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Insight into the Phytoremediation Capability of *Brassica juncea* (v. Malopolska): Metal Accumulation and Antioxidant Enzyme Activity

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Abstract:

Metal hyperaccumulating plants should have extremely efficient defence mechanisms, enabling growth and development in a polluted environment. *Brassica* species are known to display hyperaccumulation capability. *Brassica juncea* (Indiana mustard) v. Malopolska plants were exposed to trace elements, i.e., cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn), at a concentration of 50 μM and were then harvested after 96 hours for analysis. We observed a high index of tolerance (IT), higher than 90%, for all *B. juncea* plants treated with the four metals, and we showed that Cd, Cu, Pb and Zn accumulation was higher in the above-ground parts than in the roots. We estimated the metal effects on the generation of reactive oxygen species (ROS) and the levels of protein oxidation as well as on the activity and gene expression

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of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). The obtained results indicate that organo-specific ROS generation was higher in plants exposed to essential metal elements (i.e., Cu and Zn), compared with non-essential ones (i.e., Cd and Pb), in conjunction with SOD, CAT and APX activity and expression at the level of encoding mRNAs and existing proteins. In addition to the potential usefulness of *B. juncea* in the phytoremediation process, the data provide important information concerning plant response to the presence of trace metals.

Key words: oxidative stress, antioxidative system, Brassicaceae family, heavy metals,

1. Introduction

Trace metal element contamination in soils is one of the world's major environmental problems, posing significant risks to human health as well as to ecosystems (Chen et al., 2014). Metals such as zinc (Zn), iron (Fe) and copper (Cu) are essential micronutrients required for a wide range of physiological processes in all plant organs, and the processes are based on the activities of various metal-dependent enzymes and proteins. However, they can also be toxic at elevated levels. Metals such as arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pd) are nonessential and potentially highly toxic (Dalvi and Bhalerao, 2013). Trace metal element toxicity includes changes in the chlorophyll concentration in leaves and damage of the photosynthetic apparatus, inhibition of transpiration and destruction of carbohydrate metabolism as well as nutrition and oxidative stress, which collectively affect plant development and growth (Molas, 2002; Krämer and Clemens, 2005; Bhardwaj et al., 2009; Bankaji et al., 2014; Małecka et al., 2015).

Biological organisms are incapable of degrading metals, so they persist in their body parts and environment, leading to health hazards (Khan et al., 2015). Metal accumulation and other abiotic stresses cause excess ROS generation, leading to oxidative stress (e.g., Małecka

et al., 2015). Plant cells are equipped with enzymatic mechanisms to eliminate or reduce oxidative damage that occurs under metal accumulation. The antioxidative defence system includes SOD, CAT and APX, which are regarded as responsible for maintaining the balance between ROS production and scavenging (Bankaji et al., 2015).

The Brassicaceae family includes many genera abundant in metallophytes, such as *Thlaspi*, *Brassica* and *Arabidopsis*. They accumulate a wide range of heavy metals, especially Zn, Cd, nickel (Ni), thallium (Tl), chromium (Cr) and selenium (Se) (Babula et al. 2012). The term hyperaccumulator is used for plants that accumulate 1000 mg per kg of dry matter of any aboveground tissue when grown in their natural habitat (Eapen, 2005; Singh et al., 2016). As of 2013, approximately 500 metal hyperaccumulator plant species were described (Rascio, Navari-Izzo 2011, Ent et al., 2013), and the number is increasing. *B. juncea* exhibits some traits of a metal hyperaccumulator – this species can take up significant quantities of Pb, Cd (Jiang, Liu, and Hou 2000; Meyers et al. 2008) and Cr, Cu, Ni, Pb and Zn (Prasad and Freitas 2003; Babula et al., 2012), although its translocation ability is not as efficient as shown for other known hyperaccumulators. Metal hyperaccumulating plants should have extremely efficient defence mechanisms, enabling growth and development in a polluted environment. Therefore, the objective of the present study was to estimate the contribution of the *B. juncea* (v. Malopolska) enzymatic antioxidant system to combating the oxidative stress induced by essential (Cu, Zn) and non-essential (Pb, Cd) metal elements to allow survival under adverse environmental conditions. The analysis included trace metal accumulation, level of stress parameters and antioxidant enzyme activity as well as estimation of encoding mRNA and enzyme protein levels.

2. Result

2.1 Levels of metal accumulation

Research using laser ablation combined with plasma mass spectrometry (LA-ICP-MS) made it possible to determine the levels of metal accumulation in *B. juncea* organs (Fig. 2). The analyses were performed for roots, stems and leaves. In the case of roots, Pb constituted approximately 60% of all accumulated metals. In addition, approximately 4 times higher levels of accumulated Cu and Zn as well as more than 140 times higher levels of Cd were found in roots compared to control plant seedlings. In the stems and leaves, high levels of Cu and Zn were observed to be approximately 20 times higher than in control plants. The data allowed for calculation of the amount of accumulated Cu, Cd, Zn and Pb in the above-ground parts, which were 58%, 55%, 52% and 38% higher, respectively, than the amount in the roots. The results indicated that *B. juncea* is a good accumulator of trace metals, especially Cd.

