PHYSIOLOGICAL RESPONSES TO CADMIUM, NICKEL AND THEIR INTERACTION IN THE SEEDLINGS OF TWO MAIZE (*ZEAMAYS* L.) CULTIVARS

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In the leaves of maize seedlings, cultivars Premia and Blitz, the relatively low 2 µmol/L concentration of cadmium (Cd), nickel (Ni), or both metals acting simultaneously (Cd + Ni) for 72 h, induced a significant metal accumulation, decrease in total K⁺ content, reduction of light-induced membrane electrical potential (E_M) repolarisation in mesophyll cells and changes of ascorbic acid (AsA), dehydroascorbic acid (DHA) and glutathione (GSH) content. Shoot growth and the values of resting E_M did not change significantly. Increased K⁺ leakage, from the leaves, and lipid peroxidation accompanied by increase of TBA-reactive substances (TBARS) were found only in cv. Blitz exposed to Cd + Ni. This indicates a capability of high leaf-cell anti-oxidant pool to ameliorate the toxic effects on plasma membrane of single ions in both cultivars, and of Cd + Ni only in cv. Premia. The decreased total content of K⁺ in leaves in all variants indicated repressing the K⁺ uptake and/or distribution to the shoots. Under anoxia, the magnitude of the repolarisation obtained after switching on the light was smaller in Cd-treated cultivar Premia than in the controls, and this also occurred in Ni- and Cd + Ni-treated cultivar Blitz.

Key words: maize leaves, heavy metals (Cd, Ni), plasma membrane integrity, photoelectric response, antioxidants

Heavy metals (HMs) such as cadmium (Cd) and nickel (Ni) are naturally occurring in the earth's crust. Cd is a non-essential element that negatively affects plants (Tran & Popova 2013) while Ni in low concentrations fulfils a variety of essential roles in plants as a constituent of several metallo-enzymes, such as urease (Ahmad & Ashraf 2011). However, uptake of higher quantities of Ni may turn toxic to plants (Seregin & Kozhevnikova 2008). Due to anthropogenic and industrial activities, high soil concentrations of Cd and Ni can occur simultaneously.

In agriculture, this may be caused by using sewage sludge, decayed and composted wastes to enhance soil fertility (Alegria *et al.* 1991; Chopra *et al.* 2009) or by industrial waste water irrigation (Sharma *et al.* 2007).

Various physiological processes are impaired by Cd²⁺ and by excess Ni²⁺ resulting not only in common but also in specific symptoms of metal toxicity. Briefly, the common toxic effects are primarily manifested as plant growth inhibition, morphological alterations, decrease in biomass production,

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membrane distortion leading to decrease of essential mineral nutrients, changes in nitrogen metabolism, disturbance of water status, production of toxic metabolites and reactive oxygen species (ROS). These may react with lipids, proteins, pigments, and nucleic acids and initiate lipid peroxidation. Ultimately, all of these altered processes result in reduced yields of agricultural crops (Tran & Popova 2013; Ahmad & Ashraf 2011).

The plasma membrane (PM) of living cells represents the major barrier between cytoplasm and environment and is the first functional site of contact of the cells with any ion. In fact, PM contains potential Cd- and Ni-sensitive enzyme systems, such as PM $H^+ATPase$ (Ros *et al.* 1992). An increase in permeability related to membrane damage was observed in plants subjected to Cd and Ni stress (Sanz *et al.* 2009; Fiala *et al.* 2015), resulting in disruption of membrane integrity and ionic homeostasis of cells. On the other hand, ROS generated by Cd and Ni are generally reported to increase lipid peroxidation in plants (Wu *et al.* 2003; Gajewska *et al.* 2012). However, as soon as any HM penetrates the cells, antioxidative endogenous defence mechanisms are stimulated that can remove, neutralise or scavenge oxygen radicals and their intermediates by antioxidant enzymes or non-protein thiol groups, especially GSH and AsA (Pandey *et al.* 2009).

