Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin
Akademicka, 20-950 Lublin, Poland
e-mail: justyna.lesniowska@up.lublin.pl

MICHAŁ NOWAK ⓘD, JUSTYNA LEŚNIOWSKA-NOWAK ⓘD
MAGDALENA SOZONIUK ⓘD

Regulation of mitochondrial manganese superoxide dismutase (MnSOD) gene expression in cereals by copper and manganese excess

Zmiana ekspresji genu mitochondrialnej manganowej dysmutazy ponadtlenkowej (MnSOD) w zbożach pod wpływem miedzi i manganu

Summary. Within many different cytotoxic activities of heavy metals in plant cells, one of the most important is connected with reactive oxygen species (ROS) generation. Mechanism of plant cell defense against reactive oxygen species and free radicals has a comprehensive character. The aim of presented paper is characterization of changes in mitochondrial manganese superoxide dismutase (MnSOD) gene transcript level that occurred in bread wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) seedlings during copper and manganese treatment. Our results show down-regulation of MnSOD expression in most cases after the oxidative burst evoked by copper excess. Manganese treatment, on the other hand, caused differential reaction of tested material indicating the substantial impact of cultivar genetic background in molecular response to the same stress-inducing conditions.

Key words: heavy metal stress, mitochondrial manganese superoxide dismutase (MnSOD), *Triticum aestivum* L., *Hordeum vulgare* L.

INTRODUCTION

Heavy metal accumulation in soils is serious problem for agriculture and food production due to its effects on crop growth as well as food safety and marketability. Plants growing in metal-polluted sites exhibit altered metabolism that consequently leads to growth reduction and lower biomass production. Heavy metal toxicity in plants varies depending on plant species, specific metal, its concentration, chemical form and soil chemical properties [Nagajyoti et al. 2010]. The primary way of a heavy metal ions absorption by plants is their root system, however a direct influence of the particles trans-

ferred by air and deposited on the leaves also occurs. The main agricultural sources of heavy metal contamination are fertilizers, pesticides, liming, sewage disposal and irrigation [Li et al. 2013, Sheng et al. 2015].

Some heavy metal ions in small doses are essential micronutrients, which play important role in plant developmental and metabolic processes. Their main functions in live cells are participation in redox reactions and being an integral part of some enzymes particles. Copper is an electron donor in the photosystem I of plant photosynthesis [Mahmood and Islam 2006], it is also a component of cytochrome oxidase of respiratory electron transport chain [Demirevska-Kepova et al. 2004]. Moreover copper is a cofactor of oxidases, oxygenases and enzymes involved in the elimination of superoxide radicals (superoxide dismutase and ascorbate oxidase). Manganese participates in water splitting at photosystem II during photosynthesis and is a cofactor of manganese superoxide dismutase [Nagajyoti et al. 2010].

Excess of these metals ions concentration in soil is a cytotoxic factor which induces plants stress. Both copper and manganese in toxic doses cause inhibition of respiration process, have negative influence on nitrogen and protein metabolism, decrease polysaccharides content and some enzymes activity, reduce chlorophyll content, weaken a photosynthesis process in leaves and can damage chloroplasts [Singh et al. 2007, Li et al. 2013, Sheng et al. 2015].

Within many different cytotoxic activities of heavy metals in plant cell, one of the most important is associated with reactive oxygen species (ROS) generation. Metals are involved in the direct or indirect generation of free radicals and ROS by: direct electron transfer in single electron reduction, disturbance of metabolic pathways, inactivation and down regulation of the antioxidative enzymes and depletion of low molecular weight antioxidants [Aust et al. 1985].

Level of cell damage caused by reactive oxygen species is dependent on both; ROS amount and the detoxification and repairing mechanisms. Mechanism of plant cell defense against ROS and free radicals has a comprehensive character. The major enzymes involved in this process are superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). In details scavenging of ROS in plant cell was revised by Mittler [Mittler 2002], whereas comprehensive characterization of SODs can be found in review of Miller [Miller 2012].

 Studies concerning influence of heavy metals on antioxidant enzymes in plants showed that metal ions can directly modify their activity as well as change expression of the genes encoding them. There are many papers in literature describing SODs activity modification by heavy metals in cereals [Tamás et al. 2004, Olteanu et al. 2013, Sheng et al. 2015, Kacienė et al. 2017]. Comparatively, information on SODs gene expression changes during exposure to heavy metals are more limited.

