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The effect of EDDS chelate and inoculation with the arbuscular mycorrhizal fungus *Glomus intraradices* on the efficacy of lead phytoextraction by two tobacco clones

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Abstract

Two pot experiments were conducted to investigate the effect of inoculation with the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* on Pb uptake by two clones of *Nicotiana tabacum* plants. Non-transgenic tobacco plants, variety Wisconsin 38, were compared in terms of Pb uptake with transgenic plants of the same variety with inserted gene coding for polyhistidine anchor in fusion with yeast metallothionein. Bioavailability of Pb in experimentally contaminated soil was enhanced by the application of a biodegradable chelate ethylenediaminedissuccinate (EDDS).

EDDS addition (2.5 and 5.0 mmol kg^{-1} substrate) increased Pb uptake from the substrate and enhanced Pb translocation from the roots to the shoots, with shoot Pb concentrations reaching up to 800 mg kg^{-1} at the higher chelate dose. Application of a single dose of 5 mmol kg^{-1} proved to be more efficient at increasing shoot Pb concentrations than two successive doses of 2.5 mmol kg^{-1}, in spite of a marked negative effect on plant growth and phytotoxicity symptoms. Pb amendment (1.4 g kg^{-1} substrate) connected with either dose of EDDS decreased significantly plant biomass as well as reduced the development of AM fungi. AM inoculation promoted the growth of tobacco plants and partly alleviated the negative effect of Pb contamination, mainly in the case of root biomass.

No consistent difference in Pb uptake was found between transgenic and non-transgenic tobacco plants. The effect of AM inoculation on Pb concentrations in plant biomass varied between experiments, with no effect observed in the first experiment and significantly higher root Pb concentrations and increased root–shoot ratio of Pb concentrations in the biomass of inoculated plants in the second experiment. Due to probable retention of Pb in fungal mycelium, the potential of AM for phytoremediation resides rather in Pb stabilisation than in phytoextraction.
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1. Introduction

Lead is an element occurring naturally in the earth's crust; however, its concentrations can be highly elevated due to industrial activities, including mining and smelting of metalliferous ores, burning of leaded gasoline and the use of fertilizers, pesticides, sewage sludge and Pb-based paints. As a soil contaminant, Pb is one of the more persistent heavy metals, and it is extremely difficult to remediate because it is strongly bound to both organic and inorganic ligands and has therefore only limited bioavailability in the soil

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(Adriano, 2001). Conventionally, excavation and landfilling of the contaminated soil is used for the clean-up of Pb-contaminated soils. However, phytoextraction using plants to remove toxic metals from soil is emerging as an environmentally sound and cost-effective alternative to conventional methods for surface soil contamination over large areas (Huang et al., 1997; Cunningham and Berti, 2000; Macek et al., 2004).

In phytoextraction biotechnology, the highest possible proportion of soil Pb should be taken into plant roots, translocated into the shoots and finally removed by plant harvesting. To accomplish this goal, different methods and approaches facilitating increase in Pb bioavailability, Pb uptake into plant roots and Pb translocation to plant shoots can be combined. First, Pb is released from soil bonds by the application of different chelating agents. Then increased Pb solubility can be combined with root to shoot translocation to large accumulations of Pb in the shoots of plants (McGrath et al., 2001; Piechalak et al., 2003; Cui et al., 2004). Huang et al. (1997) reported that increase of shoot Pb concentration from less than 500 mg kg^{-1} to more than 10 000 mg kg^{-1} can be induced by the addition of selected chelates to soils, and 10 000 mg kg^{-1} is the value targeted for large-scale industrial phytoextraction. However, side effects such as leaching of Pb and other metals into groundwater raise environmental concerns (Grčman et al., 2001; McGrath et al., 2001) so that the demand for environmentally safer chelates has emerged. Grčman et al. (2003) introduced a promising biodegradable organic chelate for induced Pb phytoextraction – ethylenediaminedissuccinate (EDDS) and showed that it causes much lower Pb leaching than the most frequently used non-degradable synthetic chelate, ethylenediaminetetraacetic acid (EDTA).

Target plants for metal phytoextraction should meet two main requirements: (1) high biomass yield and (2) accumulation of high concentrations of metal(s). However, yet known plants species accumulating high levels of heavy metals in their biomass such as *Thlaspi caerulescens*, *T. goesingense* or *Cardaminopsis halleri* are slow growing, low biomass-producing species of no value for agronomic use (Cunningham et al., 1995; McGrath et al., 2001). Therefore, development of transgenic crop plants with hyperaccumulating capability due to either introducing/overproducing genes encoding heavy metal binding peptides and proteins or overexpressing metal transporter proteins has been proposed as a promising tool for use in phytoremediation efforts (Pilon-Smits and Pilon, 2002). Recent studies have shown that such transgenic plants can translocate and accumulate more Cd in the aboveground plant parts (Hasegawa et al., 1997; Janoušková et al., 2005a).

