ORGANOSPECIFIC REACTIONS OF YELLOW LUPIN SEEDLINGS TO LEAD

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ABSTRACT

Changes caused by lead, supplied in the form of $\text{Pb}(\text{NO}_3)_2$, in roots and hypocotyls of 4 day old yellow lupin (*Lupinus luteus* L. cv. *ventus*) seedlings have been analyzed using a transmission electron microscope and polyacrylamide gel electrophoresis (PAGE). The cells of all examined parts of the roots growing in the presence of Pb^{2+} contained many lead deposits (mainly in the cell walls and vacuoles) and the increased amount of polypeptides of molecular weight close to 16 kDa have been observed. Similar changes were detected in the area of hypocotyl adjoining the root. However, in upper regions of the hypocotyl only a slight amount of lead deposits was visible and the 16 kDa polypeptide content was comparable to the control cells. The obtained results indicate a relationship between the presence of lead deposits in cells and accumulation of polypeptides of ~ 16 kDa. The results seem also to indicate that in the analyzed parts of the seedlings, both the amount of accumulated polypeptides of MW ~ 16 kDa and the amount of lead decreased from root tip to hypocotyl.

KEY WORDS: transmission electron microscope, polyacrylamide gel electrophoresis, polypeptide pattern, root, hypocotyl, Pb.

INTRODUCTION

Despite extensive research on lead influence on plants (Huang et al. 1974; Koeppe 1977; Burzyński 1987a, 1987b and 1988; Wierzbicka 1987; Woźny 1987; Książek and Woźny 1990; Woźny 1995; Samardakiewicz et al. 1996), defense reactions of plants to this metal are not still completely known. One of the often mentioned mechanisms of lead detoxification in plant cells is based on its precipitation mainly with phosphates and sulfates and deposition in the vacuoles and cell walls (Lane and Martin 1982; Woźny et al. 1982; Książek et al. 1984; Przymusiński and Woźny 1985; Wierzbicka 1995; Woźny 1995), inside the middle lamella and in the intercellular spaces (Wierzbicka 1984) and in the dictyosomes, endoplasmic reticulum and the nuclear envelope (Woźny et al. 1982; Książek et al. 1984; Idzikowska 1988). Peptides of phytochelatin type (Grill et al. 1985; Schat and Kalff 1992; Tukendorf 1993; Woźny 1995), proteins known as plant metallothioneins (Tomsett et al. 1989; Lane et al. 1987; Tukendorf 1993) and heat shock proteins (Orzech and Burke 1988; Neumann et al. 1994) which bind lead and other heavy metals in non-toxic complexes (Tukendorf 1989; Rauser 1990; Reddy and Prasad 1990) have a similar function. Increased tolerance for lead and other heavy metals can be connected with the raised level of the so called scavengers of free superoxide radicals (Liczmański 1988), mainly superoxide dismutases (SOD). Earlier research (Przymusiński and Gwóźdź 1994; Przymusiński et al. 1995) proved that lead and other toxic and stress factors create a major increase in accumulation of polypeptide of MW 16 kDa in lupin roots. This

polypeptide has many features in common with SOD (Przymusiński et al. 1995) and consequently may play an important role in the limitation of lead toxic influence on plants.

The hitherto existing research demonstrated that the polypeptide of MW 16 kDa was present only in apical parts of lupin roots (Przymusiński et al. 1991). The aim of this work was to find out whether this polypeptide occurs also in higher regions of roots and in hypocotyls and if there is any relationship between the amount of lead detected with the microscope and the accumulation of 16 kDa polypeptide.

