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Enhancing phytoremediative ability of *Pisum sativum* by EDTA application

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Abstract

The aim of our research was to demonstrate how the presence of EDTA affects resistance of pea plants to Pb and Pb–EDTA presence, and to show the effectivity of lead ions accumulation and translocation. It was determined that EDTA not only increased the amount of Pb taken up by plants but also Pb ion transport through the xylem and metal translocation from roots to stems and leaves. It can be seen in the presented research results that addition of the chelator with Pb limited metal phytotoxicity. We also demonstrated a significant effect of EDTA not only on Pb accumulation and metal transport to the aboveground parts but also on the profile and amount of thiol compounds: glutathione (GSH), homoglutathione (hGSH) or phytochelatins (PCs), synthesized by the plants. We observed a significant effect of the synthetic chelator on increasing the level of Pb accumulation in roots of plants treated with Pb including EDTA (0.5 and 1 mM). *Pisum sativum* plants treated only with 1 mM $Pb(NO_3)_2$ accumulated over 50 mg Pb × g^{-1} dry wt during 4 days of cultivation. Whereas in roots of pea plants exposed to Pb + 0.5 mM EDTA 35% more Pb was observed. When 1 mM EDTA was applied roots of pea accumulated over 67% more metal. The presence of EDTA also increased metal uptake and transport to the aboveground parts. In pea plants treated only with 1 mM lead nitrate less than 3 mg Pb × g^{-1} dry wt was transported, whereas in *P. sativum* treated with Pb–EDTA doubled amount of Pb was observed in stems and leaves.

Keywords: *Pisum sativum*; Phytoremediation; Detoxification; Lead ions; EDTA; Glutathione; Phytochelatins

1. Introduction

Small amounts of metals constantly circulate between bio, geo-, hydro- and atmospheric systems. Increasing pollution of the environment caused by heavy metals (HM) is most often the result of antropogenic activities, e.g. metallurgical industry, mining, agriculture and waste utilization (Table 1). A high level of metal accumulation in the soil and in ground and surface waters poses a threat to normal functions of plants and both directly (alimentary canal) and indirectly (the respiratory system) in men. An increase in the level of the phytoavailability of necessary (Cu^{2+}, Zn^{2+}, Mn^{2+}) and nonessential (Cd^{2+}, Pb^{2+}, Hg^{2+}) metals is harmful. Phytotoxic properties are shown by Cu, Ni, Zn ions, whereas Pb and Cd ions are most dangerous for men. Plants can take up and accumulate significant amounts of Pb and Cd without any visible changes in their habit or yield, thus introducing these metals into the food chain. Depending on soil reaction mobile Pb forms can make up 9–12% of total Pb content and Cd forms—even up to 30% of its total content (Dąbrowska-Naskręt, 2002). Bioavailable forms of Pb are mainly complex ions, $PbOH^+$ and $Pb(CO_3)_2^{2-}$ in alkaline soils, while in acid soils Pb^{2+} and $PbHCO_3^+$ prevail (Kabata-Pendias and Pendias, 1999). Microorganisms and plants have a number of various mechanisms which protect them against the toxic effect of heavy metals. Here we can include active translocation of metals, synthesis of peptides able to bind metal ions, e.g. metallothioneins or phytochelatins, storage of the metals in the vacuole and many other. In the recent years, intense research on the use of plant properties in the remediation process of the environment polluted with heavy metals has been conducted. Techniques using green plants for the removal or inactivation of organic compounds or heavy

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Table 1
Anthropogenic sources of toxic metals (ref. Nriagu and Pacyna, 1988) in tons per year

Metal	Fuel, power and metallurgy	Agriculture	Manufacturing	Waste disposal
Lead	325.88	192.00	9.30	58.57
Cadmium	16.44	3.67	2.45	25.47
Cooper	26.95	411.00	33.74	68.88
Nickel	239.90	60.25	7.44	60.97
Zinc	162.87	824.94	85.02	168.82

metals are commonly referred to as phytoremediation. Traditional methods of cleaning polluted areas such as excavation or burial are very expensive, require specialized equipment and are mainly associated with disappearance of all life activity (Wójcik, 2001; Cunningham and Berti, 2000). Application of plants is much less expensive and less invasive for the environment (Cunningham and Berti, 2000). Unfortunately, phytoremediation techniques are very time-consuming and its effects are visible after only several years. Plants ideal for phytoremediation should posses multiple traits. They must be fast growing, have high biomass, deep roots, be easy to harvest and tolerate and accumulate a range of heavy metals in their aerial parts (Clemens, 2002). Out of various phytoremediation methods phytoextraction, phytostabilization and rhizofiltration are the most effective in removing or inactivating metal ions. Phytoextraction is the most important method of removing heavy metals from the soil. Initially, research on using plants in phytoextraction focused on plants, which can grow in an extremely polluted environment and accumulate up to several percent of a metal in their leaves and shoots. Such plants are called hyperaccumulators and the process is classified as natural phytoextraction. The best known Pb hyperaccumulators are, for instance: *Thlaspi caerulescence*, *Thlaspi rotondifolium* or *Alyssum lesbiacum* (Baker and Brooks, 1989). However, all plants which are well-adapted to life in a heavily polluted environment are slow growing and produce little biomass. Therefore, some researchers think that these plants will not fulfil all expectations related to removal of metals from the environments. One of the special plants is *Sesbania drummondii*, a leguminous shrub (Sahi et al., 2002). Shoot concentrations of > 4% Pb were obtained from *Sesbania* plants grown on modified Hoagland's solution containing 1 g $Pb(NO_3)_2$/l.

Plants used for Pb extraction should exhibit accumulation at the level of 1% of metal concentration in the aboveground organs and 20 t × ha^{-1} × $year^{-1}$ of aboveground biomass production (Huang and Cunningham, 1996). A substantial part of the present research focuses on such plants as Indian mustard, corn, pea, oats or barley which exhibit high tolerance to environment contaminated with heavy metals and, simultaneously, show high biomass growth (Salt et al., 1997; Huang et al., 1998; Ebbs and Kochian, 1998; Wu et al., 1999). In order to compensate for the relatively low level of metal uptake by these plants some authors suggest application of synthetic chelators, which facilitate the uptake of heavy metals, and their translocation to the aboveground parts. In 1974 Wallace suggested for the first time that metal–EDTA complexes formed in the soil can increase solubility and phytoavailability in metals. It was shown that among the studied synthetic compounds—EDTA (ethylenediaminetetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), CDTA (*trans* - 1,2 - diaminocyclohexane - *N,N,N′,N′* - tetraacetic acid) and EGTA (ethyleneglycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid)—the most effective Pb ion chelator is EDTA (Huang et al., 1997). Due to its application a 1.5% increase of Pb accumulation was observed in aboveground parts of white mustard growing on soil containing 600 mg Pb × kg^{-1} with almost 100 mg × kg^{-1} to (Blaylock et al., 1997), and for corn—from less than 500 mg × kg^{-1} to 2% on soil containing 2500 mg × kg^{-1} Pb (Huang et al., 1997). It was determined that EDTA not only increases the amount of soil Pb taken up by plants but also metal transport through the xylem and Pb translocation from roots to shoots and leaves (Huang et al., 1997; Epstein et al., 1999).

