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THE EFFECT OF CADMIUM-NICKEL INTERACTIONS ON SUPEROXIDE PRODUCTION, CELL VIABILITY AND MEMBRANE POTENTIAL (E_M) IN ROOTS OF TWO MAIZE CULTIVARS

Roderik Fiala,[1] Vladimír Repka,[1] Milada Čiamporová,[1]
Michal Martinka[1,2] and Ján Pavlovkin[1]*

[1]Institute of Botany, Slovak Academy of Sciences,
Dúbravská cesta 9, SK-84523 Bratislava, Slovak Republic
[2]Department of Plant Physiology, Faculty of Natural Sciences, Comenius University,
Mlynská dolina B-2, SK-84215 Bratislava, Slovak Republic

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Effects of $CdCl_2$, $NiCl_2$ or both on superoxide production, viability and membrane potential (E_M) of root cells in meristematic (MZ) and differentiation (DZ) zones of two maize cultivars (cv. Premia and cv. Blitz) were studied. Plants were supplied with 10 and 100 μM concentrations of heavy metals (HM). The responses in the studied parameters to HM were concentration- and time-dependent, and were found only in the cells of MZ. The treatment of roots with Cd-stimulated massive superoxide production, although to different extent depending on the cultivar, root zone, and metal concentration. The stimulating effect of Ni on oxidative burst in Cd-treated maize roots was related to an increased Cd-induced superoxide production. The cell death appeared between 24 and 48 h and between 12 and 24 h of the 10 μM and 100 μM metal treatments, respectively. This was in accordance with Cd-induced ROS (superoxide) production and the E_M decline in the corresponding time periods. Cell viability, E_M changes and partially superoxide production indicate that the impact of the metals on the studied parameters declined in the order Cd+Ni > Cd > Ni and that cv. Blitz tends to respond more sensitively than cv. Premia.

Keywords: Cd and Ni – superoxide production – cell viability – membrane potential (E_M)

INTRODUCTION

The presence of elevated concentrations of cadmium (Cd) and nickel (Ni) in the soil is one of the major causes of limiting crop production. A number of physiological processes are impaired by Cd and by excess Ni resulting not only in common but also in specific symptoms of metal toxicity [6, 34]. Ultimately, all of these altered processes result in reduced yields of agricultural crops when they encounter excessive Cd and Ni concentrations [34].

Cd is a toxic, non-essential metal rapidly taken up by roots and accumulated in various plant tissues [34], which also hampers the crop growth and productivity worldwide [15]. This metal disrupts the uptake of macro and micro elements [16] and carbohydrate metabolism leading to chlorosis and photosynthetic rate reduction [24].

*Corresponding author; e-mail adresses: jan.pavlovkin@savba.sk; janpav@azet.sk

Numerous data also indicate that Cd induces accumulation of reactive oxygen species [31] affecting various cellular processes, mainly the functioning of membrane systems [17]. Cd is a non-redox metal unable to perform single electron transfer reactions and does not produce reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet -}$), but generates oxidative stress by interfering with the antioxidant defence system [4]. Interestingly, the occurrence of ROS has also been observed in plants treated with HM belonging to redox inactive metals, such as Cd and Ni [20, 32]. $O_2^{\bullet -}$ plays a crucial role because it can easily arise as a by-product from many electron transport processes and can directly inhibit or modify some proteins [5]. Several publications showed that the decreased antioxidant capacity of cells, especially during a long term exposure to Cd, was responsible for ROS accumulation [28].

Ni, in low concentrations, fulfils a variety of essential roles in plants. It is a constituent of several metallo-enzymes [14]. Therefore, Ni deficiencies in plants reduce urease activity, disturb N assimilation and reduce scavenging of the superoxide free radical [11]. On the other hand, high Ni concentration retards growth, decreases biomass production, induces leaf spotting, and produces Fe deficiency that leads to chlorosis and foliar necrosis. Additionally, excess of Ni also affects nutrient absorption by roots, impairs plant metabolism and inhibits photosynthesis and transpiration and many other processes [1].

