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THE IMPACT OF COPPER IONS ON OXIDATIVE STRESS IN GARDEN CRESS *Lepidium sativum*

WPŁYW JONÓW MIEDZI NA STRES OKSYDACYJNY U PIEPRZYCY SIEWNEJ *Lepidium sativum*

Abstract: Normal oxygen metabolism is an endogenous source of reactive oxygen species (ROS). The source of ROS are also many environmental factors including heavy metals. In certain concentration range, the presence of ROS is necessary to maintain proper cell function. Thus, cells have many mechanisms, which role is focused on maintaining a constant concentration of ROS. Imbalance between the formation of ROS and action of a protective antioxidant system leads to oxidative stress. This may results with a damage to the structure of proteins, lipids and nucleic acids, which in turn can lead to disturbances in the functioning of the cell and even to the death. The aim of the study was to evaluate the effect of copper ions on the metabolic activity of garden cress *Lepidium sativum* L. The action of copper ions with different concentrations was treated seeds. After four, six and eight days after planting in the leaves of garden cress were determined the specific activity of guaiacol peroxidase (GPOX), lipid peroxidation and protein content. Additionally intake of copper ions was determined using adsorption spectrometry technique. The results revealed that the applied doses of copper ions affected the activity of guaiacol peroxidase. The highest enzyme activity was found in plant material, which was treated with dose of copper ions 1000 mg/dm^3 regardless of day. In the same samples the lowest level of lipid peroxidation was found. The highest concentrations of total proteins was found in samples treated with the highest dose of copper ions. The copper content in the tested plant material is correlated with the applied dose of copper ions. Our results indicate reliable correlations between copper content and values of oxidative stress biomarkers in plant tissues.

Keywords: *Lepidium sativum*, copper, oxidative stress

Introduction

Reactive oxygen species (ROS) have important roles in cell signaling and are a natural product of the normal oxygen metabolism in the cell. This is an endogenous source of ROS. The source of ROS are also many environmental factors, exogenous sources, which can be divided into two groups: abiotic and biotic. Abiotic sources of ROS are including UV radiation, high temperature, freezing, drought and xenobiotics such as drugs or heavy metals. Biotic sources are pathogens such as fungi or bacteria. In certain concentration range, the presence of ROS is necessary to maintain proper cell function such as regulation of gene expression governing such processes as cell cycle, growth and development.

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Furthermore, ROS is involved in plant signal systems. The production of reactive oxygen species is normally carefully controlled by the plant. Thus, cells have many mechanisms, enzymatic and non-enzymatic, which role is focused on maintaining a constant concentration of ROS. Plants possess specific antioxidant enzymes such as peroxidase, catalase and superoxide dismutase and low-molecular weight antioxidants like ascorbate, glutathione, tocopherols, flavonoids and carotenoids. Imbalance between the formation of ROS and action of a protective antioxidant system leads to oxidative stress, especially when plants are exposed to abiotic stress. This may results with a damage to the structure of proteins, lipids and nucleic acids, which in turn can lead to disturbances in the functioning of the cell and even to the death [1, 2].

Copper is classified as heavy metal but, contrary to cadmium, lead or mercury, is an essential micronutrient for plant, playing an important role in maintaining plants natural metabolism and growth. Copper is a common cofactor for many enzymes including oxidases and certain proteases, is included in the composition of several proteins, which participate in numerous processes crucial for vital functions of the cell. It is known that Cu binds to chromosomes participating in the maintenance of their structure. On the other hand copper in excess is also an inhibitor of various physiological functions, for example root growth inhibition. Above an optimal level becomes toxic (above 30 ppm). Copper as a transition, very active metal has ability to produce reactive oxygen species and thus may be responsible for oxidative stress in plants [3, 4]. Copper is widely distributed in nature, mainly because of human activities. Use of Cu-bearing compounds in agriculture for combating soil pathogens results in its accumulation in the soil and plants. The effect of Cu excess is clearly manifested in suppression of plant growth and disturbance of photosynthesis. Furthermore, excess Cu in plants caused changes in activity and the content some components of antioxidant system, such as specific antioxidant enzymes, low-molecular nonprotein antioxidant and metal-binding SH proteins [2].

The aim of the study was to evaluate the effect of copper ions on the metabolic activity of garden cress *Lepidium sativum*.

