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Micronucleus frequency and lipid peroxidation in *Allium sativum* root tip cells treated with gibberellic acid and cadmium

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Abstract Gibberellic acid (GA_3) is a very potent hormone whose natural occurrence in plants controls their development. Cadmium is a particularly dangerous pollutant due to its high toxicity and great solubility in water. In this study, the effect of GA_3 on *Allium sativum* root tip cells was investigated in the presence of cadmium. *A. sativum* root tip cells were exposed to $CdNO_3$ (50, 100, 200 μM), GA_3 (10-3 M), both $CdNO_3$ and GA_3. Cytogenetic analyses were performed as micronucleus (MN) assay and mitotic index (MI). Lipid peroxidation analysis was also performed in *A. sativum* root tip cells for determination of membrane damage. MN exhibited a dose-dependent increase in Cd treatments in *A. sativum*. GA_3 significantly reduced the effect of Cd on the MN frequency. MN was observed in GA_3 and GA_3 + 50 μm Cd treatments at very low frequency. MI slightly decreased in GA_3 and GA_3 + Cd treatments. MI decreased more in high concentrations of Cd than combined GA_3 + Cd treatments. The high concentrations of cadmium induce MN, lipid peroxidation and lead to genotoxicity in *A. sativum*. Current work reveals that the effect of Cd on genotoxicity can be partially restored with GA_3 application.

Keywords Lipid peroxidation · *Allium sativum* · Gibberellic acid · Cadmium · Micronucleus

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Introduction

Plant hormones play important roles in all aspects of the growth and development of plants and in their interactions with the environment and other organisms. Many of the developmental and physiological processes regulated by plant hormones are of agronomic importance. Gibberellic acid (GA_3) is the most widely available compound, which is a fungal product. GA_3 also plays important roles in many cellular processes including promoting stem elongation, seed germination, fruit setting and growth, sex expression (Paroussi et al. 2002; Davies 2005; Tuluce and Celik 2006). The levels of this hormone placed into the environment may soon exceed those of insecticides (Tuluce and Celik 2006; Mickel 1978).

Cadmium (Cd) is hazardous heavy metal for plants, animals, and humans (Kim et al. 2003). In plants, some metal compounds are known to be responsible for genotoxic effects (Radetski et al. 2004). The involved mechanism is unclear. In plants, there are insufficient data about the possible involvement of a putative reactive oxygen species (ROS)-mediated

mechanism in the genotoxicity of metals (Radetski et al. 2004; Kasai et al 1992). Cd^{2+} induces genotoxicity and programmed cell death (Behboodi and Samadi 2004; Garnier et al. 2006; Ünyayar et al. 2006). Exposures to high Cd concentration have been found to be carcinogenic, mutagenic, and teratogenic for a large number of animal species (Degraeve 1981; Waalkes 2000). Some researchers revealed that low micromolar concentrations of Cd are efficient in inducing DNA breaks in human blood lymphocytes (Depault et al. 2006).

Micronucleus (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 (Heddle et al. 1991; Cotello et al. 1999; Fenech et al. 1999). MN results from chromosomal fragments or whole chromosome lagging during cell division. Several studies showed that MN assay is an effective technique in assessing the genotoxic damage occurring by environmental pollutant and toxic substances, such as pesticides and 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; Hazen and Guiterrez Gonzalvez 1988; Khora et al. 1997; Yi et al. 2005; Çelik et al. 2003a,b, Abou-Eisha et al. 2004; Martinez et al. 2004).

Although GA_3 are used for a wide variety of crops is, there is no information about the combined effects with cadmium. The aim of the study was to evaluate whether GA_3 and cadmium treatments have any effects on the micronucleus and lipid peroxidation in the *Allium sativum* root tip cells.

Materials and methods

Treatment solution and root tip preparations

Healthy and equal-sized *A. sativum* gloves were selected. The dry scales of the garlic bulbs were removed, and bulblets were suspended in test tubes containing 0.5 N Hoagland's nutrient solution. 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 mmol/m^2/s photons (day/night 14/10 h) for 10 days. The Hoagland's nutrient solution consisted of 0.821 g/l Ca $(NO_3)_2{\cdot}4H_2O$, 0.506 g/l KNO_3, 0.136 g/l KH_2PO_4, 0.120 g/l $MgSO_4{\cdot}7H_2O$, 50 g/l $C_6H_5FeO_7{\cdot}5H_2O$, 1.80 g/l $MnCl_2{\cdot}H_2O$, 2.90 g/l H_3BO_3, 0.12 g/l $ZnCl_2$, 0.05 g/l $CuCl_2{\cdot}2H_2O$ at pH 5.5 (Hoagland and Arnon 1938). One group of seedlings was transferred into a container containing $CdNO_3$ (Merck, purity= 99%) added to concentrations 50, 100, and 200 μM for 48 h. One group was transferred into another container containing GA_3 (10^{-3} M; IMPA, 20 g/l). The other group of seedlings was exposed both $CdNO_3$ and GA_3. The positive control group was suspended over the solution containing 5 mg/ml cyclophosphamide. Three plantlets from each treatment were harvested based on uniformity of size and color 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.

