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The effect of a growth regulator Ribav-Extra on winter wheat seedlings exposed to heavy metals

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Abstract

We studied the influence of a natural plant growth regulator Ribav-Extra and ions of heavy metals (HM) Pb^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} on the physiological and biochemical indices of the winter wheat (*Triticum aestivum* L.) cultivar 'Mironovskaya 808'. The seeds of wheat were treated with Ribav-Extra (10 ppm) and grown for 7 days on heavy metal salt solutions (10 µM or 1 mM). After that we recorded heavy metals accumulation, growth, generation of superoxide anion ($^{\bullet}O_2^-$), lipid peroxidation (LPO), and catalase (CAT) and ascorbate peroxidase (APOX) activity. It was found that 1 mM of HM in growth media increased heavy metals accumulation in wheat plants and inhibited the growth of roots and shoots. Both low and high concentrations of heavy metals stimulated $^{\bullet}O_2^-$ production and oxidative damage in wheat seedlings. In most treatments, the toxic effect of heavy metals enhanced at higher concentration of metals. Pre-sowing treatment of seeds with Ribav-Extra decreased the negative impact of heavy metals by reducing oxidative stress which led to suppressed $^{\bullet}O_2^-$ generation, lipid peroxidation intensity and catalase activity. This suggests that the wheat plants, whose seeds had been treated with Ribav-Extra, were more heavy metal-resistant than the untreated ones.

Key words: antioxidative enzymes, heavy metals, oxidative stress, Ribav-Extra, *Triticum aestivum*.

Introduction

Soil contamination with various heavy metals and their impacts on plant health and productivity are extensively reported (Anjum et al., 2015 a). Once inside plants, even low levels of heavy metals are phytotoxic. In plants, heavy metals cause various disorders in physiological and metabolic processes. They can impair plant growth, development and productivity (Duchovskis et al., 2006; Башмаков, Лукаткин, 2009; Gangwar et al., 2014; Anjum et al., 2015 a). Elevation in the generation of reactive oxygen species (ROS), such as superoxide radicals ($^{\bullet}O_2^-$), hydroxyl radicals ($OH^{\bullet}$), perhydroxyl radicals ($HO_2^{\bullet}$), alkoxy radicals ($RO^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), has been considered as the major factor of plant response to low and high levels of beneficial metals (Hossain et al., 2012; Anjum et al., 2015 a). Notably, if not metabolized, ROS can imbalance cellular redox homeostasis and oxidize lipids and proteins (Anjum et al., 2015 b), and finally halt cellular metabolism (Anjum et al., 2012). Plants tend to counteract ROS-accrued consequences by employing antioxidant defence system comprising antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX) and glutathione reductase (GR), and non-enzymatic antioxidants, such as ascorbate, glutathione, tocopherol, carotenoids and flavonoids (Anjum et al., 2012; 2016). Toxic effects of heavy metals strongly depend on the metal, its concentration, plant species and plant phenophase as well as on the environmental (edaphic, climatic, etc.) factors (Sharma et al., 2017).

Modern agriculture seeks to achieve the maximum productivity of agricultural plants and to obtain a yield that does not contain substances toxic to humans and animals (Lazauskas et al., 2012). Bioactive compounds are extensively reported to confer tolerance to environmental stresses in plants (Asgher et al., 2015; Miliauskienė et al., 2016). Thus, exogenous application of plant growth regulators (PGRs) can significantly

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modulate both enzymatic and non-enzymatic components of the antioxidant defence system, neutralize the negative impact of heavy metals such as alterations in the activity of enzymes of radical scavenging system, and are capable of improving plant growth or/and biomass accumulation, chlorophyll and carotenoid content, the rate of photosynthesis, heavy metals accumulation in yield, etc. (Bashmakov et al., 2012; Gangwar et al., 2014; Asgher et al., 2015; Xia et al., 2015, Gruznova et al., 2017).

Synthetic PGRs like brassinosteroids (Fariduddin et al., 2013; Hayat et al., 2014; Bashmakov et al., 2016), cytokinin-like substances (Bashmakov et al., 2012; Sazanova et al., 2012), exogenous gibberellins (Gangwar et al., 2011 a), synthetic auxins (Gangwar et al., 2011 b; Zhu et al., 2013), salicylic acid (Zhang et al., 2011; Ali et al., 2015) or methyl jasmonate (Singh, Shah, 2014) are used as protectors against stress induced in plants by heavy metals.

Natural growth regulators are a complex of bioactive compounds of plant origin or products of the metabolism of microorganisms. They can be used in low concentrations and are non-toxic to plants and to the environment. The application of some natural products to plants showed plants resistance to biotic (Sakalauskienė et al., 2012), abiotic stresses (Lukatkin, Pogodina, 2012), heavy metals and herbicides (Asgher et al., 2015).

