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Communication

Cadmium-induced changes in antioxidant enzymes in suspension culture of soybean cells✪

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Cadmium (Cd), similarly to other heavy metals, inhibits plant growth. We have recently showed that Cd^{2+} either stimulates (1–4 μM) or inhibits (≥ 6 μM) growth of soybean (*Glycine max* L.) cells in suspension culture (Sobkowiak & Deckert, 2003, *Plant Physiol Biochem.* 41: 767–72). Here, soybean cell suspension cultures were treated with various concentrations of Cd^{2+} (1–10 μM) and the following enzymes were analyzed by native electrophoresis: superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APOX). We found a significant correlation between the cadmium-induced changes of soybean cell culture growth and the isoenzyme pattern of the antioxidant enzymes. The results suggest that inhibition of growth and modification of antioxidant defense reactions appear in soybean cells when Cd^{2+} concentration in culture medium increases only slightly, from 4 to 6 μM.

The increasing amount of cadmium (Cd) in the environment affects various physiological and biochemical processes in plants (Sanitia di Toppi & Gabbrielli, 1999). The most pronounced effect of heavy metals on plant development is growth inhibition, which is inseparably connected with cell division. However, the mechanisms involved in those processes are still not completely understood. Among other effects, Cd causes production of reactive oxygen species (ROS) in plant and animal cells (Olmos *et al.*, 2003; Szuster-Ciesielska *et*

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Abbreviations: APOX, ascorbate peroxidase; CAT, catalase; POX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

al., 2000). Cadmium, unlike other heavy metals, such as Cu, seems not to act directly on the production of ROS (*via* Fenton and/or Haber-Weiss reaction) (Sanitia di Toppi & Gabbrielli, 1999). Irrespective of the production pathway, ROS are highly cytotoxic and their level within plant cells must be controlled by enzymatic and non-enzymatic antioxidant defense systems.

We have recently showed that, depending on their concentration, cadmium ions (Cd^{2+}) either stimulate (1–4 μM) or inhibit ($\geq$ 6 μM) growth of soybean cells in suspension culture (Sobkowiak & Deckert, 2003). In the present study we were interested in establishing a correlation between cadmium-induced changes of soybean cell culture growth and isoenzyme pattern of the following antioxidant enzymes: superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7) and ascorbate peroxidase (APOX, EC 1.11.1.6).

MATERIALS AND METHODS

Cell culture. Suspension culture of soybean (*Glycine max* L. cv. Naviko) cells was established from hypocotyl cells of 5-day old seedlings and maintained as previously described (Sobkowiak & Deckert, 2003). Cells were grown in liquid culture in modified B5 Gamborg medium, supplied with 20 $g \cdot L^{-1}$ sucrose, 1 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid at pH 5.5 and maintained at 24°C in darkness on a rotary shaker (Certomat) at 150 r.p.m. At 7-day intervals, the culture was subcultured. For cadmium treatment experiments, freshly diluted subcultures were grown for 4 days as described above, followed by addition of $CdCl_2$. The final concentrations of Cd^{2+} in various cultures were as follows: 1, 2, 3, 4, 6, 7, 8, 9 and 10 μM. After 7 days the cultured cells were collected by vacuum filtration, followed by a quick freeze in liquid N_2 and then used for protein isolation.

Protein extraction, native gel electrophoresis and enzyme activity staining. Cell suspension (0.5 g) was homogenised with a mortar and pestle in 50 mM Tris/HCl (pH 7.5) on ice. For the APOX assay, proteins were extracted in 0.1 M sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate and 1 mM EDTA. The slurry was centrifuged for 15 min at 12 000 $\times$ ***g***. The supernatant was stored at –80°C. Protein concentration in the samples was estimated according to Bradford (1976).

Samples of crude soybean extracts were electrophoresed in 10% (SOD, POX and APOX) or 8% (CAT) (w/v) polyacrylamide slab gel at pH 8.9 under nondenaturing conditions according to Davis (1964). Isoenzymes of antioxidant enzymes were visualised in gels by the methods of Beauchamp & Fridovich (1971) for SOD, Woodbury *et al.* (1971) for CAT, Ros Barceló (1987) for POX, and Mittler & Zilinskas (1993) for APOX.

RESULTS AND DISCUSSION

The aim of this work was to establish a correlation between cadmium-induced changes of the isoenzyme pattern of antioxidant enzymes and the previously described effect of this metal on soybean cell culture growth (Sobkowiak & Deckert, 2003). The Cd^{2+} concentrations used corresponded to those observed in natural conditions in contaminated soil (Sanita di Toppi & Gabbrielli, 1999).

Isoenzyme patterns of antioxidant enzymes were analyzed in soybean cell suspension cultures cultivated in the presence of various concentrations of Cd^{2+} (1–10 μM). Cd-dependent changes in isoenzyme patterns were observed for superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APOX). Total SOD activity was not considerably affected by different concentrations of Cd^{2+} (not shown), but the isoenzyme pattern was significantly dif-

ferent at the growth-inhibiting concentrations of Cd^{2+} ($\geq 6\,\mu M$) (Fig. 1) compared with

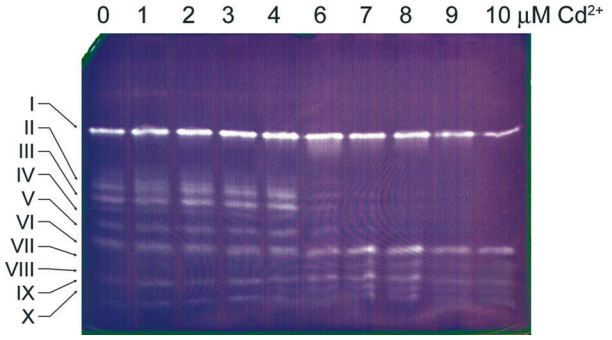

Figure 1. Isoenzyme pattern of SOD in soybean cells treated for 7 days with various concentrations of Cd^{2+} (1–10 μM).