Materials and methods

Plant materials and treatments

Lepidium sativum L. is commonly known as garden cress. Its a small, fast growing annual herbaceous plant, that is native to Egypt and west Asia, but now is cultivated in the entire World. The edible is the whole plant and seeds, which have health promoting properties, so is functional food ingredient. This plant is a rich source of vitamins, minerals, unsaturated fatty acid and phenolic compounds [5, 6].

Seeds of *L. sativum* (5 g) were treatment with different concentrations of Cu^{2+} supplied in $CuSO_4$ solution: 0 (control), 500 and 1000 mg/dm^3. Each treatment was made in five replicates. After 12 hours of Cu^{2+} treatment the seeds were washed with sterile water and sowed on Petri dishes. The cultivation was carried out in growth chamber in a photoperiodic system day/night 14/10 hours at temperature 20/16°C respectively and 70% relative humidity. Irrigation with Knap solution was provided in sufficient quantities for plant growth. After four, six and eight days after planting in the leaves of garden cress were determined protein content, the specific activity of guaiacol peroxidase (GPOX), lipids peroxidation and copper content.

Guaiacol peroxidase extraction and measuring of activity

The specific activity of guaiacol peroxidase (GPOX) was determined spectrophotometrically using guaiacol as the substrate and H_2O_2 as the hydrogen donor. By the oxidation of guaiacol is formed coloured tetraguaiacol (extinction coefficient 26.6 $mM^{-1}cm^{-1}$).

For enzyme extraction 1 g of shoots was homogenized using a chilled mortar and pestle with 2 cm^3 of 0.1 M sodium phosphate buffer (pH = 7.0). The extraction procedure was carried out on ice. The homogenate was centrifuged for 20 min at 10 000 rpm (revolutions per minute) at 4°C. The supernatant was used of protein and enzyme activity measurement. Guaiacol peroxidase activity GPOX was determined according to the modified method by Zaharieva et al. [7]. The reaction mixture (5.0 cm^3) consist of 0.1 M phosphate buffer (pH = 7.0), 38 mM H_2O_2 and 4 mM guaiacol. The reaction was started by the addition of 0.2 cm^3 supernatant (enzyme extract). Absorption intensity of extractions was determined after 1 minute in wave length 470 nm. The enzymatic activity of GPOX was calculated in terms of the protein content of the sample and was expressed in µmol per minute and milligram of protein.

Determination of protein content

The amount of total protein of shoots was measured by Lowry method using bovine serum albumin (BSA) as standard [8]. Absorption intensity of extractions was determined in wave length 750 nm and the results were reported according to mg/g f.w. (fresh weight).

Determination of lipid peroxidation

Lipid peroxidation was measure as the amount of malondialdehyde (MDA) determined by thiobarbituric acid (TBA) reaction as described by Heath and Packer [9]. Briefly, 0.3 g of shoots were homogenized in 4 cm^3 of 0.25% (w/v) thiobarbituric acid (TBA) in 10% (w/v) trichloroacetic acid (TCA) using mortar and pestle. The homogenate was heated at 95°C for 30 min, quickly cooled in an ice bath and then centrifuged at 10 000 rpm for 10 min. The absorbance of supernatant was recorded at 532 and 600 nm. The blank was 0.25% TBA in 10% TCA. The MDA concentration was calculated by subtracting the absorbance at 600 nm (nonspecific turbidity) using the extinction coefficient of 155 $mM^{-1}\,cm^{-1}$ and expressed in terms of µmol/g f.w. [10].

Determination of copper content

The *L. sativum* samples (dry mass each of them was 0.400 ± 0.001 g) were mineralized in the mixture of nitric (V) acid and hydrochloric acid (HNO_3 65% : HCl 37% = 1 : 3) using a Speedwave Four made by Berghof, DE microwave oven. The mineralization process temperature was 180°C. MERCK company reagents were used to prepare solutions [11]. Copper was determined with the atomic absorption spectrometer iCE 3000 made by Thermo Electron Corporation (USA). The Instrument Detection Limit (*IDL*) and the Instrument Quantification Limit (*IQL*) for copper were 0.0045 and 0.033 mg/dm^3, respectively. The highest concentration of the calibration standard available from ANALTYIKA Ltd., Czech Republic, namely 5 mg/dm^3, was assumed the upper limit of the linear relation between the concentration of the analyte and the instrument signal [12].

The quality control of measurements was assured by test analyses of the BCR 414 *plankton* and BCR-482 *lichen* reference materials from the Institute for Reference Materials and Measurements in Belgium. The obtained results are summarized in Table 1.

