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Arbuscular mycorrhiza decreases cadmium phytoextraction by transgenic tobacco with inserted metallothionein

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Abstract

The effect of arbuscular mycorrhiza (AM) on the phytoextraction efficiency of transgenic tobacco with increased ability to tolerate and accumulate cadmium (Cd) was tested in a pot experiment. The tobacco plants bearing the yeast metallothionein *CUP1* combined with a polyhistidine cluster were compared to non-transgenic tobacco of the same variety at four Cd concentrations in soil, non-inoculated or inoculated with two isolates of the AM fungus *Glomus intraradices*. Mycorrhizal inoculation improved the growth of both the transgenic and non-transgenic tobacco and decreased Cd concentrations in shoots and root to shoot translocation. Differences were found between the two AM fungal isolates: one isolate supported more efficient phosphorus uptake and plant growth in the soil without Cd addition, while the other isolate alleviated the inhibitory effect of cadmium on plant growth. The resulting effect of inoculation on Cd accumulation was dependent on Cd level in soil and differed between the more Cd tolerant transgenic plants and the less tolerant non-transgenic plants. Mycorrhiza mostly decreased the phytoextraction efficiency of transgenic plants while increased that of non-transgenic plants at Cd levels in soil inhibitory to tobacco growth. Mechanisms of the observed effects of inoculation on growth and Cd uptake are discussed as well as the possible implications of the results for the exploitation of AM in phytoextraction of heavy metals from contaminated soils.

Abbreviations: AM – arbuscular mycorrhiza; ERM – extraradical mycelium; HisCUP – transgenic tobacco; HM – heavy metal; WSC – non-transgenic tobacco

Introduction

The ability of plants to extract ions from the soil and concentrate them in their tissues leads to elevated concentrations of heavy metals (HM) in the biomass of plants growing on HM contaminated soils. This represents a severe environmental risk as increased amounts of these toxic elements enter the food chain. On the other hand, this ability of plants can be utilised in HM phytoextraction, when metal-accumulating plants are grown on a contaminated site and the HMs are removed within their harvestable parts (Macek et al., 2004).

In comparison to physico-chemical methods applied for the clean up of HM contaminated soils, phytoextraction represents an economically

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feasible alternative, which preserves the soil structure and biological function (McGrath et al., 2001). Vegetation cover can, moreover, decrease further dispersal of the contaminants due to erosion and their leaching to the groundwater (Ernst, 1996). The technology of phytoextraction, however, has still several limitations. Among them the long duration of the process, the HM uptake only to the rooting depth of plants, or the restriction of the HM uptake due to HM sorption or slow diffusion in the soil (Khan et al., 2000; McGrath et al., 2001).

One field of research focused at improving the efficiency of the phytoextraction process is identification of appropriate plants. Plants naturally hyperaccumulating heavy metals usually produce low biomass, have shallow rooting, difficult handling and predominant specificity to one metal only (Cunningham et al., 1995). High biomass crops or fast-growing trees with reasonably high HM uptake ability and deeper rooting are therefore considered (Kumar et al., 1995; Keller et al., 2003). Their use is regarded as promising especially in connection with genetic engineering further enhancing their heavy metal tolerance and uptake (Krämer and Chardonnens, 2001).

Another approach considers rhizospheric processes and interactions in order to increase the HM tolerance and uptake of plants (McGrath et al., 2001). Arbuscular mycorrhizal (AM) symbiosis is an almost ubiquitous rhizospheric interaction (Smith and Read, 1997), and its possible effects on the phytoextraction process have been repeatedly suggested (Leyval et al., 1997; Chaudhry et al., 1998; Khan et al., 2000; McGrath et al., 2001).

Symbiosis with AM fungi has been shown to increase plant tolerance to HMs (Hetrick et al., 1994, Díaz et al., 1996). This is an important factor if the establishment of vegetation cover or high biomass yields are required on soils with toxic HM levels. On the other hand, mycorrhiza can reduce the HM uptake or translocation to the aerial parts of plants (Loth and Höfner, 1994, Ricken and Höfner, 1995) – an effect that is undesirable from the point of view of phytoextraction. Other authors (e.g., Killham and Firestone, 1983; Rivera-Becerril et al., 2002), however, found increased HM concentrations in the shoots of mycorrhizal plants as compared to non-mycorrhizal plants. Moreover, the extraradical mycelium (ERM) of AM fungi has been shown to transport HMs into roots from root free soil increasing the depletion zone of the plants (Guo et al., 1996; Joner and Leyval, 1997). The effect of AM fungi on the HM uptake of their hosts can depend, among other factors, on plant species (Malcová et al., 2003a) or even genotype (Rivera-Becerril et al., 2002), on the HM concentration in soil (Heggo and Angle, 1990) and on the AM fungal species or isolate used for inoculation (Malcová et al., 2003b).

Mycorrhizal association is unlikely to influence the HM uptake of natural hyperaccumulaters, which often belong to non-mycotrophic family Brassicaceae (McGrath et al., 2001). However, the results reported on the interaction of mycorrhizal plants with HMs, as reviewed by Leyval et al., (1997), indicate that mycorrhiza must be taken into account if the phytoextraction potential of mycotrophic plants is assessed.