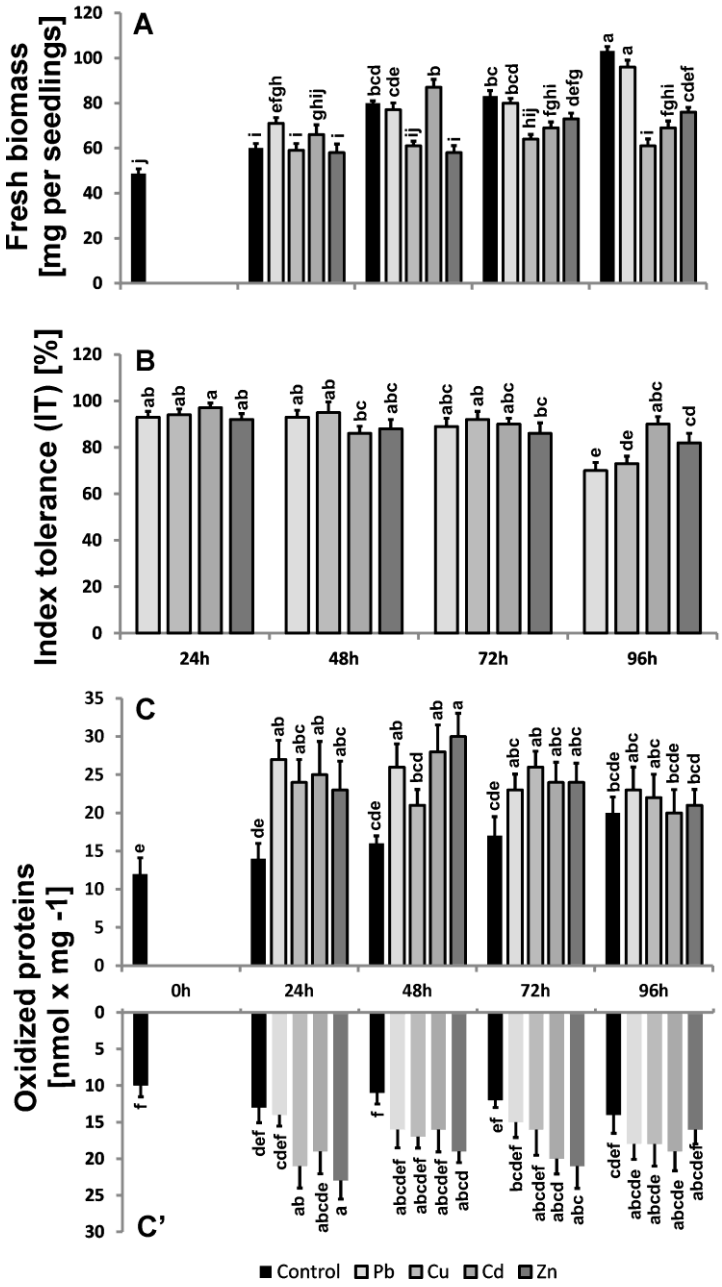
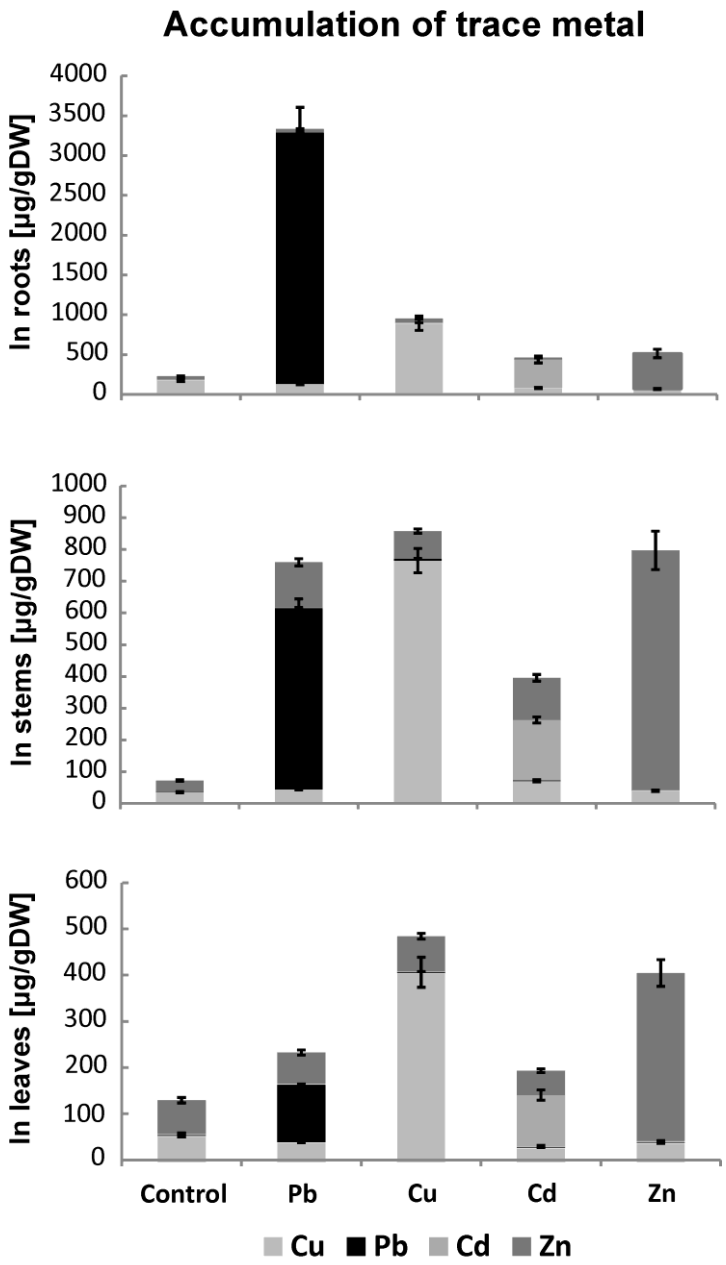


Figure 1. Accumulation of Pb, Cu, Cd and Zn in the roots, stems and leaves of *Brassica juncea* var. Malopolska seedlings grown in Hoagland's medium and treated with lead, cooper, cadmium and zinc ions. Metal solutions $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, and $ZnSO_4$ were applied at a 50 μM concentration. Mean values of three replicates (±SD).

2.2 Biomass and morphological changes

The metals used in the research did not dramatically increase *B. juncea* (v. Malopolska) seedling biomass (Fig. 1). The highest inhibition of biomass growth was observed for seedlings exposed to Cu. After 96 hours of treatment, the seedling biomass was approximately 34% lower than that of control plants. The weakest effect was observed for seedlings treated with Pb, as after 96 hours of treatment, the seedlings were approximately 10% lighter compared to control plants. The metals used in the study also did not appreciably inhibit the increase in root length. The value of the index of tolerance (IT), based on average root length, also did not change dramatically (Fig. 1). After 96 hours of treatment, we observed the lowest IT value for Pb (70%) and the highest IT value for Cd, i.e., 90,4%. We observed the occurrence of necrotic spots on leaves and the inhibition of leaf blade surface growth with respect to control seedlings in the above-ground parts of seedlings. Moreover, in Cd-treated seedlings, leaves were slightly twisted, whereas Cu caused strong chlorosis and shortening of the end of leaves. The smallest morphological changes were observed for seedlings treated with Zn.



111

112 **Figure. 2** Stress parameters in *Brassica juncea* seedlings treated with trace metals: Pb, Cu, Cd
113 and Zn. The results are expressed as the mean ± standard deviation (n=3). Metal solutions
114 $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, and $ZnSO_4$ were applied at a 50 µM concentration. Mean values of
115 three replicates (±SD).

116

117 *2.3 Production and localization of ROS*

The metal-treated seedlings increased $O_2^{\cdot-}$ production at levels comparable for shoots and roots compared to control seedlings, but the fluctuation in the production observed for control plants was maintained (Fig. 3). In the roots, the highest values were mainly observed in the first 72 hours (over 30%), whereas in the aboveground parts, the highest values were observed for 48 hours (over 30-40%). After 96 hours, the levels of $O_2^{\cdot-}$ decreased, which may indicate high activity of the SOD enzyme. The highest level of $O_2^{\cdot-}$ in roots was observed for plants treated with Zn compared with shoots treated with Zn and Cd.

The profile of the changes in the H_2O_2 level was similar for control roots and shoots, but the levels were distinctly higher in roots. The highest H_2O_2 amount was observed in roots treated with Cu, Cd and Zn. For metal-treated samples, a significant increase in H_2O_2 occurred between 48 and 72 hours of treatment, and the observed profile of H_2O_2 changes was more homogenous for shoots. We noticed a large difference in the level of H_2O_2 in roots after 96 hours of treatment, reaching approximately 20-50% higher compared to the control. As in the case of $O_2^{\cdot-}$, H_2O_2 levels were also confirmed by confocal microscopy (Fig. 4). The most intensive fluorescence DHE, indicating the presence of $O_2^{\cdot-}$, was observed for the *B. juncea* roots treated for 24 hours with 50 μM Cd and Zn. The highest amount of H_2O_2 generated was observed in roots treated with 50 μM Cu, Cd and Zn.

