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Cadmium-induced genotoxicity, cytotoxicity and lipid peroxidation in *Allium sativum* and *Vicia faba*

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Cadmium (Cd) is one of the most toxic environmental pollutants affecting cytogenetically the various organisms. The cytogenetic damage in root tip cells exposed to cadmium nitrate ($CdNO_3$) solutions at four different concentrations (1, 10, 100 and 200 μM) was evaluated with biological tests based on micronucleus (MN) assay in two plant species, *Allium sativum* and *Vicia faba*. Additionally to the cytogenetic analysis, lipid peroxidation analyses were performed in both *A.sativum* and *V.faba* roots. Cd enhanced the MN frequency in both *A.sativum* and *V.faba* root tip cells, but no dose-dependent. Induction of MN is not depending on $CdNO_3$ concentrations. Besides, high concentrations of Cd decreased the mitotic index and caused the delay in mitosis stages in both plants, mainly in *V.faba*. On the other hand, lipid peroxidation was significantly enhanced with external Cd in *V.faba*. The results clearly indicate that high concentrations of cadmium induce the lipid peroxidation resulting in oxidative stress that may contribute to the genotoxicity and cytotoxicity of Cd ions.

Introduction

Cadmium (Cd) is released into the environment by human activities such as phosphate fertilizers, disposal of household, municipal and industrial wastes. These sources may cause enhanced soil and hence crop Cd levels, which may lead to increases in dietary Cd exposure. Cd is a particularly dangerous pollutant due to its high toxicity and great solubility in water (1,2). At low concentrations, Cd is not toxic to plants but at higher concentrations it is toxic. Exposures to high Cd concentration have been found to be carcinogenic, mutagenic and teratogenic for a large number of animal species (3). Characteristically inhibits root growth and cell division in plants such as onion, tradescantia, *Vicia faba* (4), *Nicotiana tabacum* (5) and *Allium sativum* (6). Numerous experimental studies have shown the genotoxicity of Cd salts (5,7). Moreover, in plants Cd accumulation causes reductions in photosynthesis, diminishes water and nutrient uptake and results in visible symptoms of injury in plants, such as chlorosis and growth inhibition (8).

Cd was found to decrease the mitotic index (MI) and also induced chromosomal aberrations, micronucleus (MN) formation in plant root cells (9,10). In several studies, it is indicated that Cd damaged the nucleolar structure, DNA and RNA in both animal and plant cells (11–13). Some researchers reported that the Cd salts are not directly genotoxic in rodent cell lines. According to the International Agency for Research on Cancer classified Cd is suspected as co-mutagen and human carcinogen (14). Plant species respond differently to exposure of the same metal depending on genetic structures (9,15). *A.sativum* has considerable abilities to remove Cd from solutions and accumulate it. The Cd content in roots of *A.sativum* increased with increasing concentrations of Cd^{+2} (2,16).

A.sativum is well known for its characteristic resistance to biotic and abiotic environmental stresses, such as viral, bacterial and oxidative stress (16). Cd was recognized as causing oxidative stress in plants, so *A.sativum* may have a strong resistance to Cd, but little is known about the effects of Cd on lipid peroxidation and genotoxicity of *A.sativum* seedlings. In this study, we investigated lipid peroxidation [malondialdehyde (MDA) content], MI and MN induction in roots of *A.sativum* and *V.faba* seedlings after Cd exposure. MN induction was chosen as the cytogenetic endpoint, due to the fact that MN scoring and slide preparation are less time-consuming and expensive than other endpoints, such as observation of chromosome aberrations. The mechanism underlying the MN formation is well understood owing to several researchers (17–19). MN results from chromosomal fragments or whole chromosome lagging during cell division. Several studies showed that MN assay is an effective technique to assess the genotoxic damage occurring by environmental pollutant and toxic substances, such as pesticides, heavy metals, in both plant cells and animal cells such as mammalian cell (human peripheral blood lymphocytes, exfoliated buccal cell and rat bone marrow cells) (20–26).