A natural plant growth regulator Ribav-Extra® is a product of the metabolism of mycorrhizal fungi isolated from ginseng (*Panax ginseng* C.A. Mey) roots (Рязин и др., 2005; Толмачева, Михеева, 2008). It stimulates the synthesis of phytohormones, growth processes, and it can increase resistance to stressors (Толмачева, Михеева, 2008). Earlier it was shown that Ribav-Extra increased plant tolerance to temperature stress (Lukatkin, Pogodina, 2012). Currently, studies on the effect of Ribav-Extra on the crop plants are extremely few; therefore, the study on the effectiveness of Ribav-Extra application on medium containing heavy metals is of practical interest.

This study aimed to evaluate the significance of Ribav-Extra in the control of growth traits, $^{\bullet}O_2^-$ level, lipid peroxidation intensity, CAT and APOX activity, heavy metals tolerance and accumulation in winter wheat under Cu^{2+}, Ni^{2+}, Zn^{2+} and Pb^{2+} exposure. Hence, this study investigated the physiological and biochemical efficacy of pre-sowing seed treatment with Ribav-Extra solutions.

Materials and methods

Plant culture. Commercial seeds of the winter wheat (*Triticum aestivum* L.) subspecies *lutescens* cultivar 'Mironovskaya 808' were used for this experiment.

Plant growth regulator. A natural plant growth regulator Ribav-Extra® ("Selhozekoservis", Russia) is a product of the metabolism of mycorrhizal fungi isolated from ginseng (*Panax ginseng* C.A. Mey) roots. Ribav-Extra (RE) is a 60% alcohol extract, which contains a complex of natural amino acids and biological active substrates. Ribav-Extra is usually used in field or greenhouses for seed treatment, spraying plants or processing cuttings. It stimulates plant resistance to unfavourable environment (Толмачева, Михеева, 2008).

Heavy metals (HM) exposure. Wheat seeds were treated with 0.5% $KMnO_4$ for 5 min to surface sterilize then with 10 ppm Ribav-Extra for 8 h (control seeds were treated with distilled water), and then germinated in plastic pots (50 seeds per pot) for 7 days in water (50 ml per pot) supplemented with 10 µM (low dose) or 1 mM (high dose) $Pb(NO_3)_2$, or $CuSO_4 \times 5H_2O$, or $NiSO_4 \times 7H_2O$ or $ZnSO_4 \times 7H_2O$, at temperature 22–24°C, photoperiod 16/8 h (day/night), and the photosynthetic photon flux density (PPFD) was about 80 µmol m^{-2} s^{-1}. In 7-day seedlings we measured heavy metals accumulation in shoots and roots, length of axial organs, superoxide ($^{\bullet}O_2^-$) generation in leaves, lipid peroxidation (LPO) intensity, and catalase (CAT) and ascorbate peroxidase (APOX) activities.

Biometric measurments. The length of axial organs in 30 selected seedlings per single experiment was measured. To assess the tolerance of seedlings to heavy metals we applied a Wilkins tolerance index (WTI):

WTI = $(l_{me}/l_c) \times 100\%$, where l_{me} is the root length in a metal ion solution, l_c – the root length in the control material (Wilkins, 1978).

Heavy metals accumulation in biomass was determined using atomic absorbance spectrometer AA-7000 (Shimadzu, Japan). The heavy metals content in plant samples was calculated in µg g^{-1} of air-dry weight (Bashmakov et al., 2015). To assess the heavy metals transition in aerial parts, we used an index of the metal translocation (I_{HMt}):

$I_{HMt} = (C_s/C_r)$, where C_s is the metal concentration in the shoots, C_r – the metal concentration in the roots.

Superoxide ($^{\bullet}O_2^-$) generation in leaf disks was detected using a method that is based on the capacity of $^{\bullet}O_2^-$ to oxidize epinephrine to adrenochrome (Lukatkin, 2002 a). A total of 300 mg of leaves was homogenized with 15 ml of distilled water. The solution was centrifuged for 15 min at 3000 rpm; 0.1 ml of adrenaline solution (0.01%) was added to 3 ml of homogenate and incubated for 45 min at ambient temperature and 200 µM photons m^{-2} s^{-1}. Immediately after incubation, the optical density of the adrenochrome that formed was measured against homogenate in water on a spectrophotometer UVmini 1240 (Shimadzu, Japan) at $\lambda = 480$ nm. The rate of $^{\bullet}O_2^-$ generation was calculated by adopting a molar extinction coefficient ($\varepsilon = 4020$ M^{-1} cm^{-1}) in µM g^{-1} min^{-1}.