The most common symptoms of Cd and Ni toxicity are structural and functional alterations in photosynthetic apparatus due to reduced uptake of essential elements (Maximović *et al.* 2007). Previous studies also showed that photosystem II (PSII) was extremely sensitive to Cd and Ni, and its function was inhibited to a much greater extent than that of PSI (Ghasemi *et al.* 2012; Duan *et al.* 2010). It seems that there are no data available, concerning the action of Cd and Ni on E_M with regard to dark/light signals in terrestrial plants. Some results on these parameters are known from the cells of maize leaves treated with lead (Pazurkiewicz-Kocot & Pietruszka 2000) and of macroalga *Nitellopsis* treated with cadmium and lead (Kurtyka *et al.* 2011).

Most of our knowledge is based on the effects of Cd and Ni acting as individual ions on plethora of plant physiological processes. However, under natural environments, the plants must cope with combined action of several HMs. Differential re-

sponses particularly due to metal ion interactions were shown after Cd, Ni and their joint application in maize roots (Artiushenko *et al.* 2012) and in elodea leaves (Maleva *et al.* 2012) while no substantial interactions were found with regard to phytochelatins biosynthesis in tobacco cells (Nakazawa *et al.* 2001) or transport processes and transpiration in Ni hyperaccumulator *Leptoplax emarginata* (Bartoli *et al.* 2012).

The goal of this study was to extend our knowledge on the responses of leaf cell E_M and plasma membrane integrity, to relatively low 2 $\mu\text{mol/L}$ concentration of Cd and Ni acting as single ions as well as both metals together, and the role of antioxidants AsA and GSH in stress response of two commercially available maize cultivars Premia and Blitz.

MATERIAL AND METHODS

Plant material and growth conditions

Two Ukrainian maize (*Zea mays* L.) cultivars Premia FAO 190 and Blitz FAO 160 available commercially (<http://maize.com.ua/catalog>) were used. The seeds were soaked in running water for 1 h, surface sterilised with 5% NaClO for 10 min, washed for 10 min and germinated in moistened filter paper rolls at 25°C for 72 h. The seedlings with the first leaf were exposed to aerated hydroponic treatments of distilled water (control), and 2 $\mu\text{mol/L}$ concentrations of Cd^{2+} ($3 \text{ CdSO}_4 \times 8 \text{ H}_2\text{O}$) or Ni^{2+} ($\text{NiSO}_4 \times 6 \text{ H}_2\text{O}$) or to their combination for 72 h at laboratory conditions. Control and metal solution pH was adjusted to 5.7. The relatively low metal concentrations were selected as they reduced but not completely stopped the root growth (Artiushenko *et al.* 2012). To obtain dry matter values, the leaves of the control and treated plants were washed thoroughly, surface-dried with filter paper, dried at 70°C for 72 h and weighed.

Determination of Cd and Ni contents

The leaves of the seedlings were dried at 80°C and used for Cd and Ni contents determination according to Angelova *et al.* (2004). The HMs concentration was determined using atomic absorption spectrophotometer S-115U, SelmiOAO, (Ukraine) and related to the fresh weight (Borzou & Azizinezhad 2012).

Electrophysiological measurements

In the segments excised from the first leaf of the 72-h-treated seedlings, the E_M was measured using standard microelectrode technique (Pavlovkin *et al.* 1986). After rinsing the leaf segments with 0.5 mmol/L CaSO_4 , they were mounted in 4 mL volume Plexiglas chamber and were constantly perfused (10 mL/min) with a bathing solution containing 0.2 mmol/L KCl, 1 mmol/L NaH_2PO_4 , 0.2 mmol/L $\text{Ca}(\text{NO}_3)_2$ and 0.5 mmol/L MgSO_4 (control medium) adjusted to pH 5.7 with 0.1 mol/L HCl. The HM-containing bathing solutions consisted of the control medium supplemented with 2 $\mu\text{mol/L}$ concentrations of Cd, Ni, or both metal ions together. The E_M was then measured using micropipettes filled with 3 mol/L KCl. The tip diameter was 0.5 μm , tip potential -5 to -15 mV. Micromanipulator was used to insert micropipettes into single leaf mesophyll cells under microscope control.

