Martina Janoušková

Effects of Arbuscular Mycorrhiza on Cadmium Uptake by Tobacco (*Nicotiana tabacum* L.)

Ph.D. Thesis

Charles University
Faculty of Science
Department of Plant Physiology

Academy of Sciences of the Czech Republic
Institute of Botany
Department of Mycorrhizal Symbioses

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<u>Supervisor</u>   Mgr. Miroslav Vosátka, Ph.D.
Academy of Sciences of the Czech Republic, Institute of Botany

<u>Consulting supervisor</u>

Assoc. Prof. Jana Albrechtová, Ph.D.
Charles University, Department of Plant Physiology
Academy of Sciences of the Czech Republic, Institute of Botany

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Abstract

The effects of arbuscular mycorrhiza (AM) on Cd accumulation by tobacco (*Nicotiana tabacum* L.) were investigated in a series of cultivation experiments. The main aims were i) to characterise how AM symbiosis influences the Cd uptake, growth and mineral nutrition of tobacco in Cd contaminated and non-contaminated soils and ii) to assess which physiological processes and factors are involved in the mycorrhizal effects on Cd accumulation by tobacco. Mycorrhizal effects were also studied on a transgenic tobacco clone with enhanced Cd uptake and improved Cd tolerance.

Mycorrhiza consistently decreased the Cd shoot concentrations of tobacco over broad range of Cd availabilities in soil, but increased them when Cd availability in soil was very low. At toxic Cd concentrations in soil, mycorrhiza alleviated the Cd-induced growth inhibition in tobacco both at shoot and root level. The effect of mycorrhiza on the total Cd uptake by tobacco depended on the mycorrhizal growth response of the plants, which ranged from positive over neutral to negative. However, mycorrhiza increased the total Cd content per plant only when Cd induced severe growth inhibition in the non-mycorrhizal plants, which was alleviated by mycorrhiza.

Consistently lower Cd concentrations in mycorrhizal tobacco as compared to non-mycorrhizal tobacco could not be fully explained by the biomass dilution effect or decreased Cd translocation from roots to shoots in the mycorrhizal plants. However, Cd was more effectively immobilised in the rhizosphere of mycorrhizal tobacco as compared to non-mycorrhizal tobacco and plant-mediated effects of mycorrhiza on rhizospheric properties were probably responsible for the difference rather than Cd sorption by AM fungal ERM.

Mycorrhiza decreased Cd accumulation by the transgenic tobacco clone with higher Cd uptake relatively to the wild type clone. It is suggested that mycorrhiza could be useful in decreasing Cd concentrations in the leaves of commercially produced tobacco, but an application of mycorrhiza to enhance Cd accumulation in tobacco shoots for the purposes of phytoextraction can be excluded based on the results of this thesis.

Acknowledgements

Declaration

I hereby declare that the work presented in this manuscript is my own and was carried out entirely with help of literature and aids cited in the manuscripts. The work or its major part has not been submitted to acquire any other academic degree.

Martina Janoušková

Important abbreviations

AM	arbuscular mycorrhiza
Basma BEK	commercial variety of Oriental tobacco
EcM	ectomycorrhiza
ERM	extraradical mycelium
HisCUP	clone of tobacco variety Wisconsin 38 bearing a transgene coding for a polyhistidine cluster combined with yeast metallothionein *CUP1* (see 4.1 and 4.2)
HM	heavy metal
K326	commercial variety of flue-cured tobacco
TN90	commercial variety of Burley tobacco
WSC	wild type clone of tobacco variety Wisconsin 38

Contents

1 <u>Introductory comments</u>

The main principles of the functioning of arbuscular mycorrhiza (AM) have been generally described and accepted. However, AM influences plant physiology in such a complex way that mycorrhizal plants often differ from non-mycorrhizal plants in parameters seemingly little related to the main functions of the symbiosis. Among others, considerable body of information has been collected on how AM can affect the survival, growth and heavy metal (HM) uptake of plants. However, the understanding of the interaction is hampered especially by contradicting reports on the effects of AM symbiosis, difficulties in the reproducibility of the obtained results and lack of knowledge on the responsible mechanisms.

The main idea of this thesis was therefore to dedicate more time and efforts to a defined experimental system, and to not only show how AM may affect the interaction of the host plant with HMs in soil, but also to describe when and why, i.e. in which conditions, and which responses to AM symbiosis are involved. Cd is a suitable heavy metal for this purpose due to its high toxicity, high mobility in the soil-plant system and relatively abundant knowledge on its behaviour in soil and plants in comparison with other HMs. Moreover, it should not be forgotten that Cd contamination of soils by human activities concerns vast areas especially of industrialised regions and is aggravated by the above-mentioned characteristics of Cd, which facilitate its entering into the food chain. Tobacco, the selected model plant, is an important crop plant and its Cd accumulation characteristics are of interest from the point of view of plant-product safety. Moreover, it also enabled to test the often repeated but rather theoretic idea that AM could be applied to support phytoremediation of HM contaminated soils. Not only that tobacco is a candidate plant for Cd phytoextraction from soils, but also a transgenic clone of tobacco was available for testing, which displayed enhanced ability to tolerate and accumulate Cd.

I am aware that a second thesis could follow to further explore the "when" and "why" of mycorrhizal effects on Cd uptake by tobacco and to bring more direct evidence for some hypothesis resulting from the presented study. However, I also believe that the presented results help to understand the complexity of the mechanisms and factors, which govern the interaction of AM with HMs in soils. Last but not least, the results give a clear idea about when it would be useful to support AM of tobacco and when not to optimise Cd accumulation in tobacco.

2 <u>Aims and scopes</u>

- To determine **how AM affects Cd accumulation** by tobacco. Specifically, the role of the following factors:

    - mycorrhizal effects on growth and Cd tolerance
    - mycorrhizal effects on Cd concentrations in biomass
    - mycorrhizal effects on Cd translocation from roots to shoots
    - Cd concentration in soil.

- To assess, **which physiological responses to AM symbiosis contribute to the mycorrhizal effects on Cd uptake** by tobacco, especially the role of:

    - mycorrhizal effects on biomass production (the biomass dilution effect)
    - mycorrhizal contribution to Cd immobilisation in roots
    - differences in Cd availability between mycorrhizal and non-mycorrhizal rhizosphere.

- To determine **how mycorrhiza affects Cd accumulation by a transgenic tobacco with improved Cd tolerance and higher Cd uptake** in comparison with wild type plants.

- To evaluate **whether the mycorrhizal effects on Cd accumulation by tobacco could be exploited**

    - in the phytoremediation of Cd contaminated soils
    - in decreasing Cd concentrations in the leaves of commercially produced tobacco.

3 <u>Literature review</u>

This chapter presents and overview on the current state of knowledge on the interaction of Cd with (AM) plants. Generally valid results obtained on other HMs or other types of mycorrhizal symbiosis are mentioned as well, but efforts have been made to point out the specific features of the Cd - AM interaction. Two more applied aspects of the interaction, which are related to the focus of this thesis, are reviewed in the last subchapter.

3.1 <u>Cd in soils</u>

3.1.1 Speciation and bioavailability

Bioavailability can be defined as the fraction of the total chemical compound that can interact with a biological target (Vangronsveld and Cunningham 1998), and depends on the chemical's speciation in soils. Free and complexed HM ions in the soil solution are in dynamic equilibrium with ions adsorbed on inorganic and organic soil constituents as well as with precipitates such as carbonates, phosphates and sulphides. The uptake by organisms and toxicity of HMs in soil mostly correlate best with the HM concentration in soil solution, especially with the activity of free, non-complexed ions (Parker and Pedler 1997). In addition, the non-specifically adsorbed, exchangeable ions contribute to the bioavailable fraction (Schachtschabel et al. 1992).

Cd is a transition, class B metal, which is almost always divalent in its stable compounds. It is, similarly to Zn, relatively mobile in soils in comparison with most other HMs such as Pb, Cu or Cr. In general, the higher the clay and/or organic matter content and pH of soils, the more firmly bound are HMs (Greger 1999). Clay minerals, Fe, Al and Mn oxides as well as organic matter reduce Cd bioavailability mainly by increasing the cation exchange capacity (CEC) of soils (Adriano 2001). Formation of cadmium sulphide restricts Cd bioavailability at low redox potential (Bingham et al. 1976).

Cd, similarly to Zn, tends to be sorbed by the soil components rather by non-specific ion exchange processes than by specific interactions (Ross 1994) and is therefore relatively easily replaceable from sorption sites by competing divalent cations or by H^+. Soil pH is therefore the most important single soil property affecting Cd bioavailability (Adriano 2001), which increases with decreasing pH not only due to the decreasing effective CEC but also due to dissolution of precipitates. Chelation of Cd by soluble organic matter plays a minor role in

Cd immobilisation in soil, as organic complexes of Cd are considerably less stable than those of Cu and Pb (Stevenson 1976). In contrast, formation of soluble Cd-organic matter complexes may contribute to solubilisation of particulate-bound Cd into the soil solution and increase Cd mobility in soil (Mench and Martin 1991). The formation of complexes with Cl^- has a similar effect, so that salinity increases Cd uptake by plants (e.g. Norvell et al. 2000, Usman et al. 2005).

In addition to soil characteristics, the proportion of bioavailable Cd depends also on the total Cd concentration in the soil. The Cd fraction in soil solution increases with increasing total Cd concentration (Prokop et al. 2003). In contaminated soils, larger proportion of Cd is associated with more labile fractions such as the exchangeable fraction than in non-contaminated soils (Chlopecka et al. 1996).

3.1.2 Biotic effects on Cd speciation and bioavailability

Metabolically active roots influence HM speciation and bioavailability by creating the rhizosphere, a zone of few millimetres of soil around roots of different physical, chemical and biological conditions than the bulk soil (Wenzel et al. 1999). E.g., Cd concentration in radish better correlated with Cd concentration in the rhizospheric solution than in the non-rhizospheric solution or total Cd concentration in soil (Lorenz et al. 1997).