Similar developments have been predicted in the case of Pb, and transgenic plants with an improved affinity for Pb have been proposed (Kos and Leštan, 2003); however, only a limited number of studies has yet been published on this subject. Li et al. (2001) reported a higher Pb resistance and accumulation in transformed *Petunia* plants with introduced metallothionein gene. Gisbert et al. (2003) succeeded in achieving increased tolerance and doubling the accumulation of Pb and Cd in *Nicotiana glauca* transgenic plants expressing the wheat gene for phytochelatin synthase. In this case, seedlings were tested even in mining soil containing high levels of Pb (1572 ppm). Another example of preparation of transgenic plants for Pb remediation is based on overexpression of a bacterial analogue of ATPase *Znt*A in *Arabidopsis thaliana* (Lee et al., 2003b). Transgenic plants showed increased Pb and Cd resistance, probably due to the exclusion of heavy metals at the cellular level by pumping them from the plasma membrane to the extracellular space, but accumulated less Cd and Pb in the leaves.

Growth rate of, as well as metal accumulation by, target plants can be considerably modified by mutualist arbuscular mycorrhizal fungi that associate with the roots of the majority of vascular plants. The influence of these soil microorganisms on the processes of metal extraction/stabilisation as well as their compatibility with the intended remediation procedures should be tested. From the view of phytoextraction biotechnology, the inoculation with AM fungi may be advantageous if total Pb amount extracted from the soil is enhanced, either by promoting plant growth or by increasing the retention of metals in plant tissues (or both). However, studies of AM-mediated effect on heavy metal uptake are inconsistent, and results showing different responses of the inoculation on Pb concentrations in plant biomass have been reported. In some cases, lower Pb concentrations both in the shoot and roots of inoculated plants were reported (Kaldorf et al., 1999; Malcová et al., 2003), and other authors reported reduced Pb concentrations in the shoots of mycorrhizal plants without assessing root Pb concentrations (Díaz and Honrubia, 1995; Karagiannidis and Hadjisavva-Zinoviadi, 1998; Karagiannidis and Nikolaou, 2000). Recently, Hovsepyan and Greipsson (2004) reported higher Pb concentration in the shoots of maize plants grown in the soil treated with a fungicide (benomyl) that suppressed AM colonisation. On the other hand, Weissenhorn et al.

(1995) did not observe any difference in Pb shoot and root concentrations between non-mycorrhizal and mycorrhizal maize plants, and both Killham and Firestone (1983) and Rydlová and Vosátka (2003) found higher Pb concentrations in the shoots of mycorrhizal plants than in non-mycorrhizal ones. It seems probable that the effect of AM inoculation depends – among other factors – on host plant species. For example, Malcová et al. (2003) showed significantly reduced Pb concentrations in shoots and roots of maize plants inoculated with two *Glomus intraradices* isolates, while Pb concentrations in *Agrostis capillaris* plants were not changed or were even increased by the same inoculation treatment. It is therefore clear that the role of AM in Pb accumulation and translocation should be tested for particular fungus-plant combinations, and that possible potential of AM inoculation for Pb phytoextraction would depend on the balance between AM effect on biomass yield and Pb uptake.

Two cultivation experiments were conducted with the aims (1) to find out how EDDS chelate influences development of AM symbiosis and Pb phytoextraction by tobacco plants, (2) to elucidate if inoculation with AM fungus changes the growth of target tobacco plants and their Pb tolerance and Pb accumulation and (3) to assess whether the line HisCUP of tobacco with an inserted gene coding for yeast metallothionein gene CUP1 in fusion with a polyhistidine cluster accumulates and tolerates more Pb than non-transformed plants of the same variety.

2. Materials and methods

2.1. Plant and fungal material

Tobacco (*Nicotiana tabacum* L.) is a fast-growing plant species with a high biomass yield that has been shown to possess an exceptional capacity to accumulate Pb, Cd and Zn in high metal exposure conditions (Angelova et al., 2004) and translocate considerable proportion of heavy metals to the shoots (Wenger et al., 2002; Keller et al., 2003). Two clones of tobacco plants were used in these experiments: (1) non-modified plants of the variety Wisconsin 38 (WSC) and (2) genetically modified plants of the same variety bearing a transgene coding for a polyhistidine cluster in fusion with yeast metallothionein (HisCUP, Macek et al., 2002). The transgenic tobacco plants HisCUP are characterized by expressing yeast metallothionein gene *CUP*1 in fusion with a polyhistidine chain (Macek et al., 1996, 2002) that is used worldwide for immobilised metal affinity chromatography protein purification. The yeast metallothionein CUP1 itself is known for its metal binding properties, its fusion product with polyhistidine anchor proved to have increased affinity towards Cd in previous experiments (Macek et al., 2002; Pavlíková et al., 2004; Janoušková et al., 2005a,b): HisCUP plants showed greater tolerance to Cd concentrations and accumulated more Cd in above-ground parts as did normal tobacco plants. As far as the usefulness of the HisCUP expressing tobacco in phytoremediation studies with Cd-contaminated soils has been already proved, we expected accordingly also a positive effect in the case of Pb. Several metal binding domains have been described to bind both Cd (II) and Pb (II), e.g. considerable increase of Cd and Pb accumulation occurred in *Escherichia coli* overexpressing MerR from *Shigella* (Song et al., 2004). Razmiafshari et al. (2001) studied metal-histidine binding in zinc-finger transcription factors using two-dimensional NMR spectroscopy. Their findings provided strong evidence that environmental metals such as Pb, Cd, and Hg can selectively bind to the zinc finger motif.

