

## Effects of cadmium on root growth, cell division and micronuclei formation in root tip cells of *Allium cepa* var. *agrogarum* L.

Efectos del cadmio en el crecimiento radical, división celular y formación de micronúcleos en células apicales radicales de *Allium cepa* var. *agrogarum* L.

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**Abstract.** The effects of various cadmium (Cd) concentrations (10, 50 and 100  $\mu\text{M}$ ) on root growth, cell division and micronuclei formation in root tip cells of *Allium cepa* var. *agrogarum* L. were investigated to better understand the processes of Cd-induced apoptosis. The results indicated that 10  $\mu\text{M}$  of Cd had an obviously passive influence on root growth during the 24 h treatment, and that the influence was even more serious with increasing Cd concentrations and duration of treatments. The mitotic index decreased with increasing Cd concentration and duration of treatments. Cadmium induced c-mitosis, chromosome bridges, chromosome stickiness and micronuclei. The frequency of cells with these disorders varied with prolonging the duration of treatments and increasing Cd concentrations. Micronuclei (MN) are biomarkers of genotoxic events. Our analyses proved the presence of a number of cells carrying micronucleus, and the elimination of the exceeding material itself, after exposing *A. cepa* var. *agrogarum* L. root tips to the Cd treatments. A number of micronuclei and nuclear anomalies were noticed at 10  $\mu\text{M}$  Cd. Inferences about the implications of the elimination of genetic material from micronuclei, such as cell viability and apoptosis, are also presented.

**Keywords:** *Allium cepa* var. *agrogarum* L.; Cadmium (Cd); Chromosome disorder; Micronuclei.

**Abbreviations:** MN, Micronuclei; CBMNcyt, cytokinesis-block micronucleus cytome; NPB, nucleoplasmic connection; Cd, Cadmium.

**Resumen.** Los efectos de varias concentraciones de cadmio (Cd; 10, 50 y 100  $\mu\text{M}$ ) se investigaron en el crecimiento de raíces, división celular y formación de micronúcleos en células de ápices radicales de *Allium cepa* var. *agrogarum* L. para entender mejor los procesos de apoptosis inducidos por Cd. Los resultados indicaron que 10  $\mu\text{M}$  de Cd tuvo una influencia obviamente pasiva en el crecimiento de raíces durante el tratamiento de 24 h, y que la influencia fue más seria con el incremento de las concentraciones de Cd y la duración de los tratamientos. El Cd indujo c-mitosis, puentes entre cromosomas, cromosomas pegajosos y micronúcleos. La frecuencia de células con estos desórdenes varió prolongando la duración de los tratamientos e incrementando las concentraciones de Cd. Los micronúcleos (MN) son biomarcadores de los eventos genotóxicos. Nuestros análisis probaron la presencia de un número de células transportando micronúcleos, y la eliminación del material excedente después de la exposición de ápices radicales de *A. cepa* var. *agrogarum* L. a los tratamientos de Cd. Un número de micronúcleos y anomalías nucleares se notaron a 10  $\mu\text{M}$  Cd. También se presentan inferencias acerca de las implicancias de la eliminación de material genético desde los micronúcleos, tal como la viabilidad celular y la apoptosis.

**Palabras clave:** *Allium cepa* var. *agrogarum* L.; Cadmio (Cd); Desorden de cromosomas; Micronúcleos.

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## INTRODUCTION

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Cadmium (Cd) is non-essential but toxic for biology, especially high Cd concentrations in soil (Schutzendubel & Polle, 2002; He et al., 2011; Rascio & Navari-Izzo, 2011). This heavy metal, which most likely enters the cell through the existing mineral uptake machinery, also constitutes a serious threat to human health (Peralta-Videa et al., 2009; Straif et al., 2009; Lin & Aarts, 2012). It is well documented that Cd exposure, for example, can cause growth inhibition related to reduction of mitotic activity, induction of chromosome disorders and nuclear abnormalities in the apical meristems (Liu et al., 2003/2004; Zhang et al., 2009; Qin et al., 2010). But genotoxicity of Cd has not been fully evaluated.