The presence of Pb^{2+} ions in the environment leads to many disturbances in metabolic processes essential for the plant, which results in a lowered rate of plant growth and a decrease in fresh and dry mass. The toxic effect Pb^{2+} consists mainly in the ability of Pb to react with function groups: sulfhydryl –SH, carboxyl –COOH and amine $–NH_2$, which leads to a decrease in or ever the loss of activity of many enzymes which are important for the cell functions. Plants have a whole set of universal mechanisms which are used to protect them against abiotic factors including metals, both non-essential (Hg^{2+}, Cd^{2+}, Pb^{2+}) and essential for the plant (Cu^{2+}, Fe^{2+}, Zn^{2+}) if occurring in excessive amounts. Cells of the root tip secrete various substances, e.g. base sugars, aminoacids, organic acids, phenol compounds, and also water-insoluble gels with a high content of polysaccharides and mucus containing large amounts of polyuronic acids (Seregin and Ivanov, 1998). These compounds may play the role of a cation-exchange column affecting the modification of cation mobility within the rhizosphere. It was observed (Przymusiński and Woźny, 1985; Piechalak et al., 2002) that lead can be accumulated and bound within the cell wall by among other things polysaccharides (Seregin and Ivanov, 1998). It was determined that the same plants prevent Pb penetration of the cell by intensification of calose synthesis (β-1,3-gluconate), which itself is not able to chelate metal ions but it constitutes a kind of a "mechanical barrier" before the plasmalemma (Samar-

dakiewicz et al., 1996). Moreover, it was shown that plants can also actively remove Pb from the cell. Malone et al. (1974) showed that Pb can be translocated from the cell to the cell wall in dictiosome vesicles, whereas Wierzbicka (1998) found out active Pb secretion into the apoplast by plasmotubules in *Allium cepa*.

Within the cytoplasm lead ions can be chelated by compounds containing the –SH groups, e.g. phytochelatins, glutathione, organic acids and other ligands. It was shown that the synthesis of the low-molecular peptides—called phytochelatins (class III metallothioneins)—takes place in most plants except metallothioneins under the influence of heavy metal presence. Phytochelatins occur as a result of the enzymatic reaction in which the function of the substrate is played by glutathione or its homolog (Grill et al., 1985; Tomaszewska, 2002). The main function of phytochelatins is maintaining the homeostasis of trace metals in the cell; these peptides chelate metal ions and form the low-molecular complexes (LMW–HM). These complexes enter the vacuole, where they gain acid-labile sulphur and form "high molecular weight complexes" (HMW) (Sanita di Toppi et al., 2002). HMW complexes occur within the vacuole as aggregates with crystalline CdS "nucleus" surrounded with phytochelatins. Due to a higher S content, it is possible to chelate metal in HMW more effectively, the presence of S also stabilises the complexes (Cobbett, 2000). Metal transport in the plant and the regulation of its concentration in the cytosol are integrally connected with plant growth and development. Transport proteins play an essential role in the homeostasis of heavy metals. Supposedly, translocation of metals through two phospholipid layers takes place by means of protein carriers which are integral proteins of the membrane. The driving force of this transport is the proton gradient controlled by H^+-ATP-ases localized within the cell membrane. They belong to a large group of phosphorylating enzymes, which also includes type P ATP-ases, which form phosphorylated intermediates by K^+-induced ATP hydrolysis. It is commonly assumed that type P ATP-ases are responsible for the translocation of both necessary (e.g. Cu^{2+}, Zn^{2+}, Mn^{2+}) and nonessential metals (Cd^{2+}, Pb^{2+}, Hg^{2+}) through the biological membranes (Rensing et al., 1998; Williams et al., 2000). Soliz and Vulpe (1996) named P-ATP-ases responsible for heavy metal transport as CPx-ATP-ases, because they possess one conservative transmembrane domain which consists of the CPx motif that may include 3-aminoacid fragments involved in translocation of metals:

Cys-Pro-Cys, Cys-Pro-His or Cys-Pro-Ser

Nowadays, the way in which metal ions are translocated in the plant is especially intensively studied because of a more and more common application of plants in the processes of remediation of heavy metal contaminated waters and soils.

2. Results

2.1. The effect of EDTA on Pb accumulation in various plant parts

The research that has been conducted on *Pisum sativum* (Piechalak et al., 2000, 2002) has shown that these plants exhibit average ability to take up and accumulate Pb in tissues and also a low level of translocation of the metal uptake to aboveground parts. This property may have special significance in the possible use of pea as a remediator. It seamed reasonable to conduct experiments with the use of a synthetic compound able to chelate Pb^{2+} ions, the property which would not only increase the amount of Pb uptake accumulated by the plant but would also increase the level of Pb translocation to the aboveground parts. Based on the literature data, EDTA, a derivative of acetic acid, was chosen. *P. sativum* plants were grown in three experimental variations:

1. with 1 mM $Pb(NO_3)_2$
2. with 1 mM $Pb(NO_3)_2 +$ 0.5 mM EDTA
3. with 1 mM $Pb(NO_3)_2 +$ 1 mM EDTA

It was determined that addition of 1 mM lead nitrate + 0.5 mM EDTA leads to an increase in the total amount of Pb taken up by plants by 35%, while doubling the EDTA amount increases lead uptake by 67.6% in comparison to plants grown only with lead nitrate. EDTA supplied with the metal in equimolar concentration had the strongest effect on the growth of accumulation and Pb transport in plants. It was determined that roots of pea cultivated with the addition of 1 mM $Pb(NO_3)_2$ + 1 mM EDTA accumulated 60% more Pb (81 mg Pb $\times$ g^{-1} dry wt) (Fig. 1a) than roots of plants treated with only 1 mM Pb^{2+} (50.5 mg/g dry wt). In shoots of plants treated with 1 mM Pb^{2+} + 1 mM EDTA for 4 days a double increase in the amount of accumulated Pb was observed, which was 4.197 mg Pb $\times$ g^{-1} dry wt (Fig. 1b), and similarly in leaves, which was 2.29 mg Pb $\times$ g^{-1} dry wt (Fig. 1c). Addition of 2 times lower amount of the chelator (0.5 mM) resulted in a 60% increase in the amount of the metal transported to shoots and leaves in comparison with plants cultivated with only 1 mM $Pb(NO_3)_2$. 27% more Pb were accumulated in shoots of the treated plants, whereas in leaves the increase in the amount of Pb uptake was 163%.

The results obtained with the use of a synthetic chelator show that EDTA does not exert such a dramatic effect on Pb accumulation growth in pea plants as it had

been expected. However, the application of EDTA (especially at 1 mM concentration) significantly improved the level of Pb translocation to the aboveground parts, and consequently, leaves contained 4% and shoots 10% of total metal uptake after 4 days of cultivation (Fig. 2).