Lipid peroxidation (LPO) intensity in leaf disks was detected using a method that is based on the accumulation of malonic dialdehyde (MDA) (Lukatkin, 2002 a). A total of 300 mg of leaves was homogenized with 10 ml of medium containing 0.1 M Tris-HCl buffer, pH 7.6, with 0.35 M NaCl. Two ml of thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) was added to 3 ml of homogenate, heated in a boiling water bath for 30 minutes and filtered. The optical density was recorded against isolation medium on a spectrophotometer UV mini 1240 at $\lambda = 532$ nm. The concentration of MDA was calculated by adopting a molar extinction coefficient ($\varepsilon = 1.56 \times 10^5$ M^{-1} cm^{-1}) in µM g^{-1} of fresh leaves weight.

Catalase (EC 1.11.1.6) activity detection. One g of leaf disks was homogenized with 10 ml of 50 mM phosphate buffer (pH 7.0). The homogenate was filtered and centrifuged for 10 min at 8000× *g*; 2.9 ml of phosphate buffer (pH 7.0) was added to 25 µl of enzyme extract. Directly before the measurement, 90 µl of 3% hydrogen peroxide (H_2O_2) was added to the solution. The optical density decrease during 1 min was measured on a spectrophotometer UV mini 1240 at $\lambda = 240$ nm. The activity of CAT was calculated by adopting a molar extinction coefficient ($\varepsilon = 39.4$ mM^{-1} cm^{-1}) in µM g^{-1} min^{-1} (Lukatkin, 2002 b).

Ascorbate peroxidase (EC 1.11.1.11) activity detection. One g of leaf disks was homogenized with 10 ml of 50 mM phosphate buffer (pH 7.6), in the cold. Polyvinylpyrrolidone (0.3 g) was added to the homogenate, which was filtered and then centrifuged for 10 min at 12000× *g*. The reaction mixture contained 50 µl of 0.1 mM ethylenediaminetetraacetic acid (EDTA), 50 µl of 0.05 mM ascorbate (AsA), 50 µl of 0.1 mM H_2O_2, 2.55 ml of 50 mM phosphate buffer (pH 7.6) and 300 µl of plant extract, which was obtained after centrifugation of the homogenate. The optical density was measured on a spectrophotometer UVmini 1240 at $\lambda = 290$ nm against the control mixture devoid of the enzyme extract. The contribution of nonenzymatic AsA oxidation was neglected, since it did not exceed 5%. A decrease in the optical density for the first 30 s of the reaction was taken as a measure of the enzyme activity, which was expressed in µM AsA g^{-1} min^{-1} using a molar extinction coefficient ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) (Lukatkin, 2002 b).

Statistical analysis. All experiments were conducted in triplicate, and each experiment consisted of at least 150 seedlings. For all measurements averages and standard errors were calculated in *MS Excel*. Differences between means were assessed by the Duncan's test at $P = 0.05$.

Results and discussion

Accumulation of heavy metals in the axial organs of winter wheat. Heavy metals are toxic to plants in high doses (Anjum et al., 2015 a). Due to the fact that only absorbed metal ions can affect plants, it is necessary to determine the accumulation of heavy metals in various organs of the plants. The concentration of metals in the roots and shoots of wheat was determined 7 days after exposure of plants to heavy metals. In the control plants, the heavy metal concentrations were very low, probably indicating the initial content of heavy metals in the seeds. The heavy metal-treated plants exhibited much higher contents of heavy metals as compared to the control plants (Table 1).

Table 1. The influence of Ribav-Extra (RE) on the metal accumulation (µg g^{-1} DM) and translocation in winter wheat plants treated with heavy metal (HM) ions

Treatment		Cu	Ni	Pb	Zn
Roots					
Without RE	0 (control)	7 ± 1 a	6 ± 1 a	14 ± 1 a	13 ± 0 a
	10 µM	281 ± 11 g	28 ± 2 c	131 ± 14 c	104 ± 17 b
	1 mM	432 ± 2 h	226 ± 19 f	3997 ± 139 f	1576 ± 205 e
10 ppm RE	0 (control)	7 ± 1 a	6 ± 1 a	14 ± 1 a	12 ± 1 a
	10 µM	36 ± 0 c	43 ± 0 c	119 ± 4 c	308 ± 28 d
	1 mM	54 ± 7 d	194 ± 11 f	2714 ± 138 e	1430 ± 19 e
Shoots					
Without RE	0 (control)	11 ± 3 a	10 ± 2 ab	15 ± 1 a	13 ± 2 a
	10 µM	19 ± 2 b	62 ± 1 d	110 ± 14 bc	159 ± 51 bc
	1 mM	90 ± 5 f	142 ± 19 e	365 ± 95 d	256 ± 77 cd
10 ppm RE	0 (control)	12 ± 1 ab	10 ± 1 b	15 ± 1 a	13 ± 1 a
	10 µM	16 ± 0 b	36 ± 4 c	80 ± 11 b	83 ± 1 b
	1 mM	78 ± 2 e	56 ± 5 d	294 ± 9 d	216 ± 5 c
Index of HM translocation ($I_{HMt} = C_{shoots}/C_{roots}$)					
Without RE	0 (control)	1.57	1.67	1.07	1.00
	10 µM	0.07	2.22	0.84	1.53
	1 mM	0.21	0.63	0.09	0.16
10 ppm RE	0 (control)	1.72	1.67	1.08	1.08
	10 µM	0.44	0.84	0.67	0.27
	1 mM	1.45	0.29	0.11	0.15