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 14°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 ultra-distilled 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*, 6,000 cells per seedling were scored to calculate MN frequency. The cells with MN were evaluated under 1,000× magnification with the use of a light microscope (Olympus, Japan). The following criteria for MN analyses were used in *A. sativum* root tip cells (Tolbert et al. 1992). MN should: (1) be almost one third the diameter of the main nucleus; (2) be on the same plane focus; (3) have a chromatin structure similar to that of the main nuclei; (4) be smooth, oval, or round-shaped; and (5) be clearly separated from the main nucleus.

Mitotic index

Mitotic Index (MI) was determined by counting the number of mitotic cells among the total amount of scored cells per seedlings. MI was scored from 3,000 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 malondialdehyde (MDA) according to Ohkawa et al. (1979). Each experiment was run with three replications. 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 rpm 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×*g* 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^{-1} cm^{-1}.

Statistical analysis

Data were evaluated by analysis of variance using SPSS for Windows software package. Multiple comparisons were performed by least significant difference test. A $P<0.05$ was considered as level of significance.

Results

MDA contents, MN frequency, and MI values are presented in Fig. 1. Cd adversely induced the MN formation in *A. sativum* (Fig. 2). The severity of the response was directly related to increasing Cd concentrations. There is no significant difference between GA_3, GA_3 + 50 μM Cd, GA_3 + 100 μM Cd treatments and negative control groups. There is no significant difference between PC and 200 μM Cd. MN increased depending on the Cd concentrations. Highest MN frequency was observed in 200 μM Cd treatment. GA3 significantly reduced the effect of Cd on the MN frequency. MI significantly decreased in Cd treatments, especially 100 and 200 μM Cd treatments ($p<0.001$). MI slightly decreased in GA_3 and GA_3 + 50 μM Cd treatment compared with negative control. MI decreased more in high concentrations of Cd than combined GA_3 + Cd. There was no significant difference between GA_3 + 50 μM Cd treatment and negative control for the MI.

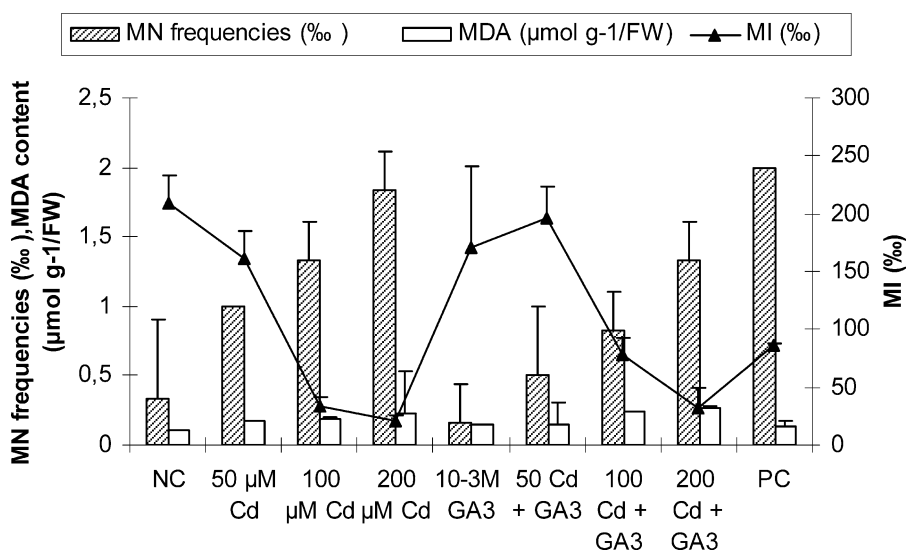

Fig. 1 Effects of cadmium and/or GA_3 on MDA contents, MN frequency, MI in *A. sativum* root tips

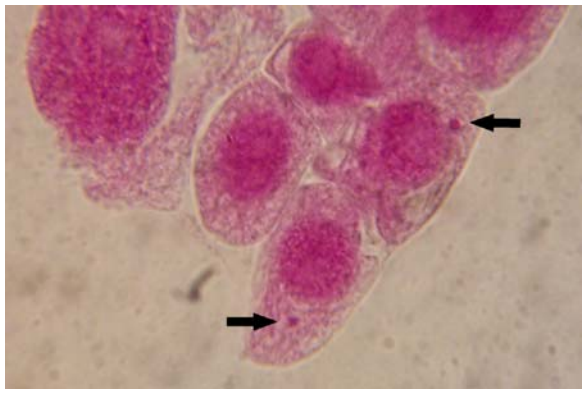

Fig. 2 Micronucleated cells in cadmium- and/or GA_3-treated *A. sativum* root tips

Membrane lipid peroxidation in roots, measured as the content MDA, is given in Fig. 1. Cd treatments significantly increased MDA content of root tip cells. MDA is the final product of membrane lipid peroxidation and accumulates when plants are subjected to oxidative stress. MDA contents in GA_3 and GA_3 + Cd treatments slightly increased compared with negative control. However, it is determined that the MDA content remarkably increased depending on Cd concentrations. Also, the many cells with abnormal morphology in high Cd concentrations were observed. GA_3 treatments increased the MDA contents compared with negative control.