The aim of presented paper is characterization of changes in mitochondrial manganese superoxide dismutase (MnSOD) gene transcript level that occurred in bread wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) seedlings during copper and manganese treatment. Moreover, the importance of material genetic background in gene expression analysis is discussed.

MATERIALS AND METHODS

Plant material. The plant material consisted of ten common wheat (*Triticum aestivum* L.) and ten barley (*Hordeum vulgare* L.) cultivars. Within tested common wheat

cultivars five were of spring growth habit ('Bombona', 'Hewilla', 'Histra', 'Kosma', 'Żura') and five were of winter growth habit ('Finezja', 'Fregata', 'Nutka', 'Rywalka', 'Satyna'). Similarly, five of tested barley cultivars we spring ('Atol', 'Blask', 'Granal', 'Poldek', 'Ryton') and five were winter cultivars ('Bartosz', 'Bażant', 'Bursztyn', 'Gregor', 'Horus').

Growth conditions. Kernels of analyzed forms were surface-sterilized with 1.5% sodium hypochlorite and rinsed three times with sterile distilled H_2O. Then kernels were placed on a filter paper soaked in sterile distilled H_2O on Petri dishes and placed in growth chamber at 25°C. After five days seedlings were placed on nets and transferred to plastic trays containing 4 l of copper or manganese sulphate solution, in a way that their roots were immersed in solution. Two solutions of different concentrations for each heavy metal were used – 1 mM and 10 mM for copper ($CuSO_4 \cdot 5H_2O$) and 0.1 mM and 1 mM for manganese ($MnSO_4 \cdot 5H_2O$). Control batch was transferred to plastic trays filled with sterile, distilled H_2O. Trays were placed in phytotron with photoperiod of 16 h light and 8 h darkness and temperature of 24°C. Each treatment consisted of two replicates and each replicate contained ten plants. Plant material for gene expression analyses was collected after 48 h and after 7 days of treatment. Immediately after collection, plant material was frozen in liquid nitrogen and total RNA was extracted.

Total RNA extraction. Before RNA isolation mortars and pestles, tubes and pipette tips were rinsed with DEPC solution (0.1%) and sterilized in autoclave (121°C, 30 min) to avoid RNase contamination. Plant tissue frozen in liquid nitrogen was ground to a fine powder using mortar and pestle. Total RNA was extracted with "Total RNA" kit (A&A Biotechnology) according to manufacturers' instructions.

cDNA synthesis. Reverse transcription was performed with "TaqMan[®] Reverse Transcription Reagents" kit (Life Technologies). As a template 200 ng of total RNA was used. Reaction was carried out with random hexamer primers in total volume of 10 μl. For reverse transcription the following thermal profile was applied: incubation at 25°C for 10 min, reverse transcription at 48°C for 30 min and reverse transcriptase inactivation at 95°C for 5 min.

Real-time PCR reaction. The transcript level of MnSOD gene was assessed by qPCR based on SYBR[®] Green chemistry. As an additional reference dye ROX was used. Reactions were prepared in final volume of 25 μl and contained 20 ng of template cDNA, 200 nM of each primer, 12.5 μl of 2 × Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) and 0.375 μl of ROX dye (diluted 1 : 500). Primers for MnSOD were taken from Baek and Skinner [Baek and Skinner 2003]. After preliminary evaluation of putative reference genes, *Ubi* [Kobayashi et al. 2005] for wheat samples and *GAPDH* [Burton et al. 2004] for barley samples were chosen as internal controls to normalize the data. All samples were analyzed in three replications using Mx3005P apparatus (Agilent Technologies). Real-time PCR thermal profile consisted of initial step of 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 60 s at 55°C and 30 s at 72°C. In order to confirm reaction correctness, dissociation curves analyses were carried out. Results analysis and interpretation were based on the $\Delta\Delta C_T$ method [Livak and Schmittgen 2001] and for this purpose MxPro 4.10 software (Agilent Technologies) was used.

Statistical analysis. Statistical analysis of obtained results was based on relevance of differences between ΔC_T values of control form and tested sample, and carried out according to procedure described by Yuan et al. [Yuan et al. 2006]. For this purpose a non-parametric Wilcoxon test was utilized with the significance level of 0.05. For statistical analysis SAS 9.2 software (SAS Institute) was used.

