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Oxidative Stress in Cucumber (*Cucumis sativus* L.) Leaf Cells: Short-Term Influence of Heavy Metals (Lead and Copper)

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ABSTRACT

The patterns of oxidative stress expression in the short-term (up to 4 hrs) influenced by heavy metals (lead and copper) was investigated in experiments with cucumber (*Cucumis sativus* L. cv. 'Vjaznikovskij 37') leaf disks. Both metals caused a non-linear intensification of superoxide anion and an increase of TBA-reacting products.

Keywords: leaf disks, lipid peroxidation, superoxide anion
Abbreviations: HM, heavy metal; **LPO**, lipid peroxidation; **MDA**, malondialdehyde; **ROS**, reactive oxygen species; **TBA**, tiobarbituric acid

INTRODUCTION

Environment factors are very different and dynamic, and can often reach dangerous levels for plants. The plant life-cycle undergoes considerable changes and oscillations in response to human activity. Heavy metal (HM) pollution of soils has received considerable attention as a consequence of increased environmental pollution from industrial, agricultural, energetic and municipal sources. This is relevant for many industrial regions in Russia and around the world, as it quali- and quantitatively affects plant production (Bezel *et al*. 1994).

Metal trace elements represent only a small portion of the solid part of soil but their influence on its fertility is well known (Kabata-Pendias 2001). The trace elements (and HMs as a sub-set of this group) are very persistent in soil and can interact by adsorbing to soil particles and therefore increase the risk of long-term soil pollution and the risk of toxic effects on organisms (including humans) (Tarradellas *et al*. 1997; Love and Babu 2006).

All trace metals are distinguished by two groups of elements (Punz and Sieghardt 1993). The first group consists of elements that are necessary for life, including that of plants (Fe, Cu, Mo, Zn, Ni, Co, Mn, V). The bioavailability of these elements is therefore of fundamental importance. A small available amount of these elements can cause deficiencies in plants. However, in the case of pollution, a large available amount of these elements can be detrimental to plant growth (Mitsios and Danalatos 2006). The second group includes the non-essential (toxic) elements (Ag, Cd, Cr, Hg, Pb). So elements of the first and second group are potentially toxic for plants in above-optimal concentrations. The accumulation of some HMs in leaves can be considered as a means to assess dust contamination in urban environments (Mingorance and Rossini Oliva 2006).

The most toxic element to plants in first group is copper, Cu. Cu, as an essential biological element, interferes with numerous physiological functions. It is a constituent micronutrient of the protein component of several enzymes, mainly of those participating in electron flow, catalyzing redox reactions in mitochondria, chloroplasts, cell walls and in the cytoplasm of plant cells. However, excessive concentrations can be toxic to plants (De Vos *et al*. 1991). A well-known harmful effect of Cu is the inhibition of growth and alteration of plasma membrane permeability (Ouzounidou 1995).

Lead (Pb) is not needed biologically and is toxic to most living organisms exposed to high concentrations. Pb might interfere with physiological functions of plant tissues (growth, photosynthesis, respiration and others) (Fargasova 2004; Li *et al*. 2004; Bertrand and Poirier 2005). These two elements are interesting for investigation as representative members of the two different groups of trace elements and are thus the choice of HMs for this study.

HMs influence oxidative stress (Sharma *et al*. 1999), which is the state of shifted balance between reactivated oxygen species (ROS) and antioxidants in plant cells (Scandalios 1990; Dubey *et al*. 2005). This can result in serious cell damage, causing a variety of metabolic imbalances and destruction of biomolecules (Bertrand and Poirier 2005; Clemens 2006).

Induction of the oxidative burst by HMs is still poorly characterized despite numerous studies. The induction of an oxidative response to HMs by different plant species is explained in **Table 1**. Severe oxidative stress was induced by different HMs (Cd, Cu, Zn, Pb, Ni, etc.) and was visible by ROS generation, glutathione and ascorbate oxidation, enhancement of lipid peroxidation (LPO), accumulation of hydrogen peroxide (H_2O_2), and inactivation (or activation) of antioxidative enzymes. However, all these events were species-, cultivar-, concentration- and time-dependent. For example, in suspension culture roots of *Panax ginseng* grown in bioreactors Cu induced changes in oxidative metabolism at 50 μM Cu, but not at 5 and 20 μM Cu (Ali *et al*. 2006).