To register the photoelectric changes of E_M , the leaf segments were illuminated with a light intensity of 120 $\mu\text{W/cm}$ for 4 h and then the leaf cell responses to dark or light were continuously recorded for 30–60 min. All experiments were carried out at 22°C. For E_M measurements in the dark, a green filter was placed in front of the light source during micropipette impalement of the cells.

Potassium determination

For potassium determination, the plants were cultivated in the solution without HMs (control) or supplemented with 2 $\mu\text{mol/L}$ concentrations of Cd, Ni and Cd + Ni for 72 h. The leaf segments (1 g) were rinsed briefly with 0.5 mmol/L CaSO_4 , and the K^+ was extracted by boiling the tissue in distilled water for 10 min. Alternatively, K^+ leakage was analysed from leaf segments treated for 72 h in the medium containing HMs at the concentration of 2 $\mu\text{mol/L}$ and the contents of K^+ were measured in the leaf surrounding media. The K^+ concentrations in both cases were determined with an ion-meter CyberScan 2100 (Eutech Instr. PTE Ltd., Singapore) and were related to fresh weight.

Content of thiobarbituric acid-reactive substances (TBARS)

The content of TBARS was measured according to Hodges *et al.* (1999). The tissue homogenate prepared with 0.1 mol/L potassium phosphate buffer

containing 0.25 mol/L sucrose was centrifuged at 8,000g for 20 min. To 0.5 mL of the supernatant 2 mL H_2O , 1 mL of 17% trichloroacetic acid and 1 mL of 0.8% thiobarbituric acid were added. Then the mixture was heated in a boiling water bath for 20 min. Immediately after incubation, the mixture was cooled down in an ice bath. Absorbance at 532 nm wavelength was read in the supernatant against a blank.

Extraction and analysis of AsA and DHA

Leaves (1 g) were homogenised in 5% metaphosphoric acid at 4°C. The homogenate was centrifuged at 18,000g and the supernatant was then used. The AsA and DHA contents were determined according to Kampfenkel *et al.* (1995) with modifications by de Pinto *et al.* (1999).

Content of GSH

The glutathione pool was assayed according to previously described methods of Beutler *et al.* (1963) modified by Gryshko and Syshchykov (2002) and the optical density was measured at 412 nm using spectrophotometer SF-2000 UV/Vis (OKB Spectr, Russia).

Statistical analysis

Data were analysed using one-way ANOVA with $P < 0.05$ or 0.01 (Prism 5, GraphPad Software Inc., USA). Means and standard deviations were calculated from three independent experiments ($n = 3$ –30 leaf segments). Each experiment was repeated at least three times.

RESULTS AND DISCUSSION

Dry matter accumulation

Dry weight accumulation was slightly higher in the control and treated leaves of the cultivar Blitz than in Premia. However, there were not statistically significant differences between control and metal-treated seedlings (data not shown). In contrast to roots (Martinka, personal communication), the dry weight of the leaves was not significantly affected by metal treatments (Table 1). Likewise, unaffected dry matter accumulation was found also in the rice shoots exposed to Cd or Ni (Rubio *et al.* 1994). This might be explained by the lower metal concentra-

tions in shoots compared to roots as has been reported for maize (Maksimović *et al.* 2007) and by higher level of antioxidants in leaves than in roots (Jozefczak *et al.* 2015), which can better protect the leaves from damage.

Metal accumulation

Both maize cultivars accumulated Cd and Ni in their leaves when the metals were present in the root medium during 72 h (Figure 1). As could be expected, Cd or Ni concentrations in the leaves corresponded with the presence of the individual metal in the root medium. Comparing the cultivars exposed to Cd or Ni alone as well as to both metals together, significantly greater amounts of the respective metals were accumulated in Premia than in Blitz. Within each cultivar, the exposure to combined Cd + Ni treatment lowered the accumulation of either Cd or Ni comparing to their concentrations when the respective metals were administered separately. This could indicate antagonistic relations manifested in decreasing the metal entry into the cells ow-

ing to competition between Cd and Ni as suggested for maize roots (Jozefczak *et al.* 2015) and *Elodea* leaves (Maleva *et al.* 2012). The amounts of Cd and Ni accumulated in leaves were substantially lower than the concentrations detected in the roots of the same maize cultivars (Artiushenko *et al.* 2012). However, the tendencies of accumulating more Ni than Cd as well as the antagonistic relations were similar in both plant parts.

