Membrane potential differences and viability of grapevine root cells treated with $HgCl_2$

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ABSTRACT

The effect of mercury (Hg) on the electrophysiological and permeability properties of grapevine adventitious root cells was examined. In short-term experiments, the apical segments of adventitious roots were treated with different concentrations of mercury (0.01 μmol/L to 2 μmol/L $HgCl_2$), and trans-membrane electrical potential differences (E_M) were monitored in root cortical cells, localized in distinct root zones. Based on Hg-induced decrease of E_M, we can confirm that the depolarization of the membrane is an instant Hg-response and the extent of E_M decrease is not only time- and concentration dependent, but also related to the developmental stage of cells and their localization on root axis. The sensitivity of root cells to Hg declined in the direction of cell division zone > cell elongation zone > absorption zone. Long-term treatment of roots with 2 μmol/L mercury showed that Hg-induced decrease of E_M was accompanied by an increase of K^+ efflux and a decrease of the diffusion potential (E_D) values. Application of fusicoccin (H^+-ATPase activator) to the root medium caused an immediate hyperpolarization of the membrane in control and Hg-treated cells. Laser scanning confocal microscopy analysis confirmed that Hg reduced cell viability, which was accompanied with the occurrence of cell death hallmarks, like condensation of protoplasts, nuclei fragmentation and deposition of granular material.

Keywords: *Vitis vinifera* L. cv. Limberger; cell death; K^+-efflux; trans-plasma membrane electrical potential difference

With the advent of the industrial revolution, heavy metal contamination has become an ever increasing concern. Thus, it is of major importance to understand the extent of heavy metal toxicity in plants and animals and the consequences arising from the ingestion of contaminated food.

Mercury (Hg) is known to be a metal, which can be easily modified into several oxidation states and to be spread through many ecosystems (Boenning 2000, Clarkson and Magos 2005). Due to the recurrence of Hg pollution and also because of the lack of knowledge about the effects of this heavy metal on plants, it is urgent to evaluate and understand the mechanisms of Hg-induced phytotoxicity.

Studies regarding Hg toxicity are mostly performed on animals and humans, as the toxicity is linked to autoimmune diseases (Pheng et al. 2003). Yet, the interaction between Hg and plant systems is of particular importance, due to Hg use in seed disinfectants, fertilizers and herbicides (Cavallini et al. 1999).

At the cellular level, heavy metals damage the structure and function of cell membranes. These include composition and fluidity, transport of essential ions, activity of H^+ATP-ase, displacement or substitution of metal ions from molecules (such as Mg from chlorophyll), denaturing or inactivation of proteins and, finally, disruption of cell membranes

Supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and Slovak Academy of Science, Grant No. VEGA 2/0023/13 for V. Repka, R. Fiala, J. Pavlovkin and ITMS 26240220040 for M. Čarná.

or organelles (Patra et al. 2004). In addition, heavy metals can be dangerous by blocking important molecules (e.g., enzymes and polynucleotides). At the cellular level mercury toxicity includes DNA damage, alteration of protein structure (due to its high affinity to sulphydryl, SH groups), induction of free radical formation, alteration of ion and water transport by cell membranes (Patra and Sharma 2000, Patra et al. 2004).

Hg chloride was shown to reduce elongation of *Zea mays* primary roots, as well as inhibition of the gravimetric response of the seedlings (Patra et al. 2004). The same authors also described a Hg-induced decline in the respiration rate of *Vigna radiata* seedlings, total nitrogen, sugars, DNA and RNA levels. Exposure to Hg can also reduce photosynthesis, chlorophyll synthesis, transpiration rate, and water uptake, as well as potassium, magnesium, and manganese loss and accumulation of iron in roots (Boenning 2000).

The aim of this study was to analyse the effect of Hg^{2+} on some electrophysiological parameters and viability of grapevine root cortical cells grown in hydroponic cultures. In particular, the effect of Hg^{2+} on the trans-membrane electrical potential differences and potassium fluxes was analyzed and correlated to structural cell changes, determined by using confocal microscopy.

MATERIAL AND METHODS

Plant material and growing conditions. Grapevine (*Vitis vinifera* L., cv. Limberger) shoots were taken from production vineyards of Slovakian region Ruban. After stratification in cold room (4°C) for one month, nodal explants (10 cm) with single axillary bud were used for hydroponic cultivation. These grapevine explants were grown in magenta jars, filled to 60 mL with aerated half strong MS media (Murashige and Skoog 1962) at 25 ± 1°C under 14-h photoperiod. Two-month old adventitious roots were cut off the stem and used for all experiments described.

