Carpathian Journal of Earth and Environmental Sciences, November 2013, Vol. 8, No. 4, p. 83 - 92

COPPER-INDUCED GENOTOXIC EFFECTS IN ROOT MERISTEMS OF *TRITICUM AESTIVUM* L. CV. BETI

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Abstract: Copper is one the most abundant heavy metals in agricultural soils and its excess in soil comes from the largely use of this heavy metal in industry and agriculture (as fungicide). Mitotic index, rate and categories of ana-telophase chromosome aberrations, as well as the frequency and types of metaphase disturbances were scored in root tip meristems of *Triticum aestivum* L. cv. Beti after seed exposure to copper, provided as copper acetate and copper citrate, at four concentrations (10, 25, 50, and 100 µM) containing 0.64, 1.59, 3.18, 6.35 µg ml^{-1} Cu^{2+}, and 1.91, 4.77, 9.53, 19.06 µg ml^{-1} Cu^{2+}, respectively. Except the mitostimulant effect of 25 µM concentration, all the other concentrations of copper acetate and copper citrate showed mitodepressive action. The copper genotoxicity is expressed in the increased level (1.5 – 5-fold higher than in control) of the rate of chromosome aberrations in mitotic ana-telophases of copper-treated variants. Chromosome bridges, laggards and complex aberrations are the most numerous, although multipolarity, fragments and micronuclei are present, but with lower frequency and not in all copper-treated variants. Concerning the rate of metaphase disturbances, copper acetate augmented 2 – 3 times the rate of abnormalities in all variants, whereas only variant treated with 25 µM copper citrate exceeded the control in a substantial manner. Metaphases with chromosomes expulsed from equatorial plate are numerically preponderant, followed by C-metaphases. These observations constitute a signal about the risks of the widespread and increasing presence of some heavy metals into environment. The results reported here could be considered in a future evaluation of copper effects on other organisms, even on human health, due to large use of copper compounds, inclusively as fungicides.

Key words: aneugenic effects; clastogenic action; genotoxicity; mitotic index; wheat

1. INTRODUCTION

Heavy metal pollution largely affects the biosphere, particularly in areas with high anthropogenic activity (United States Environmental Protection Agency, 1997, cf. Jadia & Fulekar, 2009). Volcanoes, natural weathering of rocks, the mining, the sewage and the combustion of fossil fuels are sources permanently releasing heavy metals into environment. Metal concentrations in soil range from less than 1 mg/kg to high as 100,000 mg/kg (Peralta et al., 2000). Kabata-Pendias & Pendias (2001) stated that the most toxic metals for both higher plants and micro organisms are Hg, Cu, Ni, Pb, Co, Cd, and possibly Ag, Be, and Sn.

Copper, like other bivalent metal cations, is among the most abundant metals in agricultural soils. Excess Cu^{2+} in soils comes from its use in industry and agriculture (as fungicide, algicide, or bacteriocide) (Posmyk et al., 2008; Souguir et al., 2008). Most often copper is used as electrical conductor. Copper acetates are used as reagents for the synthesis of various inorganic and organic compounds, while copper alloys are used in jewelry, bronze sculptures and for coins. Its concentration can reach 500 mg kg^{-1} in vineyard soils, even 5800 mg kg^{-1} in the vicinity of copper-nickel smelters (Souguir et al., 2008).

Copper is an essential micronutrient required in trace amount to plants (Kabata-Pendias & Pendias, 2001). It is involved in several physiological processes in plants, such as protein and carbohydrate metabolism, detoxification of free radicals, cell wall lignification, photosynthesis,

respiration, seed germination (Peralta et al., 2000), disease resistance (Kabata-Pendias & Pendias, 2001) and it is cofactor of enzymes such as plastocyanin, cytochrome c, and Cu/Zn superoxide dismutase (Cu/Zn-SOD) (Sudo et al., 2008).

The high concentrations of copper are phytotoxic and can induce leaf chlorosis, root growth suppression or root malformation (Pandey, 2008). Copper excess affects key cellular processes and can induce the formation of reactive oxygen species (Qi et al., 2006; Sudo et al., 2008) which damage the biological macromolecules such as DNA, proteins, and lipids. Toxic concentrations of copper and other trace elements in plant tissues are very difficult to establish. Copper is considered to be excessive or toxic for agronomic crops in the range of 20 - 100 ppm/dry weight, the normal level being 5 - 30 ppm/dry weight (Kabata-Pendias & Pendias, 2001). Susceptibility to copper stress varies with plant species: alfalfa and barley are highly tolerant, rice and potato are less tolerant, while wheat is very sensitive to copper deficiency (Sudo et al., 2008).

Copper-induced genome alterations consist in DNA single and double strand breaks, DNA-DNA cross-links, protein-DNA cross-links, modified bases, abasic sites, intra-strand dimerization of adjacent purine bases, formation of SCE, resulting in incorrect DNA replication and changes in mitotic division, as well as in occurrence of chromosome aberrations (Jiang et al., 2001; Kašuba et al., 2004; Unyayar et al., 2006; Liu et al., 2009). The wide-spreading of heavy metals and their potential to induce genome damage require the extent of the evaluation of their genotoxicity on a larger spectrum of plant species. Plant systems are good candidates in monitoring of cytotoxic and genotoxic effects of various environmental chemical and physical stressors, but sometimes, as with copper it happens, the results are contradictory because of many negative as well as positive findings, depending on species, different growth conditions, copper concentration, types of copper compounds (Navari-Izzo et al., 1998; Jiang et al., 2001; Posmyk et al., 2008; Souguir et al., 2008).