Intensive ROS formation and, as a consequence, activation of LPO are early nonspecific reactions of vegetative crops to some stress factors, including HMs (Lukatkin 2002; Choudhury and Panda 2004; Chaneva *et al*. 2006). Thereof, analysis of parameters such as oxidative stress, generation of $O_2^{\cdot-}$ as well as LPO intensity, are indispensable to establish the legitimacy of the claim that HMs induce oxidative stress.

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Table 1 Oxidative responses induced by heavy metals in different plant species.

Species	Experimental conditions	HM applied	Concentration of HMs	Oxidative events	Antioxidants	References
Arabidopsis thaliana	Plants grown for 3 weeks	Cd	1 or 10 μM	Lowering of reduced GSH, rise in GSSG and decrease in GSH/GSSG.	Enhanced mRNA level for GSH-synthesizing enzymes, lowering GR transcripts. Increase of GR, APX, CAT and SOD activity.	Semane *et al*. 2007
Arabidopsis thaliana	Plants treated 7 days with HMs	Cd, Cu	5-100 μM	Increased lipid peroxide content and MDA + hydroalkenals level.	GST activity enhanced in Cu-stressed plants and in 100 μM Cd-treated plants.	Skorzynska-Polit *et al*. 2010
Cucumis sativus	Hydroponic culture	Cd	Up to 100 μM	Lowering of chlorophyll content, enhancing of MDA level, H_2O_2 and O_2^- in chloroplasts.	Decrease of SOD, APX and GR activity, as well as glutathione and AsA level (in chloroplasts).	Zhang *et al*. 2003
Eruca sativa	Greenhouse	Zn	0.25–2 mg g^{-1} dried growth medium	LPO increased in proportion with an increase in Zn.	Decrease of SOD and CAT activities at increased Zn; increase of APX and POD activities. Increase of non-protein thiols and AsA content.	Ozdener and Aydin 2010
Helianthus annuus roots	Hydroponic culture	Cu	50 μM	Not specified	Decrease of SOD activity, enhance of CAT and POD activity.	Jouili and El Ferjani 2003
Helianthus annuus	Callus cells	Cd	150 μM	Enhanced LPO and protein oxidation.	Decline in both GSH and GSSG levels with concomitant reduction in the GSH/GSSG ratio.	Gallego *et al* 2005
Hordeum vulgare	Hydroponic culture	Cd	25 μM	Enhanced LPO (measured by MDA content).	Increased APX and CAT activity.	Metwally *et al*. 2003
Lycopersicon esculentum	Hydroponic culture in greenhouse	Zn	Not specified	Increase in MDA level.	Increased SOD, CAT and GPX activity, decreased POD activity. APX activity enhanced in first 3 day then decreased; GR activity lowered in first 5 day then rose. AsA and GSH levels increase.	Ding *et al*. 2005
Nicotiana tabacum	Cell suspension cultures	Cu	Up to 100 μM	Fast and concentration-dependent H_2O_2 accumulation.	Not specified	Raeymaekers *et al*. 2003
Oryza sativa roots	Hydroponic culture, 24 h Cd treatment	Cd	10,100, 1000 μM	Rise of LPO intensity, H_2O_2 level and O_2^- generation.	Increase of SOD, CAT, POX and GR activity; enhanced AsA and glutathione levels. At high Cd concentration the glutathione level decreased.	Choudhury and Panda 2004
Oryza sativa	Cd-sensitive mutants	Cd	0.5 mM	Rapid H_2O_2 increase.	Lowered CAT activity in leaves and enhanced POD activity in roots.	Chen *et al*. 2007
Panax ginseng	Suspension culture roots grown in bioreactors	Cu	50 μM (not 5 and 20 μM)	Increase of MDA content, O_2^- accumulation, AsA and GSH oxidation.	Inhibition of CAT, glutathione peroxidase and glutathione metabolism enzymes activity.	Ali *et al*. 2006
Pisum sativum	Seedlings were planted on Knop's solution in pots	Cd	50 and 100 μM	Increase in H_2O_2 and MDA level in leaves.	Not specified	Chaneva *et al*. 2006
Pisum sativum	Hydroponic culture	Cd	50 μM	O_2^- and H_2O_2 hyperaccumulation.	Decrease of GSH and AsA as well as CAT, GR, CuZnSOD and POD activity. Enhanced MnSOD activity.	Rodriguez-Serrano *et al*. 2006
Pisum sativum roots	Hydroponic culture	Pb	0.1 and 0.5 mM	Rapid increase in O_2^- formation and H_2O_2 and MDA level, proportional to Pb concentration.	GSH/GSSG dropped proportionally to lead stress intensity. SOD, CAT and APX activities changed.	Malecka *et al*. 2009
Saccharum officinarum leaves	Hydroponic culture	Cd	2 and 5 mM	Not specified	CAT activity decreasing, GR activity increasing. SOD activity not changed, but disappeared one isozyme Cu/ZnSOD.	Fornazier *et al*. 2002
Secale cereale	Hydroponic culture	Pb or Ni	10–1000 μM	LPO increase (measured by MDA); decrease in O_2^- generation at high concentration of Pb.	Not specified	Lukatkin *et al*. 2009
Sesbania drummondii	Callus cultivated 4 week on media supplemented with Cd	Cd	Up to 250 μM	Not specified	GSH level and GSH/GSSG enhanced (up to 50 μM Cd), then dropped. SOD, APX and GR activity changed analogous to glutathione.	Israr *et al*. 2006
Triticum durum	Greenhouse	Cd	Up to 40 μM	Enhanced LPO, protein oxidation; H_2O_2 overproduction.	Decrease in the ascorbate redox state; activity of the ASC–GSH cycle enzymes significantly increased.	Paradiso *et al*. 2008
Zea mays	Hydroponic culture	Zn or Ni	1 and 5 mM	Not specified	Increase of APX activity proportional to Zn concentration, and decreased at 5 mM Ni.	Lukatkin *et al*. 2007

