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Effect of cadmium on growth, proton extrusion and membrane potential in maize coleoptile segments

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Abstract

Cd accumulation, its effects on elongation growth of maize coleoptile segments, pH changes of their incubation medium and the membrane potential of parenchymal cells were studied. The Cd content increased significantly with exposure to increasing cadmium concentrations. Coleoptile segments accumulated the metal more efficiently in the range 10 - 100 μM Cd, than in the range 100 - 1000 μM Cd. Cd at concentrations higher than 1.0 μM produced a significant inhibition of both growth and proton extrusion. 100 μM Cd caused depolarization of the plasma membrane (PM) potential in parenchymal cells. The simultaneous treatment of maize coleoptile segments by indole-3-acetic acid (IAA) and Cd, counteracted the toxic effect of Cd on growth. Moreover, our data also showed that 100 μM Cd suppressed the characteristic IAA-induced hyperpolarization of the membrane potential, causing membrane depolarization. These results indicate that the toxic effect of Cd on growth of maize coleoptile segments might be, at least in part, caused *via* reduced PM H^+-ATPase activity.

Additional key words: auxin, cadmium, elongation growth, indole-3-acetic acid, membrane depolarization, *Zea mays*.

Introduction

Cadmium, when present in excess, is one of the most phytotoxic metals (reviewed in Sanità di Toppi and Gabbrielli 1999, Seregin and Ivanov 2001). Cd ions are easily taken up by the plant root system and translocated to the shoot where they finally accumulated in the leaves (Salt *et al.* 1995, Di Cagno *et al*. 1999, Haag-Kerwer *et al*. 1999, Wójcik and Tukiendorf 2005). High concentrations of Cd strongly inhibited photosynthesis, respiration, growth and plant development, causing even plant death (Steffens 1990, Greger *et al*. 1991, Krupa *et al*. 1993, Burzynski and Buczek 1994, Krupa and Baszyński 1995, Haag-Kerwer *et al*. 1999, Baryla *et al*. 2001, Linger *et al*. 2005, Dražić *et al.* 2006). Plant growth and development are tightly regulated by phytohormones among which indole-3-acetic acid (IAA) plays a key role (Moore 1989, Davies 2004). It is noteworthy that over the last decade extraordinary progress has been made towards elucidation of the IAA-induced signal transduction pathway, whereas our knowledge of how IAA regulates growth of plant cells in the presence of heavy metals is still very limited.

One important aspect of IAA action, studied in maize coleoptile segments, is its effect on both cell elongation and proton extrusion (Kutschera and Schopfer 1985a,b, Lüthen *et al.* 1990, Peters and Felle 1991a,b, Claussen *et al*. 1996, Karcz *et al*. 1990, 1995, Karcz and Burdach 2002). According to the "acid-growth theory" (Rayle and Cleland 1970, 1992, Hager *et al.* 1971, 1991) auxin-induced proton pumping causes cell wall acidification, which in turn results in enhanced elongation growth. In maize coleoptile segments auxin-induced H^+ extrusion is mediated by increase in either the activity or the amount of plasma membrane (PM) H^+-ATPase (Hager *et al.* 1991, Frias *et al*. 1996).

The goal of the present study was to investigate interrelations between the action of IAA and cadmium on growth of plant cells by: *1*) determining Cd accumulation in maize coleoptile segments which were incubated in medium containing Cd or Cd together with IAA; *2*) studying the effects of Cd on growth in the presence or absence of IAA, where growth and pH changes of the incubation medium were measured simultaneously; *3*) establishing membrane potential changes in parenchymal cells treated with Cd or Cd applied together with IAA.

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Abbreviations: d.m. - dry mass; E_m - membrane potential; IAA - indole-3-acetic acid; PM - plasma membrane; SGR - spontaneous growth response.

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Materials and methods

Seeds of maize (*Zea mays* L. cv. K33 × F2) were soaked in tap water for 2 h, sown on wet wood wool in plastic boxes and placed in a growth chamber at 27 ± 1 °C. The experiments were carried out with 10 mm long coleoptile segments cut from 4-day-old etiolated maize seedlings. The segments with the first leaves removed were excised 3 mm below the tip and collected in water. An aqueous stock solution (1 mM) of IAA (*Serva*, Heidelberg, Germany) was prepared. Cd was added to the incubation medium as $CdCl_2$.

