Vitis **54**, 175–182 (2015) DOI: http://dx.doi.org/10.5073/vitis.2015.54.175-182

Early cadmium-induced effects on reactive oxygen species production, cell viability and membrane electrical potential in grapevine roots

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Summary

Cadmium (Cd) is one of the most worldwide concerned metal pollutants. It is able to induce reactive oxygen species production through indirect mechanisms causing oxidative stress. *Vitis vinifera* roots were treated with 100 µM Cd for 0-180 min or 20-100 µM Cd for 24 h. Fluorescence confocal microscopy showed elevated hydrogen peroxide and superoxide levels in the apical root segments. Two phases (after 30 min and 24 h) of the superoxide raised levels were observed. This was accompanied by the decrease in root cell viability. Cd in concentrations between 0.005-10 mM induced significant, but different changes in membrane electrical potential (E_M) of the root epidermal cells. The low concentrations of Cd (0.005-0.01 mM) caused transient E_M hyperpolarization followed by depolarization, whereas by higher concentrations (0.05-5.0 mM) E_M was depolarized. In any case, the depolarization or hyperpolarization were only transient up to 5 mM Cd concentration indicating that the plasma membrane function was not irreversibly destroyed. Hyperpolarization of E_M induced by fusicoccin (FC) was completely suppressed only in the presence of 10 mM Cd pointing to the inhibition of H^+-ATPase. The results suggest that the Cd interactions, depending on cellular development, result in activation of a complex of various mechanisms such as peroxide and hydrogen peroxide production, which in turn may be a more probable reason for the root cell responses to Cd toxicity than the transient E_M changes.

K e y w o r d s : grapevine; 'Limberger'; cadmium; reactive oxygen species; cell viability; membrane electric potential.

Introduction

Cadmium, although a non-essential element, can be accumulated by plants from mineral fertilizers, pesticides, sewage sludge or as an environmental pollutant resulting from various industries, e.g. mining and smelting (Nazar *et al.* 2012). Cd is considered to be highly toxic (Pál *et al.* 2006), easily taken up by plants (Lee *et al.* 1998), then entering the food chain and resulting in serious health issues for animals and humans (Horiguchi *et al.* 2010). In plants Cd can induce complex changes at the genetic, biochemical and physiological levels (Gallego *et al.* 2012) leading to a number of phytotoxic effects including inhibition of plant growth and development, chlorosis, necrosis, reduction of chlorophyll content and photosynthetic rate, imbalance in mineral nutrient uptake and affection of enzymatic activities (Tran and Popova 2013). Rupp *et al.* (1985) found transport of iron from roots to leaves to be nearly completely inhibited by Cd (1-10 ppm), forcing the iron accumulation in roots. Cd thus may cause iron deficiency chlorosis in grapevines. *Vitis vinifera* cell suspension cultures grown in the presence of 1 mM $CdCl_2$ were found to have increased (in a time and concentration dependent manner) contents of α-tocopherol, which is the major compound of vitamin E found in leaf chloroplasts and may be involved in Cd tolerance and hyperaccumulation (Cetin *et al.* 2014).

When released in plant environment, Cd is predominantly accumulated by plant roots. Yuan-peng *et al.* (2012) showed that almost all of the absorbed Cd remains in the roots of grapevine, with a majority accumulated in the cell wall fraction. Du *et al.* (2012) showed in grapevine that absorbed Cd was mostly distributed to underground organs (roots and rhizomes) or below a graft position.

It is well established that Cd induces reactive oxygen species (ROS) generation in many plant species. Briefly, ROS such as superoxide anion ($O^{\cdot-}_2$) and hydrogen peroxide (H_2O_2), were initially recognized as toxic by-products of aerobic metabolism (particularly formed in the electron transport chain in mitochondria), removed by means of antioxidants and antioxidative enzymes (Romero-Puertas *et al.* 2007, Paradiso *et al.* 2008). It is known that various environmental stress stimuli induce excess ROS production causing widespread damage to cells. On the other hand, $O^{\cdot-}_2$ plays a role in the immune system (Guzik *et al.* 2003). H_2O_2 has important roles as a signalling molecule involved in regulating a variety of biological processes such as root growth, programmed cell death and response to biotic and abiotic environmental stimuli in plants (Veal *et al.* 2007, Dat *et al.* 2000). The controlled, harmless accumulation of specific ROS, like H_2O_2, acts as a cellular signal for the onset of grapevine berry ripening (Pilati *et al.* 2014). H_2O_2 is mainly detoxified by catalase in glyoxysomes and peroxisomes and by ascorbate peroxidase in chloroplasts, mitochondria and peroxisomes (Shigeoka *et al.* 2002). When

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H_2O_2 concentrations are raised by stress, it can become toxic. H_2O_2 thus may act both as a stress-induced ROS and, on the other hand, it is involved in defence mechanisms against oxidative stress, by increasing antioxidant enzymes associated gene expression like in grapevine buds (Vergara *et al.* 2012, Ozden *et al.* 2009).