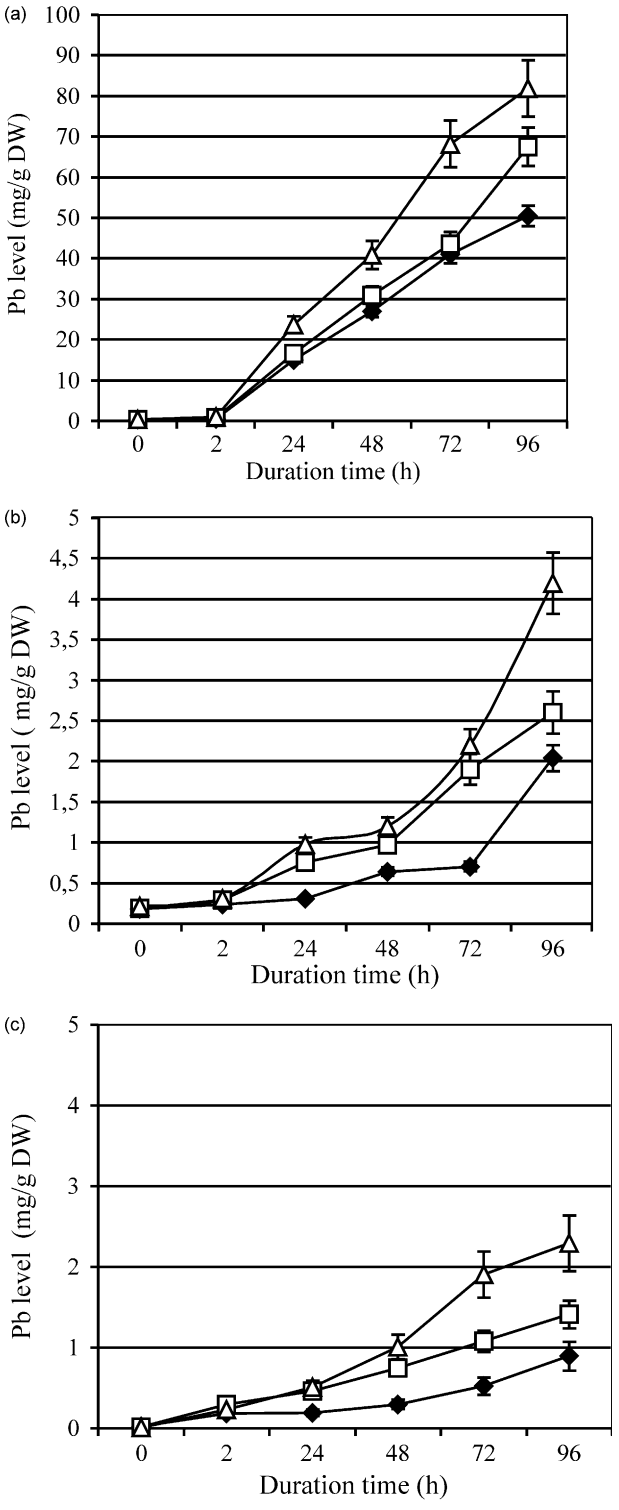

Fig. 1. Changes in Pb content (mg × g^{-1} DW.) in (a) roots of *Pisum sativum* plants, (b) stems of *Pisum sativum* plants, (c) leaves of *Pisum sativum* plants, grown hydroponically in Hoagland medium in the presence of ◆ 1 mM $Pb(NO_3)_2$, □ 1 mM $Pb(NO_3)_2$ + 0.5 mM EDTA and △ 1 mM $Pb(NO_3)_2$ + 1 mM EDTA for 96 h.

2.2. The effect of EDTA on the tolerance of P. sativum plants to Pb

No negative effect of EDTA presence on the rate of root elongation growth of pea plants or on plant biomass growth was observed. During 4 days of cultivation in a medium with the addition of 0.5 and 1 mM EDTA root elongation growth remained at the level similar to the control, i.e. 96% and 80%, respectively (Table 2). It was determined that addition of 1 mM EDTA caused a decrease in the biomass of pea plants by 13% in comparison with the control. Differences of the habits were observed between plants of pea exposed to 1 mM $Pb(NO_3)_2$ and treated with 1 mM $Pb(NO_3)_2$ supplemented with 1 mM EDTA. Inhibition of root elongation growth and browning of roots occurred in plants cultivated only with lead nitrate addition. However, when the chelator was added to the medium a clear decrease in the Pb toxic effect on plants was observed. The addition of EDTA eliminated to a great degree the inhibition of root elongation growth, lowered roots browning and resulted in a growing number of side roots. Moreover, the fresh weight of plants treated with Pb–EDTA was much higher in comparison with plants treated only with Pb and remained at the slightly lower level than the biomass of the control plants, which were grown only in Hoagland medium. When 1 mM Pb + 0.5 mM EDTA or 1 mM EDTA was used the fresh weight of plants was 71% and 85% of the control plant biomass, respectively (Fig. 3).

Indexes of tolerance were also determined for plants cultivated only with EDTA application. After 4 days of exposition IT values of these plants were above 80% (Table 2). These data indicate that EDTA addition,

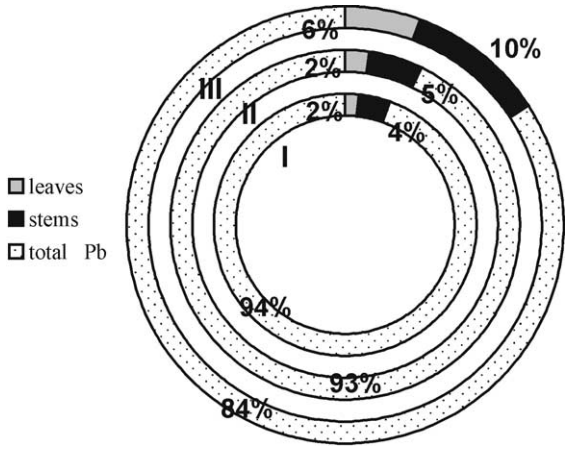

Fig. 2. Changes in Pb translocation from roots to the aboveground parts of *Pisum sativum* treated with Pb + EDTA after 4 days. I: 1 mM Pb, II: 1 mM Pb + 0.5 mM EDTA, III: 1 mM Pb + 1 mM EDTA.

Table 2
Index of tolerance—estimated by means of the Wilkins' test for *Pisum sativum* roots grown hydroponically in Hoagland medium in the presence of 1 mM $Pb(NO_3)_2$, 1 mM $Pb(NO_3)_2$ + 0.5 or 1 mM EDTA and 0.5 or 1 mM EDTA during 4 days

	Index of tolerance (%)
0.5 mM EDTA	96.1
1 mM EDTA	80.7
1 mM $Pb(NO_3)_2$ + 0.5 mM EDTA	84.4
1 mM $Pb(NO_3)_2$ + 1 mM EDTA	94.3
1 mM $Pb(NO_3)_2$	39.1

especially at 0.5 mM concentration, does not have a negative effect on plant growth.