Abbreviations: APX, ascorbate peroxidase; **AsA**, ascorbic acid; **CAT**, Catalase; **GPX**, glutathione peroxidase; **GR**, glutathione reductase; **GSH**, glutathione reduced; **GSSG**, glutathione oxidized; **GST**, glutathione-*S*-transferase; **H_2O_2**, hydrogen peroxide; **LPO**, lipid peroxidation; **MDA**, malone dialdehyde; **O_2^-**, superoxide anion; **POD**, guaiacol peroxidase; **POX**, peroxidase; **SOD**, superoxide dismutase

Table 2 Effect of duration of cucumber leaf discs exposition in $Pb(NO_3)_2$ solutions on $O_2^{\cdot-}$ generation, μM· (g fresh weight)$^{-1}$.

$Pb(NO_3)_2$ concentration	Prior exposure	After exposure at indicated duration			
		1 h	2 h	3 h	4 h
H_2O	4.91 ± 0.80 ef	5.80 ± 0.47 ef	6.60 ± 0.34 e	4.98 ± 0.59 f	4.18 ± 0.40 f
10 mM		4.19 ± 0.72 f	4.09 ± 0.63 f	4.16 ± 0 .46 f	2.40 ± 0.22 g
100 μM		2.44 ± 0.53 g	2.40 ± 0.21 g	2.48 ± 0.26 g	4.87 ± 0.31 f
10 μM		4.96 ± 0.62 f	15.70 ± 0.31 bc	13.22 ± 0.80 c	5.87 ± 0.62 ef
1 μM		22.31 ± 1.31 a	14.00 ± 0.58 c	14.12 ± 0.86 c	10.72 ± 0.83 d
0.1 μM		11.62 ± 0.77 cd	13.17 ± 0.71 c	16.52 ± 0.88 bc	17.40 ± 1.61 b

Different letters within a column indicate significant differences at $P \leq 0.05$ according to Tukey's test.

