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Influence of arbuscular mycorrhiza on the growth and cadmium uptake of tobacco with inserted metallothionein gene

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Abstract

The effect of arbuscular mycorrhiza (AM) on the growth and cadmium (Cd) uptake of transgenic tobacco with increased ability to accumulate Cd was assessed. The transgenic tobacco bearing yeast metallothionein gene combined with a polyhistidine cluster was compared to non-transgenic tobacco in two pot experiments with different substrates – soil and river sand – amended or unamended with Cd.

The development of AM did not differ between the transgenic and non-transgenic plants in either experiment. AM improved the phosphorus nutrition of the tobacco plants in both experiments, their biomass production, however, was increased only in sand, while in soil, it was lower or remained unchanged compared to non-mycorrhizal plants. AM decreased the Cd uptake of the tobacco plants per unit of shoot biomass in both experiments and decreased the Cd accumulation in the shoots of the transgenic tobacco relatively to the non-transgenic tobacco. It is concluded that AM symbiosis is likely to influence the heavy metal (HM) accumulation ability of plants targeted by transgenosis. Thus, AM must be considered in testing the transgenic plants as it can change the relative performance of the transgenic plants compared to the non-transgenic plants.

Keywords: Heavy metals; Genetic engineering; Phytoremediation; Soil microflora; *Glomus*

1. Introduction

Phytoextraction of heavy metals (HM) from contaminated soils, i.e. their removal by means of green plants, has been recognised as a possible cost-effective alternative to physical soil remediation techniques (Salt et al., 1995). Plants, which naturally hyperaccumulate heavy metals are the most obvious

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tools for phytoextraction purposes, but the small biomass production of most and the common specificity to one metal only limit their application potential (Krämer and Chardonnens, 2001). The preparation of high biomass crops with increased ability to tolerate and accumulate heavy metals by introduction of metal-binding proteins or peptides is therefore considered and tested as an alternative (Mejáre and Bülow, 2001).

Insertion of genes for metal-binding proteins has been reported to modify plant tolerance and HM uptake in hydroponic or agar-based cultivation, but few data are available from soil based cultivation experiments or studies under field conditions (Krämer and Chardonnens, 2001). Moreover, virtually no attention has been paid so far to the effect of soil microflora on the performance of transgenic plants with increased ability to accumulate heavy metals.

Arbuscular mycorrhiza (AM) represents an almost ubiquitous relationship between soil microflora and plants. The fungal symbiont increases its host's uptake of nutrients and can improve its growth and resistance to environmental stresses (Smith and Read, 1997). AM symbiosis can also modify the response of plants to excess HM in soil (as reviewed by Leyval et al., 1997), e.g. increase the heavy metal tolerance of plants (Hildebrandt et al., 1999), increase (Rivera-Becerril. et al., 2002) or decrease (Heggo and Angle, 1990) their HM uptake per unit of biomass or reduce their HM translocation from roots to shoots (Loth and Höfner, 1994).

Gaur and Adholeya (2004) suggested that inoculation of HM hyperaccumulating plants with AM might enhance the potential of the phytoremediation technology. Nevertheless, AM fungi have been also found in roots of plants growing in soils heavily contaminated with HM (e.g. Turnau, 1998). Therefore, the effect of mycorrhiza on the phytoextraction process has to be taken into account, even if no targeted inoculation of plants is performed. Leyval et al. (1997) pointed out that attention needed be paid to the susceptibility of genetically modified plants to mycorrhiza in view of the beneficial effects, which the symbiosis can have on plant growth and nutrition.

This study attempts to contribute to the missing knowledge on the effect of AM on plants, which had been genetically engineered to accumulate higher amounts of HM. The development of AM symbiosis on the transgenic and non-transgenic tobacco plants as well as the influence of AM symbiosis on their growth and Cd uptake were compared in two experiments with different substrates and modes of Cd application.

2. Material and methods

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) in two pot experiments conducted in greenhouse conditions, each experiment using a different cultivation substrate. Both experiments were designed as $2 \times 2 \times 2$ factorial with the following factors:(1) plant treatment (WSC and HisCUP plants), (2) Cd treatment (with or without Cd application to soil) and (3) mycorrhizal treatment (non-mycorrhizal, mycorrhizal). Each combination of the factors involved five replicates, each represented by one plant in a 5 l pot.

The HisCUP plants bore a transgene coding for a polyhistidine cluster combined with yeast metallothionein *CUP1*, under the control of CaMV 35S promoter (Macek et al., 2002). The best Cd accumulating line T–HisCUP–X was chosen from the transgenic tobacco lines (Macek et al., 2002) and its increased Cd uptake and tolerance had been confirmed in a sand-based cultivation test (Pavlíková et al., 2004). In vitro cultivated vegetatively multiplied aseptic plantlets were used as starting material, transplanted to the experiments at the size of about 10 cm (after 6 weeks of growth).