Table 1

Measured and certified values of Cu concentration in the BCR 414 *plankton* and the BCR 482 *lichen* reference material

BCR 414 *plankton*					**BCR 482 *lichen***				
Certified value	**±Uncertainty**	**AAS**		***D*** *	**Certified value**	**±Uncertainty**	**AAS**		***D*** *
		Mean	**±*SD***				**Mean**	**±*SD***	
[mg/kg d.m.]				**[%]**	**[mg/kg d.m.]**				**[%]**
29.5	1.3	27.8	1.9	–5.8	7.03	0.19	6.54	0.18	–7.0

* Deviation - a difference between a measured value and a certified value, divided by the certified value

Statistical analysis

All experiments were carried out in five replications and mean values ± standard error were presented. One-way analysis of variances (ANOVA) and t-Student's test were used to determine statistical differences between treatment samples and control using Excel Data Analysis. Differences were considered significant at the level of $p < 0.05$. In order to assess the interdependence of copper content in *L. sativum* shoots and the tested oxidative stress markers a correlation analysis was performed using Excel Data Analysis.

Results

Protein content

In the present study, the highest content of total proteins was found in samples treated with the highest dose of Cu^{2+} (1000 mg/dm^3). While the content of total proteins in samples treated with lower dose of Cu^{2+} (500 mg/dm^3) was similar to a protein content in control samples. It is notable that for samples treated with copper ions, the highest content protein statistically significant was found in samples tested the fourth day (with the exception of dose of copper 1000 mg/dm^3). The protein content in the subsequent days decreased (Fig. 1).

Guaiacol peroxidase activity

The highest specific activity of guaiacol peroxidase was found in plant material, which was treated with the highest dose of Cu^{2+} (1000 mg/dm^3), about 7 times more compared with the control sample. In the case of lower doses of Cu^{2+} (500 mg/dm^3) also increased significantly activity of enzyme compared to the control samples. The highest activity of GPOX was found in samples tested the fourth day, and then in the subsequent days statistically significant decreased (ANOVA, $p = 0.002$). In the case of the highest dose of Cu^{2+} (1000 mg/dm^3), enzyme activity was similar regardless of the date of tests (Fig. 2).

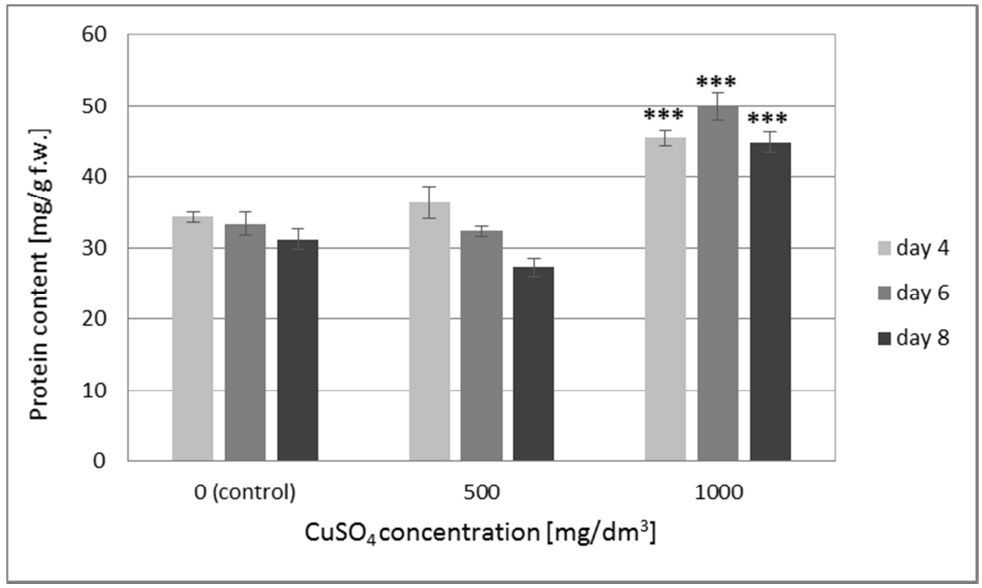

Fig. 1. Effects of different copper concentration on protein content in *Lepidium sativum* shoots (bars represent standard errors of the means, $n = 5$). *** - significance level $p < 0.001$ (Student's test) in relation to the control

