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ORIGINAL ARTICLE

Exposure of *Vicia faba* and *Pisum sativum* to copper-induced genotoxicity

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Abstract The potential genotoxicity of Cu^{2+} was investigated in *Vicia faba* and *Pisum sativum* seedlings in hydroponic culture conditions. Cu^{2+} caused a dose-dependent increase in micronuclei frequencies in both plant models. Cytological analysis of root tips cells showed clastogenic and aneugenic effects of this heavy metal on *V. faba* root meristems. Cu^{2+} induced chromosomal alterations at the lowest concentration used (2.5 mM) when incubated for 42 h, indicating the potent mutagenic effect of this ion. A spectrum of chromosomal abnormalities was observed in *V. faba* root meristems, illustrating the genotoxic events leading to micronuclei formation.

Keywords Chromosomal aberrations · Genotoxicity · Heavy metal · Micronuclei · Root tips

Abbreviations
MCN micronuclei
MH maleic hydrazide

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Introduction

Heavy metal deposition has increased dramatically over the last decades as a result of anthropogenic activities leading to heavily polluted areas worldwide. One of the major problems of this environmental pollution is copper over-accumulation in soils. Its concentration can reach 500 mg kg^{-1} in vineyard soils (Deluisa et al. 1996; Brun et al. 2001) or even more in the vicinity of copper-nickel smelters in the northern hemisphere, with 5800 mg kg^{-1} being measured in the organic layer of the strongest polluted places (Helmisaari et al. 1995). Copper is an essential micronutrient for higher plant growth and metabolism (Maksymiec 1997). However, its high bioavailability in soils make it a potentially toxic substance causing inhibition of growth and oxidative injuries (Hall 2002; Schützendübel and Polle 2002; Yruela 2005).

The formation of micronuclei (MCN) in root tips has been widely described and used as a bioassay for the evaluation of in vivo mutagenic effects of environmental pollutants on plants (Grant and Owens 1998; Knasmüller et al. 1998; Türkoglu 2007). MCN can be composed of small chromosome fragments resulting from chromosome breaks caused by clastogenic activity. The failure of entire chromosomes to migrate during anaphase as a result of the aneugenic effects of genotoxic agents can also lead to MCN formation (Krishna and Hayashi 2000; Çava and Ergene-Gözükara 2003).

In this study, we analysed and compared the potentially genotoxic effects of high Cu^{2+} concentrations on two closely related Fabaceae species, *Vicia faba* and *Pisum sativum*. Cytological analysis of *V. faba* meristematic root cells showed the potential clastogenic and aneugenic effects of Cu^{2+} on *V. faba* chromosomes. The different types of mitotic chromosomal abnormalities could be grouped into

two classes, according to the structural and/or numerical disturbances that might lead to MCN formation.

Materials and methods

The genotoxicity of an essential heavy metal for meristematic root cells of two related Fabaceae species, *V. faba* and *P. sativum*, was analysed. Both species have a low chromosome number (2n=12 and 2n=14, respectively), making them suitable for cytogenetic studies. Seeds of *V. faba* var. Aguadulce and *P. sativum* var. Douce de Provence were germinated on saturated paper at 25°C for 4 to 5 days, and transferred to a hydroponic support in the following nutrient solution (pH 7): 3.9 mM $Ca(NO_3)_2$, 6.5 mM KNO_3, 2 mM $MgSO_4$, 0.9 mM KH_2PO_4 plus micronutrients: 90 μM Fe-ethylene diamine tetra-acetic acid, 2.7 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 4.5 μM H_3BO_3, 4 μM $CuSO_4$ and 2.0 μM $Mo_7O_{24}(NH_4)_6$. Once roots had reached a length of 2–3 cm, additional concentrations of CuSO4 (1 to 50 mM) were added to the hydroponic solution for 42 h at 25°C with a light/dark photoperiod of 16:8 h. All tests were repeated three times, using a negative control (containing no additional Cu^{2+}), and nutrient solution containing 4×10^{-3} M maleic hydrazide (MH) as a positive control. MH is a herbicide known to be a mutagenic and clastogenic agent (Marcano et al. 2004).

Root tips (meristem zones) were cut and placed overnight in the dark in the Carnoy fixation solution containing ethanol and glacial acetic acid (3:1) at 4°C, and then stored in 70% ethanol. Root tips were rinsed with distilled water and hydrolysed with 1 N HCl for 10 min. The root cap was removed before squashing root tissues, and samples were stained with orcein. The slides were examined under a Zeiss microscope. At least three slides were stained per replica and at least 1000 cells were scored from each slide. Therefore, the analysis was conducted on an average of 9000 cells per treatment. MCN frequency was calculated from the number of MCN scored divided by the total cells scored, and expressed in terms of MCN/1000 cells.