Aseptic plantlets of both lines were multiplied from cuttings on Linsmeier and Skoog medium (from liquid concentrate supplied by SIGMA), with 20 g l^{-1} of sucrose (solidified by 8 g l^{-1} of agar). After 6-week growth on the medium, plantlets were released and agar from their roots was washed out by rinsing with tap water. Before planting, plantlets were left for 3 days in uncovered vessels containing tap water and sprayed in regular intervals with water to get gradually acclimatized to the lower air humidity present under greenhouse conditions.

For AM inoculation, *G. intraradices* Schenck and Smith, isolate PH5 originating from the Pb-contaminted waste disposal site in the proximity of Pb-smelter near Příbram (Czech Republic) was chosen. Pb concentrations in the substrate of isolate origin reached (in mg kg^{-1}, determined by different extractants): 14776 (1 M ammonium acetate–0.1 M EDTA), 2333 (1 M ammonium acetate), 573 (0.005 M DTPA–0.01 M $CaCl_2$–0.1 M triethanolamine) and 34 (0.1 M $Ca(NO_3)_2$). Since isolation via trap and multispore cultures, the isolate has been cultured continuously in the original Pb-contaminated substrate to maintain its potential to tolerate heavy metal contamination.

2.2. Experimental design

The first experiment was designed as factorial with the following experimental factors: (1) tobacco clone (WSC and HisCUP plants), (2) inoculation (non-inoculated plants and plants inoculated with *G.*

intraradices PH5), (3) Pb application (0 and 1.4 g Pb kg^{-1} substrate). Plants were planted into plastic pots, each filled with 2.5 kg of dry soil sterilised previously by γ-irradiation (5 Mrad). The soil used in the experiment had the following parameters: Chernozem, pH_{KCl} 7.2 ± 0.2, organic matter (C_{ox}) 18.3 ± 4.1 g kg^{-1}, cation exchange capacity 258 ± 4 cmol kg^{-1}, available P content 132 ± 5 mg kg^{-1}, total Pb content 29.3 ± 2.0 mg kg^{-1}. Due to a high demand of tobacco plants for N, 2.5 g of N in the form of NH_4NO_3 solution in distilled water were mixed into the substrate prior to planting. In order to partly re-establish the original microbial community in soil, all pots received also 10 ml of a filtrate from the non-sterile soil obtained by passing 1:10 suspension of soil through filter paper to remove AM fungal spores and mycelia. Every pot in mycorrhizal treatments received 10 ml of inoculum in the form of suspension containing colonised root segments, extraradical mycelium and spores. All non-inoculated plants were treated with the same amount of inoculum that had been autoclaved twice at 121 °C for 25 min. To equalise possible differences in microbial communities between inoculated and non-inoculated treatments, non-inoculated pots were irrigated with a filtrate from a non-sterile inoculum (prepared as above). Half of the pots received 1.4 g Pb kg^{-1} substrate (referred to as Pb1.4 treatment), applied as $Pb(NO_3)_2$, whereas the second half (Pb0) was treated with NH_4NO_3 so that final N contents in both Pb1.4 and Pb0 treatments were equal. A 10 ml of either solution in distilled water were injected to the soil and manually thoroughly mixed.

Two months after experiment had been established, the fourth factor was incorporated into experimental design as EDDS chelate at three different levels (Ch0 = 0 mmol kg^{-1} substrate, Ch2.5 = 2.5 mmol kg^{-1} 1 and Ch5 = 5 mmol kg^{-1}) was applied to the pots in order to increase Pb bioavailability. The plants in Ch2.5 and Ch5 treatments were irrigated with the corresponding amount of chelate dissolved in 150 ml of distilled water whereas the pots in Ch0 treatment were irrigated with the same amount of distilled water. Due to EDDS application, average soil Pb concentrations (1 M NH_4NO_3-exctractable) were 0.5 mg kg^{-1} in Ch0 in comparison with 102 and 189 mg kg^{-1} in Ch2.5 and Ch5 treatments, respectively, at the end of the experiment. In addition to EDDS application to the Pb1.4 pots, the pure effect of EDDS at the same doses was tested also in the pots without Pb amendment. The biodegradable chelate EDDS (ethylenediaminedissuccinate) was chosen because it has been reported as environmentally safer for the enhanced Pb phytoextraction than, e.g. more frequently used ethylendiaminetetraacetic acid (Grčman et al., 2003). Moreover, EDDS was proved to be less phytotoxic as well as less detrimental to soil microorganisms, including AM fungi. Each combination of the factors involved four replicates, resulting in a total number of 96 plants in the experiment.

The second experiment adopted a similar factorial design as Experiment 1, again involving two different tobacco clones (WSC and HisCUP), Pb application (0 and 1.4 g Pb kg^{-1} substrate) and two inoculation treatments (non-inoculated control plants and plants inoculated with *G. intraradices* PH5). Based on the results of the previous experiment, only the lower dose of EDDS chelate (2.5 mmol kg^{-1} substrate) was chosen, however, two consecutive applications after 8 and 10 weeks of plant growth were performed and the chelate was applied to all pots in Pb1.4 treatment. At the harvest, 1 M NH_4NO_3-exctractable Pb concentrations were 99 mg kg^{-1} in Pb1.4 treatment, compared with 0.6 mg kg^{-1} in Pb0. Each treatment involved eight replicate plants.