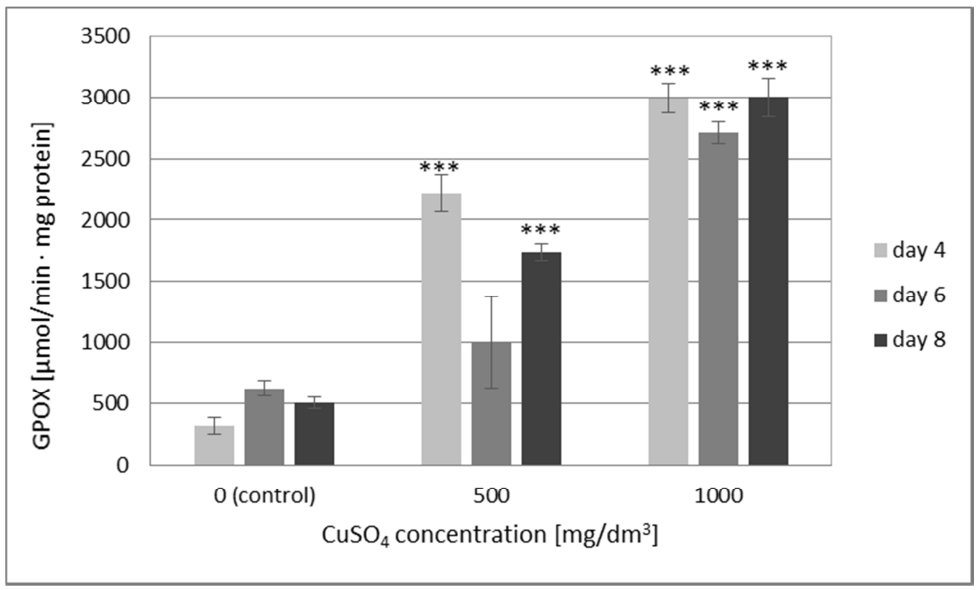

Fig. 2. Effects of different copper concentration on the specific activity of guaiacol peroxidase (GPOX) in *Lepidium sativum* shoots (bars represent standard errors of the means, $n = 5$). *** - significance level $p < 0.001$ (Student's test) in relation to the control

Lipid peroxidation

Lipid peroxidation was measured as the amount of MDA determined by thiobarbituric acid reaction, which products are coloured. The results revealed that MDA concentrations in control samples and samples treated with lower dose of Cu^{2+} (500 mg/dm^3) were similar, without statistically significant differences. But very interesting is that the lowest level of lipid peroxidation was found in plants treated with dose of Cu^{2+} 1000 mg/dm^3. This indicates that in plants treated with dose of Cu^{2+} 1000 mg/dm^3 the very high peroxidase activity reduced lipid peroxidation (Fig. 3).

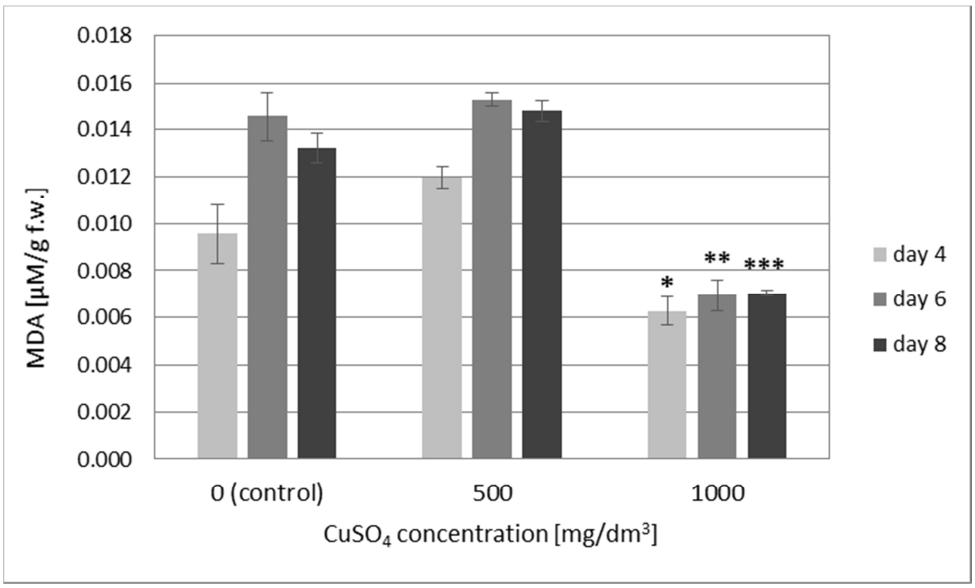

Fig. 3. Effects of different copper concentration on malondialdehyde (MDA) content in *Lepidium sativum* shoots (bars represent standard errors of the means, n = 5). * - significance level of $p < 0.05$ (Student's test) in relation to the control, ** - significance level $p < 0.01$ (Student's test) in relation to the control, *** - significance level $p < 0.001$ (Student's test) in relation to the control