One of the early events induced by Cd in plants have been shown to be H_2O_2 and $O^{\cdot-}_2$ release (Olmos *et al.* 2003, Garnier *et al.* 2006, Ortega-Villasante *et al.* 2005, 2007). At the subcellular level the plasma membrane (PM) is considered a source of H_2O_2 and $O^{\cdot-}_2$ (Romero-Puertas *et al.* 2004, 2007). In four grape cultivars treated with 0.5 mM $CdCl_2$, Shao *et al.* (2009) found the root mitochondrial H_2O_2 contents increasing in the order of the cultivars Kyoho > Ze Xiang > Muscat Hamburg > Long Yan. The root activity of Kyoho was easier to be inhibited under Cd stress, while that of Long Yan was least affected by $CdCl_2$. The H_2O_2 levels increased in response to Cd treatment in *Arabidopsis thaliana* (Maksymiec and Krupa 2006). Such increase in H_2O_2 accumulation changes the redox status of the cell and induces the production of antioxidants and the activation of antioxidant mechanisms (Mittler 2002, Paradiso *et al.* 2008, Cetin *et al.* 2014).

Cd-induced oxidative stress leads to impairment of PM permeability and electrolyte leakage in leaf and root cells by triggering strong lipid peroxidation (Hatata and Abdel 2008). There are few reports on the effect of Cd on the electrolyte leakage from root cells (De Filipis 1979) or membrane potential changes (Karcz and Kurtyka 2007, Sanz *et al.* 2009, Kurtyka *et al.* 2011). There are several references on PM alterations as the primary events of Cd toxicity, such as the modification of PM composition and in turn the inhibition of various protein functions including PM H^+-ATPase activity (Janicka-Russak *et al.* 2008).

In this work we studied Cd-induced early changes in grapevine roots. Because the root cell PM is the first barrier encountered by Cd during its soil/plant transfer we focused on determining the changes of the membrane electrical potential (E_M) and relating these findings to oxidative stress as a consequence of Cd excess treatments. Intracellular H_2O_2 and $O^{\cdot-}_2$ as oxidative stress indicators, as well as cell viability by means of cell membrane integrity were detected using confocal laser scanning microscopy (CLSM).

Material and Methods

Plant material and growth conditions: Grapevine (*Vitis vinifera* L. 'Limberger') shoot cuttings were taken from production vineyards of the Slovak region Rúbaň. After stratification in a cold room (4 °C) for one month, nodal explants (10 cm) with a single axillary bud were used for hydroponic cultivation. The explants were grown in magenta jars filled to 60 mL with aerated half strong MS medium (Murashige and Skoog 1962) at 25 ± 1 °C under 14 h photoperiod. Data presented in this paper were obtained on excised adventitious roots.

Cd treatments: Two-month-old explants with adventitious roots were transferred to aerated solutions containing 0.1 mM KCl and 0.1 mM $CaCl_2$, pH 5.8 supplemented with 0 (control), 0.02, 0.06 or 0.1 mM $CdCl_2$ for 24 h in the concentration-dependence experiments, and with 0 (control) and the highest 0.1 mM $CdCl_2$ concentration for 10, 30, 60 or 180 min in the time-dependence experiments. The roots were then stained as described below.

Confocal laser scanning microscopy: Propidium iodide (PI, Fluka, Switzerland) was used to counterstain the cell wall and nuclei of ruptured cells (Oh *et al.* 2010). 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was used as an indicator for H_2O_2 accumulation in cells. To monitor real time $O^{\cdot-}_2$ production in the root tips we used Superoxide Detection Kit (Enzo Life Sciences, USA). Apical 0.5 cm long root segments were stained 2 min with 10 $\mu g \cdot mL^{-1}$ PI in water, 15 min with 50 µM H_2DCFDA in 50 mM phosphate buffer pH 7.5 or 15 min with superoxide staining solution (following the manufacturer's manual), washed for 2 min in distilled water (for superoxide just briefly) and observed in confocal microscope Olympus FV1000 (Olympus, Japan). PI and H_2DCFDA were excited at 488 nm and fluorescence was detected using emission barrier filters 560-660 nm for PI or 505-550 nm for H_2DCFDA. The superoxide stain excitation wavelength was 543 nm and the emission was detected using 560-660 nm barrier filter. The confocal microscopy images represent at least 3 roots and were selected from at least 3 different images of each root. Fluorescence signal intensity (CTCF, corrected total cell fluorescence) was measured and calculated by the open source analyzing software Image-J2/Fiji (http://imagej.net/Fiji).

