Notulae Botanicae Horti Agrobotanici Cluj-Napoca

Zinc-Induced Genotoxic Effects in Root Meristems of Barley Seedlings

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Abstract

The pollution increase, as a result of the release into environment of genotoxic chemicals, including heavy metals, largely affects the ecosystems and the health of living organisms. Although zinc is not considered highly phytotoxic, its excess becomes noxious. In literature, the reports on zinc genotoxicity are equivocal. Therefore, the objective of this work was to evaluate the amplitude of cytogenetic damage induced in *Hordeum vulgare* L. cv. 'Madalin' after seed treatment with different concentrations (10, 100, 250, 500 μM) of Zn^{+2}, provided as zinc sulphate and zinc acetate. The mitostimulatory effect was present at all concentrations of both zinc compounds. The rate of ana-telophase aberrations exceeded by 2 - 3 times the control, and the frequency of metaphase disturbances was 5.0-10.0 times higher than the control. The results indicate the clastogenic and aneugenic potential of zinc in barley and constitute a signal about the risks of its increasing presence into the environment, with repercussions on living systems, even on human health, due to the extensive use of zinc compounds including as pesticides.

Keywords: aneugenic effect, chromosome aberrations, clastogenic action, heavy metals

Introduction

In addition to its undeniable positive effects, the progress of human society had disastrous repercussions on biological systems due to the increase of the pollution level. Chemical pollution by heavy metals resulting from anthropogenic or natural sources is only one of the factors enhancing the risks for the environment and living organisms because they tend to accumulate in soil, water and plant and animal tissues. Despite the measures taken in many countries to counteract their release into the environment, this phenomenon has been continuously increasing. In Romania, in 2004, of the 0.9 million chemically polluted ha, 0.2 million ha were excessively contaminated with heavy metals that exceeded the maximum allowable limits (3 - 30 times for Pb, 2 - 32 times for Cd, 2 - 3 times for Zn, 2 - 4 times for Cu); high amounts were detected in plant leaves (Sanitation Country Profile, 2004).

Zinc is an essential nutrient for living organisms, representing the 23rd most abundant element on earth (Broadley *et al.*, 2007) and the 2nd most abundant transition metal, after iron (Jain *et al.*, 2010). Environmental Zn accumulation is due to mining and refining of nonferrous metals, chemical industry, burning of fossil fuels, and agricultural utilization of fertilizers and herbicides (Zn chloride and Zn sulphate) (Päivöke, 2003). The total Zn content of soils varies between 10 and 300 μg/g (Tewari *et al.*, 2008), or from 4 to 270 μg/L, in soil solutions, with a maximum of 17,000 μg/L in the highly contaminated soils (Kabata-Pendias and Pendias, 2001). Zn is redox-stable

under physiological conditions, being considered as one of the least toxic heavy metals (Codina *et al.*, 2000; Steinkellner *et al.*, 1998). It is a structural stabilizing factor of cell membrane and DNA-linking proteins (Salama and El Fouly, 2008) and it plays a significant role in the control of gene expression and DNA transcription (Päivöke, 2003). Zn excess becomes toxic to plants by altering the physiological processes (Jain *et al.*, 2010). The toxicity limits for Zn depend on plant species, genotype, and growth stage. Hence, about 300 ppm Zn is reported to be toxic to young barley (Davis *et al.*, 1978). Most commonly, the upper toxic levels range between 100 and 500 ppm (Macnicol and Beckett, 1985), whereas Paschke *et al.* (2006) emphasized that EC_{50} varies between 43 and 996 mg Zn/L.

As Zn forms stable complexes with nucleic acids, it can negatively influence their stability, so producing errors in the genetic information system (Patra *et al.*, 2004). The evaluation of the genotoxic impact of xenobiotics by investigating the extent of chromosome damage represents an effective method for biomonitoring studies. The interaction between Zn and DNA is little known in the light of its involvement in carcinogenesis. The studies on Zn genotoxicity and mutagenicity conducted in a variety of test systems resulted in non convincing results. Therefore, chromosomal aberrations, single-strand breaks or SCEs have been observed in rats and mice following *in vivo* exposure to Zn (Banu *et al.*, 2001; Kowalska-Wochna *et al.*, 1988), but there was no convincing evidence on the clastogenic effect in human lymphocytes exposed to Zn chloride (Deknudt and Deminatti, 1978). Also, Zn acetate did not

show mutagenic activity in *Salmonella* assays, but induced positive responses in *in vitro* cytogenetic CHO assay (Thompson *et al.*, 1989) and in *Vicia faba* micronucleus assay (Kumari *et al.*, 2012).