The rhizosphere pH often differs from that of the bulk soil and is better correlated with HM availability to plants than the bulk-soil pH, as was shown for Cu (Youssef and Chino 1989a) or Zn (Youssef and Chino 1989a, Loosemore et al. 2004). Various metabolic processes in roots may modify soil pH but the most significant one is the release of charges carried by H^+ or OH^- to compensate for an unbalanced cation-anion uptake, mainly in connection with N availability as NH^{4+}/NO_3^- (Hinsinger et al. 2003). However, in non-artificial conditions when N is available as both cation and anion, pH changes in the rhizosphere also substantially depend on the pH of the bulk soil. Acidification is more likely or pronounced in alkaline soils and alkalinisation in acidic soils (Youssef and Chino 1989b).

Rhizodeposition, the release of organic compounds from roots, is another process that creates specific conditions in the rhizosphere in comparison with the bulk soil. Low-molecular-weight organic acids of root-origin increase Cd solubility and bioavailability in soil by formation of soluble complexes (Mench and Martin 1991, Krishnamurti et al. 1996, Cieslinski et al. 1998, Nigam et al. 2001, Quartacci et al. 2005, do Nascimento et al. 2006). Carboxylic acids seem more effective than amino acids (Nigam et al. 2001). The effect of

organic acids on Cd bioavailability is therefore opposite than reported for Al, as the release of organic acids is regarded as a tolerance mechanism against Al-toxicity (Pellet et al. 1995, Ma et al. 2001). Furthermore, soil Cd can be also mobilised by phytosiderophores, non-proteinogenic amino acids secreted by graminaceous species in response to Fe deficiency (Awad and Römheld 2000, Römheld and Awad 2000). However, this effect of phytosiderophores has not been consistently reported (e.g. Shenker et al. 2001). Hill et al. (2002) even suggested that phytosiderophores released in response to Cd-induced Fe deficiency might reduce Cd uptake by maize.

Plant roots influence the soil properties also via enhanced abundance and activity of soil microorganisms in the rhizosphere (Wenzel et al. 1999). Bacterial and fungal cells possess high sorption capacity for Cd (Kurek et al. 1982), though Cd is bound less than other HMs (Mullen et al. 1992, Ledin et al. 1999). On the other hand, there are also indications that certain bacteria could increase Cd availability to plants (Salt et al. 1995). The availability of Cd in soil, however, is less affected by bacterial activities than the availabilities of HMs, which can be transformed into more or less soluble oxidation states such as Cr or Mn.

The rhizosphere of plants associated with mycorrhizal fungi is sometimes referred as to the mycorrhizosphere. In addition to occupying a greater soil volume than the rhizosphere of non-mycorrhizal plants (including also soil influenced by the extraradical mycelium of the mycorrhizal fungi), it may also differ in physical, chemical and microbial properties from the rhizosphere of non-mycorrhizal plants (Linderman 1988). HM speciation, mobility and availability can be modified especially by the presence and action of the fungal mycelium. The extramatrical mycelium and mantle hyphae of ectomycorrhizal (EcM) fungi were shown to accumulate several HMs including Cd (Denny and Wilkins 1987, Wilkins 1991, Turnau et al. 1994). The ERM of AM fungi may also immobilise high amounts of HMs. Joner et al. (2000) reported high Cd binding capacity of excised ERM. Chen et al. (2001) obtained unusually high Zn concentrations in intact ERM. Gonzalez-Chavez et al. (2002) related Cu sorption by the ERM of different AM fungal isolates to their CEC. In addition, Cu can be immobilised in soils by glomalin, an insoluble protein released into the soil by the extraradical hyphae of AM fungi (Gonzalez-Chavez et al. 2004). On the other hand, EcM and ericoid mycorrhizal (ErM) fungi can solubilise Cd, Cu and Zn phosphates (Martino et al. 2003, Fomina et al. 2005) and thus increase the availability of these HMs in soil. This effect is attributed to the release of organic acids such as oxalate, citrate and malate by EcM fungi (Griffiths et al. 1994, Sun et al. 1999). Release of organic acids, however, could not be

confirmed for AM fungi (Allen et al. 1996) and it seems rather improbable that these fungi solubilise phosphates or other precipitates in soil (Smith and Read 1997).

Li and Christie (2001) reported lower Zn concentrations in the rhizospheric soil solution of AM plants in comparison with non-mycorrhizal plants. Their result was confirmed and extended to Cd by Shen et al. (2006). In both studies, the lower HM levels in the soil solution were associated with higher pH in the mycorrhizosphere, an effect of mycorrhiza, which was observed also by other authors (Bago and Azcón-Aguillar 1997, Marschner and Baumann 2003, Chen et al. 2004). The results of Marschner and Baumann (2003) suggested that the mycorrhizal effects on rhizosphere pH are plant-mediated. Differences in HM availability between rhizosphere and mycorrhizosphere can thus result also from the complex effects of mycorrhiza on the physiology of the host plant. Moreover, presumably mycorrhizal effects can reflect interactions of AM fungi with soil bacteria. Synergic effect of AM symbiosis and certain bacterial isolates improved the growth of plants and decreased Cd availability in a contaminated soil (Vivas et al. 2003a).

3.2 Cd in mycorrhizas

The term mycorrhiza refers to the plant and its mycorrhizal symbiont(s). Thus, it reflects the reality that most plants are mycorrhizal in natural conditions. However, plants and mycorrhizal symbionts react to environmental stresses, such as elevated HM levels in soils, both independently and in interaction, which is respected in the structure of this chapter.

3.2.1 Uptake and translocation

3.2.1.1 Uptake and translocation by plants

Baker (1987) proposed three strategies of HM uptake in plants. Excluders maintain low HM concentrations in tissues across a wide range of HM availability in soil up to a critical level, above which the uptake increases exponentially probably due to the toxic action of the HM. Accumulators concentrate the HM in their tissues to high concentrations already at low HM availability, but do not increase their uptake substantially at high HM availability. The HM uptake of indicator species increases linearly with the HM availability in soil. A small group of plants, the hyperaccumulator species, which constitute less than 0.2 % of angiosperms, accumulate HMs to 10-1000-fold higher concentrations than usual (Alkorta et

al. 2004). Most of the known hyperaccumulators concentrate nickel in their biomass (300 species), while hyperaccumulation of Cd is rare (Prasad 2003).

Similarly to other HMs, Cd is first taken into the root apoplast by a non-metabolic, passive process. There, it is bound to negatively charged sites of the cell wall components by cation exchange processes. The density of the negatively charged sites and thus the CEC of cell walls is higher in dicotyledonous than in monocotyledonous species (Ross and Kaye 1994). The cell-wall bound fraction of Cd in roots was estimated to 20-25% in soybean (Cataldo et al. 1983), to 4-15% in maize and *Agrostis gigantea* (Rauser 1987) and to 30% in white lupin (Costa and Morel 1993). However, all these studies worked with about 10-days old seedlings and limited exposure times.

Cd uptake into the root symplast is a metabolic process mediated by transporters with broader substrate specificity (Hall and Williams 2003). IRT1, an iron transporter from the ZIP family, may facilitate the uptake of Cd (Cohen et al. 1998). Another iron transporter responsive for Cd uptake into root cells has been identified in the Nramp family (Thomine et al. 2000). Association of Cd transport with Ca transport is suggested by the identification of the LCT1 transporter of wheat, which mediated the uptake of both Ca and Cd when expressed in yeast cells (Clemens et al. 1998). Cadmium can also enter cells via Ca channels was been suggested for stomatal guard cells (Perfus-Barbeoch et al. 2002).

Cd is readily translocated from roots to shoots in comparison with Cu or Pb (Greger 1999). In general, roots retain at least twice the Cd concentration found in vegetative tops (Koeppe 1977). However, *Nicotiana tabacum* often accumulates higher Cd concentrations in the leaves than in the roots (Lugon-Moulin et al. 2004). Little is still known about the processes controlling the acropetal transport of HMs. Cd accumulation in leaves is driven mainly by mass flow due to transpiration and appears to be independent from root uptake (Salt et al. 1995). Controlled transport from the root symplast as well as apoplastic transport may both contribute to xylem loading. AtHMA4, a member of the P_{1B} ATPase subfamily from *Arabidopsis thaliana*, was suggested to play a role in the xylem loading of Zn and Cd (Mills et al. 2003, Verret et al. 2004). An early study indicated that Cd is transported in the xylem sap complexed by organic ligands (White et al. 1981). Salt et al. (1995) reported Cd interaction with O and N in the xylem of *Brassica juncea*. Citric or other small organic acids were suggested to complex, at least partly, Cd in xylem (Senden et al. 1995, de la Rosa et al. 2004). Other authors proposed Cd translocation as free Cd^{2+} ion (Hardiman and Jacoby 1984, Leita et al. 1996). Gong et al. (2003) found evidence for root-to-shoot transport of phytochelatins and phytochelatin-dependent transport of Cd.

3.2.1.2 *AM effects on plant uptake and translocation*

AM symbiosis enhances the acquisition of the essential HMs Zn and Cu by plants (Clark and Zeto 2000) and it can even alleviate deficiencies of these micronutrients e.g. in linseed (Thompson 1994). Studies using compartmented pots also confirmed that AM fungal ERM takes up and transports the non-essential element Cd into plant roots (Guo et al. 1996, Joner and Leyval 1997, Hutchinson et al. 2004, Lee and George 2005). The contribution of the fungal symbiont to the Cd uptake by plants was estimated to up to 37% (Guo et al. 1996), 10% (Hutchinson et al. 2004) or 20-40% (Lee and George 2005). The latter work, however, also showed that this contribution might be significantly lower than in the case of essential HMs such as Cu and Zn.

The ability of AM fungi to enhance the uptake of Cd (and other elements) in plants is related to their ability to grow into root-free-soil as demonstrated for Zn by Bürkert and Robson (1994). However, little is known about the cellular mechanisms of element uptake and translocation in AM fungi and at the plant-fungus interface. Elements are transported in the ERM by cytoplasmic streaming (Cox et al. 1980). They can be stored in vacuoles in the intraradical mycelium or released by an efflux transporter or channel at the arbuscules, which are assumed to be the site of nutrient transport from the fungal to the plant partner (Ferrol et al. 2002). Selective enrichment of HMs in the intraradical mycelium of AM fungi was detected by Turnau et al. (1993) by electron energy loss spectroscopy and Turnau (1998) by rhodizoniate staining and scanning electron microscopy with EDS. Kaldorf et al. (1999) observed enrichment of some HMs in those parts of mycorrhizal roots, which contain the fungal cells, by three different micro-beam techniques.

