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The impacts of heavy metals on oxidative stress and growth of spring barley

Research Article

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Abstract: Oxidative stress is accepted to play a significant role in stress symptoms, caused by different stressors in a variety of organisms. In this study seedlings of spring barley (*Hordeum vulgare* L.) were exposed to a wide range of copper, zinc, chromium, nickel, lead and cadmium concentrations in order to determine the relationships between heavy metals-induced oxidative stress and plant growth inhibition. All investigated heavy metals induced an essential increase in lipid peroxidation and a reduction of dry biomass along with an increase in metal concentration in the nutrient solution. A very close and statistically significant exponential relationship between lipid peroxidation and growth inhibition was detected in this study. According to the results of analysis of variance (ANOVA), the intensity of nonspecific oxidative stress is identified as the main factor of barley growth inhibition, explaining 75% of total variance. Almost 10% of growth inhibition is attributed to the specific impact of heavy metals. The most pronounced increase of malondialdehyde content and growth inhibition was observed in Cu and Cd treatments, whereas the lowest changes in observed indicators were detected after exposure to Zn and Pb.

Keywords: *Heavy metals • Oxidative stress • Lipid peroxidation • Malondialdehide • Growth inhibition*

1. Introduction

Heavy metals are continuously released into the environment from both natural and anthropogenic sources. Natural concentrations of heavy metals in soil depend on the weathering of the bedrock and volcanic activity and usually do not cause a negative impact to plants [1]. Rapidly increasing anthropogenic environmental pollution from agriculture (fertilizers and pesticides), metallurgy (mining and foundry works), energy production and fuel burning, microelectronic production, and waste disposal has resulted in an essential increase of heavy metals' concentrations in soil. Contamination of soil with heavy metals is one of the most serious anthropogenic environmental stressors determining inhibition of plant growth and productivity [2-4].

When plants receive heavy metals in small amounts, mechanisms of avoidance *e.g.* translocation, complexation and sequestration are sufficient to overcome the negative impact. However, when excess of metals in soil is achieved, heavy metals become highly phytotoxic [5,6].

It is generally considered that the negative impact of heavy metals is based on several mechanisms. First of all, inhibition of enzymes occurs when heavy metal ions react with functional groups of proteins [1]. Secondly, heavy metals can inactivate biomolecules by displacement of essential metal ions from specific binding sites [1,7,8]. Inactivation of enzymes and biomolecules are traditionally characterized as the main causes of heavy metals toxicity [9].

However, the most recent investigations provide an increasing amount of evidence that oxidative stress is the major damaging factor in plants subjected to different environmental stressors, including heavy metals [10-13]. Reactive oxygen species are produced when triplet oxygen (O_2) is partly reduced or activated by energy transfer [14,15]. Highly reactive singlet oxygen (O_2^1) is created after spin conversion, which usually

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occurs when triplet oxygen absorbs the energy of excited chlorophyll [16], whereas monovalent reduction produces partly reduced forms of oxygen: superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$) [14,17]. Enhanced concentration of reactive oxygen species (ROS) causes oxidative damage of lipids, proteins, pigments and nucleic acids [10,18].

Heavy metals, according to their redox-capacity, can participate in several ROS-generating mechanisms [9]. Cu and Cr, as redox-active metals, can participate in a Haber-Weiss reaction, producing hydroxyl radicals that are the most toxic form of ROS [7,19,20]. However, oxidative stress is attributed not only to the redox-active heavy metals [9,18,21]. The metals without redox capacity, such as cadmium, lead, zinc, nickel, *etc.,* interfere with photosynthetic process causing the enhanced production of singlet oxygen and superoxide in cells [7,8].

Apart from generation of ROS, heavy metals suppress the antioxidative system mostly by depletion of glutathione and bonding with sulfhydryl groups of antioxidative enzymes such as superoxide dismutase, reductases and catalases [1,8,19,22]. As noted by these different authors the intensity of heavy metal-induced oxidative stress is species-dependent and can vary in different genotypes, tissues and/or the stage of development. Usually, the metal-resistant genotypes show weaker symptoms of oxidative stress, whereas a strong oxidative damage can be detected in sensitive plants [9,10,18].