In Romania, 0.9 million hectares were affected by chemical pollution in 2004, 0.2 million hectares being excessively polluted with heavy metals, acid rains etc. In severely polluted areas, some heavy metals surpassed the maximum allowable limits: 3 - 30 times for Pb, 2 - 32 times for Cd, 2 - 3 times for Zn, 2 - 4 times for Cu, and excessive amounts were detected in leaves of sugar and forage beet, maize, potatoes and winter wheat (Sanitation Country Profile, Romania, 2004).

On copper polluted soils, the microbial activity is negatively influenced and the soil physicochemical profile is deeply changed, fact having an indirect impact on plants, animals and humans because all of these are constitutive parts of the trophic chain. Copper is not biodegradable and it accumulates in the environment so reaching hazardous levels of concentration. The most of the studies, inclusively those realized in Romania, confirm that the major part of copper in soil is originated in anthropogenic sources, because this heavy metal is present in excessive levels in the areas with specific industrial activities and in the agricultural zones where copper-containing fertilizers and pesticides are applied (Damian et al., 2008, 2010; Lăcătuşu et al., 2007; Secu et al., 2008; Mihali et al., 2013). Out of the investigations on the environmental heavy metal levels, these researches were focused on the negative effects of the atmospheric emissions on soils, vegetation, water and animals both in industrial areas and in urban and peri-urban zones (Lăcătuşu & Lăcătuşu, 2008; Secu et al., 2008).

The continuous production and release of chemicals into the environment has led to the need to assess their genotoxicity. Plant systems are largely used for assessing heavy metal bioavailability because they allow the evaluation of genotoxic events in root meristems, and this fact is valuable especially for Cu^{2+} which accumulates substantially in the roots of plants.

Wheat is a plant of a worldwide economic importance, a main link in the trophic chain. Although useful as a livestock feed, wheat is used mainly as a human food, rich in carbohydrates, valuable protein, minerals, and vitamins. About 1/3 of the world's people depends on wheat for their nourishment. Balanced by other foods supplying certain amino acids (lysine), this crop is an efficient source of protein. It contains a number of antioxidants (vitamins C and E, beta-carotene, phenolic compounds, and trace elements such as selenium, copper, zinc and manganese) which lower the carcinogenic risk by reducing the level of free radicals in the human body. For example, lignans from wheat bran show antitumor activity against colon cancer SW480 cells (Qu et al., 2005). In Romania, wheat represents ~ 25% of arable surface and 40% of cereal areas, winter cultivars occupying 99% of wheat cultivated surface.

For these reasons, it is necessary to deeply know the consequences of action of heavy metals, inclusively copper, on genetic apparatus and phenotype expression. The overall objective of this work is to evaluate and to complete the knowledge on copper genotoxicity, by quantification of cytogenetic

damage caused by the exposure to different concentrations of Cu^{2+}, provided as acetate and citrate, expressed in mitosis indices, rate of ana-telophase chromosome aberrations and frequency of metaphase disturbances in meristematic cells of *Triticum aestivum* L. cv. Beti root tips of young seedlings obtained by germination of copper-treated seeds.

2. MATERIAL AND METHODS

Plant material and treatment conditions. *Triticum aestivum* L. cv. Beti seeds were used in the experiments. Beti is a commercial cultivar of winter common wheat obtained at Agricultural Research and Development Station of Podu-Iloaiei - Iasi, Romania. It has a three years average yield of 5600 kg/ha, with 1000-grain weight=42-46 g (Ioan & Ciolpan 2004). The wheat grains were surface-disinfected with 1% sodium hypochlorite for 5 min and vigorously rinsed with distilled water. Then they were 4 hours treated with:

1. $(CH_3COO)_2Cu.H_2O$ (copper acetate monohydrate), molecular weight = 199.63 g mol^{-1}
2. $Cu_3(C_6H_5O_7)_2$ (copper citrate), molecular weight = 568.84 g mol^{-1}

Four concentrations (10 µM, 25 µM, 50 µM, 100 µM) have been prepared for each copper compound and were used for seed treatment. The copper concentration (µg ml^{-1}) is 0.64, 1.59, 3.18, 6.35 µg ml^{-1} Cu^{2+} in copper acetate solutions, and 1.91, 4.77, 9.53, 19.06 µg ml^{-1} Cu^{2+} in copper citrate solutions. Control was set up by seed immersion in distilled water. The treated seeds were placed on moist filter paper in covered Petri dishes and then maintained in dark, at 23°C, in order to germinate.

Cytogenetic analysis. After seed germination, roots were grown until they reached 10-15 mm in length, then the root tips (meristematic zones) were cut and placed overnight in the fixation solution containing ethanol and glacial acetic acid (3:1) for 24 h, at room temperature. After washing, the roots were stored in 70% ethyl alcohol, at $+4^0$C. The plant material was 25 min hydrolyzed in 50% HCl and then stained in modified charbol fuchsin solution (Gamborg & Wetter 1975). To prepare the slides, the root tips were cut off, macerated and squashed in 45% acetic acid. Five replicate samples (three individual root tips from each germinated seed) were made. Ten microscopic fields were microscopically analyzed on every slide at 20x objective. A Nikon Eclipse 600 light microscope was used for this analysis. Photos were taken with a Nikon Cool Pix 950 digital camera, 1600x1200 dpi resolution.