This study was therefore aimed at describing the effect of AM symbiosis on Cd uptake of genetically modified tobacco plants, which had been previously shown to tolerate higher Cd levels in substrate and to accumulate up to 80% more Cd in shoots than non-transgenic tobacco plants of the same variety (Pavlíková et al., 2004). The growth and Cd uptake of the transgenic and control plants were compared in four Cd concentrations in soil, non-inoculated or inoculated with two isolates of the AM fungal species, *G. intraradices*.

Materials and methods

Plants and AM fungi

Tobacco, *Nicotiana tabacum* L., var. Wisconsin 38, with a genetic modification increasing its cadmium accumulation (HisCUP), was compared to non-modified plants of the same variety (WSC). The HisCUP plants were prepared by Macek et al., (2002) and bore a transgene coding for a polyhistidine cluster combined with the *CUP1* yeast metallothionen under the CaMV35S promoter from cauliflower mosaic virus. The best Cd accumulating line T-HisCUP-X was chosen from the transgenic tobacco lines based on previous experiments (Macek et al., 2002). Its enhanced ability to tolerate and accumulate Cd

was also confirmed in a sand-based cultivation test (Pavlíková et al., 2004). In vitro vegetatively multiplied aseptic plantlets were used as starting material, grown on medium according to Linsmeier and Skoog (1965), from liquid concentrate supplied by SIGMA (Sigma-Aldrich, St. Louis MO, USA), with sucrose 20 g l^{-1} and solidified by agar 8 g l^{-1}.

After 6 weeks of growth (at the size of about 10 cm), the roots of the plantlets were washed from agar by rinsing under lukewarm tap water, and the plantlets were hardened by keeping them in open vessels in tap water for 3 days before planting.

Two isolates of the AM fungal species *Glomus intraradices* Schenck and Smith were used for inoculation: The isolate PH5 was isolated from a Pb-contaminated waste disposal site of a Pb smelter near Příbram, Czech Republic (Malcová et al., 2003a), while the isolate BEG75 originates from a non-polluted agricultural site in Switzerland. PH5 had been maintained and multiplied in greenhouse conditions in pot cultures using exclusively its soil of origin sterilised by autoclaving, BEG75 had been maintained in inert substrates (sand and zeolite).

Experimental design

The experiment was designed $4 \times 3 \times 2$ factorial with the following factors: (1) Cd treatment (0, 20, 40 and 60 mg kg^{-1} of soil); (2) inoculation treatment (non-inoculated, inoculated with *G. intraradices* PH5 and inoculated with *G. intraradices* BEG75), (3) plant treatment (WSC and HisCUP plants). Each combination of the factors involved 5 replicates, each represented by one plant in a 2.8-L plastic pot. Chernozem from the locality Prague-Suchdol with the following characteristics was used for the experiment: pH_{KCl} 7.2 ± 0.2; total Cd content (Cd_T) 0.321 ± 0.065 mg kg^{-1}; available Cd of Cd_T 0.3% (extracted by 0.01 mol l^{-1} CaCl2 (1:10, w/v) according to Novozámský et al., 1993); organic matter (C_{ox}) 1.83 ± 0.41%, cation exchange capacity 25.8 ± 0.4 cmol(+) kg^{-1}. Each pot was filled with 2.5 kg of dry soil, previously sterilised by γ-irradiation (50 kGy), and amended with 2.5 g of nitrogen as NH_4NO_3. Cd was applied as $Cd(NO_3)_2$ solution to the treatments with Cd application and the treatments with Cd 0, 20 and 40 mg kg^{-1} received nitrogen as NH_4NO_3 in amounts that equalised the nitrogen added to the level of the highest $Cd(NO_3)_2$ application. Both NH_4NO_3 and $Cd(NO_3)_2$ were dissolved in 10 mL of deionised water each and thoroughly mixed with the soil. The pots of the inoculated treatments received 10 mL of inoculum suspension of the corresponding AM fungus, containing colonised root segments, extraradical mycelium (ERM) and spores. The non-inoculated treatments received the same amount of autoclaved inoculum. In order to equalise the microbial conditions, all pots were irrigated with 10 mL of bacterial filtrate obtained by passing soil suspensions from the 2 cultures used for inoculation through a filter paper (Whatman No. 1) and mixing the filtrates. The plants were grown in a greenhouse with light supplement (12 h, metalhalide lamps, 400 W) for 12 weeks (from August to October).

Harvest

The shoot and root biomass was determined after drying at 80 °C for 24 h. The plant material was ground and decomposed by a dry ashing procedure. The Cd 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 accuracy of the analyses was estimated by comparison with reference material CRM 281 Rye Grass with a certified content of 0.120 ± 0.003 mg Cd kg^{-1} and 2300 ± 20 mg P kg^{-1} dry mass for which contents of 0.135 ± 0.011 mg Cd kg^{-1} and 2248 ± 109 mg P kg^{-1} dry mass were obtained.

Mycorrhizal colonisation was evaluated on root samples stained with 0.05% trypan blue in lactoglycerol (Koske and Gemma, 1989). Percentage of mycorrhizal colonisation was assessed using the grid-line intersect method (Giovannetti and Mosse, 1980). The ERM length in soil was estimated using a modified membrane filtration technique (Jakobsen et al., 1992). A small sample of homogenised substrate (approx. 2 g of dry weight) was placed in a household blender with 500 ml of distilled water and blended for 60 s. One ml of the supernatant was vacuum filtered through a membrane filter (24 mm diameter) and the filter was stained with 0.1% trypan blue in lactoglycerol. The hyphae retained on the filter

surface were counted under a microscope at 100 × magnification using an ocular grid as scale. Three samples were prepared and evaluated from every pot. The values were expressed as meters of hyphae in 1 g of air-dried substrate, and for every pot, the average of the three samples was calculated. The average background length of mycelium was assessed on samples from three non-inoculated pots per Cd treatment and the values calculated per Cd treatment were subtracted from the corresponding values obtained in the inoculated treatments.