Heavy metals-induced generation of reactive oxygen species affects various cellular processes. However the membrane system, including the membranes of chloroplasts, very often is considered as the main target of oxidative impact of heavy metals [9,23]. Polyunsaturated fatty acids, the main components of membrane lipids, are extremely sensitive for oxidation. The reaction of lipid peroxidation is initiated by hydroxyl radicals and leads to formation of highly reactive lipid peroxyl radicals that can react with another fatty acid [24]. The chain reaction can be terminated after combination of two radicals into a non-radical compound; usually malondialdehyde (MDA) is a final product of the process [22,25]. Therefore, an increase of MDA concentration is generally considered as the main biomarker of the intensity of oxidative stress [8,9,26].

Barley is one of the most popular crop species in the Northern Hemisphere, used for human consumption and especially for animal feed and malting [27]. Besides, it is the most common spring crop in Lithuania. The impact of various heavy metals on barley plants has also studied by other researchers. For example, the accumulation properties of copper, zinc, lead and cadmium were investigated by Rajcakova *et al.* [28]. Tamas *et al.* [12] detected that cadmium, nickel and mercury triggered root growth inhibition and specific antioxidative answer of barley. Wu *et al.* [10] investigated the differences in lipid peroxidation and antioxidative enzymes in several barley cultivars exposed to cadmium stress.

In this study a Lithuanian cultivar 'Aura DS' of spring barley (*Hordeum vulgare* L.) was chosen as a research object, because it has high sensitivity to the impact of environmental stressors, as established in our previous researches [29-31]. The impact of six heavy metals – copper (Cu), zinc (Zn), chromium (Cr), nickel (Ni), lead (Pb) and cadmium (Cd) was investigated. Three of them (Cu, Zn and Ni) are considered as an essential elements, while Cd, Pb and Cr are not-essential and toxic for plant metabolism [7]. The investigated heavy metals differ in their oxidative capacity: Cu and Cr are redox-active heavy metals, whereas Cd, Pb, Ni and Zn do not have redox activity [7,25].

Plant growth inhibition due to an excess of heavy metals is well documented and is considered as a general phenomena associated with the impact of heavy metals [3,7,29,32]. Therefore, the aim of this study is to investigate the impact of heavy metals-induced oxidative stress on growth of spring barley and to check the hypothesis that oxidative stress is mostly responsible for heavy metal caused growth inhibition. Comprehensive experiments included using six different heavy metals and investigating their impact on growth and lipid peroxidation in spring barley.

2. Experimental Procedures

2.1 Plant cultivation and heavy metal treatment

Experiments were carried out in a controlled environment room with a 14/10 h light/dark photoperiod, average temperature of 22ºC, relative humidity 65%, light intensity of 14000 Lx, provided by Philips MASTER Green Power CG T 600 W lamps in combination with luminescence lamps. The plants, after seed sterilization and germination were grown for five days in an aerated nutrient solution (0.4 mM $CaCl_2$, 0.65 mM KNO_3, 0.25 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $(NH_4)_2SO_4$, 0.04 mM NH_4NO_3 [33,34] supplemented with different amounts of heavy metal salts. Twenty four germinated seeds were planted in each vegetation vessel and three replicates for each heavy metal treatment were used.

The following salts were used for the experiments – $CuSO_4 \cdot 5H_2O$; $CdSO_4 \cdot 8/3H_2O$; Pb SO_4; Ni $SO_4 \cdot 6H_2O$; Zn $SO_4 \cdot 7H_2O$. Taking into account that metals differ in their toxicity, the particular range of investigated concentrations was chosen for every heavy metal in

order to achieve the gradual reduction of dry biomass to approximately 30% of the control value. The concentrations applied into nutrient solutions were as follow: 8, 10, 50, 250, 500, 1000 µM of copper; 3, 15, 22, 75, 525, 1500 µM of cadmium; 0.1, 4, 90, 410, 1000, 1600 µM of lead; 2, 4, 8, 25, 350, 1000 µM of nickel and 0.1, 4, 25, 800, 1600, 3000 µM of zinc.

