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What is the crucial difference between the metabolic consequences of cadmium and zinc treatment of the plants?

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ABSTRACT Heavy metal stress induced alterations in the activities of several representatives of the enzymatic antioxidant defense system such as guaiacol peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) were comparatively studied in barley seedlings and alfalfa aldose reductase overexpressing transgenic tobacco treated by Zn and/or Cd. Although roots were the main sites of metal accumulation, metals induced oxidative damage mainly in the leaves. Our experiments clearly show that the investigated heavy metals have quite different effects on plant metabolism and induce the protective enzymatic system in different ways. Transgenic tobacco plants showed increased tolerance against cadmium induced stress.
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KEY WORDS

heavy metals
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Plants cannot avoid permanent response to the fluctuations in their environment in order to succesfully adjust to their altered environments including the extreme concentration of different heavy metals. Heavy metals interfere with several biochemical processes of plants either directly interacting with certain cellular processes or through a seria of consecutive processes mediated by different signalling pathways (Clemens 2001). One of the most important elements of this latter modulations is the generation of active oxygen species (AOS), such as superoxide radicals ($O_2^{\bullet}$), hydrogene peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$; Salin 1987).

The redox-modulated changes that follow central events in cellular responses to the oxidative stress from environmental resources are functioning inevitably to a certain extent in plant cells even under non-stressed conditions. Plants have evolved various protective enzymatic and non-enzymatic antioxidant mechanisms to eliminate or reduce AOS species (Elstner 1982; Salin 1987; Mittler et al. 2004). One of them is the enzymatic antioxidant system, including SOD (superoxide dismutase), POD, CAT and APX. These enxymes are localized in various cell compartments and act either sequentially or simultaneously. Superoxide dismutases (SOD; E.C.1.15.1.1.) are located in the cytosol, chloroplasts and mitochondria and catalyze the disproportiation of two $O_2^{\bullet}$ radicals to O_2 and H_2O_2 (Salin 1987), that can be eliminated by peroxidases (POD, APX) or catalase (CAT; Elstner 1982). Each of these enzymes has a physiological function under non-stressed conditions, but their activity/or quantity is significantly altered under oxidative stress.

We posed the question, how this antioxidative enzymatic system (POD, APX, CAT) functions in seedlings exposed to two heavy metals – Zn and Cd – similar elements in chemical terms but profoundly differing from each other in their metabolic role of the physiology of living organisms. While Zn is an essential microelement that is indispensable for normal plant growth at low concentration and is toxic only at high concentration, Cd has no vital function in the metabolism of plants developing under "natural" conditions. Tobacco plants overexpressing alfalfa aldehyde reductase (ALR) proved to be efficient in counterbalancing the effects of active oxygen species induced by several stress factors including low temperature, drought, UV-B radiation, viruses etc. (Oberschall et al. 2000; Hideg et al. 2003; Hegedűs et al. 2004). The experiments were extended to reveal whether these transgenic plants are able to eliminate the heavy metal generated AOS as well.

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Materials and Methods

Plant materials

Barley seedlings (*Hordeum vulgare* L. cv. Triangle) were grown on moisture filter paper in dark at 20°C for four days. Then they were further grown in hydroponics of a half strength Hoagland solution (Terry 1980) and put into a growth chamber (Conviron S10H) operating with 12/12 hours light/dark cycles at the light intensity of 150 μmole/s/m^2 PPFR at constant temperature of 23°C. The seven-day-old seedlings were transferred into fresh medium supplemented with different concentrations of $CdCl_2$ or $ZnSO_4$ and plants were further grown at the growth conditions described above. After incubation for time intervals as indicated, samples were taken and analyzed.

Tobacco (*Nicotiana tabacum* cv. Petit Havanna line SR1) plants were transformed by alfalfa aldose reductase cDNA attached to the viral CaMv35S constitutive promoter. Wild type (SR1) and two transgenic lines (ALR1/5 and ALR1/9) which were previously shown by Western hybridization to overexpress the alfalfa ALR protein (Oberschall et al. 2000). Seedlings were selected on kanamycin containing medium then they were grown in a Conviron S10H growth chamber (Conviron Ltd., Winnipeg, Canada) operating with 12/12 hours light and dark cycles at 23°C. The PPFD was 150 μmol m^{-2} s^{-1} and the relative humidity was 75%.

