and cadmium (Cd) heavy metal ions on root tip cells of *Phaseolus vulgaris* L. For this aim, we used the germination percentage, root lenght, weight gain and micronucleus (MN) frequency as indicators of cytotoxicity, and supported these data with statistical analysis. Additionally to the cytogenetic analysis, DNA analyses were performed from root tip meristem of *P. vulgaris* seeds treated with Zn and Cd. The test material was used the seeds of *P. vulgaris*.The seeds were divided into three groups: control, Zn and Cd treatment groups. They were treated with two dose levels (30 and 70 ppm) of Zn and Cd during 7 days. The initial and final weights of all seeds were measured by sensitive balance in order to investigate the effect of heavy metal ions on the weight gain of seeds. The results indicated that there was an alteration in the germination percentage, root lenght, weight gain and MN frequency depending on dose in seeds exposed to Zn and Cd ions when compared with control. Both doses of Zn and Cd ions significantly decreased the germination percentage, root lenght and weight gain in seeds all treatment groups. However, MN rate showed an increase. Besides, the investigated all these paremeters (except MN frequency) was higher in seeds exposed to Zn than seeds treated with Cd. In other words, Cd was more a toxic metal than Zn. In conclusion, Zn and Cd metal ions had important cytotoxic effects on *P. vulgaris* root tip cells and the parameters such as germination percentage, root lenght, weight gain and MN frequency can be used for biomonitoring of these effects.

Key words: Cadmium, cytotoxic effect, micronucleus frequency, *Phaseolus vulgaris* L., zinc

ÇİNKO VE KADMİYUM METAL İYONLARININ *PHASEOLUS VULGARİS* L. (FABACEAE) KÖK UCU HÜCRELERİ ÜZERİNE SİTOTOKSİK ETKİLERİ

Özet: Bu çalışma *Phaseolus vulgaris* L. kök ucu hücreleri üzerine çinko (Zn) ve kadmiyum (Cd) ağır metal iyonlarının farklı konsantrasyonlarının sitotoksik etkilerini değerlendirmek için tasarlandı. Bu amaçla, çimlenme yüzdesi, kök uzunluğu, ağırlık kazanımı ve mikronukleus (MN) sıklığını sitotoksikitenin indikatörleri (belirteçleri) olarak kullanıldık ve bu verileri istatistiksel analizler ile destekledik. Sitolojik analizlere ilaveten, Zn ve Cd ile muamele edilen *P. vulgaris* tohumlarının kök ucu meristemlerinden DNA analizleri de gerçekleştirildi. Test materyali olarak *P. vulgaris* tohumları kullanıldı.. Tohumlar kontrol, Zn ve Cd uygulama grubu olmak üzere üç

gruba ayrıldı. 7 gün süresince Zn ve Cd'nin iki dozu ile muamele edildiler (30 ve 70 ppm). Tohumların ağırlık kazanımları üzerine ağır metal iyonlarının etkilerini araştırmak amacıyla, hassas terazi tarafından tüm tohumların başlangıç ve son ağırlıkları ölçüldü. Sonuçlar gösterdiki, kontrol grubu ile karşılaştırıldığında Zn ve Cd iyonlarına maruz kalan tohumlarda doza bağlı olarak çimlenme yüzdesi, kök uzunluğu, ağırlık kazanımı ve MN sıklığında bir değişim vardı. KULTİGİN Sayfa 2 25.05.2009Zn ve Cd'un her iki dozunda, tüm uygulama grubu tohumlarda çimlenme yüzdesi, kök uzunluğu ve ağırlık kazanımı önemli oranda azaldı. Fakat MN oranı ise bir artış gösterdi. Ayrıca, araştırılan tüm bu parametreler (MN sıklığı hariç) Cd ile muamele edilen tohumlarda Zn ile muamele edilen tohumlara göre daha yüksekti. Diğer bir ifadeyle, kadmiyum (Cd) çinkoya (Zn) göre daha toksik bir metaldi. Sonuç olarak, Zn ve Cd metal iyonları *P. vulgaris* kök ucu hücrelerinde önemli sitotoksik etkilere sahipti ve çimlenme yüzdesi, kök uzunluğu, ağırlık kazanımı ve MN sıklığı gibi parametreler bu etkilerin izlenmesi için kullanılabilirdi.