In Experiment 1, non-contaminated chernozem with the following characteristics was used as cultivation substrate: 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%; organic matter (C_{ox}) 1.83 ± 0.41%, cation exchange capacity 25.8 ± 0.4 cmol(+) kg^{-1}. Each pot contained 5 kg of dry soil sterilised by γ-irradiation (50 kGy) and amended with 1.5 g of nitrogen as ammonium nitrate at the beginning and further 1 g in the middle of the cultivation period. Cd was applied in the corresponding treatments to the concentration of 40 mg kg^{-1} soil as cadmium nitrate at the beginning, and further 15 mg kg^{-1} soil in the middle of the cultivation period. The control treatments without Cd addition received the equivalent amount of nitrogen as ammonium nitrate. Both

ammonium nitrate and cadmium nitrate were added in solution.

In Experiment 2, the plants were cultivated in river sand supplied three times a week with the modified White's nutrient solution P2N3 (Gryndler et al., 1992), 140 ml in the first half and 280 ml in the second half of the cultivation period. The solution was enriched with phosphorus (P) to a weekly dose of 4.2 mg. Each pot contained 7 kg of dry sand sterilised by heating at 120 °C for 8 h in 2 consecutive days. In the corresponding treatments, Cd was added to the nutrient solution in the amount of 0.6 mg l^{-1}, which equals to the total dose of 4.5 mg Cd per plant for the whole cultivation period.

The mycorrhizal treatments were inoculated with a *Glomus intraradices* Schenck and Smith, isolate originating from a Pb-contaminated waste disposal site near Příbram, Czech Republic (PH5) (Malcová et al., 2003). This isolate had been selected due to its quick development and ability to develop well in different soil conditions. The fungus was added as 10 ml of inoculum suspension containing colonised root segments, extraradical mycelium (ERM) and spores. The non-inoculated treatments received the same amount of autoclaved inoculum. All pots were irrigated with 5 ml of filtrate from the non-sterile inoculum to equalise the microbial conditions.

The plants of each experiment were harvested after 12 weeks. The dry weights of the shoots and roots were recorded after drying at 80 °C for 24 h. The plant material was ground, decomposed by a dry ashing procedure and the Cd concentrations were determined by atomic absorption spectrometry (Varian SpectrAA-300) with flameless atomization. For the determination of P, the Scalar (San System) segmented continuous flow analysis with photometric detector was used.

Root colonisation by the AMF was assessed using the grid-line intersect method (Giovannetti and Mosse, 1980) after staining with 0.05% trypan blue in lactoglycerol (Koske and Gemma, 1989). The ERM length of the AMF was estimated using a modified membrane filtration technique (Jakobsen et al., 1992). The average background length of the ERM from five non-inoculated treatments was subtracted from all values obtained in the inoculated treatments.

The results of the experiments were analysed using analyses of variance (ANOVA). Where necessary, data were logarithmically transformed [$y = \ln(x + 1)$] to meet the requirements of ANOVA. Comparisons between means were performed using Duncan's multiple range test at the significance level of $P < 0.05$.

3. Results

Inoculation of the tobacco plants with the *G. intraradices* isolate resulted in successful establishment of mycorrhiza in both experiments, without any difference in the evaluated mycorrhizal parameters between WSC and HisCUP plants. In Experiment 1 (soil), root colonisation reached 95% in average, the ERM length 3.9 m g^{-1}. In Experiment 2 (sand), both percentage of colonised roots and ERM length were lower than in Experiment 1 (59% and 0.8 m g^{-1}). No significant inhibition of intraradical or extraradical growth of the fungus has been observed in the Cd amended substrates.

The Cd concentrations in tissues were decreased by mycorrhiza in both experiments (Tables 1 and 2). HisCUP plants had significantly higher Cd shoot and root concentrations than WSC plants only in Experiment 1 (Table 1). The Cd root to shoot translocation ratio was neither significantly influenced by mycorrhiza nor differed between WSC and HisCUP plants (data not shown).

The Cd accumulation in shoots, expressed as Cd shoot content per plant, was significantly influenced by the interaction of the factors Cd in soil and transgene in both experiments: the effect of mycorrhiza differed between WSC and HisCUP plants. Mycorrhiza reduced the Cd shoot content of HisCUP plants whereas it had no effect on that of WSC plants. The reduction comprised 39% in Experiment 1 and 29% in Experiment 2.