Determination of Cd and Zn content content

One g of plant tissue was dehydrated at 105°C for 24 hours and 0.1 g of powdered dry material was dissolved in 2 ml of HNO_3:H_2O_2 mixture (1:1 v/v). The metal content of the filtered solution was determined by atomic absorption spectroscopy (ICAP 61E Plasma Spectrometer, Thermo Jarell Ash Corporation, Franklin, Mass., USA; Horváth et al. 1996).

Chlorophyll determination

The chlorophyll content was determined in 80% acetone extract of 0.1 g leaf as described by Arnon (1949).

Preparation of tissue extract

One g of plant tissue was homogenized with three fold excess of buffer containing 0.05 M Tris (pH 8.0), 0.004 M citric acid, 0.008 M cysteine, 0.005 M ascorbic acid, 0.01 M $MgCl_2$ and sucrose (Arulsekar and Parfitt 1986). The filtered tissue extract was centrifuged at 10000xg for 30 min. The whole procedure was carried out at 4°C. The supernatant was used for further analyses.

Assays of enzyme activities

POD and CAT activities were determined using guaiacol and H_2O_2 substrates, respectively as described by Chance and Maehly (1955), while the APX activity was measured according to Nakano and Asada (1981) and the activities were expressed in μkat/g.fr.w.

Statistical analysis

All measurements were repeated in 6 to 9 independent experiments and the determination of enzyme activities was performed with 3 parallels in all cases. Standard deviations were calculated from 18-27 samples.

Results and Discussion

The dose- and time-responses of Zn and Cd accumulation in the leaves and roots of barley seedlings are summarized in Figure 1. Both heavy metals have been accumulated in the plants; the accumulation was proportional to with the progress of incubation time and metal concentration of culture medium. The primary site of the metal accumulation was the root. Similar correlations and accumulation patterns were observed both in SR1 and ALR overexpressing transformants

The characteristic visual symptom of heavy metal accumulation was quite different in the Zn and Cd treated seedlings. While with the enhanced Cd accumulation the reduction of the chlorophyll content of leaves could be observed, there was no substantial, visible sign of the Zn treatment. This observation was strengthened by the determination of the chlorophyll content of leaves (Fig. 2). In Cd treated seedlings 0,3-1 mM Cd concentrations resulted in 60% reduction after a 4 day treatment, but even the highest (1 mM) Zn concentration and the longest (7 day) treatment had no effect on the chlorophyll content of leaves. The dramatic reduction in the chlorophyll content after Cd treatment is not surprising, as Cd has been proved to be an aggressive, oxidative damage inducing agent especially harmful to photosynthetic processes involving the various complexes of chloroplast membranes (Stobart et al. 1985; Sanitá di Toppi and Gabbrielli 1999). Zn-ions at high concentration also induce oxidative damage (Chaoui et al. 1997), but at the same time they are essential cofactors of chlorophyll-synthetizing δ-aminolevulinic acid dehidratase. Our observation can be explained at least in two different ways. For one the intracellular localisation of Zn is different from that of Cd, for the other the antioxidative protective system can overcome the detoriating effect of Zn. In the case of ALR transformant tobacco plants the decrease of chlorophyll content was slightly smaller than that in the parent SR1 plants.

To answer these questions we have determined the activity of characteristic antioxidative enzymes. Among various enzymes involved in the abolishment of AOS, guiacol peroxidase can be considered as one of the key ones, since both of its extra- and intracellular forms participate in the breakdown of H_2O_2 (Van Assche and Clijsters 1990).

The POD activity was determined in Zn- and Cd-treated seedlings, and the data are presented in Figure 3. Isoenzyme analysis showed a general increase in the intensity of individual bands. Under all examined experimental conditions and plant systems there was increase in POD activity , although to different extent. It was the highest in Cd treated leaves, despite the fact, that the heavy metals are primarily accumulated in the roots. Cd-treatment even at the lowest concentration resulted in a substantial increase of enzyme activity, while Zn provoked considerable enzyme activity increase only at the highest concentration and after longer incubation time (7 day).