Electrophysiology: Measurements of transmembrane electrical potential were performed on single epidermal cells within the root zone located 200-1500 µm from the root tip of 20 mm long root segments using standard microelectrode technique as described in detail by Pavlovkin *et al.* (2006). After rinsing with 0.5 mM $CaSO_4$, the roots were mounted in a 4 mL volume Plexiglas chamber and constantly perfused (4 $mL \cdot min^{-1}$) with the bathing solution containing 0.1 mM KCl and 0.1 mM $CaSO_4$. The initial changes of E_M induced by 0.05-10 mM $CdCl_2$ were measured after addition of $CdCl_2$ to the perfusion solution. Subsequently, the effect of short-term Cd treatments was registered after the cells attained a resting potential with an equimolar $CaCl_2$ solution by exchanging $CaCl_2$ with $CdCl_2$ in the perfusion solution, to avoid the effect of the counterion. The E_M of the roots treated for several hours (up to 24 h) with $CdCl_2$ was also measured using the same solution, containing 0.1 and 1 mM $CdCl_2$.

Fusicoccin (FC), a PM H^+-ATPase stimulator, was used to monitor the functionality of the PM H^+-pump (Marrè 1979) at the final concentration of 30 µM in 0.1 % ethanol.

Results

Cell viability and ROS production – the concentration dependence: Microscopic examination of grapevine roots revealed considerable differences between control and Cd-treated plants. After

the 24 h treatment we observed a concentration- dependent progression of cell death in the roots determined by CLSM using PI as a fluorescent marker. The root tip cells treated with 60 and 100 µM Cd were relatively intensively loaded with PI indicating that the cell membrane integrity was disturbed (Fig. 1). In control roots PI stained only cell walls indicating that the cells were viable, with intact cell membranes.

Following the 24 h treatment with 20, 60 and 100 µM Cd, concentration-dependent production of ROS was observed using ROS-specific fluorescent probes. As for $O^{\cdot-}_2$, the control cells exhibited just a very weak fluorescence in the membranes. In comparison with control, elevated amounts of $O^{\cdot-}_2$ were accumulated in the cells treated with 20 or 60 µM Cd for 24 h while the highest fluorescence was observed in the cells treated with 100 µM Cd (Fig. 1). As for H_2O_2, the cells treated with 20 µM Cd exhibited just a slight increase in its amount in comparison with the control while the treatment with 60 and 100 µM Cd induced a substantially higher H_2O_2 production that is indicated by an intensive green fluorescence (Fig. 1).

Cell viability and ROS production - the time dependence: To obtain more detailed information about the effect of 100 µM Cd on the cell viability and ROS production a time course analysis was performed. After the treatment of the apical root segments with 100 µM Cd for increasing time periods, the onset and extent of the cell death were determined. As shown in Fig. 2 the treatment with 100 µM Cd for 10 min had no obvious effect on the cell viability. In contrast, in roots treated for 30 to 180 min the PI stain was localized also in the cell nuclei indicating the cellular death.

The time course analysis of ROS production in the Cd-treated roots revealed interesting results. As expected, a very weak $O^{\cdot-}_2$ production was observed in the control cells. The strongest orange fluorescence indicating the $O^{\cdot-}_2$ production was observed after 30 min of the treatment with 100 µM Cd. Interestingly, no remarkable $O^{\cdot-}_2$ accumulation was detected after 10 min as well as after 60 and 180 min of the treatment (Fig. 2). The H_2O_2 staining revealed a very weak fluorescence signal in the control cells. The exposure to 100 µM Cd for 10 or 30 min showed a weak punctuate pattern of the H_2O_2 production. Almost equally higher H_2O_2 accumulation was detected in the roots treated for 60 and 180 min. The H_2O_2 specific signal was located near cell membranes (Fig. 2).