The growth experiments were carried out in an apparatus, which allowed (using the same tissue sample) simultaneous measurements of elongation growth and pH of the incubation medium (Karcz *et al*. 1990, Karcz and Burdach 2002). In this set-up the optical system used for growth measurements (shadow graph method) permitted recording of the longitudinal extension of a stack of 21 segments (10 mm in length each). The volume of the incubation medium (1 mM KCl, 0.1 mM NaCl, 0.1 mM $CaCl_2$; initial pH 5.8-6.0) in the elongation and pH-measuring apparatus was 6.3 cm^3 (0.3 cm^3 $segment^{-1}$). It is noteworthy that in this apparatus the incubation medium also flowed through the lumen of the coleoptile cylinders (Karcz *et al*. 1995). This feature permits the treatment solutions to be in direct contact with the interior of the segments, which significantly enhances both IAA-induced growth of the coleoptile segments and acidification of their medium (Karcz *et al*. 1995). Some growth experiments were also performed with an angular position transducer (*TWK Electronic*, Düsseldorf, Germany) which allowed high resolution measurements of growth rate (Karcz and Burdach 2002). In this system six unabraded coleoptile segments, 10 mm in length each, were strung on a stainless steel needle and inserted vertically in an intensively aerated solution (30 cm^3) with the same composition as that used in the apparatus for simultaneous measurements of elongation and pH of the incubation medium (first system). The length of the segments was sampled every 3 min by a *CX 721* converter (*Elmetron*, Zabrze, Poland) and analysed with *Statistica* program. It should be emphasised that in experiments with an angular position transducer the volume of the incubation medium per coleoptile segment was significantly larger (5 cm^3 $segment^{-1}$) than in the first system (0.3 cm^3 $segment^{-1}$). The temperature of all solutions in the elongation-measuring system was 25 ± 1 ºC. Measurements of pH were performed with a pH-meter (type *CP-315*, *Elmetron*) and pH electrode *OSH 10-10* (*Metron*, Gliwice, Poland). Growth and pH were read every 30 min under the same conditions.

The electrophysiological experiments were performed on intact, 10 mm long, coleoptile segments. The standard technique was used, as previously described by Stolarek and Karcz (1987) and Karcz and Burdach (2002). Briefly, the membrane potential (E_m) was measured by recording the voltage between a 3 M KCl-filled glass micropipette inserted into the parenchymal cells and a reference electrode in the bathing medium containing the same composition as used in growth experiments. Before the electrophysiological experiments the coleoptile segments were preincubated for 2 h in an aerated bathing medium. After this period one of them was transferred into a perfusion plexiglass chamber, which was mounted on a microscope stage. The flow of the medium was driven by a peristaltic pump (type *PP 1B-05A*, *Zalimp*, Warszawa, Poland), which allowed a change of the bathing medium in the chamber (usually fourfold within less than 2 min). The microelectrodes were inserted into the cells under the microscope by means of a micromanipulator (*Hugo Sachs Elektronik*, March-Hugstteten, Germany). Micropipettes were prepared as previously described by Karcz and Burdach (2002).

The concentrations of Cd in maize coleoptile segments were measured by emission spectrometry with excitation by argon inductively coupled plasma technique (ICP-AES). Before chemical analysis, 200 coleoptile segments were split along the long axis and preincubated for 2 h in an intensively aerated growth medium (solution with the same composition as that used in growth experiments). The volume of the incubation medium was 60 cm^3 (0.3 cm^3 $segment^{-1}$). After 2 h of preincubation, Cd (at concentrations 10, 100, 1000 μM) or Cd together with IAA was introduced (for the next 5 h) to the incubation medium. After 5 h of incubation in the presence of cadmium the halves of the segments were removed from the solution and washed 3 times with distillated water, whereupon they were dried at 80 ºC to obtain a stable mass. In addition, accumulation of Cd at 100 μM Cd in the medium was studied as a function of time. For Cd analyses, dry plant tissue was digested with ultra-pure concentrated nitric acid (*Merck*, Darmstadt, Germany). All experiments concerning accumulation of Cd were replicated at least three times.