The continuous production and release of chemicals into the environment emphasize the necessity to assess their genotoxicity. Plants, which constitute a main link in the food chain, are often used to evaluate the genotoxic and mutagen potential of risk factors, due to the highly preserved structure of their genetic material. Barley is a plant of worldwide economic importance, which also has cancer preventive properties due to lunasin, a recently discovered peptide (Lumen, 2008). It has 14 large chromosomes and is often used as monitoring system in the evaluation of possible genetic risks of heavy metals.

The main purpose of this paper is to evaluate the genotoxic potential of Zn (provided as Zn sulphate and Zn acetate), expressed as chromosomal damage and mitotic irregularities, by analyzing the clastogenic and aneugenic effects induced in root meristems of barley seedlings.

Materials and methods

Seeds of early, autumn, six-row *Hordeum vulgare* L. cv. 'Madalin' provided by the Center for Agricultural Research and Development from Secuieni were used. This Romanian commercial variety having the grain yield potential over 6000 kg ha^{-1} (1000-seed weight = 42 - 45 g) was created at National Institute of Agricultural Research and Development - Fundulea (Patent 111732 B1 - 1994).

The barley grains were 3 min surface sterilized with freshly prepared solution of 2% NaOCl, operation followed by several washes to remove any trace of disinfectant. The seeds were then subjected for 3 hours to the treatment with solutions of 10, 100, 250, and 500 μM ZnSO$_4$.7H$_2$O (MW = 287.5799 g/mol) and (CH$_3$COO)$_2$Zn.2H$_2$O (MW= 219.5286 g/mol) containing 0.654, 6.54, 16.35, and 32.70 μg ml^{-1} Zn^{2+}, respectively. Controls were free from any heavy metal. After chromium treatment and several rinses with running tap water, the seeds were transferred on moistened filter paper, in order to germinate. Petri dishes were covered and incubated in dark at 20°C.

For cytogenetic analysis, barley roots (10 - 15 mm in length) were fixed for 24 h in ethyl alcohol:glacial acetic acid (3:1, v/v), at room temperature, then washed and stored in 70% ethyl alcohol. The plant material was hydrolyzed for 10 minutes in 50% HCl, and then stained in modified charbol fuchsin (24 hours, at +4°C (Gamborg and Wetter, 1975). To prepare the slides (five per variant) - the meristematic regions were squashed into 45% acetic acid (Raicu *et al.*, 1973). 10 fields were microscopically analyzed on each slide. A Nikon Eclipse 600 light microscope was used for this analysis. Photos were taken with a Nikon Cool Pix 950 digital camera, 1600×1200 dpi.

The cytogenetic indicators were calculated according to the following formulas: *Mitotic index (MI) (%) =* TDC × 100/TC; *prophase index (PI%) =* prophase cells × 100/TDC; *metaphase index (MeI%) =* metaphase cells × 100/TDC; *anaphase index (AI%) =* anaphase cells × 100/TDC; *telophase index (TI%) =* telophase cells × 100/TDC, where TDC: total dividing cells and TC: total cells (dividing and non-dividing).

The rate of *ana-telophase chromosome aberrations (A-T$_{CA}$%)* and the rate of *metaphase abnormalities (M$_{abn}$%)* were also calculated in relation to the number of cells in mitosis: A-T$_{CA}$% = A-T$_{CA}$ × 100/TDC, and M$_{abn}$ %= M$_{abn}$ × 100/TDC.

The data were expressed as mean ± standard error of the means for all groups of investigated parameters. The Microsoft Office Excel 2003 software of Windows XP operating system was used to calculate and graphically represent the statistical parameters.

Results and discussion

Mitotic index and frequency of cell division stages

In Patra's classification (2004) based on the severity of the effects on mitosis, the heavy metals are grouped in three classes, Zn being included in the class with marked impact on cell division, together with Al, Mn, Fe, Se, Sr, Sb, Ca, and Ti. Contrary to the opinions expressed in other studies (Jain *et al.*, 2010; Powell *et al.*, 1986), in this research Zn augmented MI as compared to the control, the stimulatory effect resulting in increases of approximately 23 - 57% in Zn acetate treated variants, and 10 - 80% for Zn sulphate (Tab. 1; Fig. 1). In Zn sulphate treated variants, the highest average value of MI was registered for the 100 μM concentration. From this point, a progressive MI decrease occurs in variants exposed to 250 and 500 μM concentrations, but the level still remains over the control. In Zn acetate treated variants, a descending trend from 10 μM to 250 μM was noticed, followed by a MI increase at the maximum tested concentration. Whereas some previous studies (El-Ghamery *et al.*, 2003; Shaymurat *et al.*, 2012) revealed a dose-dependent inhibition of MI in Zn treatments, Somesh *et al.* (2005) confirmed the mitodepressive potential of Zn acetate and Zn sulphate only at concentrations over 500 ppm, while the lower concentrations, comparable with those tested in our study, stimulated MI.