2.3. Harvests, plant analyses, mycorrhizal parameters

The plants were harvested after 12 weeks of cultivation (Experiment 1 from May to July 2003 and Experiment 2 from July to September 2004) in a temperature-controlled greenhouse under natural light conditions with supplementary 12-h illumination provided by metal halide lamps (400 W). Dry mass of the shoots and roots were recorded after drying at 80 °C for 24 h. The plant material was ground and decomposed by dry ashing, and Pb concentrations in the roots and shoots were determined by atomic absorption spectrometry (Varian SpectrAA-300) with flameless atomisation. For the determination of P, the Scalar (San System) segmented continuous flow analysis with photometric detector was used. The quality of the analyses was verified using the CRM CTA-OTL-1 Tobacco leaves reference material.

To assess AM colonisation in plant roots, root samples were stained with 0.05% trypan blue in lactoglycerol (Koske and Gemma, 1989) and examined under a microscope at 100× magnification using a modified grid-line intersect method (Giovannetti and Mosse, 1980). The substrate from each pot was mixed thoroughly and three samples were taken to evaluate the length of extraradical mycelium of fungi using the aqueous extraction and membrane filtration technique (Jakobsen et al., 1992). The substrate was blended with

300 ml of distilled water for 30 s, 1 ml of the supernatant was then pipetted onto a membrane filter (24 mm diameter, 0.40 μm pore size), vacuum filtered and the extraradical mycelium retained on the filter was stained with 0.05% trypan blue in lactoglycerol. The total length of extraradical mycelium was assessed using the grid-line intersect method under a microscope at 100× magnification, and expressed in m hyphae g^{-1} of dry substrate. The negligible background lengths of fungal mycelium observed in non-inoculated treatments were averaged, and the mean was then subtracted from the values found in the inoculated treatments. Small samples of mycelium extracted from a homogenised substrate by wet sieving were used for the evaluation of NADH-diaphorase activity in extraradical mycelium (Sylvia, 1988; Hamel et al., 1990). The tubes with extraradical mycelium were incubated with NADH-staining solution at room temperature in the dark for 14 h and the proportion of viable extraradical mycelium containing red precipitate (NADH-diaphorase activity) was then assessed under a microscope at 400× magnification.

2.4. Statistical treatment

The results of the experiments were analysed using Statistica 6.0 software. The effects of experimental factors were evaluated by the analysis of variance (ANOVA) and comparisons between means were carried out using the Duncan's multiple range test at the significance level of $P < 0.05$. Proportional data (percentage of root colonisation and NADH-diaphorase activity) were arcsine transformed prior to the statistical analysis.

3. Results

3.1. Experiment 1

3.1.1. Plant growth

WSC plants generally had significantly greater shoot and root dry mass (by approximately 35%) than HisCUP plants (Table 1). Pb addition into the soil led to a significant decrease in shoot dry mass, but in the case of root biomass the negative effect of Pb was only marginally significant ($P = 0.053$). However, detrimental effect of Pb on plant growth was significant only when EDDS chelate in either dose was applied into the soil. When Pb1.4 pots were not treated with the chelate, the effects of Pb on shoot and root dry mass were not statistically significant ($P = 0.105$ and 0.099, respectively). Application of the chelate decreased significantly both shoot and root dry mass (Table 1) but its negative effects were observed not only in Pb1.4 treatment but also on root biomass of Pb0 plants ($P = 0.016$). Necrotic lesions were observed on the leaves of chelate-treated plants in Pb1.4 treatment as well as on plants not exposed to Pb, though the latter were less prominent. In spite of the lack of differences in the growth of Ch2.5 and Ch5-treated plants in Pb1.4 treatment, visual symptoms of EDDS-toxicity and EDDS-mediated Pb-toxicity were much more frequent and pronounced at the higher chelate dose. Inoculation of plants with AM isolate did not affect shoot dry mass of tobacco plants but increased the biomass of their roots by 20%. In Pb1.4 treatment, inoculation alleviated the negative effect of Pb on root biomass so that the inoculated plants showed similar root dry mass as the plants in Pb0 treatment (significant interaction Pb × inoculation at $P < 0.05$).

Table 1
Effects of Pb, inoculation with *G. intraradices* PH5, EDDS chelate and clone on shoot and root dry mass of tobacco plants (Experiment 1)

Factor	Treatment	Shoot dry mass (g)	Root dry mass (g)
Pb	0	13.3 ± 0.4 a	2.4 ± 0.1 a
	1.4	11.4 ± 0.5 b	2.1 ± 0.1 a
Inoculation	NM	12.4 ± 0.5 a	2.0 ± 0.1 b
	M	12.3 ± 0.4 a	2.4 ± 0.1 a
Chelate	0	13.6 ± 0.5 a	2.6 ± 0.1 a
	2.5	11.9 ± 0.6 b	2.1 ± 0.1 b
	5	11.5 ± 0.6 b	2.0 ± 0.1 b
Clone	WSC	14.2 ± 0.4 a	2.6 ± 0.1 a
	HisCUP	10.5 ± 0.4 b	1.9 ± 0.1 b
F-level/significance			
Pb		14.8^{***}	3.9 ns
Inoculation		0.0 ns	8.3^{*}
Chelate		6.6^{**}	7.0^{**}
Clone		54.9^{***}	20.1^{***}

The mean ± S.E. followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$; $n = 4$. Pb – 0: 0 g Pb kg^{-1} substrate; 1.4: 1.4 g Pb kg^{-1} substrate. Inoculation – NM: non-inoculated plants; M: inoculated plants. Chelate – 0: 0 mmol kg^{-1} substrate; 2.5: 2.5 mmol kg^{-1}; 5: 5 mmol kg^{-1}. Clone – WSC: non-transformed tobacco plants; HisCUP: tobacco plants of the same variety with inserted transgene coding for a polyhistidine cluster in fusion with yeast metallothionein. Significant effect at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: non-significant effect.