The obtained data prove that pea plants cultivated with the addition of Pb–EDTA show high resistance to the phytotoxic effect of metals. The results obtained from the cultivation with EDTA addition were compared with the results obtained after exposing pea plants to 1 mM concentration of $Pb(NO_3)_2$. Adding 0.5 mM EDTA raised the IT value twice (Table 2), whereas after adding a higher concentration of the chelator (1 mM) root elongation growth of pea was close to the control plants and their fresh biomass was only 9% lower (Fig. 3).

2.3. The functioning of the detoxicative system in *P. sativum* plants cultivated with the addition of Pb–EDTA

The presence of Pb^{2+} ions leads to a number of cell detoxication mechanisms which, among other things, include peptides of high sulphur content, e.g. glutathione and its homologs, phytochelatins and homophytochelatins. Due to the presence of –SH groups these compounds show the ability to bind heavy metal ions and antioxidative properties.

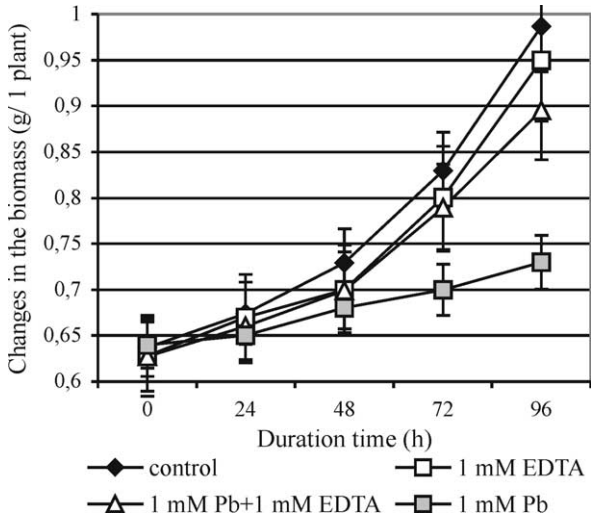

Fig. 3. Changes in fresh weight of *Pisum sativum* plants treated with 1 mM $Pb(NO_3)_2$ 1 mM EDTA and 1 mM $Pb(NO_3)_2$ + 1 mM EDTA for 96 h.

In roots of pea treated only with EDTA the level of GSH remained at the same level as in the control (Fig. 4). Changes in the dynamics of GSH level occurred between roots exposed to 1 mM $Pb(NO_3)_2$ and roots incubated with 1 mM Pb + 0.5 and 1 mM EDTA. The level of glutathione in roots of the control plants remained at the stabile level of 650–800 nmol SH × g^{-1} fr wt during 4 days of cultivation. Under lead ion stress conditions caused by adding 1 mM $Pb(NO_3)_2$ the level of GSH increased rapidly after 8 h after the moment of metal application (Fig. 4). A gradual decrease was observed during further cultivation. However, completely different profiles of changes of reduced glutathione were observed in roots treated simultaneously with Pb–EDTA. A slight increase in glutathione amount in comparison to the control plant roots (Fig. 4) was observed in the roots of pea plants treated with 1 mM Pb + 1 mM EDTA. During the first 24 h after Pb + EDTA addition the level of GSH remained at the level of the control. A gradual increase in glutathione amount was shown after 48 h and after 4 days of cultivation 48% more GSH was determined in roots of plants incubated with 1 mM Pb + 0.5 mM EDTA, and 68% more when 1 mM Pb + 1 mM EDTA were added (Fig. 4).

The level of homoglutathione in roots of the control plants remained at the stabile level of 100 nmol SH × g^{-1} fr wt during 3 days of cultivation. In roots of plants incubated with lead ion and similar with 1 mM EDTA was observed gradual increase in the level of hGSH (Fig. 5). Under stress conditions caused by adding 1 mM $Pb(NO_3)_2$ + 0.5 or 1 mM EDTA the level of hGSH

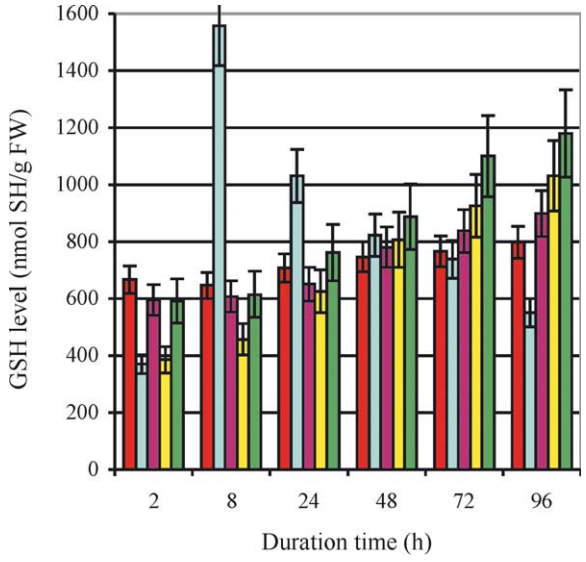

Fig. 4. The effect of lead accumulation on changes of glutathione contents in roots of *Pisum sativum* treated for 96 h. Thiol concentration is expressed in nmol SH × g^{-1} FW. control 1 mM Pb 1 mM EDTA 1 mM Pb + 0.5 mM EDTA 1 mM Pb + 1 mM EDTA.

increased rapidly (even 6–9 times) during the 3 days of cultivation (Fig. 5).

It was demonstrated that in roots of pea plants treated with Pb–EDTA first phytochelatins appeared only after 24 h of incubation while in roots of plants treated with Pb and without the chelator induction of PCs synthesis took place much earlier—just after 2 h of incubation (Fig 6). EDTA application caused a visible delay in the induction of PCs synthesis and thereby in the activation of phytochelatin synthase by Pb ions. Most Pb occurs in the form of complexes with EDTA. The maximum level of PCs in pea cultivated with 1 mM $Pb(NO_3)_2$ was observed after 72 h (PC = 4 500 nmol SH/g^{-1} fr wt.), while in plants treated with 1 mM $Pb(NO_3)_2$ + 0.5 mM EDTA the amount of PCs increased during 4 days of cultivation to reach 9 800 nmol SH × g^{-1} fr wt after 96 h (i.e. 2.5 times more than in plants grown only with lead ions after 96 h). Also in plants grown with 1 mM $Pb(NO_3)_2$ + 1 mM EDTA a significant increase in PCs amount was observed after 4 days of exposition. The maximum level of PCs was determined after 96 h and it was 8700 nmol SH × g^{-1} fr wt. (over 2 times more PCs than in plants with 1 mM of lead nitrate). Although application of EDTA delayed the synthesis of PCs but their total amount was over 2 times higher. The amount of synthesized hPCs was also over 6 times higher than in pea plants treated only with $Pb(NO_3)_2$ (Fig. 6).