Catalase, which is located mostly in peroxysomes and participates in the breakdown of the photorespiratory H_2O_2 (Foyer et al. 1994) could not be detected in roots and showed no significant alterations in either experimental system (Fig. 4). This is suprising as peroxisomes can have key role in the

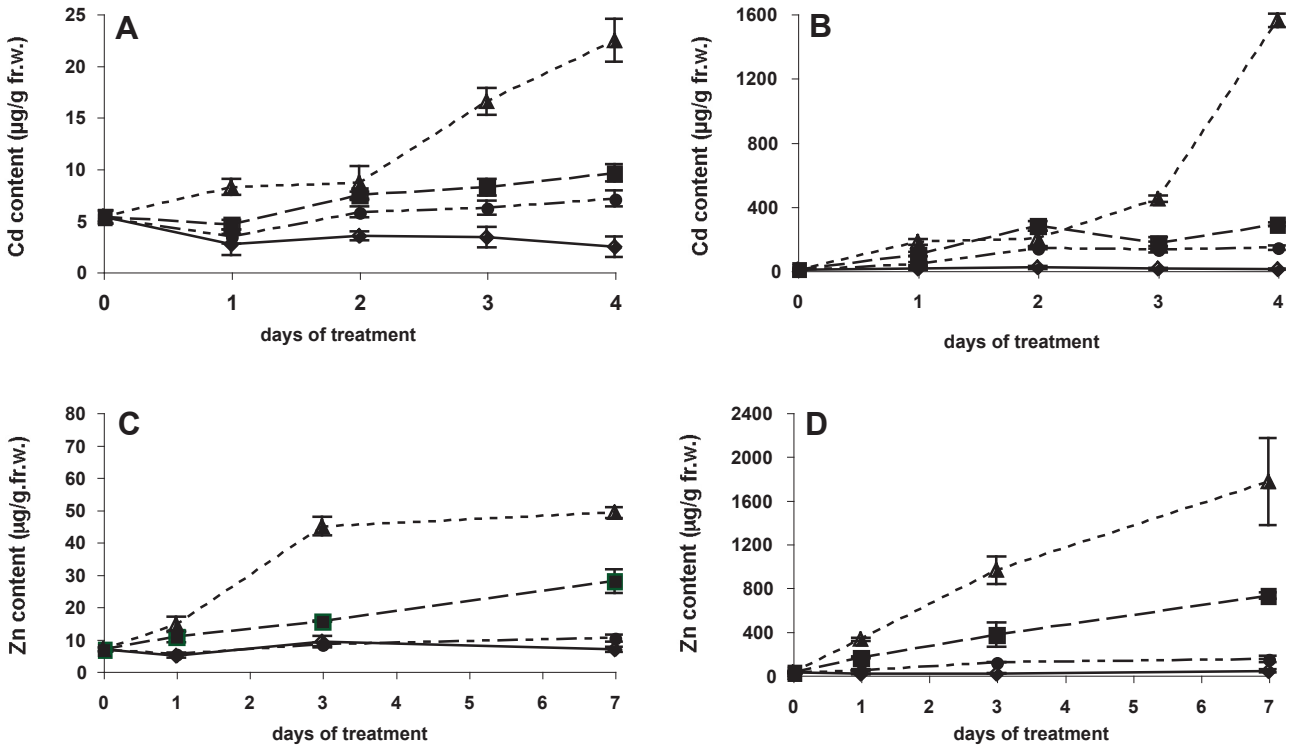

Figure 1. Cd (A, B) and Zn (C, D) accumulation in leaves (A, C) and roots (B, D) in barley seedlings. (♦) control; (●) 0,1 mM; (■) 0,3 mM; (▲) 1 mM Zn and Cd respectively. (Data presented as mean ± S.D., n=18).