Among other detrimental effects of Cd and Ni, direct effects on plasma membrane properties have been reported. Thus, alterations of the membrane permeability (increasing efflux rates) or of E_M, and hence a decrease of ion influx rates, may explain the metal effects on lowered nutrient uptake. There are several reports on the effect of Cd and Ni on these parameters, but they have been studied separately and in different plants [2, 22, 23, 27, 30]. These metals, in short term experiments, depolarized cell membranes and it has been suggested that this effect could be involved in metal toxicity.

The tolerance limits for Cd and Ni toxicity are specific for every species [10] and even for varieties or cultivars of cultural plants [18, 19]. The results reported by various authors differ and are rather contradictory, as the nature of HM effect depends also on the age of plants, on plant organs, tissue fractions, in addition to metal concentrations, stress duration, physical and chemical properties of contaminants.

Plant cultivation in contaminated soils is problematic due to accumulation of these metals in cereals. Evidence of the phytotoxic effects of Cd and Ni, or both metals together prompted us previously to investigate their short-term effects on maize seedlings [9]. These investigations were focused on the effects of Cd and Ni on root tip cell plasma membranes [i.e. respiration, membrane potential (E_M) and membrane conductivity]. Here we extend our knowledge on the longer-term effects of both metals acting individually or together on the apical part of the roots of the same maize cultivars, with new data on superoxide production, viability and E_M, of the cells in MZ and DZ root zones.

MATERIALS AND METHODS

Plant material and growth conditions

Two maize cultivars, Premia FAO 190 and Blitz FAO 160 grown in Ukraine were used in our experiments. Seeds were surface-sterilized with sodium hypochlorite (1% available chlorine) for 2 min and rinsed three times in sterile distilled water for 2 min. The seeds germinated in the rolls of moistened filter paper in the thermostat in the dark at 21 °C for 3 days. The 3-day-old seedlings were used for experiments. All physiological parameters were analyzed in the cells of MZ located < 1 mm, and DZ 5 to 10 mm from the root tip. Cell viability was detected also in the root cap cells up to 0.5 mm from the root tip.

Superoxide production

To monitor real time superoxide production we used Superoxide Detection Kit (Enzo Life Sciences, USA). Roots of the seedlings were exposed to 100 μmol/L $CdCl_2$ and/or 5 mmol/L $NiCl_2$ in a solution containing 0.1 mmol/L KCl and 0.1 mmol/L $CaCl_2$ adjusted to pH 5.5 for 3, 8, 16 or 24 h. Then apical 5 mm long primary root segments were stained for 15 min, in 1 × Wash buffer (included in the detection kit) containing 4 μL of Superoxide Detection Reagent (Orange) in final dilution 1 : 2500, briefly washed and observed in the confocal laser scanning microscope (Olympus FV1000, Japan). The excitation wavelength was 543 nm and the emission was detected using 560 to 660 nm barrier emission filter. The relative superoxide levels were calculated based on the fluorescence intensities (softwares Fluoview FV1000 ver. 3.0, Olympus, Japan and Excel 2003, Microsoft, USA).

Cell viability test

To counterstain cell walls and nuclei of ruptured cells [25] the fluorescent dye propidium iodide (PI, Fluka, Switzerland) was used. The roots were treated with 10 or 100 μmol/L Cd and/or Ni for 0 to 48 h. Apical primary root segments (5 mm) were stained in 10 μg/mL PI for 4 min, washed in distilled water for 2 min and investigated in the confocal microscope. The dye was excited at 488 nm and the fluorescence was detected using 560 to 660 nm barrier filter.

Electrophysiological measurements

Measurements of E_M were performed on single outer cortical cells of the 25 mm long apical primary root segments using standard microelectrode techniques [27]. After rinsing the roots with 0.5 mmol/L $CaSO_4$, the roots were mounted in a 4 mL volume

plexiglass chamber and were constantly perfused (5 mL min^{-1}) with bathing solution containing 0.1 mmol/L KCl, 0.1 mmol/L $CaCl_2$ adjusted to pH 5.5 using 0.1 mmol/L HCl and 0.01 mmol/L or 0.1 mmol/L $CdCl_2$, $NiCl_2$ or the same concentrations together (Cd+Ni measurements). However, since the measurements were performed in a zone of active growth, it was not possible to keep the electrode impaled into the same cell for longer time. Therefore, plants were treated with the metals (10 and 100 µmol/L) for several hours (until 48 h) and then their E_M was measured at 21 °C.