Cd effects on the membrane electrical potential: In order to detect immediate cell responses of the root apex to Cd, E_M was recorded before and during Cd application. The initial resting E_M in the epidermal cells revealed distinct properties in different root developmental zones. The meristematic (MZ), elon-

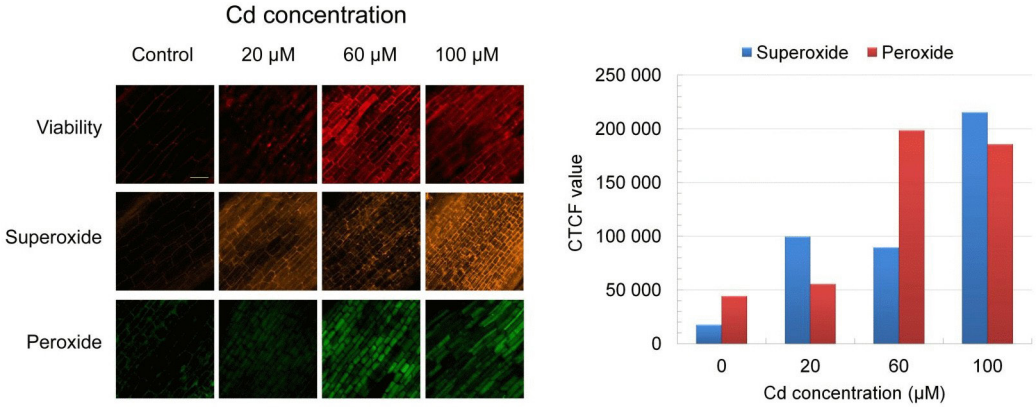

Fig. 1: Cell viability and superoxide and hydrogen peroxide production detected with fluorescent probes in the root tip cells of grapevine exposed for 24 h to various Cd concentrations. Confocal microscopy micrographs are supplemented with the bar chart expressing fluorescence signal intensity values (CTCF). Appropriate stainings and metal concentrations are indicated. Bar = 30 µm.

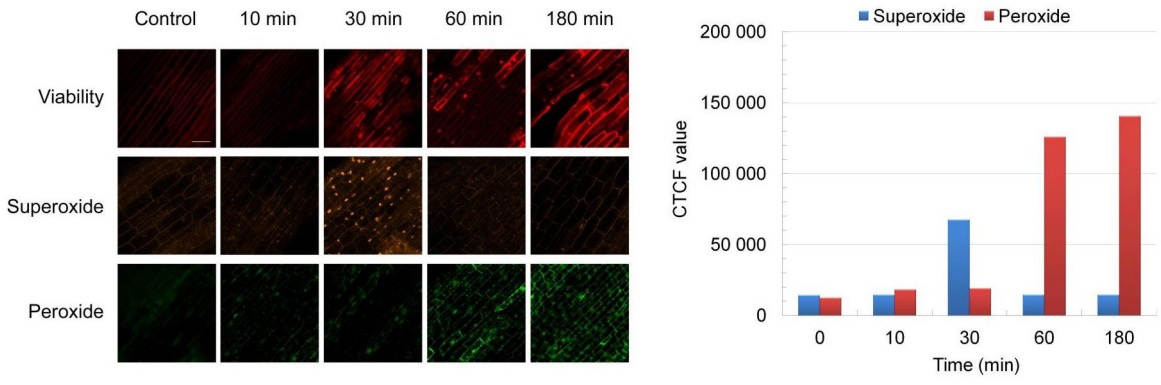

Fig. 2: Cell viability and superoxide and hydrogen peroxide production detected with fluorescent probes in the root tip cells of grapevine exposed to 100 µM Cd. for increasing time periods. Confocal microscopy micrographs are supplemented with the bar chart expressing fluorescence signal intensity values (CTCF). Appropriate stainings and exposure times are indicated. Bar = 30 µm.

gation (EZ), and differentiation (DZ) zones were located 180-300 µm, 430-930 µm and > 930 µm behind the root tip, respectively. The E_M of untreated epidemal cells varied between -116 mV and -125 mV (-120 ± 4.7 mV, n = 19) in MZ, between -117 mV and -135 mV (-129 ± 5.9, n = 22) in EZ and between -115 mV and -127 mV in DZ (-121 ± 5.6, n = 14) (Fig. 3). Based on this electrophysiological characteristic we further performed experiments to determine the sensitivity of the three developmental zones to 1 mM $CdCl_2$ exposure (Fig. 3). In all three developmental zones E_M depolarization was induced within 1 min after adding Cd. However, E_M depolarized to a greater extent in the epidermal cells of MZ (ΔE_M = 38.4 ± 1.95 mV, n = 6) and EZ (ΔE_M = 40.0 ± 3.2 mV, n = 9) than in the cells of DZ (ΔE_M = 31.9 ± 3.1 mV, n = 11) (Fig. 3). Thus all the following E_M measurements were performed in the epidermal cells located 500 µm behind the root tip.