Anahtar kelimeler: Kadmiyum, sitotoksik etki, mikronukleus sıklığı, *Phaseolus vulgaris* L., çinko

INTRODUCTION

The pollution of environment by heavy metals as a result of human, agricultural and industrial activities is rather widespread in nowadays (ARAVIND & PRASAD 2005). Among these heavy metals, especially cadmium (Cd) and zinc (Zn) are the most abundant pollutants in the terrestrial environment (CHANEY & RYAN 1994, FORSTNER 1995). Cd is a quite toxic metal for plants (SIROKA et al. 2004). Although it is not an essential elements for plant cells and no known biological function, it can be easily absorbed by soil and accumulated in different parts such as root, steam and leaf of plant (SUZUKI 2005). But, Zn is an indispensable trace element for plants. It is necessary for detoxification of reactive oxygen species (ROS), activation of enzymes, reducing the production of free radicals by superoxide radical producing enzymes. Besides, Zn has protective effect againist photoxidative damage catalysed by ROS in chloroplasts (BAGCI et al. 2007). However, like other heavy metals, when Zn present in excess in plant tissues, it causes significant alterations in plant tissues. It was reported that Zn and Cd metals can induce anomalies such as reduce of chlorophyll content, inhibition of root elongation and plant growth, reduce of photosynthesis and chlorophyll biosynthesis, the formation of reactive oxygen species (ROS), increase of cell toxicity and induction of oxidative stress which caused oxidative modifications of proteins in plants (STOYANOVA & DONCHEVA 2002). Hence, when the soils were contaminated with Zn and Cd, may be observed to a significant decrease in the amount of harvest which a serious problem for agricultural economies (JOHNSON & EATON 1980, OHKI 1984). Despite regulatory measures carried out in many countries, these metals continue to increase in the environment.

Consequently, Zn and Cd are major environmental pollutants that inhibited to plant growth. Although many cytological studies have been carried out to detect the effects of heavy metals on plants, the mechanisms of heavy metal toxicity in plants are still poorly understood. The aim of this work was to evaluate the cytotoxic effects of Zn and Cd metal ions in *P. vulgaris* seeds.

MATERIALS AND METHODS

Preparation of root tips: In this study, 30 and 70 ppm concentrations of Zn and Cd metal ions were used. Cadmium (Merck, 1.19777) and Zinc (Merck, 1.19806) were dissolved in water to prepare the treatment solutions. The all solutions were freshly prepared in distilled water before use (pH 6.7). Healthy and proximate equal-sized *P. vulgaris* seeds were selected. The seeds were washed in ultradistilled water for 24 h. Controls were placed in tap water only. The treatment group seeds were placed on two sheets of filter paper (Whatman No. 1) in 9 cm diameter Petri plates. The 50 seeds were placed in each petri dish and treated with test solutions during the one week in an incubator at 23°C. Petri dishes were controled and added with 2 ml of Zn and Cd solutions during a 24-h period. For the the cytogenetic analysis, when the roots attained a length of approximately 1-2 cm, they were washed with distilled water, and temporary squash preparations were made (WEI 2004).

Determination of root length, weight gain and germination percentage: The root lengths of the germinated seeds were measured by a milimetric ruler. The root lengths were determined by radicula formation bases of *P. vulgaris* seeds non-exposed and exposed to different doses of Cd and Zn. The weight gains were determined by measuring the weights of seeds before and after treatment with a sensitive balance. Seed germination percentages in Cd and Zn treatment groups were calculated using Equation 1.

Germination (%) = Germinated seeds/total seeds x 100 (1) (ATIK vd. 2007).

MN assay: The root tips were fixed for 6 h in a Clarke's fixator (3: acetic acid glaciale / 1: distilled water), washed for 15 min in ethanol (96%) and stored in ethanol (70%) in the fridge at +4 ^{o}C until making the microscope slides. Root tips were hydrolyzed in 1N HCl at of 60 ^{o}C for 17 min, treated with 45% CH_3COOH solution for 30 min. For microscopic observation the root tips were stained with acetocarmine for 24 h. After staining, the root meristems were separated and squashed in 45% CH_3COOH solution (STAYKOVA et al. 2005, WEI 2004).
For MN analysis, 1000 cells were scored for each slide to calculate MN frequency. Micronucleated cells were evaluated under a binocular light microscope (Japan, Olympus BX51) at X500 magnification. For the scoring of MN the following criteria were adopted from FENENCH et al. (2003): (i) the diameter of the MN should be tenth of the main nucleus, (ii) MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary, (iii) MN should have similar staining as the main nucleus.