Statistics

Data were analyzed using 3-way ANOVA with $P<0.05$ or 0.01 (Prism 5, GraphPad Software Inc.). Means and SD were calculated from three independent experiments.

RESULTS

Production of superoxide

Control cells remained unstained or just with very weak fluorescence in the course of the experiment (Fig. 1A). After metal treatments the membranes, nuclei and/or cytoplasm became stained orange indicating elevated superoxide levels. Upon metal treatment for 16 h the superoxide production was above the control level in both cultivars. The same maximum levels of superoxide production were recorded in Cd-treated roots of both cultivars after 16 or 24 h exposure (Fig. 1A). When Ni or Cd+Ni treatments were applied the fluorescence of the root cells of both cultivars reached its peak after 16 h with a slight (Premia) or considerable (Blitz) decline after 24 h (Fig. 1A). When comparing the treatments, the strongest superoxide production was induced by Cd after 24 h and Cd+Ni after 16 h exposures. The Blitz seedlings treated with Cd or Ni for 16 h exhibited higher superoxide levels compared to those of Premia. Because Ni and Cd+Ni treatment gave very similar fluorescent images only Cd+Ni pictures are presented for simplification.

The relative superoxide levels are shown in Fig. 1B. As for Premia, there were no significant differences ($P<0.05$) in the relative superoxide production either among the metal treatments or the different time points. The relative superoxide production in Blitz revealed a more complex pattern. Comparative data analysis revealed significant differences ($P<0.05$) among the respective metal treatments in Blitz after 16 h. In Blitz the most potent inductors of superoxide production were Cd+Ni and Cd after 16 and 24 h, respectively. This response was also time-dependent.