Note. Different letters within each column mean significant differences between treatments (assessed by the Duncan's test at $P = 0.05$).

Higher concentrations of heavy metals in solutions resulted in higher accumulation of metals in the wheat seedlings. However, in the roots of plants exposed to 10 µM, metal concentrations increased from 4.6 to 40 times (in the sequence Ni < Zn ≈ Pb << Cu), and in shoots up to 12.2 times (in the sequence Cu < Ni ≈ Pb < Zn). When the heavy metals concentrations in the growth medium were maximal (1 mM), the metal concentrations increased even more: from 37.6 to 277 times (in the sequence Ni < Cu < Zn < Pb) in the roots and from 8.2 to 24.3 times (in the sequence Cu < Ni < Zn < Pb) in the shoots.

We found that the metal concentrations in the aerial biomass were lower than those in the roots (except for 10 µM Ni^{2+}). The ability of the roots to bind heavy metals reduces their translocation to the aerial organs (Sharma et al., 2017). At the same time, it was established that the number of heavy metals in stems and leaves increases with an increase in the metal concentration in the environment and with an increase in their content in the roots. This indicates that protective mechanisms and barriers at root cell and tissue levels are not able to completely prevent the heavy metals translocation into shoots (Seregin, Kozhevnikova, 2008).

Index of heavy metals translocation (I_{HMt}) showed that at low concentration it was small in the case of Cu^{2+}, but exceed 1 in treatments with Ni^{2+} and Zn^{2+}. Higher concentration of heavy metals led to sharp diminution of heavy metals translocation (except Cu^{2+}). Presowing treatment with Ribav-Extra modified both the heavy metals uptake into the roots and heavy metals translocation into shoots significantly. Thus, in the medium containing 10 µM Ni^{2+} or Zn^{2+}, Ribav-Extra enhanced the ion uptake into the roots by 1.5 or 3 times, respectively; but it reduced significantly the Cu^{2+} uptake, most in the medium containing 1 mM

HM ions. In the shoots of RE-pre-treated plants, heavy metal concentrations reduced from 14% to 59% against untreated plants. However, as a rule, the efficiency of the regulator decreased slightly as the concentration of heavy metals in the medium increased. Moreover, in all treatments of the experiment Ribav-Extra significantly increased (almost by 7 times) the roots to shoots transport of Cu, but lowered Ni transport from root shoots, as was shown by I_{HMt}. In the case of Pb and Zn, the I_{HMt} level was invariable in wheat seedlings pre-treated with Ribav-Extra (Table 1).

The influence of heavy metal ions and Ribav-Extra on winter wheat seedling growth. Growth is an integral characteristic of the plant state, indicating disturbances in physiological processes at the organism level. Disturbances in growth and morphogenesis are visible symptoms of plant exposure to stress factors. Also, the heavy metals toxicity can be clearly traced on growth inhibition, as shown for a wide range of plant species (Wilkins, 1978; Gangwar et al., 2014; Singh et al., 2016; Hussain et al., 2017). In this experiment, we demonstrated the growth parameters of wheat plants exposed to heavy metal ions. Seven days after wheat seedlings exposure to 10 µM of all HM ions (except for Zn^{2+}), the root lengths exceeded those in water control plants by 25% to 38% (Table 2). One mM of Ni^{2+}, Cu^{2+} and Pb^{2+} inhibited root growth significantly (by 92, 70 and 40 %, respectively, in comparison to water control); Zn^{2+} both concentrations did not affect the root growth. Also, 10 µM HM (except for Ni^{2+}) stimulated the growth of wheat shoots. When the plants were exposed to the higher concentration (1 mM) of Cu, Ni or Pb, shoot growth was inhibited, especially at Ni exposure (76% to water control). However, the growth of shoots was more resistant to heavy metals in comparison to roots.