DNA isolation protocol: Modified DNA isolation protocol was applied according to ARUN et al. (2002). For DNA isolation the plant material was grinded in liquid nitrogen (2 g of fresh tissue). The grinded material was transferred to a solution containing 1M Tris-HCl, 0.5 M EDTA, 5M NaCl, 1M β-merkaptoetanol, dH_2O and incubated at 65^{o}C for 30 min. Centrifugation at 6800 rpm at room temperature for 15 min was applied and the supernatant carefully transferred into a fresh polypropylene tube and 5M potassium acetate was added. The solution was incubated in ice-bath and

centrifugated at 15000 rpm at room temperature for 15 min. An equal volume of chloroform-isoamylalcohol (24:1) was added onto supernatant and mixed by inversion for about 1 min. After centrifugation at 15000 rpm the aqueous phase transferred into a new polypropylene tube and ethanol : sodium acetate (2:1) was added. The mixture was incubated for 40 min at –20^{o}C and centrifugated at 15000 rpm. The pellet was washed with 80% ethanol, air-dried for 30 min and dissolve in 0.5 mL TE buffer. The DNA solutions were runned on a 0.8% agarose gel, and molecular imaging and DNA concentration were achieved by "Biovision+100/26MX" analyzer.

Statistical analysis: For the statistical analysis, data were analysed using the SPSS for Windows software, Version 10.0 (SPSS Inc., Chicago, USA). Statistically significant differences between groups were compared using analysis of "Paired Samples and Independent T-Test". The data are displayed as means ± standard deviation (SD), and P-values less than 0.05 are considered significant.

RESULTS

Effects of Cd and Zn ions on Germination Percentage

As shown in Table 1, the germination percentages of seeds treated with Zn and Cd were rather different from the control group ($P<0.05$). The highest germination percantage was observed in the control group seeds (in proportion as 96%). Zn and Cd metals caused a significantly decrease in the germination percentage at all doses. Besides, the germination percentage of Zn treatment group was higher than those of Cd group. 30 ppm and 70 ppm doses of Zn and Cd caused 30%, 44% and 40%, % 56 decreases in the seed germination, respectively. These results showed that the effects of Zn and Cd on the germination percentage depending on their doses.

Table 1. The effects of Zn and Cd on germination percentage of *P. vulgaris* seeds

Treatment time (day)	Groups	Concentrations (ppm)	Number of seeds	Number of germinated seeds	Number of not germinated seeds	Germination percentage (%)
7	Control	–	50	48	2	96
7	Zn1	30	50	35	15	70
7	Zn2	70	50	28	22	56
7	Cd1	30	50	30	20	60
7	Cd2	70	50	22	28	44

Effects of Cd and Zn ions on Root Lenght and Weight Gain

The results related to the weight gain and root lenght are given in Table 2, 3 and 4. These data showed that Zn and Cd treatments significantly prevented the root length and weight gain of seeds. A correlation was determined between heavy metal doses with the root length and weight gain. The highest root length and weight gain was observed in seeds of control group at the end of experimental period. The least root length and weight gain was observed in seeds treated with 70 ppm dose of Zn and Cd. In the

control group, the final weights of all seeds increased about 0.98 g according to initial weight. The root lengths of control seeds were measured as 6.07±1.51 cm at the end of experimental period. The final weights of seeds exposed to 70 ppm dose of Zn and Cd increased about 0.21 g and 0.11 g according to initial weight, respectively. Cd treated seeds showed a lower root length and weight gain than Zn treated seeds, but differences were not statistically significant (P>0.05). For all that, there were significant differences among control and treatment groups, and differences were statistically significant (P<0.05).