Materials and methods

Treatment solution and root tip preparations

Healthy and equal-sized *A.sativum* gloves and *V.faba* (broad bean) were selected. For the *A.sativum* test, the dry scales of the garlic bulbs were removed and bulblets were suspended in test tubes containing 0.5 N Hoagland's nutrient solution. For the *V.faba* test, dry broad bean seeds were soaked for 24 h in ultradistilled water and allowed to germinate between two layers of moist cotton at 24 ± 2°C. When the newly emerged roots were of 1.00–2.00 cm in length, 10 seedlings for each treated group were selected and transferred into container (10 cm tall, 30–20 cm) with 0.5 N Hoagland's nutrient solution at 26/22°C (day/night) temperature on 65 ± 5% relative humidity in a growth chamber with 480 $\mu mol.m^2/s$ photons (day/night 14/10 h) for 10 days. The Hoagland's nutrient solution consisted of (27) 0.821 g/l $Ca(NO_3)_2.4H_2O$, 0.506 g/l KNO_3, 0.136 g/l KH_2PO_4, 0.120 g/l $MgSO_4.7H_2O$, 50 g/l $C_6H_5FeO_7.5H_2O$, 1.80 g/l $MnCl_2.H_2O$, 2.90 g/l H_3BO_3, 0.12 g/l $ZnCl_2$, 0.05 g/l $CuCl_2.2H_2O$ at pH 5.5. Seedlings were transferred into container containing $CdNO_3$ (Merck, purity = 99%) added to concentrations 1, 10, 100 and 200 μM for 48 h. The positive control group was suspended over the solution containing 5 μg/ml cyclophosphamide. Three plantlets from each treatment were harvested based on uniformity of size and colour after 48 h of incubation. The roots were rinsed in ultradistilled water to remove traces of nutrient and Cd ions on the surface. They were used in the tests.

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Table I. Frequency of MN, MI values and MDA contents by cadmium nitrate ($CdNO_3$) in *V.faba* and *A.sativum* root tip cells

Test substance	Concentration (mM)	MN frequencies (‰) (mean ± SD)		MI (‰) (mean ± SD)		MDA contents (μmol g^{-1} FW) (mean ± SD)	
		A.sativum	*V.faba*	*A.sativum*	*V.faba*	*A.sativum*	*V.faba*
Negative Control Hoagland's nutrient solution	0	0.00 ± 0.00	0.00 ± 0.00	97.6 ± 6.65	101.9 ± 5.54	19.87 ± 3.59	10.17 ± 3.29
Cadmium nitrate solution (μM)	1	0.30 ± 0.34	1.12 ± 0.35*	52.6 ± 9.07***	70.4 ± 1.40***	19.35 ± 1.02	25.55 ± 7.76**
	10	1.30 ± 0.00***	2.40 ± 0.34***	43.9 ± 12.2***	64.0 ± 4.35***	20.25 ± 1.35	25.03 ± 3.50**
	100	0.95 ± 0.40***	1.65 ± 0.40**	55.0 ± 7.81***	4.66 ± 1.15***	22.45 ± 9.06	22.21 ± 3.10**
	200	0.95 ± 0.40***	1.65 ± 0.40**	24.0 ± 5.29***	3.66 ± 1.52***	23.15 ± 5.80	22.45 ± 3.92**
Cyclophosphamide (positive control) (μg/ml)	5	5.12 ± 0.35***	10.82 ± 1.28***	49.3 ± 6.42***	62.0 ± 4.58***	24.64 ± 11.30	17.29 ± 5.50

*** $P < 0.001$, ** $P < 0.01$,* $P < 0.05$ compared with negative control.

MN test

After 48 h of incubation, the roots were fixed with acetic acid–ethanol [1:3 (v/v)] solutions for 24 h. The solutions were freshly prepared before use. Both positive and negative control samples were fixed at +4°C the same time. For slide preparation and microscopic examination, the rinsed root tips were hydrolyzed in 1 M HCl at 60°C for 8 min. After staining with Feulgen, they were washed in ultradistilled water. An aliquot of 1 mm of the mitotic zone from well-stained root tips were immersed in glycerin–gelatin (1/7/7(v/v/v) gelatin/ultra distilled water/Glycerin) on a clean slide and squashed under a cover glass.