Results

Cadmium content in maize coleoptile segments: Prior to the growth experiments, in which the effects of various Cd concentrations on growth of maize coleoptile segments were studied, the accumulation of cadmium in this model system had been determined (Fig. 1). At 10 and 100 μM Cd in the medium, cadmium content in maize coleoptile segments, within 5 h, increased to 176.8 ± 18.4 mg kg^{-1}(d.m.) and 1900.8 ± 178.6 mg kg^{-1}(d.m.) (*ca*. 10-fold increase of Cd content), respectively. This indicates that the content of Cd in coleoptile segments

increased proportionally with increasing cadmium concentration in the incubation medium. However, a total Cd content of 4401.2 ± 190.3 mg kg^{-1} (d.m.), after 5 h, was found in maize coleoptile segments treated with 1000 µM Cd, which means that the increase of Cd concentration in the incubation medium from 100 to 1000 µM caused only a 2.3-fold increase of the Cd content in the tissue. In turn, at 100 µM Cd its content in maize coleoptile segments increased non-linearly (biphasic relation) in time (Fig. 1, inset). IAA did not change the content of Cd in the coleoptile segments (Fig. 1).

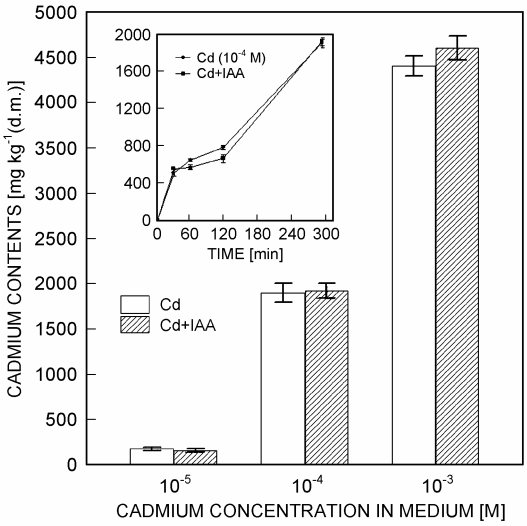

Fig. 1. Cd content in maize coleoptile segments exposed (for 300 min) to either 10, 100 and 1000 µM Cd or Cd added together with (10 µM) IAA. The segments were firstly preincubated (for 2 h) in control medium, whereupon Cd or Cd with IAA was added. The inset shows the time-dependent accumulation of Cd in maize coleoptile segments which were incubated for 30, 60, 120 and 300 min at 100 µM Cd. Results are the means of three independent experiments ± SE.

Effect of Cd in absence or presence of IAA on growth: Cd added to the incubation medium (after 2 h of segment's preincubation in control medium) at concentrations higher than 0.1 µM diminished growth of maize coleoptile segments (Fig. 2). For example, Cd added at a final concentrations 100 and 1000 µM inhibited (over 5 h) elongation growth of maize coleoptile segments by 41 and 59 %, respectively, as compared to the growth in control medium (941.6 ± 44.8 µm cm^{-1}). However, in the presence of 1.0 and 10 µM Cd the inhibition of the segment elongation did not exceed 15 %. Application of IAA together with Cd at concentrations higher than 0.1 µM conteracted the toxic effect of cadmium on growth, *i.e.* at low Cd concentrations growth in the presence of IAA was even greater than growth in control (941.6 ± 44.8 µm cm^{-1}) (Fig. 2). It was observed 40 % decrease of the IAA-induced growth rate at 100 and 1000 µM Cd (Fig. 3). Interestingly, at 0.1 µM Cd stimulation (by 10 - 15 %) of growth (in the absence or presence of IAA) was observed (Fig. 2).