RESULTS

MnSOD gene expression changes induced by copper ions treatment. Majority of cultivars (both wheat and barley) treated with 1 mM Cu^{2+} for 48 h reacted with a decline in MnSOD transcript level as compared to their respective controls. Significant increase in MnSOD expression was observed only for wheat cultivar 'Finezja' and barley cultivars 'Atol' and 'Bartosz' (Fig. 1, Tab. 1). Similarly, decrease in MnSOD transcript level was observed in most cultivars treated with higher Cu^{2+} dose (10 mM) for 48 h. Significant increase in analyzed gene transcription was observed only for barley cultivar 'Ryton' (Fig. 2, Tab. 1).

Prolonged exposure (7 days) of tested forms to 1 mM Cu^{2+} resulted in upregulation of MnSOD transcription in wheat cultivars 'Kosma' and 'Nutka', while its downregulation was still observed in cvultivars 'Histra' and 'Rywalka'. MnSOD expression in other wheat forms reached the level observed in control plants. Nevertheless, comparison of data obtained for wheat forms at both time points revealed that in all samples (except for 'Finezja') MnSOD transcript level was greater after 7 days of experiment than after 48 h of treatment. For analyzed barley cultivars a clear trend of response was observed as majority of forms showed continuous decrease of transcript level. However, MnSOD mRNA level observed in barley cultivar 'Atol' remained increased (Fig. 1, Tab. 1).

Analysis of results obtained after 7 days of exposure to higher Cu^{2+} dose showed diverse reaction within wheat as well as barley cultivars (Fig. 2, Tab. 1). In most tested cereal forms MnSOD transcript level remained unchanged or declined, when compared to their respective controls. Two of wheat cultivars ('Histra' and 'Finezja') and three of barley cultivars ('Atol', 'Bartosz' and 'Gregor') showed increase in MnSOD mRNA after one week of experiment.

MnSOD gene expression changes induced by manganese ions treatment. After 48 h of 0.1 mM Mn^{2+} exposure most tested wheat forms showed decline in MnSOD gene transcript level. Among them only two ('Histra' and 'Kosma') showed increased transcript amount. Different pattern of response was noticed for barley, where MnSOD expression either increased or remained unchanged relative to respective controls. For four out of five cultivars, where enhancement of analyzed gene transcription was noticed, reaction for stressful conditions was fairly strong (over two-fold increase in MnSOD mRNA amount was observed). Decrease in analyzed gene expression was shown only for barley cultivar 'Bartosz' (Fig. 3, Tab. 1).

Similar reaction of tested forms was observed after 48 h of 1 mM Mn^{2+} treatment. All analyzed wheat cultivars (but for 'Bombona' and 'Kosma' where no changes were detected) revealed declined level of MnSOD transcripts when compared to respective controls. Contrasting results were obtained for barley, which showed enhancement of analyzed gene transcription in most of tested forms. Downregulation of MnSOD expression was observed only in barley cultivars 'Bartosz' and 'Gregor' (Fig. 4, Tab. 1).

Analysis of samples collected after 7 days of experiment showed that majority (0.1 mM Mn^{2+} treatment) or half of tested wheat forms (1 mM Mn^{2+} treatment) reacted with declined MnSOD expression. Barley cultivars, regardless of Mn^{2+} concentration, displayed no clear trend of response, as either decrease, no change or increase in MnSOD transcripts levels were noticed (Fig. 3, 4, Tab. 1).

Table 1. Fold change of the MnSOD gene mRNA quantity in comparison to control forms