Statistical treatment

The effects of the factors Cd in soil, inoculation treatment and plant treatment on each parameter were determined by three-way analysis of variance (ANOVA).

The effect of Cd on the mycorrhizal parameters (colonisation, ERM length) as well as on the growth response of tobacco plants to inoculation was also tested for each plant-isolate combination separately by one-way ANOVA. Similarly, the effect of Cd on shoot and root dry weight was tested for each combination of plant and inoculation treatment separately. The shoot contents were compared on each Cd level in soil by one-way ANOVA to determine the best performing combination of plant and inoculation treatment at the given Cd level. Comparisons between means were carried out using the Duncan's multiple range test.

The data of the parameters percentage of root colonisation and percentage of biomass (growth response) were arcsine transformed prior to the statistical analysis. The data of the parameters Cd shoot concentration, Cd root concentration and Cd shoot to root concentration ratio were square root transformed in order to obtain normal distribution of the data.

Results

Development of mycorrhiza

Both *G. intraradices* isolates reached high root colonisation levels at the end of the experiment (average values per treatment from 75–94%). According to three-way ANOVA (Table 1), colonisation significantly differed between the isolates and was influenced by the addition of Cd. No significant differences in colonisation level were found between HisCUP and WSC plants. The BEG75 isolate reached higher colonisation levels than the PH5 isolate over all treatments and colonisation was higher at Cd 20 and 40 mg kg^{-1} than at Cd 60 mg kg^{-1} and the soil without Cd amendment according to Duncan's multiple range test of the factor Cd. The factor Cd, however, did not significantly influence the colonisation of any plant-isolate combination when each combination was tested separately by one-way ANOVA (Table 1).

The ERM length was significantly influenced by the factors Cd addition and isolate as well as by their interaction (Table 1). The BEG75 isolate generally produced less ERM than the PH5 isolate and its ERM length was significantly decreased by the highest Cd soil addition of 60 mg kg^{-1} in combination with either plant. The ERM length of the PH5 isolate, on the contrary, was even higher at Cd 40 and 60 mg kg^{-1} than at Cd 20 mg kg^{-1} and the treatment with no Cd addition (Table 1).

Plant growth and P uptake

The shoot and root dry weights of the tobacco plants were generally influenced by all factors (Cd addition, inoculation treatment and plant) (Table 2). The factors Cd and inoculation significantly interacted in their effects on both shoot and root dry weight. In the case of root dry weight, the effect of inoculation differed between HisCUP and WSC plants.

Addition of Cd decreased the shoot and root dry weights of both WSC and HisCUP plants, but the effect differed between the inoculation treatments. This interaction is demonstrated more in detail by determining the effect of Cd addition on each plant-isolate combination separately by one-way ANOVA (Table 2): The shoot and root growth of non-inoculated WSC and HisCUP plants was significantly inhibited by Cd 40 mg kg^{-1}. The shoot growth of plants inoculated with the BEG75 isolate was significantly inhibited only at the highest Cd level of 60 mg kg^{-1}, while root growth inhibition was significant already at lower Cd levels in soil:

Table 1. Colonisation and length of extraradical mycelium (ERM) of the *Glomus intraradices* isolates BEG75 and PH5 in association with transgenic and non-transgenic tobacco in soil with four cadmium (Cd) concentrations

Isolate	Plant	Cd0	Cd20	Cd40	Cd60	*Signif. (F)*
			Colonisation (%)			
BEG75	HisCUP	89 (3)	93 (2)	91 (2)	92 (1)	*n.s. (0.96)*
	WSC	88 (3)	93 (3)	94 (2)	91 (2)	*n.s. (1.09)*
PH5	HisCUP	82 (4)	90 (2)	92 (2)	84 (4)	*n.s. (2.48)*
	WSC	75 (6)	87 (5)	86 (3)	86 (3)	*n.s. (1.67)*
		Significance (F-value) of three-way ANOVA				
		Cd (A)	** (4.76)	A × B	n.s. (0.49)	
		Isolate (B)	***(15.19)	A × C	n.s. (0.22)	
		Plant (C)	n.s. (0.43)	B × C	n.s. (1.29)	
				A × B × C	n.s. (0.90)	
			ERM (m.g^{-1})			
BEG75	HisCUP	1.2 (0.1)^a	1.2 (0.1)^a	1.3 (0.1)^a	0.6 (0.1)^b	*** *(10.73)*
	WSC	1.4 (0.2)^a	1.0 (0.1)^a	1.1 (0.2)^a	0.5 (0.1)^b	** *(7.37)*
PH5	HisCUP	1.0 (0.1)^b	1.0 (0.1)^b	1.4 (0.1)^a	1.7 (0.1)^a	*** *(10.17)*
	WSC	0.9 (0.1)^b	1.0 (0.1)^b	1.7 (0.2)^a	1.4 (0.1)^a	** *(6.36)*
		Significance (F-value) of three-way ANOVA				
		Cd (A)	** (5.36)	A × B	*** (24.19)	
		Isolate (B)	** (11.31)	A × C	n.s (0.84)	
		Plant (C)	n.s. (0.31)	B × C	n.s. (0.29)	
				A × B × C	n.s. (1.78)	

The values are given as means of 5 replicates (S.E.). Significances and uppercase letters in italics refer to the effects of Cd addition as evaluated for each plant–fungus combination separately by one-way ANOVA and Duncan's multiple range test at the level $P < 0.05$: Means in lines followed by the same letters are not significantly different. Significant effects: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; n.s. non-significant effect.