The effect of cadmium on pH changes of the incubation medium: When the coleoptile segments are incubated in control medium pH initially increased, then within 2 h it usually reached near neutral value, and decreased to 5.5 - 5.7 after 7 h. Addition of IAA to the control medium (after 2 h of preincubation) caused an additional drop of pH to 5.2 - 5.3. Expressing the

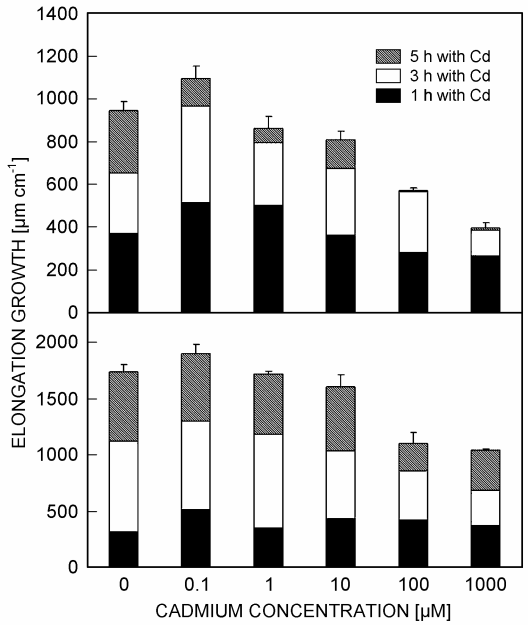

Fig. 2. Effect of Cd (0.1 - 1000 µM) on the growth of maize coleoptile segments incubated in the absence of IAA (*above*) or presence of IAA (*below*). The coleoptile segments after 2 h of their preincubation in control medium were treated 5 h with Cd. The growth of maize coleoptile segments after 1, 3 and 5 h is shown (see legend). Results are the means of 6 to 10 independent experiments ± SE.

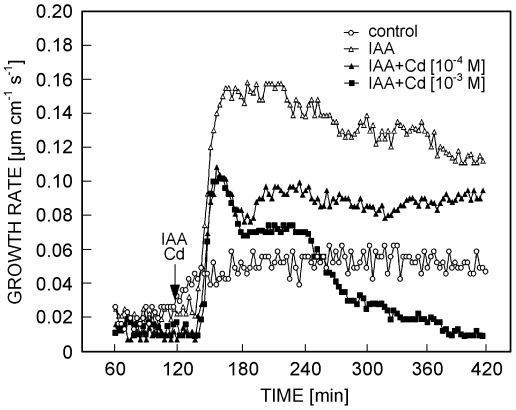

Fig. 3. Effect of Cd (100 - 1000 µM) on growth rate of maize coleoptile segments incubated in the presence of IAA. The growth rate of six coleoptile segments (10 mm in length) was recorded in an intensively aerated solution (5 cm^3 $segment^{-1}$) by means of an angular transducer. The coleoptile segments were first preincubated (for 2 h) in control medium (solution without IAA and Cd), whereupon Cd together with IAA was added (*arrow*). Representative curves are shown.

acidification of the external medium as the difference between pH at 420 min and 120 min (ΔpH; Figs. 4, 5), suggested that cadmium at concentrations higher than 10 µM significantly suppressed proton extrusion during growth in the absence or presence of IAA.

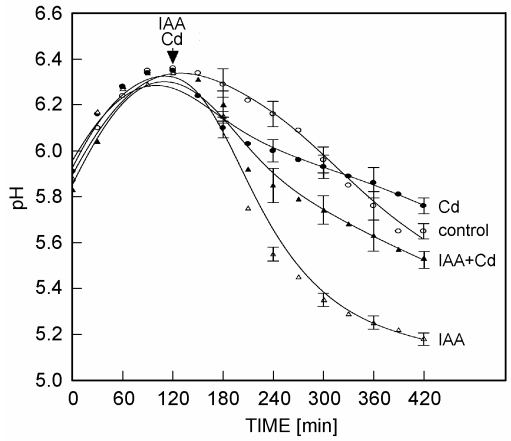

Fig. 4. Effect of Cd (100 µM) on pH changes in the incubation medium measured simultaneously (using the same tissue sample). The segments were first preincubated (for 2 h) in control medium, whereupon IAA, Cd or Cd together with IAA were added. Representative curves for each treatment are shown. Adequate mean values are indicated in Fig. 5.