Species	Cultivar	1 mM CuSO$_4$		10 mM CuSO$_4$		0.1 mM MnSO$_4$		1 mM MnSO$_4$	
		48 h	7 days	48 h	7 days	48 h	7 days	48 h	7 days
Triticum aestivum L.	'Bombona'	−0.568*	0.018	−0.914*	−0.282	0.168	−1.390*	−0.266	−1.810*
	'Hewilla'	−0.391	−0.235	−1.720*	−0.478*	−1.920*	−0.766*	−0.971*	−0.430*
	'Histra'	−1.880*	−1.710*	−0.108	1.000*	2.570*	−1.160*	−1.140*	−0.110
	'Kosma'	−1.150*	1.630*	−0.448*	−0.823*	0.346*	−1.070*	0.083	−0.855*
	'Żura'	−0.783*	0.287	−0.387	−0.675*	−0.604*	0.214	−0.353*	−0.585*
	'Finezja'	1.040*	−0.161	0.005	2.080*	−1.140*	0.897*	−0.348*	0.620*
	'Fregata'	0.032	0.162	−2.380*	−3.530*	−1.170*	0.688*	−0.437*	−0.147
	'Nutka'	−0.477*	5.270*	−0.911*	−5.980*	−1.190*	−0.625*	−3.060*	−0.494*
	'Rywalka'	−0.734*	−0.668*	−0.263	0.072	−0.154	−0.552*	−0.500*	1.620*
	'Satyna'	−0.927*	−0.392	−6.780*	−2.360*	−1.470*	−0.754*	−1.420*	1.510*
Hordeum vulgare L.	'Atol'	0.929*	1.150*	0.290	0.843*	2.550*	−0.586*	0.622*	−0.572*
	'Blask'	−0.749*	−1.250*	−8.060*	−1.010*	0.370*	0.892*	1.270*	−0.200
	'Granal'	−0.397	−2.550*	0.063	−1.720*	−0.151	−0.443*	1.290*	−0.885*
	'Poldek'	−0.590*	−0.910*	−1.330*	−2.180*	0.083	0.286	−0.329	0.181
	'Ryton'	0.083	−1.520*	0.593*	0.080	2.050*	−1.030*	1.080*	0.088
	'Bartosz'	2.060*	−1.720*	−0.110	1.460*	−0.990*	0.107	−2.000*	−0.903*
	'Bażant'	−1.730*	−0.765*	−3.460*	−1.310*	3.370*	1.770*	1.980*	0.890*
	'Bursztyn'	−0.371	−0.674*	−0.309	−0.751*	2.290*	0.081	2.340*	0.442
	'Gregor'	−1.470*	−2.360*	−1.190*	1.360*	0.004	−1.700*	−1.140*	0.034
	'Horus'	−1.590*	−0.959*	0.156	−0.212	0.282	0.844*	0.433	2.840*