2.2 Determination of dry biomass

Dry biomass of plants (shoots and roots) and MDA concentration in leaves were detected after 5 days of treatments with different concentrations of investigated metals. Ten randomly sampled plants were taken from each vessel and dried in an electric oven at 70ºC for 24 hours to determine the mean of dry biomass per plant.

2.3 Assay of lipid peroxidation

The concentration of malondialdehyde (MDA), the end-product of lipid peroxidation, was used as a biomarker of membrane oxidative damage. MDA content was determined by reaction with a thiobarbituric acid (TBA) giving the pink-colour compound after heating. The sample of leaf tissue was homogenized with Tris-HCl buffer solution containing 1.5% of PVPP (pH 7.4) and centrifuged at 10000x*g* for 30 min at 4ºC. Equal amounts of tissue extract and 0.5% TBA in 20% trichloroacetic acid (TCA) (w/v) was mixed and heated at 95ºC for 30 min. The reaction was stopped by transferring tubes to ice. After centrifugation of the reaction mixture at 10000x*g* for 15 min. the absorbance of the coloured supernatant was measured at 532 nm and corrected for unspecific turbidity by subtracting the value of absorbance at 600 nm. The concentration of MDA was expressed in nmol g^{-1} fresh weight using an extinction coefficient of 155 $mM^{-1}cm^{-1}$ (24).

2.4 Statistical analyses

Software STATISTICA 6 was applied for statistical analysis and presentation of the data. The mean values are presented with the standard errors (SE) of three replicates. The model of exponential regression was applied in order to examine the relationships between heavy metal-induced lipid peroxidation and biomass reduction of barley plants. The contribution of heavy metal specific influence to growth inhibition and its dependency from lipid peroxidation was evaluated by ANOVA using SPSS (version 13) software. The logarithm of MDA values was used in order to achieve the normality of the data. The analysis included dry biomass as dependent variable and categorized MDA values, heavy metal species and interaction between MDA and metal species as the main factors, *i.e.* $SST=SS_{MDA}+SS_{metal}+SS_{MDA*metal}+SS_{error}$. The effect of these factors was assessed *via* classical eta-squared (η^2), which represent the proportion of total variation of the dependent variable, attributable to the factor: $\eta^2_F=SS_F/SST$ [35].

3. Results and Discussion

The concentration of malondialdehyde (MDA) in the leaves and the average dry biomass of barley plants exposed to different concentrations of heavy metals are presented in Table 1. Even the lowest concentrations of copper, lead and chromium resulted in a statistically significant ($P<0.05$) increase in MDA concentration as compared to control. The concentration-dependent increase of MDA content was induced by all investigated heavy metals. However, a statistically significant ($P<0.05$) reduction of lipid peroxidation was registered when plants were treated with the highest concentration of nickel (1000 µM), compared to the treatment with considerably lower concentration (350 µM).

Statistically significant reduction of dry biomass was detected along the concentration gradient of all investigated metals. Even the lowest concentrations of heavy metals (except nickel) caused statistically significant ($P<0.05$) decrease in dry biomass of barley plants. The increase of concentration of heavy metals in nutrient solutions led to gradually more pronounced inhibition of barley growth.

3.1 Oxidative stress induced by different heavy metals

For more obvious comparison of heavy metals impact on lipid peroxidation, MDA concentrations were expressed as a percentage of control value and metal concentration values were log-transformed (Figure 1).