Shoot contents are also determined by biomass production. Cd addition significantly decreased plant shoot and root dry weight of both the WSC and HisCUP plants in Experiment 1 (Table 1). Mycorrhiza improved the P nutrition of the plants as indicated by the consistently higher P concentrations in the shoots of mycorrhizal plants. However, mycorrhiza had negative effect on tobacco growth in the control soil with no Cd amendment and no growth effect at all in the soil with Cd added. The factor transgene neither

Table 1
Shoot and root dry weights (DW) of tobacco plants, phosphorus (P) concentrations in shoots, cadmium (Cd) concentrations in shoots and roots and total Cd content in shoots per plant in Experiment 1

Treatment	DW (g)		P ($\mu g\ g^{-1}$)	Cd ($mg\ g^{-1}$)		Cd (mg)
	Shoots	Roots	Shoots	Shoots	Roots	Shoots
0/NI/WSC	46.4 (0.5)	4.7 (0.3)	1145 (38)			
0/NI/HisCUP	44.7 (1.7)	5.1 (0.4)	1040 (49)			
0/G.i./WSC	42.0 (1.0)	3.7 (0.3)	1518 (156)			
0/G.i./HisCUP	40.2 (1.7)	4.0 (0.4)	1562 (108)			
Cd/NI/WSC	34.2 (0.8)	3.3 (0.3)	819 (40)	134 (7)	100 (9)	4.6 (0.3)
Cd/NI/HisCUP	35.8 (1.6)	3.3 (0.2)	859 (11)	208 (21)	141 (47)	7.6 (0.6)
Cd/G.i./WSC	35.7 (0.6)	3.4 (0.1)	2018 (41)	117 (18)	93 (4)	4.2 (0.7)
Cd/G.i./HisCUP	34.4 (1.2)	3.0 (0.3)	2038 (31)	134 (13)	102 (14)	4.6 (0.3)
Significances (F-values) of three-way ANOVA						
Cadmium (A)	90.13^{***}	29.66^{***}	4.98^{*}			
Inoculation (B)	6.49^{*}	7.95^{**}	242.64^{***}	8.39^{*}	0.691[a] n.s.	10.47^{**}
Plant (C)	0.85 n.s.	0.00 n.s.	0.00 n.s.	8.30^{*}	0.000[a] n.s.	9.92^{**}
A × B	6.60^{*}	4.69^{*}	49.94^{***}			
B × C	0.74 n.s.	0.56 n.s.	0.38 n.s.	3.25 n.s.	ND[a]	6.14^{*}

Abbreviations: 0, no Cd added to soil; Cd, Cd added to soil (55 mg kg^{-1}); NI, non-inoculated; G.i., inoculated with *G. intraradices* PH5; WSC, non-transgenic tobacco; HisCUP, transgenic tobacco. The values are given as means of five replicates (s.e.). Significant effects according to three-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; n.s., non-significant effect and ND, not determined. The non-significant interactions between factors were omitted.

[a] Evaluated by non-parametric Kruskal–Wallis test, as data did not meet the requirements of ANOVA.

influenced the growth of the tobacco nor interacted with the factors Cd addition to soil and inoculation for the plant growth parameters (Table 1).

In Experiment 2, Cd addition had no influence on shoot dry weight, while root dry weight was even higher in the Cd amended treatments (Table 2). Improved P nutrition of the mycorrhizal plants was accompanied by a positive growth response of both WSC and HisCUP plants to mycorrhiza. Similarly to Experiment 1, WSC and HisCUP plants did not differ

Table 2
Shoot and root dry weights (DW) of tobacco plants, phosphorus (P) concentrations in shoots, cadmium (Cd) concentrations in shoots and roots and total Cd content in shoots per plant in Experiment 2