Abbreviations: NM = non-inoculated, BEG75 = inoculated with *G. intraradices* BEG75, PH5 = inoculated with *G. intraradices* PH5, HisCUP = transgenic plants, WSC = control plants, Cd0, Cd20, Cd40, Cd60 = soil with Cd added in concentrations 0, 20, 40, 60 mg kg^{-1}.

20 mg kg^{-1} (HisCUP plants) and Cd 40 mg kg^{-1} (WSC plants). No significant growth inhibition by Cd was found for PH5-inoculated WSC and HisCUP plants. The interaction between Cd addition and inoculation treatment produced differential effects of inoculation with the two isolates at each Cd level: at Cd levels in soil non-inhibitory to the growth of non-inoculated plants, plants inoculated with the BEG75 isolate grew better than non-inoculated and PH5-inoculated plants according to Duncan's multiple range tests to the factor inoculation, while inoculation with either isolate improved plant growth at the two higher Cd levels (40 and 60 mg kg^{-1}).

The significant differences in shoot and root dry weight between WSC and HisCUP plants resulted in higher dry weights of HisCUP plants over all treatments. The difference was most pronounced when comparing non-inoculated WSC and HisCUP plants at the highest Cd concentration in soil 60 mg kg^{-1}: WSC plants produced only 35% of the shoot biomass and 43% of the root biomass produced by HisCUP plants. The difference was much lower when comparing inoculated WSC and HisCUP plants at the highest Cd concentration or non-inoculated plants at lower Cd concentrations in soil. The differences in root dry weight between WSC and HisCUP plants also depended on inoculation treatment: inoculation generally decreased the root dry weight of HisCUP plants but increased that of WSC plants.

The effects of inoculation on shoot growth at the different Cd levels in soil is summarised in Figure 1. Inoculation increased shoot biomass significantly more at Cd 40 and 60 mg kg^{-1} than in the soil without Cd addition, with the exception of BEG75-inoculated HisCUP plants. The effect

Table 2. Shoot and root dry weights of transgenic and non-transgenic tobacco as affected by cadmium (Cd) addition to soil and inoculation

Inoculation	Plant	Cd0	Cd20	Cd40	Cd60	*Signif. (F)*
		Shoot dry weight (g)				
NM	HisCUP	6.5 (0.7)^{a}	5.9 (0.5)^{ab}	3.7 (0.6)^{c}	4.3 (0.2)^{bc}	** *(6.30)*
	WSC	6.6 (0.3)^{a}	5.8 (0.3)^{a}	2.6 (0.7)^{b}	1.5 (0.3)^{b}	*** *(27.43)*
BEG75	HisCUP	9.8 (0.6)^{a}	8.3 (0.7)^{a}	8.5 (0.4)^{a}	5.4 (0.2)^{b}	*** *(9.43)*
	WSC	8.2 (0.6)^{a}	7.1 (0.4)^{a}	6.8 (0.4)^{a}	4.6 (0.4)^{b}	*** *(13.40)*
PH5	HisCUP	6.8 (0.9)	6.9 (0.5)	7.7 (0.2)	6.4 (0.6)	*n.s. (0.72)*
	WSC	6.2 (1.0)	5.9 (0.4)	6.9 (0.5)	5.0 (0.4)	*n.s. (1.62)*
		Significance (F-value) of three-way ANOVA				
		Cd (A)	*** (28.75)	A × B	*** (7.99)	
		Inoculation (B)	*** (51.25)	A × C	n.s. (0.92)	
		Plant (C)	*** (23.24)	B × C	n.s. (0.28)	
				A × B × C	n.s. (1.24)	
		Root dry weight (g)				
NM	HisCUP	0.92 (0.12)^{a}	0.84 (0.08)^{a}	0.38 (0.09)^{b}	0.46 (0.04)^{b}	*** *(18.75)*
	WSC	0.99 (0.05)^{a}	0.91 (0.11)^{a}	0.27 (0.14)^{b}	0.20 (0.04)^{b}	*** *(9.76)*
BEG75	HisCUP	1.70 (0.08)^{a}	1.17 (0.09)^{a}	0.98 (0.05)^{bc}	0.79 (0.08)^{c}	** *(7.00)*
	WSC	1.15 (0.11)^{a}	0.95 (0.09)^{ab}	0.78 (0.09)^{bc}	0.59 (0.06)^{c}	*** *(26.43)*
PH5	HisCUP	1.11 (0.14)	1.15 (0.14)	1.06 (0.11)	1.12 (0.24)	*n.s. (0.67)*
	WSC	0.84 (0.08)	0.84 (0.05)	0.80 (0.11)	0.68 (0.08)	*n.s. (0.05)*
		Significance (F-value) of three-way ANOVA				
		Cd (A)	*** (27.09)	A × B	*** (5.98)	
		Inoculation (B)	*** (32.02)	A × C	n.s. (0.48)	
		Plant (C)	*** (27.55)	B × C	* (3.87)	
				A × B × C	n.s. (1.13)	