Results and discussion

Micronuclei induction

In order to assess the genotoxic effects of Cu^{2+}, different concentrations were applied for a transient period of 42 h in hydroponic cultures. The frequencies of cells with Cu^{2+}-induced MCN in meristematic root tips of both plant species are presented in Fig. 1. Low MCN frequencies were detected in root tips of control plants. However, as expected, MH dramatically increased the frequency of MCN in root meristems as compared to control plants, showing that this herbicide can be used as a positive control for genotoxicity studies.

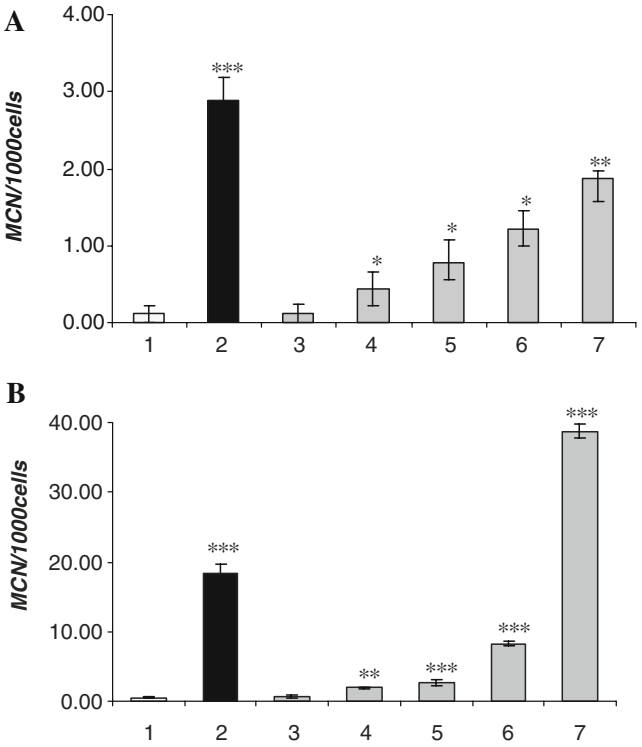
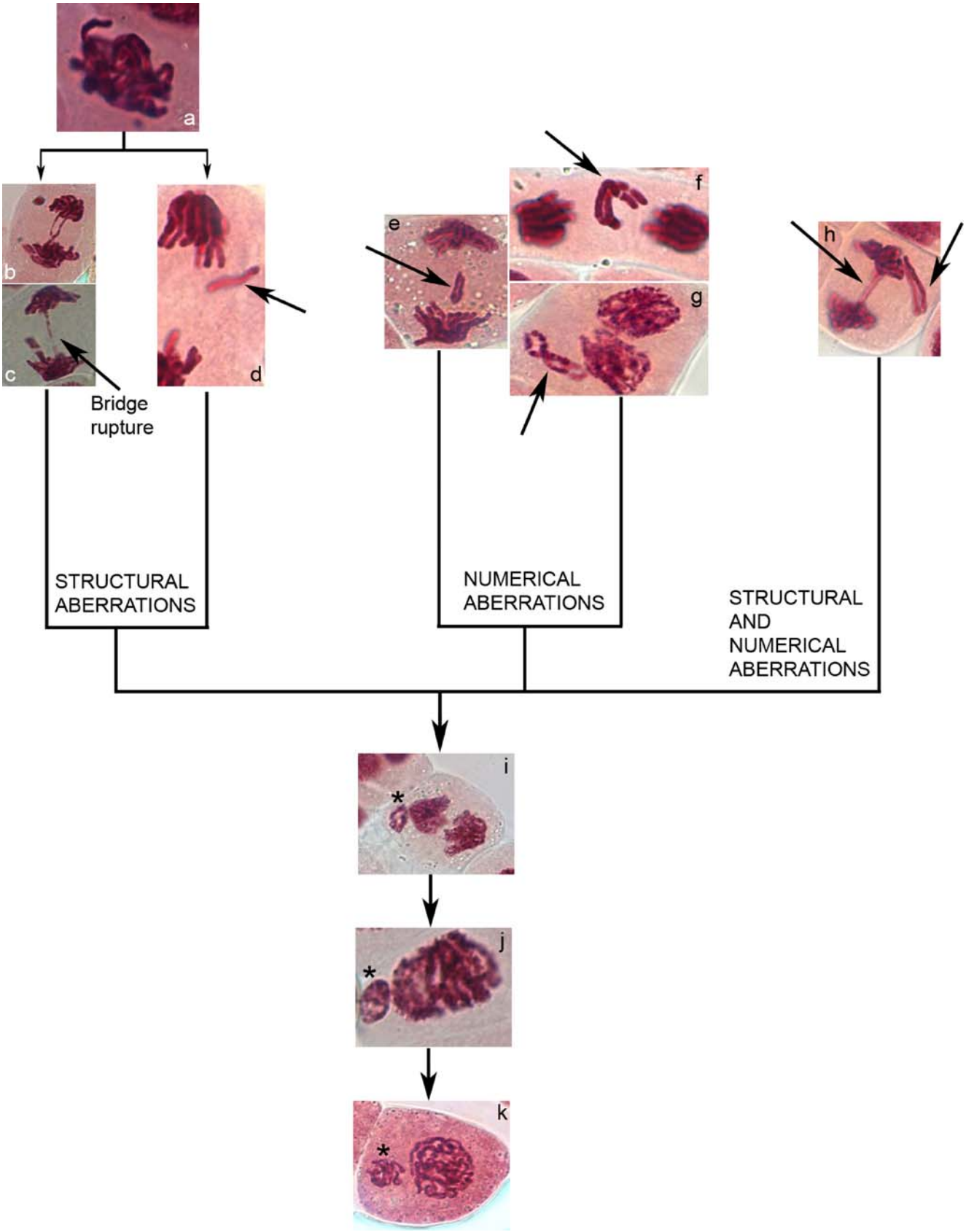