Copper content

The highest copper content was found in plants, which were treated with dose of copper ions 1000 mg/dm^3 (even 200-fold higher than in control plants). Relatively lower copper content was in the shoot treated with dose of copper ions 500 mg/dm^3 (Fig. 4).

Furthermore, the copper content in plants treated with lower dose of Cu^{2+} (500 mg/dm^3) were significantly reduced in the subsequent days ($p < 0.01$, ANOVA). In contrast, the copper content in plants treated with 1000 mg/dm^3 were increased in the subsequent days ($p < 0.01$, ANOVA). In the control sample, the copper content did not change.

It may be assumed, that in plant treated with dose of copper ions 1000 mg/dm^3 was abnormal (disturbed) transport processes and elimination of copper ions. In plant samples treated with lower dose of copper ions, copper was involved in metabolic processes and its excess was effectively eliminated from the cell.

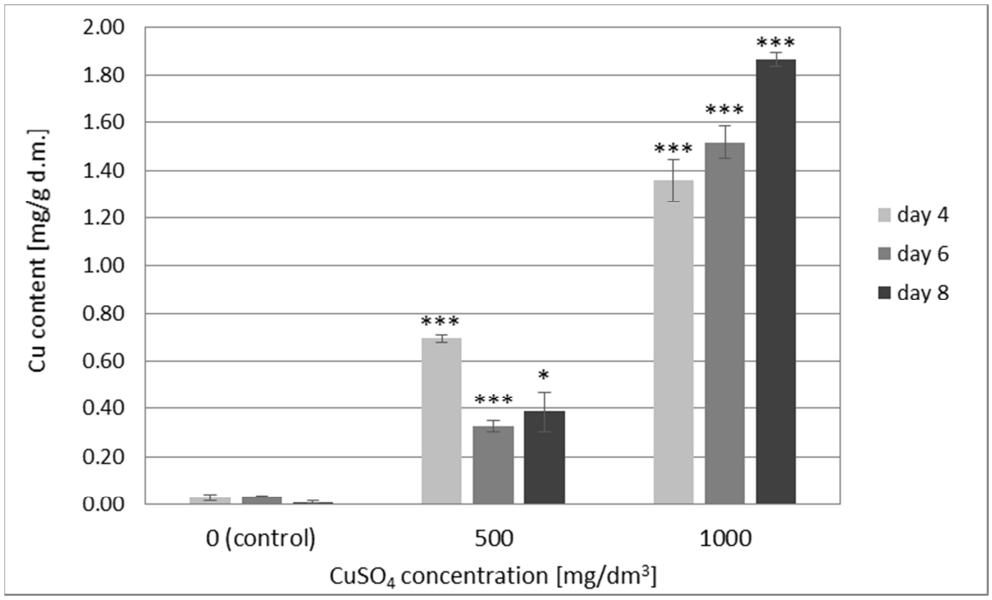

Fig. 4. Copper content in *Lepidium sativum* shoots under Cu^{2+} stresss (bars represent standard errors of the means, $n = 5$). * - significance level of $p < 0.05$ (Student's test) in relation to the control, ** - significance level $p < 0.01$ (Student's test) in relation to the control, *** - significance level $p < 0.001$ (Student's test) in relation to the control

The correlation between copper content in shoots of *L. sativum* and the concentration of oxidative stress markers: MDA, protein and guaiacol peroxidase activity was investigated (Table 2). The analysis revealed a negative correlation between copper content and MDA concentration in shoots, particularly evident on day 6 and 8 of the study. In addition, a strong positive correlation between copper content in plant and GPOX activity and protein content were found.