The values are given as means of 5 replicates (S.E.). Significances and uppercase letters in italics refer to the effects of Cd addition as evaluated for each plant-fungus combination separately by one-way ANOVA and Duncan's multiple range test at the level $P < 0.05$: Means in lines followed by the same letters are not significantly different. Significant effects: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; n.s. non-significant effect. For abbreviations see Table 1.

of inoculation at Cd 40 and 60 mg kg^{-1} was more pronounced for WSC plants than for HisCUP plants: PH5-inoculated WSC plants produced 233% more biomass than non-inoculated WSC plants at the highest Cd level, while the difference was only 49% in the case of HisCUP plants.

The P concentrations in shoots and roots were significantly influenced by Cd in soil, inoculation treatment and interaction between both factors (Table 3). The highest P shoot and root concentrations were found in BEG75 inoculated plants, the lowest in non-inoculated plants according to multiple range test of the factor inoculation. Both parameters varied with Cd in soil, but no consistent increase or decrease with increasing Cd concentration in soil was apparent. The effect of inoculation on P concentrations also depended on Cd addition to soil as both factors significantly interacted. The P concentrations did not differ between HisCUP and WSC plants, but the plant factor significantly interacted with the factor Cd addition for the parameter P shoot concentrations.

Cadmium concentrations in shoots and roots

In the treatments without Cd addition to soil, average shoot Cd concentrations per treatment ranged between 1.5 and 3.3 $\mu g\ g^{-1}$, the average root concentrations between 0.5 and 1.1 $\mu g\ g^{-1}$ (data not shown).

Cd concentrations in shoots were influenced by each of the three tested factors separately (Cd, inoculation and plant) as well as by all their interactions (Table 4). Cd concentrations increased

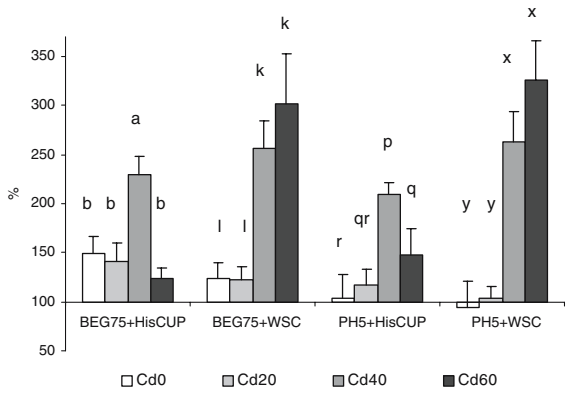

Figure 1. Growth response of transgenic and non-transgenic tobacco to inoculation with two *G. intraradices* isolates, expressed as percentage of the average biomass of non-inoculated plants at the corresponding Cd level. The values are given as means of 5 replicates (+S.E.). Columns indicated by different letters within one plant-fungus combination are significantly different according to one-way ANOVA and Duncan's multiple range test at the level $P < 0.05$. For abbreviations see Table 1.

with Cd addition, were decreased by inoculation and were higher in HisCUP plants than in WSC plants. At the highest Cd addition, however, all treatments had similar Cd shoot concentrations except non-inoculated HisCUP plants, which had about 90% higher Cd shoot concentrations than the other treatments.

Cd concentrations in roots significantly increased with Cd addition and were higher in HisCUP plants than in WSC plants (Table 4). Significant interaction of the factors inoculation and plant shows that the differences in root dry weight between WSC and HisCUP plants depended on inoculation treatment: while HisCUP plants had higher Cd root concentrations than WSC plants if non-inoculated, they tended to have lower Cd root concentrations when inoculated.

The Cd translocation from roots to shoots, expressed as shoot to root Cd concentration ratio, generally decreased with increasing Cd concentration in soil and was lower in plants inoculated with either of the two isolates isolate than in non-inoculated plants (Table 4). No significant difference in root to shoot translocation was observed between WSC and HisCUP plants.

Cadmium contents in shoots

The Cd shoot content represents the amount of Cd actually extracted from soil by one plant and depends on both plant growth and Cd shoot concentrat n. Generally, the Cd shoot contents increased with increasing Cd added and were higher in HisCUP plants than in WSC plants. The effect of inoculation treatment depended on Cd addition and on plant. All interactions between factors significantly influenced the parameter, resulting in different Cd accumulation

Table 3. Phosphorus (P) concentrations in the shoots and roots of transgenic and non-transgenic tobacco as affected by cadmium (Cd) addition to soil and inoculation

Inoculation	Plant	P shoot conc. (μg g^{-1})				P root conc. (μg g^{-1})			
		Cd0	Cd20	Cd40	Cd60	Cd0	Cd20	Cd40	Cd60
NM	HisCUP	1398 (120)	1236 (48)	1182 (30)	1084 (120)	1400 (102)	1307 (83)	1830 (149)	1283 (91)
	WSC	1261 (60)	1368 (129)	1059 (114)	1632 (197)	1376 (147)	1336 (136)	2214 (400)	1355 (101)
BEG75	HisCUP	1612 (71)	1831 (88)	1716 (55)	1772 (89)	1726 (53)	1804 (92)	1871 (56)	1954 (81)
	WSC	1462 (115)	2043 (37)	1761 (35)	2206 (167)	1684 (130)	1987 (115)	2055 (189)	2097 (179)
PH5	HisCUP	1671 (139)	1670 (106)	1397 (57)	1462 (73)	1603 (61)	1770 (109)	1891 (81)	1767 (88)
	WSC	1633 (120)	1607 (43)	1321 (75)	1583 (75)	1599 (121)	1905 (79)	1707 (100)	1650 (61)