E_M

Cd and Ni have been shown to inhibit photosynthesis, namely photosystem II activity and affect the structure and function of protoplasmic membranes (Maksimović *et al.* 2007; Duan *et al.* 2010; Ghase-mi *et al.* 2012). However, most of the studies on the effects of HMs on photosynthesis have been carried out on isolated chloroplasts and little information is presently available on their effect on E_M and photoelectric reaction of leaf parenchyma cells. In this study, we, for the first time, report on the effects

T a b l e 1

Shoot dry weight of Premia and Blitz seedlings grown in the root media containing distilled water (Control), 2 $\mu\text{mol/L}$ Cd^{2+} , 2 $\mu\text{mol/L}$ Ni^{2+} , or 2 $\mu\text{mol/L}$ Cd^{2+} + 2 $\mu\text{mol/L}$ Ni^{2+} for 72 h. (Mean weights in $\text{mg} \pm \text{SD}$; the same letters indicate no statistically significant differences at $P < 0.05$).

Treatments	Leaf dry weight [mg]			
	Control	Cd	Ni	Cd + Ni
Premia	16.92 ± 1.923^a	17.31 ± 1.846^a	15.58 ± 1.154^a	15.19 ± 1.923^a
Blitz	21.35 ± 2.5^b	21.92 ± 2.885^b	17.69 ± 2.5^{ab}	21.54 ± 2.673^b

T a b l e 2

Concentration of TBARS, increase of K^+ concentration in the surrounding medium leaked from the leaf segments, expressed as % of control (dash = no increase), and K^+ content in the leaf segments of maize seedlings, cv. Premia and cv. Blitz, control and treated with 2 $\mu\text{mol/L}$ Cd, 2 $\mu\text{mol/L}$ Ni and 2 $\mu\text{mol/L}$ Cd + 2 $\mu\text{mol/L}$ Ni for 72 h. Values are means $\pm$ SD ($n = 3$). * = values significantly different from control.

	TBARS [μM TBA mg^{-1} protein]		Increase of K^+ concentration [% of control]		K^+ content [μM K^+ g^{-1} FW]	
	Premia	Blitz	Premia	Blitz	Premia	Blitz
Control	2.52 ± 0.341	2.23 ± 0.096	—	—	28.2 ± 3.1	26.1 ± 3.2
Cd	2.60 ± 0.126	2.48 ± 0.153	—	—	$20.8 \pm 2.9^*$	$21.5 \pm 2.6^*$
Ni	2.07 ± 0.154	1.89 ± 0.161	—	1.2 ± 1.1	$22.7 \pm 2.2^*$	$20.2 \pm 2.5^*$
Cd + Ni	2.70 ± 0.282	$2.50 \pm 0.05^*$	—	$9.7 \pm 3.1^*$	$23.9 \pm 2.6^*$	$23.1 \pm 2.4^*$

Figure 1. Accumulation of Cd and Ni in the shoots of Premia and Blitz seedlings grown in the root media containing 2 $\mu\text{mol/L}$ Cd^{2+} , 2 $\mu\text{mol/L}$ Ni^{2+} , or 2 $\mu\text{mol/L}$ Cd^{2+} + 2 $\mu\text{mol/L}$ Ni^{2+} for 72 h. (Mean amounts in $\mu\text{g/g}$ fresh weight with bars representing standard deviations; the same letters indicate no statistically significant differences at $P < 0.05$).

of Cd, Ni and Cd + Ni on photoelectric reaction in maize leaf mesophyll cells.