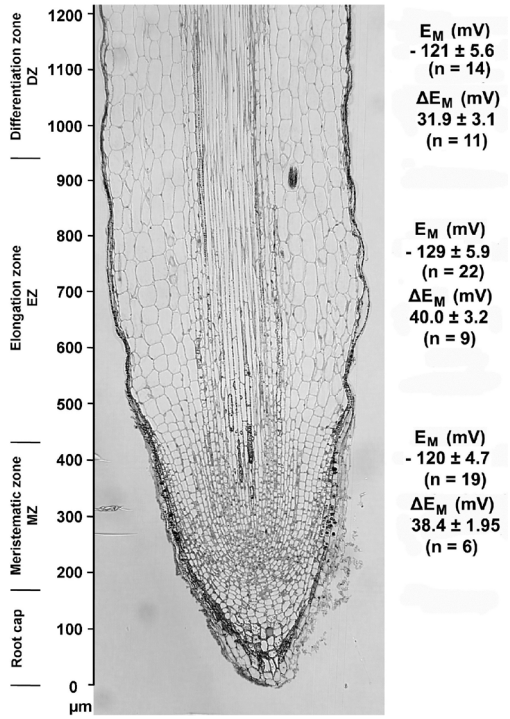

Fig. 3: Longitudinal section of the adventitious root apex of *Vitis vinifera* 'Limberger'. The root developmental zones are marked with the distances from the root tip (left). The resting membrane electrical potential (E_M) values and the maximal E_M depolarizations (ΔE_M) induced by 1 mM $CdCl_2$ in the epidermal cells are indicated for the individual root zones (right) (mean ± SD, n = 6-22).

In an early phase after adding Cd to the perfusion solution, immediate changes in E_M of root epidermal cells were induced, and their pattern depended on the Cd concentration (Fig. 4-6). Addition of 0.005, 0.01 and 0.02 mM $CdCl_2$ caused a small hyperpolarization (4.6 ± 1.4 mV, n = 9), thereafter, the membrane slowly depolarized and after 5-15 min reached maximal depolarization (5.6 ± 1.4 mV, n = 9). Then the membrane repolarized back within a few minutes to the initial level before the treatment (Fig. 4). With the increasing Cd concentration the hyperpolarization magnitude decreased and, vice-versa, the depolarization magnitude increased. No E_M hyperpolarization was recorded with the higher (0.05-10 mM) Cd concentrations (Fig. 5a-e). In the presence of 0.05, 0.5 and 1 mM Cd E_M strongly depolarized within 90-120 min to minimal values followed by a 4-7 h long phase of repolarization with E_M gradually returning to the initial values recorded before Cd treatments. The 5 mM Cd concentration triggered a rapid membrane depolarization within 1 h (Fig. 5d) and the complete E_M repolarization was reached after 12 and more hours (data not shown). Similarly to 5 mM concentration, 10 mM Cd depolarized E_M to the values of the diffusion potential within 50-70 min and this level remained unchanged within the next 24 h (Fig. 5e).

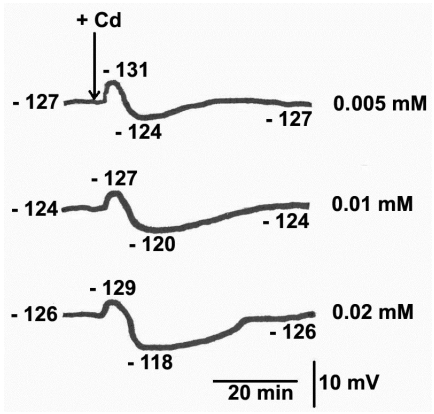

Fig. 4: Time course of the early effects of $CdCl_2$ concentrations on the membrane electrical potential (E_M) of grapevine adventitious roots epidermal cells located 500 µm from the root tip. Cd concentrations are indicated on the right. Addition of Cd is indicated by an arrow.