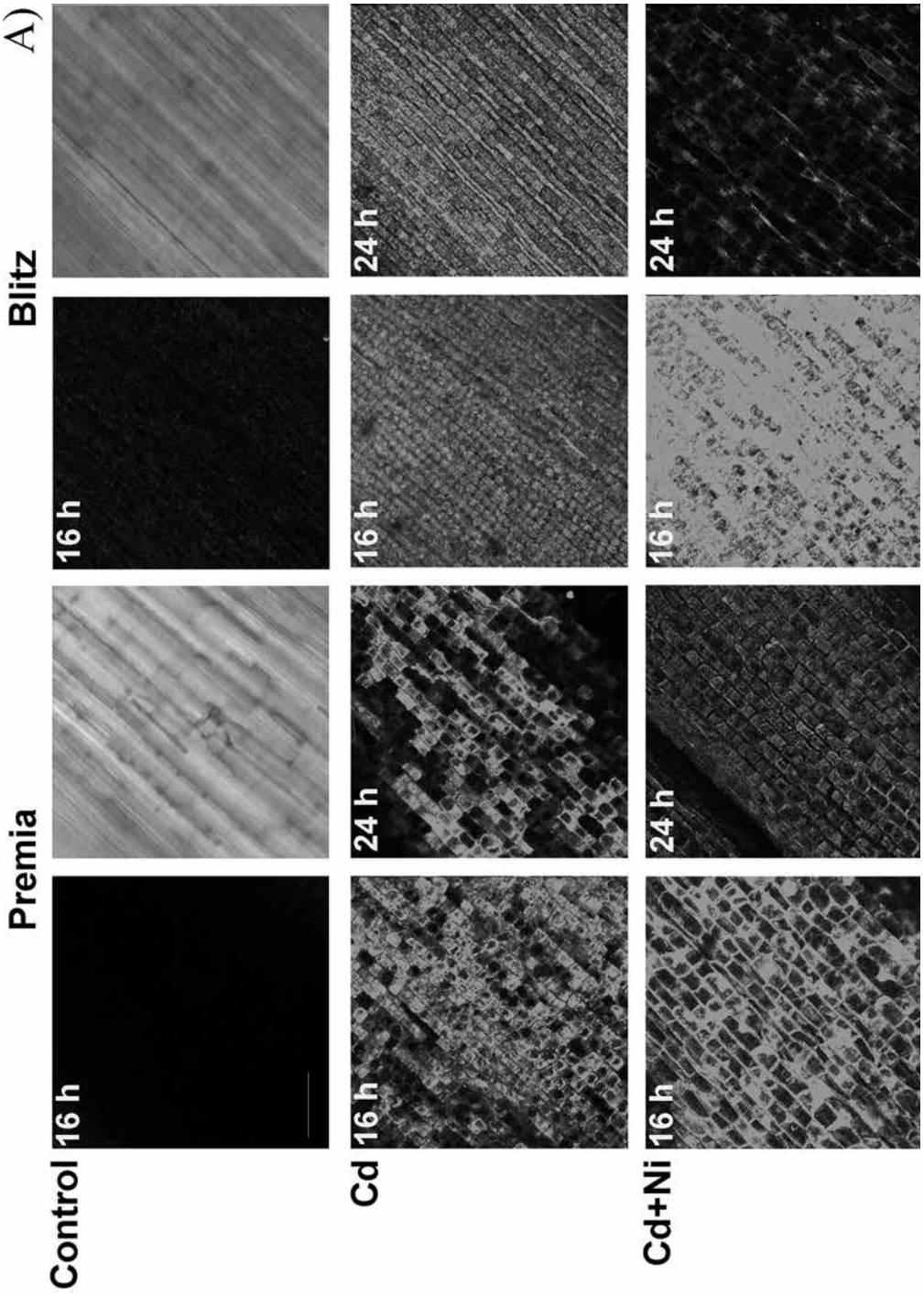


Fig. 1 (continued on p. 197)