The dividing cells and aberrations types were grouped according to modified *Allium* test introduced by Fiskesjö (1985). Mitotic index (MI), frequencies of division stages (prophase, metaphase, anaphase, and telophase indices), rate of ana-telophase chromosome aberrations and of metaphase disturbances were calculated and were used as endpoints for determination of copper-induced genotoxic effects. These indicators were calculated according to the following formulas: MI% (mitotic index) = TDC x 100/TC; PI% (prophase index) = prophase cells x 100/TDC; MeI% (metaphase index) = metaphase cells x 100/TDC; AI% (anaphase index) = anaphase cells x 100/TDC; TI% (telophase index) = telophase cells x 100/TDC, where TC = total (interphases + mitotic cells) cells, and TDC = total dividing cells. The rate of chromosome aberrations in ana-telophases (CA_{A-T}%) and the rate of metaphase disturbances (M_{abn}%) were also evaluated in relation to the number of cells in mitosis: $CA_{A-T}\% = CA_{A-T}$ x 100/TDC; $M_{abn}\% = M_{abn}$ x 100/TDC. The inhibitory/stimulatory rate (%) was calculated by the equation: (1 – x/y) 100, where *y* is the average value detected in the control and *x* is one of each treated samples (Liu et al., 2005).

Data were expressed as means±standard error of the means ($\bar{x} \pm SE$). To calculate and to graphically represent the statistical parameters, the Microsoft Office Excel 2003 software of Windows XP operating system was used.

3. RESULTS AND DISCUSSION

Mitotic index. In this study, the lowest tested concentration (10 µM) of copper acetate and copper citrate resulted in the decrease of dividing cells, mainly for copper acetate (rate of inhibition is more than 30%, compared to the control) (Fig. 1). Exposure to 25 µM enhanced the proportion of dividing cells for both copper compounds: stimulatory rate is +11.55% for copper acetate and +6.93% for copper citrate. With 50 µM concentration, a progressive decline of average level of dividing cells is visible with increase of copper concentration, MI% being in this case smaller than control. The trend continues to be descendant in the maximum tested concentration (100 µM), so that MI% is lower than control with 15.68% in copper acetate treated variant, and with 20.08% in plants exposed to copper citrate. Although in the work solutions of copper citrate the metal level is 3-fold greater than in copper acetate solutions, the differences between average values of MI% are not significant at correspondent concentrations, and the profile of this cytogenetic parameter is similar.

Experimental control of some heavy metal toxicity stated the following decreasing order: Hg > Cr > Cd ~ Cu ~ Zn > Ni, for cytotoxicity, and Hg > Cr > Cu ~ Cd ~ Ni > Zn, for genotoxicity (Codina et al., 2000). Mitotic index (MI%) is not only a parameter of intensity of cell division, but also an indicator of cytotoxicity. Based on the severity of effects induced on mitosis, Patra et al., (2004) classified the heavy metals in three groups, and Cu^{2+} is included in the category the heavy metals having very important effects, together Cd, Hg, Cr, Co, Ni, and Be.