The purpose of this study was to reveal the effects of two HM ions (Cu^{2+} and Pb^{2+}) on the development of oxidative stress (dynamic changes of ROS and LPO) in cucumber seedlings. As sub-sets of this broader objective, three other purposes were defined: a) to establish the concentrations of Pb^{2+} and Cu^{2+} that would generate $O_2^{\cdot-}$ in cucumber leaf discs; b) to reveal the relation between $O_2^{\cdot-}$ generation in cucumber cells and the duration of exposure of leaf discs to Pb^{2+} and Cu^{2+} at different concentrations; c) to evaluate the dynamic changes of LPO intensity in cucumber leaf discs at different concentrations of Pb^{2+} and Cu^{2+}.

MATERIALS AND METHODS

Young (ages: 10–17 days) cucumber plants (*Cucumis sativus* L.) var. 'Vjaznikovskij 37' were used.

The plants were grown in pot culture. Seeds were superficially sterilized (10–15 min) in a 0.5 % potassium permanganate solution were sown in vessels with soil (degraded Chernoziom, 2 kg per vessel) and grown at 22–25°C, photon flux density (PFD) approx. 200 μM m^{-2} s^{-1} (illumination provided by luminescent lamps), soil humidity = 60–80% from full moisture capacity, and 12-hr photoperiod.

The upper leaves were detached from 10–17-day-old plants and leaf discs, made by an 0.8 cm diameter cork borer, were immersed in Petri dishes with 10 ml of HM solutions of different concentrations (10 mM up to 0.1 μM) and incubated from 1–4 hrs. The control was maintained for same time in 10 ml of distilled water at room temperature. When incubation ended the $O_2^{\cdot-}$ generation rate and LPO intensity were determined.

$O_2^{\cdot-}$ generation rate was determined by a method based on the capacity of this radical to oxidize epinephrine in adrenochrome (Lukatkin 2002). 300 mg of a leaf discs was homogenized in 15 ml of distilled water. The homogenate was centrifuged for 15 min at 8000 × *g*. 100 μl epinephrine solution (pH 7.2–7.8) was added to 3 ml of homogenate and incubated for 45 min at room temperature and PFD = 80 μM m^{-2} s^{-1}. Absorbance was measured immediately at 480 nm against a homogenate with water using an SF-46 (LOMO, Russia) spectrophotometer. $O_2^{\cdot-}$ concentration was calculated as μM $O_2^{\cdot-}$ g^{-1} of fresh weight (FW) with a molar extinction coefficient ε = 4020 M^{-1} cm^{-1} (Lukatkin 2002).

LPO intensity was estimated in leaf discs by accumulation of an LPO product, malondialdehyde (MDA), in a staining reaction with thiobarbituric acid (TBA) (Lukatkin 2002). About 300 mg of leaf discs was homogenized in isolation medium (0.1 M tris-HCl buffer at pH 7.6 using 0.35 M NaCl). 2 ml TBA in 20% trichloroacetic acid was added to 3 ml of a tissue homogenate, heated in a boiling water bath within 30 min then filtered through white filter paper BFS (Filter paper laboratory, average filtering, Volzhsk, Russia). Absorbance was measured with an SF-46 spectrophotometer at 532 nm against isolation medium with a reactant (without plant material). MDA concentration was calculated with molar extinction ε = 1.56 × 10^5 M^{-1} cm^{-1}. The quantity of MDA in leaves was calculated as μM MDA g^{-1} FW.

The following reagents were used: TBA and Tris from Merck KGaA (Darmstadt, Germany); epinephrine (adrenaline hydrochloride) solution from Moscow Endocrine Plant, Russia; HCl and NaCl from NPO "EKROS", St. Petersburg, Russia.

All experiments were conducted in triplicate, and each experiment consisted of 10-16 separate seedlings for each experimental variant. For all measurements, averages and standard errors were calculated by standard mathematical methods 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 separation of means using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

$O_2^{\cdot-}$ generation in cucumber leaf discs

One of the main toxic ROS to plant cells is the superoxide anion radical $O_2^{\cdot-}$ (Scandalios 1990), and numerous articles have shown a rise in $O_2^{\cdot-}$ generation in almost all environmental stressors (e.g., Elstner and Osswald 1994; Apel and Hirt 2004).

To estimate the level of a superoxide, relatively stable ROS are used in a reaction with epinephrine, resulting in the formation of adrenochrome. In experiments investigating the effects of Pb and Cu on the generation of $O_2^{\cdot-}$, the contents of superoxide in control leaf discs (maintained in distilled water) was low (4.2–6.6 μM g^{-1}) and their changes over time were insignificant (**Table 2**).