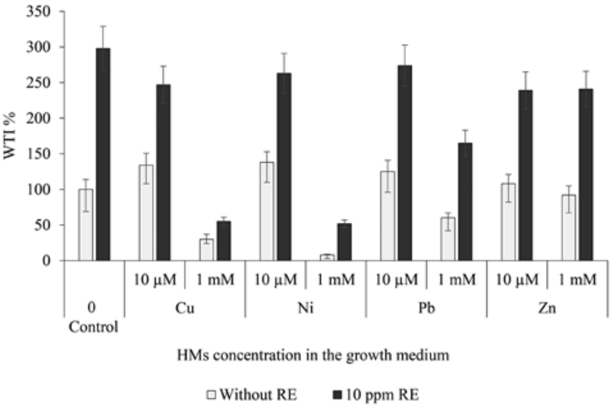
Table 2. The influence of Ribav-Extra (RE) on root and shoot length (mm) of winter wheat plants exposed to heavy metal ions

Treatment		Roots		Shoots	
		without RE	10 ppm RE	without RE	10 ppm RE
Control		33 ± 3 e	99 ± 2 j	90 ± 3 e	154 ± 4 i
Cu	10 µM	45 ± 4 f	82 ± 2 h	109 ± 3 f	122 ± 2 gh
	1 mM	10 ± 2 b	18 ± 1 cd	74 ± 3 d	44 ± 2 b
Ni	10 µM	46 ± 2 f	88 ± 3 hi	61 ± 4 c	120 ± 3 g
	1 mM	3 ± 0 a	17 ± 0 c	21 ± 2 a	86 ± 2 e
Pb	10 µM	42 ± 3 f	91 ± 3 i	105 ± 6 f	158 ± 3 i
	1 mM	20 ± 1 d	55 ± 2 g	72 ± 4 cd	129 ± 3 h
Zn	10 µM	36 ± 3 ef	80 ± 3 h	105 ± 5 f	155 ± 4 i
	1 mM	31 ± 3 e	81 ± 2 h	89 ± 8 de	135 ± 2 h

Note. Different letters mean significant differences between variants (separately for roots and for shoots).

The addition of Ribav-Extra significantly increased the length of winter wheat roots and shoots (except for 1 mM Cu^{2+} and Ni^{2+}) relative to water control (Table 2). Root growth in water (control seedlings) was enhanced by three times. When Ribav-Extra was applied in combination with heavy metals, the root length increased with respect to treatments without Ribav-Extra pre-treatment. Maximal growth increases were noted at 1 mM of Ni^{2+} and Pb^{2+} (5.6 and 2.7 times, respectively). Seed treatment with Ribav-Extra stimulated growth of above-ground parts in all experimental treatments (except for 1 mM Cu^{2+}). The treatment with Ribav-Extra increased shoot length by 1.5–4.1 and by 1.1–1.9 times (for 1 mM and 10 µM of HM, respectively), as compared to heavy metal treatments without growth regulator. As a result, Ribav-Extra treatment led to reduced negative effect of heavy metals on shoot growth. But in the case of 1 mM of Cu^{2+} there was no effect of Ribav-Extra treatment, because of significant decreases in shoot length by 41%. Since the roots first react to heavy metals concentration in soils, the Wilkins tolerance index (WTI) was calculated to assess the heavy metal-tolerance of seedlings (Fig.).

As can be seen from the Figure, wheat plant WTI was about the control level at 10 µM of HM, but decreased by 8–92% at 1 mM HM. This indicates the resistance of wheat plants to suboptimal concentrations and their sensitivity to sub-lethal concentrations of heavy metals. Pre-sowing Ribav-Extra treatment promoted an increase in WTI relative to water control and heavy metal-treated plants. Thus, Ribav-Extra contributed to a partial reduction or complete removal of the toxic effect of heavy metal ions.



White columns – without Ribav-Extra (water or metal), black columns – metal + Ribav-Extra

Figure. The effect of Ribav-Extra (RE) on Wilkins tolerance index (WTI) in winter wheat seedlings treated with heavy metals (HM)

The influence of heavy metal ions and Ribav-Extra on superoxide ($^{\bullet}O_2^-$) generation and lipid peroxidation (LPO) in winter wheat leaves. To assess the heavy metals toxicity, we studied the $^{\bullet}O_2^-$ generation in plant cells exposed to stress environment (Mittler, 2002). It is known that the stress-induced increase in $^{\bullet}O_2^-$ generation is temporary, it was shown in plants exposed to both chilling (Lukatkin, 2002 a) and heavy metals

(Lukatkin et al., 2010; Lehotai et al., 2011; Hossain et al., 2012; Thakur et al., 2017). We found that chronic metal impact on plants induced the varying intensity of $^{\bullet}O_2^-$ generation in the leaves. So, 10 µM of the HM ions (except for 10 µM of Ni^{2+}) did not change the $^{\bullet}O_2^-$ generation, but at 1 mM HM in the medium, Zn^{2+} or Pb^{2+} led to an insignificant decrease and Ni^{2+} – to a slight increase in $^{\bullet}O_2^-$ generation (Table 3).