Generally, the dark/light signals cause an electrical reaction in plant cells, depending on plant species, cell type and physiological status of the cells (Ullrich-Eberius *et al.* 1983; Shabala & Newman 1999; Pazurkiewicz & Pietruszka 2000). In maize leaf segments of 6-day-old seedlings, control and treated with HMs for 72 h, the E_M values in mesophyll cells under light and dark conditions were -174 ± 7.1 mV ($n = 20$) in Premia and -173 ± 7.1 mV ($n = 16$) in Blitz. Upon switching the light off (D) and on (L) in control seedlings, only transient depolarisation and hyperpolarisation, respectively, occurred in the leaves (Figure 2, control trace), similarly as in the leaves of majority of higher plant species (Pavlovkin *et al.* 1986). In the seedlings undergoing HM treatments for 72 h, the pattern and velocity of E_M changes upon switching the light off (D) and on (L) showed differences, depending on both metal and maize cultivar (Figure 2). The treatment with Cd enhanced the dark-induced E_M depolarisation, the magnitude of which was double of that in control leaf cells in both cultivars. After switching the light on, namely within the first 30 min, the pattern of E_M was altered in both cultivars. The phase of hyperpolarisation of E_M in Premia was much more reduced in comparison to control and Blitz. Comparing to control, Ni had no effect on either pattern or magnitude of E_M changes in Premia while in Ni-treated cv.

Blitz, the magnitude of dark-induced depolarisation enlarged two-fold and, after switching the light on, the E_M increased only slightly (Figure 2, Ni). The dark-induced depolarisations were greater after Cd + Ni treatment in comparison to single Cd or Ni in both cultivars and, the change was more conspicuous in Blitz in comparison to Premia (Figure 2, Cd + Ni). However, in plants treated by Cd + Ni, the E_M values after repolarisation, in any case, did not reach the E_M values recorded after repolarisation in plants treated with single metals (Figure 2). This clearly indicated a synergic effect of the metals on plasma membrane responses. The combined Cd + Ni treatment enhanced the dark-induced E_M depolarisation 2.5 times compared to control in both cultivars and the repolarisation was reduced particularly in Blitz. Switching the light on only weakly increased the E_M within 30–60 min (Figure 2, Cd + Ni). However, it is important to mention that following further 6 h, the E_M values recorded either in the dark or in the light returned to the control values (data not shown). This E_M recovery in light probably resulted in compensation of ATP source for H^+ -ATPase from oxidative phosphorylation, rather than from photophosphorylation. However, considering single metal effects on the repolarisation, Cd was more effective in Premia and Ni in Blitz.

To determine which energy sources contributed to the maintenance of E_M in maize leaf mesophyll cells in the light, i.e. mitochondrial phosphorylation, or photophosphorylation, we applied anoxia (N_2) in another set of experiments. Perfusion with N_2 -saturated solution alone in the light caused transient membrane depolarisation of a few millivolts (3.1 ± 1.1 mV, $n = 7$, mean $\pm$ SD) in control and HM-treated cells (data not shown). N_2 in the dark depolarised the E_M to the values between -62 and -73 mV (-69 ± 4.9 mV, $n = 13$, mean $\pm$ SD) in both cultivars in control as well as HM-treated cells (due to inhibition of oxidative phosphorylation). After depolarisation by N_2 in the dark, the E_M in control cells of both cultivars recovered completely within 15–20 min following the change from N_2 /dark to N_2 /light (Figure 3, control trace) (as a result of energy supply from photophosphorylation). Similar velocity was recorded also upon switching from N_2 to air in both control and HM-treated plants (indicating the energy supply from oxidative phosphorylation).

The E_M recovered completely within 15–30 min in both cultivars (data not shown).

In HM-treated plants, under N_2 /light, the E_M repolarised similarly but not as completely as in control, within the first 30 min, in dependence of metal treatment and cultivar (Figure 3). Following 30 min, the magnitude of E_M repolarisation was the highest in Ni-treated, lower in Cd- and Cd + Ni-treated Premia. In the leaf cells of Blitz, the magnitude of repolarisation was similar after Cd or Ni treatments and the lowest after Cd + Ni treatment. Comparing the cultivars, Premia seems to be more sensitive to Cd, and Blitz to Ni. After 180 min under N_2 /light, the values of E_M repolarisation were not statistically different from those in control regardless of the HM treatment (data not shown).