Fig. 1 Dose effect of Cu^{2+} on MCN induction in *Pisum sativum* (**A**) and *Vicia faba* (**B**) root cells. *Empty bar*: *1*: Control, *2*: MH, *filled bar*: $CuSO_4$ (*3*: 1 mM, *4*: 2.5 mM, *5*: 5 mM, *6*: 10 mM, *7*: 50 mM). Values are the means of three replicas. *Bars* indicate SD. *$P<0.05$; **$P<0.01$;*** $P<0.001$

Cu^{2+} significantly enhanced the frequency of MCN formation in the root tips of *V. faba* and *P. sativum* ($p\geqq 0.05$). MCN induction was significant ($p<0.05$) when 2.5 mM Cu^{2+} was used. In both plant species, the frequency of MCN formation was proportional to the Cu^{2+} concentration added to the hydroponic solution. In our experiments, the highest frequency of cells with MCN was detected in the presence of 50 mM Cu^{2+}. At this high Cu^{2+} concentration, the MCN frequency in root tips was dramatically higher in *V. faba* than in *P. sativum* (20 times

Fig. 2 Cytological analysis of chromosomal aberrations in *Vicia faba* meristematic root cells, illustrating the different origins of MCN formation. The mitotic chromosomal abnormalities can be grouped into two classes: structural and numerical aberrations. Both structural and numerical aberrations may be observed in mitotic cells. *a*: stickiness in metaphase, *b* and *c*: chromosome bridges in anaphase, *d*: chromosome breaks in telophase, *e* and *f*: laggard chromosomes in telophase, *g*: isolated chromosome in telophase, *h*: acentric chromosome and anaphase bridge, *i* and *j*: micronuclei in telophase and interphase, respectively, *k*: micronuclei in prophase. *Arrows* indicate the chromosomal aberrations and *Asterisks* the micronuclei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higher), suggesting that *V. faba* is more sensitive than *P. sativum*. The potential tolerance of *P. sativum* to chemicals has been demonstrated with MH treatment and other chemicals (Jain and Sarbhoy 1987a, b). As previously suggested (Ma et al. 1995), a greater total length of the diploid complement and higher number of metacentric chromosomes may result in greater sensitivity to DNA damage-inducing agents like heavy metals. *Vicia* contains long and thin chromosomes suitable for genotoxicity analysis, while *Pisum* contains shorter chromosomes (Blixt 1972). Further investigation is required to determine whether metacentric chromosomes present a potential target for heavy metal action.