Significance (F-value) of three-way ANOVA

	P shoot conc.				P root conc.		
Cd (A)	*** (7.06)	A × B	** (4.12)	Cd (A)	*** (8.61)	A × B	*** (4.83)
Inoculation (B)	*** (57.74)	A × C	*** (7.15)	Inoculation (B)	*** (25.87)	A × C	n.s (0.41)
Plant (C)	n.s. (3.59)	B × C	n.s. (1.32)	Plant (C)	n.s. (0.69)	B × C	n.s. (0.74)
		A × B x C	n.s. (0.97)			A × B x C	n.s. (0.56)

The values are given as means of 5 replicates (S.E.). Significant effects: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; n.s. non-significant effect. For abbreviations see Table 1.

Table 4. Cadmium (Cd) concentrations in the shoots and roots of transgenic and non-transgenic tobacco as affected by Cd addition to soil and inoculation

Inoculation	Plant	Cd shoot conc. ($\mu g.g^{-1}$)			Cd root conc. ($\mu g.g^{-1}$)			Shoot/root conc. ratio		
		Cd20	Cd40	Cd60	Cd20	Cd40	Cd60	Cd20	Cd40	Cd60
NM	HisCUP	118 (8.4)	191 (21.1)	240 (10.9)	45 (1.9)	101 (22.1)	213 (56.0)	2.6 (0.1)	2.1 (0.4)	1.5 (0.3)
	WSC	110 (11.1)	150 (5.3)	127 (9.0)	29 (6.3)	60 (3.9)	104 (27.0)	4.2 (0.9)	2.5 (0.1)	1.5 (0.5)
BEG75	HisCUP	87 (4.5)	115 (15.8)	130 (5.7)	35 (1.9)	72 (6.6)	110 (14.6)	2.5 (0.2)	1.6 (0.2)	1.3 (0.2)
	WSC	71 (2.0)	106 (7.2)	121 (6.3)	41 (4.4)	78 (8.1)	141 (19.3)	1.8 (0.2)	1.4 (0.1)	0.9 (0.1)
PH5	HisCUP	67 (6.7)	130 (9.4)	125 (6.7)	35 (3.7)	86 (7.7)	151 (18.6)	1.9 (0.1)	1.5 (0.1)	0.9 (0.1)
	WSC	56 (3.5)	107 (6.4)	118 (7.0)	23 (1.8)	93 (10.3)	167 (13.5)	2.4 (0.1)	1.2 (0.2)	0.7 (0.0)

Significance (F-value) of three-way ANOVA

Cd shoot conc.		Cd root conc.		Shoot/root conc. ratio	
Cd (A)	*** (1168.14)	Cd (A)	*** (299.06)	Cd (A)	*** (29.98)
Inoculation (B)	*** (73.89)	Inoculation (B)	n.s. (0.64)	Inoculation (B)	*** (8.62)
Plant (C)	*** (35.48)	Plant (C)	* (4.98)	Plant (C)	n.s. (0.39)
A × B	*** (7.17)	A × B	n.s. (1.17)	A × B	n.s. (0.46)
A × C	** (5.46)	A × C	n.s. (0.48)	A × C	n.s. (1.81)
B × C	** (7.46)	B × C	*** (9.77)	B × C	n.s. (1.03)
A × B × C	*** (5.03)	A × B × C	n.s. (1.74)	A × B × C	n.s. (1.47)

The values are given as means of 5 replicates (*S.E.*). Significant effects: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; n.s. non-significant effect. For abbreviations see Table 1.

patterns among the treatments at each Cd level in soil (Figure 2).

At Cd 20 mg kg^{-1}, only inoculation with the PH5 isolate decreased the Cd contents of plants in comparison with the non-inoculated treatment. The highest shoot contents were therefore found in non-inoculated and BEG75-inoculated HisCUP plants as well as in non-inoculated WSC plants. The lowest shoot contents were found in PH5 inoculated WSC and HisCUP plants.

At Cd 40 mg kg^{-1}, inoculation showed the trend of increased Cd shoot contents of both WSC and HisCUP. Non-inoculated WSC and HisCUP plants had significantly lower Cd shoot contents than HisCUP plants inoculated with either BEG75 or PH5 and than WSC plant inoculated with PH5.

At Cd 60 mg kg^{-1}, the shoots of non-inoculated WSC plants contained only 17% of the amount of Cd found in the shoots of non-inoculated HisCUP plants. Inoculation with either of the two isolates, however, decreased the Cd shoot contents of HisCUP plants and increased those of WSC plants, so that the differences between inoculated WSC and HisCUP plants were much lower.

Discussion

The introduction of the HisCUP construct increased the tolerance of the tobacco variety to high Cd concentrations in soil and its Cd uptake. This confirms the results of previous tests conducted with the same plant material in sand supplied with nutrient solution (Macek et al., 2002; Pavlíková et al., 2004) and in an unpolluted soil (Pavlíková et al., 2004).