MATERIAL AND METHODS

Plant material

Seeds of *Lupinus luteus* L. cv. *ventus*, similar in their morphological features, were sterilized in 0.2% solution of HgCl_2 , washed in bidistilled water and germinated in Petri dishes on two-layer filter paper moistened with sterilized bidistilled water. When the roots were 6-8 mm long, 25 randomly selected seedlings were put in the holes of plastic sieves hanging on hooks in crystallizing dish filled with 250 ml of $\text{Pb}(\text{NO}_3)_2$ water solution at the concentration of $9 \text{ mg} \cdot \text{l}^{-1}$ Pb^{2+} . The incubation in dark at the temperature $21 \pm 1^\circ\text{C}$ lasted 48 h.

Electron microscope

All selected regions of roots and hypocotyls (Fig. 1, regions II, III, IV, V, VI) were analyzed with transmission electron microscope. The parts of organs (1 mm long) were fixed for 5h in room temperature in the mixture (1:1, v:v) of 4% glu-

taraldehyde and 4% paraformaldehyde in 0.05M cacodylate buffer, pH 6.8. Postfixation of the material was carried out in 1% OsO₄ in 0.05M cacodylate buffer, pH 6.8 for 15h at 4°C. Then the material was stained in 2% water solution of uranyl acetate for 24h at 4°C and dehydrated in a series of acetone solutions of increasing concentration. The material was embedded in epoxide resin of low viscosity according to Spurr (1969). Ultrathin sections were obtained with ultramicrotome Ultratome III (LKB Sweden). They were examined in electron microscope JEM 7A (JOEL Co Japan) without additional staining.

Fig. 1. Diagrammatic representation of lupin seedlings with marked regions examined in the transmission electron microscope and polyacrylamide gel electrophoresis:

A – amount of lead deposits

B – accumulation of polypeptides of ~ 16 kDa

I, II, III, IV, V, VI – number of marked regions.

Electrophoretic analysis of polypeptides

Parts of roots and hypocotyls (1 mm long) were taken from the specified regions (Fig. 1, regions I, III, IV, V, VI) and homogenized in 0.05M buffer Tris-HCl, pH 7.5 at 4°C, centrifuged for 10 min. at 8 800 g at 4°C. The supernatant was mixed at the ratio 2:1 (v:v) with the solubilization buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue in 62.5 mM Tris-HCl pH 7.5), heated up to 100°C for 2 min. and used for electrophoretic analysis after cooling down. The samples containing the same level of protein (50 µg), defined according to Bradford's method (1976), were separated. The separation of supernatant proteins was carried out in 15% polyacrylamide gel with SDS according to Leammli (1970) at 30 mA. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 with 0.1% CuSO₄ · 5H₂O, 30% methanol and 10% acetic acid.

RESULTS

1. Lead influence on dynamics of roots and hypocotyls growth

The influence of lead on the growth of roots and hypocotyls was examined after 48h incubation in lead solutions in the concentrations of 10 and 50 mg · l⁻¹ Pb²⁺. It was established that lead limited root growth proportionally to the concentration of this element in the solution (Fig. 2). As a result, the roots were shorter than hypocotyls because the growth of the latter underwent smaller changes under the influence of the same concentrations of lead. The measurements allowed to establish a concentration which limited root growth by 50%. The concentration was 9 mg · l⁻¹ Pb²⁺ and was used in further research.

Fig. 2. Length of roots and hypocotyls of four days old lupin seedlings. Seedlings grown in water – control (0), or in Pb(NO₃)₂ water solution (10 mg · l⁻¹, 50 mg · l⁻¹ Pb²⁺). Mean values ± SD from 25 plants per condition, 2 repetitions.