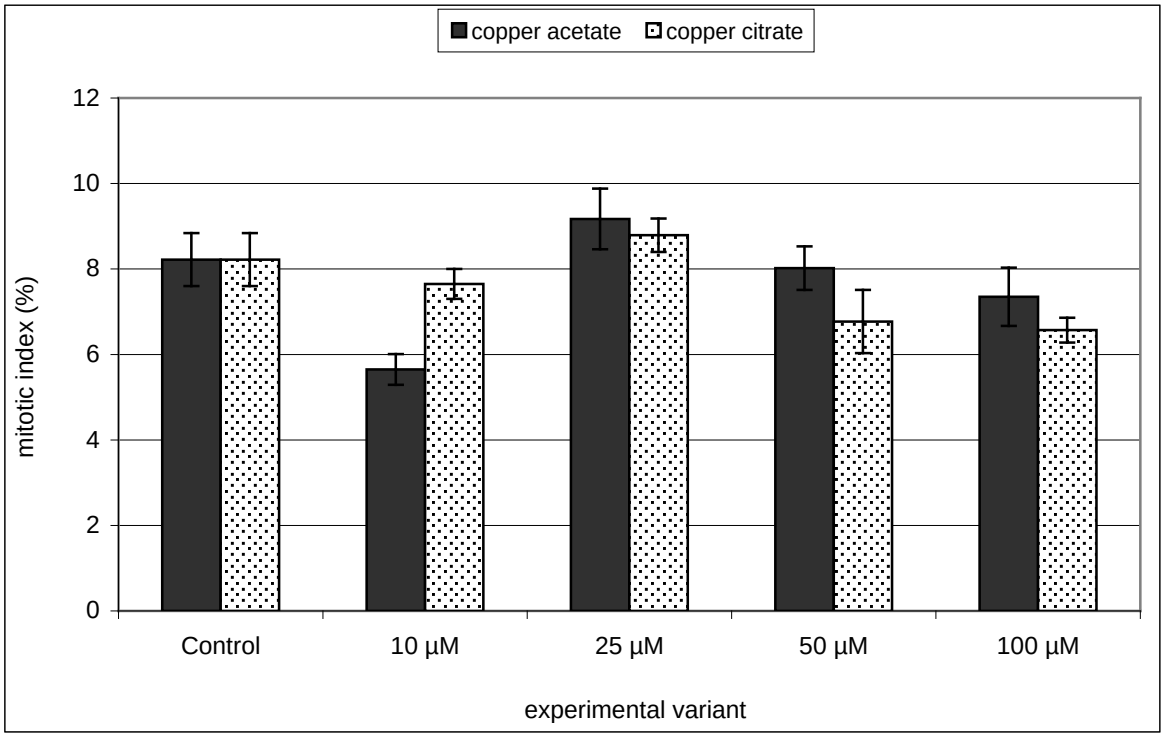

Figure 1. Profile of mitotic index in wheat root meristems, after copper exposure (bars represent standard errors of the means).

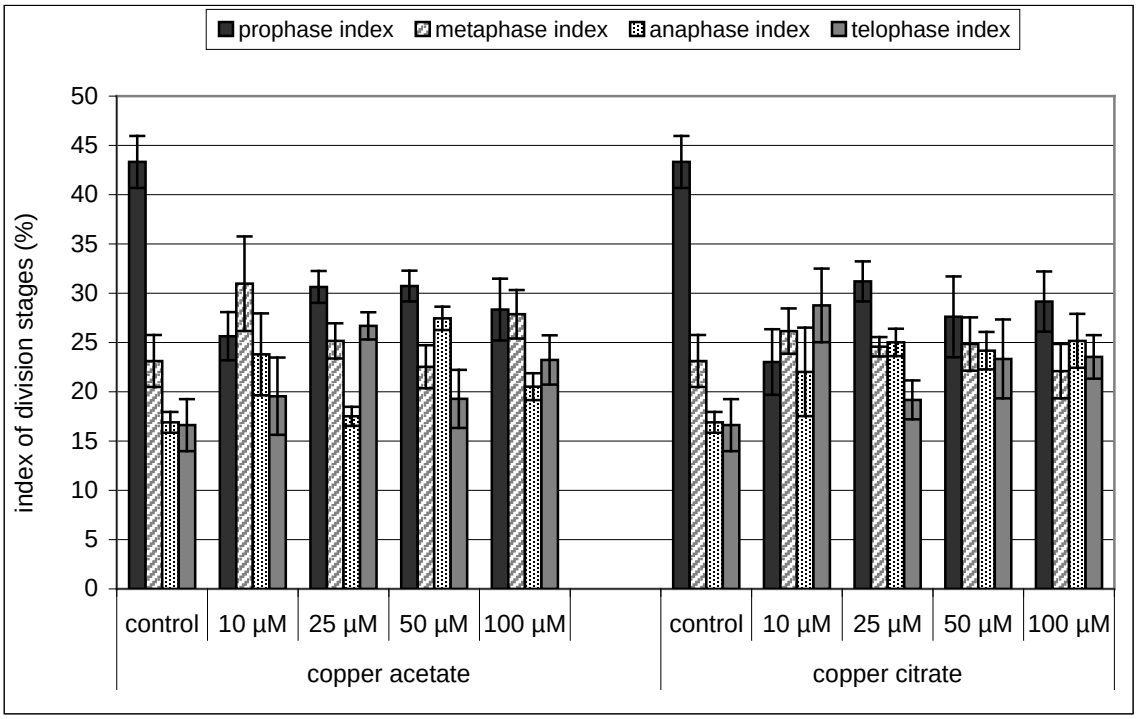

Figure 2. Percentual incidence of mitotic phases in wheat root meristems, after copper exposure (bars represent standard error of the means).

In literature, data on copper effects on cell division intensity are different, depending on species, form in which copper was provided, copper concentration, treatment duration. Cu^{+2} (given as copper sulphate, copper acetate or copper chloride) displayed mitoinhibitory effects in root meristems of *Zea mays*, in *Helianthus annuus*, *Vicia faba*, and *Allium sativum*, generally in relation to concentration increase (Jiang et al., 2001; Inceer et al., 2003; Pandey & Upadhyay, 2008; Posmyk et al., 2008; Liu et al., 2009), but the tested concentrations and duration of copper exposure differed between experiments.

The precise role of Cu^{+2} in cell proliferation is still unsolved. It could be in connection with changes in chromatin structure and DNA degradation caused by Cu^{2+} (Jiang et al., 2001). Reduction in mitotic activity could be due to the interference of heavy metal in progress of normal mitosis. So, the inhibition of DNA-polymerase, necessary for the synthesis of DNA precursors, as well as the changes in biosynthesis of some cell cycle key proteins directly involved in spindle assembly or orientation, could explain the mitodepressive effect induced by copper treatment (Yildiz et al., 2009).

Concerning the percentual distribution of mitotic stages, compared to the control, all copper-treated variants show decreases of PI%, more marked for the lowest concentration (10 µM), both for copper acetate and copper citrate, accompanied by slight increases of MeI% and consistent rises of AI% + TI% indices (with 30 – 35%, compared to the control) (Fig. 2).