The first hPCs appeared 24 h after application of Pb and Pb + EDTA. The maximum level of hPCs was observed 96 h after stress factor application in all variations of the experiment. However, in plants treated with Pb + EDTA we determined 6–7 times higher level of PCs, i.e. from 1800 to 2200 nmol SH × g^{-1} fr wt (Fig. 6).

Similarly as in the roots of the examined plants the first PCs appeared 24 h (Fig. 7) after stress factor application in leaves of pea plants treated with Pb–EDTA. However, the level of PCs observed in leaves during the whole time of exposition was more than ten times, and even several dozen lower than in the roots. For the sake of comparison Fig. 7 presents data con-

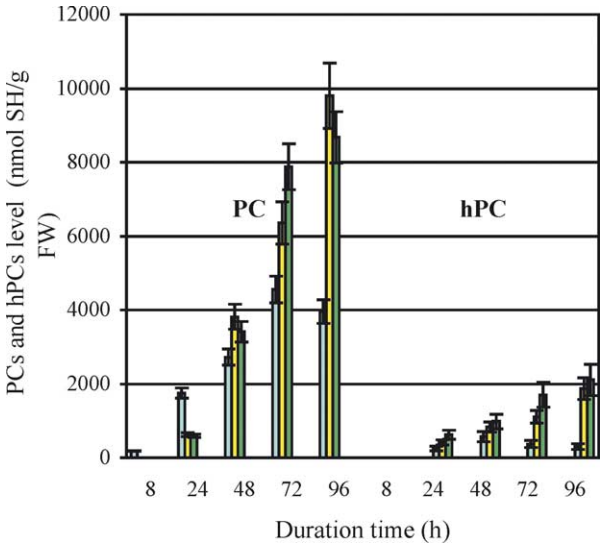

Fig. 6. The effect of lead accumulation on changes of phytochelatin and homophytochelatin contents in roots of *Pisum sativum* treated for 96 h. Thiol concentration is expressed in nmol SH × g^{-1} FW. 1 mM Pb 1 mM Pb + 0.5 mM EDTA 1 mM Pb + 1 mM EDTA.

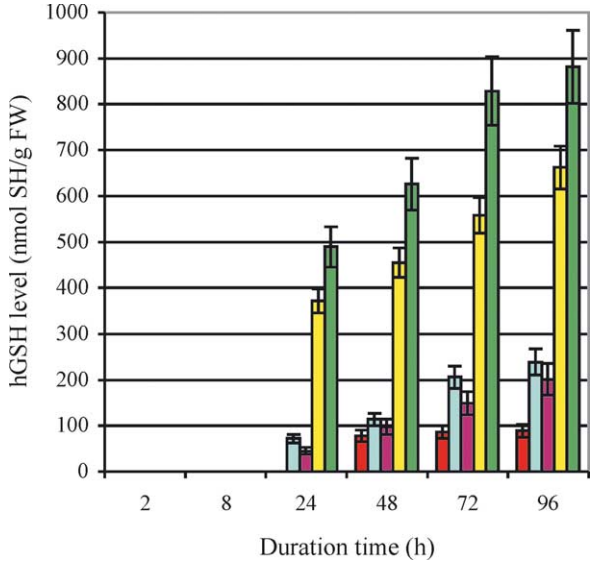

Fig. 5. The effect of lead accumulation on changes of homoglutathione contents in roots of *Pisum sativum* treated for 96 h. Thiol concentration is expressed in nmol SH × g^{-1} FW. control 1 mM Pb 1 mM EDTA 1 mM Pb + 0.5 mM EDTA 1 mM Pb + 1 mM EDTA.

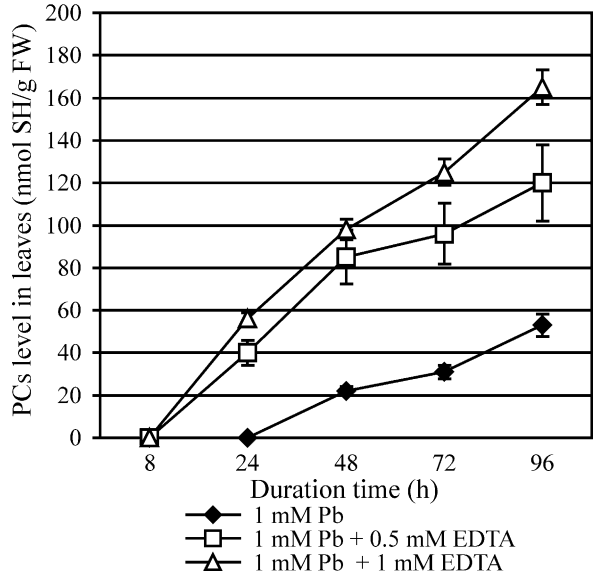

Fig. 7. The effect of lead accumulation on changes of phytochelatin contents in leaves of *Pisum sativum* treated with 1 mM $Pb(NO_3)_2$ and 1 mM $Pb(NO_3)_2$ + 0.5 or 1 mM EDTA for 96 h. Thiol concentration is expressed in nmol SH × g^{-1} FW.

cerning the level of PCs in leaves of pea plants treated with only 1 mM concentration of Pb. It was demonstrated that under these conditions PCs appeared 24 h later, and their concentration was 2–3 times lower than in leaves of plants cultivated with Pb + EDTA. The obtained results prove that PCs synthase is present in leaves of the examined plants, and its activation depends on the rate of Pb translocation from roots to shoots, which was markedly enhanced by chelate application.

Pea plants were also treated with 0.5 or 1 mM EDTA for 4 days, but in roots and leaves of this plants were no detectable PCs. The lack of changes in metabolism of thiol compounds in pea plants exposed to EDTA may be caused by too short exposition time.

2.4. HPLC–MS application

Analysis of the plant material by means of the HPLC method connected *on line* with mass spectroscopy confirmed identification of thiol compounds: cysteine, glutathione, homoglutathione, phytochelatins (PC_2, PC_3, PC_4) and homophytochelatins (hPC_2, hPC_3) based on their molecular weight. Presence of cysteine, γ-glutamylcysteine, glutathione and homoglutathione was determined in the root extract of plants cultivated under control conditions. Whereas in roots of plants treated with lead nitrate presence of cysteine, γ-glutamylcysteine, glutathione, homoglutathione and their polymers PC_2, PC_3, PC_4 phytochelatins and hPC_2, hPC_3 homophytochelatins.