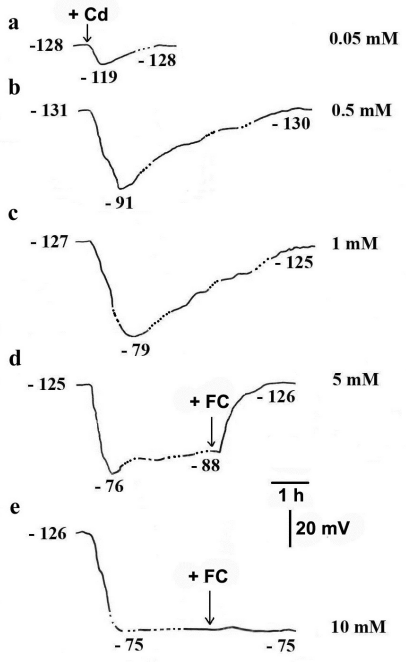

Fig. 5: Time course of the membrane electrical potential (E_M) responses to increasing $CdCl_2$ concentrations (**a**-**e**, indicated also on the right) in grapevine adventitious root epidermal cells located 500 µm from the root tip. The functionality of the plasma membrane H^+-ATPase was monitored (**d**, **e**) after adding of 30 µM fusicoccin (FC, arrows).

In a separate set of experiments FC was used to monitor the functionality of the PM H^+-ATPase. FC rapidly and permanently hyperpolarized the membranes of the epidermal cells of control roots and ΔE_M varied between 25-29 mV (data not shown). Addition of 30 µM FC to the perfusion solution following 5 mM Cd-induced membrane depolarization repolarized the membrane back during 1 - 2 h, to the level close to its resting potential (Fig. 5d). By contrast, no significant change of E_M occurred after the FC treatment of the depolarized state of the membrane induced by 10 mM Cd (Fig. 5e). These results indicate that Cd at 10 mM permanently affected the H^+-ATPase activity. The extent of Cd-triggered E_M depolarization was concentration-dependent (Fig. 6).

In addition, in a later phase, up to 24 h after adding of Cd, no significant differences were recorded between E_M values of the control cells before the treatment, and those treated with 0.05, 0.5 and 1 mM Cd (data not shown).

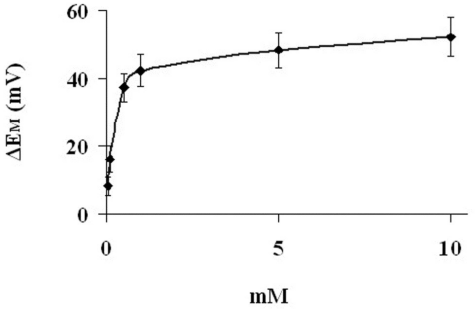

Fig. 6: Concentration-dependence of the membrane depolarization magnitude (ΔE_M) in the epidermal cells located 500 µm from the root tip of the grapevine adventitious roots (mean ± SD, n = 3-5).

Discussion

Although occupying a relatively small territory, Slovakia belongs to the countries, which have regions with severely polluted environments including vineyards. Distribution of Cd in the soils of vineyard regions in Slovakia varies between 0.1 to 8.9 mg·kg^{-1} (Šefčík 2008). Cd amounts are subject to geogene origin or cross-border atmospheric soil contamination, however, particularly to anthropogenic activities. Long-term and improper application of high doses of industrial fertilizers and pesticides, wasteful disposal of animal excrements as well as improper land management have markedly contributed to water and soil contamination, decrease of air quality and biodiversity as well as erosion, acidification, and eutrophication of the environment within the vineyard regions (Šalgovičová 2009). Although developmental trends in the 1990s led to the alleviation of agricultural pressure on the environmental contamination with heavy metals (HM), particularly Cd, the negative impacts are still persisting. Due to closing ineffective industrial productions and an extensive reconstruction of separatory technologies, Cd emissions gained a decreasing tendency (9.9 metric tons in 1990; 9.28 metric tons in 2006). However, the industrial combustion processes and the refuse incineration still represent a serious problem of vineyard regions contamination (Šalgovičová 2009). It has been shown that the metal content in grape clearly reflects that in the soil on which the grape was cultivated (Laštincová *et al.* 2009). Though in the last decades raised attention has been paid to the negative effects of HM on plants and humans, agricultural commodities like cereals are in the main focus (Bielek *et al.* 1998). There are no available data concerning the effects of HM on grapevine root cell plasma membrane and only a few data regarding the influence of Cd on the oxidative stress. This prompted us to study the effect of Cd on the roots of the one of the most grown grapevine cultivars in Slovakia - 'Limberger'.

Oxidative stress can seriously disturb normal metabolism *via* oxidation of lipids, proteins and nucleic acids. This may lead to changes in selective permeability of membranes and membrane leakage as well as to changes in the activities of membrane bound enzymes (Mittler 2002).