Discussion

Cadmium is a particularly dangerous pollutant due to its high toxicity and great solubility in water (Lockwood 1976; Jiang et al. 2001). Although Cd is a non-essential heavy metal and is not toxic to plants at low concentrations, Cd accumulation in soil and water now pose a major environmental and human health problem (Jiang et al. 2001). In recent years, a significant increase in the use of GA_3 against harmful agricultural pests, giving rise to losing product, has been observed in Turkey and the rest of the world (Tuluce and Celik 2006). In this investigation, combined effect of GA_3 and Cd was preferred because information on their combined physiological and genotoxic effects on plants is not known.

We found that Cd induced the MN formation and decreased MI. Although we reported that increase of MN frequencies in *A. sativum* was not dose-dependent in the previous our study (Ünyayar et al. 2006), in this study, MN frequency increased with increasing Cd concentration compared with negative control. It may act inconsistent factors like treatment time, setting of studies, purity of chemicals, etc. MN was observed in GA_3 and combined GA_3 + Cd (50 μM) at very low frequency. This might be explained by fact that MDA contents and MI were close to negative control. GA_3 restrains the rise in reactive oxygen species (Rosenvasser et al. 2006) and may help to prevent oxidative damage to trigger of the low concentration of cadmium.

Cd has been shown to be a genotoxic metal (Fojtova´ and Kovařik 2000; Zhang and Yang 1994). High Cd concentrations delayed mitosis more and led to increase of MN frequency than combined treatments. In study performed by Rosa et al. 2003, 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 *Vicia faba*. Zhang and Xiao (1998) and Ünyayar et al. (2006) also found that Cd reduced MI in *Hordeum vulgare* and *A. sativum*, respectively. In several studies, Cd enhanced the effects of other mutagens (Fatur et al. 2003). Nehéz et al. (2000) reported that other heavy metal Pb enhanced genotoxicity of pesticides in rats. Garnier et al. (2006), and Rojas et al. (1999) reported that Cd induces genotoxicity and programmed cell death. This results in triggered membrane peroxidation by cadmium.

It has been determined that lipid peroxidation elevates in tissue soon after exposure to Cd (Ünyayar et al. 2006; Muller 1986; Bagchi et al 1997). Karbowning et al. (2001) also reported that cadmium toxicity resulted in lipid peroxidation in hamster organs. We also determined that the MDA contents increased in Cd treatments. In eucaryotes, it was proposed that the genotoxicity of metals results from non-direct formation of ROS (Radetski et al. 2004; Kasai et al. 1992). ROS can convert fatty acids to toxic lipid peroxides, destroying biological membranes. This causes the MDA formation, damaging membrane permeability (Zhang et al. 2005). MDA formation is used as the general indicator of the extent of lipid peroxidation resulting from oxidative stress. GA_3 treatment induced lipid peroxidation in 50 μM Cd-treated or untreated roots when compared with negative control. This inducing was lower than those of single Cd treatments. However, GA_3 in high Cd

concentration significantly increased the MDA content. GA_3 treatment may stimulate the changes in ROS levels and initiate the cell death program under high levels of Cd treatments. Several researchers reported that GA-treated cells lose their ability to scavenge ROS and that this loss ultimately results in oxidative damage and cell death (Fath et al. 2001; Bethke and Jones 2001; Maya-Ampudia and Bernal-Lugo 2006). In contrast, Rosenvasser et al. (2006) suggest that GA_3 acts to inhibit leaf senescence of *Pelargonium* cuttings, reducing ROS levels. Whether ROS levels are reduced by GA3 is still not clear.

In our experiment, it was determined that high Cd concentrations reduced the effect of GA_3 on the MDA contents. Cd toxicity may result in increased membrane permeability and MDA content, which is well correlated with severity of Cd toxicity treatment. The effect of GA_3 on lipid peroxidation is still unknown.

The present study concludes that GA_3 reduced the genotoxic effect of high levels of Cd because the effect of Cd on genotoxicity can be partially restored with GA_3 application. These results advocate the need for additional research for elucidating relationship among heavy metal + GA_3, antioxidant defense system, ROS, and genotoxicity in plants.

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