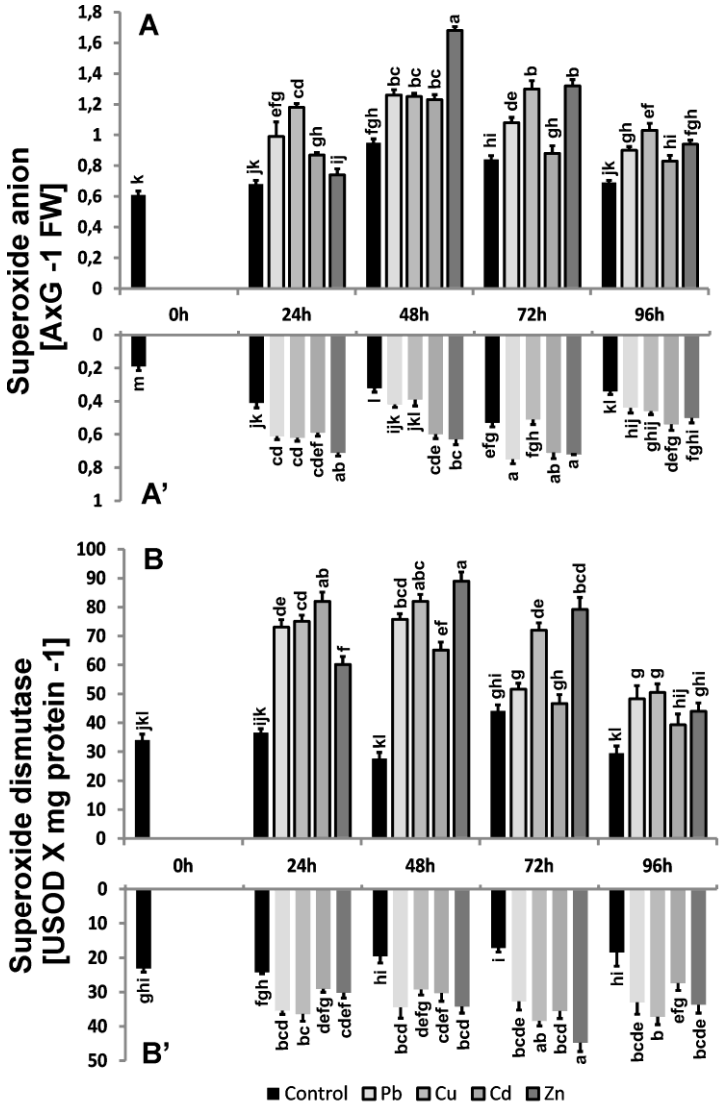


Figure 3. Superoxide anion (A580 g^{-1} FW) level and SOD (USOD mg^{-1} protein) activities in roots and above-ground parts of *B. juncea* var. Malopolska seedlings grown in Hoagland's medium and treated with lead, cooper, cadmium and zinc ions. Metal solutions $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, and $ZnSO_4$ were applied at a 50 μM concentration. Mean values of three replicates (±SD).

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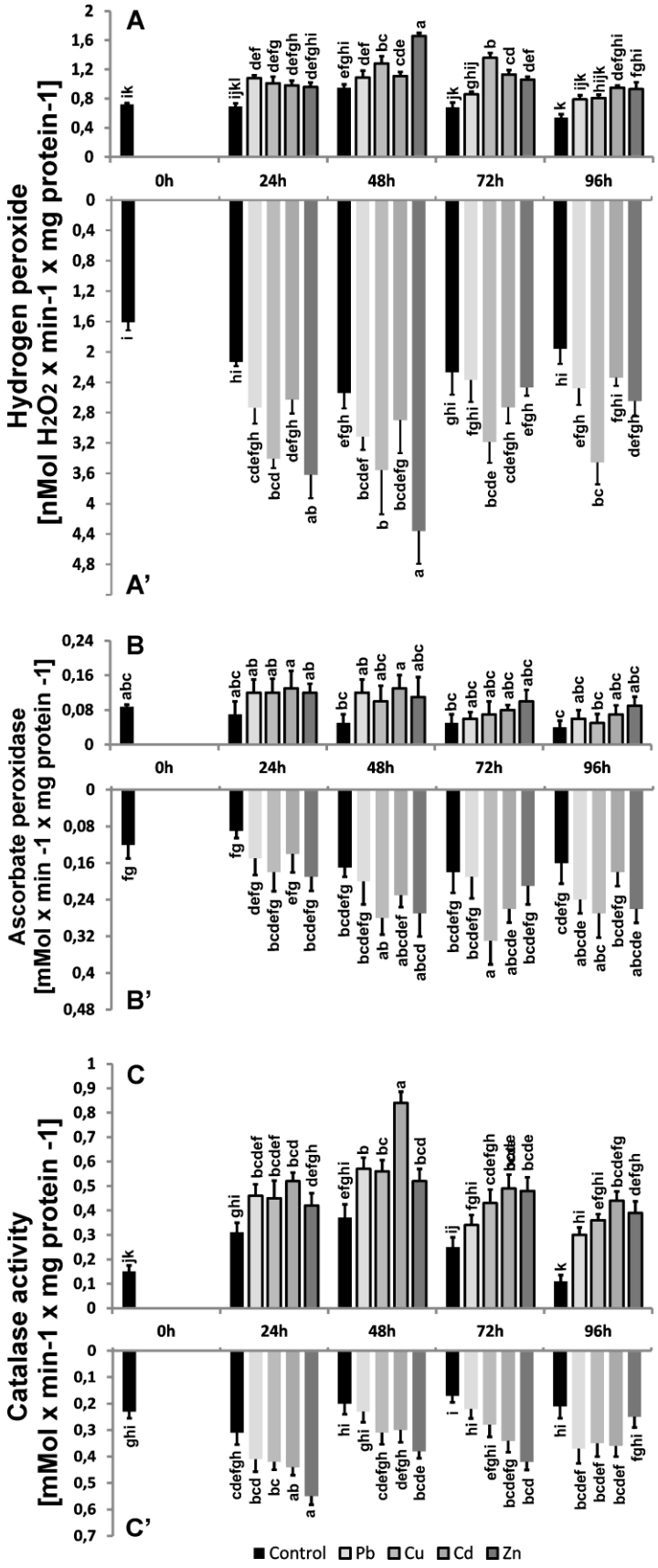
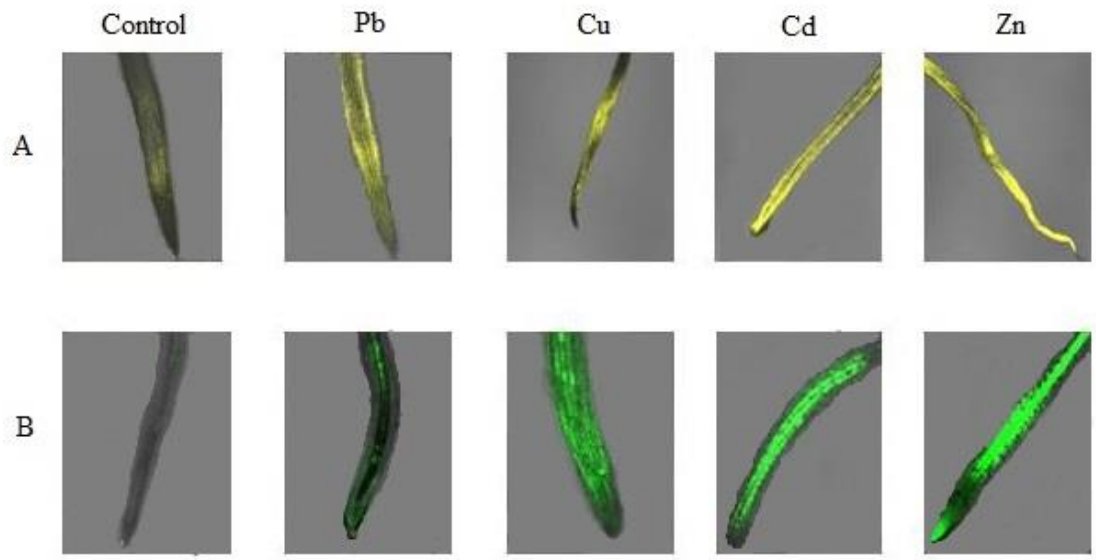