Proteins were electrophoresed in non-denaturing polyacrylamide gel and the isoenzymes of SOD were visualised as described in Materials and Methods.

the non-inhibiting ones. The most visible effect was the gradual disappearance with increasing Cd^{2+} concentration of isoenzymes: II, III, IV and V, with concomitant appearance of isoenzymes VI, VII, VIII, IX and X. The activity of isoenzyme I was the same in control cells and those treated with various concentrations of Cd^{2+}.

Total CAT activity was lower in soybean cells treated with growth-inhibiting concentrations of Cd^{2+} ($\geq 6\,\mu M$) compared with control cells or those treated with low concentrations of Cd^{2+} (1–4 μM) (Fig. 2). CAT is represented by one isoenzyme (I) in cells growing in the presence of 0–4 μM Cd^{2+} and another (II) within cells treated with higher concentrations of Cd^{2+} ($\geq 6\,\mu M$). The activity of CAT I increased gradually between control cells and those grown at 1–4 μM Cd^{2+}, whereas the activity of CAT II was highest at concentration of 6 μM Cd^{2+} and decreased at higher concentrations of Cd^{2+} (7–10 μM).

No detectable differences were observed in APOX isoenzyme pattern and total activity between control cells and those treated with various concentrations of Cd^{2+} (1–10 μM) (not shown).

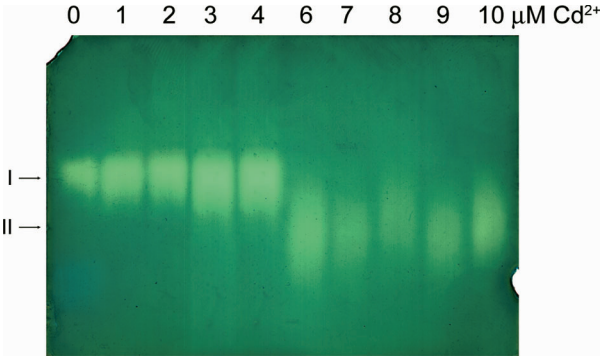

Figure 2. Isoenzyme pattern of CAT in soybean cells treated for 7 days with various concentrations of Cd^{2+} (1–10 μM).

Proteins were electrophoresed in non-denaturing polyacrylamide gel and the isoenzymes of CAT were visualised as described in Materials and Methods.

In contrast to APOX, POX activity and isoenzyme pattern were strongly affected by higher concentrations of Cd^{2+} ($\geq 6\,\mu M$), at which new isoenzymes, designated as III, V and VI appeared. The activities of other isoforms (I, II, IV) were enhanced compared to control cells or those treated with lower Cd^{2+} concentrations (1–4 μM).

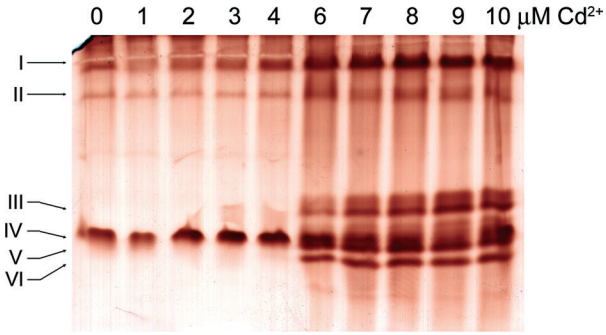

Figure 3. Isoenzyme pattern of POX in soybean cells treated for 7 days with various concentrations of Cd^{2+} (1–10 μM).

Proteins were electrophoresed in non-denaturing polyacrylamide gel and the isoenzymes of POX were visualised as described in Materials and Methods.

The available data indicate that depending on the plant species, tissue tested and the dose of the metal, Cd^{2+} can either stimulate or inhibit the activities of several antioxidant enzymes (Shaw, 1995; Gallego *et al.*, 1996;

Chaoui *et al.*, 1997; Vitoria *et al.*, 2001). Here we show that Cd^{2+}-induced changes of the isoenzyme patterns of SOD, CAT and POX are accompanied with the recently described inhibition of soybean cell culture growth (Sobkowiak & Deckert 2003). We suggests that the isoenzymes of SOD, CAT and POX present in soybean cells growing in the presence of 0–4 μM of Cd^{2+} are required to remove ROS produced during normal, physiological processes. When the concentration of Cd^{2+} is $\geq$ 6 μM, the level of ROS (produced indirectly by Cd^{2+}) becomes too high to be dealt with by the existing antioxidant isoenzymes. Thus, soybean cells switch on additional antioxidant enzyme-dependent defense reactions, which involve novel isoenzymes. The induction of stress-related isoenzymes is probably related to the level of ROS, which cause oxidative damage of various cellular components, such as proteins, membrane lipids and nucleic acids (Halliwell & Gutterdge, 1989). Many authors have suggested that heavy metal-induced oxidative stress may be responsible for inhibition of plant growth processes (Rucińska *et al.*, 1999; Sandalio *et al.*, 2001; Vitoria *et al.*, 2001). The present paper shows for the first time that the switch between physiological oxidative response and a stress-related one occurs at a very narrow range of the intensities of the stress factor, i.e. Cd^{2+} concentration. In the study presented here such a change takes place between 4 and 6 μM Cd^{2+}.

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