3.1.2. Pb accumulation in plants

The application of EDDS chelate increased considerably Pb concentrations in both shoots and roots as well as Pb translocation from plant roots to shoots (Table 2). When the plants were grown in Pb1.4 substrate without EDDS addition, Pb concentrations in their shoots and roots reached on average only 34 and

Table 2
Effects of inoculation with *G. intraradices* PH5, EDDS chelate and clone on Pb concentrations and contents in shoot and root biomass of tobacco plants (Experiment 1)

Inoculation	EDDS chelate	Clone	Pb concentration			Pb content		
			Shoots (mg kg^{-1})	Roots (mg kg^{-1})	Roots/shoots	Shoots (mg)	Roots (mg)	Total (mg)
NM	0	WSC	36 ± 3 c	344 ± 51 b	10.0 ± 2.0 a	0.6 ± 0.09 cd	0.8 ± 0.11 bc	1.4 ± 0.2 bc
NM	0	HisCUP	40 ± 5 c	449 ± 37 b	11.7 ± 2.0 a	0.5 ± 0.13 cd	0.6 ± 0.12 bc	1.1 ± 0.3 c
NM	2.5	WSC	145 ± 22 bc	462 ± 30 b	3.6 ± 0.8 b	1.8 ± 0.20 bc	0.8 ± 0.12 bc	2.6 ± 0.1 bc
NM	2.5	HisCUP	552 ± 354 ab	279 ± 60 b	1.1 ± 0.4 b	4.0 ± 2.23 ab	0.5 ± 0.17 c	4.5 ± 2.2 abc
NM	5	WSC	351 ± 135 ab	558 ± 32 b	2.2 ± 0.5 b	4.1 ± 1.05 ab	1.2 ± 0.20 bc	5.3 ± 1.0 ab
NM	5	HisCUP	749 ± 353 ab	699 ± 110 b	1.5 ± 0.4 b	4.6 ± 1.82 ab	0.7 ± 0.15 bc	5.3 ± 1.7 ab
M	0	WSC	32 ± 3 c	357 ± 59 b	11.1 ± 1.2 a	0.4 ± 0.04 cd	1.1 ± 0.15 bc	1.5 ± 0.2 bc
M	0	HisCUP	29 ± 9 c	343 ± 102 b	13.9 ± 3.0 a	0.3 ± 0.09 d	0.8 ± 0.22 bc	1.1 ± 0.3 c
M	2.5	WSC	129 ± 21 bc	529 ± 152 b	4.3 ± 1.5 b	1.7 ± 0.21 bc	1.3 ± 0.34 ab	3.0 ± 0.4 bc
M	2.5	HisCUP	131 ± 15 bc	565 ± 163 b	4.5 ± 1.4 b	1.2 ± 0.28 bc	0.9 ± 0.24 bc	2.2 ± 0.4 bc
M	5	WSC	231 ± 78 ab	531 ± 175 b	2.7 ± 0.7 b	2.7 ± 0.87 ab	1.3 ± 0.46 ab	4.0 ± 1.1 bc
M	5	HisCUP	806 ± 409 a	1392 ± 740 a	2.1 ± 0.6 b	6.1 ± 2.22 a	1.8 ± 0.40 a	7.9 ± 2.5 a
Inoculation			0.6 ns	1.3 ns	3.1 ns	0.6 ns	9.0^{**}	0.0 ns
Chelate			6.8^{**}	3.6^{*}	52.7^{***}	12.2^{***}	3.4^{*}	13.5^{***}
Clone			4.3^{*}	1.3 ns	0.0 ns	1.9 ns	2.1 ns	1.0 ns

The mean ± S.E. followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$; $n = 4$. Non-significant interactions are not shown. Inoculation – NM: non-inoculated plants; M: inoculated plants. Chelate – 0: 0 mmol kg^{-1} substrate; 2.5: 2.5 mmol kg^{-1}; 5: 5 mmol kg^{-1}. Clone – WSC: non-transformed tobacco plants; HisCUP: tobacco plants of the same variety with inserted transgene coding for a polyhistidine cluster in fusion with yeast metallothionein. Significant effect at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: non-significant effect.

373 mg kg^{-1}, respectively, whereas 239 and 459 mg kg^{-1} were observed in Ch2.5 treatment and 534 and 795 mg kg^{-1} in Ch5 treatment, respectively. Without chelate application, Pb concentrations more than ten times higher were observed in the roots than shoots but EDDS strongly decreased the amount of Pb retained in the roots and supported Pb translocation to aerial parts, thus resulting in much lower root–shoot ratios of concentrations.