The effect of Cd and IAA on the membrane potential (E_m): The E_m of the parenchymal cells, before being changed in response to Cd, was 124.6 ± 3.8 mV. The addition of Cd to the incubation medium (Fig. 6) caused depolarization of E_m during which the membrane potential became by 59 ± 6.3 mV more positive than the original potential. However, when Cd was added together with IAA a transient hyperpolarization followed by a rapid depolarization of the E_m by 65.6 ± 6.8 mV. By contrast, IAA alone (10 µM) caused a transient depolarization of E_m followed by a delayed hyperpolarization, during which the membrane potential became by 15.6 ± 3.6 mV more negative than the original potential.

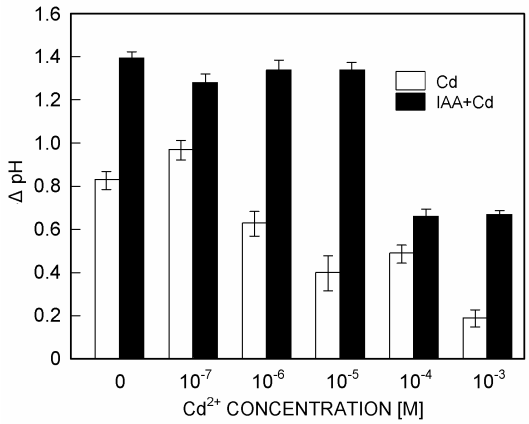

Fig. 5. Effect of Cd (0.1 - 1000 µM) on the medium pH. After preincubation of the coleoptile segments for 2 h in control medium Cd or Cd together with IAA were added. ΔpH - absolute values of difference of pH at 420 min and 120 min. Bars indicate ± SE.

Discussion

Despite the considerable research devoted to the responses of plants to Cd, the mechanism by which Cd affects growth of plant cells remains unsolved. The main objective of this work was to evaluate the effect of Cd on growth of maize coleoptile segments and pH changes of their incubation medium measured simultaneously with growth. Moreover, we have also determined the capacity of coleoptile segments to accumulate this metal and the effect of Cd on the membrane potential of parenchymal cells.

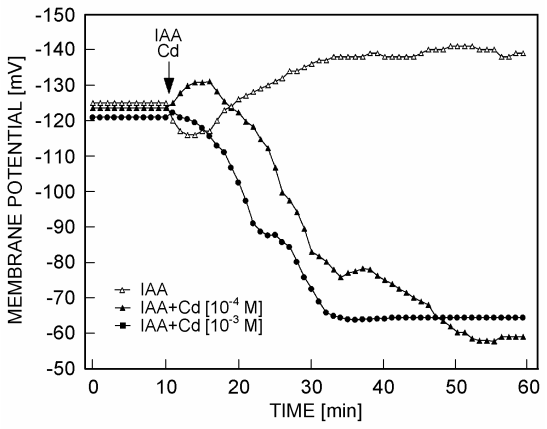

Fig. 6. Membrane potential (E_m) changes of parenchymal coleoptile cells upon addition of Cd (100 µM), IAA (10 µM) and Cd together with IAA. At time 10 min (*arrow*) the control medium was changed for a new one, at the same salt composition, containing in addition Cd, IAA or Cd together with IAA. Mean curves for each treatments are shown.

Our data concerning Cd accumulation clearly show that the accumulation of this metal by maize coleoptile segments is a concentration and time-dependent process. In the range 10 - 100 µM Cd, coleoptile segments accumulated the metal more efficiently than in the range 100 - 1000 µM Cd. Our results on the accumulation of Cd in maize coleoptile segments support the idea that the Cd-influx is firstly linear (associated with the accumulation of Cd in the apoplast), and then non-linear (saturable component associated with transporter-mediated Cd-influx across the plasma membrane) (Cohen *et al*. 1998, Hart *et al.* 1998).

