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# Prooxidative and antioxidative properties of cucumber (*Cucumis sativus* L.) callus *in vitro* and young *in vivo* plantlets in response to copper ions

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**ABSTRACT**

The effects of different concentrations of copper ions ($Cu^{2+}$ in the form of CuSO4 × 5H2O) on *in vivo* cucumber (*Cucumis sativus* L. 'Edinstvo') seedlings as well as on *in vitro* hypocotyl-derived callus were considered. Callus induction from hypocotyls was more prolific than from roots or cotyledons. Thus, callus obtained from hypocotyls of 7-day-old cucumber plants was cultured for 5 weeks on Murashige and Skoog medium containing 4 mg $dm^{-3}$ 2,4-D + 1 mg $dm^{-3}$ BA supplemented with 0.01 mM, 0.1 mM or 1.0 mM of $Cu^{2+}$. Biochemical indices related to oxidative stress were assessed. $Cu^{2+}$ at 0.01 mM stimulated callus induction but 1.0 mM $Cu^{2+}$ negatively affected callus formation and growth. LPO intensity was significantly lower than the control at all concentrations of $Cu^{2+}$ but significantly higher than the control in plants exposed to 0.01 or 0.1 mM $Cu^{2+}$. A similar trend was observed for the generation of the superoxide radical in both callus and plantlets. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APOX) activity increased in both callus and plantlets, but the level of increase in these antioxidant enzyme systems depended on the $Cu^{2+}$ concentration. $Cu^{2+}$ ions had a stronger (i.e., more negative) influence on oxidative stress in cucumber seedlings than on cucumber callus.

Key words: ascorbate peroxidase, callus culture, catalase, lipid peroxidation, seedlings, superoxide anion, superoxide dismutase

**Abbreviations:**

2,4-D - 2,4-dichlorophenoxyacetic acid, BA - 6-benzyladenine, APOX - ascorbate peroxidase, CAT - catalase, GPOX - guaiacol peroxidase, GR - glutathione reductase, GSH - glutathione reduced, GSSG - glutathione oxidized, HM - heavy metal, LPO - lipid peroxidation, MS - Murashige and Skoog medium, NBT -nitrobluetetrazolium, POX - peroxidase, ROS - activated oxygen species, SOD - superoxide dismutase, TBA -thiobarbituric acid, TCA - trichloracetic acid.

## INTRODUCTION

Copper (Cu) is an essential nutrient for plants, but at high concentrations it can be very toxic to plants. Environmental pollution with HMs results from mining and metallurgic industries and from the application of chemicals in plant disease control programs (Shaw et al. 2006). Consequently, risks related to HM toxicity affecting human health and

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the environment need to be well studied (Manevaet al. 2009). Excess Cu causes a range of deleterious effects on plants, including the inhibition of photosynthesis, pigment synthesis, damage to plasma membrane permeability and other metabolic disturbances (Andrade et al. 2010). The assimilation of high quantities of Cu inhibits biomass synthesis which in turn brings about a reduction in growth (Maneva et al. 2009). Toxic effects of Cu-treated plants were reflected by a reduction in fresh weight, shoot and root length, chlorophyll and carotenoids contents (Fariduddin et al. 2009, Mourato et al. 2009, Elobeid and Polle 2010). Copper-induced oxidative damage in treated plants was revealed by enhanced levels of malondialdehyde (MDA) and accompanied by higher lipoxygenase activity, coupled with a higher content of hydrogen peroxide ($H_2O_2$) and superoxide anions (Panda 2008, Madejón et al. 2009). The activity of antioxidant enzymes catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) and the content of proline increased in plants grown under Cu stress (Fariduddin et al. 2009, Mourato et al. 2009). However, Cu in appropriate doses is an essential micronutrient for plants and plays an irreplaceable role as a component of several enzymes, many of which are involved in electron flow in mitochondria and chloroplasts (Marschner 1995). So both a deficit and excess of Cu can cause damage.

Different mechanisms leading to HM tolerance in some plant populations and species exist (Landberg et al. 2011, Zacchini et al. 2011, Shankar et al. 2012). However, in some cases, the mechanisms underlying the tolerance to HMs are not fully understood. *In vitro* tissue culture constitutes an important tool to study the physiological and biochemical mechanisms that operate in response to stress conditions at the cellular level (Gatti 2008, Niknam et al. 2011, Rai et al. 2011). Furthermore, plant tissue culture allows the control of stress homogeneity and the characterization of cell behavior under stress conditions, independent of the regulatory systems that are in place at the whole plant level (Hossain et al. 2012). Some callus and suspension cell cultures provide clues to the dynamics of such systems under extreme stress conditions, such as HMs (Drążkiewicz and Baszyński 2008). For example, callus of *Trema orientalis* plants derived from both contaminated and uncontaminated sources were tested *in vitro* for their relative tolerance to chromium (Cr) and nickel (Ni). The callus derived from contaminated sources was HM-tolerant and showed better growth than those obtained from uncontaminated plants. The specificity of HM tolerance shown by the parent material was maintained in the callus (Samantaray et al.1999). Sunflower (*Helianthus annuus* L.) callus growing in the presence of Cd developed increased resistance to oxidative stress. The capacity to grow in the presence of Cd was related to the ability of tissues to maintain high intracellular levels of the main low molecular antioxidant, glutathione (GSH) (Gallego et al. 2005).