Figure 4. Hydrogen peroxide level (nMol H_2O_2 x min^{-1} x mg $protein^{-1}$, CAT (µmol min^{-1} mg^{-1} protein) and APX (µMol x min^{-1} x mg $protein^{-1}$) activities in roots and above-ground parts of

144 *B. juncea* var. Malopolska seedlings grown in Hoagland's medium and treated with lead, cooper,
145 cadmium and zinc ions. Metal solutions $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, and $ZnSO_4$ were applied at
146 a 50 μM concentration. Mean values of three replicates (±SD).
147



148
149 **Figure 5**. Trace metals induced $O_2^{-\bullet}$ and H_2O_2 production in *B. juncea* var. Malopolska roots.
150 Fluorescent images of *B. juncea* roots grown in Hoagland's medium in the presence of 50 μmol
151 of $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$ and $ZnSO_4$ for 24 hours and control roots of plants stained with
152 DHE for 12 h (A) and DCFH-DA for 4 h (B). The bar indicates 1 μm.
153
154 *2.4 Levels of oxidized proteins*
155 The levels of protein oxidative modification imposed by the metal treatment were 12 to 44%
156 higher for roots and above-ground parts compared to control plants (Fig. 1). The level of
157 oxidized proteins reached a maximum after 48 hours and was three-fold higher than in the
158 shoots of control plants.
159

160 *2.5 Enzyme antioxidant activity*

161 SOD activities were 25 to 50% higher in the roots of plants treated with trace metals. In the
162 aboveground parts, greater differences in SOD activity between research variants, ranging from
163 8 to 70%, were observed. However, the general activity of SOD was higher in roots and shoots
164 compared to control seedlings (Fig. 3) and changed differently for the seedling parts. In the case
165 of roots, the activity level and profile were comparable to those of control seedlings, whereas
166 for shoots, after the initial increase, the activity decreased significantly after 96 hours. The
167 generation of H_2O_2 caused a rapid increase in CAT activity within 24 hours of cultivation, i.e.,
168 from 30 to 70% in the roots of plants treated with trace metals, especially in plants treated with
169 Zn (Fig. 4). In the next days, we observed a slight decrease (approximately 12 to 55%), but this
170 decrease remained higher than that in control plants. The highest CAT activity was observed
171 above ground in the first 48 hours of cultivation (56%) in plants exposed to Cd. Activities of
172 APX, a second enzyme involved in the dismutation of hydrogen peroxide, systematically
173 increased in roots exposed to metals during the cultivation period, especially in plants grown in
174 the presence of Cu and Zn, which had approximately 10-43% higher levels than those observed
175 in the control (Fig. 4). In the aboveground parts of *B. juncea* cultured in the presence of trace
176 metals, we observed an increase in the intensity of APX during the first 48 hours, reaching a
177 maximum in plants treated with Cd for 48 hours, approximately 62% higher than in the control,
178 and then a slight decrease, but the activities were approximately two-fold higher than those in
179 control plants. The activity profiles of CAT and APX differed between the control roots and
180 shoots (Fig. 4). The metal treatment increased the activity of both enzymes, and the CAT
181 activity profile appeared to be maintained in roots and shoots. However, the APX profile did
182 not differ from that of the control plants with respect to treated shoots, whereas in treated roots,
183 the APX activity profile was variable and metal-dependent, although comparable for Cu and
184 Zn.

2.6 Levels of gene transcripts

To estimate possible changes at the level of CuZnSOD and MnSOD encoding gene transcripts, we used an electrophoretic separation technique and the CpAtlas programme (Fig. 6). In the case of CuZnSOD, a decrease in the expression of the gene encoding CuZn-SOD was observed in the roots of plants treated with trace metals after 4 and 24 hours of cultivation, with the exception of the roots of *B. juncea*-treated Cu. Induction of the gene in the aboveground parts was visible, with an approximate two-fold increase in the level of the transcript in plants after 8 hours of copper treatment and an approximate 2-fold decrease in plants after 4 hours of zinc treatment. The results indicate that the presence of cadmium ions had no significant effect on the induction of CuZnSOD gene expression because no significant changes in the level of the transcripts was observed in either the roots or above-ground parts of *B. juncea* plants.

When analysing changes in the expression of the gene encoding MnSOD, a decrease in the expression was observed in the roots and above-ground parts of plants after 4 hours of treatment with lead ions; in the remaining research variants, there were no significant differences in transcript levels compared to control plants. An approximate two-fold increase in the level of the transcript was found in plant roots after 24 hours of Pb and Zn treatment in comparison to the control. The greatest decrease in expression was observed after 24 hours in the aboveground parts of plants treated with Cu, which was almost fivefold higher than that in the control (Fig. 6).