*change statistically significant at p = 0.05 level in comparison to respective control form
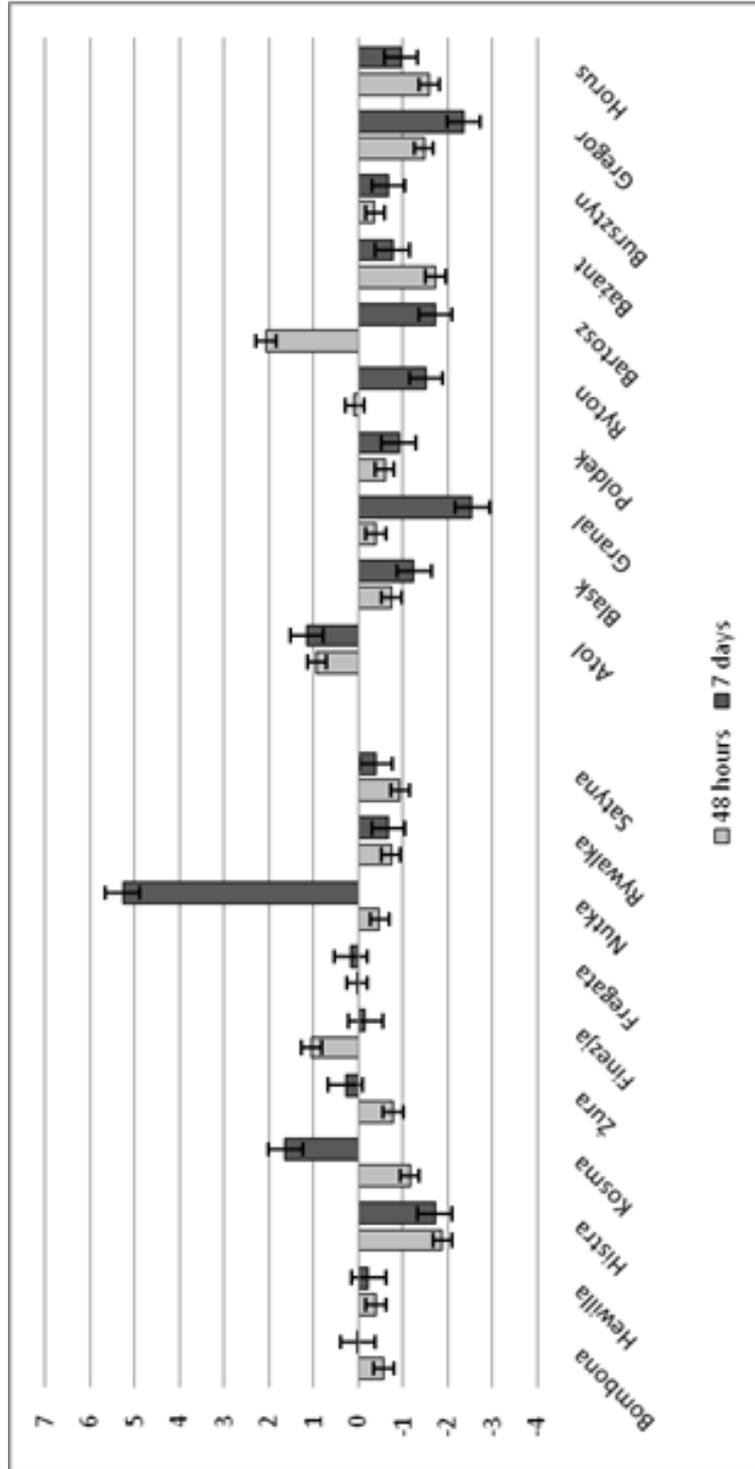
Fig. 1. Fold change of the MnSOD gene transcript level in common wheat and barley cultivars after 48 h and 7 days of 1 mM $CuSO_4$ solution treatment
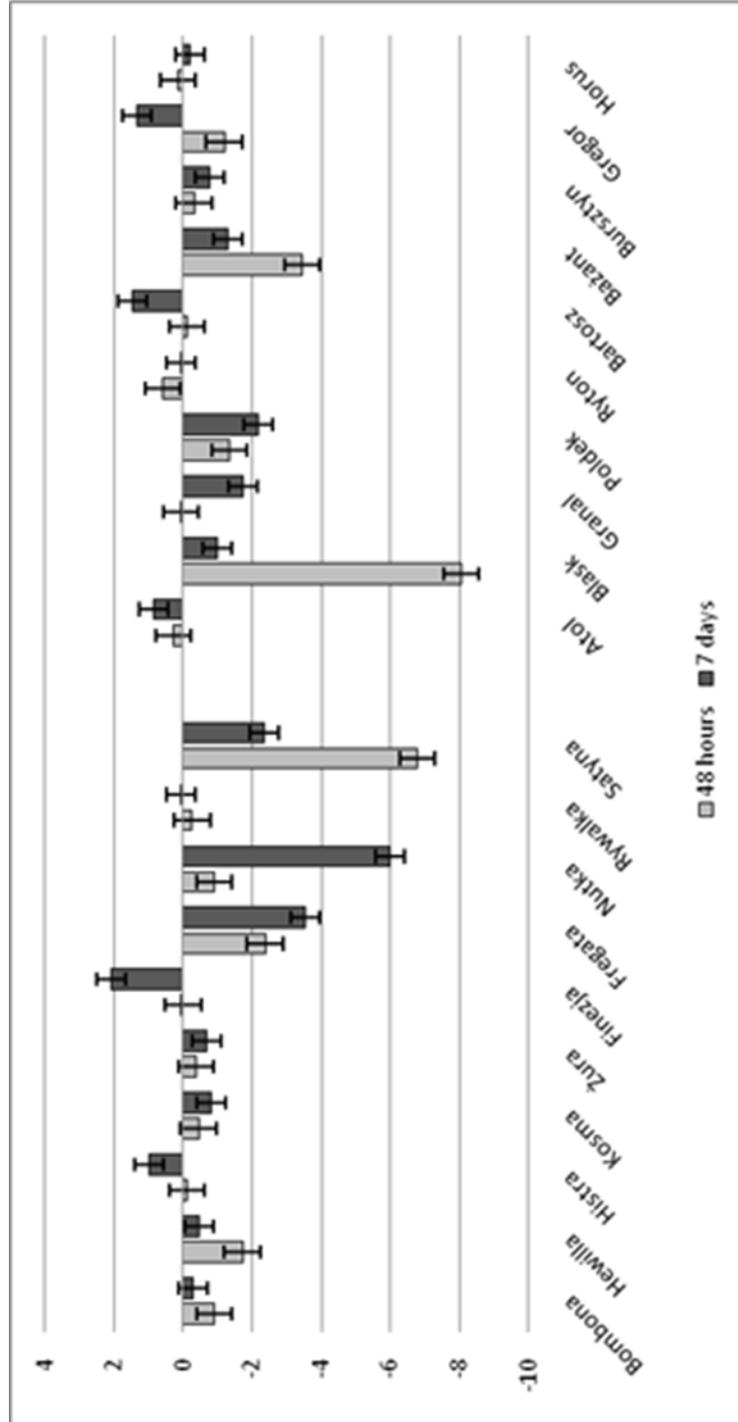