Prophase number decline can be in relation to the copper action which prevents the cells from division onset by blocking interphase cells to enter into prophase, whereas higher level of metaphases and ana-telophases could be the result of copper interaction with division spindle, with blocking of mitotic cells in these stages.

A modification of proportion of mitotic phases compared to the control was evidenced in *Helianthus annuus* root meristems after copper exposure, expressed in increased metaphase stages and decreased ana- and telophase stages (Inceer et al., 2003).

Ana-telophase chromosome aberrations. Cytogenetical analysis of biological material subjected to copper action revealed a wide range of disturbances in ana-telophases which have not been observed in root meristematic cells of wheat control plants. In all copper-treated variants, the genotoxicity of the heavy metal is expressed in high values of $CA_{A\text{-}T}$% which exceed ~1.6 – 3.3-fold the control in copper acetate, and 3 – 5-fold in copper citrate (Fig. 3). The highest preponderance belongs to chromosome bridges (Figs 4, 5a, b) followed by laggards (Figs 4, 5d), and complex aberrations (Figs 4, 5c, f, i), in proportions depending on copper compound and concentration.

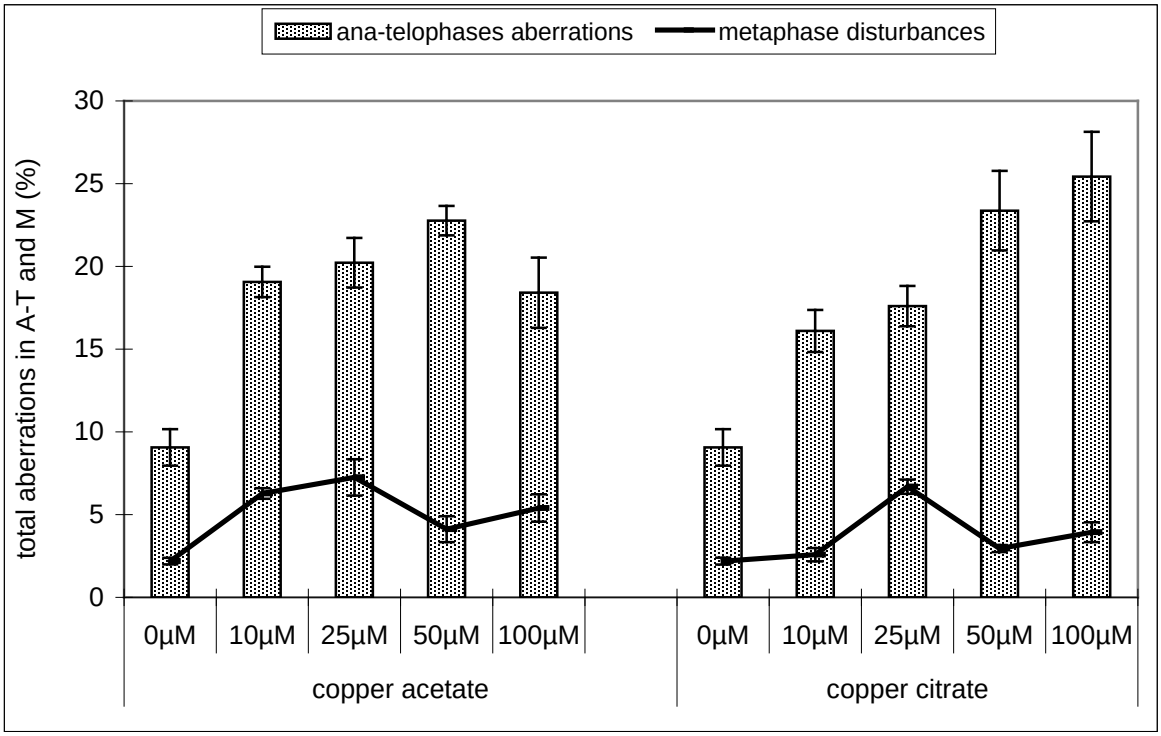

Figure 3. Mean rates of total ana-telophase chromosome aberrations and total metaphase abnormalities induced by copper in wheat root meristematic cells (bars represent standard errors of the means).