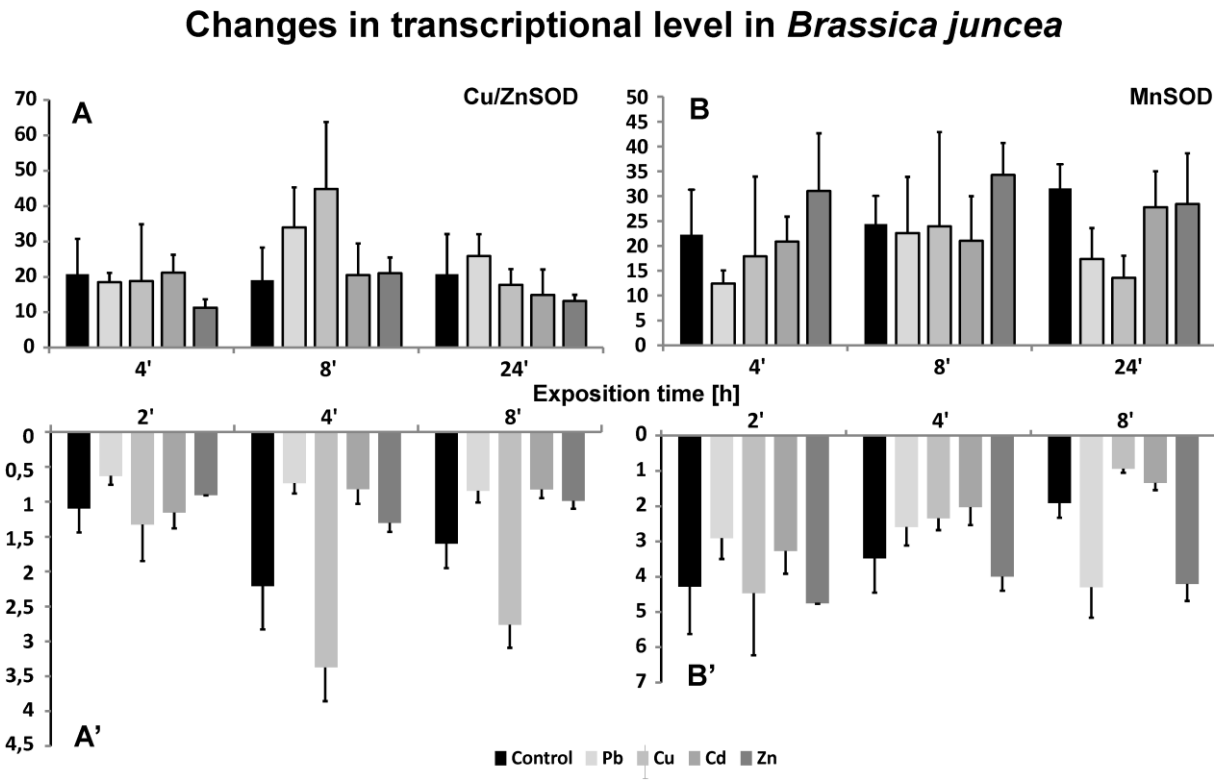


Figure 6. Transcriptional levels of genes encoding antioxidative enzymes in roots and above-ground parts of *B. juncea* var. Malopolska seedlings grown in Hoagland's medium and treated with lead, cooper, cadmium and zinc ions. Metal solutions $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, and $ZnSO_4$ were applied at a 50 μM concentration. Enzymes chosen for the experiment were amplified using semi-quantitative RT-PCR with primers designed for *A. thaliana* genes. CSD1 for CuZnSOD and *MSD1* for MnSOD.

2.7 Identification of enzyme forms

To distinguish between the enzyme forms, Western blot analysis was performed for protein extracts from roots and above-ground seedling parts in the absence and presence of the metal treatment (Fig. 7). This allowed for the detection of MnSOD (25 kDa) and CuZnSOD (15 and 20 kDa) subunits. The obtained signal was similar for both the treated and control seedlings. Thus, the metal presence likely did not change the levels of the CuZnSOD and MnSOD proteins.

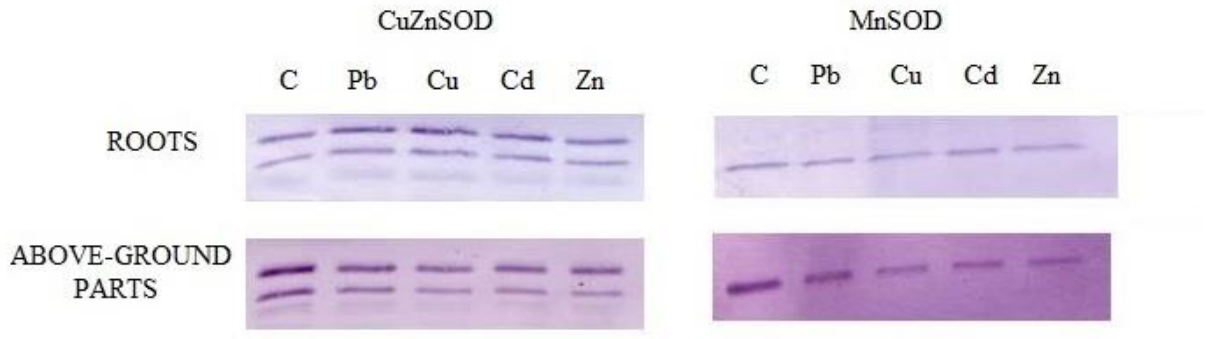


Figure 7. Effects of 50 μM Pb, Cu, Cd and Zn for 24 h on the CuZnSOD and MnSOD of roots and above-ground parts of *B. juncea* var. Malopolska seedlings. The protein content was evaluated by Western blot using specific antibodies.

3. Discussion

Trace metals are one of the most important abiotic stress factors affecting the natural environment. As a result of anthropogenic activities, we can observe their increasing levels from year to year. Metal toxicity results in effects at physiological and cellular levels, leading to distorted metabolism, including plant metabolism (Hossain et al., 2012). Abiotic stresses, including the presence of trace metals in soil, are estimated to be the main cause of global crop yield reduction of ca. 70% and thus are considered a great constraint to crop production. This situation has worsened due to disturbed equilibrium between crop production and human population growth. Therefore, it is especially important to understand plant responses to such stress factors. This also applies to trace metals (Singh et al. 2016). In the present study, this was clearly visible in the growth of plant biomass, which significantly decreased during the culture in the presence of heavy metals. Copper and zinc ions are essential for the normal growth and development of all organisms but can be toxic to plants at excessive levels. Lead and cadmium are nonessential elements and are toxic to plants even at low levels (Khan et al., 2015). Essential and nonessential trace elements, when exceeding the threshold limits, can cause different

physiological, morphological, and genetic plant anomalies, including reduced growth, mutations, and increased mortality (Khan et al., 2015). Therefore, plants suitable for phytoremediation are at present of great importance.

In our study, we noticed that in the case of *B. juncea* v. Malopolska, all the mentioned metals used at 50 μM concentration displayed moderate phytotoxic properties. The biomass increments ranged between 96 mg for Pb-treated plants and 61 mg for Cu-treated plants, and the values were approximately 7% and 41% lower, respectively, than those in control plants. Several studies have shown that high concentrations of trace metals in the soil cause plant growth impairment (Malecka et al., 2014; Bankaji et al., 2015). In *Sesbania drummondii*, a reduction in seedling biomass was caused by Pb -21%, Cu-46,3%, Ni-31,5% and Zn- 25,2% (Israr et al., 2011). The inhibition of shoot growth by trace metals may be due to a decrease in photosynthesis, as trace metals disturb mineral nutrition and water balance, change hormonal status, and affect membrane structure and permeability (Sharma and Dubey 2005). Trace metals might cause an inhibition of root growth that alters water balance and nutrient absorption (Singh et al., 2016) and decrease calcium uptake in root tips, leading to a decrease in cell division or cell elongation (Liu et al., 2009; Marshner 2012; Bankaji et al., 2015). According to Marshner (2012), Cd-induced mineral stress can reduce plant dry weight accumulation. Other authors have shown a negative influence of Pb (Zaier et al. 2010), Cu (Yadav et al., 2018), Cd (Irfan et al., 2014) and Zn (Israr et al., 2011). Despite the inhibitory effect caused by trace metals on the growth of the biomass of *B. juncea*, we observed a high IT amounting to approximately 90% resistance of the plants to trace metals.

The bioaccumulation of trace metals is different for various plant species, reflected by their growth, reproduction, occurrence, and survival in metal-contaminated soil because the mechanisms of elemental uptake by plants are not the same for all species. The capacity of plants to take up trace metals is different for different metals, and the same trace metal can be

accumulated at different ratios in different plant species (Singh et al. 2010b). Metal bioavailability is also affected by the presence of organic compounds of that metal in plants (Khan et al., 2015). The ICP-MS results we obtained indicate that the accumulation of trace metals was higher in above-ground parts than in roots, especially for cadmium, lead and zinc. The metal concentrations followed an order of Pb>Cu>Zn>Cd in roots, Zn>Cu>Pb>Cd in the stem and Zn>Cu>Cd>Pb in leaves (Kutrowska et al., 2017). Based on the obtained results, it can be concluded that *B. juncea* is a hyperaccumulator of Cd, Zn and Pb. Cherif and co-authors (2011) reported that Zn induced a decrease in Cd uptake and a simultaneous increase in Zn accumulation, indicating a strong competition between these two metals for the same membrane transporters. In our earlier study (Kutrowska et al., 2017) in *B. juncea* plants treated with a binary combination of metals, namely, PbCu, PbCd, PbZn, CuZn, CuCd and ZnCd, at a concentration of 25 μM of each, a synergistic response between Zn and Pb was observed, resulting in an increased accumulation of the two metals. The accumulation results obtained for plants treated with Cu are different from those of other researchers. Purakayastha and others (2008) showed that Cu is accumulated mainly in above-ground parts of *B. juncea*. This difference may result from different exposure durations of the plant to the metal, other metal concentrations and different plant ages at the time of analysis of the collected metal. Quaritacci et al. (2006) reported that *B. juncea* was identified as a species able to take up and accumulate metals in its above-ground parts, such as Cd, Cu, Ni, Zn, Pb and Se. It has been observed that this species concentrated Cu, Pb and Zn in its above-ground part in amounts much higher than those detected in the metal soluble fractions present in a soil contaminated by acidic water and pyritic slurry (Quaritacci et al., 2006).

