Changes in peroxidase activity and isoenzymes in spruce needles after exposure to different concentrations of cadmium

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Abstract

We studied the guaiacol peroxidase activity, isoenzyme pattern and metal content in the needles of 2-year-old spruce grown on soils supplemented with cadmium concentrations from 1 to 21 mg kg$^{-1}$. Following exposure to cadmium, an initial increase and subsequent decrease in the activity of the soluble fraction was observed. A parallel change of their isoenzyme pattern occurred. An increase of the cell wall-bound peroxidase activity under prolonged metal treatment was evident. The results obtained show that peroxidase activity and isoenzyme pattern could be used to evaluate the capacity of one part of the defense system in spruce seedlings to withstand metal stress. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Physiological processes in plants are affected by increased heavy metal contents in soils surrounding the plant. One of the consequences of the presence of toxic metals in cells is the formation of free radical species, which can be initiated directly or indirectly by metals, and consequently cause severe damage to different cell components. Antioxidative systems, consisting of several enzymatic and enzymatic mechanisms, are activated in the cell as a response to different types of damaging conditions (Rabe and Kreeb, 1979; Hendry and Crawford, 1994; Hippeli and Elstner, 1996), and in particular to heavy metals (De Vos et al., 1992; Gallego et al., 1996; Chaoui et al., 1997; Ručinska et al., 1999; Sanita di Toppi and Gabrielli, 1999). Amongst them, peroxidases, both intracellular and extracellular (soluble and cell wall-bound) have an important role in the antioxidative response of plant cells (Castillo, 1986; Siegel and Siegel, 1986). Peroxidases can function as effective quenchers of reactive intermediary forms of oxygen and peroxy radicals induced by increased metal levels in the cell...
The peroxidase reaction is unspecific towards the type of stress. Product of peroxidase-catalyzed polymerization of phenolic alcohols (coniferyl, synapyl, p-coumaryl alcohol) in the cell wall is a lignin polymer, the increase in lignin quantity being an indication of stress intensity (Castillo, 1986; Siegel and Siegel, 1986; Pandolfini et al., 1992; Polle and Chakrabarti, 1994). The capacity to synthesize peroxidases in tree leaves was used as a parameter for monitoring and mapping the defense of air pollution (Keller, 1974). The mechanism of peroxidase action in plants exposed to elevated concentrations of heavy metals has not been completely elucidated, nor has the evaluation of the phytotoxicity of metal contaminated soils been performed completely either.

In the published literature the effect of stress induced by heavy metals has been predominantly studied in plants either taken from the field (Polle and Chakrabarti, 1994) or grown in controlled conditions on hydroponic nutrient solutions (Brune and Dietz, 1995; Ouzounidou et al., 1995; Arduini et al., 1996; Weckx and Clijsters, 1996). Amongst them spruce grown in controlled environmental conditions on the soil has not been studied. In this work we studied the activity of intracellular and extracellular soluble peroxidases, and the activity of cell wall-bound peroxidases and the metal content in needles of spruce grown on soil supplemented with different cadmium concentrations. More generally, the results obtained could particularly contribute to the knowledge on the effect of cadmium on the plant antioxidative enzymes, which response is activated in parallel with the synthesis of phytochelatins, another major quencher of heavy metals (Steffens, 1990; De Vos et al., 1992). It could be expected that such an approach may contribute to the understanding of the plant response to more complex combination of external factors. An additional aim of this study was to determine whether it may be possible to use the peroxidase activity as one of the parameters for assessment of the plant antioxidative state. Due to the presence of many modifying environmental factors, it is almost impossible to study the response of spruce needles to cadmium in adult plants in the field. Therefore, we have examined the response of spruce seedlings to a range of Cd concentrations in controlled environmental conditions. We tried to show that under such conditions, there is a parallel change in general peroxidase activity and increase of cadmium in the needles of the spruce seedlings in response to cadmium stress.