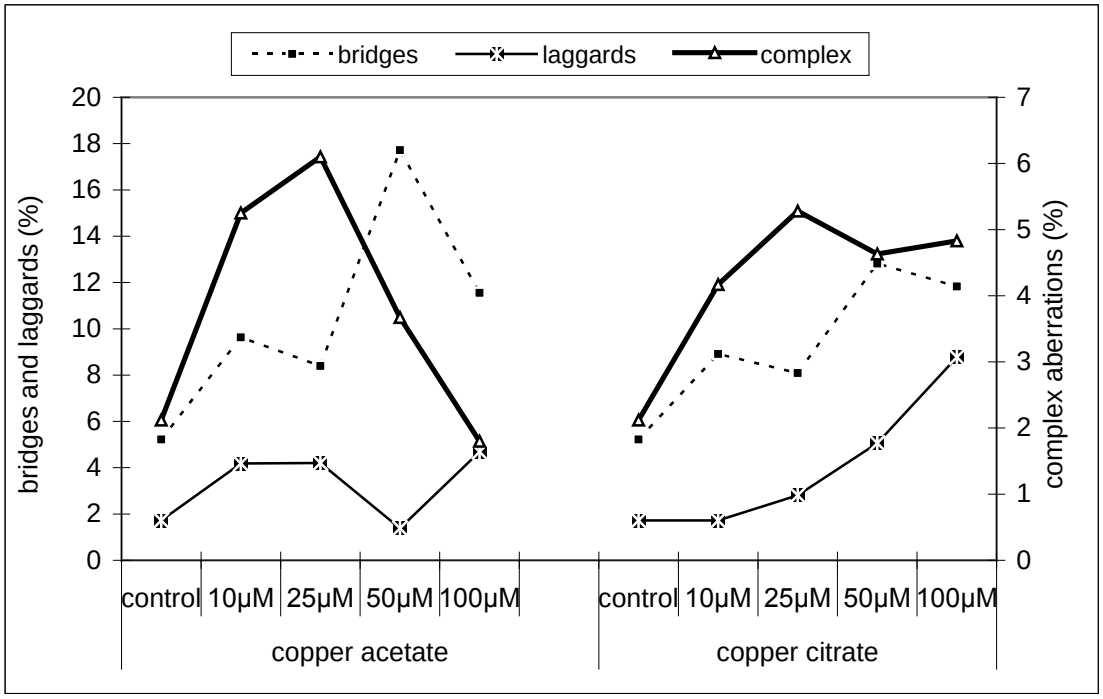

Figure 4. Graphic representation of the percentual values of main categories of ana-telophase aberrations induced by copper in wheat root meristematic cells

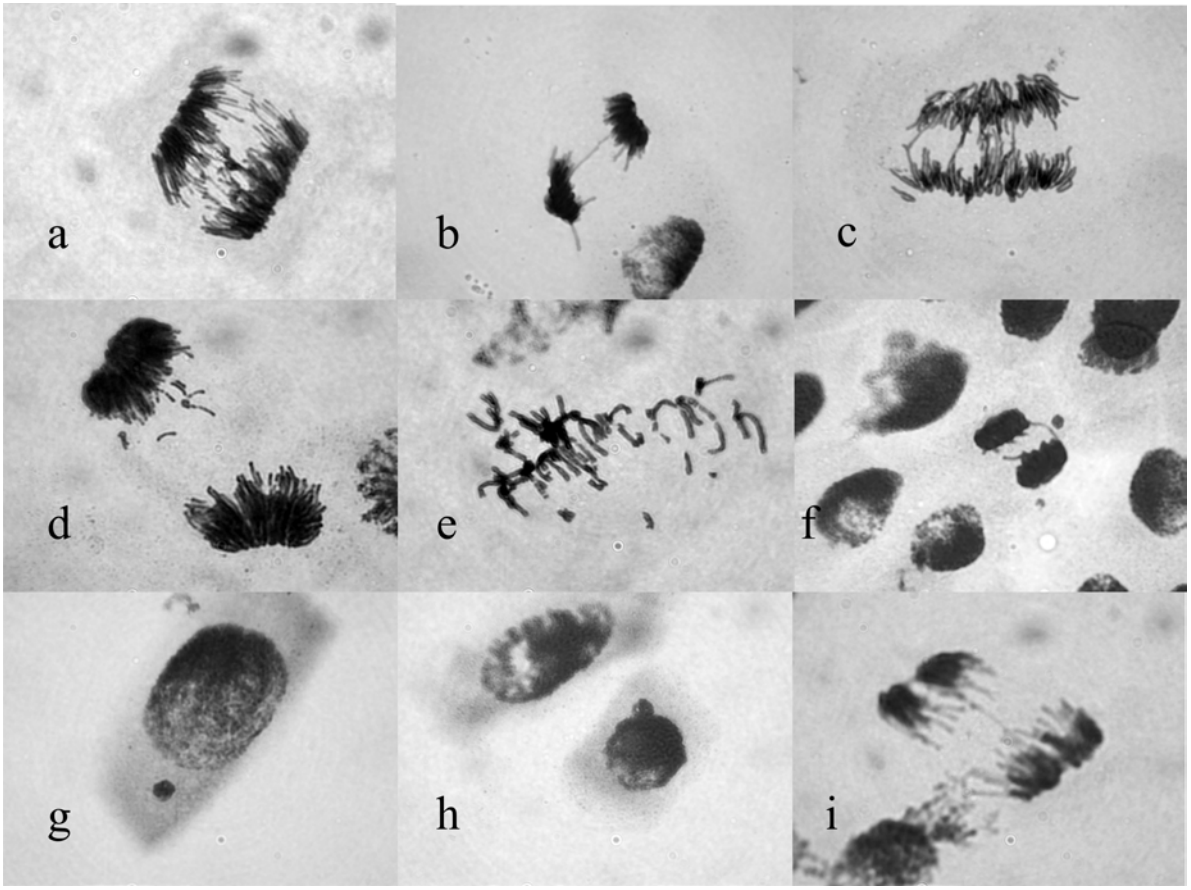

Figure 5. Types of ana-telophase chromosome aberrations [a) multiple bridges – 25μM copper acetate; b) single chromosome bridge – 100 μM copper acetate; c) multiple bridges and expulsed chromosomes – 100 μM copper acetate; d) lagging chromosomes – 50 μM copper citrate; f) chromosome bridge and micronucleus – 100 μM copper acetate; i) multipolarity + bridge – 25 μM copper acetate], metaphase disturbances [e). fragments and expulsed chromosomes – 25 μM copper citrate] and micronuclei in interphase [g, h – 50 μM copper citrate] induced by copper in root tip meristems of wheat seedlings.