As concerns the frequency of mitotic phases, the decreasing order of the division stages was generally the following: PI% > MeI% > TI% > AI%. Except for 500 μM Zn sulphate and 250 μM Zn acetate, where the average PI% is close to the control, in the other variants the number of prophases exceeds the control. The MI increase is the result of the accumulation of prophases and metaphases, or of telophases, as it happens in 10 μM Zn acetate (Tab. 1; Fig. 2). Alterations in the frequency of mitotic phases as compared to the control have also been reported in wheat and *Nigella sativa* after Zn supply (El-Ghamery *et al.*, 2003).

Tab. 1. Behaviour of the cytogenetic parameters in barley root tip meristems, after seed short term exposure to different concentrations of zinc sulphate and zinc acetate

Variant		Analyzed cells		MI (%)*	PI%	MeI%	AI%	TI%
		Number	x±SE*					
Zinc sulphate	0 μM	9694	1938.8±246.46	3.88±0.18	29.27	27.42	20.84	22.45
	10 μM	6732	1346.4±29.51	4.26±0.38	35.54	25.73	17.77	20.93
	100 μM	4153	830.6±49.82	7.04±0.52	34.73	21.12	20.38	23.73
	250 μM	7864	1006.4±51.00	5.16±0.38	36.67	28.39	18.31	16.59
	500 μM	6138	1227.6±126.34	4.42±0.42	28.22	31.35	18.84	21.57
Zinc acetate	0 μM	9694	1938.8±246.46	3.88±0.18	29.27	27.42	20.84	22.45
	10 μM	6100	1220.6±100.20	5.90±0.54	30.27	26.15	17.24	26.32
	100 μM	5202	1040.4±49.55	5.55±0.24	32.67	30.12	18.46	18.73
	250 μM	5677	1135.4±32.11	4.77±0.35	28.62	29.07	19.77	22.54
	500 μM	5750	1150.0±54.29	6.08±0.62	34.97	24.00	18.36	22.67

*mean ± standard error of the means; MI = mitotic index; PI% prophase index; MeI% = metaphase index; AI%= anaphase index; TI%= telophase index (mitotic phases values were calculated considering mitotic index as 100%)
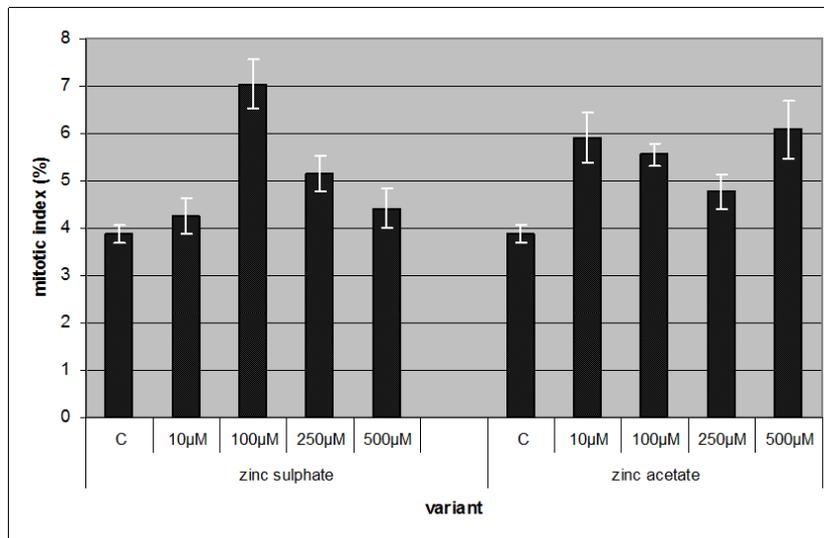