For the analysis of micronuclei, in *A.Sativum* and *V.faba*, 6000 cells per seedling were scored to calculate MN frequency. The cells with MN were evaluated under 1000× magnification with use of a light microscope (Japan, OLYMPUS). The following criteria for MN analyses were used in *A.sativum* and *V.faba* root tip cells (28). MN should: (i) be almostly one-third the diameter of the main nucleus; (ii) be on the same plane focus; (iii) have a chromatin structure similar to that of the main nuclei; (iv) be smooth, oval or round shape; and (v) be clearly separated from the main nucleus.

MI

MI was determined by counting the number of mitotic cells among the total amount of scored cells per seedlings. Approximately the frequencies of prophase, anaphase, metaphase and telophase were scored from 3000 cells of three separate seedlings for each treatment and control group. Each experiment was run with three replications.

Lipid peroxidation

Lipid peroxidation was determined by measuring the amount of MDA according to Karabal *et al*. (29). A total of 0.2 g of root tissues from control and treated plants were cut into small pieces and homogenized by the addition of 1 ml of 5% trichloroacetic acid (TCA) solution. The homogenates were then transferred into fresh tubes and centrifuged at 12 000 r.p.m. for 15 min at room temperature.

Equal volumes of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA solution (freshly prepared) were added into a new tube and incubated at 96°C for 25 min. The tubes were transferred into ice bath and then centrifuged at 10 000 r.p.m. for 5 min. The absorbance of the supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm, 0.5% TBA in 20% TCA solution was used as the blank. MDA content was determined using the extinction coefficient of 155/mM/cm.

Statistical analysis

Data were evaluated by ANOVA using SPSS for Windows software package. Multiple comparisons were performed by Least Significant Difference (LSD) test. $P < 0.05$ was considered as level of significance.

Results

The MN frequencies and MI values are depicted in Table I. Cd induced the MN formation in both *A.sativum* and *V.faba* (Figure 1). There is a statistically significant difference between treatment and control groups ($P < 0.001$) for MN frequency and statistical results refer a statistical difference between 1 and 10 μM and, 100 and 200 μM Cd ($P < 0.001$, $P < 0.01$, respectively) in *A.sativum*. But, there is no significant difference between 10 and 100 and 200 μM concentration of Cd ($P < 0.05$, $P = 0.125$) for *A.sativum*. Results obtained from statistical analysis indicate that there is a statistically significant difference between 1 and 10 μM Cd ($P < 0.05$), but there is no significant difference between 10 and 100 and 200 μM Cd ($P > 0.05$, $P = 0.149$) for *V.faba*. It is observed that the increase in MN frequency is not dose-dependent.

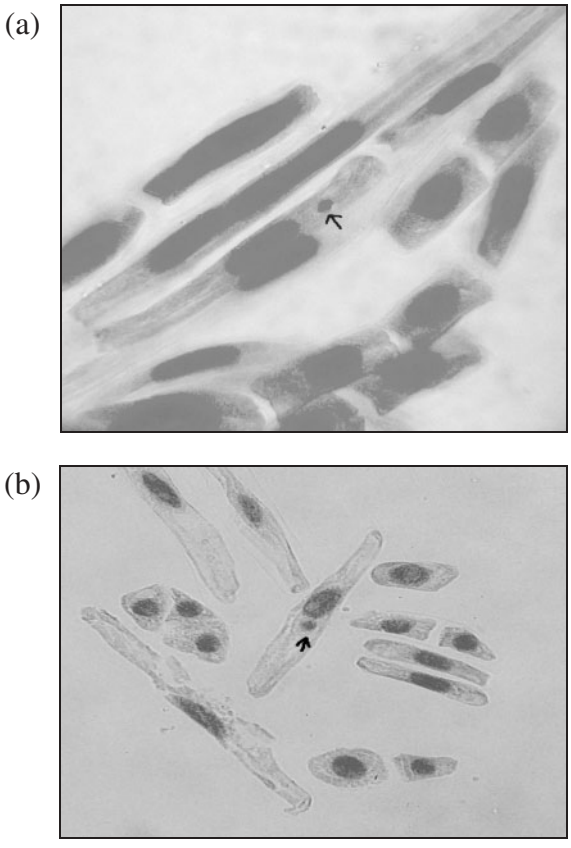

Fig. 1. Micronucleated cells in root tips. (**a**) *A.sativum* (**b**) *V.faba*.