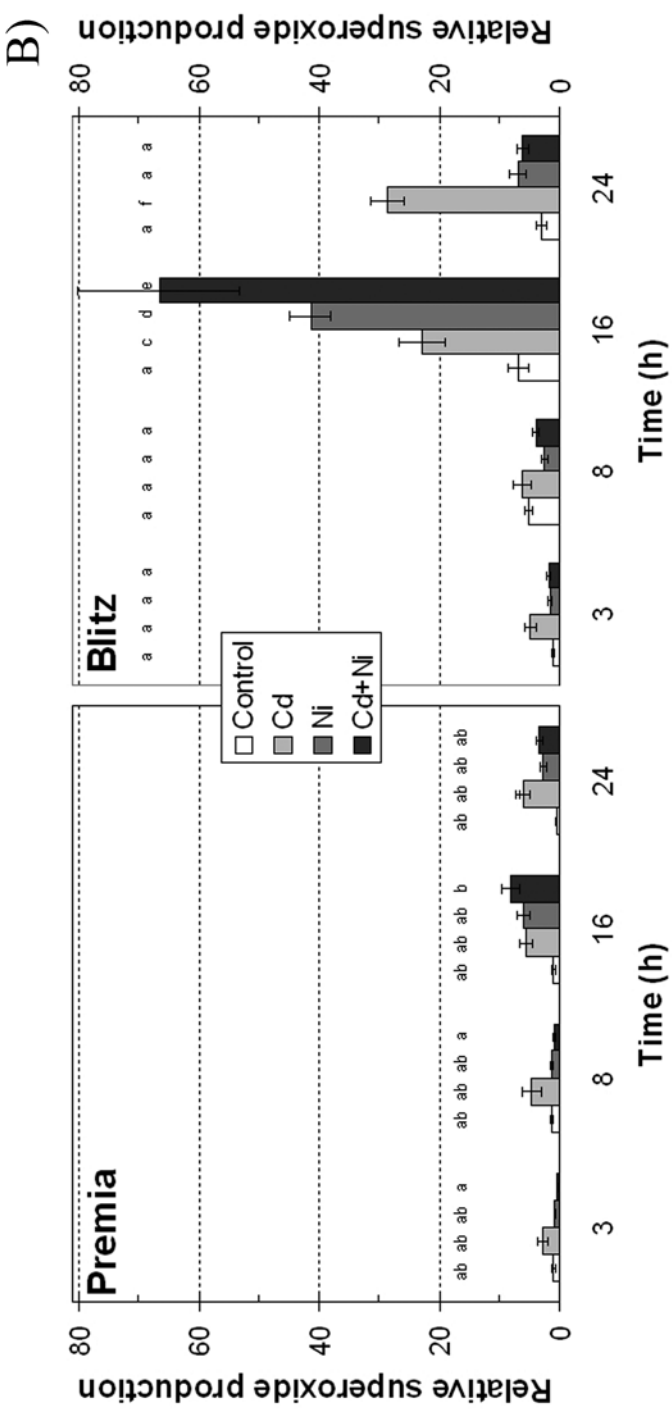
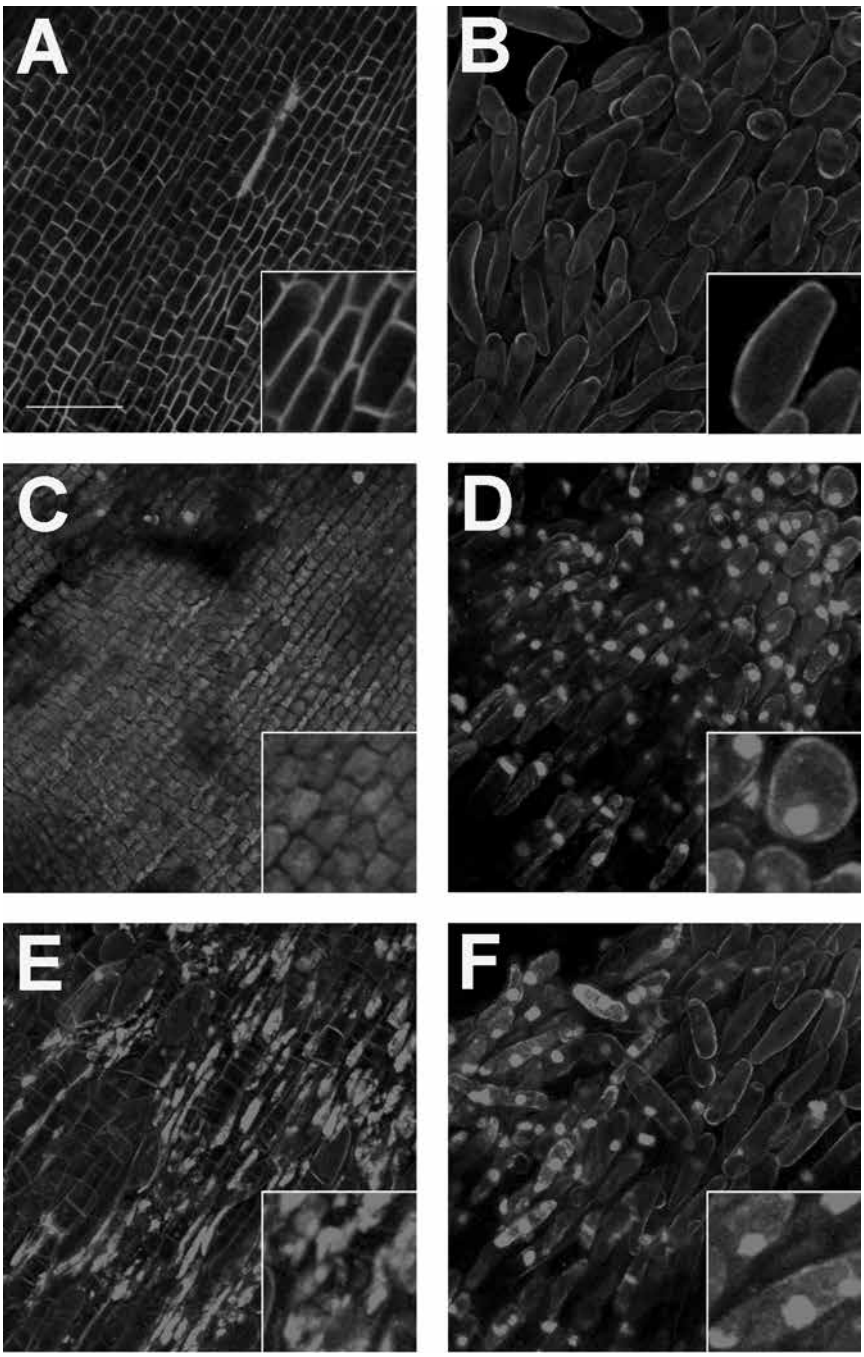