2. Ultrastructure of root and hypocotyl cells

The root parts 2 mm below the transition region (region II, Fig. 1) and the parts adjacent to the hypocotyl (region III, Fig. 1) were analyzed with transmission electron microscope. In both parts of the root lead deposits were located mainly in the cell walls of the cortex parenchyma (Fig. 4), quite often in the vacuoles (Fig. 5) and intercellular spaces and seldom in the Golgi apparatus (Fig. 6) and endoplasmic reticulum (Fig. 7). The frequency of lead deposits decreased towards the cylinder axis and towards the hypocotyl. Moreover, in the cortex parenchyma of the mentioned root parts, in large number of the cells a damage in the cytoplasm structure was observed, ranging from increased vacuolization to a complete granulation. The majority of the cells with a well-preserved cytoplasm were usually observed in deeper parts of the cortex parenchyma. The cells of the vascular cylinder in both examined parts of the root were well-preserved and only a few lead deposits were found in the cell walls and intercellular spaces, dictyosomes and endoplasmic reticulum of some cells and sporadically in the vessels in the pits area (Fig. 8).

A much bigger variety in the number of lead deposits was observed in the examined parts of the hypocotyl (regions IV-VI). In the part of hypocotyl adjacent to the root (region IV, Fig. 1) the number of lead deposits was relatively high and

their localization was similar to that observed in the root itself. However, the number and size of lead deposits in this region was distinctly lower as compared to the root (Fig. 9). In the middle region (region V, Fig. 1) and the upper part of the hypocotyl (region VI, Fig. 1) there were hardly no lead deposits. They were present only in the vacuoles (Fig. 10) and the intercellular spaces (Fig. 11). Moreover, in all the examined parts of the hypocotyl a large number of cells located mainly in the area of the cortex parenchyma showed various degree of damage, such as vacuolization.

3. Changes in accumulation of root and hypocotyl polypeptides

Lead supplied in the form of lead nitrate influenced the polypeptide pattern of proteins separated both from roots and hypocotyls. The most pronounced change was an increase accumulation of polypeptides of molecular weight close to 16 kDa (Fig. 12). In this area, when using 15% polyacrylamide gel, three polypeptides situated close to each other were observed. At a high level of accumulation, they looked as one wide strip or, if two lower weight polypeptides merged, as one wide strip and a third polypeptide just above it. In the presented research the strongest accumulation of polypeptides of 16 kDa area under lead influence was found in the apical parts of the root (region I, Fig. 1). Additionally, in this region of the root a large increase of polypeptides of 38 kDa and 33 kDa and a decreased accumulation of MW 85 kDa, 50 kDa and 31-29 kDa polypeptides could be observed.

In the base part of the root (region III, Fig. 1) and the adjoining to the root part of the hypocotyl (region IV, Fig. 1) an intensified accumulation of polypeptides of MW ~ 16 kDa occurred, but it was not as clear as in the apical part of the root (Fig. 12). The lowest level of 16 kDa polypeptides accumulation occurred in the part of hypocotyl that was adjacent to the root (region IV). Moreover, in both parts of the seedlings there was a distinctive decline in the 88 kDa polypeptide accumulation. However, in the upper parts of the hypocotyl (region V and region VI) accumulation of polypeptides close to 16 kDa was similar to that observed in the control cells (Fig. 12). Changes in the level of accumulation of the remaining polypeptides were not observed, except for the part situated in the middle-length of the hypocotyl (region V) where the amount of polypeptides of MW 54 and 38 kDa slightly increased.

DISCUSSION

Most of the lead taken up by plants remains in the root (Schierup and Larsen 1981; Burzyński 1987a and 1988; Wierzbicka 1991). Due to this fact, roots were the preferred research material (Woźny et al. 1982; Wierzbicka 1984; Idzikowska 1988; Książek and Woźny 1990; Woźny 1995). In our research we did not include an analysis of lead influence on the ultrastructure of the apical part of lupin root, as it was described in detail by Przymusiński and Woźny (1985). Little was known about the presence of lead in the basal part of the root and in the hypocotyl. Our observations indicate that in all examined regions of the root and in the hypocotyl adjacent to the root, the amount of lead decreases towards hypocotyl and then epicotyl. The trace amount of this metal in higher parts of the hypocotyl may result from its immobilization in root tissue and relatively small mobility comparing to other metals (Koepe 1977). This is supported by the differences in growth rate between roots and hypocotyls. Lead reduced the growth rate of roots much more than of hypocotyls.