Fig. 2. Fold change of the MnSOD gene transcript level in common wheat and barley cultivars after 48 h and 7 days of 10 mM CuSO$_4$ solution treatment
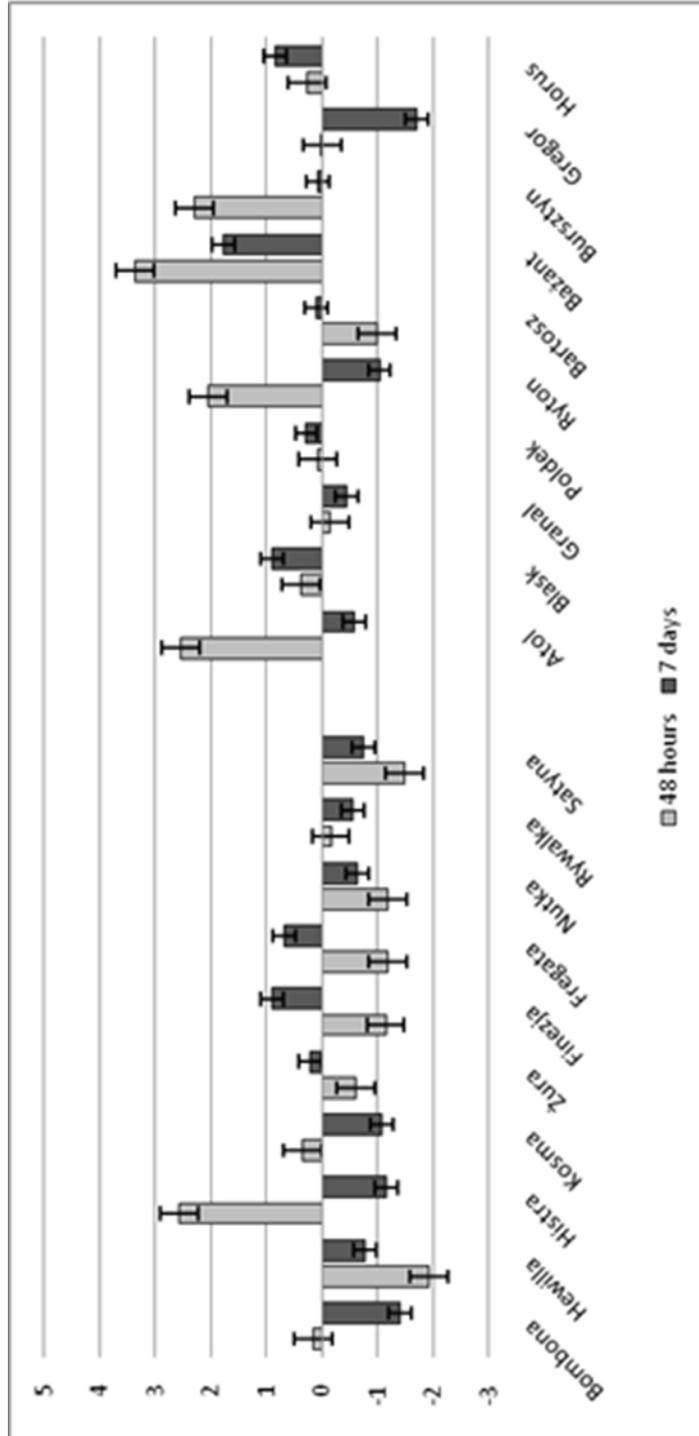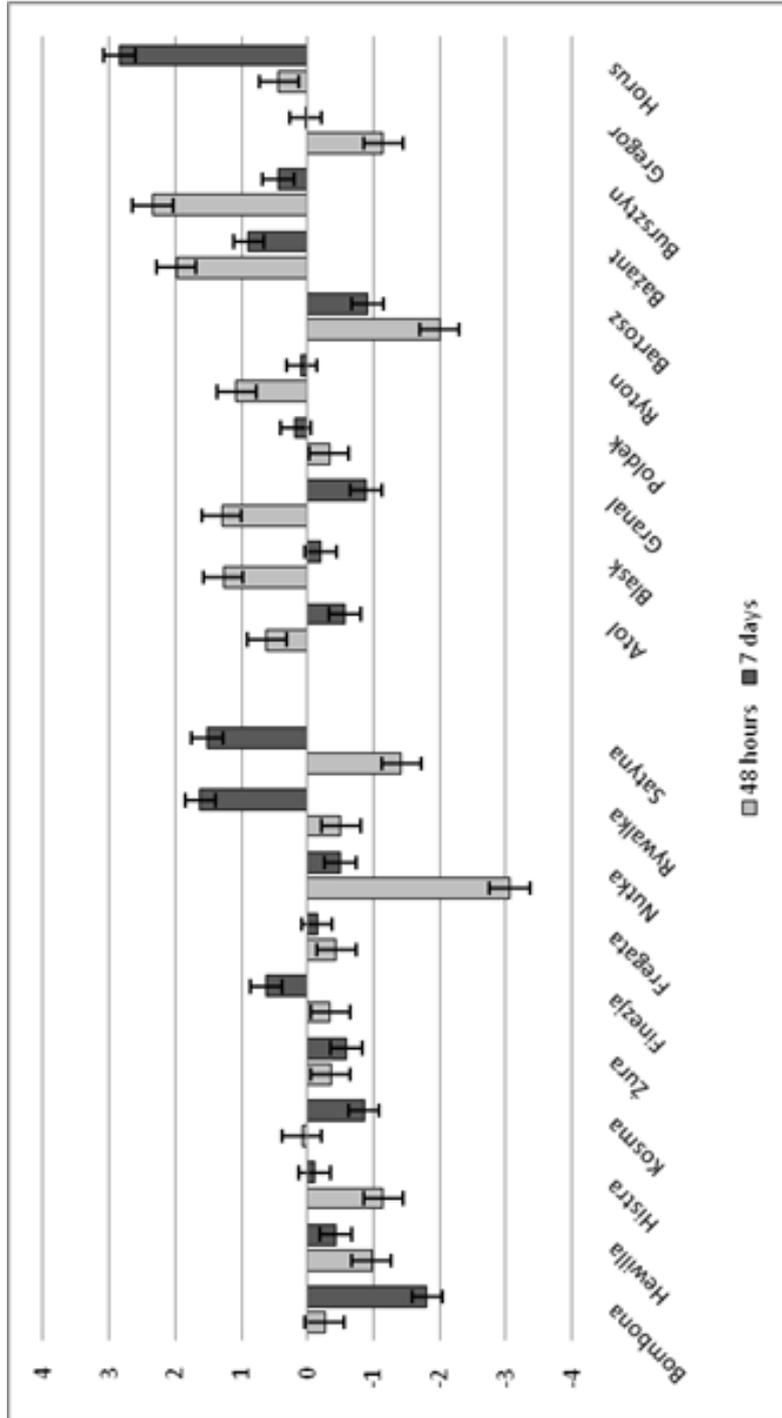
Fig. 3. Fold change of the MnSOD gene transcript level in common wheat and barley cultivars after 48 h and 7 days of 0.1 mM $MnSO_4$ solution treatment