Increased Cd tolerance has been repeatedly reported for tobacco with inserted mammalian metallothionein genes (Maiti et al., 1989; Hattori et al., 1994; Elmayan and Tepfer, 1994). The increased tolerance was mostly accompanied by decreased Cd translocation to the leaves, which 'clearly limits the use of metallothioneins in phytoremediation' as concluded by Krämer and Chardonnens (2001). On the other hand, increased tolerance associated with higher Cd translocation to shoots has been found in cauliflower with introduced *CUP1* gene (Hasegawa et al., 1997). Similarly, the introduction of the *CUP1* gene considerably increased the copper tolerance and uptake of tobacco, though it did not affect its Cd tolerance and uptake (Thomas et al., 2003). The main mechanism of tolerance

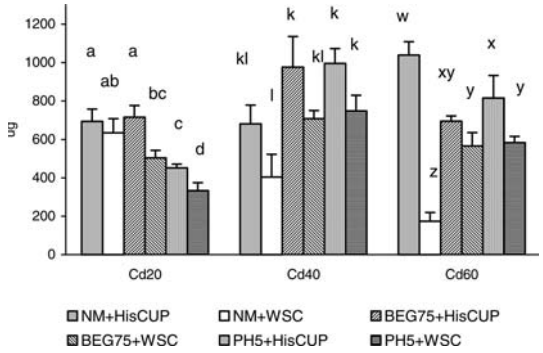

Figure 2. Cadmium contents in tobacco shoots. The values are given as means of 5 replicates (+S.E.). Three-way ANOVA of the data provided following significant effects of the factors and their interactions (*F*-value): Cd in soil (A) *** (139.14), Inoculation (B) n.s. (1.92), Plant (C) *** (52.50), A × B *** (7.45), A × C *** (9.88), B × C * (3.26), A × B × C *** (4.86). Significant effects: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; n.s. non-significant effect. Columns indicated by different letters within one Cd concentration in soil are significantly different according to one-way ANOVA and Duncan's multiple range test at the level $P < 0.05$. For abbreviations see Table 1.

consisted apparently in more effective Cd immobilisation in the roots in the former reports, while improved HM chelation and detoxification in the leaves can be assumed in the latter reports as well as in this study.

However, not only the transgene, but also inoculation with AM fungi improved the growth of the tobacco plants in Cd amended soil. At the two higher Cd levels in soil, which inhibited the growth of non-mycorrhizal plants, inoculation improved tobacco growth more substantially than in the soil without Cd. Moreover, the more Cd sensitive non-transgenic plants responded better to inoculation at these Cd levels in soil than the less sensitive transgenic plants. In contrast to the effect of the transgene, the improved growth of mycorrhizal plants was mostly accompanied by decreased Cd concentrations in shoots and lower Cd translocation rates from roots to shoots. Similar effects of mycorrhiza on plant growth and HM uptake have been previously reported by Heggo and Angle (1990) and Díaz et al., (1996).

The effects of inoculation did not principally differ between the two tested isolates. However, inoculation with the BEG75 isolate only improved plant growth in the soil without Cd amendment and at the lowest Cd level in soil, while the growth of PH5-inoculated plants was not affected by Cd. Additionally, the BEG75 isolate better supported P acquisition of the tobacco plants. It seems therefore that the BEG75 isolate was more effective in supporting plant growth in conditions of no or low Cd stress, while the PH5 isolate protected plants more effectively against Cd stress. Cd did not decrease the root colonisation of either isolate, but it differentially affected their ERM growth: The ERM growth of the BEG75 isolate was inhibited by the highest Cd concentration in soil, while the ERM length of the PH5 isolate was even higher at Cd 40 and 60 mg kg^{-1} than in the soil without Cd amendment. The BEG75 isolate reacted in accordance with the suggestion of Vidal et al., (1996) that the extraradical phase of AM fungi may be more sensitive to high HM concentrations in soil than the intraradical phase. The PH5 isolate was clearly more tolerant to Cd than the BEG75 isolate, but the stimulation of ERM growth in Cd amended soil is difficult to explain based on the analysed data. The tolerant AM fungus may have profited from Cd-induced changes in the soil microbial community or may have reacted to Cd toxicity with increased production of extraradical hyphae.

The PH5 isolate originates from an industrial site contaminated predominantly by lead, but containing also elevated concentrations of Cd and other heavy metals. It displayed higher tolerance to lead than the BEG75 isolate from a non-polluted agricultural area in an in-vitro test (Malcová et al., 2003a). Isolates originating from HM contaminated soils repeatedly demonstrated higher HM tolerance than isolates from non-contaminated soils (Gildon and Tinker, 1981; Weissenhorn et al., 1993; Malcová et al., 2003b). They were also shown to promote the growth and/or decrease the HM uptake of their hosts in contaminated soils more effectively than isolates from non-contaminated soils (Díaz et al., 1996; Kaldorf et al., 1999; Hildebrandt et al., 1999), which indicates higher ability to protect plants against HM stress. These results, however, do not seem to have general validity as they were not confirmed e.g., by Weissenhorn et al., (1995) and Weissenhorn and Leyval (1995). Moreover, the effects observed in our study do not correspond with the results of

Malcová et al., (2003a), who tested the PH5 and BEG75 isolates under simulated Pb stress: the BEG75 isolate more effectively improved phosphorus acquisition by *Agrostis capillaris* but not by *Zea mays*, and it failed to improve the growth of either of the two plants. Their study shows as well that the influence of mycorrhiza on plant growth and HM uptake is specific to plant species, which may partly explain the discrepancies of the results.