Mycorrhizal plants may have higher root-to-shoot ratios of Cd than non-mycorrhizal plants in uncontaminated soils (Loth and Höfner 1994) as well as in contaminated soils, which is supported by more evidence (Loth and Höfner 1994, Ricken and Höfner 1996, Tonin et al. 2001, Hutchinson et al. 2004). This effect of mycorrhiza was interpreted as Cd immobilisation in the intraradical fungal structures by the corresponding authors. Joner and Leyval (1997) demonstrated, using compartmented pots, that Cd translocated into maize roots by AM fungal ERM is almost exclusively immobilised in roots. However, no enhanced Cd immobilisation in the roots of mycorrhizal plants was observed in their study when both ERM and roots had equal access to Cd. In contrast, Tonin et al. (2001) reported more than 6-fold Cd root concentrations and similar Cd shoot concentrations in mycorrhizal clover in comparison with non-mycorrhizal clover in a contaminated soil. This study showed also that AM fungal isolates differ in their capacities to accumulate Cd in the intraradical structures and that the

compatibility of an isolate with the cultivation conditions is an important factor (Tonin et al. 2001). Moreover, the effect may also depend on plant species: Guo et al. (1996) showed that Cd transported by ERM from root-free compartments may be efficiently sequestered in the roots of one plant species (bean), but translocated into shoots in another plant species (maize).

The effects of AM symbiosis on the concentrations of Cd and other HMs in plant shoots are variable (as reviewed by Leyval et al. 1997). Higher Cd shoot concentrations of mycorrhizal plants were reported e.g. by Rivera-Becerril et al. (2002), Yu et al. (2005) and Andrade et al. (2005). However, mycorrhizal plants can also have lower Cd concentrations in shoots (e.g. Gildon and Tinker 1983, Weissenhorn et al. 1995a, Vivas et al. 2003a, Vivas et al. 2003b, Vogel-Mikus et al. 2006). The effect of mycorrhiza on Cd uptake by plants seems to depend on the Cd concentration in soil: Heggo et al. (1990) reported higher Cd, Zn and Mn concentrations in mycorrhizal soybean in soils with low levels of these HMs, but an opposite effect at high levels. Concentration dependent effects of mycorrhiza were also reported by Chen et al. (2003) for Zn uptake and Toler et al. (2005) for Cu uptake. Similarly to the situation with other mycorrhizal effects, the identity of the plant and the fungus play a role. E.g., Andrade et al. (2005) reported differential effects of three AM fungal species on the Cd concentration in the shoots of jackbean. This may be related to the ability of the fungi to form ERM (see Bürkert and Robson 1994). Malcová et al. (2003a) observed different reactions to inoculation in terms of Pb uptake in two plant species. Differences can be expected also between genotypes of one plant species as shown by Rivera-Becerril et al. (2002). Furthermore, the results of pot experiments may be influenced by the experimental set-up and cultivation conditions, i.e. by factors such as light intensity (Weissenhorn et al. 1995a), root density and the length of the cultivation period (Joner and Leyval 2001).

The HM uptake of mycorrhizal and non-mycorrhizal plants is often difficult to compare due to the better growth of mycorrhizal plants. The bigger the root absorption area, the more effective is the element uptake, however, the tissue concentration will decrease due to biological dilution (Marschner 1995). Dilution effects were suggested to explain lower HM concentrations in the shoots of mycorrhizal plants growing in HM contaminated soils e.g. by Díaz and Honrubia (1995), Weissenhorn et al. (1995a), Hutchinson et al. (2004) and Shen et al. (2006).

3.2.2 Toxicity

3.2.2.1 Toxicity to plants

At the molecular level, Cd toxicity primarily inhibits enzyme activities, either by the interaction with SH groups or by displacement of divalent cations in positions important for the enzyme activities (Seregin and Ivanov 2001). Cd generates oxidative stress in plant cells by disturbing metabolic pathways, inactivating antioxidant enzymes and depleting the pool of the antioxidant glutathione. The presence of free radicals and reactive oxygen species leads to peroxidation of membrane lipids, oxidative breakdown of chlorophyll and carotenoids as well as to oxidative damage of nucleic acids (Dietz et al. 1999). The functionality of biological membranes is also decreased by Cd-induced changes in the biosynthetic pathways leading to qualitative and quantitative changes in membrane lipids (Devi and Prasad 1999). The effects of Cd at the molecular level lead to a wide range of secondary effects, structural and ultrastructural changes as well as to disorders in many physiological processes.

As a rule, inhibition of root elongation is the first visible effect of Cd toxicity. This can result from both inhibition of root cell division and decreased cell expansion in the elongation zone (Barceló and Poschenrieder 1999). For example, root growth was inhibited at an internal Cd concentration of only 86 ng.g^{-1} in bean after 48 h of exposition due to inhibition of cortical cell division (Vázquez et al. 1992). Root growth of monocotyledonous species seems to be more resistant to Cd than that of dicotyledonous species (Inouhe et al. 1994). Root branching is less inhibited than root elongation, which makes the root system of Cd-stressed plants more compact as a general response (Ivanov et al. 2003). However, lateral root formation may be also increased or decreased by Cd (Koeppe 1977, Punz and Sieghardt 1993).

Cd toxicity produces disorders in the acquisition of mineral nutrients and ionic balance. Direct competition at membrane transporters may affect the uptake, translocation or compartmentation of Zn and Ca, while metal-induced disorders in the cell metabolism, changes in membrane enzyme activities and membrane structure may affect the balance of all nutrients (Seregin and Ivanov 2001). E.g., Cd restricted the uptake of nitrate and disturbed nitrate metabolism in barley (Boussama et al. 1999), and decreased the biomass concentrations of P, K, Ca or Mn in various plants (e.g. Gussarsson et al. 1996, Hernandez et al. 1996, Ouariti et al. 1997, Ghnaya et al. 2005).

Cd-stressed plants often demonstrate wilting, decreased transpiration rates and water contents as well as increased stomatal resistance up to stomatal closure. Early effects of Cd toxicity in roots, decreased diameter of xylem vessels as well as CO_2 accumulation in

photosynthetic tissues due to inhibition of photosynthesis may be responsible for these effects (Poschenrieder and Barceló 1999). At the cellular level, Cd stress lowers the contents of the compounds maintaining cell turgor and cell wall plasticity thus decreasing the water potential of cells, which is an important factor of cell expansion (Seregin and Ivanov 2001). Inhibition of photosynthesis is a well-known effect of Cd, based on a large variety of primary effects, e.g. the interference of Cd with the synthesis of pigments, especially of chlorophylls, changes in the structure of the thylacoid membranes, inhibition of the enzymes of the Calvin cycle or lack of CO_2 due to stomatal closure (Prasad and Strzalka 1999). Toxic effects of Cd on the catabolism are often less obvious. Increase in respiration rates may be a consequence of increased energy demand for processes associated with Cd exclusion or repair of Cd-induced damage, while decreased respiration rates indicate inhibition of sensitive metabolic steps of the catabolic pathways (Lösch and Köhl 1999).

3.2.2.2 Toxicity to AM fungi

Up to date, evidence has been collected on the occurrence of AM fungi in HM contaminated soils and the inhibitory effects of high HM concentrations on the survival and development of AM fungi in their different life phases. It can be assumed that the basic mechanisms of Cd toxicity at the cellular level are similar as in plants. However, virtually nothing has been reported on this topic for AM fungi.

HMs added into soil with sewage sludge decreased spore numbers and AM fungal species diversity in a long-term field experiment (del Val et al. 1999a). In other studies, however, spore diversity and root colonisation by AM fungi in contaminated agricultural soils correlated rather with the P status of the host plants than with HM availability (Weissenhorn et al. 1995b, c). As summarised by Meharg and Cairney (1999), AM fungal populations are common and sometimes diverse even in highly contaminated mine spoils. There is, however, evidence that AM fungal isolates originating from non-contaminated habitats are much more sensitive to HMs than isolates originating from contaminated sites. This has been shown for Cd and Zn on spore germination (Weissenhorn et al. 1993, 1994), for Pb on the re-growth of hyphae from colonised root segments (Malcová et al. 2003a) and for Mn on root colonisation and ERM growth (Malcová et al. 2003b).

Vidal et al. (1996) suggested that mycorrhiza formation might be more sensitive to HMs than plant growth. While this assumption seems to be true for Mn (Arines et al. 1992, Malcová et al. 2003b), the evidence for Cd is inconclusive. Weissenhorn and Leyval (1995) reported that Cd eliminated root colonisation by both a supposedly tolerant AM fungal isolate

(originating from a contaminated soil) and a reference isolate at Cd concentrations that still enabled maize growth. In the study of Rivera-Becerril et al. (2002), however, a *Glomus intraradices* isolate originating from a non-contaminated habitat proved more tolerant to Cd than even a Cd-tolerant genotype of pea.

The germination of AM fungal spores has been used as a convenient parameter to assess the tolerance/sensitivity of different AM fungal isolates to Cd or other HMs (Weissenhorn et al. 1993, 1994, Bartolome-Esteban and Schenck 1994). The former authors observed that Cd inhibited spore germination more than the extension of pre-symbiotic hyphae from spores once a spore had germinated. Pawlowska and Charvat (2004) confirmed this result, but concluded also that spore germination is far more Cd tolerant than other aspects of the AM fungal life cycle, such as symbiotic mycelial expansion or sporulation.

In the symbiotic life phase of AM fungi, ERM growth was repeatedly shown to be more sensitive to HMs than the development of intraradical colonisation (Vidal et al. 1996, del Val et al. 1999b, Andrade et al. 2005). Pawlowska and Charvat (2004) observed that HMs might differentially affect sporulation and ERM growth, but none of the processes was distinctly more sensitive in their study. Karagiannidis and Nikolaou (2000) observed both reduction of AM fungal spore density and of root colonisation in a soil supplemented with Cd. The intraradical colonisation by AM fungi may be not only decreased or eliminated, but also morphologically altered as effect of HM stress (Gildon and Tinker 1983). Davies et al. (2002) observed reduced arbuscule formation in the presence of hexivalent Cr, but no difference in the frequency of intraradical hyphae or vesicles. Similarly, Repetto et al. (2003) reported higher Cd-sensitivity of arbuscule formation than of the spread of intraradical hyphae. In contrast, arbuscule formation was largely unaffected by Cd and Cu in mycorrhizal root segments in the study of Vidal et al. (1996), while the frequency of mycorrhizal root segments decreased. Generally, the effect of HMs on AM fungi in the symbiotic phase may be mediated by the HM tolerance of the host plant (Repetto et al. 2003).