2. Materials and methods

In the experiments we used 2-year-old spruce seedlings (Picea abies L.) grown in the greenhouse. Seedlings were transferred from the nursery to the greenhouse on the 5th of November and grown singly in pots of 300 ml volume, in daylight and temperature between 20 and 26°C. The seedlings were grown on a soil composed of peat and compost (4:1 v/v) mixed with quartz sand (3:1 v/v). pH value of the soil was 6.6 before treatment. Soil was analyzed for the presence of cadmium prior to the planting and no increased concentrations of cadmium were detected. The treatment of the seedlings started on the 10th of March. During 2 weeks before application of cadmium, 50 mM Na–acetate buffer pH 4.3 was supplied to the soil in the pots where plants were grown, in order to buffer the soil and lower its pH value. After this period, different cadmium concentrations from 1 to 21 mg kg\(^{-1}\) were added to the soil, in the form of CdSO\(_4\) in 50 mM Na–acetate buffer, pH 4.3. After treatment the soil was watered with the same buffer instead of water. Seedlings were harvested after 1, 2, 15, 30 and 60 days of Cd treatment. Plants used for the experiments were healthy, without any exogenous infection detected. Needles from the whole plant were used as experimental material. The influence of metal ions on the metal content, peroxidase activity and isoenzyme pattern in the needles were monitored during 2 months after treatment, using atomic absorption spectroscopy, isoelectrofocusing and UV–VIS spectrophotometry.

Extraction of soluble peroxidases was performed by homogenization of needles in 0.1 M Tris–HCl buffer, pH 7.8, containing 1 mM dithiotreitol and 1 mM EDTA, according to the procedure of Mocquot et al. (1996), Weckx and
The homogenate was squeezed through eight gauze layers and centrifuged at 12,000 × g and 4°C for 10 min. The supernatant was used for peroxidase activity measurements. The pellet was washed with distilled water and 50 mM Na–acetate buffer pH 5.5. After centrifugation at 200 × g for 5 min, the pellet was sonicated for 5 s in the same buffer and centrifuged at 200 × g for 5 min, according to the procedure of Otter and Polle (1994), Takahama and Oniki (1994). Obtained pellet was suspended in Na–acetate buffer and used for the measurement of the activity of the peroxidase bound fraction.

Peroxidase activity was determined with guaiacol as the substrate in a total volume of 3 ml. The assay mixture contained 50 mM acetate buffer, pH 5.5, 92 mM guaiacol, 18 mM H₂O₂ and variable amounts of the enzyme preparations. The turnover of guaiacol was monitored using Shimadzu UV-160 spectrophotometer at 470 nm. Reaction rate was calculated from the extinction coefficient for guaiacol of 25.5 mM⁻¹ cm⁻¹. The activity was referred to the protein content of each enzymic fraction, except for the whole activity of the cell wall fraction referred to the cell wall dry weight.

Protein concentration of the enzymic fractions was determined by Lowry assay (Lowry et al., 1951) with bovine serum albumin as the standard.

Soluble peroxidase isoenzymes were separated by isoelectric focusing in a pH gradient from 3 to 9 (using 3% ampholite solution) on a 5% polyacrylamide gel by staining with 20 mM guaiacol and 5 mM H₂O₂ in Na–acetate buffer pH 5.5 for 10 min at 25°C.

For metal determination, needles were dried at 70°C for 12 h, and ashed in a muffle furnace at 500 ± 50°C for 6 h. The ash was dissolved in 2 ml HNO₃ (1:3 v/v), evaporated and dried at 500 ± 50°C for 1 h. For measurement the ash was dissolved in 2 ml HCl (1:3 v/v) and metal content was determined by flame atomic absorption spectrophotometry. The calculated detection limit was 1 ± 0.5 mg Cd kg⁻¹ dry needles.

Each experimental variant and control were represented by three to five seedlings, which were treated as replicates. Statistical analysis of the enzyme activity data was performed using the Kruskal–Wallis ranking test, at the 0.05 level of significance.