Location of lead deposits in the cells of the examined organs was similar to that described by other authors (Woźny et al. 1982; Książek et al. 1984; Wierzbicka 1984; Idzikowska 1988; Woźny 1995). Large amount of lead deposits in the cell walls and in the vacuoles confirms that lead is neutralized there by immobilization in structures, which are probably most of all predisposed to that function (Przymusiński and Woźny 1985, Poulter et al. 1985). The presence of lead deposits in the cell walls, intercellular spaces and sometime in the area of pits may indicate that the apoplast is the main route of lead movement in the root, which was previously suggested e.g. by Książek et al. (1984). On the other hand, smaller amount of lead in the vascular cylinder as compared to the cortex may result from the central position of the cylinder (Przymusiński and Woźny 1985) as well as from the small mobility of lead. Analyses with light and electron microscopes have shown that at an early stage of seedlings development, which was the case in our research, the endodermis is still lacking and so there are no morphological barriers inhibiting lead migration towards the vascular cylinder.

In the light of the ultrastructural research described above it was interesting to examine the changes in the polypeptide pattern of the root and hypocotyl proteins. Special attention was paid to the presence of the polypeptide of MW 16 kDa. Its intensive accumulation has been observed so far in the apical part of lupin roots exposed to many stress factors such as lead, cadmium, zinc, copper, sodium chloride, sodium nitrite and heat shock (Przymusiński et al. 1991; Przymusiński and Gwóźdź 1994). In our experiments an intensive accumulation of a wide band of polypeptide of MW about 16 kDa was found not only in the apical part of the root, where its highest level was observed, but also in the basal part of the root and in the part of hypocotyl adjacent to the root. Moreover, using 15% polyacrylamide gel we were able to distinguish three polypeptides in the area of 16 kDa, which could be merged in one wide band at high accumulation level of this polypeptide. By means of immunological methods Przymusiński et al. (1994) have found that one of the three polypeptides of 16 kDa is immunologically related to cytosolic form of Zn, Cu superoxide dismutase (SOD). This enzyme is found in all living organisms and plays a crucial role as a free radical scavenger (Kwiatkowski 1988), which are generated for instance in redox reactions (Liczmański 1988). It seems that various stress factors such as heavy metals, salt stress and high temperature accelerate formation of free radicals in cells, in removal of which SOD is involved (Przymusiński et al. 1995).

The ultrastructural and electrophoretal research suggests that there is a relation between the level of the polypeptides of MW ~ 16 kDa and the amount of lead deposits in the cells. It is confirmed by the fact that both the amount of lead and the level of polypeptides of ~ 16 kDa decrease towards hypocotyl (Fig. 1). In higher parts of the hypocotyl the level of the polypeptides of ~ 16 kDa did not increase, which corresponds to a very small amount of lead in the cells of these parts. However, we could not exclude the possibility that synthesis of these polypeptides was not intensified. The results of the experiments by Przymusiński (not published), with isolated cotyledons of ripening seeds of lupin, incubated in lead solution, may prove this thesis. Polypeptide of MW 16 kDa was detected only after using leucine C¹⁴ and fluorography. A clear band corresponding to the polypeptide of 16 kDa was detected in spite of the fact that lead deposits were not found in the cotyledons tissue during ultrastructural research. By using this method in the case of the hypocotyl it may be

Fig. 12. SDS-PAGE patterns of soluble proteins from roots and hypocotyls of lupin seedlings grown on H_2O or $9 \text{ mg} \cdot \text{l}^{-1} \text{ Pb}^{2+}$. Following 48h cultivation different part of lupin seedlings (regions I, III, IV, V, VI at Fig. 1) were homogenized with 50 mM Tris-HCl pH 7.5. After centrifugation for 10 min 8,800 g samples of supernatant fractions were subjected to SDS-polyacrylamide gel electrophoresis, and stained with Coomassie Brilliant Blue R-250. M – marker proteins of known mol. wt. in kDa: 94, phosphorylase; 67, bovine serum albumin; 43, ovalbumin; 30, carbonic anhydrase inhibitor; 20, trypsin inhibitor; 14, lactalbumin.