Considering the effect of chelate on total Pb contents in plant tissues, higher amounts of Pb were phytoextracted from the substrate due to EDDS application, in spite of its negative influence on plant growth. Inoculation of plants with the AM isolate did not influence Pb concentrations in their shoots and roots (Table 2). A trend to a higher retention of Pb in the roots of inoculated plants was observed though the effect of inoculation on root–shoot ratios of Pb concentrations was not statistically significant ($P = 0.089$). Due to the higher root biomass of inoculated plants, significantly higher Pb contents were also observed in their roots in comparison to non-inoculated plants. Nevertheless, since root biomass represented only a small proportion of the total biomass, the effect of inoculation on root Pb contents was not reflected in total amount of Pb extracted from the substrate by the inoculated plants (Table 2). When comparing both tobacco clones, HisCUP plants showed significantly higher Pb concentrations in the shoots than their non-transformed counterparts but neither root Pb concentrations nor root–shoot ratios of Pb concentrations differed between both clones.

3.1.3. Development of mycorrhizal symbiosis

In general, high levels of mycorrhizal colonisation (more than 80%) were observed in the roots of tobacco plants, with no significant difference between tobacco clones. No influence of EDDS chelate on mycorrhizal colonisation was observed in non-contaminated soil whereas the length of extraradical mycelium and its NADH-diaphorase activity were reduced by EDDS application even in the treatment without Pb addition. When Pb was mixed into the soil, all mycorrhizal parameters were significantly higher in control treatment without chelate application than in either EDDS dose (Table 3).

3.2. Experiment 2

3.2.1. Plant growth

There was a marked difference in the growth of the two tobacco clones, with WSC plants showing consistently greater shoot and root dry mass and larger leaf area. Pb application associated with EDDS addition into the soil considerably reduced shoot and root dry mass as well as total leaf area of tobacco plants (Table 4). Control plants had 22% and 30% higher root

Table 3
The effect of Pb addition and different doses of EDDS chelate on the development of *G. intraradices* PH5 (Experiment 1)

Pb	EDDS chelate	Root colonisation (%)	Extraradical mycelium length (m g^{-1})	NADH-diaphorase activity (%)
0	0	83 ± 3 ab	3.0 a	66 ± 4 a
0	2.5	81 ± 2 ab	2.3 ab	52 ± 4 b
0	5	82 ± 3 ab	2.3 ab	50 ± 4 b
1.4	0	88 ± 2 a	2.9 a	66 ± 5 a
1.4	2.5	83 ± 3 ab	1.7 b	48 ± 4 b
1.4	5	79 ± 2 b	1.6 b	41 ± 3 b
Pb		0.7 ns	3.6 ns	1.5 ns
Chelate		2.3 ns	7.2^{**}	14.9^{***}
Pb × chelate		1.4 ns	0.5 ns	0.5 ns

Mean ± S.E. marked with different letters are significantly different according to Duncan's multiple range test at $P < 0.05$; $n = 4$. Pb – 0: 0 g Pb kg^{-1} substrate; 1.4: 1.4 g Pb kg^{-1} substrate. Chelate – 0: 0 mmol kg^{-1} substrate; 2.5: 2.5 mmol kg^{-1}, 5: 5 mmol kg^{-1}. Significant effect at $^{**}P < 0.01$, $^{***}P < 0.001$, ns: non-significant effect.

and shoot biomass, respectively, and 8% larger leaf area than the plants grown in the soil with Pb amendment. Similarly, inoculation with AM fungus had a highly significant effect on plant growth, with about 10% increase in all evaluated parameters in inoculated plants. The significant triple interaction (Pb × inoculation × clone) for leaf area pointed out a different effect of AM inoculation on the alleviation of Pb-toxicity in the two tobacco clones: the detrimental effect of Pb addition was reduced by AM in HisCUP more than in WSC. The similar interactions were near significance also for the other parameters evaluated, i.e. shoot biomass ($P = 0.081$) and root biomass ($P = 0.051$).

3.2.2. Pb accumulation in plants

Inoculation with AM fungi significantly increased Pb concentrations in root biomass, root-to-shoot ratio of Pb concentrations, and Pb contents in the roots (Table 5). However, the effect of inoculation on total Pb content was not statistically significant ($P = 0.063$). No difference in Pb concentrations and root-to-shoot Pb translocation was found between the two tobacco clones. WSC plants accumulated greater higher total Pb content due to their higher shoot and root biomass.

3.2.3. Development of mycorrhizal symbiosis

Pb addition combined with EDDS application into the substrate resulted in a significantly lower percentage of root colonisation in comparison to the control plants. Although the effect of Pb was highly significant ($P < 0.001$), the percentage of colonised roots in Pb1.4 treatment was still high, and the difference between Pb1.4 and Pb0 plants was surprisingly small (96 ± 0.5% versus 89 ± 1.2%, respectively). Similarly,

Table 4
Effects of Pb, inoculation with *G. intraradices* PH5 and clone on shoot and root dry mass and leaf area of tobacco plants (Experiment 2)