The greatest levels of bridges are encountered in variants treated with 50 μM copper acetate and 50 μM copper citrate (17.72%, and 12.82%, respectively, compared to 5.22 % for control). Except for 50 μM variant, in the other three concentrations of copper acetate, the level of laggards is ~2.5-fold greater than in control.

The increment is also important in the plants treated with the maximum tested concentrations of copper citrate (5.06% in 50 μM, and 8.77% in 100 μM, compared to the control percentage – 1.72%). Lagging chromosomes are a potential source of aneuploidy because they lost the ability to attach by spindle fibres; they do not participate to the normal division and cause genetic disequilibriums between daughter cells. This type of aberration is more likely irreversible (Yildiz et al., 2009).

Laggards and bridges are the result of aneugenic effect of copper action which alters the normal function of mitotic spindle, so that the chromosome movement to the cell poles is disturbed.

Chromosome gaps (50 μM copper citrate), micronuclei, fragments (25 μM copper citrate), expulsed chromosomes, and multipolarity (25 μM copper acetate; 10, 25 and 50 μM copper citrate) were also observed, but these disturbances are lesser extent as frequency. Chromosome gaps, which represent losses of chromatin material, may be consecutive to damage of the protein part of the chromosome rather than the whole chromosome. The most micronuclei were induced by copper citrate at 50 μM concentration, but their presence was also noticed in 10 μM copper citrate and 100 μM copper acetate (Fig. 5 g, h). Micronuclei might be formed by acentric fragments (clastogenic action), chromosomal losses or malformation of the mitotic spindle (aneugenic action) (Sudhakar et al., 2001) or even by the elimination of amplified genetic material (Fernandes et al., 2007). In the studied wheat cultivar, the micronuclei observed after copper exposure are of small sizes.

According to Leme & Marin-Morales (2009), micronucleus size can be an effective parameter to assess the clastogenic and aneugenic effects. So, large micronuclei would indicate an aneugenic effect resulting from a chromosome loss, whereas small micronuclei may prove a clastogenic action resulting from chromosome break. Micronucleus assay performed on three biological systems (*Tradescantia, Allium cepa, Vicia faba*) evidenced a lower capacity of copper to induce micronuclei, compared to other heavy metals: $As^{3+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Cu^{2+}$ (Steinkellner et al., 1998).

Our results on copper-induced aberrations are in agreement with literature data. So, in *Zea mays, Helianthus annuus, Vicia faba,* and *Allium sativum,* copper induced, at different concentrations and exposure times, anaphase bridges, micronuclei, laggards, chromosome stickiness and broken nuclei (Jiang et al., 2001; Inceer et al., 2003; Posmyk et al., 2008; Liu et al., 2009). The ability of some chemical stressors to induce chromosome aberrations in root tips of some species (*Cymbopogon citratus, Allium cepa*) after short-term treatments, without recovery, was reported by many authors (Williams & Omoh, 1996; Kalcheva et al., 2009) who observed genetic damage after a 3 h exposure to the tested compounds. A non linear relationship was noticed in this study between the frequency of chromosome aberrations and the copper concentration, except laggard number which increased in a concentration-dependent manner in copper citrate treated variants. In copper acetate, small progressive increases of total level of ana-telophase aberrations were noticed from 10 to 50 μM. Although Cu^{2+} concentration in solutions of copper citrate is 3-fold higher than in copper acetate, the genotoxic effects, expressed in $CA_{A\text{-}T}$% rate, are not substantially different.

Many authors consider the high incidence of ana-telophase chromosome aberrations to be, at least in part, the consequence of copper-induced oxidative stress and generation of reactive oxygen species (Navari-Izzo et al., 1998). Reactive oxygen species and their intermediary products interfere with genetic material and alter its normal condensation, replication or repair process, so influencing in a negative manner the biological state of nucleic acids.

Metaphase disturbances. The two copper-containing compounds determined different rates of total metaphase disturbances, as well as different proportions of aberration categories (Figs. 3, 6). Compared to the control, it was evidenced a significant increase (2 – 3-fold) of the M_{abn}% rates in all copper acetate treated variants, whereas only variant treated with 25 μM copper citrate exceeded the control. Except for 25 μM copper acetate where C-metaphases are predominant, in all other variants the chromosomes expulsed from equatorial plate are numerically preponderant in the total level of metaphase disturbances (Fig. 5e). C-like metaphases resulting from aneugenic action of copper are configurations with thick and shorter chromosomes without equatorial orientation, similar to those induced by colchicine. Spindle dysfunction is mainly due to the reactivity of metal ions with thiol groups of tubulin, a protein in which cysteine radicals are actively implied in regulation dynamics of microtubule assembly.