3. Results

There were no visible symptoms of the injury of seedlings exposed to 1–7.5 mg kg⁻¹ Cd. Wilting of the needles treated with 15 and 21 mg kg⁻¹ Cd was observed after 30 and 60 days of treatment.

After 1 day of treatment with 7.5–21 mg kg⁻¹ Cd and 2 days of treatment with 7.5–15 mg kg⁻¹ Cd, 0.8–1.5 mg kg⁻¹ DW Cd was detected in the needles, which is within the calculated detection limit. In case of 2-day treatment with 21 mg kg⁻¹ Cd in the soil, 2 mg kg⁻¹ Cd was detected in the needles, which is above detection limit (Fig. 1). As for long term treatments, in case of 7.5, 15 and 21 mg kg⁻¹ Cd, 2–5.5 mg kg⁻¹ DW Cd was detected in the needles treated 15–60 days, while in the case of lower metal concentrations there was no detectable amount of cadmium in the needles (Fig. 1).

Fig. 2 presents the activity of both soluble and bound peroxidase fraction in the needles of plants exposed to different cadmium concentrations in the soil, in comparison with metal untreated plants, for various durations of treatment. Time change of activity of both peroxidase fractions,
for particular applied cadmium concentrations, is presented by histograms (Fig. 3). Soluble peroxidases, including intracellular and extracellular apoplastic enzymes, were found to have notably higher activity in comparison with cell wall-bound ones (Figs. 2 and 3).

Comparing short and long term treatments, it is worth noting a considerable decrease in the activity of soluble, and an increase in the activity of the bound peroxidase fraction, in both metal treated plants and metal untreated ones but grown on the acidified soil, during 15, 30 and 60 days, in comparison with 1- and 2-day treated ones (Figs. 2 and 3). The increase in soluble peroxidase activity with exposure time was considerably more pronounced than the decrease of the bound fraction activity. There was an increase of the total protein concentration in the soluble fraction of the long-term treated plants in comparison with the short term treated ones, independent of the metal exposure (Fig. 5).

There was no significant difference in activity of both 1- and 2-day treated needles, in comparison with untreated ones, in case of either soluble and cell wall-bound fraction, for all metal concentrations investigated (Fig. 2a). The exception are the seedlings treated for 2 days with 15 mg kg\(^{-1}\) Cd, where a statistically significant decrease of activity in relation to the untreated plants was found.
The change of peroxidase activity of both soluble and cell wall-bound fraction (Figs. 2 and 3) occurred in parallel with the raise of cadmium content in the needles exposed to long-term treatments with increasing cadmium concentrations in the soil (Figs. 1 and 4). A significant change in the activity of soluble peroxidase fraction was detected in time after treatment with 7.5, 15 and 21 mg kg\(^{-1}\) Cd, in comparison with untreated trees (Fig. 2). Lower metal concentrations did not induce significant activity changes in time. In the case of addition of 7.5–21 mg kg\(^{-1}\) Cd to the soil, an increase of soluble peroxidase activity was found in comparison to the control 15 days after treatment. In 30-day treated plants significant activity increase of the soluble fraction was observed in the case of 7.5 and 15 mg kg\(^{-1}\) Cd in the soil.

After 60 days of treatment there was no significant difference in the soluble peroxidase activity in treated seedlings from the control plants. The effect was more pronounced 15 days after treatment (Fig. 2b). In case of cell wall-bound peroxidase fraction, a significant activity change in time occurred after treatment with 7.5 and 15 mg kg\(^{-1}\) Cd in the soil (Fig. 2b). There was no significant effect in case of lower metal concentrations, as well as after addition 21 mg kg\(^{-1}\) Cd (Fig. 2b and Fig. 3). The increase in the activity of cell wall-bound peroxidase fraction, in comparison with the control, was statistically significant after treatment with 7.5, 15 and 21 mg kg\(^{-1}\) applied cadmium 15 days after treatment, as well as in case of 3, 7.5 and 21 mg kg\(^{-1}\) Cd 60 days after treatment. After 30 days of treatment there was no significant difference from the control (Fig. 3b).