Fig. 1. Mitotic index in barley root meristems, after zinc treatment (the bars represent the standard errors of the means)
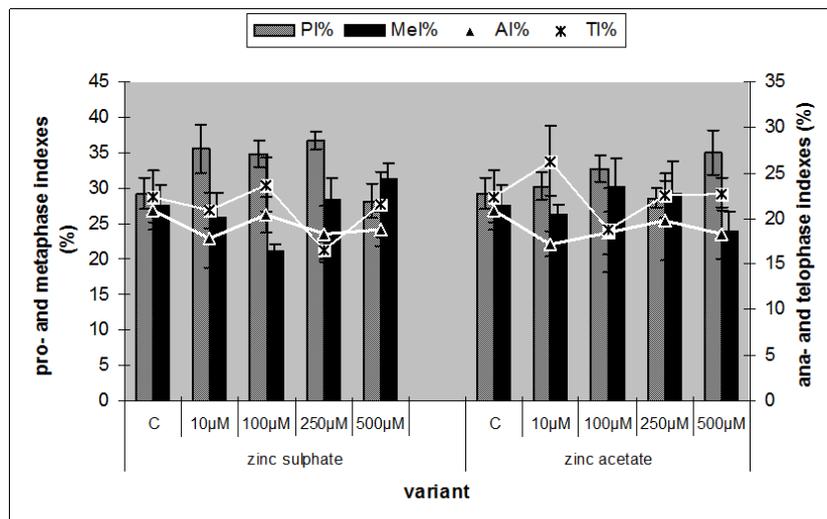

Fig. 2. Frequency of the mitotic stages in barley root meristems, after zinc treatment (the bars represent the standard errors of the means)

Frequency of abnormal metaphases

M_{abn}% is much higher as compared to A-T_{CA}%. Hence, Zn sulphate determined 5.4 - 8.3 times more abnormal metaphases, whereas Zn acetate induced 7.0 - 10.0 times more metaphase disturbances, as compared to the control (Fig. 3). Concerning disturbance categories, C-metaphases, which indicate an effect of zinc similar to that induced by colchicine, are numerically preponderant in all Zn-treated variants (Fig. 4).

Metaphase stickiness (Fig. 6h) occurred within a 3 to 11% variation range (the highest value was observed for 250 µM Zn acetate). In all Zn-treated variants, chromosomes expulsed from equatorial plates have been detected. Their level was 3.0 - 5.5 times higher than the control in Zn sulphate treated variants, and 3.5 - 7.0 times higher for Zn acetate (Fig. 4).

The high values of C-metaphases, scattered and expelled chromosomes (Fig. 6d, e, i) prove the aneugenic action of Zn compounds, probably due to the perturbation of calmodulin, a small Ca^{2+}-binding protein involved in the chromosome moving by the control of microtubule polymerization/depolymerization (Zou *et al.*, 2006).

Frequency of ana-telophase chromosome aberrations

All concentrations of Zn-containing compounds induced important increases of the rate of ana-telophase chromosome aberrations (Fig. 3). A-T_{CA}% in root tip meristems of Zn-treated variants of barley exceeded by 2 - 3 times the control, in a progressive manner up to 250 µM, in Zn sulphate treated variants, and up to 500 µM, in Zn acetate treated variants.

Chromosome bridges (Fig. 5 and 6c) surpass by 2.2 - 2.6 times the control in Zn sulphate treatments, and by 1.6 - 3.3 times in Zn acetate treated variants. The highest levels were encountered in 250 and 500 µM Zn acetate.
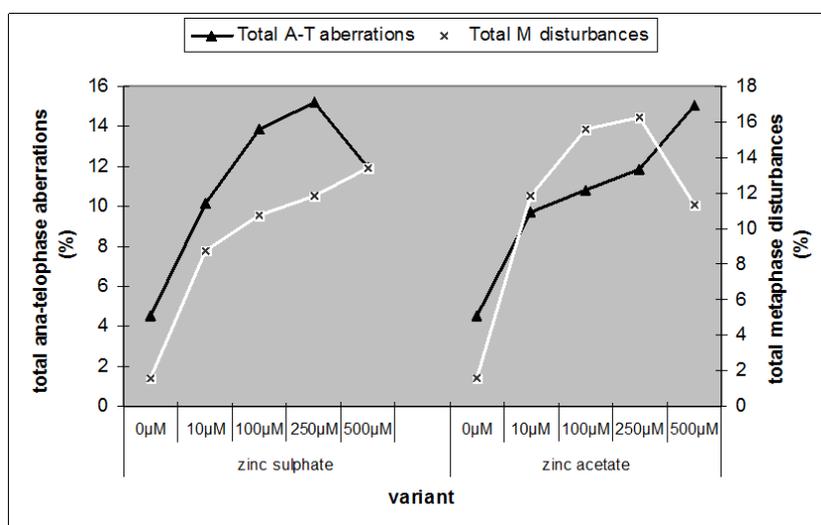