3. Discussion

Lead belongs to the group of chemical elements which are not necessary for the life of plants, nor for any other living organisms, including man. It is a fact of great importance to understand mechanisms due to which plants can live unharmed in an environment contaminated with lead compounds. Particular species, and even plant varieties differ as to the tolerance to the harmful effect of heavy metals and their ability to accumulate trace metals (Kabata-Pendias and Pendias, 1999). Plant characteristics change according to the environmental conditions, plant age and its general physical condition and the vegetation period. There are several possible areas through which lead can penetrate plants. However, it is commonly that roots are the main pathway through which trace metal ions penetrate plants. It was determined that in plants metal uptake at first stopped on root surface and then a portion of ions which penetrate roots is bound in cell walls and the rest is accumulated in the intercellular space (Malone et al., 1974; Wierzbicka, 1987). It was determined that in the examined pea plants roots are the main accumulation site of Pb^{2+} (Figs. 1 and 2). After 4 days of cultivation with 1 mM lead nitrate over 90% of Pb uptake was accumulated in the roots. During 96 h of incubation with Pb a steady increase in the level of accumulated metal up to 50.5 mg Pb × g^{-1} dry wt was observed. Comparing the level of Pb accumulation in the examined plants (Fig. 1) one could observe an almost linear increase in the amount of deposited metal during 4 days of growth with $Pb(NO_3)_2$ supplement. Our earlier studies of legumes including *Lupinus luteus* (Tomaszewska et al., 1996), *P. sativum*, *Vicia faba* and *Phaseolus vulgaris* (Piechalak et al., 2000, 2002) have also shown that most, i.e. over 90% of Pb uptake was accumulated in the roots. Comparatively, plants of the Brassicaceae family, which are thought to be good lead accumulators, the metal content in the roots was over 100 mg Pb × g^{-1} dr. wt and in the aboveground parts about 10 mg Pb × g^{-1} dry wt, i.e. 10 times less than in the roots (Kumar et al., 1995). *P. sativum* plants treated with 1 mM $Pb(NO_3)_2$ showed low ability to translocate Pb to the aboveground organs. Only 6% (3% in leaves and 3% in stems) of Pb taken up by plants was deposited in shoots of pea. It was demonstrated that the layer of cuticle and waxes in leaves usually forms an effective barrier against atmospheric Pb penetration. This protective layer stops Pb^{2+} ions on the surface of leaves and does not let them into the tissues (Little and Martin, 1992; Piechalak et al., 2002).

EDTA application made it possible to raise the level of metal translocation to aboveground parts from 6% to 16% (6% leaves and 10% stems). This is not much if we consider that Huang et al. (1997) obtained over a 100-fold increase in the amount of Pb accumulated in the aboveground parts of soil pea cultivation. Besides pea the authors also tested other plants including corn and sunflower. However, Pb amount in leaves and stems of pea was, for instance, over 3 times higher than in corn. In this experiment the Pb content in the soil was 2500 mg/kg and EDTA 0.5 g/kg. It is difficult to say explicitly why EDTA application in our experiment did not bring the expected result. There could be several reasons: the species of pea, the stage of plant development, and above all, the short, 4 days long, time of cultivation. Most experiments using EDTA are long-time cultivations, the shortest ones being 7–10 days (Wójcik and Tukendorf, 1999a; Wu et al., 1999; Sahi et al., 2002). Most often experiments last 2–3 weeks (Blaylock et al., 1997; Wójcik and Tukendorf, 1999b; Geebelen et al., 2002; Wu et al., 2003) and 8–9 weeks (Ebbs and Kochian, 1998; Begonia et al., 1998). There is a lot of literature on increasing Pb accumulation in the aboveground plant parts, among others, in *Brassica juncea* (Blaylock et al., 1997; Vassil et al., 1998; Epstein et al. 1999), corn (Huang et al., 1998; Wu et al., 1999), bean (Seret et al., 2001). It was demonstrated that Pb is taken up from the medium or the soil in the form of EDTA

complexes and in this form it enters the plant more easily (Vassil et al., 1998; Wu et al., 1999). Obviously, the results obtained from the hydroponic cultivations may differ from the data concerning soil cultivation. There are additional active factors which are absent in hydroponics, for instance: soil chemistry and the presence of different types of organic compounds playing the role of ligands for trace metals. Moreover, there are microorganisms which exert a significant effect on the activity of many metals including Pb, and which are able to conduct metylation of chemical elements (Kabata-Pendias and Pendias, 1999).

Differences in growth rate of root length are an important indicator of plant resistance to heavy metals—one of the most commonly used measurement methods is one described by Wilkins (1957). This method consists in comparing the growth of roots growing with the addition of the stress factor with the growth of roots of control plants. The obtained results is described as the Index of tolerance (IT). The lowered root elongation growth of pea plants observed after application of 1 mM $Pb(NO_3)_2$ corresponds with the results described earlier by many authors. The negative effect of 1 mM $Pb(NO_3)_2$ on root elongation growth of lupin, pea, bean and horse-bean was described by us earlier (Tomaszewska et al., 1996; Piechalak et al., 2002). Geebelen et al. (1999, 2002) also showed Pb phytotoxicity. These authors described a noticeable reduction of root length of *P. vulgaris* (L. cv ''Limburgse vroege'') already at 0.08 μM $Pb(NO_3)_2$, whereas the application of 5 times higher (0.4 μM) concentration of lead nitrate caused a decrease in the amount of bean total fresh weight by as much as 90%.

Disturbances in root growth caused by the lead effect were described also for other plants, e.g. onion (Wierzbicka, 1989, 1995), lupin (Przymusiński et al., 1991; Tomaszewska et al., 1996), Indian mustard (Liu et al., 2000), cucumber (Talanova et al., 2000). Mechanisms initiating inhibition of root elongation are not well-understood. This must be a complex process that is affected by many factors. It is thought that the process is correlated with a decrease in dry and fresh weight of plants (Gabara and Gołaszewska, 1991). It is assumed that one of the reasons can be the disturbance of plant water balance. The resulting water deficit is one of the main factors which cause limitations of growth and plant development in an environment contaminated with heavy metal ions (Wierzbicka, 1994). The remaining factors are: disturbances in forming microtubules and consequently inhibition of cytokinesis (Wierzbicka, 1989), a lower number of mitotic divisions in the root meristematic zone (Wierzbicka, 1989; Przymusiński et al., 1991) or disturbances of *c*-mitosis (Wierzbicka, 1995; Liu et al., 2000).

Limitation of root elongation growth is also caused by other heavy metals: Cd and Cu ions in broad bean (Karavaev et al., 2001), Cd ions in Indian mustard cultivation (Zhu et al., 1999), pea (Dixit et al., 2002) and corn (Wójcik and Tukendorf, 1999a, b) and Cu and As ions in *Holcus lanatus* cultivation (Hartley-Whitaker et al., 2001). This was probably caused by suberization or intensified production of phenol compounds (Punz and Sieghardt, 1993; Woźny et al., 1995), which is a non-specific plant response also to the presence of many heavy metals, including Cd (Howden et al., 1995) and Cu (Chongpraditum et al., 1992).