*In vitro* cell cultures are also useful for improving tolerance to HMs (or to other abiotic stresses) in plants through selection of tolerant cell lines that are able to regenerate plants. Selection and characterization of various cell cultures tolerant to HMs have been reported (Gatti 2008). Callus from 15 cotton (*Gossypium hirsutum* L.) genotypes were screened for HM resistance and two of them were resistant to Cd, Cu and Ni (Biçakçi and Memon 2005). *In vitro* breeding and somaclonal variation were used as tools to improve the potential of Indian mustard (*Brassica juncea* L.) to extract and accumulate toxic HMs. When callus from *B. juncea* was cultivated on modified Murashige and Skoog (MS) medium supplemented with 10-200 μM Cd or lead (Pb), new *B. juncea* somaclones with improved tolerance to Cd, zinc (Zn) and Pb regenerated from HM-tolerant callus cells (Nehnevajova et al. 2007). An *in vitro* system provides a good way to select stress-tolerant clones under strict controlled environmental conditions without the interference of biotic and abiotic factors as would normally occur in greenhouse or field trials. Cell cultures are useful for obtaining, in a relatively short time, stress-tolerant cell lines (Samantaray et al. 1999).

Zn-tolerant and intolerant ecotypes of the metallophyte *Silene vulgaris* were examined for their suitability to efficiently form callus. Highly tolerant lines (i.e. plants derived from callus) could regenerate in the presence of 1750 μM Zn in media while in sensitive lines could regenerate only with 500 μM Zn. This study demonstrates the stability of Zn-tolerant lines and the potential to use callus to study the genetic basis of tolerance to Zn and other HMs in species in which it is possible to naturally select a high level of tolerance (Jack et al. 2005).

In *Nicotiana tabacum* L. 'BEL W3', Cu in concentrations greater than 50 μM significantly inhibited callus growth and shoot regeneration (Gori et al. 1998). After 5–6 months of culture only a few morphogenic callus lines survived in the presence of 100 μM Cu. This callus could regenerate shoots through successive subcultures on medium containing 100 μM Cu. Plants regenerated

from Cu-tolerant callus lines were able to grow in the presence of 100 μM Cu. In Cu-tolerant plants, dry matter production was higher than in controls, particularly in roots.

Different environmental stresses (salinity, drought, heat/cold, light and other hostile conditions) may trigger oxidative stress in plants, generating the formation of reactive oxygen species (ROS) (Lukatkin 2002a, Zhao et al. 2011). These species are partially reduced or activated derivatives of oxygen, comprising both free radical ($O_2^{-}$; $OH\cdot$; $OH_2\cdot$) and non-radical ($H_2O_2$) forms, leading to cellular damage, metabolic disorders and senescence (Apel and Hirt 2004, Ciríaco da Silva et al. 2011). In order to overcome oxidative stress, plants have developed two main antioxidant defense mechanisms that can be classified as either non-enzymatic or enzymatic. The first class (non-enzymatic) consists of small molecules such as vitamin A, C and E, glutathione, carotenoids and phenolics that can react directly with ROS by scavenging them. The second class is represented by enzymes, among them SOD, POX and CAT which have the capacity to eliminate superoxide and $H_2O_2$. Under various abiotic constraints, plants undergo changes in basic antioxidant metabolism (Hossain et al. 2012).

Oxidative stress in the form of HMs also has primary effects on plants. Some studies investigated oxidative and antioxidative events in callus cultures as affected by HMs (Drążkiewicz and Baszyński 2008). The antioxidant defence system of two types of sunflower callus responded differently to 150 μM Cd(II), Al(III) and Cr(III), resulting in fewer low molecular weight antioxidants under HM stress (Gallego et al. 2002). The antioxidant enzymes responded differently according to the HM and treatment used. Ascorbate POX (APOX) activity diminished with all HM ions assayed, guaiacol peroxidase (GPOX) activity decreased in response to Cd and Cr, glutathione reductase (GR) was only decreased by Cd, and SOD and CAT activities were less than in Cd-tolerant cells. These results suggest the involvement of an antioxidant defense system in the adaptive response of sunflower cells to HM ions.

CAT and SOD activities were analysed in sugarcane (*Saccharum officinarum* L.) *in vitro* callus cultures exposed to $CdCl_2$. CAT was possibly the main antioxidant enzyme metabolizing $H_2O_2$ (Fornazier et al. 2002). *Leucaena leucocephalla* callus derived from contaminated (Cr, Ni and Zn) sources were tolerant to these HMs and showed better growth on MS basal medium supplemented with 0.015 mM Cr, 0.13 mM Ni or 0.053 mM Zn than those derived from uncontaminated seeds. Callus derived from *L. leucocephalla* growing on contaminated soil had higher CAT and POX activities than callus of plants derived from uncontaminated soil (Rout et al. 1999).