Fig. 4. Fold change of the MnSOD gene transcript level in common wheat and barley cultivars after 48 h and 7 days of 1 mM MnSO$_4$ solution treatment

DISCUSSION

Superoxide dismutase (EC 1.15.1.1) is an enzyme which forms the first line of cell defense against ROS [Gill et al. 2015]. At the early stage of stress in plant cells the large quantities of ROS are generated, what is described as a "oxidative burst" [Bhattacharjee 2005]. Many previous studies confirmed relationship between heavy metals presence and ROS generation as well as plant enzymatic antioxidative system induction [Garnier et al. 2006, Sgherri et al. 2007, Li et al. 2012b, Sheng et al. 2015].

Upregulation of MnSOD gene expression was observed by Li et al. [Li et al. 2012a] in perennial ryegrass between 4 h and 24 h of 3,2 mM Pb treatment. However, after 48 h of exposure decrease in transcript level was found. Also Luo et al. [Luo et al. 2011] reported significant induction of MnSOD in perennial ryegrass subjected to 0,2 mM Cd during first 4 h of stress. However, afterwards decline in MnSOD transcript level was noticed. On the other hand, Cd– stressed common wheat, as reported by Karimi and Mohsenzadeh [2017] exhibited increased MnSOD expression after 6 days of stress.