Micronuclei (MN) are biomarkers of genotoxic events and chromosomal instability. The genome damage event can be measured simultaneously in the cytokinesis-block micronucleus cytome (CBMNcyt) assay. This method is considered one of the most promising tests for evaluating environmental mutagenicity, since it is efficient, simple and fast (Cardozo et al., 2006). Micronuclei can originate during anaphase from lagging acentric chromosomes or chromatid fragments caused by mis- or unrepaired DNA breaks. Malsegregation of whole chromosomes at anaphase may also lead to MN formation as a result of hypomethylation of repeated sequences in centromeric and pericentromeric DNA, defects in kinetochore proteins or assembly, dysfunctional spindle and defective anaphase checkpoint genes (Fenech, 2000; Fenech et al., 2011). But the mechanism responsible for the formation of MN has not been fully explained (Gökalp Muranli et al., 2011).

*Allium cepa* is well known and commonly used in many laboratories because it is an excellent plant and a useful biomarker for environmental monitoring. Its use has many advantages such as low cost, a large number of roots, short testing time, easiness of storage and handling, large cells with excellent chromosome conditions, and easiness of observation of abnormal phenomena on chromosomes, nuclei, and micronuclei affected during mitosis (Liu et al., 1995; Qin et al., 2010). Conventionally, *Allium cepa* as a plant model system has been used to evaluate DNA damage in terms of chromosome disorders and disturbances in the mitotic cycle (Garaj-Vrhovac et al., 2013). Limited information is available on the effects of Cd on root growth, cell division and micronuclei formation in root tip cells of *Allium cepa* var. *agrogarum* L. Our objective was to study the effects of Cd on root growth, cell division and its potential to induce micronuclei formation and other cell alterations in root tips of *Allium cepa* var. *agrogarum* L.

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## MATERIALS AND METHODS

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**Culture condition and cadmium treatment.** Healthy and similar-size onion bulbs (*Allium cepa* var. *agrogarum* L.)

which had not started any growth were selected as materials. Before starting the experiments, we removed the dry scales of bulbs, and cleared them. We germinated and grew the bulbs in plastic containers by dipping the base in tap water and cultivating them in a growing chamber (day/night temperature 25/20 °C; relative air humidity 55/75%; photoperiod 14 h). Tap water was exchanged every 12 h. The bulbs were germinated in distilled water at 25 °C, producing roots reaching about 0.8 cm length. After that, they were placed in Petri dishes with 10, 50 and 100 µM Cd for 24 h, 48 h and 72 h, while distilled water was used as a control. The test liquids were changed regularly every 24 h. The Cd was provided as cadmium chloride.

**Cytological study.** Fifteen roots in each group were cut and fixed in 95% ethanol and 99.8% acetic acid (3:2) for 1 h, and hydrolyzed in 1 M hydrochloric acid, 95% ethanol and 99.8% acetic acid (5:3:2) for 5 min at 60 °C at the end of each time interval (24 h). For the observation of changes in cell division, chromosome disorder and micronucleus formation, 10 root tips were squashed in Carbol Fuchsin solution (Li, 1989).

Mitotic index and frequency of cells with chromosome disorders were used for the investigation. With this purpose, the number of dividing cells, and the number of different disorder types per 1000 observed cells were determined (Fiskesjö, 1985).

**Statistical analysis.** Each treatment was replicated 5 times for statistical validity. Analysis of variance was done with Sigma Plot 8.0 software. For statistical analysis, one-way analysis of variance (ANOVA) and t-test were used to determine the significance at  $p < 0.05$ .

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## RESULTS

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**Macroscopic effects of Cd on root growth.** The effects of Cd on root growth of *Allium cepa* var. *agrogarum* L. varied with concentration and treatment time (Figs. 1, 2). Studies have revealed that excess Cd inhibits root elongation and morphological changes. At 10 µM Cd there was obviously negative effect on root growth during the whole treatment course. Cadmium inhibited root growth significantly ( $p < 0.05$ ) at 10 µM Cd. At 50 µM Cd concentration, obvious toxic effects appeared after 24 h of treatment, and Cd inhibited root growth significantly ( $p < 0.05$ ). There was no root growth under 100 µM Cd during the whole treatment, except at 24 h (Fig. 2).