Table 3. The effect of Ribav-Extra (RE) and heavy metal ions on superoxide ($^{\bullet}O_2^-$) generation and on malonic dialdehyde (MDA) content in winter wheat leaves

Treatment		$^{\bullet}O_2^-$ generation µM g^{-1} min^{-1}		MDA concentration µM g^{-1}	
		without RE	10 ppm RE	without RE	10 ppm RE
Control		3.4 ± 0.4 c	1.6 ± 0.1 b	0.8 ± 0.03 c	0.6 ± 0.04 ab
Cu	10 µM	3.6 ± 0.7 cd	1.3 ± 0.03 a	1.4 ± 0.1 d	0.7 ± 0.1 bc
	1 mM	4.3 ± 0.3 d	1.8 ± 0.3 b	2.1 ± 0.3 e	0.7 ± 0.1 bc
Ni	10 µM	4.6 ± 0.4 d	3.2 ± 0.1 c	1.3 ± 0.1 d	0.5 ± 0.03 a
	1 mM	8.1 ± 1.0 e	2.3 ± 0.3 bc	1.5 ± 0.1 d	0.9 ± 0.1 c
Pb	10 µM	2.8 ± 0.6 bc	2.0 ± 0.2 bc	0.7 ± 0.1 bc	0.8 ± 0.2 abc
	1 mM	3.0 ± 0.5 c	2.0 ± 0.2 bc	0.8 ± 0.1 bc	0.7 ± 0.1 bc
Zn	10 µM	3.4 ± 0.7 cd	2.6 ± 0.7 bc	0.8 ± 0.1 bc	0.6 ± 0.01 b
	1 mM	2.2 ± 0.3 bc	1.5 ± 0.3 ab	0.7 ± 0.1 bc	0.8 ± 0.1 bc

Note. Different letters mean significant differences between variants (separately for $^{\bullet}O_2^-$ generation and for MDA concentration).

Thus, the chronic effect of heavy metals on wheat plants slightly altered the $^{\bullet}O_2^-$ generation in the leaf cells (except for 1 mM of Cu^{2+} and both Ni^{2+} treatments). Comparison of the $^{\bullet}O_2^-$ generation rate in wheat leaves with other species (cucumber, maize) showed that this minimal increase in $^{\bullet}O_2^-$ generation is specific to wheat plants. Perhaps this is due to the initial high level of the ROS generation in wheat and lower level in cucumber or maize. Another possible explanation for this is the adaptation of metabolic processes and the normalization in the rate of ROS generation caused by prolonged (within 7 days) plant growth on a medium containing heavy metals. The amount of $^{\bullet}O_2^-$ and H_2O_2 on the 3rd day after treatment of wheat plants with 10 µM Ni^{2+} was 250% compared to the control, but at the 6th and 9th day these parameters decreased (Gajewska, Sklodowska, 2007). Short-term (2 h) treatment of pea plants with 100 µM $CdCl_2$ or $CuSO_4$ resulted in an increase in $^{\bullet}O_2^-$ generation, followed by its decrease (Lehotai et al., 2011). Obviously, the rapidly changing level of $^{\bullet}O_2^-$ generation can hardly be used as an index for the toxic heavy metals effect in chronic intoxication of wheat seedlings, despite the LPO level.

Seed treatment with Ribav-Extra resulted in a decrease in the $^{\bullet}O_2^-$ level by 54% relative to the water control (Table 3). In addition, pre-treatment of wheat seeds with Ribav-Extra reduced the rate of $^{\bullet}O_2^-$ generation in plants exposed to heavy metals, especially at Cu^{2+} (by 60–65%) and 1 mM Zn^{2+} (by 56%) treatments. Under the action Pb^{2+}, the effectiveness of Ribav-Extra was less expressed (there was only insignificant decrease in the rate of $^{\bullet}O_2^-$ generation relative to untreated plants). Heavy metals influenced differently the LPO intensity in the wheat leaves in different variants of the experiment (Table 3). The maximum MDA concentration was detected at concentrations of 10 µM and 1 mM under the impact of Cu^{2+} (164% and 256%, respectively, compared to water control) or Ni^{2+} (1.5 and 1.7 times, respectively, above the control). Pb^{2+} and Zn^{2+} did not significantly change the amount of MDA in the leaves relative to the water control.