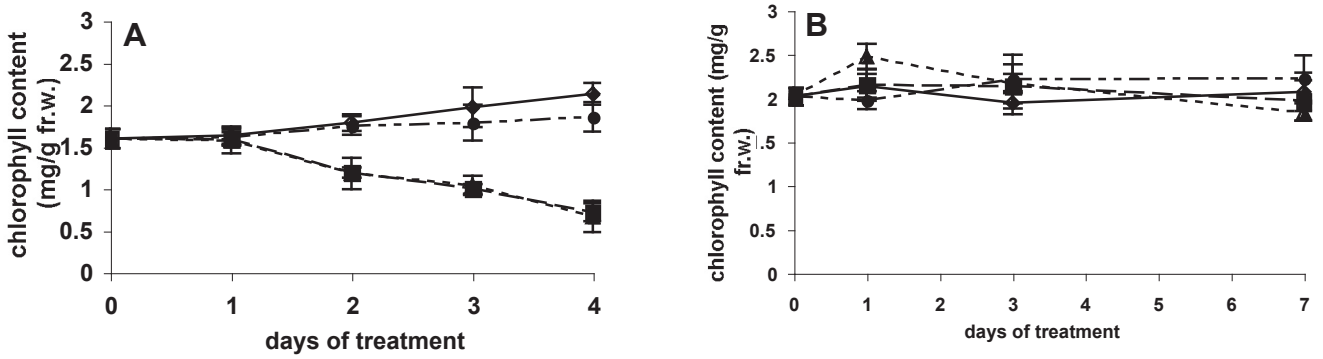

Figure 2. Changes in the chlorophyll content of leaves after Cd (A) and Zn (B) treatment. (♦) control; (●) 0,1 mM; (■) 0,3 mM; (▲) 1 mM Zn and Cd respectively. (Data presented as mean ± S.D., n=18).

elimination of AOS (Lopez-Huartes et al. 2000).

As a member of the ascorbic acid-glutathione cycle APX is one of the most important enzymes playing a crucial and regulatory signaling role in eliminating poisonous H_2O_2 from plant cells (Foyer et al. 1994; Davletova et al. 2005). In Cd-treated seedlings the APX activity of roots was proportionally increased with higher Cd concentration and further enhanced by the progress of incubation time (Fig. 5). It has to be emphasized that at all Cd concentrations the APX activity was dramatically reduced after the third day of treatment. In leaves the APX activity exhibited similar changes as those found in roots but no inhibition could be detected at the highest, 1 mM Cd concentration. Zn-treatment did not casue significant changes in APX activity (Fig. 5) at any concentrations and after any days of treatment neither in roots nor in leaves. This fact is an additional proof emphasizing the