Table 2

Correlation between copper content in shoots of *Lepidium sativum* and MDA concentration, GPOX activity and protein content

Day	Correlation coefficient *R*		
	Cu content/MDA	Cu content/GPOX activity	Cu content/Protein content
4	–0.47	0.98	0.97
6	–0.94	0.99	0.97
8	–0.93	0.94	0.91

Discussion

Effects of metals have been studied intensively at the level of biochemical-physiological and metal accumulation in plant tissue [13, 14].

One of the effects of heavy metal-induced oxidative stress is alteration in protein metabolism. Therefore, protein content is used as an important indicator of the physiological status of plants [4]. Metal stress in plants usually leads to reduction of protein content [13-16]. However, in few cases, increase protein content was noted [4, 13, 17, 18].

The significance of this phenomenon is unknown [13]. The increased protein content may be due to the synthesis of stress related protein such as phytochelatins and antioxidative enzymes [18-20].

Enzyme activities might play a central role in cellular protection against the heavy metal induces oxidative stress [13]. One of them are peroxidases, located in almost all compartment of the plant cell. The main function of peroxidases is H_2O_2 degradation. Guaiacol peroxidase (GPOX, EC 1.11.17) decomposed H_2O_2 by oxidation of co-substrates such phenolic compounds and/or antioxidants [13, 21].

Peroxidases are involved in many different plant processes, from germination to senescence, auxin metabolism, cell wall elongation, and protection against pathogens. Changes in the activity of peroxidases, especially GPOX, are used as sensitive markers of abiotic and biotic stresses in plants [22]. The activity of this enzyme generally increases under heavy metal stress [4], but in susceptible plants or from conditionally pure habitats, this enzyme are inhibited [2].

Similar to this study, stimulated GPOX was reported in Cu-exposed *Lemna gibba* and *Lemna minor* [4], *Brassica juncea* [23], *Lupinus luteus* [24], *Phaseolus vulgaris* [25], *Astragalus neo-mobayenii* [26] and *Allium sativum* [27]. In addition, it was observed, that induction of GPOX activity in copper-treated plants of *Beta vulgaris* L. was higher in young leaves than in old leaves [28]. The high activity of guaiacol peroxidase in *Lepidium sativum* shoots treated by the highest dose of Cu^{2+} suggest resistance of this plants to copper stress.

One of the most oxidative damaging effects is the peroxidation of membrane lipids. Malondialdehyde (MDA) is one of the final products of plant cell membrane lipid peroxidation and is an important sign of membrane system injury, widely used as an indicator of oxidative stress in plants cells and tissues [13].

MDA content usually increases in plants with heavy metals including copper. Increases in the level of MDA have been found in plants treated with copper such as *Eichornia crassipes* [29], *Potamogeton pusillus* [30], *Hydrilla verticillata* [17], *Spirodela polyrhiza* [4], *Brassica juncea* [23], *Astragalus neo-mobayenii* [26], *Beta vulgaris* [28], *Allium sativum* [27] and *Solanum nigrum* [31].

However, similar to results of this study, reduced MDA contents were observed in Cu-exposed *Lemna gibba*, *Lemna minor* [4] and *Rosmarinus officinalis* [32]. Decreased MDA content could be attributed to a higher activity of antioxidant enzymes, which can protect biomembranes from oxidative damage by lipid peroxidation [4, 32, 33].

The our results confirm that copper accumulation in plants depends on the concentration of metal ions in the growing medium [14, 34].

Conclusion

The highest content of total proteins and the highest specific activity of GPOX were found in plants of *L. sativum* treated with the highest dose of copper ions (1000 mg/dm^3). In the same plants the decrease MDA concentration was found. This indicates that the increased peroxidase activity reduced lipid peroxidation in plants of *L. sativum*. Such a protective effect was not observed with dose of copper ions 500 mg/dm^3.

Copper content in studied plants depends on the concentration of $CuSO_4$ solution applied to soaking *L. sativum* seeds. In addition, the copper content in plants treated with dose of Cu^{2+} 500 mg/dm^3 were significantly reduced in the subsequent days. In contrast, the

copper content in plants treated with 1000 mg/dm^3 were increased. It may be assumed that in plant treated with the highest dose of copper ions transport processes and elimination of copper ions was disturbed.

Our results indicate reliable correlations between copper content and values of oxidative stress biomarkers in plant tissues. Estimated correlation coefficients confirmed, that studied parameters strictly depends on copper ions concentration (R = 0.91-0.97). At the concentration of MDA negative correlation was found, unlike other indicators in which a clear positive correlation was showed.

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