In plant cells, antioxidants maintain ROS at low concentrations, avoiding oxidative damage while allowing them to play crucial functions in signal transduction, although little is known about the role of antioxidants during HM stress (Matamoros et al. 2010). In this work we compare the oxidative and antioxidative effects of Cu ions in cucumber cell and tissue cultures to reveal distinctions in the degree of activation of oxygen and display of oxidative stress, as well as activity of antioxidative enzymes, both in *in vitro* and *in vivo* assays.

## MATERIAL AND METHODS

### *Reagents*

The following reagents were used: ascorbic acid, TBA and Tris from Merck KGaA (Darmstadt, Germany); nitrobluetetrazolium (NBT) from Serva (Heidelberg, Germany); EDTA, riboflavin, nicotine acid, L-methionine, 2,4-dichlorophenoxyacetic acid (2,4-D) from Sigma (St. Louis, USA); meso-inositol from Fluka AG (Switzerland); 6-benzyladenine (BA) from ICN (Irvine, USA); glycine from Reanal (Budapest, Hungary); components of standard macro- and micro salt MS medium, $KH_2PO_4$, $Na_2HPO_4$, $CuSO_4 \times 5H_2O$, $KMnO_4$, HCl and NaCl from NPO EKROS (St. Petersburg, Russia); adrenaline hydrochloride solution from OAO Alvis (Moscow, Russia); thiamine from Yerevan's Chemical-Pharmaceutical Co.(Yerevan, Armenia); pyridoxine from OAO Moschimpharmpreparates (Moscow, Russia); $H_2O_2$ from ZAO Ekolab (Electrogorsk, Russia); polyvinylpyrrolidone from OAO Biochimik (Saransk, Russia); chloramines from OAO Ufachimprom (Ufa, Russia); bacterial agar from Laboratorios Conda (Madrid, Spain); sucrose from Panreak (Spain).

### *Plant material*

Seeds of *Cucumis sativus* L. cultivar 'Edinstvo' (Cucurbitaceae) were purchased from the Luhovsky cultivar-test station of the All-Russia Institute of Selection and Seed-Growing of Vegetable Cultures.

### *Cucumber callus and plant growth*

*1. Cucumber callus induction and growth*

Seeds were surface sterilized with 0.5% $KMnO_4$ for 20 min, 6% chloramines for 10min and 70% ethanol for 1 min then rinsed with sterile water

three times. Seeds were then placed on top of filter paper bridges in sterile test-tubes containing full-strength Murashige and Skoog (1962) macro- and micronutrients and vitamins, gelled with 7.5 g $dm^{-3}$ agar. 2,4-D at 1-5 mg $dm^{-3}$ and 0.5-2mg $dm^{-3}$ BA were added to all media to induce callus as well as one of three concentrations of $CuSO_4 \times 5H_2O$ (0.01 mM; 0.1 mM; 1.0 mM). Medium pH was adjusted to 5.7-5.8. Hypocotyl segments (about 5 mm length) and whole cotyledonary leaves were removed from 7-day-old seedlings and used as explants for callus induction. The cultures were maintained at constant 23±2°C in darkness for 4-6 weeks.

*2. Cucumber plant growth in vivo*

Cucumber seeds surface sterilized by the same procedure as described above were placed on filter paper bridges in test tubes on sterilized water (5 ml per seed) to test three concentrations of $Cu^{2+}$ (0.01 mM; 0.1 mM; 1.0 mM). Seedlings were grown until they were 7 days old in a 12-h photoperiod at a photosynthetic photon flux density (PPFD) of ~80 µM photons $m^{-2}$ $s^{-1}$ and at 28°C under sterile conditions. Control seedlings were cultured with water without $Cu^{2+}$ ions.

***Methods for determination of physiological and biochemical parameters in plants and callus cultures***

*1. Determination of rate of superoxide anion radical generation*

Leaf discs or callus tissue (300 mg) were homogenized in 15 ml of distilled water. This homogenate was centrifuged for 15 min at 4000 *g* and 100 µl of 0.01% adrenaline (epinephrine) solution was added to 3 ml of the supernatant, and incubated for 45 min at room temperature and a PPFD of 80 µM photons $m^{-2}$ $s^{-1}$. Optical density of the adrenohrom that formed was measured immediately after incubation against homogenate with water on a SF-46 spectrophotometer (LOMO, St. Petersburg, Russia) at 480 nm. The rate of $O_2^-$ generation was calculated in $\mu M g^{-1}$ $min^{-1}$ ($\varepsilon$ = 4020 $M^{-1}$ $cm^{-1}$) (Lukatkin 2002a).

*2. Determination of lipid peroxidation (LPO) intensity*

Samples of leaf discs or callus tissue (1 g) were homogenized in 10 ml isolation medium (0.1 M Tris-HCl buffer pH 7.6, containing 0.35 M NaCl). Two ml of thiobarbituric acid (TBA) in 20% trichloracetic acid (TCA) was added to 3 ml of homogenate, heated in a boiling water bath for 30 min and filtered. Optical density was recorded on a SF-46 spectrophotometer at 532 nm against analogously prepared isolation medium with reagents (without plant material). The concentration of MDA was calculated from the molar extinction coefficient ($\varepsilon = 1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$) and the quantity of MDA in the leaves and callus was calculated in µmole per g tissue wet weight (Lukatkin 2002a).