Numerous reports on the use of synthetic chelators in enhancing uptake and transport of heavy metals by plants have been published in the recent years. It was demonstrated that among the studied chelators: EDTA, DTPA, CDTA, EGTA—EDTA was the most effective for Pb (Huang et al, 1997; Cooper et al., 1999; Lasat, 2002). One of the most intensely studied subject at the moment is the possibility of using commercial plants exhibiting tolerance to heavy metal ions and high biomass growth in induced phytoremediation e.g.: corn, barley, bean, pea or wheat (Salt et al., 1998). Enhanced uptake and accumulation of metals by plants was obtained due to introducing a proper chelator into the environment. Cunningham and Ow (1996) determined accumulation of Pb ions at the level of 1% Pb in the aboveground parts of corn and pea cultivated with EDTA addition. Krishnamurti et al. (1997) showed that low-molecular organic acids (LMWOA) have an essential importance for the mobility and phytoaccessibility of Cd. These authors found out that these compounds enhanced Cd solubility in soil solution by creating Cd–LMWOA complexes. Wu et al. (2003) studied the effect of the presence of both LMWOA and on solubility and mobility of heavy metals (Cu, Zn, Pb, Cd). These authors showed that EDTA application raised the amount of the metals in soil solution even by 300 times for Cu and 600 times for Pb, whereas for Cd and Zn ions, 100 times and 30 times, respectively. Introduction of low-molecular organic acids into the soil resulted in a much less dramatic effect—the maximum level of the metals in soil solution raised 20–30 times.

In our experiments *P. sativum* plants were grown with the addition of 1 mM $Pb(NO_3)_2 + 0.5$ or 1 mM EDTA. The application of a synthetic chelator—EDTA—at the concentration of 1 or 0.5 mM together with Pb ions into medium resulted in a significant limitation of the metal phytotoxic effect. This concerns inhibition of root elongation growth, root colouring and the appearance of mucus (Piechalak et al., 2000). In pea plants the IT values were: 84.4% for 1 mM $Pb(NO_3)_2 + 0.5$ mM EDTA, and as much as 94.2% for 1 mM $Pb(NO_3)_2 + 1$ mM EDTA; whereas it was only 39% for pea plants cultivated without the chelator (Table 2). The IT values were also high for pea plants cultivated with 1 and 0.5 mM EDTA application these were 80.7% and 96.1%, respectively. The level of fresh weight for plants treated

with 0.5 mM EDTA was similar as in the controls, whereas 1 mM EDTA application caused a reduction of biomass by 13% during 4 days of cultivation. The similar results for pea were also obtained by Wójcik and Tukendorf (1999a, b). These authors observed the EDTA effect on limiting heavy metal phytotoxicity and showed that corn plant cultivated for 7 and 14 days with 50 μM $Cd(NO_3)_2$ + 0.2 or 0.4 mM EDTA exhibited a similar growth of fresh weight of the aboveground parts as the controls while the root elongation growth treated with Cd + 0.4 mM EDTA was even higher than in the control plants. *Zea maize* plants cultivated with the addition of cadmium nitrate for 14 days showed about 30–40% reduction of fresh weight of roots and leaf surface. Plants treated with Cd ions with EDTA by about 10–25% in comparison to the control plants. The reduction of biomass of roots and aboveground parts of corn treated only with EDTA (0.2 and 0.4 mM) obtained by the authors also corresponds to the results obtained by us. Also other authors (Greger and Lindberg, 1986; Vassil et al., 1998) observed a negative effect of EDTA alone on plant development. Probably, EDTA presence affects negatively the balance of minerals, e.g. Zn, Cu, Fe, and Ca, which leads to disturbances in cell metabolism and destabilizes biological membranes. Accumulation of EDTA in surface and drinking water is a disadvantageous property of this compound. It does not undergo degradation by means of conventional biological or physicochemical methods. This chelator is not toxic in concentrations present in water environment. However, a serious danger is connected with EDTA ability to bind heavy metals and liberate them from sediments. This could not take place under normal conditions. An additional danger is connected with the formation of EDTA chelates with ions of metals necessary in functioning of plants, which leads to disturbances in basic metabolic mechanisms.

Geebelen et al. (2002) did not find any negative changes in the amount of fresh weight of roots and leaves or length of stems in bean plants cultivated in Hoagland medium supplemented only with 0.05 and 0.2 mM EDTA. Whereas, the addition of Pb–EDTA complexes (10–200 μM) to the medium by the authors resulted in lowering the roots biomass and shortening of the stems already at 10 μM Pb–EDTA. They also observed a decrease in the fresh weight of *P. vulgaris* leaves.

There are no literature data on EDTA effect on the metabolism of thiol compounds in treated plants. Based on the presented results it can be said that the level of glutathione changes depends on the intensity of a stress factor. Glutathione synthesized intensely in leaves is most probably transported to the roots and there it is used as a metal chelator (Mehra and Mulchandani, 1995; Piechalak et al., 2002), a substrate for the synthesis of phytochelatins and as an antioxidant (Noctor et al., 1998). Glutathione transport from leaves to roots had been shown earlier by other researchers (Rauser et al., 1991; Noctor et al., 1998; Foyer et al., 2001). A steady increase in glutathione and homoglutathione levels was observed during 4 days of cultivation of pea plants with the addition of $Pb(NO_3)_2$ and EDTA. Simultaneously, it was shown that the synthesis of both phytochelatins and homophytochelatins takes place in root cells of *P. sativum* treated with Pb–EDTA. It was surprising as EDTA shows a very high affinity to Pb^{2+} ions (log $K = 17.88$ for $PbEDTA^{2-}$) and it can be assumed that Pb is taken up by plants in the form of complexes. It is assumed that phytochelatins synthesis is activated by free metal ions or their complexes with GSH (Vatamaniuk et al., 2000). This implies that the presence of chelated Pb should not affect the PCs synthesis. However, Seret et al. (2001) showed that dissociation of EDTA complexes with Pb and Zn took place in bean plants and then Pb^{2+} and Zn^{2+} ions were bound into other forms. The authors demonstrated that in leaves of *P. vulgaris* plants cultivated with Pb–EDTA application, about 60–70% of Pb was bound in the form of Pb–EDTA complexes, whereas 30–40% occurred in the form of free metal. The similar results were obtained for Zn–EDTA complexes. A partial degradation of Pb–EDTA complexes taken up by pea plants would explain activation of PCs synthesis obtained in our experiments. Phytochelatins appeared in pea root cells only 24 h after the moment of Pb–EDTA application and not after 2 h as it was the case in plants cultivated with $Pb(NO_3)_2$. Simultaneously, the amount of synthesized PCs was even 2.5 times higher than in plants treated only with lead nitrate (Fig. 6). In all experimental variation it was shown that PCs level in leaves was always markedly lower than in roots, regardless of EDTA presence. On the other hand, Wojcik and Tukendorf (1999a) showed a higher level of PCs in the aboveground parts than roots of wheat grown in Hoagland medium with 50 μM Cd, despite the fact that most cadmium was accumulated in the roots. It is well known that PCs play a significant role in the process of maintaining homeostasis of trace metals in the cell, sulphur transport, detoxification of heavy metals and also as antioxidant (Zenk, 1996; Sanita di Toppi and Gabbrielli, 1999; Cobbett, 2000; Hall, 2002; Tsuji et al., 2002). Mechanisms of Cd detoxification are best understood. Most cadmium ions in the roots is bound with thiol compounds (Cys, GSH, PCs) creating complexes transported to the vacuole. However, glutathione and PCs do not participate in Cd transport from the roots to the aboveground parts. Translocation of cadmium ions takes place through the xylem in the form of cations or in a form associated with low-molecular organic acids (Salt et al., 1998). The role of PCs in detoxification of heavy metals is confirmed by experiments with *cad 1* mutants of *Arabidopsis thaliana* conducted by Howden et al. (1995). Namely, these authors showed that mutants