Measurements of the membrane potential. Measurements of plasma membrane potential (E_M) were carried out at 22°C on outer cortical cells of 25 mm long apical adventive root segments by standard microelectrode techniques described earlier (Pavlovkin et al. 2006). After rinsing the roots with 0.5 mmol/L $CaSO_4$, apical root segments were mounted to a Plexiglas holder with a soft rubber ring and mounted in a vertical 5 mL plexiglass cuvette, which was perfused with a standard solution containing 0.1 mmol/L KCl, 1 mmol/L $Ca(NO_3)_2$, 1 mmol/L $MgSO_4$, 1 mmol/L NaH_2PO_4 and different concentrations of $HgCl_2$, adjusted to pH 5.8 using 0.1 mol/L HCl) at a flow rate of 5 mL/min. The micro-electrodes were inserted into the cortical cells under microscope by means of a micromanipulator (Leitz, Germany). Insertion and position of the microelectrode was observed under a microscope.

Fusicoccin (FC), a well-known PM-H^+-ATPase stimulator, was used (in 0.1% ethanol at a final concentration of 30 μmol/L) for monitoring the functionality of the PM-H^+ pump (Marrè 1979).

To establish anoxic conditions the perfusion solution was saturated with N_2 gas by flushing.

Potassium (K^+) determination. For K^+ determination the 25 mm long adventives apical root segments of grapevine were incubated in 25 mL aerated 0.2 mmol/L $CaSO_4$ without Hg^{2+} (control) or supplemented with 2 μmol/L Hg^{2+} (Hg-treated) in darkness. The content of K^+ in the incubation medium was determined with an ion-selective electrode (CyberScan 2100, Eutech Instruments, Singapore) and was related to the fresh mass of roots.

Laser scanning confocal microscopy (LSCM). Propidium iodide (PI, Fluka) and fluorescein diacetate (FDA, Serva) were used to counterstain the cell wall and nuclei of ruptured cells (Oh et al. 2010). Following 24 h treatment with the Hg^{2+} (2 μmol/L), adventive apical root segments of grapevine explants were sectioned (0.5 cm from the apex), stained 2 min in 10 μg/mL PI or 10 min in 5 μg/mL FDA, washed 2 min in distilled water and observed by confocal microscope Olympus FV1000 (Olympus, Tokyo, Japan). Both dyes were excited at 488 nm and fluorescence was detected using 560–660 nm for PI or 505–550 nm barrier emission filter for FDA.

Statistics. Means and standard errors were calculated from three independent experiments. Data were analyzed with a one-way analysis of variance (ANOVA) with $P < 0.05$ or 0.01.

RESULTS

Trans-membrane electrical potential. In order to detect immediate cell responses of the grapevine root to mercury, the trans-plasma membrane electrical potential of root cells was recorded before and after mercury application in individ-

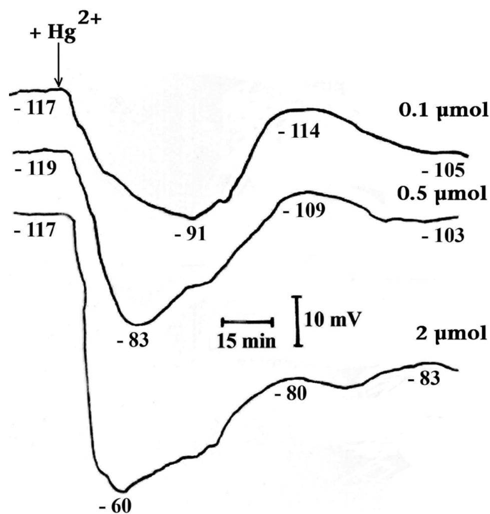

Figure 1. Effect of Hg^{2+} on the plasma membrane potential (E_M) of grapevine cortical root cells in division zone

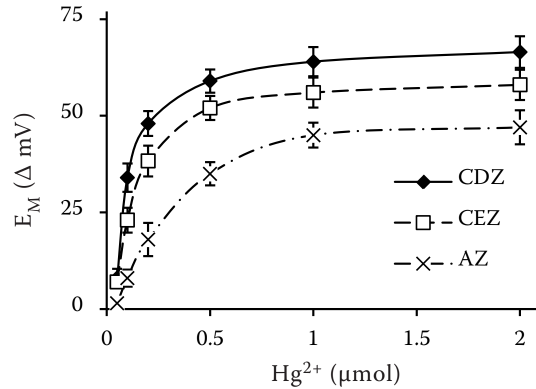

Figure 2. Changes of plasma membrane potential (E_M) in cortical root cells of grapevine roots treated with different concentrations of Hg^{2+} (values are mean ± SD, *n* = 7–16). Standard errors are shown as vertical bars. CDZ – cell division zone; CEZ – cell elongation zone; AZ – absorption zone