The effects of Cd on the morphology of roots also varied with the different concentrations of cadmium chloride in solution. At 10 µM Cd, the root tips were stunted and slightly bent after 48 h treatment. At 50 µM Cd, they were seriously stunted and bent in various directions after 24 h treatment. At 100 µM Cd, root tips were seriously rotten (Fig. 1).

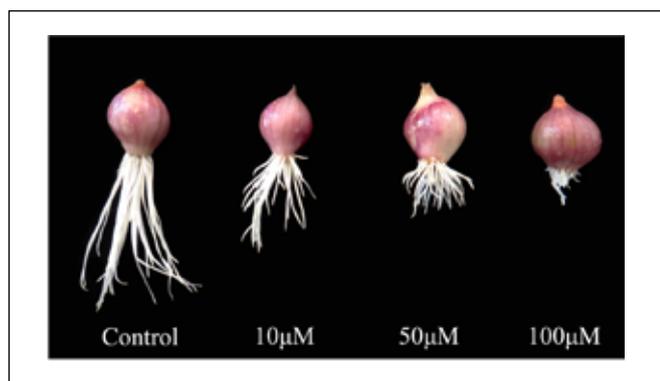


Fig. 1. Effects of various concentrations of Cd on root growth of *Allium cepa* var. *agrogarum* L. (72 h).

Fig. 1. Efectos de varias concentraciones de cadmio en el crecimiento radical de *Allium cepa* var. *agrogarum* L. (72 h).

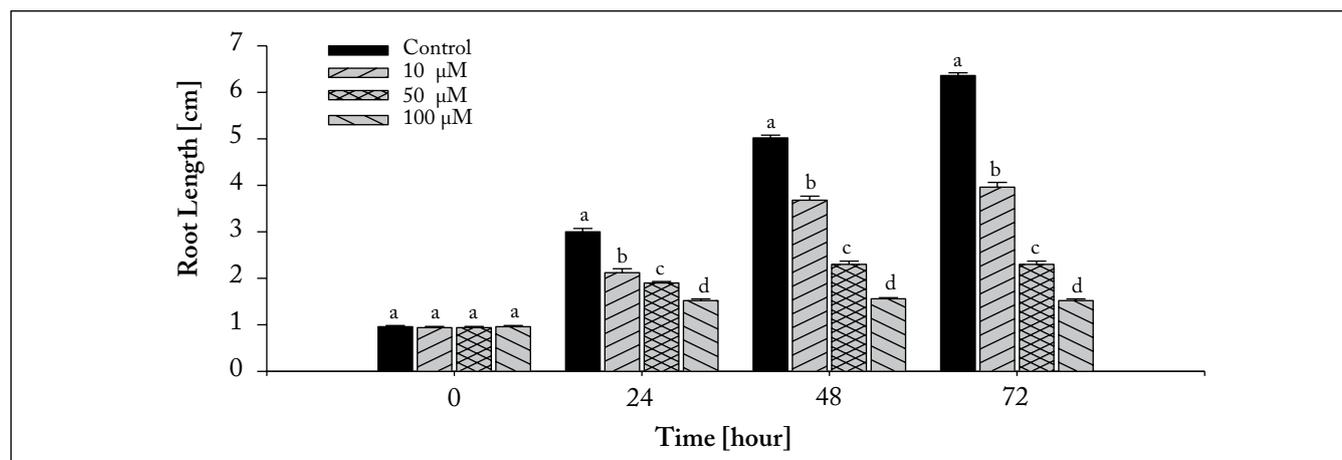


Fig. 2. Effects of different concentrations of Cd on root length of *Allium cepa* var. *agrogarum* L. Values with different letters differ significantly from each other ( $n = 5$ ,  $p < 0.05$ ).

Fig. 2. Efectos de diferentes concentraciones de cadmio en la longitud radical de *Allium cepa* var. *agrogarum* L. Los valores con letras diferentes difieren significativamente ( $n=5$ ,  $p<0,05$ ).

Table 1. Effects of Cd on cell division in the root tip cells of *Allium cepa* var. *agrogarum* L.

Table 1. Efectos del cadmio en la división celular del ápice radical de *Allium cepa* var. *agrogarum* L.