*3. Determination of SOD activity*

This method is based on the restoration of NBT by superoxide radicals with the formation of blue formazans NBT. 300 mg of leaf discs or callus tissue were homogenized in 20 ml of potassium phosphate buffer (pH 7.8) then centrifuged for 15 min at 7000 *g* and the supernatant was used as crude extract for measuring cytosolic SOD (EC 1.15.1.1). To 2.6 ml of the enzyme fraction containing SOD, the following components of the reaction medium were added in a certain order: 0.1 ml of 13 mM L-methionine, 0.05 ml of 75 mM NBT, 0.1 ml of 0.1 mM EDTA, and 0.1 ml of 2 mM riboflavin. The reaction was initiated by adding riboflavin, followed by incubation for 20 min under white light (160 µM photons $m^{-2}$ $s^{-1}$). Measurements were made against control tubes kept in the dark. Optical density was measured on a SF-46 spectrophotometer at 560 nm. SOD activity was judged by the difference in formazans formed in the absence and presence of plant material. The unit of SOD activity is 50% inhibition of formazan formation (Lukatkin 2002b).

*4. Determination of CAT activity*

CAT (EC 1.11.1.6) activity was determined spectrophotometrically. The enzyme extract was prepared as follows. One g of leaves from cucumber plants or callus tissue were ground in a mortar with 10 ml of 50 mM phosphate buffer (pH 7.0). The resulting homogenate was filtered and centrifuged for 10 min at 8000 *g*. The reaction mixture contained 2.2 ml of phosphate buffer, 100 µl of enzyme extract and 70 µl of 3% $H_2O_2$, the latter being poured directly before measuring optical density on a SF-46 spectrophotometer at 240 nm (initial and after 1 min). The control consisted of a mixture of 2.3 ml of phosphate buffer (pH 7.0) and 70 µl of 3% $H_2O_2$. Activity was calculated by the fall in optical density of the solution after 1 min and the concentration was recalculated using the molar extinction coefficient $\varepsilon$ = 39.4 $mM^{-1}$ $cm^{-1}$. Activity was calculated in mM $g^{-1}$ $min^{-1}$ (Lukatkin 2002b).

*5. Determination of APOX activity*

APOX (EC 1.11.1.11) activity was determined by the method of Lukatkin (2002b) with some modifications. Leaf discs or callus tissue weighing 1 g was homogenized in 10 ml of 50 mM phosphate buffer (pH 7.6) after the addition of 0.3 g polyvinylpyrrolidone (MW ~8000), then were filtered and centrifuged for 10 min at 8000 *g*. The reaction mixture consisted of 50 µl 0.1 µM EDTA, 300 µl 0.05 mM ascorbic acid, 50 µl 0.1 mM $H_2O_2$, 2.55 ml 50 mM phosphate buffer (pH 7.6) and 100 µl of plant extract. Optical density was recorded on a SF-46 spectrophotometer at 290 nm. The control consisted of the following mixture: 2.75 ml phosphate buffer (pH 7.6), 50 µl of EDTA, 50 µl of $H_2O_2$, 150 µl of ascorbic acid (without enzyme extract). APOX activity was calculated based on the drop in optical density of the solution over the first 30 sec of the reaction.

***Statistics***

All experiments were conducted in triplicate and each experiment consisted of 160-200 seedlings or 8-12 callus clumps for each treatment. For all measurements averages and standard errors were calculated using Microsoft Excel 2000, Biostat, and Statistica v. 2.6. The differences between means were assessed by Tukey's test at $p = 0.05$ following means separation using analysis of variance (ANOVA).

## RESULTS

***Effect of copper on cucumber callus growth***

In this study 5-weeks-old callus was used. Explants were hypocotyl and cotyledonary leaves excised from sterile 7-days-old cucumber plants that were placed on MS medium supplemented with different concentrations of two plant growth regulators, 2,4-D and BA (Tab. 1). The highest level of callogenesis (explants with callus) was noted on MS medium supplemented with 4 mg $dm^{-3}$ 2,4-D + 1 mg $dm^{-3}$ BA. Callus derived from hypocotyls was most responsive and thus most suitable as the experimental material. Thus, he effect of $Cu^{2+}$ions was investigated on 5-weeks-old callus derived from hypocotyls. The effects of $Cu^{2+}$ions on callus initiation were very different. Cu at 0.01 mM intensively stimulated callus tissue regeneration while 1.0 mM $Cu^{2+}$ negatively affected callus formation and growth (Fig. 1). Callus was small and growth was reduced.