Our CLSM results showed that treatment of the grapevine roots with Cd resulted in the decrease of root cell viability due to the disturbed plasma membrane integrity. The dead cells characteristic with PI-stained cytoplasm occurred at the concentrations 60 and 100 µM Cd after 24 h treatment (Fig. 1). The highest 100 µM Cd concentration induced the considerable decrease of cell viability already after 30-180 min (Fig. 2). The decrease in cell viability was accompanied by increased levels of $O^{\cdot-}_2$ and H_2O_2. The main increase in $O^{\cdot-}_2$ level was observed after 30 min and 24 h of the 100 µM Cd treatment. Interestingly, no increase was observed after 60 and 180 min (Figs 1 and 2) suggesting that superoxide burst was biphasic, similarly as in mechanically stressed potato tuber tissues (Johnson *et al.* 2003) or aluminium-treated *Arabidopsis thaliana* cell suspension culture (Kunihiro *et al.* 2011). The CLSM microghaphs suggested the concentration and time dependence of the H_2O_2 production manifested as a slight or more obvious increases in the H_2O_2 levels, when comparing the treatments of 20 to 100 µM Cd, and the short-time exposures for 30 to 180 min (Figs 1 and 2).Our results are in accordance with the findings of other authors. Analysis of alfalfa seedlings exposed to 3, 10 and 30 µM Cd revealed increases in cellular ROS, extracellular H_2O_2 and cell death rate after 6 to 24 h exposure (Ortega-Villasante *et al.* 2005, 2007). On the other hand, the higher, milimolar Cd concentrations caused a rapid increase of the number of dead tobacco cells within 2 h that was associated with a transient accumulation of extracellular H_2O_2 within the first 30 min (Garnier *et al.* 2006) Therefore, under extremely toxic conditions it is feasible that cells are rapidly poisoned and become metabolically non-functional, being unable to oxidize fluorescent H_2O_2 markers (Ortega-Villasante *et al.* 2005). Following a short-term (up to 180 min) Cd treatment the accumulation of $O^{\cdot-}_2$ in the epidermal cells within the elongation zone of barley root tips was accompanied by root growth inhibition and radial expansion of cortical cells (15 µM Cd), transient root growth cessation (30 µM Cd) or cell death (60 µM Cd). The generation of $O^{\cdot-}_2$ was at 15 µM Cd transient (Liptáková *et al.* 2012). Cd treatments of wheat induced the reduction of root elongation and dry biomass, and increased Cd accumulation in roots. Cd increased the levels of stress metabolites, such as

malondialdehyde (MDA), H_2O_2, and free proline content (MOUSSA *et al.* 2010). Cd-treated rice seedlings showed stunted growth, decreased length and weight, lower cell viability and less chlorophyll. The levels of H_2O_2 and $O^{\cdot-}_2$ increased in Cd-exposed plants with corresponding increase in the activity of antioxidant enzymes (SINGH and SHAH 2014). Elevated levels of lipid peroxides, increase in $O^{\cdot-}_2$ generation and a concomitant increase in the activities of glutathione peroxidase and superoxide dismutase were noticed in rice seedlings grown in the medium containing 100 and 500 μM Cd (SHAH *et al.* 2001). An increased level of H_2O_2 was found in the roots of *Brassica juncea* seedlings treated with Cd and copper (VERMA *et al.* 2008). Fluorescence and CLSM showed an over-accumulation of $O^{\cdot-}_2$ and H_2O_2 in principal and lateral roots of pea plants (RODRIGUEZ-SERRANO *et al.* 2006). SHAO and YANG (2010) working with roots and leaves of grape variety 'Ze-xiang' treated with 0.5 and 1.0 mM of $CdCl_2$, showed that the root $O^{\cdot-}_2$ generation rate and H_2O_2 and MDA contents increased significantly. At the same concentrations of $CdCl_2$ the $O^{\cdot-}_2$ generation rate in leaves was much lower while the H_2O_2 content was much higher, compared with those in roots.

Since root cell PM is the main point for Cd entering a plant, this work was performed to determine whether Cd can induce any effects at this level. E_M is widely used to report the early events associated with changes in ion permeability and H^+-ATPase activity, thus the effects of Cd on electrical parameters of grapevine epidermal root cells were examined.