3.2.3 Tolerance mechanisms

3.2.3.1 Tolerance mechanisms of plants

Traditionally, mechanisms enabling plants to survive high HM levels in soils (conferring HM resistance or tolerance) are classified into two groups: Avoidance mechanisms protect the plant externally from the influence of the stress, while "true"

tolerance mechanisms enable the plant to survive internal stress (Baker 1987). However, also most "true" tolerance mechanisms are based on avoiding the build up of toxic HM concentrations at sensitive sites at the cellular level and thus in preventing the onset of stress (Hall 2002). The mechanisms seem to be largely metal specific; there is no evidence for a single mechanism that account for tolerance to a wide range of HMs (Hall 2002).

Generally, various mechanisms preventing that HMs pass the plasmalemma of root cells have been suggested: formation of mycorrhiza (see 2.2.3.3), proliferation of roots in uncontaminated horizons, increased exudation of metal-chelating substances to decrease HM availability and toxicity in soil, changes in the metal-binding capacity of the cell wall or alteration of membrane permeability (Turner 1994). There are no indications that the release of Cd chelating agents by plants could be a mechanism decreasing Cd availability and toxicity to plants, differentially to Al (Ma et al. 2001) or Ni (Salt et al. 2000). Nedelkoska and Doran (2000) suggested delay in transmembrane uptake, accompanied by Cd accumulation in cell walls, as an important defensive strategy of *Thlaspi caerulecens* against Cd toxicity. However, the contribution of HM sorption by cell wall components to the HM tolerance of plants is questionable in realistic exposure conditions (Ernst et al. 1992).

Sequestration of HMs in roots or their allocation to old leaves may protect young leaves and reproductive organs against HM stress (Ernst et al. 1992), representing an exclusion mechanism at the organ level. Enhanced Cd sequestration in roots could be achieved by altered xylem loading: the membrane transporter AtHMA4, which is probably involved in xylem loading (Verret et al. 2004), is down-regulated by Cd in roots (Mills et al. 2003). Cd can be also sequestered or excreted by trichomes, as reported for Indian mustard by Salt et al. (1995) and described for tobacco by Choi et al. (2001) and Choi and Harada (2005).

Chelation of HMs in the cytosol is the most important mechanism involved in preventing the offset of HM stress at the cellular level. For Cd, most information is available on the role of phytochelatins. Phytochelatins are metal-binding peptides with the general structure (γ-Glu-Cys)$_n$-Gly with n = 2 to 4. They are synthesised from glutathione by the enzyme phytochelatin synthase (γ-glutamyl cystene dipeptidyl transpeptidase), and are rapidly induced in plants by HM treatments (Rauser 1995). A clear role in Cd detoxification has been supported by a range of biochemical and genetic evidence (as reviewed e.g. by Hall 2002). Low-molecular-weight cytosolic Cd-phytochelatin complexes are transported into the vacuole where high-molecular-weight complexes are formed under incorporation of S^{2-} and further Cd ions (Clemens 2001). The transport of Cd-phytochelatin complexes across the tonoplast may be mediated by a transporter of the ATP binding cassette (ABC) superfamily (Hall and

Williams 2003) while Cd^{2+}/H^+ antiport is suggested to account for the transport of free Cd ions (Salt and Wagner 1993).

Phytochelatins are sometimes considered as Class 3 metallothioneins. Class 1 and 2 metallothioneins are gene-encoded cysteine-rich polypeptides, originally discovered in animals and yeasts. More than 50 metallothionein-like sequences were found also in various plants (Clemens 2001). However, there is still a lack of information concerning the role of metallothioneins in HM detoxification in plants (Hall 2002). *Arabidopsis* metallothionein genes *MT1* and *MT2* conferred high level of Cu tolerance and intermediate Cd tolerance to yeast in a complementation assay, but an important role in Cd-chelation was questioned by the only slight induction of both genes by Cd in plants (Zhou and Goldsbrough 1994). Haag-Kerwer et al. (1999) suggested that Cd is primarily detoxified by phytochelatins in *Brassica juncea*, while metallothioneins are induced by the secondary effects of Cd toxicity and participate in maintaining HM homeostasis. Clemens (2001) concluded that a detoxification function of gene-encoded metallothioneins is indicated mainly for Cu. Two more recent studies, however, showed that the expression of some metallothionein genes is enhanced in response to Cd (Liu et al. 2002, Ma et al. 2003).

Low-molecular-weight organic acids, such as citric acid, are among other potential Cd-chelators in plants (Rauser 1999). Wagner (1993) hypothesised that citric acid might be a major Cd-ligand at low Cd concentrations. Organic acids may play a role in Cd chelation in xylem while phytochelatins participate in Cd sequestration in roots (Salt et al. 1995). However, evidence for a function of organic acids in plant metal tolerance has been difficult to obtain except for Al and Ni (Hall 2002).

In addition to HM chelation in the cytosol, other, rather general stress tolerance mechanisms may help plants to cope with HM stress. There are indications to a role of heat shock proteins in enhancing the Cd tolerance of plants. They may act as chaperons, limiting and rescuing the Cd-induced damage to proteins or protecting membranes (Prasad 1995). Similarly, HM stress may activate general protective mechanisms against oxidative stress, e.g. increase the activity of antioxidant defence enzymes (peroxidases, superoxide dismutase) and of enzymes involved in the biosynthesis of low-molecular-weight antioxidants (glutathione, ascorbate) (Dietz et al. 1999).

3.2.3.2 *Tolerance mechanisms of AM fungi*

Similarly to plants, AM fungi probably cope with elevated HM levels in substrate by restricting the HM uptake and by detoxification of the HM in the cytosol. Little is known about the concrete mechanisms in AM fungi, while more information is available on the situation in EcM fungi (as recently reviewed by Meharg 2003 or Bellion et al. 2006).

Cd binding to cell walls has been suggested, together with Cd accumulation in the vacuolar compartment, as an essential detoxification mechanism in the EcM fungus *Paxillus involutus* (Blaudez et al. 2000). Joner et al. (2000) reported much higher Cd binding by the ERM of a Cd tolerant AM fungal isolate than by other, more sensitive isolates. However, the HM-binding capacity of ERM and the HM tolerance of AM fungal isolates need not be always related as demonstrated for Cu by Gonzalez-Chavez et al. (2002). Cu was localised mainly in the mucilaginous outer hyphal wall zone and in the cell walls of extraradical hyphae (Gonzalez-Chavez et al. 2002). This corresponded with the earlier finding of Denny and Wilkins (1987) that Zn binds mainly to electronegative sites in the hyphal cell walls and the extra-hyphal polysaccharide slime of an EcM fungus. Another EcM fungus accumulated Cd, Cu and Fe mainly in electron opaque granules and in the outer pigmented layer of the cell wall, both characterized by the presence of polysaccharides and cysteine-rich proteins (Turnau et al. 1994). The results of Frey et al. (2000), however, indicate that sequestration in cell walls is not equally important as detoxification mechanism for all HMs: While Cd was almost exclusively sequestered in the cell wall, Zn also accumulated in the cytoplasm of an EcM fungus.

Two principal mechanisms of intracellular detoxification have been suggested for EcM fungi: binding by phosphate and binding by sulfhydryl compounds (Galli et al. 1994). Accumulation of HMs in polyphosphate granules in fungal vacuoles was described e.g. by Turnau et al. (1993) in mycorrhizal *Pteridium aquilinum*. However, polyphosphate granules in mycelium may be also artefacts of specimen preparation (Orlovich and Ashford 1993). Galli et al. (1994) concluded that a contribution of phosphate to HM binding within the cells of mycorrhizal fungi was not clear and no further information has been published on this topic since then.

Binding by sulfhydryl groups indicates HM detoxification by metallothionein-like proteins. This was first reported by Morselt et al. (1986) for the EcM fungus *Pisolithus tinctorius* by specific histochemical staining. The staining was most induced by Cd, but also by Zn and Cu. It seems that EcM fungi do not produce phytochelatins (Galli et al. 1993,

Courbot et al. 2004) similarly as most other fungi (Bellion et al. 2006). Metallothioneins and their induction in response to Cd were described in EcM fungi (Courbot et al. 2004, Jacob et al. 2004). In contrast, the metallothionein gene *GmarMT1* identified in the AM fungus *Gigaspora margarita* conferred Cd and Cu tolerance to yeast in complementation assays, but was induced only by Cu in the ERM of the AM fungus (Lanfranco et al. 2002). This indicates rather its role in Cu homeostasis and detoxification than in protection against Cd toxicity (Lanfranco et al. 2002). Furthermore, glutathione seems to play a major role as HM chelator in fungi in general as well as in EcM fungi (Bellion et al. 2006).

González-Guerrero et al. (2005) characterised a gene (*GintZnT1*) encoding a putative Zn transporter in the AM fungus *Glomus intraradices*. They proposed a function in Zn efflux from the cytosol or transport into the vacuole, and a role in protection against Zn stress. Enhanced Zn efflux may act as a tolerance mechanism in the EcM fungus *Suillus bovinus* (Bellion et al. 2006). Cd transport into the vacuole and association with S was suggested as a Cd detoxification mechanism in the EcM fungus *Paxillus involutus* (Ott et al. 2002).

3.2.3.3 Contribution of AM to plant tolerance

Numerous studies reported better growth of mycorrhizal plants than of non-mycorrhizal plants in HM contaminated soils (Díaz and Honrubia 1995, Weissenhorn et al. 1995a, Hildebrandt et al. 1999, Vivas et al. 2003a, Vivas et al. 2006). Unfortunately, these studies lacked control treatments in the same soils without HM stress, so that alleviation of HM stress could be only hypothesised. As adverted by Meharg and Cairney (1999), positive mycorrhizal growth response in HM contaminated soils can be also attributed to "normal" nutritional effects of mycorrhiza independent of HM stress. Control treatments without HM stress were included in other studies, which used soils artificially amended with HMs. In some of them, the positive mycorrhizal growth response can be indeed interpreted as alleviation of HM stress (Gildon and Tinker 1983, Dueck et al. 1986, Tonin et al. 2001, Rivera-Becerril et al. 2002, Chen et al. 2004). In others, however, mycorrhizal growth response was similar in the control treatments as in the treatments with HM amendment (e.g. Davies et al. 2002, Liao et al. 2003). Exceptionally, mycorrhiza even aggravated HM stress to plants (Killham and Firestone 1983). Thus, it has been demonstrated that AM symbiosis may alleviate HM stress to plants, but it is unclear under which conditions and which mechanisms are involved.