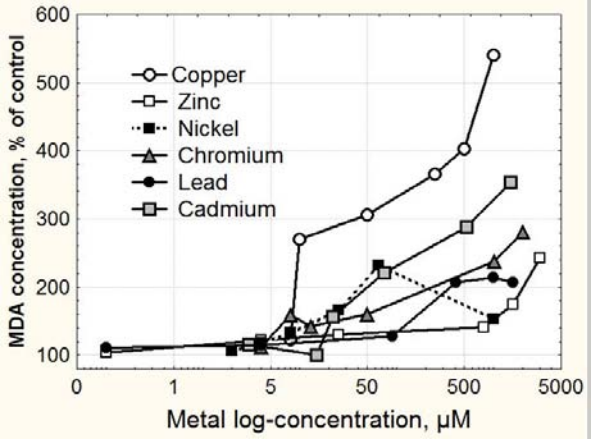

Figure 1. Dose-dependent response of lipid peroxidation to the impact of different heavy metals.

Treatment, metal conc., μM	MDA concentration, nmol g^{-1} FW	Dry biomass, mg	Treatment, metal conc., μM	MDA concentration, nmol g^{-1} FW	Dry biomass, mg
Control					
0	22,51 ± 0,42 a	34.75 ± 1.19 a			
Copper			Chromium		
8	27.80 ± 4.26 b	26.04 ± 0.71 b	4	25.20 ± 0.95 b	26.76 ± 1.97 b
10	60.84 ± 5.04 c	17.13 ± 1.34 c	8	35.85 ± 1.38 c	22.76 ± 1.18 c
50	68.88 ± 5.03 d	13.30 ± 0.34 d	13	32.01 ± 4.40 c	20.02 ± 0.16 d
250	82.39 ± 5.13 e	12.39 ± 0.53 d	50	36.06 ± 1.22 c	16.11 ± 0.90 e
500	90.67 ± 5.06 e	10.56 ± 0.67 e	1024	53.56 ± 7.57 d	10.79 ± 0.11 f
1000	121.60 ± 5.96 f	8.70 ± 0.21 f	2000	63.21 ± 2.92 d	10.23 ± 0.52 g
Zinc			Lead		
0,1	23.35 ± 1.12 a	31.44 ± 0.98 b	0,1	24.89 ± 1.32 b	26.30 ± 1.05 b
4	27.50 ± 0.95 b	29.14 ± 3.07 bc	4	25.85 ± 1.63 b	22.95 ± 1.72 c
25	29.21 ± 0.50 c	24.94 ± 1.19 c	90	28.68 ± 2.57 b	18.50 ± 0.21 d
800	31.78 ± 1.38 d	15.29 ± 1.09 d	410	46.62 ± 1.60 c	10.85 ± 0.12 e
1600	39.38 ± 1.05 e	12.48 ± 0.22 e	1000	48.18 ± 10.22 c	11.06 ± 0.18 e
3000	54.65 ± 1.52 f	11.58 ± 0.22 f	1600	46.47 ± 2.76 c	10.70 ± 0.32 e
Nickel			Cadmium		
2	24.05 ± 1.27 a	32.52 ± 1.60 a	3	26.03 ± 3.14 a	27.54 ± 2.35 b
4	26.64 ± 0.62 b	23.11 ± 0.66 b	15	22.56 ± 1.18 a	26.18 ± 1.43 c
8	29.98 ± 2.37 c	19.61 ± 1.88 c	22	35.33 ± 1.64 b	23.15 ± 3.24 c
25	37.42 ± 2.01 d	18.14 ± 1.44 c	75	49.75 ± 4.69 c	15.29 ± 0.53 d
350	52.06 ± 5.53 e	12.33 ± 0.37 d	525	64.87 ± 1.48 d	11.18 ± 0.41 e
1000	34.37 ± 5.24 d	9.18 ± 0.37 e	1500	79.60 ± 0.99 e	9.00 ± 0.27 f

Table 1. MDA concentration in the leaves and dry biomass of barley plants exposed to different concentrations of heavy metals. The data are means ± SE of three replicates. Different letters indicate significant differences ($P < 0.05$) in individual columns and the control.