Table 2. Statistically analysis of root length measured in seeds at the end of treatment period

Parameters	Control	Zn				Cd			
	Mean	30 ppm	P	70 ppm	P	30 ppm	P	70 ppm	P
Root length (cm)	6.07±1.51	4.83±0.64	+	3.46±1.85	+	2.81±0.81	+	1.77±0.81	+

*(Independent Samples T-Test)
(+) : p < 0.05 significant
(-) : p > 0.05 insignificant

Table 3. Mean weight gain of *P. vulgaris* seeds at the end of 7th day

Treatment time (day)	Groups	Concentrations (ppm)	Number of seeds	Initial weight of seeds (g)	Final weight of seeds (g)	Weight gain
7	Control	–	50	0.23±0.04	1.21±0.29	0.98
7	Zn1	30	50	0.24±0.04	0.78±0.08	0.54
7	Zn2	70	50	0.26±0.05	0.47±0.08	0.21
7	Cd1	30	50	0.23±0.03	0.63±0.08	0.40
7	Cd2	70	50	0.22±0.03	0.33±0.07	0.11

Effects of Cd and Zn ions on MN Frequency

Microscopic examination of the squashes of *P. vulgaris* root tip meristem cells showed that MN formation was not observed in the control group (Figure 1a). But, a significant increase in MN formation was observed in all seeds exposed to Zn and Cd (Figure 1b). MN frequency is indicated in Table IV. MN frequency showed an increase with rising of Zn and Cd doses. There was a certain dose-effect relationship between the MN frequency and heavy metal doses. Cd-treated seeds showed a higher frequency of MN than Zn- treated seeds. The highest frequency of MN was observed at 70 ppm dose of Cd and least frequency of MN was observed at 30 ppm dose of Zn. There was a statistically significant difference between the MN frequencies of treatment and control groups (P< 0.05). But, the increase of MN frequency in Zn and Cd treatment groups was statistically not significant (P>0.05). To determine the effect of Zn and Cd metals

on DNA concentration, DNA isolation protocol were applied from all samples. Low DNA yield were observed in samples treated with Cd. In Figure 2, it was observed that the yield of DNA in seeds treated with Zn and Cd were lower than recorded in the control. So DNA yield of Zn and Cd were run ahead on agarose gel according to control group.

Table 4. Statistically comparision of datas root length, weight gain and MN frequency determined in treatment group seeds at the end of 7th day

Parameters	***30ppm Zn***	***70ppm Zn***	***Change***	***30ppm Cd***	***70 ppm Cd***	***Change***	***P***
Root length(cm)	4.83±0.64	3.46±1.85	1.37±1.63	2.81±0.81	1.77±0.81	0.92±0.98	–
Weight gain(g)	0.78±0.08	0.47±0.08	0.31±1.23	0.63±0.08	0.33±0.07	0.30±1.14	–
MN frequency	18.80±3.43	32.70±3.30	13.90±2.69	28.60±2.22	44.50±1.58	15.90±2.56	–

*(Independent Samples T-Test)
(+) : $p < 0.05$ significant
(-) : $p > 0.05$ insignificant

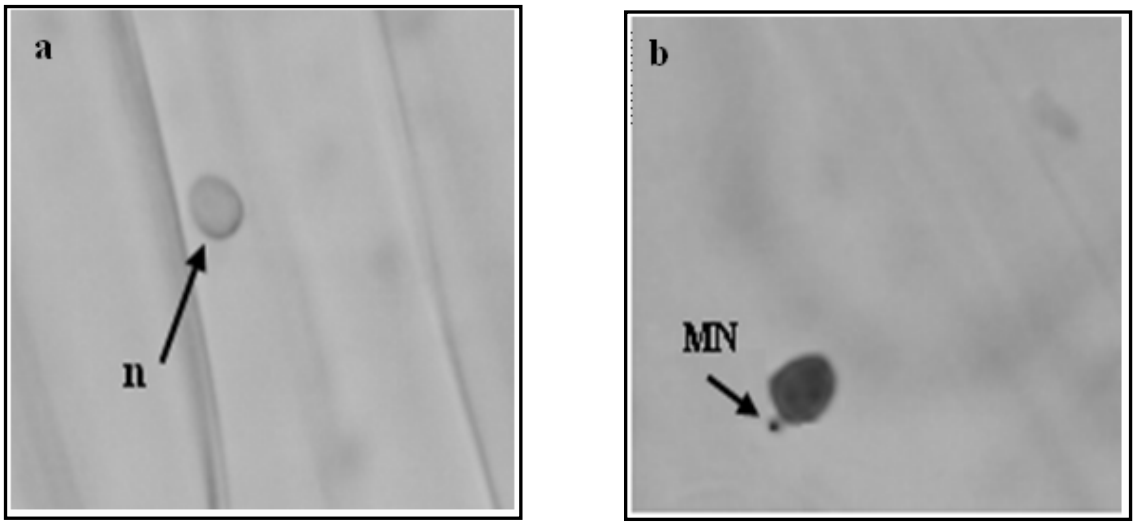

Figure 1. The appearance of nucleus (N) in control group seeds (a) and MN in treatment group seeds (b), X500