Cd solutions decreased MI in *V.faba* and *A.sativum*. Cd at the tested concentration induced a dose-dependent decrease in the MI and showed a statistically significant difference between treatment, negative and positive control groups ($P < 0.001$). MI and prophase stages decreased remarkably at

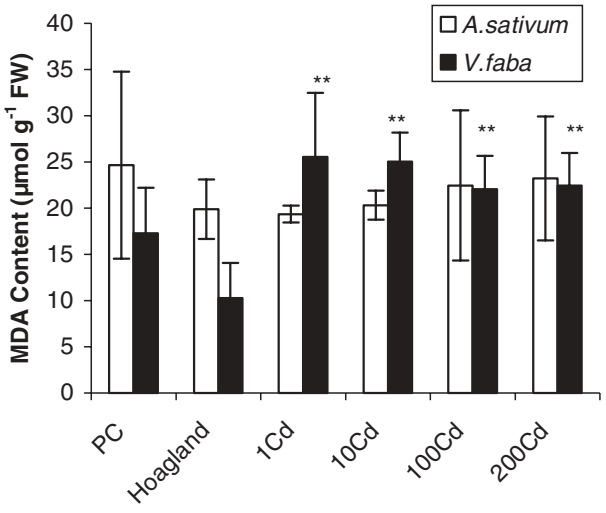

Fig. 2. Effect of Cd stress on MDA content of *A.sativum* and *V.faba*. The data represent the mean ± SE of three replicates. ** $P < 0.01$ compared with negative control.

the high concentrations (100 and 200 μM). Particularly, metaphase, anaphase and telophase stages were not observed in *V.faba* treated with 100 and 200 μM Cd concentration compared with negative control.

MI and mitosis stage slightly decreased in *A.sativum* compared with the negative control in low concentrations of Cd. The lowest MI value was detected at 200 μM Cd treated-in *A.sativum* root tips. Also, only prophase stage was observed at these concentrations.

Membrane lipid peroxidation in roots of both plants, measured as the content MDA, is given in Figure 2. MDA is the final product of membrane lipid peroxidation and accumulates when plants are subjected to oxidative stress. Lipid peroxidation was significantly higher in *A.sativum* than in *V.faba* under the unstressed conditions (Hoagland treatment). All stress conditions did not considerably change the MDA concentration in *A.sativum* while lipid peroxidation significantly increased under Cd stress in *V.faba* ($P < 0.05$). It is observed that the increase in MDA concentration is not dose-dependent. Also, 200 μM Cd resulted in increased proportion of cells with abnormal morphology in *V.faba*.

Discussion

Cadmium is one of the most toxic environmental and industrial pollutants causing DNA damage, elevating lipid peroxidation with a long biological half-life time and represents a serious environmental pollutant for animal and plants. It influences many plants, animal and human communities. Therefore, a deeper understanding of the mechanism of Cd toxicity is important.