It is known that heavy metals can induce oxidative stress (Hossain et al., 2012; Rahoui et al., 2017; Thakur et al., 2017), thereby significantly affecting the processes of LPO, leading to the destruction of cell membranes; the rate of their damage can be assessed from the accumulation of MDA (Lukatkin et al., 2010). However, in the study we found that MDA accumulation in wheat leaves was significantly dependent on the metal and much less on its concentration in the medium. The level of LPO intensity was significantly higher in Cu^{2+}- or Ni^{2+}-treated seedlings (both concentrations), but it was invariable in Pb^{2+}- or Zn^{2+}-treated plants. At the same time, differences in MDA accumulation between variants with suboptimal and sub-lethal concentrations of heavy metals in the medium were not always significant. The increase in the content of LPO products in wheat leaves affected by Ni and Cu ions indicates significant damage to membrane lipids. This can cause alterations in the membranes permeability, inhibition of membrane enzymes, and leads to the changes in cytoplasm ion balance (Anjum et al., 2015 b).

It is known that plant hormones intensively interact with ROS, thus alleviate the stress reaction of plants (Gangwar et al., 2014; Xia et al., 2015). Pre-treatment of wheat seeds with Ribav-Extra contributed to a decrease in the intensity of lipid peroxidation in plant leaves (Table 3). Comparison of RE-treated and RE-untreated plants showed that a significant decrease in the LPO intensity in the treatments at 10 µM and 1 mM with Cu^{2+} (by 36% and 70%, respectively) or Ni^{2+} (by 60% and 39%, respectively). In other treatments at both concentrations of Zn^{2+} and Pb^{2+}, Ribav-Extra effect on LPO intensity in the wheat leaves was insignificant.

The influence of heavy metal ions and Ribav-Extra on the activity of enzymatic antioxidants. A special role in the protective reactions of plants to the heavy metals action belongs to enzymatic antioxidants, whose activity increases significantly under these conditions (Hossain et al., 2012; Rahoui et al., 2017; Thakur et al., 2017). This leads to neutralization of ROS, which are formed in heavy metal-treated plants and have a damaging effect on the cells, and to increased resistance to heavy metals in plants.

Calatase (CAT) activity. One of the main enzymatic antioxidants in plants is CAT, which utilizes

H_2O_2 excess, formed during ROS metabolism (Lukatkin, 2002 b; Mittler, 2002; Anjum et al., 2016). The activity of CAT in wheat leaves increased compared to the control in all heavy metal treatments, except for 10 µM Ni^{2+} (Table 4). The maximum CAT activity was induced at 10 µM and 1 mM by Cu^{2+} (225% and 166%, respectively, compared to water control) and Pb^{2+} (227% and 164%, respectively, compared to water control). Since CAT is an inducible enzyme (its activity increases when the substrate (H_2O_2) amount rises in the medium), an increase in its activity in the presence of heavy metals indicates intensification in the ROS generation in wheat seedlings cells. CAT activity was significantly higher at 10 µM of Cu^{2+} or Pb^{2+} and at 1 mM of Zn^{2+} or Ni^{2+}. Probably, this reflected the differences in the metabolic pathways of ROS utilization under the metal action. Pre-sowing treatment of wheat seeds with Ribav-Extra promoted a decrease in CAT activity by 26% relative to water control (Table 4). In the HM-containing medium, RE-pretreatment did not change the CAT activity relative to the untreated plants, except for the following cases: in leaves of RE-treated plants, the enzyme activity decreased by 15% and 21% at 10 µM and 1 mM of Ni^{2+}, respectively, and by 10% at 10 µM of Zn^{2+}.

Table 4. The influence of Ribav-Extra (RE) on catalase (CAT) and ascorbate peroxidase (APOX) activities (µM g^{-1} min^{-1}) in winter wheat plants treated with heavy metal ions

Treatment		Catalase		Ascorbate peroxidase	
		without RE	10 ppm RE	without RE	10 ppm RE
Control		14.9 ± 0.9 bc	11.1 ± 0.2 a	0.5 ± 0.1 ab	0.2 ± 0.1 a
Cu	10 µM	33.4 ± 1.9 f	34.5 ± 1.5 f	0.3 ± 0.1 ab	0.5 ± 0.1 ab
	1 mM	24.7 ± 1.6 de	23.1 ± 0.7 de	0.3 ± 0.1 ab	0.3 ± 0.1 ab
Ni	10 µM	15.3 ± 0.7 c	13.0 ± 0.5 b	0.5 ± 0.04 b	0.5 ± 0.1 ab
	1 mM	27.5 ± 1.9 e	21.8 ± 0.2 d	0.4 ± 0.1 ab	0.5 ± 0.1 ab
Pb	10 µM	32.2 ± 0.8 f	33.0 ± 0.4 f	0.5 ± 0.1 ab	0.3 ± 0.03 a
	1 mM	24.4 ± 0.9 de	21.6 ± 0.3 d	0.3 ± 0.1 ab	0.2 ± 0.1 ab
Zn	10 µM	24.4 ± 0.7 e	21.9 ± 0.2 d	0.4 ± 0.04 ab	0.2 ± 0.1 ab
	1 mM	33.2 ± 1.1 f	31.9 ± 0.3 f	0.5 ± 0.1 ab	0.2 ± 0.1 ab

Note. Different letters mean significant differences between variants (separately for CAT and for APOX).