Cd treatment time (h)	Cd concentration (μM)	Cell number	Mitotic index (%)	Variant cells (%)				
				c-mitosis	chromosome bridge	chromosome fragment	chromosome stickiness	micronuclei
24	Control	1000	262 ± 17 a	4.7 ± 1.2 d	2.3 ± 0.6 b	0 ± 0 c	0 ± 0 c	4.0 ± 1.0 c
	10	1000	189 ± 19 b	18.0 ± 2.6 a	9.7 ± 1.2 a	0.3 ± 0.6 b	2.3 ± 0.6 c	76.7 ± 3.5 b
	50	1000	169 ± 30 bc	13.0 ± 1.0 b	3.3 ± 0.6 b	1.0 ± 0.0 a	16.3 ± 1.5 b	113 ± 8.5 a
	100	1000	139 ± 25 c	8.0 ± 1.0 c	2.0 ± 0.0 b	0.3 ± 0.6 b	63.7 ± 7 a	9.7 ± 3.0 c
48	Control	1000	241 ± 7 a	7.3 ± 1.5 b	3.3 ± 0.6 b	0 ± 0 b	1.0 ± 0.0 c	4.3 ± 1.5 c
	10	1000	167 ± 11 b	15.0 ± 1.0 a	12.7 ± 2.1 a	0.6 ± 0.6 ab	2.3 ± 0.6 c	139.3 ± 6.6 a
	50	1000	130 ± 8 c	8.0 ± 1.0 b	5.0 ± 1.0 b	1.0 ± 0.0 a	25.0 ± 5.0 b	90.7 ± 11.5 b
	100	1000	39 ± 9 d	3.9 ± 0.9 d	1.7 ± 0.6 c	0.7 ± 0.6 c	0.3 ± 0.6 ab	47.0 ± 4.0 a

Values followed by the same letters are not significantly different at ( $p < 0.05$ ). Means ± SE,  $n=3$ .

Valores seguidos por la misma letra no son significativamente diferentes ( $p < 0,05$ ). Promedios ± SE,  $n=3$ .

prolonging the duration of treatment at higher concentrations of Cd (50–100  $\mu\text{M}$ : Table 1). Chromosome bridges involving one or more chromosomes (Fig. 3d, e and f) were found at anaphase in the Cd treated groups. Chromosome bridges originated from dicentric chromosomes, which may have occurred due to the misrepair of DNA breaks, telomere end fusions, and could also be observed when separation of sister chromatids was defective at anaphase due to the failure of decatenation. The frequency of cells with chromosome bridges significantly increased ( $p < 0.05$ ) after treatment with 10  $\mu\text{M}$  Cd compared with the control, and there was no obvious effect with increasing Cd concentrations, except after exposure to 100  $\mu\text{M}$  Cd for 48 h (Table 1). Chromosome stickiness reflects highly toxic effects, probably leading to cell death (Fig. 3g, h). The frequency of cells with chromosome stickiness significantly increased ( $p < 0.05$ ) with increasing Cd concentrations (Table 1). In addition to the anomalies mentioned above, which are symptoms of genotoxicity effects of Cd, micronuclei were also found (Fig. 3i, Fig. 4). Micronuclei can originate during anaphase from lagging acentric chromosomes or chromatid fragments caused by misrepair of DNA breaks or unrepaired DNA breaks. The frequency of cells with micronuclei significantly increased ( $p < 0.05$ ) with increasing Cd (<100  $\mu\text{M}$ ) concentrations during a 24 h treatment, and significantly decreased ( $p < 0.05$ ) with prolonging treatment duration at higher concentrations of Cd (100  $\mu\text{M}$ : Table 1). The frequency of cells with micronuclei was highest at 10  $\mu\text{M}$  Cd after 48 h treatment. In addition to the disorders mentioned above, lagging chromosomes were almost not found (Table 1).

**Micronucleus formation.** It was seen that 24 h exposure to 10  $\mu\text{M}$  Cd significantly induced micronuclei (MN) formation (Table 1). Compared with controls, a significant increase in quantity of MN was observed in root tip cells of *Allium cepa* var. *agrogarum* L. after exposure to Cd (Table 1). Micronuclei formation (see Fig. 4) was considered to be the consequence of genotoxic events.