Here, we report that Cd induces the MN formation and decreases MI in *Allium* and *Vicia*. In this study, high concentrations of Cd clearly increases the MN frequencies in both plants, but not dose-dependent, compared with negative control. Also, 100 and 200 μM Cd decreased MN, compared with 10 μM Cd. This may be due to decreasing of MI, mainly in *V.faba*. Cd might delay mitosis by damaging the transport mechanism because of high lipid peroxidation in *V.faba* root cells. Lipid peroxidation induced by Cd is known to be due to the attack of free radicals on the fatty acid component of membrane lipids. These data support that enhanced lipid peroxidation in *V.faba*, in the present study may be attributed to excessive generation of reactive oxygen species (ROS) exacerbated by deficient antioxidant defenses. ROS overproduction coupled with deficiency of antioxidant defense mechanisms may be an important factor contributing to the increase in micronuclei. In some studies, it has been implied that there is the relationship between the MN formation and lipid peroxidation under several stress conditions such as diatery habits (30). Mayer *et al*. (30) have also demonstrated a positive correlation between lipid peroxidation status and genotoxicity as reflected by increased MN formation in lymphocytes. The decline of MI in *V.faba* exposed to high levels of Cd suggested that the Cd could prevent cells from going into cell division. Zhang and Yang (9) and Zhang and Xiao (10) also found that Cd reduced MI in *Hordeum vulgare*.

In many studies, Cd has been shown to be a genotoxic metal (5,10). In several studies performed by many researchers, Cd enhanced the effects of other mutagens (31). Rojas *et al*. (32) reported that Cd exerts pronounced indirect genotoxic effects; it enhanced mutagenity of UV light in several cells. The mechanism responsible for the toxicity of Cd is unclear. It has been shown that lipid peroxidation elevates in tissue soon after exposure to Cd (33,34). There are no sufficient evidences about the effects on mitosis stages concerning Cd treatment. Cd may inhibit the dissolution of nuclear membranes, mainly in *V.faba* root cells. This inhibition of nuclear membrane was found only at the highest concentration of Cd in *A.sativum*, which has considerable ability to remove Cd from solution and accumulate it (2). *A.sativum* has been shown to minimize oxygen-induced cell injury [35 and to have high antioxidant capacity under abiotic stress conditions (16)].

In eucaryotes, it was proposed that the genotoxicity of metals results from non-direct formation of ROS (36,37). Fariss (38) has depicted that free radicals scavenger and antioxidants are useful in protecting against Cd toxicity. Abiotic stresses including heavy metals result in molecular damage to plant cells either directly or indirectly (16,36,39). Protonation of radical of O_2 can produce the hydroperoxyl radical (OH^-, H_2O_2), which can convert fatty acids to toxic lipid peroxides, destroying biological membranes (16). MDA formation is used as the general indicator of the extent of lipid peroxidation resulting from oxidative stress. In the present study, MDA was not greatly affected by Cd treatment in *A.sativum* in contrast to *V.faba*. This indicates that the capabilities of *A.sativum* seedlings to adapt to lower concentration of Cd may be related to a low degree of lipid peroxidation. MDA contents in *V.faba* increased ∼2–5 times after Cd exposure. This suggests that Cd indirectly leads to excessive generation of superoxide radicals by deficient antioxidant defenses, resulting in increased lipid peroxidative products and oxidative stress in *V.faba*. These superoxide radicals induced the lipid peroxidation (16,40–42).

Fojtová and Kovaŕik (5) observed cells with aberrant morphology in tobacco cell cultures treated 50 mmol/m^{-3} of $CdSO_4$. This was supported by our finding in *V.faba*. The most common phenotype was cells with condensed protoplasts and irregular profiles, both being hallmarks of apoptosis (43). The induction of micronucleated cells following exposure to Cd indicates a potential for clastogenity. Concerning the genotoxicity parameter (micronuclei frequency), it is possible to compare the sensitivity of *Allium* and *Vicia*, since the

tests were performed at the same time and under same conditions. *V.faba* appeared to be more sensitive than *A.sativum* when exposed to Cd. In study performed by Rosa *et al*. (44), it was indicated that the MN induction may be also interpreted as a consequence of oxidative stress, upholding the view that Cd induced DNA damage is, to some extent, via generation of (intermediate) ROS in *V.faba*. This is also in agreement with previous reports on the clastogenity potential of Cd as manifested in rodent bone marrow, peripheral lymphocytes of workers exposed occupationally to Cd (45,46).

The observed inhibition of mitosis stages in root cells illustrates the cytotoxiciy of Cd. These observations imply that heavy metal resistance might be characteristic for *A.sativum* seedlings.

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