deprived of their sensibility to Cd ions even at a low concentration of 0.3 μM Cd^{2+}. Additionally, Tsuji et al. (2002) showed that a very strong induction of PCs synthesis by Zn ions takes place in sea algae *Dunatliella tertiolecta*. The authors found out that incubation (pretreatment) of algae with 100 μM $ZnCl_2$ for 12 h did not affect their future growth and, simultaneously, it enhanced their tolerance to the presence to heavy metals—Cd, Hg, Cu and Pb—hydrogen peroxide and parquat.

Phytochelatins and their homologs take part in processes of chelating and disactivating HM ions in the cell. The highest tolerance of pea plants cultivated with the addition of Pb–EDTA we have demonstrated can be associated with the increased capacity of the phytochelatin detoxication system. It is highly probable that the higher amount of glutathione and phytochelatins contributed to lowering Pb phytotoxicity, which resulted in an increased IT value (Table 2) and an increase in plant fresh weight (Fig. 3) in comparison with plants cultivated only with lead nitrate. Detoxication system is also connected with the emission of exudates into the rhizosphere, binding metals in cell walls, chelating ions of metals by organic acids or free aminoacids and many other mechanisms, with which plants defend themselves against phytotoxicity of heavy metals.

The toxic effect of heavy metals has an essential significance not only for plants but also for the existence of the whole ecosystem. Plants growing in a contaminated environment show disturbances of cell metabolism, growth reduction, lower biomass production etc. Simultaneously, these plants have also a number of various defense mechanisms enabling them to adapt to increasing environmental pollution. Plant adaptive abilities are the subject of intense research aimed at finding applications for such plants in phytoremediation.

4. Experimental

4.1. Plant material

The plant material were *P. sativum* plants (cv. "Kwestor"). Morphologically selected seeds were first sterilized in sodium hypochlorite for 5 min, washed in distilled water, then sterilized in 75% ethanol for 5 min and again washed in distilled water. The seeds were soaked in bidistilled water for 4–5 h. The seeds germinated in the dark at 22 °C for 3 d. Seedlings 4–5 cm long were placed in a germinating bed (size: 21 cm × 15 cm) containing 1700 ml 10× diluted Hoagland medium. The seedlings were cultivated for the next 72 h under controlled conditions; the photoperiod was 16/8 h, temperature—from 22 °C (at day) to 18 °C (at night), light intensity was 75 μM m^{-1} × 2 s^{-1}. After 3 days the medium was replaced by a 100× dilution and the following were added:

I—1 mM $Pb(NO_3)_2$
II—1 mM $Pb(NO_3)_2$ + 1 or 0.5 mM EDTA
III—1 or 0.5 mM EDTA

The plant material—roots, shoots and leaves—were collected after 0, 2, 8, 24, 48, 72 and 96 h of cultivation and washed in 10 mM $CaCl_2$ for 5 min, and then in bidistilled water. The plant material was frozen in liquid nitrogen and stored at −80 °C or directly used in determination.

4.2. Index of tolerance

The index of tolerance (IT) was determined according to the Wilkins' method (1957)

$$\mathrm{IT} = \frac{\text{average length of roots in tested solution}}{\text{average length of roots in control}} \times 100\%$$

The plants used in determining the IT were simultaneously weighed and measured in order to obtain information on shoot length growth and the fresh weight of treated plants.

4.3. Determining the level of Pb in plant tissues

The plant material was weighed (the degree of accuracy was 0.0001 g), dried at 100±2 °C to stable mass and again weighed with the same accuracy degree. The dried samples underwent microwave mineralization in an MDS-2000 apparatus made by CEM Corporation. Three-stage dilution was conducted in a closed system in LDV containers, using 5 ml 65% HNO_3 (max. 0.000005% Hg). After mineralization the samples were taken to 10 ml measuring flasks which were filled with deionized water. In these experiments Pb content was determined using the AAS method (atomic mass spectroscopy).

4.4. HPLC analysis of the content of thiol compounds

The plant material was homogenized in a porcelain mortar at 4 °C with 5% sulfosalicylic acid at 1:4 ratio. The homogenate was centrifuged three times at 10 000 *g* for 5 min at 4 °C. The obtained extract was used in HPLC analysis: 250 μl was put on the column. Chromatographic analysis was conducted using the Beckman apparatus, on a 4.6 mm × 45 mm precolumn and a column filled with Nucleosil 10 μm C-18, (size 4.6 × 250 mm). The separation was conducted for 45 min in a linear gradient of 70% acetonitrile at 0.1% TFA from 0 to 20%. The level of thiol compounds was determined in a postcolumn reaction connected *on line* with HPLC. In this reaction separated peptides associated with DTNB reagents (Ellman, 1959). The measurement of the amount of thiol compounds was done at wave length $\lambda = 412$ nm. The flow of eluents through the column was done at 1

ml × min^{-1} rate. The DTNB flow in the postcolumn reaction was done at 0.5 ml × min^{-1} rate (Tomaszewska et al., 1996).

4.5. HPLC–MS analysis

The plant material was homogenized in a porcelain mortar at 4 °C with 5% sulfosalicylic acid in 1:4 ratio. The homogenate was centrifuged three times at 10 000 *g* at 4 °C for 5 min. The obtained extract was used in HPLC–MS analysis; 50 μl of the extract were put on the column. Chromatographic analysis was done using a Waters/Micromass apparatus on a column filled with Nucleosil 10 μm C-18 (size: 4.6 mm × 250 mm). Separation was conducted in a linear gradient of 70% acetonitrile in 0.1% TFA from 0 to 20% for 45 min. Ionization and fragmentation of the analyzed molecules takes place in the ionization chamber of a mass spectrometer. Ionization was done using the electrospray method (ES–MS).

4.6. Statistical analysis

The statistical analysis of the results was done with the use of the Statistica software, counting arithmetic means and mean errors. The results of growth parameters are mean values of 21–25 measurements from 3–4 independent repetitions. The analysis of thiol compounds in extracts of plant organs was done 3–4 times. The measurement of Pb level in different plant organs was done twice. The analysis of thiol compounds based on molecular mass values obtained using the HPLC–MS method was done twice.

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