Taken together, the inhibition of membrane repolarisation in maize leaf cells treated with Cd, Ni or both together, cannot be considered as the more

serious HM stress symptoms under sustained depression of the driving force energising PM-ATPase (Kurtyka *et al.* 2011) because the E_M repolarised spontaneously after 3 h following switching on the N_2 /light, and N_2 /air conditions.

These results could be interpreted by assuming that HMs only transiently inhibited photophosphorylation but had no effect on oxidative phosphorylation in maize leaf cells. We suggest that the inhibition of repolarisation was due to an effect of metals on PS II system (Duan *et al.* 2010), as a result of a short-term insufficient ATP synthesis needed for standard activity of plasma membrane H^+ -ATPase, which maintains the E_M (Ullrich-Eberius *et al.* 1983).

TBARS content, K^+ efflux and K^+ content

Another symptom of membrane damage induced by Cd and Ni is an increase in membrane perme-

Figure 2. Transient E_M changes in leaf mesophyll cell (representative traces of five to seven measurements) caused by transition from light to dark (D) and dark to light (L) in control and after 72 h exposure to $2 \mu\text{mol/L Cd}^{2+}$, $2 \mu\text{mol/L Ni}^{2+}$, or $2 \mu\text{mol/L Cd}^{2+} + 2 \mu\text{mol/L Ni}^{2+}$ in the leaf media.

Figure 3. Responses of maize leaf mesophyll cell E_M (representative traces of four to nine measurements) undergoing anoxia (N) in dark, and after switching light on (L) in control and after 72 h exposure to 2 $\mu\text{mol/L}$ Cd^{2+} , 2 $\mu\text{mol/L}$ Ni^{2+} , or 2 $\mu\text{mol/L}$ Cd^{2+} + 2 $\mu\text{mol/L}$ Ni^{2+} in the leaf media.

ability with a subsequent disturbance of the cellular ionic balance, recorded as an increased K^+ efflux or conductivity HM-treated plants (Wu *et al.* 2003; Sanz *et al.* 2009; Fiala *et al.* 2015). The presence of 2 $\mu\text{mol/L}$ concentrations of the HMs in the experimental solutions did not enhance K^+ leakage from the Premia leaf segments expressed as percent of total content in the surrounding media within 72 h. Such a response was significant only in the cultivar Blitz after the treatment with Cd + Ni (Table 2) and it was reported to be associated with metal-induced lipid peroxidation in rice (Gajewska *et al.* 2012). The complete absence of K^+ leakage from the Premia leaves and from Blitz leaves treated with Cd and Ni confirms that the Premia leaf cells were able to cope with the elevated metal concentrations better in comparison to Blitz. Our results differ from those on roots of the same maize cultivars treated with high concentration of Cd, Ni and Cd + Ni (Fi-

ala *et al.* 2015), where the membrane permeability increased in all metal treatments. These differences can be explained by using high concentrations of the metals (10 μM and 100 μM) for treatments of the roots and by different sensitivity of root cells to the studied metals in comparison with the sensitivity of mesophyll leaf cells.

On the other hand, measurement of TBARS, a common product of lipid peroxidation, is regarded as a reliable method for evaluating the degree of lipoperoxidation, which may be caused by metal-induced changes in the composition of the membrane lipids and the saturation of fatty acids (Gajewska *et al.* 2012). In our experiments, the levels of TBARS in the leaves were not markedly changed, with the exception of the seedlings of maize cultivar Blitz subjected to combined Cd and Ni stress. In this variant, the contents of TBARS exceeded the control significantly (Table 2). This is in accordance with simultaneous increase in membrane permeability and TBARS content in wheat seedlings treated with Ni (Gajewska *et al.* 2012). The difference between the cultivars was probably due to differences in the ratio of unsaturated and saturated fatty acids as has previously been shown in *Silene* plants tolerant and susceptible to copper (DeVos *et al.* 1993).