The general picture of improved growth of mycorrhizal plants accompanied by lower Cd concentrations in shoots and decreased Cd root to shoot translocation seems to correspond well with two hypotheses previously suggested from studies of different HMs to explain higher HM tolerance of mycorrhizal plants in contaminated soils: The ERM of AM fungi can decrease the HM uptake of plants immobilising the HMs in soil by sorption (Joner et al., 2000; Gonzalez-Chavez et al., 2002) and intraradical fungal structures can decrease the HM translocation from roots to shoots by binding HMs within roots (Turnau et al., 1993; Joner and Leyval, 1997). Both mechanisms decrease the amounts of Cd transported to shoots and AM fungi may consequently protect their hosts against Cd stress.

However, both suggested mechanisms do not fully explain the growth response to inoculation in the treatments with Cd addition when our data on plant growth and Cd uptake are compared more in detail. If both mechanisms had been active, the improved growth of mycorrhizal plants should have been accompanied by lower Cd concentrations in shoots as a result of lower uptake due to Cd sorption on ERM and lower root to shoot translocation due to Cd immobilisation in fungal intraradical structures. Inoculation most pronouncedly improved the growth of the non-transgenic tobacco at the two higher Cd levels in soil (40 and 60 mg kg^{-1}). The effect, however, was accompanied by decreased Cd concentrations in shoots only at Cd 40 mg kg^{-1} but not at Cd 60 mg kg^{-1}. The better growth of inoculated plants in the Cd amended treatments was therefore not always correlated with lower Cd concentrations in the shoots. Similar results were obtained by Dueck et al. (1986) and Rivera-Becerril et al. (2002) from zinc and cadmium amended substrates, respectively, where improved growth of mycorrhizal plants was even accompanied by higher HM shoot concentrations.

On the other hand, the lower Cd concentrations in the shoots of mycorrhizal plants cannot be regarded as a consequence of Cd dilution in tissues by improved plant growth, only, a mechanism suggested by Malcová et al. (2003b). Plants inoculated with the PH5 isolate at the lowest Cd level and inoculated transgenic plants at the highest Cd level had not only decreased Cd tissue concentrations but also Cd contents as compared with non-inoculated plants.

Meharg and Cairney (2000) reviewed the results obtained on the interaction of AM with HMs and concluded that AM fungi probably confer little or no enhanced metal resistance to their hosts. They suggest instead that nutritional effects are responsible for better growth of mycorrhizal plants in contaminated soils, similarly to non-contaminated soils. In our study, the phosphorus acquisition of the tobacco plants was consistently improved by inoculation. However, nutritional effects only, without considering their interaction with the toxic effect of Cd, cannot explain the growth responses to inoculation at the two higher Cd levels.

Irrespective of the mechanisms involved, inoculation altered also the Cd shoot content per plant – the parameter characterising Cd extraction efficiency. This modification was comparable in its extent with the effect of the transgene. However, while the transgene increased Cd accumulation by improving growth and enhancing Cd uptake, mycorrhiza improved the growth of the plants on one hand but decreased their Cd shoot concentrations on the other hand. The resulting effect on Cd accumulation in shoots was therefore complex, depending on the Cd level in soil as well as on AM fungal isolate, and it differed between the more tolerant transgenic and the less tolerant non-transgenic plants. At the highest Cd level in soil, where the effect was most pronounced, inoculation strongly decreased the Cd shoot contents of transgenic plants but increased those of non-transgenic plants. Inoculation thus acted against the effect of the transgene, considerably decreasing the difference in Cd extraction efficiency between transgenic and non-transgenic plants. This observation supports the warning of Krä-

mer and Chardonnens (2001) that data on trace element uptake by transgenic plants collected in non-soil substrates cannot be extrapolated to soil-based experiments or even to field conditions.

The results well illustrate the multifunctional character, in which AM interacts with HMs in soil. The interaction is influenced by many factors and processes, as reviewed by Leyval et al. (1997) and Haselwandter et al. (1994). The unclear relationship between the effects of mycorrhiza on growth and Cd uptake highlights the need for identifying the mechanisms of the interaction in order to enable extrapolation and prediction of the effects of inoculation on HM accumulation by plants. From the presented results, however, it is evident that arbuscular mycorrhiza should be considered in phytoremediation strategies similarly as genetic modifications of plants. Management of microbial communities in phytoremediation programmes has been proposed by Perotto and Martino (2001), Khan et al. (2000) or Jeffries et al. (2003). This approach to increase the efficiency of the phytoextraction process deserves attention especially due to its unproblematic acceptance by the public in contrast to the use of transgenic plants. The presented results suggest, as well, that management of microbial communities may also mean mitigation of the development of AM symbiosis in certain situations.

This is the first study describing the effect of arbuscular mycorrhiza on a high biomass crop genetically engineered to accumulate more heavy metals in shoots and thus potentially exploitable in phytoextraction. Despite its model character, not including feasibility calculations of the phytoextraction process in real conditions, the study provides basic insight into possible responses of Cd accumulating plants to AM symbiosis.

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