Normally, the cell of *Allium cepa* var. *agrogarum* L. contains one nucleus (Fig. 4a). The toxic effects of Cd on MN varied with treatment time. In the presence of Cd for 24 h, nuclear blebs consisting of nuclear material, with bud-shaped excrescences on the main nucleus, protruded from the nucleus, but without an obvious constriction or bridge between the protruding nuclear material and nucleus (Fig. 4b, c and j). Gradually, nuclear buds (NBUD) (which appeared like a MN) contained a narrow nucleoplasmic connection (NPB) to the main nucleus (Fig. 4d-f and l-o) or a relatively wide NPB (Fig. 4 h). Then, the micronuclei were well separated from the main nucleus (Fig. 4g, i). The quantity of MN induced gradually increased with prolonging the treatment duration (Fig. 4m-o). Figure 4m-o shows that cell contained multiple MN with different diameters of the main

nuclei, and some of them with NPB to the main nucleus in the group treated with 10  $\mu\text{M}$  Cd for 48 h. Finally, the majority of cells with micronuclei of irregular shapes tended to apoptosis (Fig. 4p).

## DISCUSSION

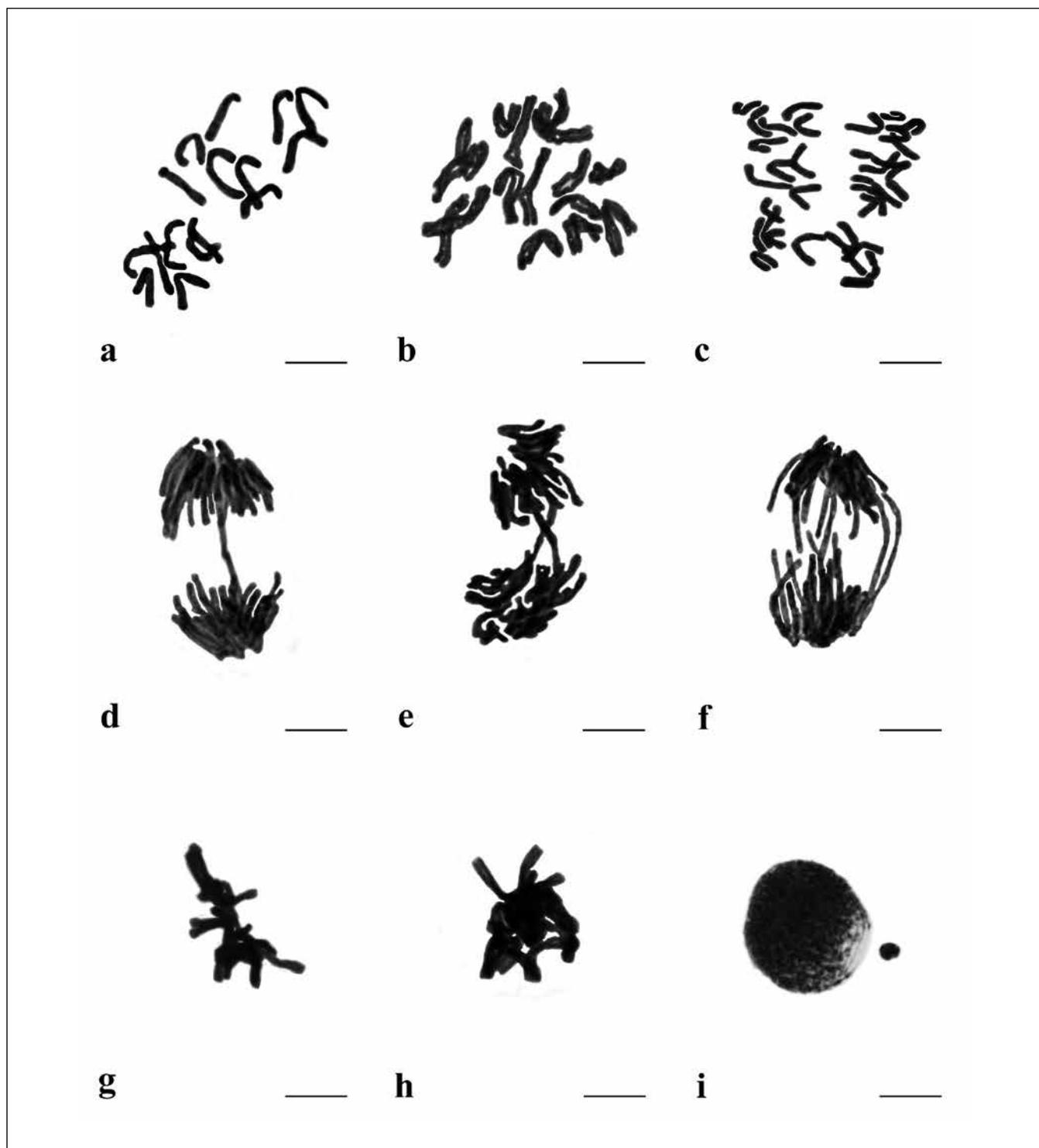
Cadmium pollution is a significant environmental problem that affects many physiological and biochemical processes (López-Millán, 2009). The most common effects of Cd toxicity in plants are stunted growth, leaf chlorosis and alteration in the activities of many key enzymes in various metabolic pathways (Zhang et al., 2009). In this investigation, reductions in root length of *Allium cepa* var. *agrogarum* L. were observed with increasing Cd concentrations.