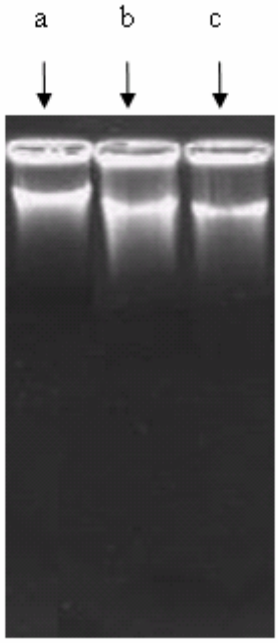

Figure 2. Isolated DNA from *P. vulgaris* resolved on 0.8 % agarose gel. a: control group, b: treatment group exposed 70 ppm Zn, c: treatment group exposed to 70 ppm Cd

DISCUSSION

A positive correlation between heavy metal doses and the germination percentage was observed in seeds. A significant decrease in the germination percentage was observed at 70 ppm doses of Zn and Cd when compared with control group. With rising of Zn and Cd doses the germination rates continuously decreased. The lowest germination rate was observed at 70 ppm dose of Cd. The results showed that the germination percentage can be considered as a sensitive indicator for Zn and Cd cytotoxicity. This information is parallel with other cytotoxic data available so far. In many studies, results clearly indicated that the test substances as heavy metal agents can lead to decrease the germination percentage in seeds of different plant species. For example, MUNZUROGLU & GECKIL (2002) reported reduce of the germination percentage with different concentrations of Hg, Co, Cu, Pb, Cd and Zn in *Triticum aes*tivum and *Cucumis sativus* plants. VERMA & DUBEY (2003) showed decrease about double of germination percentage in rice seeds exposed to high concentrations of Pb. Many similar studies were also designed to investigate the effects of heavy metals on the germination percentage of *Phaseolis vulgaris*, *Pisum sativum*, *Brassica napus* (WIERZBICKA & OBIDZINSKA 1998) and *Triticum aestivum* seeds (AYBEKE & OLGUN 2004).

The visual non-specific symptoms of heavy metal toxicity on plant are inhibition of root growth and seed weight (BURTON et al. 1984). In this study, we investigated changes in the weight gain and root growth of heavy metal-applied seeds. 30 and 70 ppm heavy metal doses inhibited the root growth. In 70 ppm dose of Cd, the root growth was about

3.43 times lower than in the control. The decrease in root growth was pronounced with the increase in Zn and Cd concentrations. The rooth growth about 10.50%, 430% decreased at 30 and 70 ppm doses of Zn and 54%, 71% decreased at 30 and 70 ppm doses of Cd, when compared with control, respectively. The root growth was more highly in Zn treated seeds when compared with those treated with Cd. This result may be explainable with become a more toxic metal of Cd according to Zn. The inhibitory effects of heavy metals on root growth were widely reported by bio-monitoring studies. In previous studies were reported that toxic concentrations of Zn, Cd, Pb, Hg, and Cu may lead to inhibition the growth of vegetative organs in some plant species (DIMITROVA & IVANOVA 2003). For example, SHAFIG & IGBAL (2006) determined decrease of root length at all concentrations (25-100 ppm) of Pb and Cd in *Cassia siamea*. ZENGIN & MUNZUROGLU (2003) investigated the effect on the root growth of Cd and Pb exposure. As a result, they showed inhibition of root growth in bean seedling treated with Hg and Cd. SYMEONIDIS & KARATAGLIS (1992) reported increase with rising concentrations of Cd, Zn and Pb metals of root length inhibition in *Holcus lanatus*. Besides, GODBALD & KETTNER (1991) observed a significant decrease in primary, secondary and tertiary root growth of *Picea abies* seedlings treated with different Pb solutions. In a similar study, OBROUCHEVA et al. (1998) demonstrated inhibition of primary root growth by heavy metals.