The measurements, conducted after leaf discs were exposed to $Pb(NO_3)_2$ solutions of different concentrations and duration, showed that the Pb ions increased the superoxide contents in disks, but this increase depended both on the exposure time and Pb concentration (**Table 2**). Exposure of leaf discs to high Pb concentrations (10 mM and 100 μM) did not influence the generation of $O_2^{\cdot-}$, or the $O_2^{\cdot-}$ level was lower than the control.

Lowering Pb concentration had an opposite effect on generation of $O_2^{\cdot-}$ in cucumber leaf discs. At 10 μM there was a sharp increase in $O_2^{\cdot-}$ level (3.2-fold) after 2 h exposure. However, subsequent exposure of leaf discs to 10 μM resulted in a gradual lowering of the $O_2^{\cdot-}$ level.

As a result of the exposure of cucumber leaf discs to 1 μM and 0.1 μM Pb^{2+} there was a significant increase (4.5- and 2.4-fold, respectively) in the level of $O_2^{\cdot-}$, even at the minimum exposure time, i.e., 1 hr. A more prolonged exposure of leaf discs to low [Pb^{2+}] solutions changed the $O_2^{\cdot-}$ level in a different way: there was a step-by-step reduction with 1 μM Pb^{2+} or a steady increase with 0.1 μM Pb^{2+}, so after 2 hrs the $O_2^{\cdot-}$ level in leaf disks was same at both concentrations; however, after 4 hrs and for an unexplainable reason, this level varied more with 0.1 μM Pb^{2+}.

Comparing the effects of different concentrations of $Pb(NO_3)_2$ on $O_2^{\cdot-}$ generation in cucumber leaf discs, the highly-toxic doses of Pb, i.e., 10 mM and 100 μM, negatively affected the $O_2^{\cdot-}$ level in cells, whereas the certainly non-toxic lead doses (0.1–10 μM) resulted in a considerable increase of levels of superoxide anions. This paradoxical behavior of Pb^{2+} ions can be explained on the basis of mechanisms of how Pb affects plant cells. Pb^{2+} ions interact with SH-groups of proteins. As a result, there is a change in protein conformation that in turn results in inactivation of enzymatic systems, including those responsible for $O_2^{\cdot-}$ formation (Seregin *et al*. 2004). Experimental data to date indicates that the main superoxide sources during oxidative burst are the enzymatic systems localized in a plasma membrane (NADPH-dependent oxidase) (Minibaeva and Gordon 2003); in mitochondria (NADH-dehydrogenase); in peroxisomes (xanthine oxidase and glycolate oxidase); in the endoplasmic reticulum (NADPH cytochrome *c* reductase, lipoxygenase); in chloroplasts (ribulose diphosphate

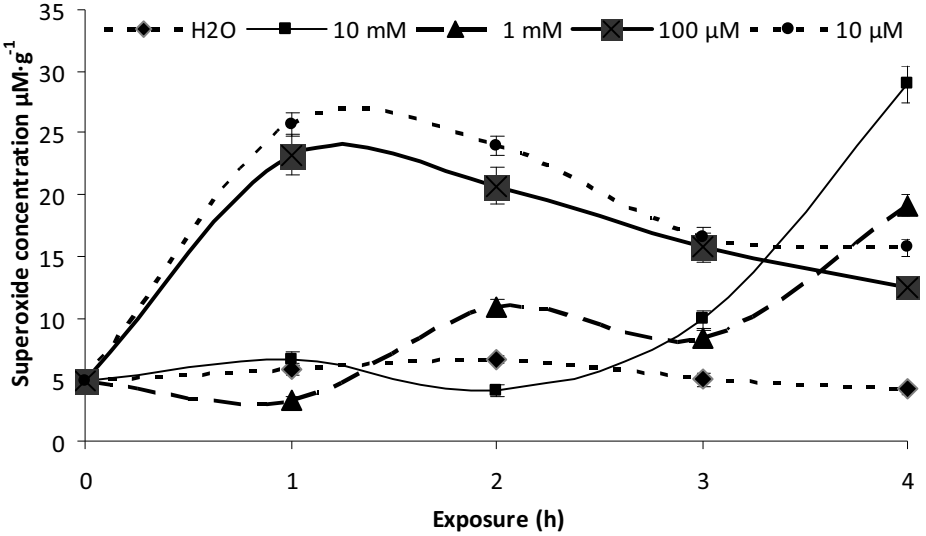

Fig. 1 Effect of duration of cucumber leaf discs exposition in $CuSO_4 \cdot 5H_2O$ solutions on $O_2^{\cdot-}$ generation, µM· (g fresh weight)$^{-1}$.