**Table 1.** Callogenesis on explants from 7-day-old cucumber in vitro on MS medium with different concentrations of plant growth regulators. Concentrations not shown indicate no callus formation

| Concentration of plant growth regulators in MS medium (mg $dm^{-3}$) | | Explant | Explant number in experiment | Explants with callus | Callus colour, consistency and growth rate |
|---|---|---|---|---|---|
| 2,4-D | BA | | | | |
| | | cotyledonary leaf | 4 | 1 | dark yellow, grainy, slow growing |
| 1 | 0.5 | hypocotyl | 6 | 3 | dark yellow, grainy, slow growing |
| | | root | 4 | - | - |
| | | cotyledonary leaf | 4 | - | - |
| 2 | 0.5 | hypocotyl | 5 | 1 | dark yellow, grainy, moderate growth |
| | | root | 4 | - | - |
| | | cotyledonary leaf | 2 | 1 | dark yellow, grainy, slow growing |
| 4 | 0.5 | hypocotyl | 2 | 2 | dark yellow, grainy, moderate growth |
| | | root | 1 | - | - |
| 1 | 1 | root | 3 | 3 | light yellow, moderate growth |
| 3 | 1 | hypocotyl | 5 | 5 | light green, fast growing |
| 4 | 1 | hypocotyl | 11 | 7 | light green, fast growing |
| 5 | 1 | root | 4 | - | - |
| | | cotyledonary leaf | 2 | 2 | light green, grainy, fast growing |
| 5 | 2 | hypocotyl | 6 | 6 | dark yellow, grainy, fast growing |
| | | root | 7 | 2 | light yellow, grainy, fast growing |
| 4 | 2 | cotyledonary leaf | 2 | 2 | light yellow, grainy, fast growing |
| | | hypocotyl | 6 | 6 | light yellow, grainy, fast growing |

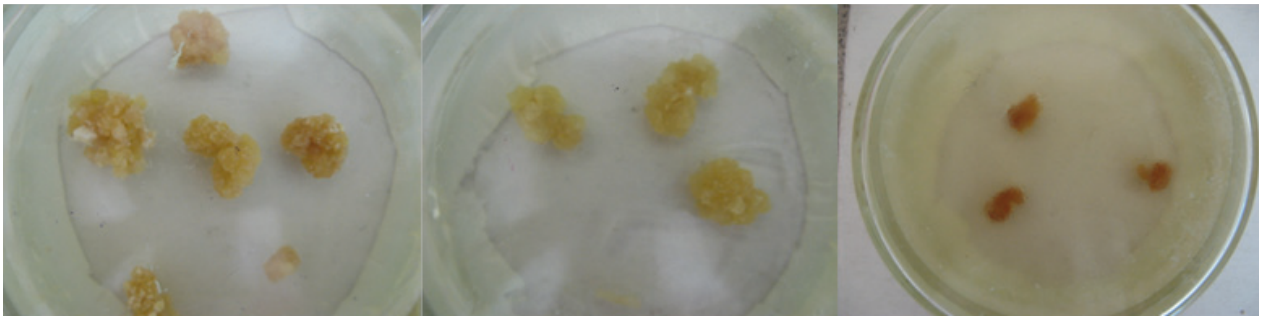

**Figure 1.** Effect of copper on cucumber callus induction and growth: 0.01 mM (left), 0.1 mM (center) and 1 mM (right)

### *Effect of copper on select biochemical properties in callus*

As copper ions have possible prooxidative actions, the rate of superoxide anion generation and LPO intensity was studied in cucumber callus. Oxidative events took place in cucumber callus but not in the control (water) treatment (Fig. 2). LPO intensity decreased 2.1-times more in callus than in the control (at 1.0 mM $Cu^{2+}$) (Fig. 2A). Similarly, lowest $O_2^-$ generation (2.7-fold lower than the water control) was observed in response to 1.0 mM $Cu^{2+}$ (Fig. 2B). SOD activity increased 7.6-fold when callus was exposed to 1.0 mM $Cu^{2+}$ but not at other concentrations (Fig. 3A). CAT activity in cucumber callus increased 1.7-fold at 1.0 mM $Cu^{2+}$ but decreased significantly at 0.1 and 1.0 mM $Cu^{2+}$ (Fig. 3B). A 1.7-fold increase in APOX activity was shown in the presence of 0.01 mM $Cu^{2+}$ (Fig. 3C). This activity was significantly lower than the control at 1.0 mM $Cu^{2+}$ (Fig. 3C).

### *Effect of copper on cucumber plants*

LPO intensity is an indicator of cell membrane damage. In cucumber plants it increased at all Cu doses in the medium compared to the water control (Fig. 4A). As $Cu^{2+}$ concentration diminished in the medium, LPO level increased, being 3.5-fold higher than the water control at a $Cu^{2+}$ concentration of 0.1 mM and 0.01 mM (Fig. 4A). The level of LPO is a consequence of ROS activation, namely the generation of $O_2^-$. The greatest increase in $O_2^-$ generation (> 4.5-fold relative to the water control) occurred in the treatment with 0.01 mM $Cu^{2+}$ (Fig. 4B). Lowest $O_2^-$ generation (1.9-fold compared to the control) was observed in the leaves of plants grown on 0.1 mM $Cu^{2+}$. At 0.01 mM $Cu^{2+}$,