All detected soluble peroxidase isoenzymes were anionic ones, with pI values between 5 and 7 (Fig. 6). There was no change in the isoenzyme pattern of soluble peroxidases, in comparison with the control, in the seedlings treated with 1 and 3 mg kg\(^{-1}\) Cd, 1 and 2 days after treatment. In the plants treated with 15 and 21 mg kg\(^{-1}\) Cd, the isoenzyme with pI about 5 disappeared after 1 day of treatment and reappeared after 2 days of treatment, while an isoenzyme with pI about 6 disappeared after 2 days of treatment (Fig. 6a). Similarly to 1- and 2-day treated plants, there was no change in the isoenzyme pattern of soluble peroxidases, in comparison with the control, in the seedlings treated with 1 and 3 mg kg\(^{-1}\) Cd 15, 30 and 60 days after treatment. In the case of 7.5 mg kg\(^{-1}\) Cd, an isoenzyme with pI 5.5 disappeared 15 days after treatment, and a new anionic isoenzyme with pI about 6 appeared 30 days after treatment, in comparison with the control. In case of 15 and 21 mg kg\(^{-1}\) Cd treatment, a new anionic isoenzyme with pI about 6 appeared 15 days after treatment, while an additional isoenzyme with pI 7 appeared 60 days after treatment. This change of isoenzyme pattern was more pronounced in the case of seedlings treated with 21 mg kg\(^{-1}\) Cd (Fig. 6b and c). The results of the isoenzyme pattern change were in accordance with the activity change in time in the plants treated with the higher metal concentrations.
Fig. 4. Parallel increase of cadmium concentration in the needles and change in peroxidase activity of soluble (a) and cell wall-bound (b) fraction with increasing cadmium concentration in the soil.

4. Discussion

Toxicity of metals in the soils depends on various physical and chemical soil parameters (pH, cation exchange capacity, chemical form of the metals, organic matter content etc.) and biological parameters, such as the presence of microorganisms that determine the metal availability to the plant. The fact that there was no effect of 1- and 2-day treatment with most of the used cadmium concentrations, on both soluble and bound peroxidase activity in spruce needles, in comparison with metal untreated plants (Fig. 2a), could be explained by a delay in metal translocation in the short-term treatments. This result is consistent with measured cadmium in the needles, which was within detection limit (Fig. 1). However, the change of the isoenzyme pattern in seedlings after 1- and 2-day treatment with 15 and 21 mg kg\(^{-1}\) Cd (Fig. 6a), as well as the fact that peroxidase
activity significantly changed in the needles 2 days treated with 15 mg kg\(^{-1}\) Cd (Fig. 2a) and metal detected in the needles treated with 21 mg kg\(^{-1}\) Cd was above detection limit (Fig. 1), shows that the plants initiated changes of antioxidative defense system to long-term stress. Since the peroxidase activity (U) of the soluble fraction in case of long-term treatments was of the same order of magnitude in comparison with short-term treatments, considerable decrease of corresponding specific activity (U mg\(^{-1}\) protein) with treatment duration (Fig. 2) was mainly due to the increase of protein concentration in this fraction (Fig. 5). The effect of soil acidification itself, without metal treatment, on the activity of both peroxidase fractions, could be also explained through the increased protein content after long-term treatments. It was shown that soil acidification can influence the exchange of mineral nutrients between plant and soil (Schulze, 1989). Under natural conditions, soil acidification increases metal uptake by plants, and as such may be a precondition of their pronounced effect on plants.