Table 3 Effect of duration of cucumber leaf discs exposition in $Pb(NO_3)_2$ solutions on LPO intensity, µM MDA· (g fresh weight)$^{-1}$.

$Pb(NO_3)_2$ concentration	Prior exposure	After exposure at indicated duration			
		1 h	2 h	3 h	4 h
Control		3.08 ± 0.11 de	3.12 ± 0.13 de	2.99 ± 0.04 e	2.93 ± 0.09 e
10 mM		3.40 ± 0.11 cd	3.38 ± 0.10 cd	2.74 ± 0.07 ef	2.65 ± 0.10 f
1 mM		3.14 ± 0.11 de	2.84 ± 0.11 ef	2.71 ± 0.06 f	1.65 ± 0.11 h
100 µM	2.91 ± 0.11e	3.89 ± 0.09 bc	3.25 ± 0.07 d	2.86 ± 0.07 ef	2.05 ± 0.09 g
10 µM		3.38 ± 0.09 cd	2.61 ± 0.09 f	3.55 ± 0.04 c	2.20 ± 0.10 g
1 µM		4.19 ± 0.08 b	4.00 ± 0.11 b	3.65 ± 0.12 bc	3.18 ± 0.11 de
0.1 µM		4.66 ± 0.15 a	4.06 ± 0.12 b	4.64 ± 0.17 a	3.70 ± 0.15 bc

Different letters within a column indicate significant differences at $P \leq 0.05$ according to Tukey's test.

carboxilase), etc. (Apel and Hirt 2004). Probably the high Pb^{2+} concentrations, through their toxicity, can inactivate these enzymatic systems. As a result, the $O_2^{\cdot-}$ level drops. The low Pb^{2+} concentrations act as the stressful factor and result in increased $O_2^{\cdot-}$ generation and in oxidative stress induction.

The exposure of leaf discs to $CuSO_4 \cdot 5H_2O$ has shown another effect, namely, the intensification of $O_2^{\cdot-}$ formation at all investigated Cu concentrations, but this $O_2^{\cdot-}$ elevation differed depending on the duration of exposure and the concentration of the solution (**Fig. 1**). At high Cu^{2+} concentrations (10 and 1 mM) the highest $O_2^{\cdot-}$ levels were noted after 4 hrs of exposure while low Cu^{2+} concentrations (100 and 10 µM) showed the highest $O_2^{\cdot-}$ levels after 1 hr exposure with a consequent lowering of the level of superoxide (although its level was always higher than the control). Thus, at high and injurious Cu^{2+} concentrations (10 and 1 mM) the level of $O_2^{\cdot-}$ was low in the first 3 hrs then increased sharply after 4 hrs of exposure. This has two possible explanations: 1) heightened $O_2^{\cdot-}$ formation, and 2) attenuated activity of radical-quenching systems.

As for Pb^{2+} ions, where high concentrations inhibited enzymatic systems of $O_2^{\cdot-}$ generation, we propose that high concentrations of Cu^{2+} ions also suppress $O_2^{\cdot-}$ formation for some time. However, prolonged exposure of leaf discs to copper solutions leads to extremely high $O_2^{\cdot-}$ generation by other $O_2^{\cdot-}$-generating systems in cucumber cells.

The superoxide content in cells is controlled by SOD activity (Bowler *et al*. 1992; Sen Raychaudhuri 2000) *via* superoxide dismutation to form H_2O_2, and activity of this enzyme is enhanced when $O_2^{\cdot-}$ concentration is high (Bowler *et al*. 1992). In our work a maximum of generating $O_2^{\cdot-}$ was at low $CuSO_4 \cdot 5H_2O$ concentrations (100 and 10 µM) after 1 hour. It is possible that different speeds of superoxide radical inactivation at miscellaneous HM concentrations are connected to the activity of antioxidative enzymes, mainly SOD. The activity of some forms of SOD increase at low Cd concentrations (up to 50 µM), but ar reduced at high Cd concentrations (up to 250 µM) (Israr *et al*. 2006) (see more in **Table 1**).