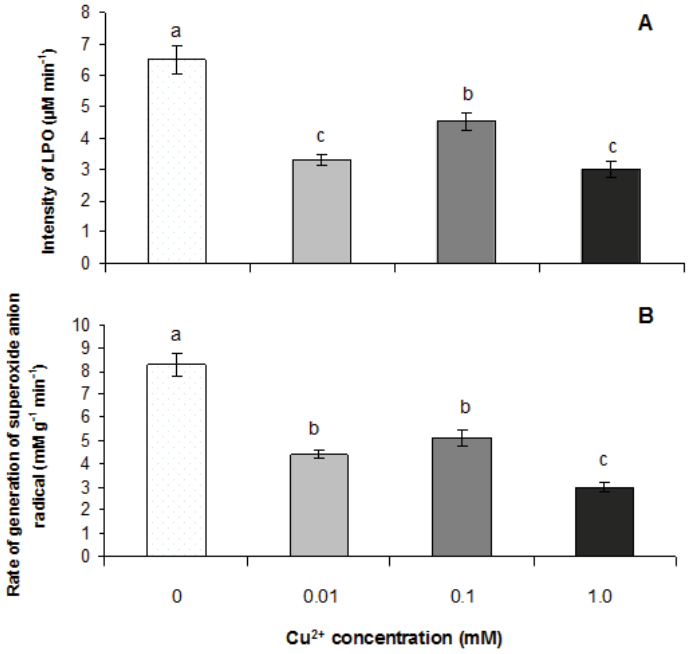


**Figure 2.** Effect of different copper concentrations in growth media on oxidative events in 5-week-old cucumber callus: LPO intensity(A) and rate of superoxide anion generation (B). Values are mean ± SE (independent series, $n = 3$). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's multiple range test

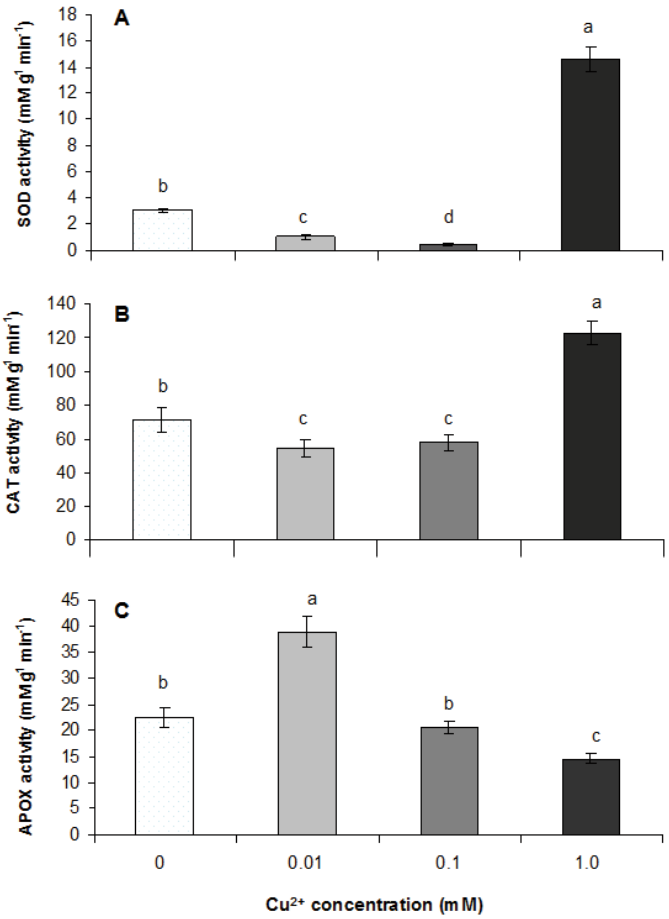


**Figure 3.** Effect of different concentration of copper ions on the activity of antioxidative enzymes in 5-weeks-old cucumber callus: SOD (top), CAT (center), APOX (bottom).Values are mean ± SE (independent series, $n = 3$). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's multiple range test

$O_2^-$ generation was enhanced, on average 2.6-fold more than the water control. SOD activity was 2.1- and 1.7-fold higher than the water control in response to 0.1 and 1.0 mM $Cu^{2+}$, respectively (Fig. 5A). CAT activity increased almost linearly in cucumber plants as $Cu^{2+}$ concentration increased, but was at all concentrations significantly higher than the control (Fig. 5B). Since the substrate for CAT I is $H_2O_2$, $Cu^{2+}$ induces an obvious increase in the formation of $H_2O_2$ in cucumber leaves that forces CAT to "function" more intensively, in this case linearly. APOX activity only increased significantly in response to 0.1 mM $Cu^{2+}$ (Fig. 5C).

## DISCUSSION

Cucumber is a good model plant for stress-related research *in vivo* and *in vitro* because it is easy to grow and is receptive to *in vitro* culture (Vasudevan et al. 2008), making it a very interesting subject for HM stress physiology studies *in vitro* and *in planta* (Burzynski and Żurek 2007, Lukatkin et al. 2010) since cell cultures can be easily established (Lukatkin et al. 2010). In these and other studies, different HMs caused a different and non-linear intensification of $O_2^-$ generation, an increase of TBA-reacting products, changes in activity of antioxidative enzymes and antioxidant levels in cucumber leaves and cotyledons, results that mirror ours but for different species and HMs. More recently, grafting in cucumber was shown to enhance tolerance to Cu by regulating nutrient uptake and the antioxidative system (Zhang et al. 2013).