Pb	Inoculation	Clone	Shoot dry mass (g)	Root dry mass (g)	Leaf area (cm^2)
0	NM	WSC	36.2 ± 1.2 b	5.7 ± 0.3 b	3075 ± 43 bc
0	NM	HisCUP	31.9 ± 1.5 c	5.2 ± 0.2 bc	2733 ± 39 d
0	M	WSC	39.8 ± 0.6 a	6.7 ± 0.4 a	3404 ± 26 a
0	M	HisCUP	34.8 ± 0.9 b	5.2 ± 0.2 bc	2870 ± 54 cd
1.4	NM	WSC	30.3 ± 0.7 c	4.5 ± 0.2 c	3013 ± 67 b
1.4	NM	HisCUP	26.0 ± 1.0 d	3.8 ± 0.1 d	2336 ± 82 e
1.4	M	WSC	30.6 ± 0.7 c	4.8 ± 0.2 c	3091 ± 77 b
1.4	M	HisCUP	30.5 ± 0.8 c	4.5 ± 0.3 cd	2753 ± 64 d
Pb			83.9^{***}	51.0^{***}	28.8^{***}
Inoculation			16.4^{***}	6.7^{*}	33.7^{***}
Clone			24.1^{***}	17.3^{***}	130.4^{***}
Pb × inoculation × clone			3.2 ns	4.0 ns	10.3^{**}

The mean ± S.E. followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$; $n = 8$. Non-significant interactions are not shown. Pb – 0: 0 g Pb kg^{-1} substrate; 1.4: 1.4 g Pb kg^{-1} substrate. Inoculation – NM: non-inoculated plants; M: inoculated plants. Clone – WSC: non-transformed tobacco plants; HisCUP: tobacco plants of the same variety with inserted transgene coding for a polyhistidine cluster in fusion with yeast metallothionein. Significant effect at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: non-significant effect.

Table 5
The effect of inoculation with *G. intraradices* PH5 and tobacco clone on Pb concentrations and Pb contents in tobacco shoots and roots (Experiment 2)

Inoculation	Clone	Pb concentration			Pb content		
		Shoots (mg kg^{-1})	Roots (mg kg^{-1})	Roots/shoots	Shoots (mg)	Roots (mg)	Total (mg)
NM	WSC	195 ± 15 a	302 ± 28 b	1.7 ± 0.3 b	5.9 ± 0.4 a	1.4 ± 0.2 b	7.2 ± 0.3 b
NM	HisCUP	204 ± 14 a	341 ± 23 b	1.7 ± 0.1 b	5.3 ± 0.3 a	1.3 ± 0.1 b	6.6 ± 0.4 b
M	WSC	204 ± 29 a	514 ± 48 a	3.1 ± 0.6 ab	6.3 ± 1.0 a	2.5 ± 0.3 a	8.8 ± 0.7 a
M	HisCUP	147 ± 27 a	547 ± 73 a	5.4 ± 1.0 a	4.4 ± 0.8 a	2.5 ± 0.3 a	6.9 ± 0.5 b
Inoculation		1.2 ns	18.5***	30.9**	0.1 ns	21.0***	3.8 ns
Clone		1.1 ns	0.6 ns	2.8 ns	3.5 ns	0.1 ns	7.3*
Inoculation × clone		2.1 ns	0.0 ns	2.9 ns	0.9 ns	0.0 ns	1.6 ns

The mean ± S.E. marked with different letters are significantly different at $P < 0.05$; $n = 8$. Non-significant interactions are not shown. Inoculation – NM: non-inoculated plants; M: inoculated plants. Clone – WSC: non-transformed tobacco plants; HisCUP: tobacco plants of the same variety with inserted transgene coding for a polyhistidine cluster in fusion with yeast metallothionein. Significant effect at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: non-significant effect.

the length of extraradical mycelium was significantly lower in the Pb-contaminated substrate ($P < 0.001$), with means reaching only 4.4 ± 0.3 m g^{-1} dry soil, compared to 7.7 ± 0.7 m g^{-1} dry soil in Pb0 treatment. NADH-diaphorase activity was decreased by Pb addition ($P < 0.001$) and significantly lower activity was observed in Pb-treated plants (42 ± 1.7%, compared 57 ± 2.3% in Pb0 treatment). None of mycorrhizal parameters (colonisation, length of extraradical mycelium, NADH-diaphorase activity) differed between the two tobacco clones.

4. Discussion

Pb addition into the substrate was not significantly detrimental to plant growth if the chelate was not applied, which confirms the generally low bioavailability of Pb. The Pb caused a significant reduction of tobacco growth only when combined with EDDS application, but without a significant difference between the lower and higher EDDS dose. In spite of that fact, the higher EDDS dose, however, appeared to be more phytotoxic based on the visual toxicity symptoms that were obvious in Pb-treated soil (and to a lesser extent also in non-contaminated substrate). Similarly, Grčman et al. (2003) reported visual symptoms of toxicity on Chinese cabbage leaves when EDDS in doses from 3 to 10 mmol kg^{-1} was applied. In contrast to our results, they did not, however, observe any effect of EDDS on plant growth, even at the highest concentration tested. As the damage of tobacco leaves by higher EDDS dose in our first experiment was fast and pronounced, we hypothesised that transpiration flux driving Pb transport through the xylem was reduced accordingly and therefore, the single dose of 5 mmol EDDS kg^{-1} soil was replaced by two successive applications of EDDS at half of the concentration in the second experiment. However, the single dose of chelate was found to be more effective in Pb phytoextraction than two successive applications, and resulted in almost three times higher average Pb concentrations in plant shoots. This result is in accordance with the findings of Grčman et al. (2003) who compared the effect of EDTA and EDDS chelates applied either in a single dose or in four partial-weekly additions. In accordance with the reported effects of other synthetic chelates (Huang et al., 1997; Wu et al., 1999), EDDS proved to increase considerably not only Pb solubility and consequently Pb uptake by tobacco plants but also changed Pb translocation to the shoots, with average ratios between root and shoot Pb concentrations ranging from 10.9 in control plants to 1.5 in plants treated with the higher EDDS dose.