Treatment	DW (g)		P ($\mu g\ g^{-1}$)	Cd ($\mu g\ g^{-1}$)		Cd (μg)
	Shoots	Roots	Shoots	Shoots	Roots	Shoots
0/NI/WSC	16.1 (0.7)	2.8 (0.2)	1370 (44)			
0/NI/HisCUP	15.2 (0.6)	2.2 (0.2)	1470 (50)			
0/G.i./WSC	20.7 (0.9)	4.2 (0.3)	1680 (41)			
0/G.i./HisCUP	19.6 (0.6)	3.5 (0.4)	1736 (30)			
Cd/NI/WSC	16.5 (0.7)	3.0 (0.3)	1307 (21)	164 (8)	120 (7)	2.7 (0.1)
Cd/NI/HisCUP	16.3 (0.7)	3.2 (0.3)	1361 (57)	168 (9)	100 (11)	2.8 (0.2)
Cd/G.i./WSC	21.1 (1.0)	4.9 (0.5)	1529 (67)	127 (3)	64 (11)	2.7 (0.1)
Cd/G.i./HisCUP	19.1 (0.6)	3.8 (0.2)	1408 (58)	106 (10)	64 (4)	2.0 (0.2)
Significances (F-values) of three-way ANOVA						
Cadmium (A)	0.51 n.s.	5.73^{*}	23.13^{***}			
Inoculation (B)	60.51^{***}	35.46^{***}	38.82^{***}	40.05^{***}	28.08^{***}	7.63^{*}
Plant (C)	3.76 n.s.	6.25^{*} n.s.	0.41 n.s.	1.21 n.s.	1.37 n.s.	4.49 n.s.
B × C	1.03 n.s.	3.03 n.s.	2.59 n.s.	2.43 n.s.	1.24 n.s.	6.75^{*}

For abbreviations see Table 1. The values are given as means of five replicates (s.e.). Significant effects according to three-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; n.s., non-significant effect. The non-significant interactions between factors were omitted.

in shoot growth, but WSC plants had generally higher root biomass (Table 2).

4. Discussion

The influence of the transgene on Cd tolerance and uptake by tobacco was less pronounced than expected from the results of previous tests (Macek et al., 2002; Pavlíková et al., 2004). In Experiment 1, Cd added to soil reached a toxic level, but the transgenic and non-transgenic plants were inhibited equally without any effect of the transgene on Cd tolerance in contrast to the results of Pavlíková et al. (2004). Higher Cd uptake per unit of biomass of the transgenic plants in comparison with the non-transgenic plants has been confirmed in Experiment 1. In Experiment 2, however, no differences have been recorded despite comparable Cd concentrations in tobacco biomass as in Experiment 1. These results show that cultivation conditions may be important for the performance of the transgenic plants.

The difference between the cultivation conditions in both experiments consisted especially in the higher level of nutrients in Experiment 1 conducted in soil, where plants produced about twice as much biomass as in Experiment 2. This can explain, why the growth response of the tobacco plants to mycorrhiza was positive in Experiment 2, while neutral to negative in Experiment 1. Smith et al. (1986) and Son and Smith (1988) recorded positive mycorrhizal growth response of plants growing in soil with low P levels, but negative growth response in conditions of high P supply. Increased inflow of P into mycorrhizal than into non-mycorrhizal plants has been recorded at both P levels, similarly to the results of this study comparing Experiments 1 and 2. The growth responses to mycorrhiza in both experiments show that mycorrhiza has higher ability of growth improvement of tobacco in conditions of low nutrient availability.

Despite the different cultivation conditions, mycorrhiza consistently reduced the Cd shoot concentrations of the tobacco in both experiments. We can, therefore, conclude that mycorrhiza decreases Cd uptake per unit of biomass in tobacco. AM symbiosis has been reported to decrease (Heggo and Angle, 1990) Cd tissue concentrations of plants growing in soils with high Cd concentrations and the effect of mycorrhiza on HM uptake seems to be specific to plant species (Ricken and Höfner, 1995).

The transgenic and non-transgenic tobacco did not principally differ in their response to mycorrhiza. However, interaction of the parameters inoculation and plant was significant in both experiments for the parameter Cd shoot content, i.e. Cd extracted per plant from soil. This interaction consisted in mycorrhiza reducing the Cd shoot contents of the transgenic plants but not those of the non-transgenic plants. The effect corresponded with a similar trend in Cd shoot concentrations, which tended to decrease as effect of mycorrhiza more in transgenic plants than in non-transgenic plants. This result is difficult to discuss as it may reflect complex physiological effects of mycorrhiza interacting with subtle differences in physiology (e.g. nutritional demands) of the transgenic plants, which have not been investigated. However, it shows that mycorrhiza alters the Cd accumulation of the transgenic tobacco relatively to the non-transgenic tobacco.

5. Conclusion

The presented results show that AM symbiosis influences the growth and Cd uptake of transgenic tobacco designed for higher HM tolerance and uptake. Mycorrhiza is not likely to increase the ability of tobacco to extract Cd from soil as Cd uptake per unit of biomass can be expected to decrease in mycorrhizal tobacco while pronounced growth improvements to compensate for this effect have not been recorded. Comparing transgenic plants with non-transgenic plants of the same variety, mycorrhiza decreased the Cd accumulation by the transgenic tobacco relatively to the non-transgenic tobacco. This result suggests that mycorrhiza should be taken into account in studies testing the performance of HM hyperaccumulating plants in soil based cultivation systems.

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