The results from the present study indicated that Cd presented mutagenic activity. This effect can be related to its ability to promote alterations in the root cells of *Allium cepa* var. *agrogarum* L., such as cells bearing c-mitosis, anaphase bridges, chromosome stickiness and micronuclei. This is in agreement with the early findings of Liu et al. (2003/2004) and Zhang et al. (2009). Compared to c-mitosis and chromosome bridges, chromosome stickiness is a more serious disturbance in cytology (Zhang et al., 2009). Also, Cd induces numerous large micronuclei that are very easily observable, mainly at lower concentrations, such as 10  $\mu\text{M}$ .

Liu et al. (2003/2004) indicated that Cd, which inhibited cell division and cell expansion growth, could interfere with CaM which locates in mitotic spindle by influencing the  $\text{Ca}^{2+}$  uptake, thus causing the abnormal process of chromosome movement leading to mitotic abnormalities.

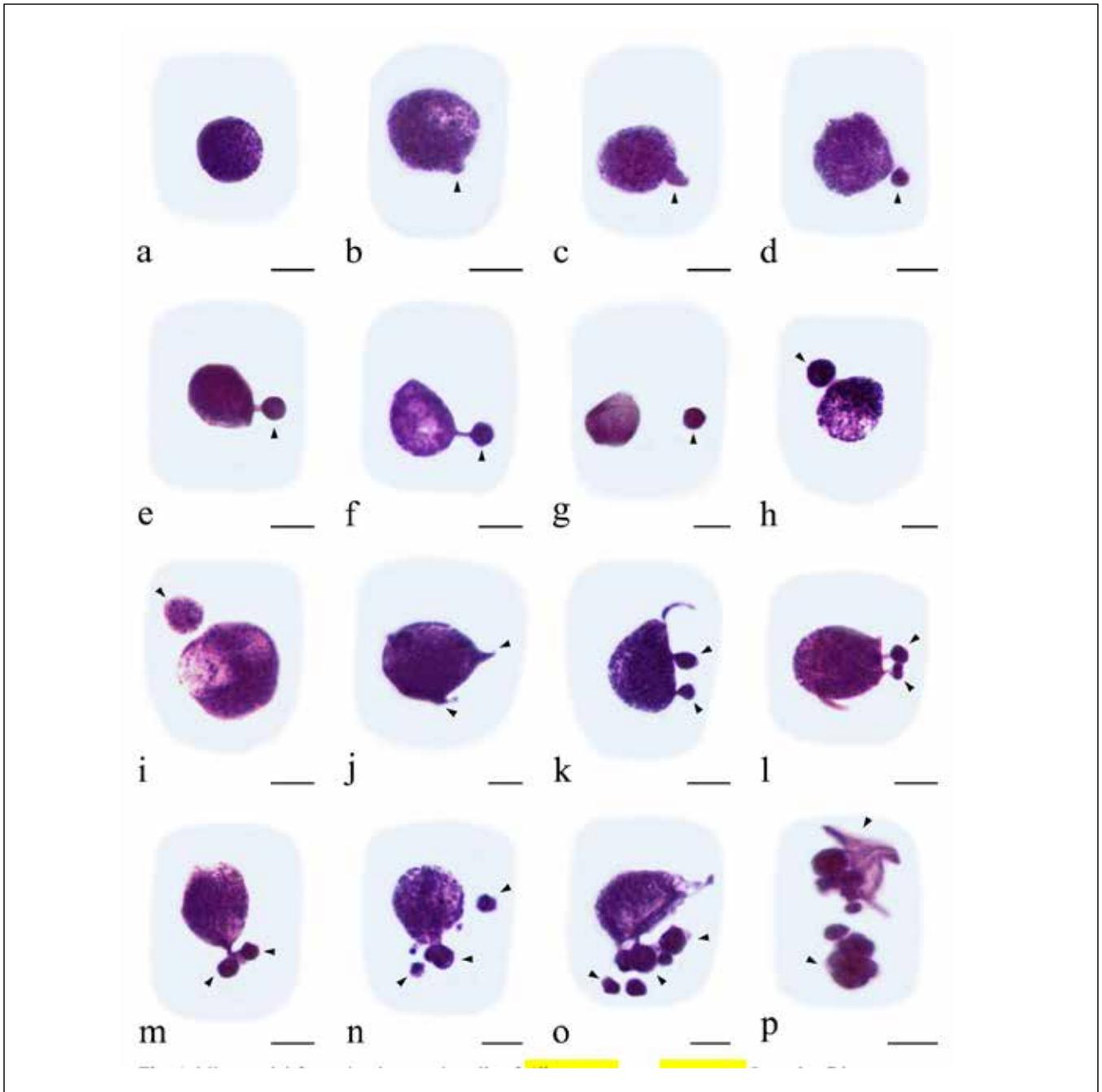
Significant increase in micronuclei formation was noticed in root tip cells of *Allium cepa* var. *agrogarum* L. exposed to Cd (Table 1). Cadmium also induced irregularly-shaped micronuclei as a genotoxicity. These structures were similar to those known as nuclear buds, found in *Allium cepa* by other authors (Fernandes et al., 2007; Leme et