Fig. 3. Incidence of the ana-telophase aberrations and of metaphase abnormalities induced by zinc in barley root meristems
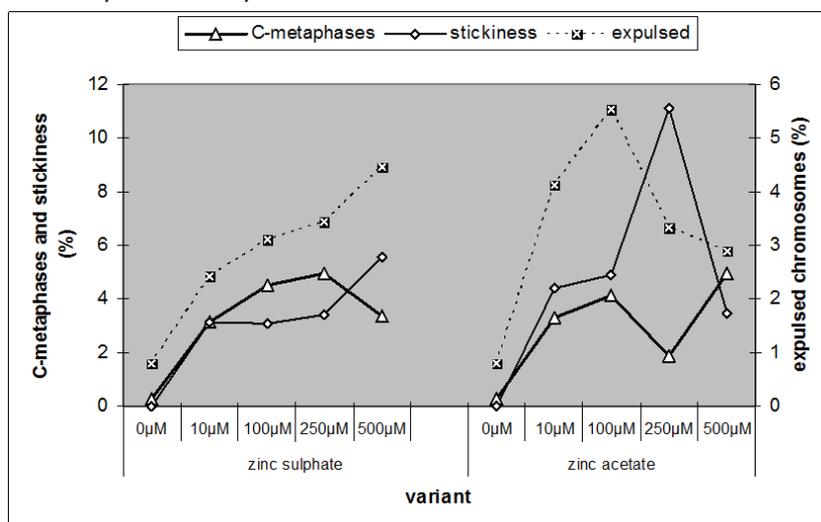

Fig. 4. Incidence of the main categories of zinc-induced metaphase disorders in barley root meristems

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Complex aberrations (multipolarity + bridges + expelled chromosomes + laggards; bridges + expelled chromosomes etc.) show high frequency (Fig. 5, 6f). They are often accompanied by major changes in the phenotype expression. In Zn acetate treatments, their levels surpassed by 2.5 - 5.0 times the control, and for Zn sulphate the complex aberrations were 1.2 - 4.2 times more numerous as compared to the control. Instead, the occurrence of laggards was 5 - 7 fold higher than the control in Zn sulphate exposures, while for Zn acetate their frequency varied between 1.16 and 1.64% (2 - 3-fold increases as compared to the control) (Fig. 5). The extent of multipolarity and expelled chromosomes was smaller.

250 μM and 500 μM Zn acetate induced lysis of chromatin material (Fig. 6g) and stickiness in all stages of mitotic division (Fig. 6h). Stickiness indicates an increased toxicity of the tested chemicals the effects of which - usually irreversible - result in cell death. The maximum Zn acetate concentration caused nuclear material fragmentation or determined the occurrence of different nuclear shapes (Fig. 6b). The presence of micronuclei was sporadically detected in 500 μM Zn sulphate; they can be acentric fragments (clastogenic response) or can result from deficient function of mitotic spindle (aneugenic response). Micronuclei were also reported at high Zn^{2+} doses in *Vicia faba* (Kumari *et al.*, 2012).
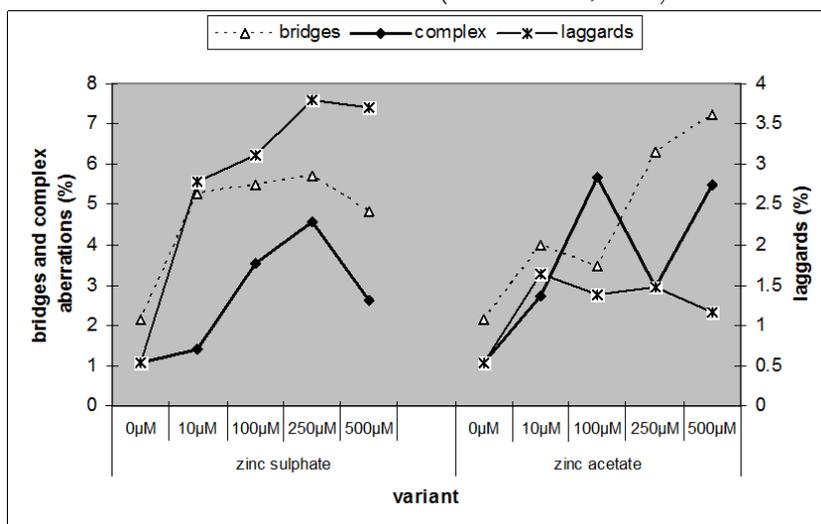