The investigations of the heavy metal impact on different plant species showed that various metals, independently of their redox-activity, caused an increase in MDA level [10,18,36-38]. In our study dose-dependent increase in MDA content was detected in the leaves of spring barley treated with all investigated metals (Cu, Cd, Cr, Ni, Pb and Zn), however, the level of MDA increase was very different. The most pronounced lipid peroxidation was induced by copper (Figure 1). The severity of Cu-induced oxidative damage coincides with other observations and can be explained by the fact that Cu, as a redox-active metal, can directly induce ROS synthesis *via* Haber–Weiss and Fenton reactions [37] and participate in peroxidation of unsaturated fatty acids leading to serious damage on membrane lipids [7,22,39,40].

Cadmium was the second heavy metal according to the level of lipid peroxidation. Although Cd is not a redox-active metal, its impact on lipid peroxidation is associated with an increase in lipoxygenase activity, leading to higher rates of lipid peroxidation and production of ROS [2,9,10,12], whereas the response of the antioxidative system to Cd stress is highly variable, and both increase and reduction in activity of antioxidative enzymes was reported [9,10,18].

The lowest increase in lipid peroxidation was observed under exposure to zinc and lead (Figure 1). Comparatively low phytotoxicity of Zn and Pb has been reported by different authors [41,42]. Zinc is one of the metal cofactors of the antioxidative enzyme superoxide dismutase (SOD). However, oxidative stress can be induced as a consequence of overloaded antioxidative capacity when Zn is applied in excess [43]. Lead, like other non redox-active heavy metals, can initiate oxidative stress indirectly. Both apoplastic and symplastic transport of this heavy metal is highly restricted in roots, leading to rather high plant resistance to lead impact [26,28,44].

A moderate increase of MDA concentration was observed in plants exposed to Ni and Cr. Although chromium is attributed to being a redox-active heavy metal, its redox activity and toxicity is highly dependent on its valency [20]. While Cr (VI) is a strong oxidative agent, the oxidative capacity of Cr (III) is much lower [11]. Dependence of Ni-induced lipid peroxidation from metal concentration did not follow a general pattern of gradual MDA increase along with increase of metal concentration. Decrease of MDA content was detected when plants were subjected to the highest concentration of Ni (Figure 1). Further studies are needed to explain these results, since there is a lack of investigations concerning Ni-mediated oxidative stress.

3.2 Growth inhibition caused by different heavy metals

Dose-dependent growth inhibition by different heavy metals is presented in Figure 2. Data were transformed as in Figure 1, *e.g.* dry biomass was expressed as a percentage of control value and the values of metal concentrations were log-transformed.

An essential dose-dependent reduction in spring barley biomass is characteristic for the impact of all heavy metals investigated in our study. It is necessary to note, however, that comparative toxicity of most of the investigated metals changed with the increase of their concentrations. Only Zn could be considered as the least toxic metal through the entire range of the investigated concentrations. In general, the most pronounced growth inhibition was recorded under treatments with Cu, Cd, and Ni and, in contrast, the least inhibition of barley growth was caused by Pb and Zn (Figure 2). As mentioned by different investigators, the growth inhibition is a general phenomenon associated with the impact of heavy metals and even essential heavy metals, usually called micro-elements (Cu, Zn, Mn, Fe), are required only in trace amounts [7,3,32]. The following sequences in decrease of heavy metals impact on plant growth have been presented by different authors: Cd>Cu>Ni>Zn>Pb>Cr [42], Ti>Cu>Ag>Hg>Cd>Zn>Pb>Co [42]. It is emphasized, however, that relative toxicity of heavy metals is species dependent [7,44] and can also be determined by soil fertility, acidity and presence of other toxic substances [42].

3.3 The relationship between heavy metal induced lipid peroxidation and inhibition of barley growth

Regression analysis was applied to approximate the relationship between heavy metal induced lipid peroxidation and inhibition of barley growth. Very close and statistically significant exponential relations between investigated indicators were detected and the coefficient of determination (R^2) fluctuated in the range of 0.945 for Cu to 0.840 for Ni exposure (Figure 3A-F).