al., 2008; Leme & Marin-Morales, 2009). Irregularly shaped nuclei and micronuclei linked to the main nucleus have been previously observed in *A. cepa* after exposure to  $\text{Pb}^{2+}$  and  $\text{Cr}^{3+}$  (Liu et al., 1992; Liu et al., 1994).

The mechanisms responsible for MN have not been yet fully understood. Fenech (2011) proposed that micronuclei can be a result of misrepair of DNA double-strand breaks. This led to symmetrical and asymmetrical chromatid and chromosome exchanges, or fragments that failed to be included in the daughter nuclei at the completion of telophase during mitosis. This was because the lack of spindle attachment during the segregation process in anaphase. Other authors that support such suggestion (no Reintegration) include the elimination of genetic material from micronuclei as mini cells and the presence of highly compacted genetic material within micronuclei (Fernandes et al., 2007).



**Fig. 3.** Effects of Cd on root tip cell division of *Allium cepa* var. *agrogarum* L. (a–c) C-mitosis (a–b. 10  $\mu$ M Cd, 48 h; c. 100  $\mu$ M Cd, 24 h). (d–f) Chromosome bridges (d. 10  $\mu$ M Cd, 24 h; e. 10  $\mu$ M Cd, 48 h; f. 100  $\mu$ M Cd, 24 h). (g–h) Chromosome stickiness (g. 100  $\mu$ M Cd, 24 h; h. 100  $\mu$ M Cd, 48 h). (i) Micronuclei (10  $\mu$ M Cd, 24 h). Scale = 10  $\mu$ m.