ual root zones. In grapevine stem adventitious roots, the cell division zone (CDZ), was located to 4 mm behind the root tip, the cell elongation zone (CEZ) 5–8 mm behind the root tip and absorption (maturation) zone (AZ) with root hairs in a distance more than 10 mm behind the root tip. In control conditions, E_M of cortical cells in the CDZ varied between –103 mV and –129 mV (–114 ± 6 mV, mean ± SD, *n* = 37), in CEZ between –102 mV and –129 mV (–122 ± 6 mV, mean ± SD, *n* = 37) and in AZ between –111 mV and –143 mV (–133 ± 9 mV, mean ± SD, *n* = 45), respectively.

Application of Hg to the root bathing solution induced an instant decrease in membrane electrical potential of cortical root cells in a concentration-dependent manner (Figure 1). The highest concentration of Hg (2 μmol/L) caused a rapid and transient depolarization of the membrane, whose magnitude reached values of 66.5 ± 4.1 mV (mean ± SD, *n* = 3) in cells of CDZ, 58 ± 3.9 mV, (*n* = 3) in cells of CEZ and 47 ± 4.4 mV, (*n* = 4) in cells of AZ. This decrease was instant, without any measurable lag period (Figure 1). The maximal depolarization was recorded in the intervals between 12 min and 30 min, and the extent of E_M decrease was more considerable in cells of CDZ and CEZ than in cells of AZ (Figure 2).

The diffusion potential (E_D) was determined in order to distinguish between passive and active, (i.e. energy-dependent), components of E_M by application of anoxic conditions. Perfusion of bathing solution with N_2 gas resulted in a rapid decrease of E_M of cortical cells in CDZ, CEZ and AZ to E_D –77 to –83 mV (–80 ± 3 mV, mean ± SD, *n* = 21), respectively (Figure 3).

In long-term experiments (24 h), the Hg-induced decrease of E_M was accompanied by an increase of K^+ efflux and a decrease of E_D values (Figures 3–4).

These electrophysiological data indicate that Hg^{2+} rapidly triggered trans-membrane ion fluxes.

The presence of 2 μmol/L Hg^{2+} in the root bathing solution induced an increase in K^+ efflux. Almost 20% increase in K^+ efflux from treated roots was recorded 3 h after Hg^{2+} application, while after 24 h the loss of K^+ was 46% (Figure 4).

Effect of fusicoccin (FC). In long-term experiments, application of FC (plasma membrane H^+-ATPase activator) to the root bathing solution caused an immediate increase of E_M in both control and treated CDZ cells with (2 μmol/L Hg^{2+} for 24 h). Due to FC, the value of E_M became more negative than the potential in control roots (32 ± 3 mV, mean ± SD, *n* = 3). However the hyperpolarization of the membrane in Hg-treated CDZ cells was very low (9 ± 3 mV, mean ± SD, *n* = 3) or completely absent (*n* = 4).

Microscopy. Microscopic examination of grapevine roots revealed considerable differences between control and Hg^{2+} treated plants. After long-term treatment (24 h) with 2 μmol/L Hg^{2+}, root tips were seriously damaged and exhibited an abnormal morphology, when compared to control roots (Figures 5a,b). FDA-stained control roots showed intensive signals, confirming that almost

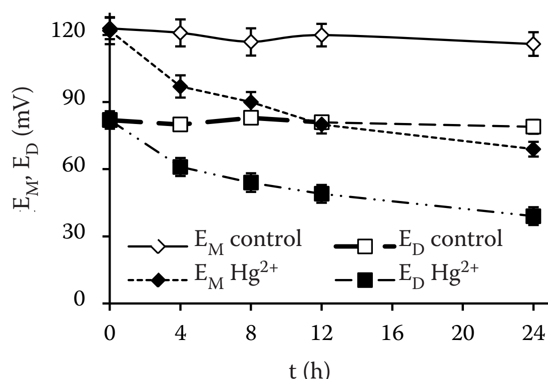

Figure 3. Time effects of 2 µmol/L Hg^{2+} on plasma membrane potential (E_M) of grapevine cortical root cells in division zone; values are mean ± SD (n = 3–5). Standard errors are shown as vertical bars