With respect to chelate toxicity to AM fungi, root colonisation was reduced by EDDS chelate only in Pb-treated soil whereas the length of extraradical mycelium and NADH-diaphorase activity were significantly decreased also by EDDS itself in the treatments without Pb application. Comparable results, i.e. low sensitivity of AM colonisation to EDDS as well as to more toxic EDTA have been reported already by Grčman et al. (2003) and Chen et al. (2004). However, they assessed only intraradical root colonisation, which is considered to be less susceptible to environmental stresses than is the extraradical mycelium (e.g. Batkhuugyin et al., 2000; del Val et al., 1999). As was the case in Experiment 1, all mycorrhizal parameters including root colonisation, the length of extraradical mycelium and NADH-diaphorase activity were also decreased in the contaminated soil in Experiment 2.

Significantly higher Pb concentrations were found in the shoots of HisCUP plants than control plants in the first experiment; however, no difference between the two clones was observed in the Experiment 2, although it was conducted in the same substrate with the same Pb contamination level. Therefore, we could not confirm our hypothesis that transgenic tobacco plants with gene coding for polyhistidine cluster combined with yeast metallothionein would accumulate and tolerate higher Pb concentrations than non-transgenic plants of the same variety. The explanation for such a discrepancy is unknown; nevertheless Janoušková et al. (2005a,b) also reported inconsistencies in Cd accumulation by the same tobacco clones in different cultivation experiments. Compared to Cd, Cu or Zn, there is little information on the mechanisms of Pb detoxification in plants. Nevertheless, reports on the induced synthesis of both phytochelatins (Gupta et al., 1995; Bajguz, 2002; Sun et al., 2005) and metallothioneins (Ma et al., 2003) in response to Pb stress have been published and transgenic plants with introduced metallothionein gene or phytochelatin synthase gene were shown to have greater higher Pb tolerance and accumulation ability (Li et al., 2001; Gisbert et al., 2003). In contrast, Kawashima et al. (2004) reported that transgenic tobacco plants overexpressing cysteine synthase which catalyses the final step of cysteine biosynthesis were significantly more tolerant to Cd, Se and Ni but not Pb and Cu, and hypothesised that these metals may have different detoxification mechanisms than phytochelatins or other thiols.

The influence of AM inoculation on tobacco growth differed between the two experiments. In Experiment 2, inoculation with AM fungi increased both shoot and root biomass, whereas a positive effect only on root biomass was observed in the first experiment. The difference could be explained by a shift in a cultivation period, and the resultant difference in cost-benefit balance for maintaining AM symbiosis. In the Pb-contaminated substrate, AM fungi showed a protective effect on plant growth, which is consistent with numerous studies showing AM-mediated alleviation of phytotoxicity of heavy metals including Pb (Díaz et al., 1996; Hildebrandt et al., 1999; Vivas et al., 2003; Chen et al., 2004).

Higher Pb concentrations in the roots of inoculated plants of both tobacco clones and increased root–shoot ratios of Pb concentrations that were observed in the second experiment suggest that Pb was retained in fungal structures, and that mycorrhizae acted as a barrier against metal transfer into plant shoots, as has been suggested earlier by Dehn and Schüepp (1989). In spite of the absence of AM effect on shoot Pb concentrations in our experiment, total amount of Pb extracted from soil was however still higher in mycorrhizal treatments than non-mycorrhizal ones due to AM-mediated plant growth stimulation.

To conclude, although EDDS chelate increased Pb uptake and translocation into tobacco shoots, even the highest concentrations of Pb achieved at 5 mmol kg^{-1} dose in transgene plants would not be sufficient for practical use as tissue Pb concentrations exceeding 1% of dry biomass would be required to reduce soil Pb concentrations by 500 mg kg^{-1} in a reasonable time span of 20–25 years using plants with high biomass yield (Huang et al., 1997; Grčman et al., 2003). It is questionable if higher Pb concentrations in plant biomass can be reached by a combination of biodegradable chelates and newly designed transgenic plants with greater demand for Pb. That was not the case with the tested HisCUP tobacco plants, whose ability to accumulate more Pb in biomass was not confirmed. The use of AM inoculation for Pb phytoextraction by mycotrophic plants seems to be reasonable rather due to AM-mediated growth stimulation of host plants resulting in a higher total amount of Pb extracted from the soil than due to increase of Pb translocation to the shoots of inoculated plants; yet potential differences among AM isolates cannot be neglected. On the other hand, the observed elevation of Pb concentrations in plant roots but not shoots of inoculated plants demonstrates a potential of AM for phytostabilisation of the soil which primarily consist in prevention of spreading of contamination in the environment by the soil erosion.

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