The effect of Cd on adventitious root cells was clearly documented by rapid changes of E_M. These changes were different in the cells in various developmental stages within MZ, EZ and DZ (Fig. 3). The 1 mM Cd concentration caused rapid PM depolarization in the cells of all three zones. The extent of depolarization, however, was much greater in the more sensitive MZ and EZ in comparison to DZ, *i.e.* the early developmental stages are more affected in comparison with the later developmental stages. This clearly indicates that the extent of Cd sensitivity as a function of cellular developmental stages should be taken into consideration. Cd, at concentrations lower than 0.02 mM (Fig. 4), induced a transient hyperpolarization which could be due to a change in the diffusion potential across the root cell membranes or increased electrogenic proton pumping (KENNEDY and GONSALVES 1987). The treatment with 0.5-1 mM Cd concentrations depolarized E_M slowly during the first 60 - 150 min nearly reaching the diffusion potential obtained in the presence of anoxia (REPKA *et al.* 2013) and then spontaneous repolarization occurred (Fig. 5). Similar repolarizations of E_M were registered with the same concentrations of Cd in rice, barley and maize roots (LLAMAS *et al.* 2000, PAVLOVKIN *et al.* 2006, SANZ *et al.* 2009). On the other hand no E_M repolarization was observed in 0.1 and 1 mM Cd-treated maize roots (KENNEDY and GONSALVES 1987) and *Impatiens* stems (AIDID and OKAMOTO 1992). The repolarizations in rice, barley and maize roots could be interpreted by assuming that Cd inhibits the active component of E_M, but a possible detoxifying mechanism present in roots would reverse the effect (LLAMAS *et al.* 2000, ARTIUSHENKO *et al.* 2014). In addition to the strong depolarization phase after Cd application, membrane permeability increased during the first hours, however the K^+ efflux rate decreased gradually thereafter (LLAMAS *et al.* 2000). Since the Cd inhibitory effect on the active component of E_M cannot be explained by changes in root respiration, LLAMAS *et al.* (2000) and SANZ *et al*. (2009) suggested a direct effect of Cd on PM H^+-ATPases, possibly by forming a complex with ATP, thus decreasing the availability of the substrate for the H^+-ATPase. Previously it has also been shown that Cd-induced E_M depolarization probably results from a decreased PM H^+-ATPase activity (PAVLOVKIN *et al.* 2006, KURTYKA *et al.* 2011). In contrast to Cd, FC enhances elongation growth by stimulating H^+ efflux driven by PM H^+-ATPase (MARRÉ 1979*)*. Simultaneously with FC-induced proton extrusion, hyperpolarization of PM was observed (PAVLOVKIN *et al.* 2006). Taking into account that FC causes effects opposite to those produced by Cd, it was interesting to study whether this phytotoxin is able to counteract the toxic effect of Cd in grapevine root epidermal cells treated with 10 mM Cd. In our experiments only the highest Cd concentration (10 mM) induced irreversible membrane depolarization evidenced by a permanent inhibition of PM H^+-ATPase that was previously published by JANICKA-RUSSAK *et al.* (2008).

Conclusion

The early effects of Cd on grapevine adventitious roots were found in cell viability and reactive oxygen species (ROS) production. Cd disturbed the plasma membrane (PM) integrity leading to the loss of the cell viability. This response was both concentration-dependent, occurring at the concentrations higher than 60 μM Cd (24 h), and time-dependent, occurring after the time period longer than 30 min (100 μM Cd). Similar concentration-dependence was observed also in the production of both superoxide anion ($O^{\cdot-}_2$) and hydrogen peroxide (H_2O_2), which, however, started to increase already at the lower (20 μM) and continued at the higher (60 and 100 μM) Cd concentrations. The continuous time-dependent increase of ROS production occurred only in the case of H_2O_2, which started to elevate before the decrease of cell viability (already after 10 min). The increase in $O^{\cdot-}_2$ production revealed a biphasic pattern being the highest after 30 min exposure. In addition to concentration- and time-dependence, the early Cd-induced changes of membrane electrical potential (E_M) depended also on the developmental stage of root epidermal cells. The E_M values of the root cells ranged between -115 and -135 mV. In the cells of meristematic (MZ) and elongation (EZ) zones E_M was considerably more negative than in older cells within the differentiation zone (DZ). The extent of the membrane depolarization by 1 mM Cd was similar in MZ and EZ and it was higher in comparison to DZ. The E_M changes reversed at Cd concentrations up to 5 mM, but did not reverse at 10 mM $CdCl_2$ even in the case of fusicoccin addition. This suggests that only the highest Cd concentration inhibited the PM H^+-ATPase.

Acknowledgements

The work was supported by Slovak Grant Agency VEGA, project No. 02/0023/13.

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Received February 19, 2015