The simultaneous measurements of elongation growth and pH of the incubation medium showed that in maize coleoptile segments Cd at concentrations higher than 10 µM produced a significant inhibition of growth (Fig. 2) as well as a reduction in proton extrusion (Figs. 4

and 5). Moreover, Cd at the concentration of 100 μM caused a depolarization of the membrane (Fig. 6). An explanation of the inhibition of growth by Cd might be suggested on the basis of the hypothesis proposed for the nature of the "spontaneous growth response" (SGR - growth acceleration of the segments observed 2 - 4 h after their excision from the seedlings). It has been proposed that this growth acceleration is caused by a stimulation of IAA synthesis in the coleoptile segments after their excision from the seedlings (Evans and Schmitt 1975, Weiler *et al*. 1981) or by a time-dependent increase in tissue sensitivity to the low endogenous IAA which remains after excision of the segments (MacDowell and Sirois 1977, Vesper and Evans 1978, Hatfield and LaMotte 1984). An alternative hypothesis has also been proposed by Hager (2003), who suggested that the SGR of coleoptile segments submerged in water is, at least in part, the result of respiratory CO_2 that accumulates in the tissue causing acidification of the cytoplasm, which in turn leads to PM H^+-ATPase activation. Also, some recent investigations, showing that the changes in extracellular proton concentration share similarity with the endogenous growth rate in excised maize coleoptile segments, suggest that the PM H^+-ATPase is involved in the induction of the SGR (Peters *et al.* 1998, Karcz and Burdach 2002). A different scenario can be proposed for the explanation of Cd inhibition of growth. Firstly, Cd decreasing IAA synthesis or tissue sensitivity to IAA may diminish PM H^+-ATPase activity. Secondly, Cd may affect respiration in maize coleoptile segments. There are data indicating that Cd inhibites respiration in different plants (Greger *et al*. 1991, Burzynski and Buczek 1994, Haag-Kerwer *et al.* 1999, Llamas *et al*. 2000). Thus, toxic effect of Cd on growth in maize coleoptile segments may result from indirect inhibition of PM H^+-ATPase activity. Both Cd-produced inhibition of proton extrusion and depolarization of the membrane potential observed here in the presence of Cd might supported this hypothesis. Our hypothesis agrees with the previous findings of other authors who also found an inhibition of PM H^+-ATPase (Fodor *et al*. 1995, Ros *et al*. 1992b) and depolarization of the membrane potential by Cd (Kennedy and Gonsalvez 1987, Aidid and Okamoto 1992, Llamas *et al.* 2000).

The growth, proton extrusion and changes in membrane potential observed here in the presence of IAA are in agreement with the results obtained with maize coleoptile segments by other authors (Kutschera and Schopfer 1985a, Felle *et al.* 1986, 1991, Lüthen *et al*. 1990, Peters *et al*. 1992, Claussen *et al*. 1996) and recently also by us (Karcz and Burdach 2002). These authors showed that in this model system IAA stimulates rapid growth, proton extrusion and a transient depolarization followed by a slow membrane hyperpolarization. To date, there is no doubt that the slow plasma membrane hyperpolarization is a consequence of a stimulated proton extrusion through the PM H^+-ATPase (Lohse and Hedrich 1992, Rücke *et al.* 1993, Hedrich *et al*. 1995).

Application of IAA together with Cd counteracted the toxic effect of cadmium on growth in maize coleoptile segments (Fig. 2). Moreover, our data also showed that cadmium at 100 μM suppressed the characteristic IAA-induced hyperpolarization of the membrane, causing depolarization of the E_m (Fig. 6). The experiments in which IAA was added showed that Cd at concentrations higher than 10 μM inhibits proton pumping, which is responsible for IAA-induced growth. However, how Cd diminishes PM H^+-ATPase activity is not entirely resolved, although it was suggested that Cd might decrease H^+-ATPase activity by the alteration of the membrane fluidity and lipid composition (Ros *et al.* 1992a, Fodor *et al.* 1995, Hernández and Cooke 1997, Ouariti *et al.* 1997) or by the binding of the metal to sulfhydryl groups of the enzyme (Stobart *et al*. 1985, Van Assche and Clijsters 1990, Lagriffoul *et al*. 1998).

In conclusion, the obtained results show that in maize coleoptile segments Cd at concentrations higher than 10 μM produced a significant inhibition of growth as well as a reduction in the proton extrusion. The treatment of maize coleoptile segments by IAA added together with Cd counteracted the toxic effect of Cd on growth. In addition, it was found that Cd at 100 μM suppressed the characteristic IAA-induced hyperpolarization of the membrane. It is suggested that the toxic effect of Cd on the growth of maize coleoptile segments might be, at least in part, caused via reduced PM H^+-ATPase activity.

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