As mentioned above (see 3.2.3.1), mycorrhizal symbiosis was suggested to alleviate HM stress to plants by restricting their HM uptake (e.g. Turner 1994, Hall 2002). This

suggestion was based on studies reporting better growth and lower HM concentrations in the biomass of mycorrhizal plants in comparison with non-mycorrhizal plants (e.g. Gildon and Tinker 1983, Weissenhorn et al. 1995a, Vivas et al. 2003a). Just recently, Vogel-Mikus et al. (2006) have proposed that mycorrhiza may reduce HM uptake even in the presumably non-mycotrophic hyperaccumulator *Thlaspi praecox* and thus function as a tolerance strategy on HM contaminated soils. Potential mechanisms, how mycorrhiza may decrease the HM uptake of plants, have been reviewed with emphasis on Cd in the previous chapters. They can be summarised as follows: i) lower Cd availability in the mycorrhizosphere due to Cd immobilisation by ERM (see 3.1.2), ii) lower Cd availability in the mycorhizosphere due to the plant-mediated effects of mycorrhiza on the physical or biological soil characteristics (see 3.1.2); iii) restricted Cd uptake into root cells due to Cd immobilisation in the intraradical fungal structures (see 3.2.1.2); iv) Cd dilution in the larger biomass of mycorrhizal plants resulting in lower Cd biomass concentrations (see 3.2.1.2). Hence, the lower HM uptake of mycorrhizal plants can be based both on direct and plant-mediated effects of mycorrhiza. In addition to "filtering" HM ions in soil or plant roots (Joner et al. 2000, Joner and Leyval 1997), AM symbiosis may decrease the HM uptake by plants, total or per unit of biomass, by altering plant growth, nutrition or root exudation.

However, the better growth of mycorrhizal plants in contaminated soils need not be always associated with lower HM concentrations in their biomass (Dueck et al. 1986, Ricken and Höfner 1996, Rivera-Becerril et al. 2002, Wang et al. 2005). This lead Meharg and Cairney (1999) to the conclusion that no systematic evidence is available that AM fungi protect their hosts from exposure to HMs by reduced assimilation. Interestingly, restricted HM uptake as HM tolerance mechanisms has been accepted for EcM plants in certain conditions (Jentschke and Godbold 2000).

It is therefore assumed that AM fungi confer HM resistance to their host plants (also) by maintaining P acquisition and other nutritional effects (Meharg and Cairney 1999). However, there is little direct evidence for this mechanism. Shetty et al. (1995) suggested improved P nutrition as the main mechanism of enhanced Zn tolerance of mycorrhizal plants growing in soils with high Zn levels. However, the difference in P status between mycorrhizal and non-mycorrhizal plants was similar at all Zn levels tested. Chen et al. (2004) supplied mycorrhizal plants with different amounts of P via ERM, but could not prove a role of improved P nutrition in their higher Cd tolerance. Mycorrhiza alleviated Cd-induced stress to pea without improving the P status of the plants in the study of Rivera-Becerril et al. (2002). Mycorrhizal plants maintained higher levels of photosynthetic activity under Cd stress despite

similar Cd shoot concentrations as non-mycorrhizal plants. This result suggested more efficient protection of the sensitive photosynthetic apparatus against Cd toxicity in mycorrhizal plants, but it is unclear by which mechanisms. The results of Andrade et al. (2005) indicated alleviation of oxidative stress by mycorrhiza: mycorrhizal jackbean plants had lower guaiacol peroxidase activity in roots despite higher Cd concentrations.

More inside into this topic will be certainly gained by studies at the molecular level in the future. Up to date, however, only few such studies have been published. Repetto et al. (2003) demonstrated by proteomics that the expression of some proteins involved in Cd responses might be modulated by mycorrhiza in pea. None of the putatively identified proteins, which expression was influenced by the interaction of Cd and mycorrhiza, belonged to proteins already well known to be involved in Cd detoxification or Cd response mechanisms. Rivera-Becerril et al. (2005) studied the effect of AM symbiosis on the expression of several genes related to Cd stress response in pea and concluded that Cd chelation pathways may not make a major contribution to metal tolerance strategies operating in the AM symbiosis. Similarly, Ouziad et al. (2005) did not find any up-regulation of genes related to Cd chelation or vacuolar transport of Cd in mycorrhizal tomato as compared to non-mycorrhizal plants. In contrast, some of the genes were down-regulated in mycorrhizal roots, which the authors explained by lower HM concentrations in the mycorrhizal roots.

3.3 **Applied aspects**

3.3.1 Genetic engineering of plants

Genetic engineering can serve as a tool to decrease the HM concentrations in crops and thus to protect consumers against HM uptake, as has been suggested for Cd and tobacco (Lugon-Moulin et al. 2004). However, the possibility of gene transfer has been more intensively explored to enhance the HM accumulation of plants for the purposes of phytoextraction (see 3.3.2).

Misra and Gedamu (1989) first reported stable genetic transformation of plants resulting in higher Cd tolerance. However, the Cd uptake by the transgenic tobacco and oilseed rape with constitutively expressed human metallothionein-II, was not tested. In further studies, transgenic plants with introduced metallothionein genes often demonstrated higher Cd tolerance (Kärenlampi et al. 2000), but their Cd uptake varied relatively to the wild type plants. Introduction of mammalian metallothioneins into tobacco decreased its Cd

concentration in the leaves and root-to-shoot translocation (Elmayan and Tepfer 1994, Hattori et al. 1994, de Borne et al. 1998). However, introduction of metallothionein genes also increased Cd accumulation by plants (Hasegawa et al. 1997, Pavlíková et al. 2004, Zhang et al. 2006). Constitutively expressed yeast metallothionein CUP1 promoted Cu accumulation, but not Cd accumulation or tolerance in tobacco (Thomas et al. 2003). However, the *CUP1* gene increased Cd accumulation by tobacco when it was fused with a polyhistidine tail (Macek et al. 2002). Generally, preparation of plants with the desired Cd uptake characteristics could be achieved by the expression of the introduced metallothionein genes under the control of root- or shoot-specific promoters (Krämer and Chardonnens 2001).

Another possibility how to improve Cd chelation in cytosol consists in increasing the rate of biosynthesis of phytochelatins or their precursor glutathione. Zhu et al. (1999a, 1999b) improved the Cd tolerance and increased the Cd shoot concentrations of Indian mustard (*Brassica juncea*) by introducing and overexpressing the *E. coli* γ-glutamylcysteine synthetase gene (*gshI*) or glutathione synthetase gene (*gshII*). In contrast, overexpression of *Arabidopsis* PC synthase gene (*AtPCSI*) in transgenic *Arabidopsis* paradoxically lead to Cd hypersensitivity, probably due to supraoptimal phytochelatin levels when compared with glutathione levels (Lee et al. 2003a). Harada et al. (2001) achieved higher phytochelatin levels in tobacco overexpressing the rice cysteine synthase gene. The transgenic plants were more tolerant to Cd and had lower Cd concentrations. In another transgenic tobacco overexpressing cysteine synthase, enhanced Cd tolerance was accompanied by higher Cd concentrations in shoots and higher root-to-shoot translocation of Cd in comparison with wild type plants (Kawashima et al. 2004).

The increased rate of synthesis of metal chelating compounds is energy expensive and requires significant amounts of S and N. It is therefore argued that a more economic strategy could consist in engineering HM vacuolar transporters, where a small amount of protein would help to detoxify large amounts of HMs (Tong et al. 2004). Overexpression of the *A. thaliana* Ca vacuolar transporter CAX2 enhanced accumulation of Cd and Mn in tobacco (Hirschi et al. 2000). Song et al. (2003) introduced the yeast vacuolar transporter YCF1 into *A. thaliana* and obtained transgenic plants with higher Cd and Pb tolerance as well as higher accumulation of these HMs in shoots. In contrast, expression of the bacterial Pb/Cd/Zn plasmalemma efflux pump ZntA in *A. thaliana* resulted in higher Cd and Pb tolerance accompanied by lower shoot concentrations of these metals (Lee et al. 2003b).

Unfortunately, the successfully transformed plants with promising HM accumulation or tolerance features have been rarely tested in soil-based cultivation of even in field-

conditions (Pilon-Smits and Pilon 2002). Brandle et al. (1993) did not find any difference in Cd accumulation between tobacco lines bearing a metallothionein-β-glucuronidase gene fusion and wild type plants in a field test. In contrast, Bennett et al. (2003) confirmed the characteristics described in transgenic Indian mustard by Zhu et al. (1999a, b) in a greenhouse cultivation experiment with contaminated soils. Similarly Pavlíková et al. (2004) proved enhanced Cd accumulation by a transgenic tobacco clone both in sand-based cultivation and cultivation in contaminated soil. Other transgenic plants displaying interesting tolerance and accumulation features in laboratory cultivation still remain to be tested in more realistic conditions.

3.3.2 Phytoremediation of contaminated sites

The concept of phytoremediation, i.e. the possibility to use plants to remediate contaminated soils, has received increasing attention in recent years and numerous reviews cover the topic and its various aspects (e.g. Cunningham et al. 1995, Chaudhry et al. 1998, Pilon-Smits and Pilon 2002, Singh et al. 2003). Generally, plants can be utilized for two main purposes in the remediation of HM contaminated soils: i) immobilisation of HMs in the soil in order to decrease their toxicity and prevent further dispersal (phytostabilisation) or ii) removal of the HMs from the soil by plant uptake and biomass harvest (phytoextraction) (Salt et al. 1995).