A comparison of heavy metal impact on lipid peroxidation and growth inhibition reveals high affinity between changes of these stress indicators in plants subjected to various heavy metals. This finding concurs with earlier studies. Cu-resistant wild carrot plants showed lower MDA and H_2O_2 accumulation in leaves, as well as higher biomass and antioxidative enzyme activities compared to sensitive plants after exposure to Cu [37]. Investigations by other authors on plant responses to the impact of cadmium showed that more sensitive barley genotypes produced higher levels of

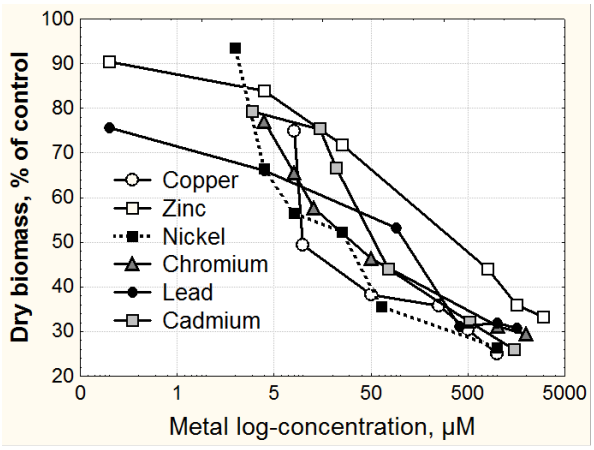

Figure 2. Dose-dependent growth inhibition by different heavy metals.

MDA and the growth inhibition of these genotypes was more expressed [10].

The shape of the exponential relationship between heavy metal-induced lipid peroxidation and inhibition of barley growth is rather different (Figure 3). The most expressed curvature (the highest values of the exponential coefficient) of this relationship is characteristic for lead and nickel treatment. The most expressed increase of biomass inhibition was detected in a certain range of lipid peroxidation, corresponding to the initial increase of MDA up to 150% of control. Further increase of lipid peroxidation had little effect on barley growth inhibition, indicating that specific mechanisms of both metal toxicity and plant tolerance might be important for the reduction of growth under the impact of these heavy metals. In the case of Cu and Cd treatments, the curvature of the lipid peroxidation – growth inhibition relationship is much less expressed and the increase in biomass reduction is rather proportional to the increase of MDA concentration along the entire range of metal concentrations. As noticed earlier, these metals caused the most pronounced lipid peroxidation and growth inhibition (Figure 1 and 2).

In order to evaluate the contribution of non specific oxidative stress (lipid peroxidation) and heavy metal specific impact on barley growth inhibition, the analysis of variance (ANOVA) was applied (Table 2). As can be seen from the presented data, the impact of both investigated factors was highly significant ($P<0.001$), although their interaction was very weak ($F=0.5$) and statistically insignificant ($P>0.05$). Moreover, the effect size of investigated factors was shown to be very different. The effect size of the investigated factors was assessed *via* eta-squared (η^2) values. Classical η^2 is determined as an additive measure of unique variation in the dependent variable and is used to determine the

Factor	Sum of squares	Degree of freedom	Mean Square	*F* criteria	Significance	Effect size (η^2)
MDA	5755,8	4	1437	112,4	<0,001	0,750
Metal	663	6	110,5	8,6	<0,001	0,086
MDA*metal	101,8	15	6,8	0,5	0,917	0,013
Error	1151,8	90	12,8			0,150
Total	7672,5	115				1,000

Table 2. Results of analysis of variance (ANOVA) indicating the impact of heavy metal-induced lipid peroxidation (MDA), heavy metal species (metal) and interaction of these factors (MDA*metal) on growth of barley plants and the effect size (η^2) of these factors.