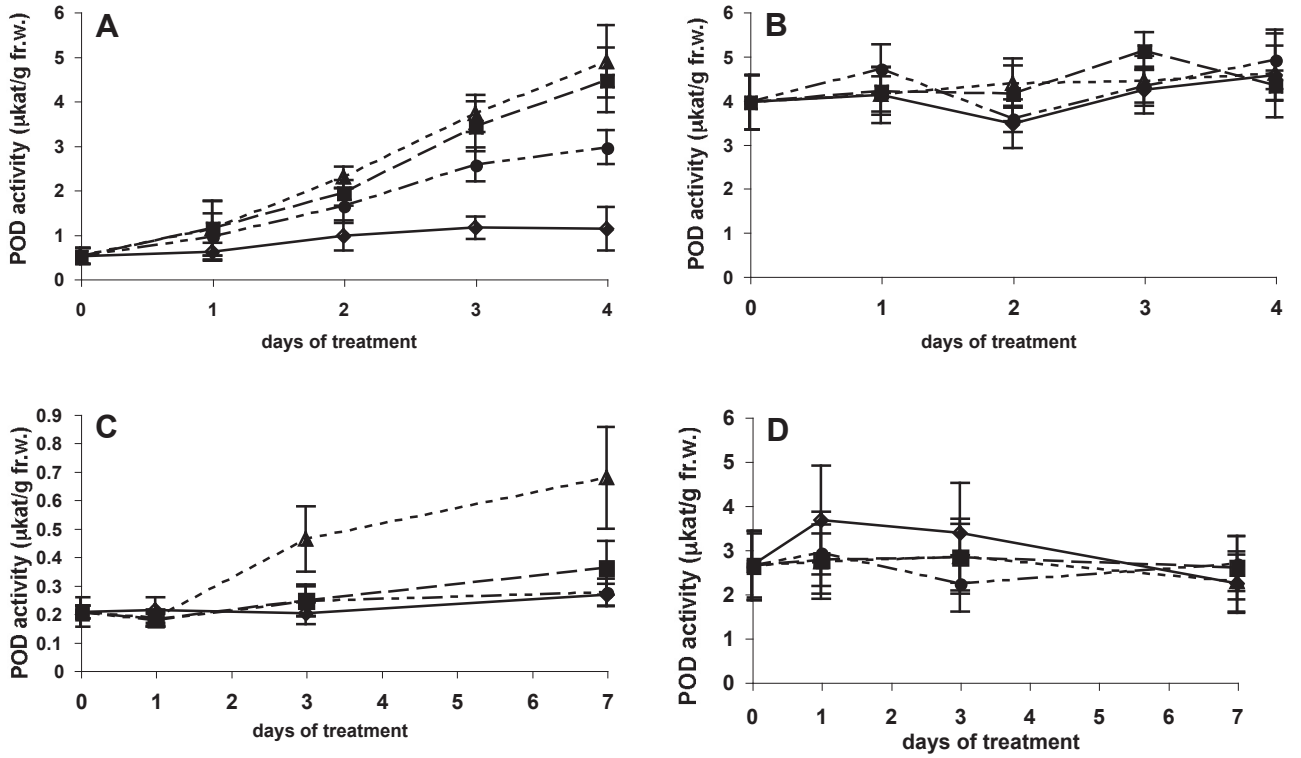

Figure 3. POD activity after Cd (A, B) and Zn (C, D) treatment in the leaves (A, C) and in the roots of barley seedlings. (♦) control; (●) 0,1 mM; (■) 0,3 mM; (▲) 1 mM Zn and Cd respectively. (Data presented as mean ± S.D., n=18).

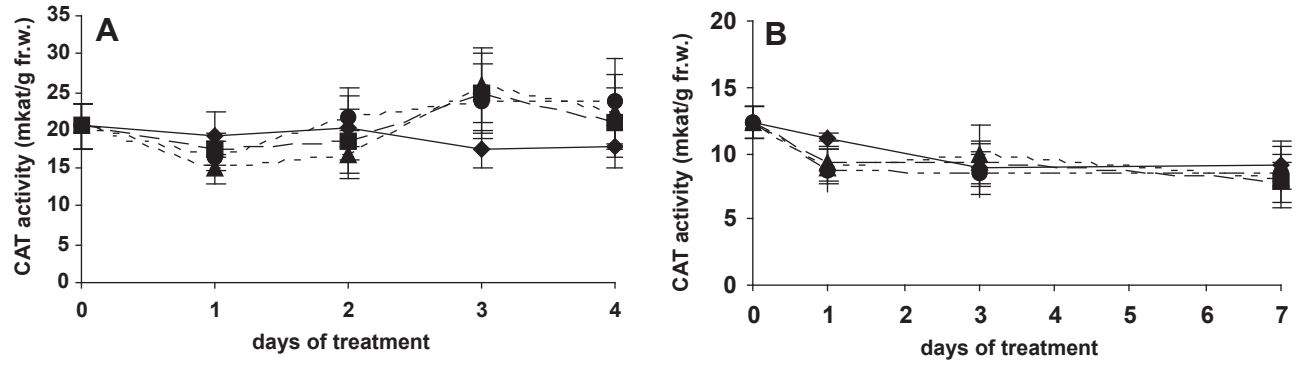

Figure 4. CAT activity after Cd (A) and Zn (B) treatment in the leaves of barley seedlings. (♦) control; (●) 0,1 mM; (■) 0,3 mM; (▲) 1 mM Zn and Cd respectively. (Data presented as mean ± S.D., n=18).

essentially different effects of the heavy metal Zn and Cd on plant metabolism.

We conclude that there is a fundamental difference in the metabolic changes induced by the heavy metal Zn and Cd. Cadmium induces drastic changes that are reflected in the decrease of chlorophyll content and the radical changes in the activity of the antioxidative enzyme system. These effects were partially counteracted in ALR transformed tobacco plants. The other tested heavy metal, zinc is an essential nutrient element in a defined concentration range. Above the optimal supply it induces much milder changes in the plant metabolism that is reflected in the constant chlorophyll content, the CAT and APX activity. The only parameter indicating the toxicity of Zn ions at high concentration was the increase of POD activity in leaves. It is in a good agreement with the observation that Zn is transported to leaves to a comparably