The findings obtained from this experiment showed that Zn and Cd metals affected the weight gain depending on doses which were used during the experimental period. Namely, the control group seeds showed an increase of 526% while Zn and Cd treated seeds showed an increase of 325%-180% and 274%-150% according to initial at the end of experimental period, respectively. The results indicated that Zn and Cd metals depressed and decreased the weight gain of plant materials. And also, the Cd treated seeds showed a lower weight gain than Zn treated seeds. In previous studies related to the weight gain, the effects of heavy metals on seed weight were not reported with deserve. Although the mechanism of Zn and Cd toxicity on the weight gain is completely unknown, it seems plausible that these metals acts as a blocking agent by interaction with the cell components. SHARMA & DUBEY (2005) reported that heavy metal ions may block the entry of cations and anions into plant tissues. They also determined that heavy metals may cause a decline in transpiration rate and water content of plant tissues. CHAOUI et al. (1997) determined to have a negative effect on mineral nutrition of excess increasing of Zn in plants. These conditions may cause significant alterations in nutrient contents of tissues, and may reduce the weight gain and growth.

In our present study, the frequency of MN was also recorded. The results showed that there was a dose-related increase in the frequency of MN in seeds treated with heavy metals. On the other hand, the frequency of MN increased with the rising of Zn and Cd doses. The highest frequency of MN was observed at 70 ppm dose of Cd, and least frequency of MN was observed at 30 ppm dose of Zn. At all doses also were observed statistically significant increases in the number of micronucleated cells according to the control group. Moreover, the frequency of MN elevated in Cd-treated seeds when compared with Zn, but difference was not statistically significant. This result may be related to a more toxic metal of Cd when compared with Zn. All these findings suggested that Zn and Cd had genotoxic activity which induced MN formation in seeds

of *P. vulgaris*. These observations are in agreement with cytotoxicity data reported by other authors so far. In most of the studies results indicated that the test substances as heavy metals induces MN formation which are the result of chromosomal damage or damage to the mitotic apparatus in the root tip cells of the plant seeds (INCEER et al. 2003). Especially, the inhibition of spindle formation has been shown to lead to severe abnormalities such as stickiness, unequal distribution, multipolar anaphase, chromosal bridges and laggards. Zn and Cd metal ions can be bound to reactive groups such as hydroxyl and sulfhydryl of biomolecules. So they cause conformation changes in biomolecules (proteins, enzymes or nucleic acids) or break the metabolic reactions (KARK 1979). In our opinion about this matter, heavy metals may enter into the cell nucleus and may bind to purine and pyrimidine bases or proteins such as spindle. These interactions may denature spindles and may cause a delay in the formation of chromosome-spindle complex. Hence, this condition may cause MN formation as a result of a decrease in chromosome number of the main nucleus. This knowledge is also in agreement with results reported by STAYKOVA et al. (2005), IVANOVA et al. (2005) and IVANOVA et al. (2008). They reported high MN frequency induced by the lagging of whole chromosomes or the immobility of large acentric fragments in *Allium cepa*. In a similar study, it was found a systematically increase in the MN rate and chromosome aberrations with increased concentration of CrO3 in *V. faba* (WEI 2004). In another study, ROSA et al. (2003) observed a significant increase in MN frequency at 20 microM dose of Cd^{++}. Consequently, it was concluded that the *P. vulgaris* MN assay may be used as an endpoint biomarker acceptable in biomonitoring environmental pollutives such as Zn and Cd.

Besides, this is the first study about a correlation between MN formation and the concentration of DNA was investigated. Therefore, we obtained DNA from root tip meristems of *P. vulgaris* and used DNA electrophoresis technique to measure the DNA concentration. As a result, it was found that DNA is rather sensitive to 70 ppm doses of Zn and Cd metals. We suggest that the length of DNA bands in Zn and Cd treated seeds were longer than recorded in the control. And also, the DNA yields in control group were higher than observed in Cd and Zn treatment groups. The DNA yield order obtained in this study was control>Zn>Cd. This diversity may be associated with a loss of genetic material. Because, MN formation is a condition which termination with loss of genetic material in nucleus and originated from chromosome fragments or whole chromosomes. This observation suggests that MN formation is related directly with the length of DNA bands, and this data has been not reported detailed by other researchers so far. This is the first report demonstrated relation between DNA amount and MN frequency in root tip cells, and that may serve as possible indicators and thereby provide new insights into the mechanisms of heavy metal toxicity.

From all these results, we concluded that Zn and Cd metals have serious cytotoxic effects on the root tip cells of *P. vulgaris*, and the parameters such as the germination percantage, root length, weight gain and MN frequency are suitable indicators for biomonitoring of these effects.

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