In addition to the above-mentioned direct effects of Cd and Ni on the PM integrity, these metals as divalent cations may compete with other essential mineral nutrients in their transport across membranes. Our results on the decrease of K^+ content in maize leaves also confirmed such competition. In the seedlings grown with roots exposed to Cd, Ni, and Cd + Ni for 72 h, the total K^+ content decreased in the leaf tissues in both cultivars (Table 2). Similar results were found by Gabbrielli *et al.* (1999) who showed that in shoots and roots of pea plants, the toxic concentrations of Ni lowered K^+ content but did not cause an increase in K^+ efflux. The authors suggest that the effect could be due to a decrease in K^+ uptake rather than by direct effect on membrane permeability. Moreover, Rubio *et al.* (1994) showed that Cd and Ni treatments also induced a decrease in K^+ , Ca^{2+} and Mg^{2+} content in rice plants, particularly in the shoots, indicating that Cd and Ni interfered not only with nutrient uptake but also with nutrient distribution into the different plant parts. In our set of experiments, we found an antagonism of metal

ions in relation to K^+ content, which was higher under joint Cd + Ni than under individual metal exposure. This resembles the competitive interactions restricting the entry of HMs into the *Elodea* leaf cells (Maleva *et al.* 2012).

AsA and DHA

In plants, antioxidative responses are largely based on the use and recycling of both AsA and

GSH, complemented by superoxide dismutase and catalase. Both AsA/DHA and GSH/GSSG are major cellular redox buffers. Changes in the ratios of the reduced and oxidised forms reflect cellular toxicity and have been associated with redox signalling (reviewed by Anjum *et al.* 2014). AsA is able to directly neutralise oxygen radicals and recover tocopherol, thus protecting the membranes. Ascorbate also

Figure 4. Contents of AsA and its oxidised forms (DHA) in the leaf tissues of maize cultivars Premia and Blitz in control and after 72 h exposure to 2 $\mu\text{mol/L}$ Cd^{2+} , 2 $\mu\text{mol/L}$ Ni^{2+} , or 2 $\mu\text{mol/L}$ Cd^{2+} + 2 $\mu\text{mol/L}$ Ni^{2+} in the root media. (Means $\pm$ SD; the same letters indicate no statistically significant differences at $P < 0.05$).

Figure 5. Contents of total glutathione (GSH) in the leaf tissues of maize cultivars Premia and Blitz in control and after 72 h exposure to 2 $\mu\text{mol/L}$ Cd^{2+} , 2 $\mu\text{mol/L}$ Ni^{2+} , or 2 $\mu\text{mol/L}$ Cd^{2+} + 2 $\mu\text{mol/L}$ Ni^{2+} in the root media. (Means $\pm$ SD; the same letters indicate no statistically significant differences at $P < 0.05$).

functions as co-substrate of plant oxidases, such as ascorbate peroxidase system, which produces dehydroascorbic acid. HMs induce changes in the AsA/DHA ratio, depending on plant species and metal stress conditions. Therefore, to understand the contribution of the non-enzymatic antioxidants in response of maize seedlings to Cd and Ni toxicity, we examined leaf concentrations of AsA, DHA and GSH.

In the leaves of *Premia* seedlings exposed to cadmium and nickel single or combined, the amounts of AsA decreased and DHA increased significantly (Figure 4). One of the causes might be neutralising free radicals by oxidation of AsA. Decreasing amount of AsA caused by Cd stress was noted in leaves of *Pisum sativum* (Romero-Puertas *et al.* 2007) and *Oryza sativa* (Chao *et al.* 2010). In the leaves of cultivar Blitz, the AsA content increased only under the exposure to single nickel (Figure 4). The single metal impact might cause activation of de novo AsA synthesis necessary for the removal of free radical reactions in the leaves. As a result of these processes, the amount of oxidised forms of ascorbate increased (Figure 4). With the combined action of Cd + Ni in both variants, there is a tendency of decreasing AsA amount while increasing that of its oxidised forms. This, in our opinion, can be explained by a decrease of AsA pool resulting from the intensification of its utilisation in hindering lipid oxidation by free radicals. Our results are in agreement with the findings of other authors, e.g. decreasing amount of AsA in the roots of *Kandelia candel* and *Bruguiera gymnorhiza* treated with heavy metal mixture (Cd^{2+} , Pb^{2+} and Hg^{2+}) in the concentration of 1.5 mg/L (Huang *et al.* 2010). The cultivar Blitz seems to be more sensitive to Ni, whereas the hybrid *Premia* to Cd ions. The results show that AsA in cultivar Blitz may play a more important role in the fight against heavy metal-induced oxidative damage than it does in the cultivar *Premia*.