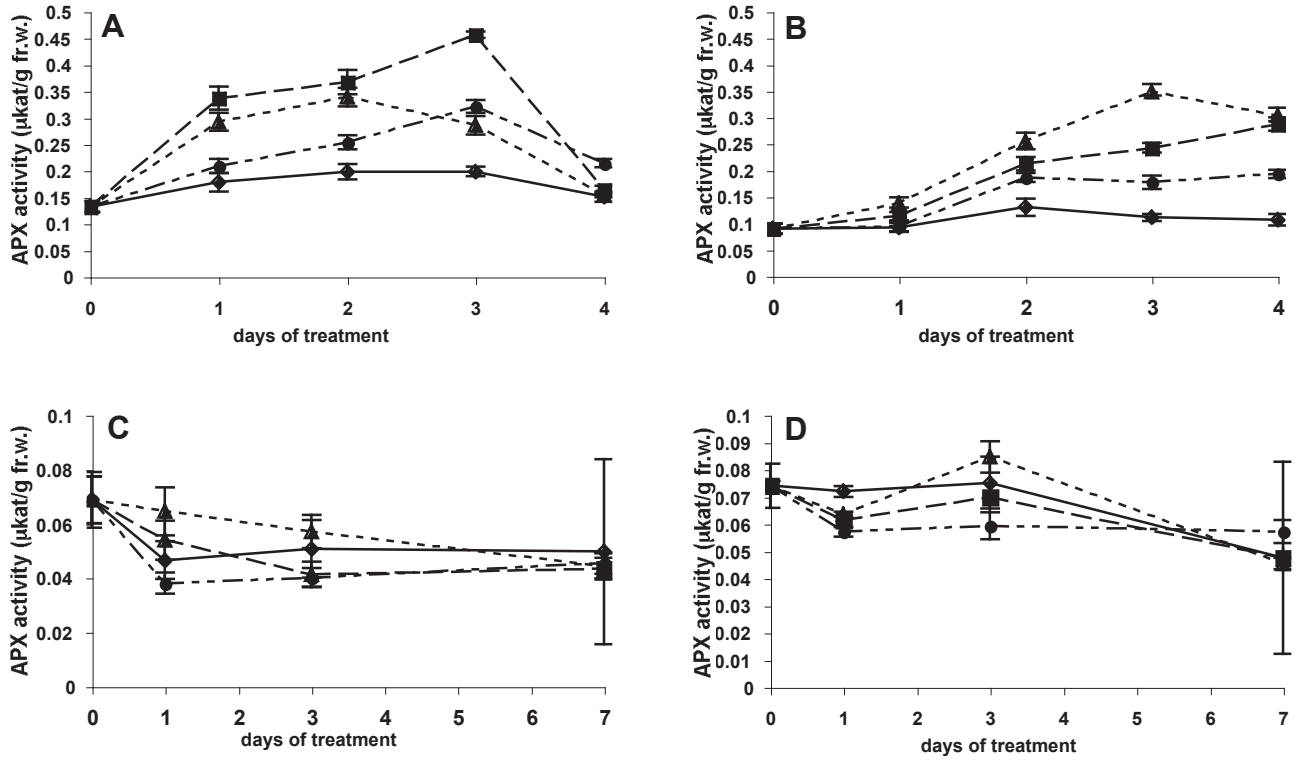

Figure 5. APX activity after Cd (A, B) and Zn (C, D) treatment in the leaves (A, C) and in the roots of barley seedlings. (♦) control; (●) 0,1 mM; (■) 0,3 mM; (▲) 1 mM Zn and Cd respectively. (Data presented as mean ± S.D., n=18).

higher extent also in other plants compared to Cd transport (Prasad 2005). We conclude that the reason of less toxicity of Zn should be either the different localization of Zn or the most effective phytochelation of the heavy metal. The mechanism of the Zn and Cd uptake, translocation and transport to and within the cells is going to be more and more clarified (Hall and Williams 2003). The understanding of the connections between plant's initial responses and the downstream events that constitutes successful adjustment to the altered environment is one of the great challenges and prosperious fields of plant biology and we can get a better insight into the consequences of heavy metal toxicity in the near future (Vranová et al. 2002).

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