The effect of stress on biological systems depends on the ratio between stress intensity and buffer capacity of the soil. We used Na–acetate buffer to decrease pH of the soil. This buffer has a pK\(_a\) value of 4.7, which means that its highest buffer capacity falls within a pH range of the soil polluted by acidic rain. Cadmium appears to be absorbed passively (Cutler and Rains, 1974) and translocated freely (Jarvis et al., 1976) in plants. Even if there is some complexation of acetate ions with cadmium, this should not influence the availability of cadmium ions in the reactions, since these complexes are not stable.

The results of long-term treatments showed that increasing cadmium concentration induced an increase of the capacity of the antioxidative defense system in the seedlings, alleviating the toxic effects of metal ions. The increase of peroxidase activity in treated seedlings is probably related to oxidative reactions corresponding to an increase in peroxides and free radicals in the plant cells. Activation of oxygen has been proposed as a general reaction on several stress factors including metals, and reactive oxygen forms could be quenched by the induction of specific enzymes, one of them being peroxidase (Vangronsveld and Clijsters, 1991, 1994; Hippeli and Elstner, 1996).

The induction of the antioxidative enzymes is one of the processes implicated in the regulation of the metal ion concentration in plants. Synthesis of phytochelatins is a parallel process that removes metal ions intracellularly (Steffens, 1990; De Vos et al., 1992). Cadmium complexed by chelating proteins was found in many higher plants (Steffens, 1990; Das et al., 1997). However, many metal-tolerant plants have not been found to accumulate phytochelatins as a response to metal stress (Steffens, 1990). This was explained by the fact that overproduction of phytochelatins seems to be an unlikely mechanism for metal tolerance, owing to the energy required for sulfate reduction to support phytochelatin synthesis.

A cadmium concentration of 3 mg kg\(^{-1}\) in the soil is considered to be a threshold value for plant toxicity, according to the criteria of the Economic board of the United Nations for Europe and International Union of Biological Sciences (International Union of Biological Sciences, 1994). Our results show that, in case of spruce seedlings, soil cadmium concentrations up to 3 mg kg\(^{-1}\) do not cause any significant changes in the part of oxidative metabolism, as measured by induced changes in guaiacol peroxidase activity and isoenzyme pattern. Higher cadmium concentrations caused an increase in oxidative metabolism, and this can be caused by an increased quantity of free radicals.

Fig. 5. Total protein concentration in the soluble fraction from the spruce leaves as a function of the applied cadmium concentration, for different duration of treatment.
change at higher cadmium concentration was followed by a change of the soluble peroxidase isoenzyme pattern, as well as with the accumulation of cadmium in the needles. Wilting of the needles was an exogenous symptom of toxicity in the plants treated with higher cadmium concentrations. Our results showed that the initial increase of one part of antioxidative defense system capacity is related to de novo synthesis of the new soluble peroxidase isoenzymes, having a role in elimination of the reactive molecules. The new soluble isoenzymes (Fig. 6) probably do not possess sufficient capacity for maintenance of protective level of oxidative metabolism. On the other hand, the increased response of the bound peroxidases in case of prolonged metal stress (Figs. 3 and 4) may be linked to their role in lignin synthesis, one of the important protective reactions in the plant cell under the stress conditions (Imberty et al., 1985; Pandolfini et al., 1992). It would be important to define the physiological threshold of different plants towards different kinds of stress, that is the stress intensity causing a decrease of oxidative metabolism and physiological functions. It has been shown that the peroxidase activity can be used as a potential biomarker for sublethal metal toxicity in examined plant species (Vangronsveld and Clijsters, 1991, 1994; Mocquot et al., 1996). Our results showed that in the spruce seedlings, even in case of 21 mg kg$^{-1}$ Cd in the soil and corresponding 4–6 mg kg$^{-1}$ Cd in the needles after 60-day treatment, mechanisms of antioxidative defense were active. Although studies on the physiology of metal toxicity are difficult, our data support the concept that monitoring of the oxidative metabolism parameters in different plant species should be an integral part of the evaluation and quantification of the effect of metal stress on plants.

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References