LPO intensity in cucumber leaf discs

It is possible to suspect that increased $O_2^{\cdot-}$ formation in plant cells influenced by HM will enhance the intensity of lipid peroxidation in tissues and that these two processes are correlated. To verify this hypothesis, we measured the contents of TBA-reacting compounds parallel with ROS determination. The base TBA-reacting compound is MDA (Lukatkin and Golovanova 1988). LPO balances plant cells in the absence of extreme endo- and exogenic factors. The concentration of LPO products remains at a constant low level under normal conditions (Lukatkin and Golovanova 1988; Merzlyak 1989). From **Table 3** it is possible to see that level of TBA-reacting compounds (= MDA content) did not change when exposed to water (control). However, exposure to different $Pb(NO_3)_2$ solutions lead to significant alterations in MDA content and therefore in LPO intensity. MDA content tended to enhance cucumber leaf discs already after 1 hr exposure to $Pb(NO_3)_2$ solutions. MDA content increased as lead concentration decreased. Maximum MDA level was noted when Pb^{2+} concentration was 0.1 µM.

Further exposure of leaf discs to $Pb(NO_3)_2$ solutions resulted in a gradual decrease of MDA content. So LPO intensity was lower or equal to the control at Pb^{2+} concentrations ranging from 10 mM to 10 µM although MDA content was higher than the control level at Pb^{2+} concentrations of 1 µM and 0.1 µM.

Exposure of cucumber leaf discs to $CuSO_4 \cdot 5H_2O$ solutions induced an initial (not always significant) increase in LPO (**Fig. 2**). Measurements conducted after the exposure of leaf discs to different concentrations of Cu solution showed that the maximum MDA level occurred at a Cu concentration of 10 mM and 1 mM (1.6- and 1.4-fold increase, respectively over the control) when exposed for the longest period, i.e. 4 hrs. At low copper concentrations, i.e.,

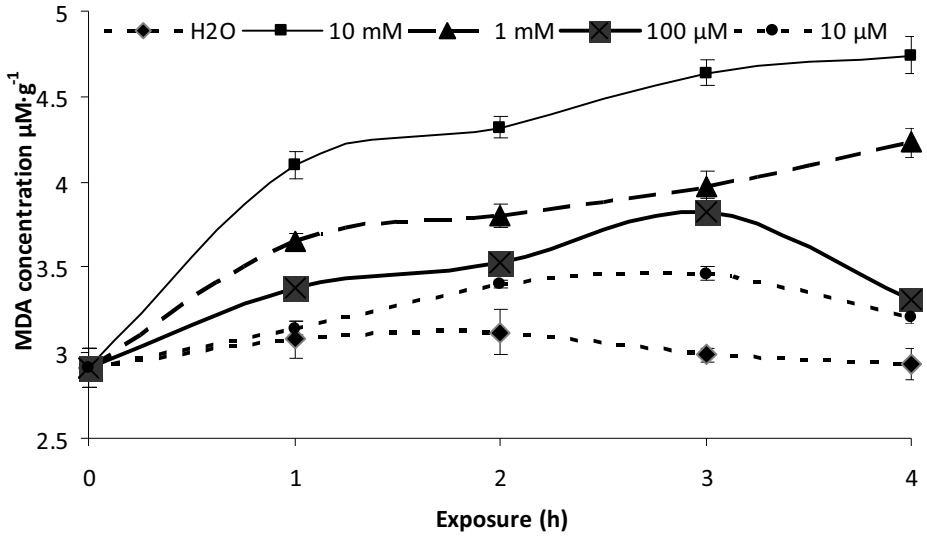

Fig. 2 Effect of duration of cucumber leaf discs exposition in $CuSO_4 \cdot 5H_2O$ solutions on LPO intensity, µM· (g fresh weight)$^{-1}$.