Our results showed that in most cases the expression of MnSOD gene was downregulated by oxidative burst caused by copper excess. The level of the MnSOD transcript remained decreased throughout the experiment. This may suggest the involvement of other acclimation mechanisms in response to subjected stress. However, it can also be noticed that prolonged exposure to copper excess in some cultivars (of both species and both growth habits) led to activation of MnSOD expression. Obtained results did not reveal relation between spring or winter growth habit and reaction to copper ions treatment for any of the analyzed cereal species.

Manganese treatment affected MnSOD transcription in various ways, as either its induction, inhibition or lack of significant change were observed. No clear tendency of response was noticed in tested material, except for winter wheat cultivars. Regardless of manganese dose, after 48 h of exposure the decrease of MnSOD expression was detected. Comparison of data obtained after 48 h and 7 days, however, shows expression induction. After initial inhibition, the MnSOD gene was activated – the transcript level exceeded that of respective controls (but for some remained below it). Interestingly, MnSOD expression induction may be noticed in most barley spring cultivars already after 48 h of manganese exposure. Prolonged treatment, however, led to its decrease to the control level or below it.

Tested cereals forms subjected to copper excess reacted mostly with decreased MnSOD expression over the time of experiment. However, those treated with manganese excess displayed differential trends of response. Profile of expression obtained in reaction to subjected treatments was clearly cultivar dependent – this can be best demonstrated in barley cultivars exposed to higher manganese dose (1 mM $MnSO_4$) in analyzed time points (48 h and 7 days). For instance, cultivar 'Granal' at first showed significantly higher level of MnSOD transcript, which at the end of experiment dropped below that observed in respective controls. Cultivar 'Bartosz' showed inhibited MnSOD expression regardless of exposure time while cv. Bażant, on the contrary, showed its induction. In cultivar 'Poldek' no significant changes in MnSOD transcript level were observed throughout the experiment.

Lack of congruence in profiles of response among tested forms indicates the substantial impact of cultivar genetic background on its response to some stressors and complexity of gene expression regulation. Therefore, an emphasis should be put on taking into account the material genetic background when interpreting gene expression analysis results. Authors performing experiments on selected research material (especially when it consists

of single cultivar/ form/ line of a given species) should be cautious when drawing conclusions and should avoid generalizations and extrapolation of their data.

Literature confirms that different genotypes of the same species may vary in their response to the same stressful conditions. Evident genotypical differences in two barley cultivars at both the gene expression and antioxidant enzyme activity levels upon severe drought stress were reported by Harb et al. [2015]. Expression profile of antioxidant enzymes specific to each wheat genotype were observed by Sheoran et al. [2015] under drought. Differences in antioxidative systems response (at the level of enzyme acitivity) among various wheat cultivars subjected to stressful conditions were shown by Chakraborty and Pradhan [2012] and Rao et al. [2013].

CONCLUSIONS

Presented paper characterizes the changes in mitochondrial manganese superoxide dismutase (MnSOD) gene transcript level that occurred in bread wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) seedlings during copper and manganese treatment. Obtained results show that the expression of MnSOD in tested wheat and barley cultivars exposed to copper excess is predominantly downregulated, thus suggesting the induction of other acclimation mechanisms. Manganese treatment, however, caused differential response among tested material indicating the substantial impact of cultivar genetic background in molecular response to the same stress-inducing conditions.

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Streszczenie. Spośród wielu różnych działań cytotoksycznych metali ciężkich w komórkach roślinnych jedno z najważniejszych wiąże się z wytwarzaniem reaktywnych form tlenu (ROS). Mechanizm obrony komórek roślinnych przed ROS i wolnymi rodnikami ma kompleksowy charakter. Celem prezentowanej pracy jest charakterystyka zmian w poziomie transkryptów genu mitochondrialnej manganowej dysmutazy ponadtlenkowej (MnSOD), które wystąpiły w siewkach pszenicy zwyczajnej (*Triticum aestivum* L.) i jęczmienia zwyczajnego (*Hordeum vulgare* L.) podczas traktowania ich miedzią i manganem. W badanym materiale w większości przypadków obserwowano obniżenie ekspresji genu MnSOD w wyniku stresu oksydacyjnego wywołanego nadmiarem miedzi. Z kolei traktowanie siewek nadmiarem manganu powodowało zróżnicowaną reakcję, co wskazuje na dużą rolę tła genetycznego badanych odmian w odpowiedzi na wywołane warunki stresowe.

Słowa kluczowe: metale ciężkie, manganowa dysmutaza ponadtlenkowa (MnSOD), *Triticum aestivum* L., *Hordeum vulgare* L.