The accumulation of trace metals in organs is dangerous for plants. In an earlier study (Hanc et al., 2016), we confirmed that plants are not adequately protected by the detoxification system

because trace metals penetrate in areas with high metabolic activity, such as the cytoplasm, mitochondria or cell membrane.

The occurrence of oxidation stress conditions in *B. juncea* treated with the trace metals Pb, Cu, Cd and Zn was confirmed by the increase in the level of oxidized proteins in the roots (approximately 7-12%) and aboveground parts (approximately 13%). Several metals, including Cd, Pb and Hg, have been shown to cause protein oxidation by depletion of protein thiol groups (Sharma et al., 2012). ROS cause protein modifications through the formation of carbonyl groups at certain amino acid residues. Such modifications were caused by the presence of heavy metals, e.g., cadmium (Romero-Puertas et al., 2002), mercury lead, aluminium, zinc, copper, cobalt, nickel, and chromium (Pena et al., 2006).

ROS also act as signalling molecules involved in the regulation of many key physiological processes, such as root hair growth, stomatal movement, cell growth and cell differentiation, when finely tuned and regulated by an antioxidative defence system (Singh et al., 2016). We showed an increase in the level of ROS compared to control plants in all plants treated with heavy metals. The $O_2^{\cdot -}$ rate after 2 hours of culture was 2 times higher than that observed in plants grown under control conditions. The high level of $O_2^{\cdot -}$ was the highest between 24 to 72 hours of the treatment depending on the research variant. The highest value of $O_2^{\cdot -}$ was measured in plants treated with Zn, while the highest H_2O_2 values were observed in plants treated with Cu and Cd. Similar results were obtained by other researchers. Markovska et al. (2009) showed a 10-fold higher level of H_2O_2 in the leaves of *B. juncea* after 5 days of treatment with Cd ions at a concentration of 50 μM. Wang et al. (2004) observed the highest levels of H_2O_2 in *B. juncea* roots treated with Cu ions for 4 days. In our research, the highest level of H_2O_2 was obtained after 4 days in plants treated with single metals. The reduction of $O_2^{\cdot -}$ and the H_2O_2 content in roots and above-ground parts of plants treated with trace metals during the cultivation period suggested that some antioxidative enzymes would work effectively in the

312 removal of ROS. To detect ROS in plant cells, we used incubation with fluorescent labels such
313 as 2'7'-difluoroscein and dihydroethidium and imaging under confocal microscopy. We
314 observed increased generation of $O_2^{\cdot-}$ and H_2O_2 in the roots of *B. juncea* treated with trace metals,
315 especially Cd, Zn (for $O_2^{\cdot-}$) and Cu, Cd and Zn (for H_2O_2).
316 The increase in ROS production in metal-treated plants was precisely associated with changes
317 in the activity of antioxidant enzymes. We always observed the induction of antioxidant enzyme
318 activity in *B. juncea* roots and leaves, although there were no significant differences between
319 the used metals. We observed increasing activity of antioxidant enzymes, i.e., 20-158% for
320 SOD, 15-147% for CAT, and 6-68% for APX. The highest activity of SOD in both roots and
321 shoots was observed in plants treated with Zn and Cu. The first line of defence against ROS-
322 mediated toxicity is through SOD, which catalyses the dismutation of superoxide anions to
323 H_2O_2 and O_2. The stimulation of SOD activity has also been reported in several plants exposed
324 to Pb, Cu, Cd, Zn, Ni and As ions (Israr et al., 2011; Malecka et al., 2012; Kanwar et al., 2015;
325 Yadaw et al., 2018). We noticed that in the roots of *B. juncea*, the most induced activity of CAT
326 was for Zn, compared with Cd in the above-ground parts. APX was definitely lower than
327 catalase, especially in the aboveground parts, which means that this enzyme complements CAT
328 catalytic activity. APX activity was significantly elevated in the metal-treated plants, which
329 suggests its role in the detoxification of H_2O_2. Enhanced CAT and APX activity has been
330 observed in various plant species after application of trace metals: Pb, Cu, Cd, Zn, Ni, and As
331 (Wang et al., 2009; Israr et al., 2011; Malecka et al., 2012; Kanwar et al., 2015, Yadaw et al.,
332 2018). APX may be responsible for controlling the levels of H_2O_2 as signal molecules, and the
333 CAT function is to remove large amounts of oxygen during oxidative stress. APX may be
334 responsible for controlling the levels of H_2O_2 as signal molecules, and the CAT function is to
335 remove large amounts of oxygen during oxidative stress (Pinto et al., 2009). Mohamed et al.
336 (2012) showed in *B. juncea* that the higher activity of antioxidant enzymes offers a greater

detoxification efficiency, which provides better plant resistance against trace metal-induced oxidative stress. Yadav and co-authors (2018) reported increases in the activities of antioxidant enzymes: SOD by 16,2%, DHAR - 27,58, GR- 35,74%, GST, GPX by 19,19% and APX by 42,75% in *B. juncea* plants treated with 0,0005 M Cu. The authors indicated that brassinostereoids can regulate the activity of the antioxidant system and help in scavenging overproduced ROS and can provide tolerance by inducing the expression of regulatory genes such as respiratory burst oxidase homologue, mitogen activated protein kinase-1, and mitogen-activated protein kinase 3, as well as activating genes involved in antioxidative defence and responses (Yadav et al., 2018). Other authors (Singh et al. 2016) have noted that brassinosteroids are a group of hormones that regulate ion uptake in plant cells and reduce trace metal accumulation in plants. An exogenous application of brassinosteroids is widely used to improve crop yield as well as stress tolerance in various plant species.