Fig. 1. Superoxide production detected with fluorescent probe in the root tip cells of maize influenced by Cd and Cd+Ni. Appropriate treatments, cultivars and exposure times are indicated. A) Representative images of five roots per treatment. The staining pattern upon Ni treatment was the same as Cd+Ni (not shown). Fluorescence intensities of membranes, nuclei and/or cytoplasm corresponding to SO levels can be compared directly, except Cd+Ni, Blitz, 16 h, where the original intensity was higher. Bar = 30 µm. B) Relative SO levels calculated based on the fluorescence intensities (mean±SD, n = 5–11). Statistically significant differences ($P<0.05$) are indicated by different small letters (a–f)



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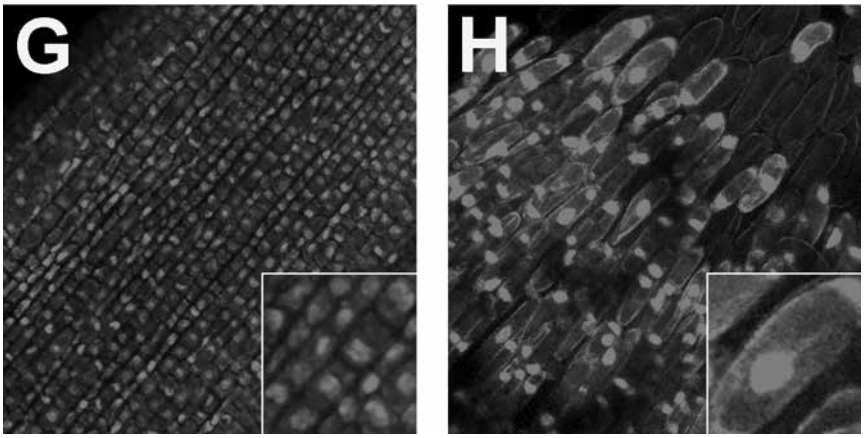

Fig. 2. Viability of the root tip cells of maize exposed to 10 μM Cd and/or Ni. Metal-treated roots were stained with propidium iodide and observed by confocal microscopy. Representative images, showing viable cells of control or treated roots with only cell wall stained (A, B) and dead cells having been influenced by Cd (C, D), Ni (E, F) or both Cd and Ni (G, H). Meristematic cells (A, C, E, G), root cap cells (B, D, F, H). In meristematic cells the red staining was seen in the cytoplasm and sometimes also in nuclei, while in root cap cells it was mostly in nuclei. Bar = 30 μm

Cell viability

In control roots as well as in the DZ of the metal-treated roots of both cultivars almost all cells were viable during the experiments, having only cell walls stained. In the metal-treated roots, PI stained cytoplasm and/or nuclei of meristematic and mostly nuclei of root cap cells indicating their death. Using this test the differences between treatments and cultivars were manifested in the time course of cellular dying. If 10 μmol/L HM concentration was applied, Cd caused cell death after 39 h in both meristematic and root cap cells of Blitz, and in root cap cells of Premia (Fig. 2C, D), while meristematic cells of Premia were alive within 48 h (Fig. 2A). Ni had none or only a slight effect on the root cell viability: there was no cell death observed in meristematic cells of Premia and root cap cells of Blitz within 48 h (Fig. 2A, B), and only a few cells were stained after 39 h in the meristem of Blitz (Fig. 2E) and root cap of Premia (Fig. 2F). When treated with Cd+Ni, both meristematic and root cap cells of Premia were dead after 36 h and in Blitz the death appeared after 30 h (meristem) or 27 h (root cap) (Fig. 2G, H).