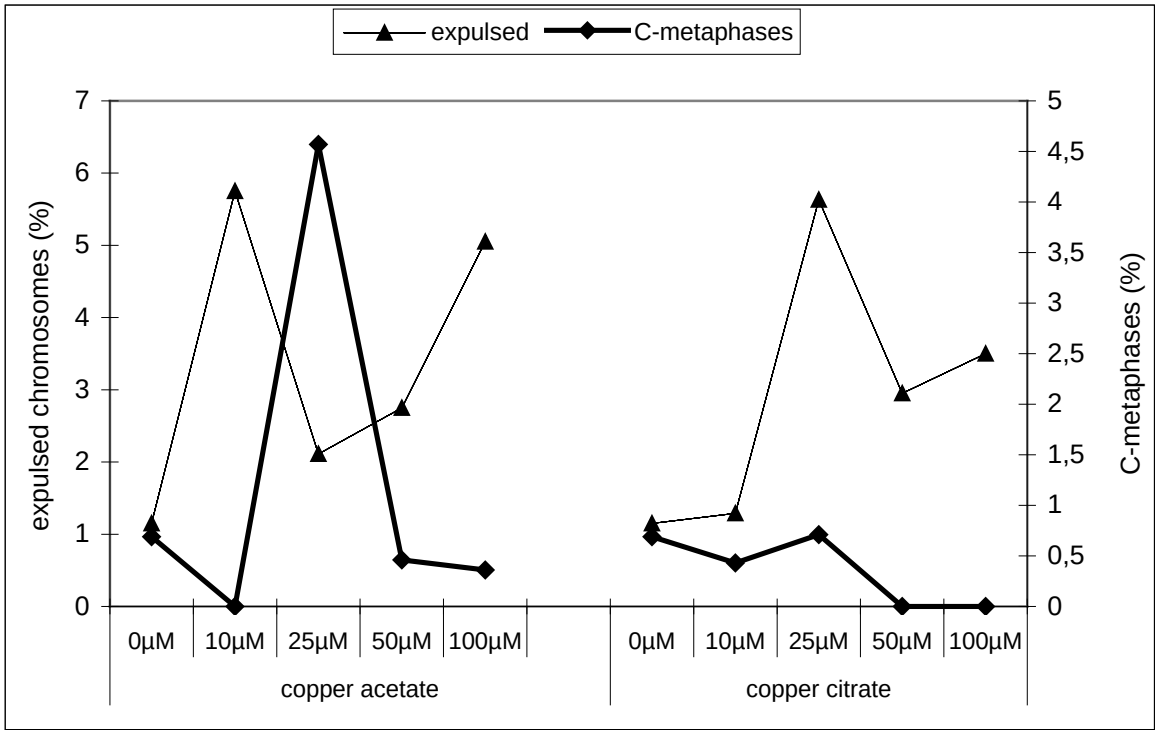

Figure 6. Graphic representation of the percentual values of main categories of metaphase disorders induced by copper in wheat root tip meristems

Formation of C-mitoses after copper treatment was also confirmed in other plant systems, such as *Allium sativum* L. (Liu et al., 2009). Inceer et al., (2000) reported that the wastes of copper mine induced some abnormalities including scattered chromosomes in the root tip cells of *Allium cepa.*

4. CONCLUSIONS

Therefore, in the present study, heterogeneous responses have been obtained concerning the behaviour of cytogenetic parameters. By the amplitude of induced disturbances, both tested copper-containing compounds exhibited a relatively high genotoxic potential. The clastogenic and aneugenic effects are reflected in the increased values of CA_{A-T}% and M_{abn}% rates. Although the problem of Cu^{2+}-induced mutagen effects remains in discussion, many authors confirmed the genotoxic potential of this heavy metal in their studies. The pathways of genotoxicity of copper and other heavy metals may involve their interaction with DNA, either directly or indirectly *via* oxidative stress, but the mechanism of this interaction is not fully understood (Cenkci et al., 2010). The amplitude of the responses to copper action in wheat investigated genotypes is enough large to conclude that it is important to get thoroughly into the studies concerning the genetic risks of copper excess by the complete knowledge of the damage induced on genetic level and of phenotype repercussions of this injury. We consider that these results prove the clastogenic and aneugenic potential of copper, and they constitute a signal about the genetic risks of increasing presence of copper into environment**,** inclusively as result of the use of this heavy metal in agriculture as pesticide. Excess of copper and other heavy metals have genetical consequences, with possible unwanted repercussions on the economically important phenotypic traits in cereals and other plants and decreases in their yields; they also negatively influence the human health, given the large presence of cereals in human diet. Therefore, specific measures have to be taken for preventing soil pollution. Thoroughgoing studies on genotoxicity of copper and other heavy metals, inclusively at molecular level (Mihasan et al., 2012), are necessary to be made on other species in order to find those plants which can absorb and accumulate the harmful chemicals so preventing/reducing soil pollution and the contamination of underground waters. This ability will allow their use in the phytoremediation programs. Also it is recommended the cultivation of some hybrid species which require a minimum of spraying with copper-containing compounds (Secu et al., 2008); another way to control the soil pollution can be the by reduction or interdiction of copper-containing chemical fertilizers and pesticides, and their replacement with bionatural "alternatives".

Acknowledgements

This research was realized in the project PN 09-36-301/2009-2011, BIOSTAR subprogram, National Program for Research and Development, Romania.

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Received at: 20. 09. 2012
Revised at: 09. 09. 2013
Accepted for publication at: 24. 09. 2013
Published online at: 27. 09. 2013