GSH

GSH is not only a central compound in sulphur metabolism but also a major component of the cellular antioxidative defence system against HM toxicity. GSH acts as disulphide reductant to protect thiol (–SH) groups on enzymes, regenerates AsA and reacts with singlet oxygen and hydroxyl radi-

cals. A number of reports are available regarding the depletion of GSH pool in Cd- and Ni-exposed plants (Sharma & Dhiman 2013).

The concentration of GSH in the leaf tissues varied in dependence of maize cultivar as well as of metal treatment (Figure 5). In control conditions, the concentration of GSH was significantly higher in the leaf tissues of *Premia* than of Blitz. Differences in GSH content were found in other species, e.g. the leaves and roots of *Arabidopsis thaliana* plants exposed to Cd (Jozefczak *et al.* 2015), in which the wild type responded with statistically higher content of GSH in comparison to the GSH-deficient mutants. Compared to control, the Cd treatment decreased GSH content in *Premia* and increased in Blitz. The Ni treatment had an opposite effect, resulting in increase of the GSH content in *Premia* and decrease in Blitz. The joint Cd + Ni treatment had no effect on GSH level in *Premia* but increased it in Blitz (Figure 5). According to these data, the cultivar Blitz seemed to respond more sensitively to Ni ions. In this case, there were no changes of GSH content in *Premia* synthesis and the pool of another antioxidant available in cells was used. The increase in GSH concentration induced by Cd in Blitz could be caused by active functioning of enzymatic system of its reutilisation. Based on the changes in GSH content, the cultivar *Premia* reveals a higher sensitivity to cadmium as the concentration of GSH decreased in Cd-treated or remained unchanged in all other variants. Most likely, in *Premia*, the obtained data can testify a primary functioning of other antioxidant systems (in particular ascorbate-dependent) for neutralisation of the negative influence of the metals. Similar results were found in rice seedlings tolerant and sensitive to Cd, in which the content of GSH decreased in the leaves of Cd-sensitive cultivar but not in the Cd-tolerant cultivar (Chao *et al.* 2011), or in the roots of the same maize cultivars (Artiushenko *et al.* 2014).

CONCLUSIONS

Our results suggest that in maize plants subjected to 2 $\mu\text{mol/L}$ Cd^{2+} , Ni^{2+} or both metals together, antioxidants (ASA, DHA and GSH) were activated, and different responses between cultivars and

metals were also observed. Concerning the metals, the interaction of Cd and Ni revealed antagonistic relations in their accumulation in the leaf tissues, while synergic effect of the metals was manifested in greater dark-induced depolarisation and light-induced repolarisation after Cd + Ni treatment than after the single ions. Concerning the cultivars, both of them revealed the tendency of accumulating more Ni than Cd while they differed in the metal-induced changes of antioxidant contents.

Generally, the data obtained from both cultivars appear to support the concept that controlling the capacity of antioxidants in different maize cultivars should be an integral part of the evaluation and quantification of the effects of Cd²⁺ and Ni²⁺ stress on plants. Moreover, it is supposed that the observed decrease in K⁺ uptake but not direct effect on membrane permeability and E_M in HM-treated plants can be considered as stress symptoms. On the other hand, the addition of the second metal to the root medium alleviated the detrimental action of individual ions owing to the enhanced K⁺ content.

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