At 100 μmol/L HM concentration the meristematic cells were dead after 20 h regardless of the metal(s) applied or the cultivar. The root cap cells of both cultivars were dead after 16 and 20 h of Cd and Cd+Ni treatments, respectively. Treatment with Ni induced root cap death after 24 h, while in Premia almost all root cap cells remained unstained within 24 h.

Electrophysiological measurements

The changes in electric potential differences between the vacuole and the medium in cortical cells of the MZ and DZ in two maize cultivars growing in the presence of Cd and Ni or Cd+Ni were determined. Under control conditions (before being exposed to HM), the E_M of the cortical root cells in MZ ranged from –128 to –146 mV (with the means –136±6.1 mV SD, n = 48) in cv. Premia and from –115 to –134 mV (with the means –127±5.6 mV SD, n = 53) in cv. Blitz. The E_M of cortical root cells in the DZ under the same control conditions varied between –134 and –155 mV (with the means –145±6.6 mV, SD, n = 42) in cv. Premia and between –132 and –154 mV (with the means –143±6.6 mV, SD, n = 37) in cv. Blitz.

During long-term experiments, the addition of Cd, Ni and Cd+Ni to the perfusion solution at either 10 or 100 µmol/L concentrations changed the values of E_M in MZ and DZ in different manner. The magnitudes of E_M changes were concentration and time dependent (Fig. 3). After an immediate decrease of E_M, the membranes of root

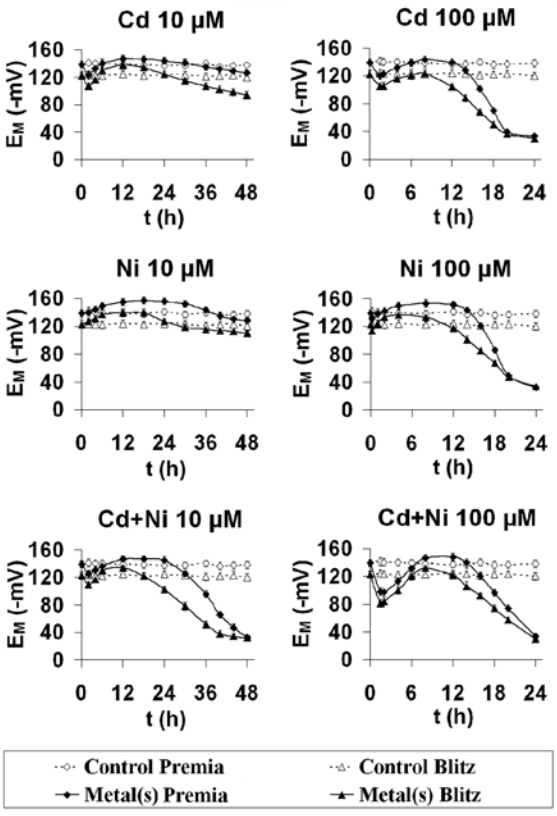

Fig. 3. Time dependent changes in membrane potential (E_M) of outer cortical cells (< 1 mm from root tip) exposed to 10 and 100 µmol/L Cd, Ni or Cd+Ni in long term experiments. Mean values±SD (n = 9–17)

cortical cells of either MZ or DZ exposed to the higher concentration of HM began to hyperpolarize, but subsequently the membranes of the cells in MZ compared to those of DZ depolarized again and, after 24 h the cells still remained in depolarized state. In contrast to the responses of the cells in MZ, the values of the more distant cortical cells in the DZ of the root treated with 10 μmol/L Cd, Ni and Cd+Ni retained the initial E_M values within 60 h (data not shown). The linear pattern of E_M depolarization indicated that the HM affected plasma membrane of the root cortical cells in changing its permeability.