A – control (H_2O), B – $9 \text{ mg} \cdot \text{l}^{-1} \text{ Pb}^{2+}$,

arrow – polypeptides of MW ~ 16kDa

1 – region I, 2 – region III, 3 – region IV, 4 – region V, 5 – region VI

possible to analyze the changes in the synthesis of proteins under influence of lead ions.

The results obtained in our research seem to point out that the reaction of lupin to the presence of lead varies in different organs. The most important changes were noticed in the root and the adjacent part of the hypocotyl. The amount of lead detected in these organs by electron microscope, decreased towards epicotyl and corresponded to the level of ~ 16 kDa polypeptides.

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Figs 3-11. Electron microscope observations.

Paraformaldehyde-glutaraldehyde/ OsO_4 , Spurr (1969). Sections were examined unstained.

Key to lettering in the Figures: CW – cell walls, D – dictyosome, ER – endoplasmic reticulum, IS – intercellular space, P – plastid, V – vacuole, Pb – lead deposits.

Figs 3, 4, 5, 6, 7 and 8. Cells of the basal part of root (region II and III at Fig. 1).

Fig. 3. Control (H_2O). x 19 000. Figs 4, 5, 6, 7 and 8. Pb^{2+} – $9 \text{ mg} \cdot \text{l}^{-1}$. The lead deposits present in cell walls (Fig. 4. x 20 000), vacuole (Fig. 5. x 21 000), dictyosome (Fig. 6. x 49 000), endoplasmic reticulum (Fig. 7. x 21 000) and in the vessels in the pits area (Fig. 8. x 6 000).

Figs 9, 10 and 11. Cells of the hypocotyle (region IV, V and VI at Fig. 1)

The lead deposits in cell walls (Fig. 9. region IV. x 17 000), vacuole (Fig. 10. region V x 13 000) and intercellular space (Fig. 11. Region VI. x 10 000).

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ORGANOSPECYFICZNA REAKCJA SIEWEK ŁUBINU ŻÓŁTEGO WYWOŁANA DZIAŁANIEM OŁOWIU

STRESZCZENIE

Stosując metody mikroskopii elektronowej i elektroforezy analizowano zmiany jakie ołów, podany w formie $Pb(NO_3)_2$, wywołuje w korzeniach i hypokotylach czterodniowych siewek łubinu żółtego (*Lupinus luteus* L. cv. *ventus*). Przeprowadzone badania wykazały, że komórki wszystkich badanych stref korzeni rosnących w obecności Pb^{2+} charakteryzowały się obecnością licznych złogów ołowiu (głównie w obrębie ścian komórkowych i wakuol) oraz podwyższonym poziomem akumulacji polipeptydów o masie cząsteczkowej zbliżonej do 16 kDa. Podobne zmiany obserwowano w strefie hypokotyli stykającej się z korzeniem. Natomiast w wyższych partiach tego organu stwierdzono niewielką liczbę złogów ołowiu i porównywalny z materiałem kontrolnym poziom polipeptydów z rejonu 16 kDa. Uzyskane wyniki zdają się wskazywać na związek pomiędzy akumulacją polipeptydów o MW ~ 16 kDa a obecnością złogów ołowiu w komórkach, a także na malejącą w kierunku hypokotyli gradację, zarówno w poziomie akumulacji polipeptydów o MW ~ 16 kDa jak i ilości ołowiu w analizowanych fragmentach siewek.

SŁOWA KLUCZOWE: transmisyjny mikroskop elektronowy, elektroforeza, korzeń, hypokotyl, Pb.