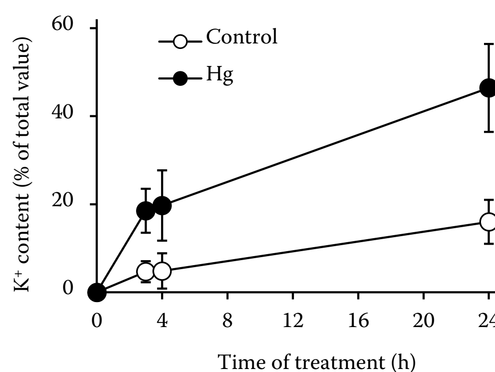

Figure 4. Changes in K^+ leakage from grapevine roots, expressed as a percentage of total K^+ content in the roots, induced by 2 µmol/L Hg^{2+} treatment

all cells were alive (Figure 5a2), while besides a few cells, Hg^{2+} treated roots remained unstained (Figure 5b2), suggesting that root cell membranes were leaky. In control roots, PI stained only cell walls, indicating that cell membranes were intact (Figure 5a4), whereas Hg^{2+}-treated roots showed intense intracellular staining, pointing to a cytoplasmic membrane damage (Figure 5b4). Higher magnification revealed a considerable cell collapse: protoplast condensation (Figure 5c1), nuclear staining, disintegration (Figure 5d) and deposition of granular material in the cytoplasm (Figure 5e).

DISCUSSION

Since Hg^{2+} can interact with a number of extra and intracellular structures many different mechanisms of Hg^{2+} toxicity have been hypothesized (Patra and Sharma 2000). These mechanisms include disruption of the plasma membrane and related transport processes that can result in plant nutritional and metabolic disorders (Azevedo and Rodriguez 2012).

Results based on the measurement of the trans-membrane electrical potential of roots show a different extent of Hg^{2+}-induced depolarization of the cell membrane. In the present experiments, the Hg^{2+}-induced membrane depolarization was concentration-dependent and partially reversible. The rapidity and the reversibility of the Hg^{2+}–induced depolarization indicate that Hg^{2+} may influence the structure and the permeability of plant cell membranes (Kennedy and Gonsalves 1987). Further evidence was provided by the work of Hendrix and Higinbotham (1974), who found that Hg^{2+} severely inhibits K^+ uptake into pea stem while having little effect on respiration. The decay of root trans-membrane electrical potential and H^+ efflux, induced by 10 µmol/L Hg^{2+}, was also observed by Kennedy and Stewart (1982), suggesting that these two effects were correlated. Our results support these findings and, in addition, specify that the root cortical cells were sensitive to Hg, in connection with their localization in the root axis and developmental stage. Comparing changes in E_M, during short-term experiments (up to 15 min), Hg^{2+} caused a rapid decrease of E_M in cells of all three root zones; however, the extent of the decrease was in the direction of CDZ > CEZ > AZ.

To characterize the instant effect of Hg^{2+} on the PM-H^+ATPase of cortical root cells, we performed a set of experiments with FC. These results show that the functional activity of root cortical cells, in division zone treated with 2 µmol/L Hg^{2+} for 24 h, were reduced which means that Hg^{2+} may counteract FC-caused hyperpolarization. The response may indicate a similar target for alteration of PM-H^+-ATPase activity by Hg^{2+} and FC. Plant PM-H^+-ATPases are known to be inhibited by sulphydryl reagents such Hg^{2+}. In addition, an ATPase in a putative plasma membrane fraction from maize roots, was also inhibited by 1 µmol/L Hg^{2+} by over 50% (Beffagna et al. 1979).

Electrophysiological experiments were corroborated by observations with confocal microscopy. Analyses demonstrate changes in the viability of grapevine root cells. In particular, our results strongly suggest that Hg^{2+} – stimulated K^+ efflux and changes in E_D value were a direct consequence of membrane damage. After long-term exposure of