*In vitro* cell cultures and plant systems, which can be used to test and select for tolerance to environmental stressors (Nehnevajova et al. 2007, Rai et al. 2011), have used callus to test the effect of HMs on other plants such as sugarcane (Fornazier et al. 2002), *Agave amanuensis* (Sugijanto et al. 2002), *Nicotiana tabacum* (Raeymaekers et al. 2003), cotton (Biçakçi and Memon 2005), and tomato (Muschitz et al. 2009).

A comparison of oxidative events and the activity of three antioxidative enzymes in cucumber

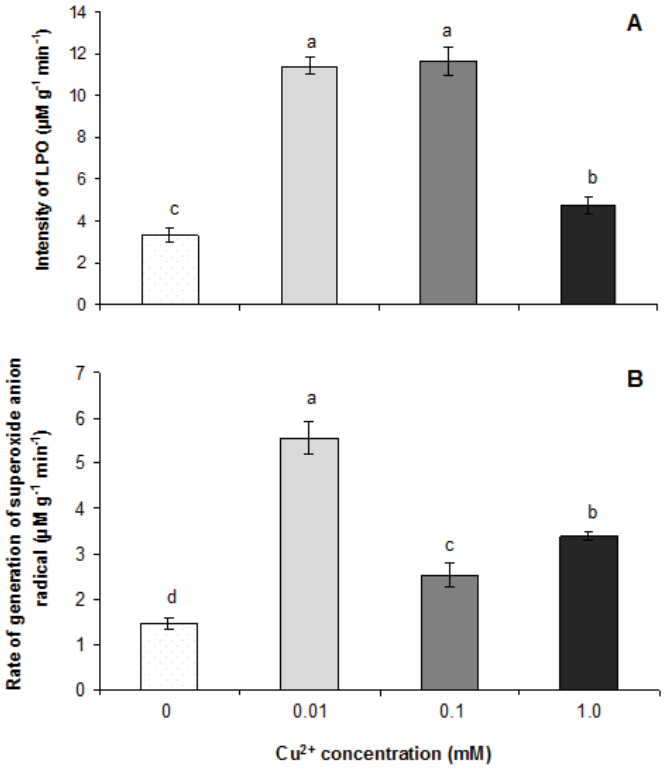


**Figure 4.** Effect of different copper concentrations in growth media on oxidative events in 7-days-old cucumber plants: LPO intensity (top); rate of superoxide anion generation (bottom). Values are mean ± SE (independent series, $n = 3$). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's multiple range test

callus and seedlings was made in this study. Absolute values in cucumber callus were higher than *in planta* while the effect of $Cu^{2+}$ treatment was irregular for both tissues. Thus, plant LPO intensity increased at all concentrations of $Cu^{2+}$, but in callus it decreased at all $Cu^{2+}$ concentrations (Fig. 2A, 4A).This indicates the unfavourable effects of $Cu^{2+}$ on cucumber plants since LPO is an indicator of considerable damage to unsaturated lipids and hence cellular membranes (Lukatkin 2002a). There was a non-linear relationship (i.e., increase) between LPO and membrane damage in cucumber cotyledonary leaves. Moreover, superoxide generation followed the same pattern as LPO intensity (Fig. 2B, 4B). It can be assumed that a long period (5 weeks) of cultivation of callus cells on nutrient medium supplemented with $Cu^{2+}$ promoted partial cell selection and subsequent stability to a HM (Cu in this case) since callus did not show a stress response to $Cu^{2+}$ ions. All concentrations of $Cu^{2+}$ induced sharp oxidative stress in the leaves of cucumber plants. A high dose of $Cu^{2+}$ (1 mM) strengthened ROS generation to a small degree and, hence, caused weak oxidative stress, while a low dose of copper ions (0.01 mM) led to greater stress. These events and differences between callus and plantlets are paradoxic.

SOD activity in plants increased at high and average $Cu^{2+}$ concentrations (Fig. 5A), although an opposite trend was observed in callus: SOD activity always (at all $Cu^{2+}$concentrations in medium) decreased in comparison to the control (Fig. 3A). A low concentration of $Cu^{2+}$ions (0.01 mM) acts as a micronutrient for cucumber plants and consequently does not activate SOD, even reducing it. This is most likely connected with direct enzyme inhibition of SOD protein by Cu. However, it remains obscure why the degree of SOD inhibition in different Cu treatments was not proportional to the concentration of $Cu^{2+}$ ions. It is not clear from our experiments too, why a stronger generation of $O_2^-$ at a low dose of $Cu^{2+}$ (0.01 mM) was accompanied by considerable suppression of SOD activity. Possibly, there was a nonspecific influence of $Cu^{2+}$ on enzyme proteins.