We previously demonstrated an increase in the activity of the antioxidant system at the physiological and biochemical levels. The next step was to determine whether trace metals influence the transcription level of genes encoding suitable defence proteins. ROS concentration at an appropriate level can promote plant development and reinforce resistance to stressors by modulating the expression of a set of genes and redox signalling pathways (Singh et al., 2016). In our research, we observed differences in the expression induction depending on the exposure time and the metal used. We observed an increase in the level of the gene coding for Cu, Zn-SOD in plants treated with copper, zinc and lead. The highest level of expression was obtained after 4 hours in roots and 8 hours in above-ground parts. Romero-Puertas and co-authors (2007) noted a drastic reduction in the expression of genes coding for CuZnSOD and no changes in MnSOD in *Pisum sativum* under conditions of stress caused by the presence of Cd. Their results showed a reduction in Cu and Zn-SOD levels in the presence of Cd, while in our study, we did not observe significant differences in the level of transcript for plants treated

with this metal in relation to control plants. We observed the induction of gene expression encoding Mn-SOD in *B. juncea* roots after 8 hours of exposure to Zn and Pb ions, compared with lead ions in above-ground parts. Other authors did not observe any changes or a low expression of genes coding for SOD, e.g., Fidlago et al. (2011) showed no differences in Mn-SOD-related mRNA accumulation in leaves and roots, but CuZn-SOD-related transcripts decreased in leaves but did not change in roots in Cd-treated *Solanum nigrum* L. Others authors (Lou et al., 2011) indicated that Cd stress induced an upregulated expression of FeSOD, MnSOD, Chl Cu/ZnSOD, Cyt Cu/ZnSOD, APX, GPX, GR and POD at 4–24 h after treatment began for *Lolium perenne* L., and their results suggested that the gene transcript profile was related to the enzyme activity under Cd stress. Romero-Puertas et al. (2007) indicated two groups of genes in pea plants treated with Cd. First, some elements of the signal transduction cascade accentuated or attenuated the Cd effect on CAT, MDHAR and CuZn-SOD mRNA expression. The second was formed by the genes Mn-SOD, APX, and GR that were not affected by these modulators during the Cd treatment because their expression was not modified compared to control plants.

The effect of Cd on the expression of CuZn-SOD was reversed by an $NO^{\cdot}$ scavenger, indicating that $NO^{\cdot}$ must be a key element in the regulation of this SOD, showing the existence of a relationship between an increase in ROS production and nitric oxide (NO). NO-dependent downregulation was also observed for Mn-SOD, while the opposite effect was found for APX and GR. This suggests that protein phosphorylation is involved in the response to Cd stress (Romero-Puertas et al., 2007). Bernard and co-authors (2015) indicate that molecular analysis (gene expression) is the first level of integration of environmental stressors, and it is supposed to respond to stressors earlier than biochemical markers.

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Our results from Western blotting indicate that the presence of trace metals does not increase the synthesis of the proteins CuZnSOD and MnSOD in the organs of *B. juncea* plants but induces an increase in their activity.

4. Materials and Methods

4.1 Plant material

Brassica juncea v. Malopolska seeds were grown in Petri dishes for 7 days under optimal conditions. Next, seedlings were cultivated hydroponically on Hoagland's medium for 7 days in a growth room with a 16/8 h photoperiod, day/night at room temperature and light intensity of 82 μmol m^2 s^{-1}. Then, the applied medium was changed into 100 x-diluted Hoagland's medium and a heavy metal solution in combination; Cu, Pb, Cd and Zn ions at a concentration of 50 μM were applied. In the cultivation, a solution of $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, Zn SO_4 was used. The roots and shoots were cut off after 0, 24, 48, 72, and 96 hours of cultivation. The roots were dipped sequentially in cold solutions of 10 mM $CaCl_2$ and 10 mM EDTA for 5 minutes each to eliminate trace elements adsorbed at the root surface. Then, roots and shoots were rinsed three times with distilled water, frozen in liquid nitrogen and stored at -80°C until molecular analysis.

4.2 Phytotoxic test

The index of tolerance (IT) was calculated according to Wilkins (1957):

$$IT = \frac{\text{average length of roots in tested solution}}{\text{average length of roots in control}} \times 100\%$$

The changes in fresh biomass of control plants and plants treated with metals were measured on a Radwag scale after 0, 24 28, 72 and 96 hours of cultivation.

4.3 Accumulation of trace metals

The determination of trace metal accumulation was performed using *ICP-MS (inductively coupled plasma mass spectrometry)* and laser ablation connected with *ICP-MS (LA-ICP-MS).* Plant material (roots, stems and leaves) was rinsed with distilled water, gently dried on blotting paper, weighed and dried at 70 ± 2 °C. The dried samples were mineralized in an MDS-2000 microwave digestor oven (CEM Corporation Matthews, NC, USA). A three-stage dilution was conducted in a closed system using 5 mL of 65% HNO_3. After mineralization, samples were transferred to 10 mL flasks filled with deionized water. An inductively coupled plasma mass spectrometer (ICP-MS) model Elan DRC II, (Perkin Elmer Sciex, Canada) was used to determine the concentration of elements in the mineralized plant tissues.

Plant roots, stems and leaves were collected after 72 hours of treatment for the analysis of metal distribution. Samples were cut into 3 mm long pieces and ablated along the pre-defined line across the cross-sections. Laser performance was optimized according to a detailed scheme (Hanć, Olszewska, and Baralkiewicz 2013) using a single variable method.

4.4 Superoxide anion determination

The superoxide anion content was determined according to Doke (1983). *B. juncea* roots (0.5 g) were placed in the test tubes that were filled with 7 mL of a mixture containing 50 mM phosphate buffer (pH 7.8), 0.05% NBT (nitro blue tetrazolium) and 10 mM of NaN_3. Next, the test tubes were incubated in the dark for 5 min, and then 2 mL of the solution was taken from the tubes, heated at 85°C for 10-15 minutes, cooled on ice for 5 min, and the absorbance was measured at 580 nm against the control.

4.5 Hydrogen peroxide content

The hydrogen peroxide content was determined using the method described by Patterson et al. (1984). The decrease in absorbance was measured at 508 nm. The reaction mixture contained 50 mM phosphate buffer (pH 8.4) and reagents, 0.6 mM 4-(-2 pyridylazo) resorcinol and 0.6

mM potassium-titanium oxalate (1:1). The corresponding concentration of H_2O_2 was determined against the standard curve of H_2O_2.

4.6. In situ detection of superoxide anion and hydrogen peroxide

The roots and shoots from plants exposed to metals for 24 hours were submerged for 12 hours in 100 µM of $CaCl_2$ containing 20 µM of dihydroethidium (DHE, pH 4.75; samples for superoxide anion radicals) or 4 µM dichlorodihydrofluorescein diacetate (DCFH-DA) (samples for hydrogen peroxide) in 5 mM dimethyl sulfoxide (DMSO). After rinsing with 100 µM of $CaCl_2$ or 50 mM phosphate buffer (pH 7.4), the roots and shoots were observed with a confocal microscope (Zeiss LSM 510, Axiovert 200 M, Jena, Germany) equipped with no. 10 filter set (excitation 450-490 nm, emission 520 nm or more).

4.7 Estimation of protein oxidation

For carbonyl quantification, the reaction with DNPH was used basically as described by Levine et al. (1994). For each determination, two replicates and their respective blanks were used. Roots and shoots (0.5 g) were incubated with isolation buffer containing 0.1 M Na-phosphate buffer, 0.2% (v/v) Triton X—100, 1 mM EDTA and 1 mM PMSF. After centrifugation at 13000 × *g* for 15 minutes, supernatants (200 μL) were mixed with 300 μL of 10 mM DNPH in 2 M HCl. The blank was incubated in 2 M HCl. After 1 h incubation at room temperature, proteins were precipitated with 10% (w/v) trichloroacetic acid (TCA), and the pellets were washed three times with 500 μL of ethanol/ethylacetate (1:1). The pellets were finally dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer (pH 2.3), and the absorption was measured at 370 nm. Protein recovery was estimated for each sample by measuring the absorption at 280 nm. The carbonyl content was calculated using the molar absorption coefficient for aliphatic hydrazones, 22 000 M^{-1} cm^{-1}.