Induction of chromosomal aberrations

We investigated chromosomal aberrations in *V. faba* root cells, since this species displayed higher sensitivity to Cu^{2+} in terms of MCN induction. In addition to MCN formation, cytological analysis of root meristems revealed drastic changes in the organization and morphology of chromosomes (Fig. 2). We could group the chromosomal abnormalities detected in meristematic cells into two classes: structural and numerical aberrations. Our results corroborate the documented MCN formation in animal cells containing chromosomal aberrations induced by exposure to genotoxic agents (Krishna and Hayashi 2000; Iarmarcovai et al. 2006). Chromosome stickiness should be the initial event that occurs in prophase cells. This has been described as chromosome agglutinations displaying a sticky appearance (Rieger et al. 1976) and a winding of chromosomal fibres created by chromatid bridges (Jiang et al. 2000). This phenomenon is regarded as a physiological effect exerted by chemical agents that may affect peripheral proteins such as DNA topoisomerase II (Gaulden 1987). According to Gömürgen (2005), this chromosomal abnormality leads to chromosome bridges, a result of the failure of free anaphase separation, unequal translocation or inversion of chromosome segments. Since stickiness holds the two chromosomes together, the separation process can lead to chromosome rupture, i.e. chromosome break formation. Therefore, chromosome stickiness, chromosome bridges and chromosome breaks are closely related. These chromosome aberrations, sticky metaphase, anaphase bridges and fragments of chromosomes in telophase (Fig. 2a–d), were detected during the mitotic phases when *V. faba* seedlings were incubated with Cu^{2+}. Since Cu^{2+} induced chromosome fragmentation, this heavy metal should be considered as a clastogenic agent.

Besides structural aberrations, Cu^{2+} also induced numerical aberrations in *V. faba* root meristems. Numerical aberrations resulting from acentric fragments or lagging chromosomes lead to aneuploidy, i.e. loss of genetic material and MCN formation. Aneugenic effects are represented by laggard chromosomes (Fig. 2e) and acentric chromosomes in telophase (Fig. 2f,g). Figure 2h illustrates that anaphase bridges and acentric chromosomes, which lead to structural and numerical aberrations, respectively, can occur simultaneously in mitotic cells. Earlier reports have demonstrated that heavy metals can cause spindle perturbance. Several authors have described the alteration of chromosome numbers by environmental aneuploidy-inducing agents that induce microtubule and kinetochore disorganization in mitotic cells (Borboa and De la Torre 1996; Voutsinas et al. 1997; Seoane and Dulout 2001; Qian 2004). Dysfunction of the spindle mechanism is mainly due to the reactivity of metal ions with thiol groups of tubulin, a multi-cysteine protein in which the cysteine residues are actively involved in regulating microtubule-assembly dynamics (Chaudhuri et al. 2001). Since Cu^{2+} has a high affinity for sulphydryl groups (Maksymiec 1997), the aneuploidy phenomenon observed in *Vicia* root meristems might be due to the high sensitivity of the microtubular apparatus to this ion.

The structural and numerical disturbances of chromosomes induced by the heavy metal during mitosis are expected to lead to MCN formation. Chromosome breaks or anaphase bridges, grouped into structural aberrations, result in chromosomal fragments segregating independently to daughter nuclei. Chromosome laggards or non-separated chromosomes, grouped into numerical aberrations, fail to migrate towards either of the daughter nuclei during telophase of the mitotic cells (Krishna and Hayashi 2000; Iarmarcovai et al. 2006). MCN formation was also obvious in *V. faba* root cells during telophase or interphase (Fig. 2i,j). Cells containing abnormal chromosomes could enter a new mitotic cycle (Fig. 2k).

To conclude, our work illustrates the genotoxic events leading to MCN formation through different pathways in the studied model plant. This is obvious even when an essential element like Cu^{2+} is used at a high concentration (>2.5 mM), causing chromosome breaks and chromosome migration impairment. This heavy metal, therefore, acts as a potent genotoxic agent.

In an environmental perspective, the bioavailability of Cu^{2+} might become an increasing concern for a range of living organisms including cultivated plants. The necessity to elucidate Cu^{2+} toxicity results from its systematic use as a fungicide, algicide or bacteriocide in agriculture. The evaluation of Cu^{2+} bioavailability is highly complex and plant species specific, since it is related not only to the physical-chemical properties of the soil but also to processes governed by plants (Hinsinger et al. 2006). Plant bioassay systems allowing the detection of cellular genotoxic events in meristems provide the best approach for assessing metal bioavailability, especially for Cu^{2+} which

accumulates substantially in the roots of plants. Another indicator of Cu^{2+} bioavailability has also been developed, in which Cu^{2+} uptake is estimated by quantifying the Cu^{2+} content of the plant (Brun et al. 2001). Combining plant bioassays and root analyses of Cu^{2+} content would allow us to define phytotoxicity thresholds in plants.

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