An increase in CAT activity in plants was associated with increased concentrations of Cu (Fig. 5B), although in callus, CAT activity increased only in response to a high $Cu^{2+}$ concentration of 1.0 mM (Fig. 3B). This indicates, indirectly, that the increase in $Cu^{2+}$ concentration caused strong oxidative stress

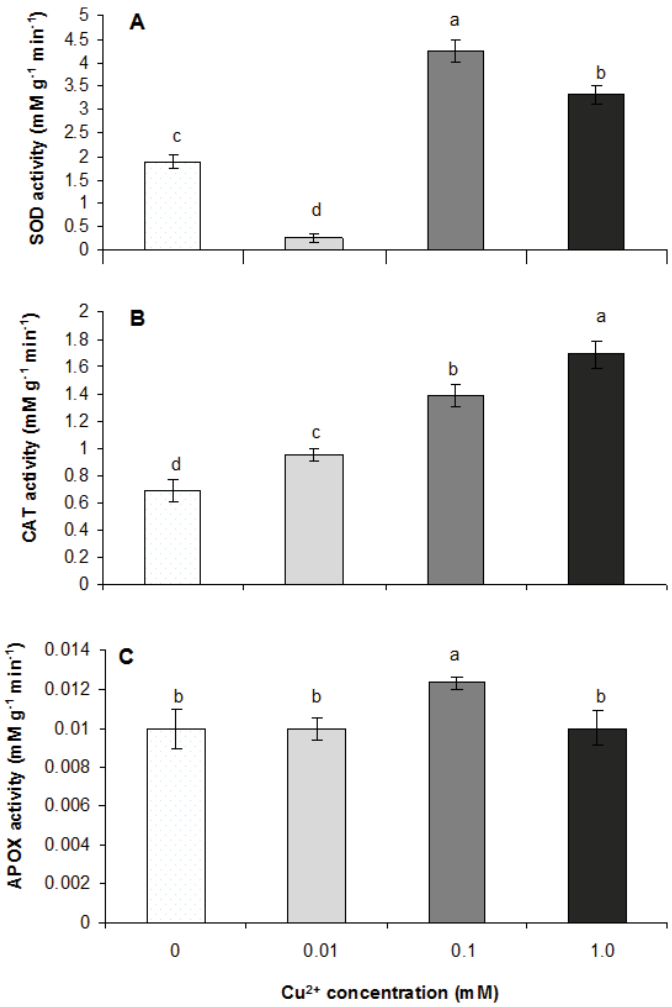


**Figure 5.** Effect of different copper ionconcentrations on the activity of antioxidative enzymes in cucumber plants: SOD (top), CAT (center), APOX (bottom).Values are mean ± SE (independent series, $n = 3$). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's multiple range test

in cucumber plants. CAT is a substrate-activated (inducible) enzyme and enzyme activity (as well as protein level) increase sharply at elevated substrate concentration in the medium (Auh and Scandalios 1997, Lukatkin 2002b).

A clear link between APOX activity and $Cu^{2+}$ concentration could not be established in cucumber *in vitro* callus or *in planta*. APOX is not sensitive to and may act independent of $Cu^{2+}$ concentration. One possible conclusion is that APOX is not involved in cucumber stress reaction in response to $Cu^{2+}$ ions.

In summary, cucumber seedlings affected by $Cu^{2+}$ have strong oxidative stress while cucumber callus has very weak or no oxidative stress. This might be connected with some peculiarities of callus *in vitro*, namely the absence of a long-distant transport of $Cu^{2+}$ ions as is present in the whole plant, the absence of regulation at the tissue and whole-plant level, both of which are affected by $Cu^{2+}$ ions in the whole plant *in vivo*, or the absence of tissues that selectively absorb HMs. Cu causes alterations in the activity of vacuolar $H^+$ transporting ATPase and vacuolar $H^+$ transporting pyrophosphatase in cucumber roots (Kabała et al. 2010). Recent studies provide some new and interesting perspectives. For example, Kojo (2012) demonstrated that oxygen is a major factor differentiating *in vivo* and *in vitro* effects of antioxidants. It may be assumed that the differential effect of copper ions on the antioxidant machinery of cucumber seedlings or callus is related to similar mechanisms. Another possibility explaining the different reaction between cucumber callus and seedlings in response to $Cu^{2+}$ ions is that callus was induced on media containing high levels of 2,4-D and BA, and both these plant growth regulators can contribute to stress reaction (Bartoli et al. 2013). The induced effects on callus might be connected with the adaptation at the cellular level to superfluous doses of HMs in the medium. This latter assumption probably can be used to screen

plants whose growth is stable in the presence of a HM (Rai et al. 2011).

## CONCLUSIONS

1. 1 mM $Cu^{2+}$ negatively affected callus formation from hypocotyls, roots or cotyledons of 7-days-old cucumber plants of 'Edinstvo' cultivar.
2. $Cu^{2+}$ at 10 μM stimulated callus induction but 1.0 mM $Cu^{2+}$ negatively affected callus formation and growth.
3. Relative to the control, lipid peroxidation decreased in callus but increased in plantlets in response to $Cu^{2+}$ and the trend was identical for the generation of the superoxide radical $O2^{-}$.
4. SOD, CAT and APOX activity increased in callus and plantlets, but the exact level of increase depended on the $Cu^{2+}$ concentration.
5. When exposed to $Cu^{2+}$ *in vitro*, cucumber seedlings display stronger oxidative stress than callus.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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