4.8. Determination of antioxidant enzyme activities

458 The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction
459 of NBT, adopting the method of Beauchamp and Fridovich (1971). The reaction mixture
460 contained 13 μM riboflavin, 13 mM methionine, 63 μM NBT and 50 mM potassium phosphate
461 buffer (pH 7.8). Absorbance at 560 nm was then measured. One unit of SOD activity has been
462 defined as the amount of enzyme that causes a 50% decrease in the inhibition of NBT reduction.
463 The activity of CAT was determined by directly measuring the decomposition of H_2O_2 at 240
464 nm for 3 min as described by Aebi (1984) in 50 mM phosphate buffer (pH 7.0) containing 5
465 mM H_2O_2 and enzyme extract (Gałgańska et al., 2008 ABB). CAT activity was determined
466 using the extinction coefficient of 36 mM^{-1} cm^{-1} for H_2O_2. The activity of APX was assayed
467 using the method described by Nakano and Asada (1981) by monitoring the rate of ascorbate
468 oxidation at 290 nm (extinction coefficient of 2.9 mM^{-1} cm^{-1}) for 3 min. The reaction mixture
469 consisted of 25- 50 μL supernatant, 50 mM phosphate buffer (pH 7.0), 20 μM H_2O_2, 0.2 mM
470 ascorbate and 0.2 mM EDTA.

471 *4.9. Isolation of total RNA and RT-PCR*

472 Roots and aboveground parts (100 mg) of *B. juncea* plants in the presence of trace metals and
473 under control conditions were collected for total RNA isolation. The RNA was isolated with
474 TRIzol reagent and tested spectrophotometrically for purity at 260 and 280 nm. Then, RNA
475 was reverse-transcribed with oligo (dT) primers using the RevertAid Reverse Transcriptase Kit
476 (Thermo Science) after DNA was treated with DNase I (Thermo Science).
477 Primer pair sequences were as follows (forward/reverse, gene accession number):
478 gtgattgcttgcagggtttt/ cagaatacggaagcaaatgtca, X54844.1 (TUB1), ggagcaagtttggttccatt/
479 aaggttattcggccagattg, U30841.1 (MnSOD), gaacaatggtgaaggctgtg/ gtgaccacctttcccaagat
480 M63003.1 (Cu,Zn-SOD). As a reference gene, the gene encoding tubulin was used. PCRs were
481 performed with 30 (BJMnSOD) and 34 (BjCuZnSOD) cycles of denaturation, 95°C for 30 s;

annealing primers, 53°C for 30 s; and elongation, 72°C for 30 s using a 1:100 diluted cDNA template and REDAllegro*Taq* DNA Polymerase (Novazym).

PCR products were separated by electrophoresis on a 1,3% agarose gel with ethidium bromide in TBE (445 mM Tris-HCL; 445 mM boric acid; 10 mM EDTA; pH 8,0), visualized under UV light and photographed using the Photo Print 215SD V.99 Vilber Lourmat Set. CP Atlas 2.0 were used for densitometric analysis of relative gene expression.

4.10. Western blot

RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) was used to lyse cells for protein extraction. The protein concentrations were determined using the Bradford method, and 20 µg of each extract was loaded onto a 12% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gel. Separated proteins were transferred to polyvinylidene fluoride membrane (ImmobilonTM-P, Millipore) at 350 mA for 1 h using the Mini Trans-BlotCell (Bio-Rad). Membranes were blocked with 1% BSA and incubated with an antibody against Cu-ZnSOD at a final dilution of 1:2500. The secondary antibody, goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, St Louis, MO, USA), was used at a 1:3000 dilution to visualize protein bands by reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Sigma-Aldrich, St Louis, MO, USA/ CALBIOCHEMV.S. and Canada) as a substrate.

4.11 Protein quantification

Total soluble protein contents were determined according to the method of Bradford (1976) using the Bio-Rad assay kit with bovine serum albumin as a calibration standard.

4.12 Statistical analyses

Each experiment was performed in three biological and technical replicates. The mean values ± S.E. are given in the tables and figures. The data were analysed statistically using IBM SPSS

Statistics (Version 22 for Windows). Significant differences among treatments were analysed by one-way ANOVA, taking $p< 0.05$ as the significance threshold, and the b-Tukey post hoc test was conducted for pairwise comparisons between treatments.

4. Conclusion

This study was conducted to determine the interactive role of Pb, Cu, Cd and Zn in metal uptake, plant growth and the antioxidative system of *B. juncea*. Plants accumulated high amounts of trace metals, i.e., more than 40% in the roots, and in the above-ground parts, the values for Cu, Cd, Zn, and Pb were 58%, 55%, 52%, and 38%, respectively. The results suggest that *B. juncea* var. Malopolska is a good hyperaccumulator of trace metals, especially Cu, Cd and Zn, and can be useful in phytoremediation. The presence of metals resulted in a considerable reduction in *B. juncea* biomass; the highest reduction was observed in plants treated with Cu and Cd. Despite the visible influence of trace metals on plant morphology, the IT coefficient was high and exceeded 90%, indicating the high resistance of *B. juncea* plants. Trace metals lead to the production of ROS, which causes an imbalance in the redox state in the plant cells and increases the level of oxidized proteins. We noticed that under the conditions of oxidative stress, the antioxidant system was activated: SOD, CAT and APX. We observed that the presence of metals influenced the increase in the activity of antioxidant enzymes, while no significant differences were observed in the levels of CuZnSOD and MnSOD transcripts and proteins. The results obtained indicate that *B. juncea* var. Malopolska has efficient defence mechanisms to cope with different metals.

Conflicts of Interest: The authors declare that they have no conflict of interests

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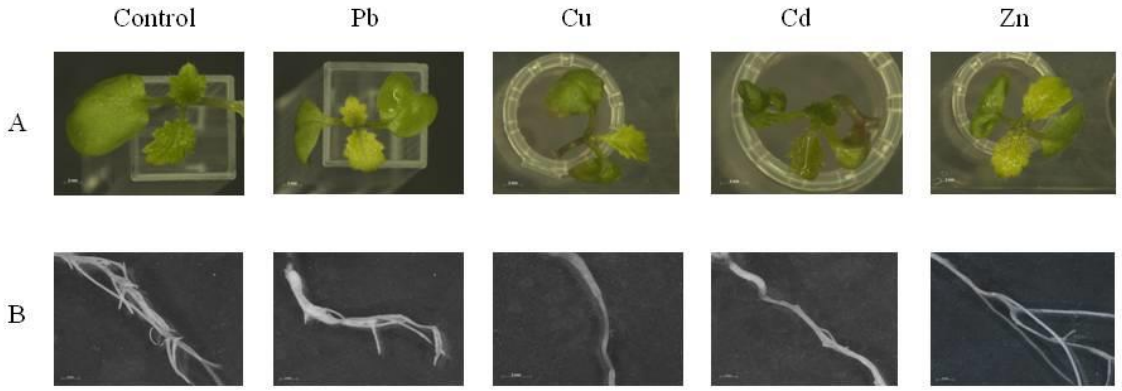

Fig.8. Morphological changes of *Brassica juncea* roots and leaves exposed to Cu, Pb, Cd and Zn metals at 50 μMol concentration for 48 hours using Zeiss stereoscopic microscope.

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