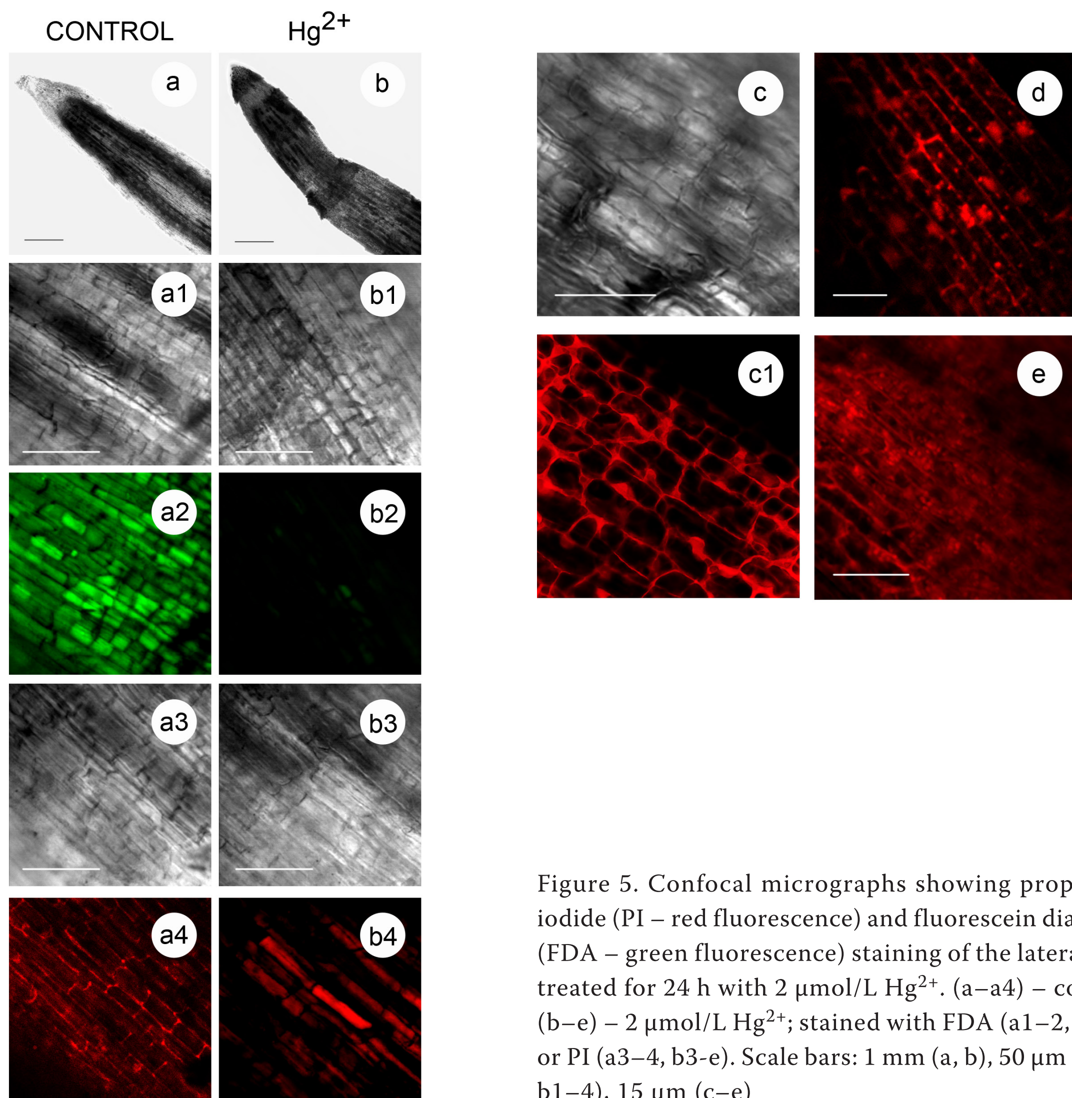

Figure 5. Confocal micrographs showing propidium iodide (PI – red fluorescence) and fluorescein diacetate (FDA – green fluorescence) staining of the lateral root treated for 24 h with 2 µmol/L Hg^{2+}. (a–a4) – control; (b–e) – 2 µmol/L Hg^{2+}; stained with FDA (a1–2, b1–2) or PI (a3–4, b3-e). Scale bars: 1 mm (a, b), 50 µm (a1–4, b1–4), 15 µm (c–e)

grapevine root to Hg^{2+}, we observed significantly reduced cell viability accompanied with cell death hallmarks, like condensation of protoplasts, nuclei fragmentation and deposition of granular material (Figure 5). A similar Hg-induced K^+ efflux was described by Coskun et al. (2012), but no protoplast condensation was seen after staining the cells with PI. Characteristic structural features, associated with plant cell death, were also detected after exposure of root cells of different plant species to aluminum (Pan et al. 2001), cadmium (Ortega-Villasante et al. 2005) or zinc (Li et al. 2012).

Based on our results, we can conclude that Hg^{2+} negatively affects both functional and structural properties of grapevine root cells. The sensitivity of the root cells is not only time- and concentration-dependent, but it is also related to the developmental stage of root cells and their localization on root axis. Hg^{2+} simultaneously decreased the E_M, E_D, and FC-stimulated E_M, while the PM-H^+-ATPase induced an efflux of K^+. The extent of membrane depolarization decreased continuously from the apex to the base of the root.

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Received on January 1, 2013
Accepted on June 18, 2013

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