Ascorbate peroxidase (APOX) activity. In the experiment, we demonstrated the influence of heavy metals on the activity of APOX in wheat leaves. APOX is the main enzyme that utilizes H_2O_2 in plants. An increase in the activity of APOX may perhaps indicate an increase in the synthesis of APOX isoenzymes, which reduce the concentration of H_2O_2 and protect against oxidative stress (Anjum et al., 2016). In our experiments, the activity of APOX was at the control level at all the heavy metals studied (Table 4). We showed that RE-pretreated plants had lower APOX activity in comparison to untreated ones in a medium containing Pb or Zn ions but it insignificantly increased in the presence of Cu or Ni.

Conclusions

1. Heavy metals (HM) in growth media lead to oxidative stress in winter wheat (*Triticum aestivum* L.) seedlings. By toxicity level to wheat seedlings, the heavy metals can be arranged in the following order: Zn ≤ Pb << Cu < Ni. There were no significant differences between essential (Zn and Ni) and non-essential (Pb) heavy metals influence on superoxide ($^{\bullet}O_2^-$) generation in wheat leaves. The increase in lipid peroxidation (LPO) products in Cu^{2+}- and Ni^{2+}-treated plants may be evidence of serious damage in membrane lipids induced by heavy metals. Heavy metal ions induced enhanced catalase (CAT) activity, too.

2. Ribav-Extra (RE) stimulated growth of roots and shoots (except for 1 mM of Cu^{2+}) in HM-treated wheat plants and significantly reduced the heavy metals accumulation in wheat seedlings exposed to Cu^{2+} or 1 mM of Pb^{2+} (in roots) and to 10 µM of Cu^{2+} or 1 mM of Ni^{2+} (in shoots). Also, Ribav-Extra treatment promoted Cu^{2+} translocation in shoots and inhibited the Ni^{2+} translocation.

3. Ribav-Extra showed the opposite effect on heavy metals action, lead to partial reduction of $^{\bullet}O_2^-$ generation in wheat leaves, most effectively in Cu^{2+}-treated plants as well as to a decrease of LPO intensity and CAT activity. The maximal effectivity of RE-treatment was noted in the case of Cu^{2+} and Ni^{2+} action.

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Augimo reguliatoriaus Ribav-Extra įtaka sunkiaisiais metalais paveiktų žieminių kviečių daigams

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Santrauka

Tirta natūralaus augimo reguliatoriaus Ribav-Extra ir sunkiųjų metalų (SM) jonų – Pb^{2+}, Cu^{2+}, Zn^{2+}, bei Ni^{2+} – įtaka žieminio kviečio (*Triticum aestivum* L.) veislės 'Mironovskaya 808' fiziologiniams ir biocheminiams rodikliams. Žieminių kviečių sėklos buvo paveiktos Ribav-Extra (10 ppm) ir 7 dienas augo sunkiųjų metalų druskų tirpaluose (10 µM arba 1 mM), po to įvertintas sunkiųjų metalų kaupimasis augaluose, augalų augimas, superoksido anijonų ($^{\bullet}O_2^-$) gamyba, lipidų peroksidacija, katalazės ir askorbato peroksidazės aktyvumas. Nustatyta, kad 1 mM SM tirpalas augimo terpėje padidino sunkiųjų metalų kaupimąsi žieminiuose kviečiuose ir slopino šaknų bei ūglių augimą. Ir mažos, ir didelės sunkiųjų metalų koncentracijos kviečių daiguose paskatino $^{\bullet}O_2^-$ gamybą ir sukėlė oksidacinius pažeidimus. Beveik visų variantų augaluose sunkiųjų metalų toksinis poveikis sustiprėjo esant didesnėms koncentracijoms. Sėklų apdorojimas Ribav-Extra prieš sėją mažino sunkiųjų metalų poveikį žieminių kviečių daigams, slopino jų sukeltą oksidacinį stresą, mažino $^{\bullet}O_2^-$ gamybą, lipidų peroksidacijos intensyvumą ir katalazės aktyvumą. Tai rodo, kad sunkiųjų metalų poveikiui buvo atsparesni augimo reguliatoriumi Ribav-Extra apdoroti žieminių kviečių daigai nei neapdoroti.

Reikšminiai žodžiai: antioksidaciniai fermentai, oksidacinis stresas, Ribav-Extra, sunkieji metalai, *Triticum aestivum*.