Phytostabilisation techniques exploit plant-associated processes to decrease the bioavailability of HMs in soils with the aim to reduce or even eliminate further environmental harm or human-health risk (Cunningham et al. 1995). Vegetation cover protects soil from wind or water erosion, litter input increases the organic matter content of soils and thus their capacity to bind HMs. Phytostabilisation represents a reasonable solution especially for heavily HM contaminated soils (Ernst 1996) and often uses species naturally occurring at the local contaminated sites (Krämer 2005). Soil additives such as lime, manganese or iron oxides may further reduce the mobility and bioavailability of HMs in soil and limit their entry into the food chain via plant uptake (Mench et al. 1994).

Mycorrhizal symbiosis was repeatedly suggested as a supporting factor to the phytostabilisation process (Leyval et al. 2002, Jeffries et al. 2003, Gaur and Adholeya 2004). As reviewed in the previous chapters, AM fungi often improve plant growth on HM contaminated soils, contribute to the immobilisation of HMs in soils and enhance HM sequestration in plant roots. HM-resistant grasses, which are proposed as the most appropriate

candidates for the revegetation of contaminated soils (Ernst 1996), also benefit from being mycorrhizal (Dueck et al. 1986, Hetrick et al. 1994, Shetty et al. 1994, Rydlová and Vosátka 2003).

Phytoextraction utilises the ability of plants to take up HMs from soils, translocate and concentrate them in their aerial parts. An ideal candidate plant should therefore i) display reasonably high HM tolerance and accumulate large amounts of one or several HMs in the shoots, ii) exhibit a high rate of biomass production, and iii) develop an extensive root system to explore a large volume of soil (Kärenlampi et al. 2000, Krämer and Chardonnens 2001). Three groups of plants provide potential candidates: natural hyperaccumulators, high biomass crops and fast-growing trees (McGrath et al. 2001a). Natural hyperaccumulators, such as *Thlaspi* sp., are mostly herbs of slow growth, small biomass production and unfavourable agronomic properties (Cunningham et al. 1995), but they may accumulate more than 50-fold higher HM concentrations in their biomass than candidate plants among high biomass crops (McGrath et al. 2001b). Despite this difference, high biomass crops may be more effective in removing HMs from soil especially if their performance is enhanced by agronomic practices that increase their shoot biomass production and/or increase the HM bioavailability in the rhizosphere (Ebbs et al. 1997). E.g., synthetic chelators such as EDTA can increase the bioavailability of Cd in soil (Blaylock et al. 1997) though their application is more effective in the case of less mobile HMs such as Pb (Lasat 2002). The HM tolerance and accumulation of high biomass crops may be also improved by genetic engineering as reviewed in chapter 3.3.1. This is a more realistic approach than attempting to increase the biomass production of hyperaccumulator species by genetic modifications (Pilon-Smits and Pilon 2002).

Exploitation of AM symbiosis in phytoextraction has been also proposed (Ernst 2000, Khan et al. 2000, Alkorta et al. 2004, Gaur and Adholeya 2004). Generally, mycorrhizal effects on the HM uptake of plants are variable, and published results suggest that mycorrhizal plants tend to retain HMs more efficiently in roots (see 3.2.1.2) despite single reports on opposite effects of mycorrhiza (Citterio et al. 2005). Mycorrhiza may accelerate the phytoextraction process mainly by increasing the soil volume explored by roots and improving plant growth in HM contaminated soils (Khan et al. 2000). The latter has been conclusively demonstrated by Vivas et al. (2003b, 2003c) on *Trifolium* plants grown in contaminated soils. The growth-promoting effect of mycorrhiza was further enhanced by co-inoculation with bacteria originating from contaminated soils in their study. The significance of mycorrhiza as a factor to be considered in phytoextraction is limited by the fact that many hyperaccumulators as well as some of the promising high biomass crops (*Brassica* sp.) belong

to family Brassicaceae, which members usually do not form mycorrhiza (McGrath et al. 2001a). However, mycorrhiza increased Ni accumulation in the shoots of the hyperaccumulator *Berkheya coddii* (Asteraceae) by improving its growth (Turnau and Mesjasz-Przybylowicz 2003). Moreover, even presumably non-mycotrophic species may become colonised by AM fungi under certain conditions and change their HM accumulation pattern upon colonisation (Vogel-Mikus et al. 2006).

As concluded by Göhre and Paszkowski (2006), it remains crucial to determine the appropriate conditions (such as plant-fungus combination) for a given contaminated site to maximise the utility of AM symbiosis. Application of mycorrhizal inoculum into the soils may either support indigenous AM fungal communities or introduce effective AM fungal isolates.

4 <u>Results</u>

The results of this thesis are presented as four publications, which form the following subchapters 4.1-4.4.

A fifth publication has been elaborated in frame of the thesis, which has not been directly included, because the described study used *Daucus carota* as host plant instead of tobacco. The abstract of this publication is presented as chapter 4.5. The study using the agar-based monoxenic cultivation system of AM fungi on Ri T-DNA transformed roots was originally planned with tobacco roots in order to investigate mycorrhizal effects at the root level without the interference of shoots and other soil microorganisms. However, efforts to establish a reliable experimental system based on tobacco roots were not successful, because both preparation of Ri T-DNA transformed roots of the Wisconsin 38 tobacco and cultivation of excised roots failed. The results obtained with *D. carota* roots are included into the General discussion (chapter 5) similarly as results of other authors.

4.1   Publication 1: Applied Soil Ecology 29, 2005

Janoušková M, Pavlíková D, Macek T, Vosátka M **(2005)** Influence of arbuscular mycorrhiza on the growth and cadmium uptake of tobacco with inserted metallothionein gene. **Applied Soil Ecology** 29: 209-214

see special file

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4.2   Publication 2: Plant and Soil 272, 2005

Janoušková M, Pavlíková D, Macek T, Vosátka M **(2005)** Arbuscular mycorrhiza decreases cadmium phytoextraction by transgenic tobacco with inserted metallothionein. **Plant and Soil** 272: 29-40

see special file

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4.3   Publication 3: Chemosphere, in press

Janoušková M, Pavlíková D, Vosátka M **(in press)** Potential contribution of arbuscular mycorrhiza to cadmium immobilisation in soil. **Chemosphere**

see special file

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4.4   **Publication 4: Applied Soil Ecology, accepted**

Effects of arbuscular mycorrhizal inoculation on cadmium accumulation by different tobacco (*Nicotiana tabacum* L.) types

Janoušková M.[1], Vosátka M.[1], Rossi L.[2], Lugon-Moulin N.[2]

[1] Department of Mycorrhizal Symbioses, Institute of Botany, Academy of Sciences of the Czech Republic, 252 43 Průhonice, Czech Republic
[2] Philip Morris International R&D, c/o Philip Morris Products SA, 2000 Neuchâtel, Switzerland

Abstract

The effect of arbuscular mycorrhiza (AM) on cadmium (Cd) uptake by tobacco (*Nicotiana tabacum* L.) was studied in a pot experiment. Three commercial varieties, Basma BEK, K326 and TN90, representing three distinct tobacco types, were each grown in soil with nutritional conditions matching as closely as possible their requirements for field production. Cd concentrations in these soils were within the background range. Each variety was either non-mycorrhizal or inoculated with one of five AM fungal isolates. Cd concentration in leaves was decreased by inoculation with selected isolates in the K326 and TN90 variety grown in acidic soils. In contrast, it was increased by inoculation with most isolates in the Basma BEK variety grown in a basic soil with low Cd availability. Besides, plants of all three varieties had significantly higher leaf concentrations of phosphorus and nitrogen in some inoculated treatments. The percentage of root colonisation was mostly low in the inoculated treatments. In the Basma BEK and TN90 variety, the tested AM fungal isolates differed in their ability to colonise roots, but no correlation was found between the root colonisation of an isolate and its effects on the Cd concentrations in tobacco leaves. One isolate influenced most pronouncedly Cd concentrations and improved mineral nutrition in all the three combinations of variety and soil despite its low colonization levels. AM symbiosis probably affected Cd uptake of tobacco by indirect mechanisms such as stimulation of root growth or mycorrhizal plant mediated changes in chemical or biological soil properties.

Keywords

agriculture, *Glomus*, heavy metals, nitrogen, phosphorus

Introduction

The non-essential element cadmium (Cd) is relatively mobile in soils and potentially highly toxic in comparison with other "heavy metals" (Schachtschabel et al., 1992; see Duffus, 2002, for a discussion of the term heavy metal). It is classified as a known human carcinogen (Class 1) by the International Agency for Research on Cancer (IARC, 1993). In many plants, a high proportion of the Cd uptake is effectively bound in roots. In contrast, *Nicotiana tabacum* (tobacco) usually accumulates more Cd per unit biomass in leaves than in roots and may display relatively high Cd concentrations in leaves (Lugon-Moulin et al., 2006). Food is the major source of Cd exposure for the general non-smoking population in most areas, but heavy smoking may represent another important source of exposure to this metal (WHO, 1992). Indeed, during smoking, some Cd is transferred to the mainstream smoke, which is directly inhaled by the smoker (Pappas et al., 2006; Smith et al., 1997).

Several strategies may be followed to decrease the Cd concentration in plant leaves. e.g. immobilisation of soil Cd by amendments or genetic modifications of plants to restrict their Cd uptake

(reviewed in Lugon-Moulin et al., 2004). Among other options, the formation of arbuscular mycorrhiza (AM) with obligatory symbiotic fungi of the order Glomeromycota may decrease Cd concentration in the shoots of plants exposed to Cd contamination in soil (Heggo et al., 1990; Weissenhorn et al., 1995; Chen et al. 2004), but the opposite effect of mycorrhiza has been reported as well (Rivera-Becerril et al., 2002). Several factors seem to play a role, especially the plant species (Joner and Leyval, 2001) or even variety (Rivera-Becerril et al., 2002), the AM fungal isolate involved (Liao et al., 2003; Wang et al., 2005) and the Cd concentration in soil (Heggo et al., 1990; Chen et al., 2004). In addition, cultivation conditions such as pot volume, light intensity or the length of the cultivation period may constitute important factors for the heavy metal uptake of plants in pot cultivation experiments (Weissenhorn et al., 1995; Joner and Leyval, 2001).