100 and 10 µM, a spike in LPO was noted after exposure for 3 hrs, and MDA level decreased when exposed further (after 4 hrs) to these $CuSO_4 \cdot 5H_2O$ solutions.

Cu^{2+} and Pb^{2+} intensity increased LPO levels and enhanced $O_2^{\cdot-}$ generation in cucumber leaf discs. However, lipid peroxidation increased superoxide generation over time. This might be explained by the participation of other ROS in LPO initiation, particularly highly reactive singlet oxygen $^1O_2$, which enters oxidizing reactions with organic compounds and initiates LPO. The mechanisms of $^1O_2$ generation are unknown in living organisms, although $^1O_2$ is a concomitant product in many enzymatic reactions, including those with ROS (Merzlyak 1989).

It is possible to explain the sharp increase in LPO intensity after the first hour of exposure to $Pb(NO_3)_2$ influence and decrease after prolonged exposure as "paradoxical effects" (Veselova *et al*. 1993). Weaker doses of a stressful factor result in stronger plant or cell responses than higher doses (in our case exposure duration and HM concentration). According to this concept a decrease in LPO is connected with cell capacity to initially disturb repair as well as the capacity of a cell to alter its stability (Veselova *et al*. 1993; Lukatkin *et al*. 1995).

In the case of $CuSO_4 \cdot 5H_2O$ treatment an increase in LPO intensity can be interpreted as a dose-dependent relation. An increase in dose (equivalent to exposition duration and HM concentration) results in proportional enhancement of cell responses (formation of LPO product which is detected from the MDA content). The increase in MDA content affected by Cu^{2+} can be explained by LPO activation via excess metals with variable valence (Merzlyak 1989).

ROS production and LPO are normal physiological processes in plant cells. ROS are involved in cell metabolism, they participate in the synthesis of some plant constituents and in the disassembly of injured membranous components. $O_2^{\cdot-}$ at low concentrations is a physiological modulator in mitochondria, regulating Ca^{2+} transport (Richter and Gogvadze 1995). ROS participate in cell wall lignification and in protection of plants against microflora (Ros Barceló 1998; Kotchoni and Gachomo 2006). Besides, cells can used the LPO process, which binds with modified membrane structures, as a tool to regulate the activity of membrane-bound enzymes. The speed constant of LPO reactions is supported by the antioxidative system. In stress conditions, in particular HMs, there is a disequilibrium of prooxidants/antioxidants in a cell; as a result, oxidative stress develops (Ding *et al*. 2005).

CONCLUSIONS

In this study, we demonstrate that cucumber cells exposed to $Pb(NO_3)_2$ and $CuSO_4 \cdot 5H_2O$ solutions lead to the generation of $O_2^{\cdot-}$ while enhancing LPO processes that can result in different physiological disturbances in plants. The level of HM-induced disorganization is a function of exposure intensity (duration and concentration) in a HM solution (Balachnina *et al*. 2005). A plant's sensitivity to a HM is probably determined by poor functioning of an antioxidative protective system as well as a low capacity to counter secondary disturbances, induced by changes at cellular and molecular levels.

The following conclusions may be drawn from our study:

1. High Pb^{2+} concentrations (10^{-2} and 10^{-4} mol/l) reduce $O_2^{\cdot-}$ generation in cucumber leaf cells by inhibiting enzymatic systems, accountable for their formation, whereas low Pb^{2+} concentrations (10^{-5}–10^{-7} mol/l) induce oxidative stress in plant cells.

2. The $O_2^{\cdot-}$ level rises in cucumber cells as the exposure of leaf discs to high concentrations of $CuSO_4 \cdot 5H_2O$ solutions increases, but decreases at low Cu^{2+} concentrations (10 and 100 µM).

3. All investigated Pb^{2+} and Cu^{2+} concentrations initially (after 1 hr) enhanced lipid peroxidation with a subsequent decrease in LPO level. The only exception was with high Cu^{2+} concentrations (10^{-2} and 10^{-3} mol/l), which monotonically increased the LPO level during the entire exposure (up to 4 hrs).

4. Oxidative stress in cucumber cells arising from the effect of HMs on leaf discs is caused by enhanced $O_2^{\cdot-}$ formation, which, together with other ROS, induces chain LPO processes.

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