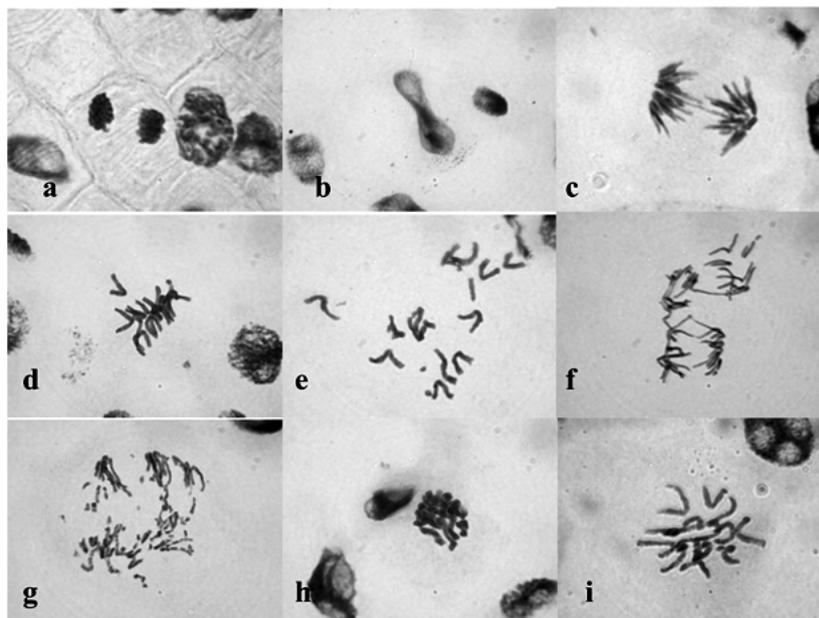Fig. 5. Frequency of the main categories of zinc-induced ana-telophase chromosome aberrations in barley root meristems

Fig. 6. Types of zinc-induced disturbances. a. polar deviation - 500 μM Zn sulphate; b. atypical nucleus in interphase - 250 μM Zn sulphate; c. bridge - 250 μM Zn acetate; d. metaphase with expulsed chromosome - 10 μM Zn sulphate; e. scattered metaphase chromosomes - 500 μM Zn sulphate; f. complex aberration - 250 μM Zn sulphate; g. chromatin lysis in anaphase - 10 μM Zn sulphate; h. stickiness - 500 μM Zn sulphate; i. C-metaphase - 500 μM Zn acetate

Contrasting and inconclusive data on the genotoxicity of Zn compounds are available in literature. The observations recorded on several herbaceous and woody species showed differentiated responses, depending on the concentration range, exposure duration, plant species, class of Zn compounds, and treatment with unary or binary solutions (Ince *et al.*, 1999; Marcato-Romain *et al.*, 2009; Patra *et al.*, 2004; Steinkellner *et al.*, 1998), but also on the number of somatic and metacentric chromosomes or on the length of the diploid complement (Ma *et al.*, 1995). Some reports state that high Zn concentrations are not strongly genotoxic (Codina *et al.*, 2000; Gómez-Arroyo *et al.* 2001; Marcato-Romain *et al.*, 2009). The aneugenic and clastogenic action of Zn was also evidenced in other species like wheat, black cumin, onion, sugarcane (El-Ghamery *et al.*, 2003; Jain *et al.*, 2010; Shaymurat *et al.*, 2012; Somesh *et al.*, 2005), but a connection between Zn concentration and aberration frequency was not always noticed.

The relatively wide range of rates of metaphase abnormalities and ana-telophase chromosome aberrations supports the genotoxic potential of zinc. The high level of chromosome aberrations offers indications on their interference with nucleic acids and on the clastogenic potential of Zn, while the disturbances implying the mitotic spindle prove its aneugenic action.

Conclusions

Although Zn holds one of the last places regarding cyto- and genotoxicity, the extent of the clastogenic and aneugenic responses to Zn action in the investigated barley genotypes is significant enough to conclude that it is necessary to extend the assessment of the genetic impact of Zn, in order to gain thorough knowledge not only of the induced chromosome damage but also of the consequences on some phenotype traits of economic interest. These results can be used to improve the management of heavy metal-containing compounds, with positive repercussions on habitats and the health of living systems.

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