The plant partner primarily benefits from AM symbiosis by improved phosphorus (P) nutrition, but acquisition of other macro- and micronutrients can be enhanced as well under certain conditions (Smith and Read, 1997). This is often accompanied by a positive growth response to AM symbiosis, which may account for decreased heavy metal concentrations in plant shoots due to the biomass dilution effect (Jarrell and Beverly, 1981; Meharg and Cairney, 2000). Receptivity to infection by AM fungi and the AM effects on plant nutrient status and growth depend on similar factors as described for heavy metal uptake: plant genotype to the variety level (Vierheilig and Ocampo, 1991; Martensson and Rydberg, 1994; Eason et al., 2001) and the colonising AM fungal isolate (e.g. Bethlenfalvay et al., 1989). Important external factors are especially P availability in soil and light supply (Graham et al., 1982; Son and Smith, 1988).

Recent studies using the tobacco variety Wisconsin 38 have shown that this tobacco readily forms AM symbiosis and responds to mycorrhiza by decreased Cd concentrations in shoots when grown in Cd contaminated substrates under a broad range of cultivation conditions (Janoušková et al., 2005a; Janoušková et al., 2005b). However, AM symbiosis of commercial tobacco varieties has apparently been little studied. As tobacco is subdivided in many different varieties, regrouped in several types, and is cultivated in a wide range of soils under different agro-climatic conditions, it is not clear to what extent AM may impact Cd uptake in commercially produced tobacco.

The principal aim of this study was to test the effect of AM symbiosis on the Cd concentration in tobacco leaves. We approached the topic by testing three distinct commercial tobacco varieties representing three major types. They were either grown non-mycorrhizal or inoculated with one of five different AM fungal isolates originating from a wide range of soil conditions including heavy metal-contaminated soils. Besides Cd accumulation in leaves, the development of AM symbiosis and its effects on tobacco growth and mineral nutrition (nitrogen, phosphorus and potassium) were also followed.

Material and methods

Plant material and substrates

Three tobacco (*Nicotiana tabacum* L.) varieties, Basma BEK, K326 and TN90, were selected for the experiment. Each variety belongs to a distinct tobacco type important in commercial tobacco production, Oriental, flue-cured and Burley (respectively). These three tobacco types differ markedly in terms of morphology, soil requirements, cultivation and curing practices (Layten Davis and Nielsen, 1999). For instance, Oriental tobacco is typically produced on low fertility soils without fertilization, and produces smaller leaves than the other two types. Flue-cured is usually grown on sandy soils while Burley tobaccos are grown on heavier-textured soils. The latter receive higher N fertilization inputs than flue-cured tobacco. Consequently, the three cultivars (types) were not compared under the same growth conditions (i.e. soil and fertilization). The goal of this study was to use soil and fertilization conditions matching as closely as possible each type's requirements. Therefore, we studied the response to AM inoculation of each type independently.

Each variety was cultivated in a soil obtained from a field where it had been produced previously (for origin and characteristics of the soils, see Table 1). The total amounts of Cd in these soils were similar. However, Cd bioavailabilities, estimated here by extraction with 0.01 M $CaCl_2$, were different, most likely due to large pH differences (Table 1). Indeed, soil acidity appears as an important factor increasing Cd bioavailability to tobacco (Tsadilas et al., 2005; review in Lugon-Moulin et al., 2004).

Fungal material

Each tobacco variety was either left uninoculated or inoculated with one of the following five AM fungal isolates (isolate name and origin in parentheses): *Glomus intraradices* Schenck & Smith (PH5; Pb contaminated waste-disposal site also containing elevated concentrations of Cd, Czech Republic), *Glomus intraradices* Schenck & Smith (BEG75; agricultural site, Switzerland), *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG25; agricultural site, United Kingdom), *Glomus claroideum* Schenck & Smith (Kb1; Cd contaminated agricultural site, Czech Republic), *Glomus geosporum* (Nicol. & Gerd.) Walker (Madag; agricultural site, Madagascar). The isolates were selected to represent different AM fungal species and soils of origin in order to obtain information on the isolate-dependent variability in the reaction of the tobacco varieties to inoculation.

The fungal inoculum originated from 4-month-old sporulating maize-clover cultures of the AM fungi maintained in greenhouse conditions in inert substrate (sand and/or zeolite). The isolates originating from contaminated soils (PH5, Kb1) were kept in their soils of origin prior to the establishment of the inoculum cultures in inert substrate.

Establishment of the experiment and cultivation conditions

Tobacco was seeded into commercial peat-based seeding substrate (Agro CZ) sterilised by autoclaving (20 min. at 120°C). At cross stage (with 4 leaves), the plantlets were transplanted into 2.8 l plastic pots (one plant per pot) filled with 2.5 kg of dry soil, previously sterilised by γγ-irradiation (50 kGy).

The pots of the inoculated treatments received 10 ml of inoculum suspension of the AM fungus consisting of colonised root segments, extraradical mycelium (ERM) and spores in water. The quality of the inocula had been checked under a binocular microscope prior to application to ensure high density of the AM fungal propagules. The non-inoculated treatments received the same amount of inoculum sterilised by autoclaving (twice for 30 min. on 2 consecutive days). In order to re-establish microbial conditions of the original soils, all pots were irrigated with 10 ml of microbial filtrate obtained by passing non-sterile soil suspension from the corresponding soil through a filter paper (Whatman No.1). Similarly, microbial filtrates were prepared from the AM fungal cultures used for inoculation, mixed and supplied in the same amount to all pots in order to equalise the microbial conditions of the inoculation treatments. Each treatment consisted of six replicates.

The experiment was grown in a greenhouse with light supplement (12 hours, metalhalide lamps, 400 W) for 16 weeks. The plants were supplied once a week with 100 ml (the first 4 weeks) or 200 ml (from the 5th week till the end of the experiment) of a fertilisation solution (see Table 1). The concentrations of macronutrients in the fertilisation solution were adjusted to the differences in nutrient demand of the three tobacco varieties.

Parameters determined at harvest

Shoot biomass was determined after drying at 80 ºC for 24 h, separated into leaf biomass and the biomass of stalks and flowers. For the Basma BEK variety, which formed suckers (regenerants on stalks), the biomass of the suckers was recorded separately. Middle leaves (No. 8-14 in Basma BEK, No. 7-12 in K326 and TN90) were ground and analyzed for their contents of Cd. Moreover, the three major nutrients typically used for tobacco fertilization were quantified: nitrogen (N), phosphorus (P) and potassium (K). Cd concentrations in roots were also determined in the inoculated treatments (and the corresponding controls) in which decreased Cd concentration in leaves was determined, to find out whether the decrease in Cd leaf concentration was accompanied by a modification of the Cd concentration in roots.

For the analysis of Cd, P and K, 500 mg of oven-dried (80 °C) tobacco leaf biomass was weighed and digested in 10 ml of concentrated nitric acid (HNO_3) in a microwave accelerated reaction system (MARS 5; CEM Corp., Matthews, NC). The element concentrations were assessed by inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7500A; Agilent Technologies, Palo Alto, CA). Blanks were introduced regularly and Certified Virginia Tobacco Leaves (CTA-VTL-2) were used as reference material (Dybczynski et al., 1997). The N concentration in leaves was determined by the combustion method with the Elemental Analyzer Carlo Erba NC 2500 using a constant flow of helium enriched with oxygen and reduction of the combustion gases with copper.

Mycorrhizal colonisation was evaluated on root samples stained with 0.05 % trypan blue in lactoglycerol (Koske and Gemma, 1989) using the grid-line intersect method (Giovannetti and Mosse, 1980). A minimum of 200 intersections were counted in every sample. The ERM length in soil was estimated by a modified membrane filtration technique (Jakobsen et al., 1992) and calculated to meters of hyphae in 1 g of air-dried substrate. The average length of background mycelium was assessed on samples from the 6 non-inoculated pots of each tobacco variety. The values (0.15, 0.07 and 0.3 m g^{-1} for the Basma BEK, K326 and TN90 variety, respectively) were subtracted from the ERM length values obtained in the corresponding inoculated treatments.

Statistial analysis

Data on root colonisation and ERM length were logarithmically transformed in order to meet the requirements of ANOVA and differences between treatments were evaluated by ANOVA and Tukey's HSD test at the significance level P<0.05. Data on shoot biomass and element concentrations were treated by one-way ANOVA with the factor inoculation and Dunnett's multiple range test for each tobacco variety separately.

Results

Mycorrhizal development

No root colonisation with AM fungi was found in the non-inoculated treatments. Percentage of root colonisation in the inoculated treatments was significantly influenced by the factors tobacco variety (F=77.21, P<0.001) and AM fungal isolate (F=30.73, P<0.001) as well as by their interaction (F=18.83, P<0.001). Generally, root colonisation was very low (<2%) in the K326 variety without significant differences between isolates (F=1.37, P>0.05) whereas colonisation was higher and significantly dependent upon isolate in the Basma BEK (F=16.77, P<0.001) and TN90 (F=59.93, P<0.001) variety (Fig. 1a). In both varieties, *G. intraradices* BEG75 reached the highest root colonisation whereas *G. mosseae* BEG25 and *G. claroideum* Kb1 reached only low colonisation levels (<10%). The ERM length was in linear relationship with root colonisation (constant ± 95% confidence interval = 0.19 ± 0.12, slope ± 95% confidence interval = 0.23 ± 0.06, R^2=0.409, P<0.001). The differences in ERM length between treatments therefore followed a similar pattern as described for root colonisation (Fig. 1b).

Effect of AM on Cd concentrations in leaves

Cd concentration in the middle leaves was affected by inoculation in all the three tobacco varieties (Fig. 2). Leaves of non-mycorrhizal Basma BEK plants contained only 0.24 μg g^{-1} Cd, and inoculation with four out of the five tested isolates significantly increased their Cd concentration (F=24.01, P<0.001). Cd concentrations in the leaves of non-mycorrhizal K326 and TN90 plants were about 10-fold higher (2.53 and 3.15 μg g^{-1}, respectively) than in the Basma BEK variety. Two and three isolates decreased the Cd concentrations in the leaves of K326 and TN90 respectively, when compared with the corresponding controls (Fig. 2). However, these AM isolates did not affect root Cd concentrations with the exception of BEG25 in TN90 (see Table 2). Higher leaf to root concentration ratios suggested that Cd translocation from roots to shoots was higher in TN90 than in K326. Inoculation tended to decrease the ratio in the TN90 variety, although it did not significantly influence this parameter in either variety (Table 2).