DISCUSSION

All over the world the agricultural soils are more or less contaminated with HM. Such contamination causes deterioration of soils, triggering several defense responses (ROS), which are responsible for damage to cells and their membranes or even for plant cell death [7, 8].

Superoxide as an important signal molecule regulates various developmental and physiological processes [12]. Here we demonstrate that the treatment of roots of two maize cultivars with Cd stimulated a massive superoxide production, although to different extents. Since redox inactive metals do not interfere directly with cellular oxygen metabolism, the question arises as to the reasons of the observed oxidative stress. The primary mechanism of Cd-induced oxidative stress may be the inhibition of cellular antioxidant systems [31]. Yakimova et al. [35] demonstrated that in tomato cells a range of antioxidants effectively blocked Cd-induced cell death, indicating that oxidative stress is instrumental in this process. Alternatively, the displacement and thereby the release of redox-active metal ions from various biomolecules by Cd may disturb the redox balance of the cell.

Garnier et al. [13] showed that oxidative burst induced by 3 mM $CdCl_2$ in tobacco BY-2 cells was a result of NADPH-oxidase-dependent H_2O_2 production, followed by superoxide accumulation. Independently, NADPH-oxidase-dependent production of ROS was identified in response to Cd in pea [28]. Our results are in line with these data; however, in both maize cultivars 100 μM Cd was sufficient to stimulate the massive oxidative burst. The stimulating effect of Ni on oxidative burst in Cd-treated maize roots was related to an increased Cd-induced superoxide production. The most intriguing question of this study is a different tolerance of maize cultivars to Cd and an augmented elicitation of this sensitivity primed by Ni ions. We hypothesize that the observed differences in sensitivity to Cd may reflect the changes in the capacity of cellular non-enzymatic scavengers of ROS such as ascorbate, glutathione, tocopherol, etc. [3, 26] and the enzymatic antioxidative system composed of SODs, peroxidases, catalases, etc. [33]. In our experiments the root cell death appeared between 12 and 24 h of the 100 μM and between 24 and 48 h of the 10 μM metal treatment what was in accordance with the E_M decline in corresponding time periods.

The plasma membrane (PM) of root cells, which contains potential metal-sensitive enzyme systems such as ATPases [25, 29], is the first living functional structure in

contact with toxic heavy metals [24]. PM can also function as a diffusion barrier, protecting the interior of the cell. Therefore, its structure and functions are susceptible to alterations inherent to interactions with metal ions, including Cd and Ni. The most important functional change occurring in the PM is the Cd and/or Ni-mediated induction of E_M. Changes in E_M appear to be correlated with changes in membrane permeability and also appear to regulate ionic flux via a membrane and signal transduction process, which is responsible for the behaviour of plants under HM toxicity [9, 30]. The shifting of E_M to HM-induced depolarization has been previously observed, but contradictory findings have also been reported. One of these studies described that after adding of 100 μM Cd the mean E_M value measured in DF zone in the interval of 10–20 h did not differ from that obtained in control cells in rice and barley roots [30]. On the contrary, an other study demonstrated that Cd elicited depolarization in maize cortical cells in the MZ [9, 27].

Here we compared the induction of E_M changes caused by Cd and Ni in cortical cells in both MZ and DZ using the roots of two maize cultivars. Our results indicate that the MZ cortical root cells are much more sensitive to Cd and Ni in comparison to the cortical root cells of DZ. Only the results obtained from the DZ cells confirmed those reported by Sanz et al. [30].

Viability, E_M changes and partially also superoxide production of the cells in MZ indicate that the impact of the metals on the studied parameters declines in the order as follows Cd + Ni > Cd > Ni, and that the cv. Blitz tends to respond more sensitively than cv. Premia. The response of the root tip meristematic cells to the presence of the used metals was concentration-dependent. The Cd-induced ROS (superoxide) production was accompanied with elevated cell death. Available data suggest that Cd, if not detoxified rapidly enough, may trigger, via disturbance of the redox control, a sequence of reactions leading to the cell death [20, 35].

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