**Fig. 3.** Efectos del cadmio en la división celular del ápice radical de *Allium cepa* var. *agrogarum* L. (a–c) C-mitosis (a–b. 10  $\mu$ M de cadmio, 48 h; c. 100  $\mu$ M de cadmio, 24 h). (d–f) puentes de cromosomas (d. 10  $\mu$ M de cadmio, 24 h; e. 10  $\mu$ M de cadmio, 48 h; f. 100  $\mu$ M de cadmio, 24 h). (g–h) Adherencia cromosómica (g. 100  $\mu$ M de cadmio, 24 h; h. 100  $\mu$ M de cadmio, 48 h); (i) Micronúcleos (10  $\mu$ M cadmio, 24 h). Escala= 10  $\mu$ m.



**Fig. 4.** Micronuclei formation in root tip cells of *Allium cepa* var. *agrogarum* L. under Cd stress. (a) ideal mononucleated cell; (b-c) nuclear blebs consisting of nuclear material protruding from the nucleus but without an obvious constriction or bridge between the protruding nuclear material and nucleus (cell containing NBUD); (d-f) NBUD that appears like a MN with a narrow nucleoplasmic connection (NPB) to the main nucleus; (g, i) cell with a MNi with 1/3 the diameter of the main nuclei within the cell. (h) cell with relatively wide NPB; (j-k) cell containing NBUDs; (l-m) NBUDs with NPB(s) to the main nucleus; (n-o) cell containing multiple MNi with different diameters of the main nuclei and some of them with NPB to the main nucleus; (p) apoptotic cell.

**Fig. 4.** Formación de micronúcleos en las células de los ápices radicales de *Allium cepa* var. *agrogarum* L. bajo estrés de cadmio. (a) célula ideal mononucleada; (b-c) protuberancias del núcleo que consisten de material nuclear saliente desde el núcleo pero sin una constricción o puente obvio entre el material nuclear saliente y el núcleo (célula conteniendo NBUD); (d-f) NBUD que aparece como un MN con una angosta conexión nucleoplásmica (NPB) al núcleo principal; (g, i) célula con un MNi de 1/3 del diámetro de los núcleos principales dentro de la célula. (h) célula con una NPB relativamente amplia; (j-k) célula conteniendo NBUDS; (l-m) NBUDS con NPB(s) al núcleo principal; (n-o) célula conteniendo múltiples MNi con diferentes diámetros de los núcleos principales y algunos de ellos con NPB al núcleo principal; (p) célula apoptótica.

One main objective of the present study was to investigate the mechanism responsible for MN induction in Cd-exposed *Allium cepa* var. *agrogarum* L. stained with Carbol Fuchsin solution.

In addition to MN, we evaluated also a number of other anomalies such as nuclear buds, bridges and cell apoptosis in the present study. While nuclear buds and bridges reflect DNA alterations and may be even more sensitive for the detection of genetic damage caused by certain exposures (Nersesyan et al., 2010), the former ones are caused by acute cytotoxic effects (Thomas et al., 2009). The mechanism triggering nuclear buds and bridge formation is unknown, but it may be related to chromosomal instability and gene amplification (Shimizu et al., 2005; Holland et al., 2008; Bonassi et al., 2011). According to our studies, we believe that if a cell undergoes a process of genetic material amplification, and such exceeding material may give rise to nuclear buds, it may result in such genetic material going from the nucleus to its membrane, and leads to consequent nucleoplasmic connection (NPB) to the main nucleus and eventually develop into micronuclei. We assumed that such MN would be the result of an event of elimination of the exceeding genetic material.

We also assumed that there was relevance between early apoptosis events and formation of micronuclei after treatment with Cd (Fig. 4) from our results. If a cell undergoes a process of genetic material amplification and such exceeding material is expelled through micronuclei, it is possible that the cells reestablish their viability, since their nuclear content would be normalized. Nevertheless, if the micronucleus is composed of chromosomal losses or chromosomal fragments, depending on the nature of the material lost, it can lead to a cell's death process (Fernandes et al., 2007).

The nuclear buds and the process of micronuclei formation is an initial process of elimination or they could lead to cell's death. Nevertheless, we cannot do any affirmation. However, it is possible to establish that the high number of cells bearing micronuclei can be an indicator of maintenance of cell physiology after exposure to Cd.

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