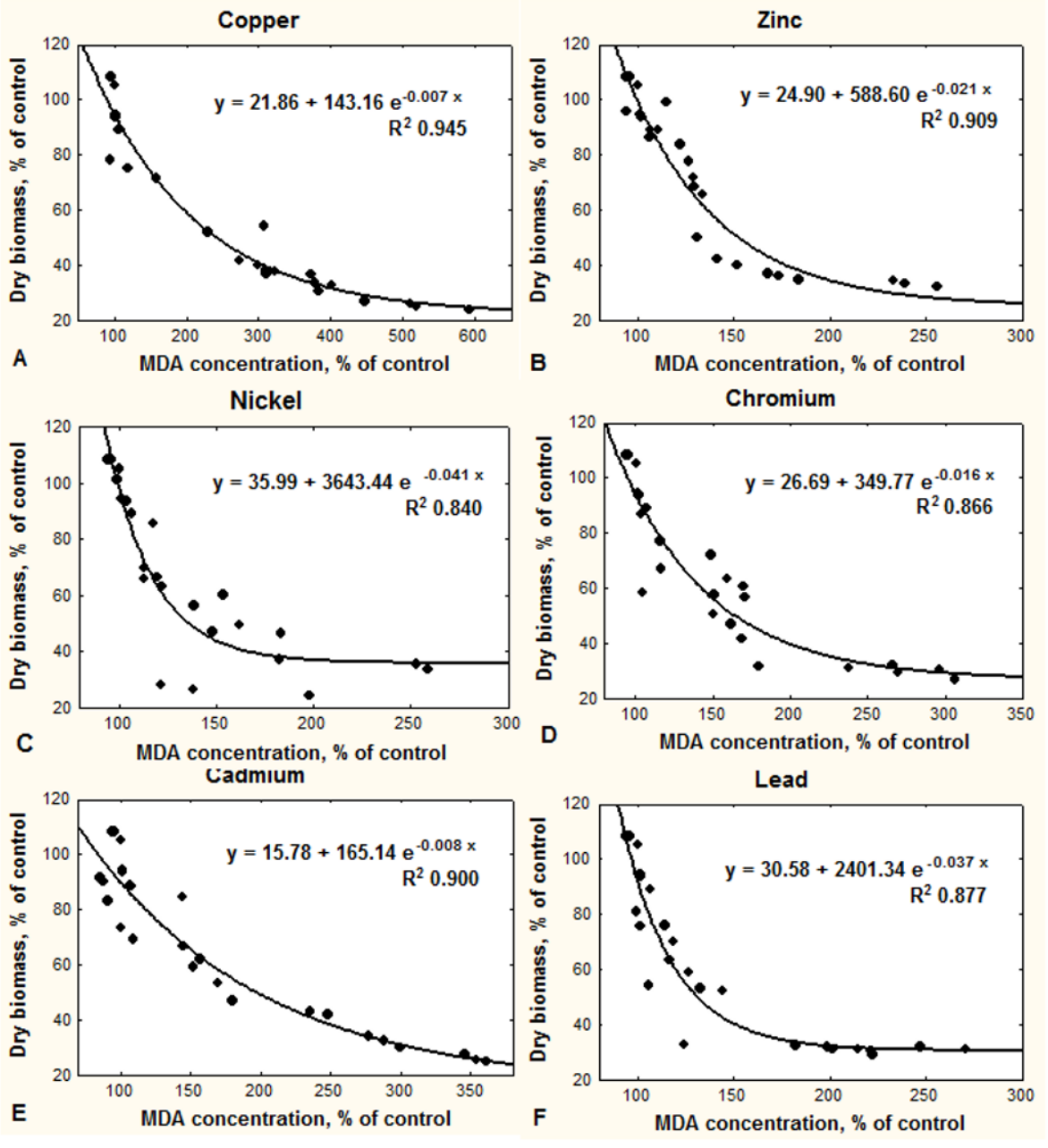

Figure 3. The relationship between heavy metal-induced lipid peroxidation and inhibition of barley growth.

strength of association between dependent variable and experimental factors [35]. Our analysis has shown (Table 2) that oxidative stress, measured as MDA concentration, was the main factor of barley growth inhibition, explaining 75% of total variance of barley growth (dry biomass), whereas 8.6% of biomass variance can be explained by specific impact of heavy metals.

According to the results of this study (Table 2), the intensity of oxidative stress, measured as MDA concentration, was the main factor of barley growth inhibition. These results could be considered as a confirmation of the initial hypothesis that when subjected to a wide range of concentrations of different heavy metals, oxidative stress is mostly responsible for growth inhibition of plants.

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