Effect of AM on tobacco biomass and NPK uptake

Inoculation with none of the AM isolates significantly increased tobacco leaf biomass in any variety (Table 3). However, inoculation with three isolates increased the regeneration capacity of Basma BEK (determined as biomass of suckers) resulting in increased total biomass in two inoculated treatments, and inoculation with one isolate increased the total shoot biomass of K326.

In the Basma BEK variety, concentrations of all three nutrients were significantly higher in three or four inoculated treatments when compared with the non-inoculated control (Table 3). Plants inoculated with *G. mosseae* BEG25 had the highest leaf concentrations of the three nutrients from all inoculation treatments. In the TN90 variety, higher concentrations of N, P, K were measured only with the BEG25 isolate. In K326, the same isolate led to significantly increased concentrations of N and P

(Table 3). Therefore, *G. mosseae* BEG25 was clearly the most effective isolate in improving leaf concentrations of N, P, K in the three "variety x soil" combinations tested.

Discussion

Inoculation with AM fungi resulted only in low levels of root colonisation in most treatments. This result was unexpected, as tobacco had been previously found to become extensively colonised with AM fungi: the *Glomus intraradices* isolate PH5 colonised 59% (in sand) or 95% (in soil) of the roots of the tobacco variety Wisconsin 38 under very similar inoculation and cultivation conditions (Janoušková et al., 2005a). The experimental design does not allow conclusions on whether the low colonisation levels were caused by low affinity of the tested tobacco varieties to mycorrhiza, as reported e.g. for some cultivars of wheat (Vierheilig and Ocampo, 1991), or by the soil or fertilisation conditions. The overall low root colonisation of the K326 variety suggests the former cause, whereas the differential development of the isolates in the roots of the other two varieties indicates rather inhibition by some external factors, which may have been better tolerated by some isolates than by others.

Root colonisation by AM fungi can also decrease during cultivation period as a result of fast root growth. However, evaluation of colonisation in some randomly selected replicates after 8 weeks (data not shown) revealed similarly low values as at harvest. Therefore, our results show that significant changes in plant Cd uptake and nutrient acquisition may be achieved by AM even at very low colonization levels. In fact, the differential effects of the isolates were not related to their development in the roots or soil. In contrast to the report of Díaz et al. (1996), no clear relationship was found between the effect of an isolate (on Cd uptake by tobacco) and its origin (i.e. from contaminated or non-contaminated soil). These results illustrate that the factors responsible for isolate-dependent variability in the effects of AM symbiosis are far from being clear.

Interestingly, the effect of inoculation on the Cd concentration in tobacco leaves differed between the cultivar-soil combinations. Inoculation with some isolates decreased Cd concentrations in the TN90 and K326 variety by up to 47% and 36%, respectively. This result was consistent with the decreased Cd concentration in the shoots of mycorrhizal tobacco of the variety Wisconsin 38 grown in substrates with high Cd availability (Janoušková et al., 2005b). The effect of mycorrhiza on the Cd concentration in Basma BEK leaves, however, sharply contrasted with the results obtained for the other two varieties: inoculation with four out of the five tested isolates increased the Cd concentration in the leaves of this variety. The Basma BEK variety was grown on a soil with low Cd availability, probably due to the high soil pH (Schachtschabel et al., 1992). This is reflected in the Cd concentration in the leaves of non-mycorrhizal Basma BEK plants, which was 11 or 13-fold lower than that of non-mycorrhizal K326 and TN90, respectively. It is noted that different tobacco varieties may show differences in Cd uptake (review in Lugon-Moulin et al., 2004). However, it is unlikely that the above >10-fold differences in Cd concentration between Basma BEK and the other two varieties would have been found if the three control varieties had been grown in the same soil conditions, although we cannot rule out that some of the difference is cultivar-based.

Our results are therefore in accordance with an assumption that mycorrhiza may increase Cd concentrations in plant shoots on soils with low Cd availability and decrease them on soils with higher Cd availability (Heggo et al., 1990). The critical Cd leaf concentration, at which the effect of mycorrhiza on Cd concentration in leaves changed from positive to negative, was between 8 and 18 μg g^{-1} for soybeans in the study of Heggo et al. (1990), whereas it was much lower (below 2.5 μg g^{-1}) for tobacco in the present study. Soil Cd bioavailability was estimated using $CaCl_2$. It is noted that chemical extractants may not always be good indicators for Cd availability to tobacco (e.g. Keller et al., 2005; Tsadilas, 2000), but the bioavailability values estimated by $CaCl_2$ extraction were consistent with the data on soil pH and Cd concentrations in the biomass of tobacco in this study if the Basma BEK variety is compared with the other two varieties.

The mechanisms behind the decreased Cd concentrations in the leaves of mycorrhizal TN90 and K326 tobacco plants are not clear. Inoculation did not increase the shoot biomass of these two varieties so that biomass dilution of Cd, observed by Weissenhorn et al. (1995), cannot be assumed. Data on Cd concentrations in roots were not in support of enhanced Cd retention in mycorrhizal roots as described e.g. by Loth and Höfner (1994), although the biomass of roots was not assessed (it was

difficult to isolate a representative proportion of the root system from the soils used because of the abundancy of fine, easily broken roots). Lower Cd availability in the rhizosphere of mycorrhizal plants due to Cd sorption on ERM has been proposed for contaminated soils as a mechanism of lower total uptake by mycorrhizal plants (Joner et al., 2000). However, the decreased Cd concentrations in some inoculated treatments were not consistently associated with higher ERM density in soil as could have been expected if ERM had played a role in our results.

It is therefore probable that the lower Cd concentration in the leaves of tobacco in some inoculated treatments was a result of indirect, plant-mediated mechanisms, which were also suggested to play a role in AM effects on Zn uptake by plants (Christie et al., 2004). Mycorrhizal plants may have higher rhizosphere pH (Marschner and Bauman, 2003; Chen et al., 2004) and an altered pattern of carbohydrate release (Jones et al., 2004) in comparison with non-mycorrhizal plants. Such differences between mycorrhizal and non-mycorrhizal rhizosphere may also result in differences in Cd availability.

In addition to altered Cd concentrations, improved P acquisition has been observed in all the three tobacco varieties after inoculation with at least one isolate despite low colonization levels. Of particular interest is the finding that improved P nutrition was accompanied by enhanced acquisition of N, a nutrient especially important in Burley tobacco (represented in our study by TN90). Despite improved mineral nutrition, there were only small positive growth effects. They can be partly related to pot size, as suggested by Zhu et al. (2001). Indeed, the available soil volume was relatively small for the large root system of tobacco towards the end of the cultivation.

In the K326 and TN90 varieties, nutrient acquisition was only improved by inoculation with the BEG25 isolate. This isolate had low root colonisation levels below 1% and an ERM length of 0.5 and 0.3 m g^{-1} soil, which is at the lowest limit of the range reported for the density of AM fungal ERM in soil (as summarised by Smith and Read, 1997). This suggests that indirect, plant-mediated effects were involved both in the improved nutrient acquisition and in the decreased Cd uptake of mycorrhizal plants. Van der Heijden (2001) observed functional AM symbiosis in *Salix repens* plants with less than 5% of their root length colonised by *Glomus mosseae*. They concluded that the mycorrhizal effects were related to the higher root length of AM plants. Stimulation of root growth by mycorrhiza has been also observed for oat (Loth and Höfner, 1994) or *Citrus* seedlings (Fidelibus et al., 2001). This mechanism may also have been responsible for the observed effects of BEG25 on the nutrient uptake of tobacco.

Conclusion

Our results show that under certain conditions the same AM isolate can decrease Cd uptake by tobacco, while in other conditions, it may increase it. While this may be accounted for by different fertilization regimes or soil characteristics, it appears that Cd bioavailability played a role in this result. Another important and intriguing result is the significant responses in terms of Cd and nutrients uptake even when the symbiosis was based on very low root colonisation levels. The small percentage of colonised roots and low amounts of ERM in most inoculated treatments suggest that plant-mediated mechanisms, such as enhanced root growth or changes in root exudation, were involved in the effects of AM symbiosis. As differences in efficiency between isolates were to a certain degree consistent among the three combinations of variety x soil, the selection of one or more effective isolates for a broader range of tobacco genotypes and soil conditions may be possible. However, there is a need to better understand the precise mechanisms by which different AM and tobacco varieties respond to each other under different environments.

Acknowledgments

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Table 1: Characterisation of the soils used for cultivation of three tobacco varieties (Basma BEK, K326, TN90) and concentrations of nutrients in the applied fertilisation solutions.

	Basma BEK	K326	TN90
Soil characteristics			
Origin	Elbasan (Albania)	Rio Grande do Sul (Brazil)	Rio Grande do Sul (Brazil)
pH (H_2O)	7.6	4.9	5.3
pH (KCl)	7.2	4,3	4.7
C (%)	1.36	0.85	1.76
N (%)	0.12	0.11	0.16
P (Olsen), (mg kg^{-1})	34.7	37.0	9.8
CEC	281.5	133.6	182.4
Cd total (mg kg^{-1})	0.13	0.10	0.20
Cd (0.01 M $CaCl_2$), (mg kg^{-1})	0.004	0.058	0.023
Fertilisation solution			
N (g l^{-1}) as $Ca(NO_3)_2$	0.06	0.12	0.18
P (g l^{-1}) as KH_2PO_4	0.03	0.06	0.09
K (g.l^{-1}) as KCl, KH_2PO_4	0.69	1.38	2.07
Mg (g.l^{-1}) as $MgSO_4$	0.12	0.25	0.37
Ca (g l^{-1}) as $Ca(NO_3)_2$	0.08	0.17	0.25
Cl (g.l^{-1}) as KCl	0.59	1.19	1.78
S (g.l^{-1}) as $MgSO_4$	0.16	0.33	0.49

Table 2: Cadmium concentrations in the middle leaves and roots of two tobacco varieties (K326 and TN90) and the ratio of leaf to root concentration, as affected by inoculation with selected AM fungal isolates.

		Leave conc. (µg g^{-1})	Root conc. (µg g^{-1})	Conc. ratio leaves/roots
K326	PH5	#1.6 (0.16)	1.0 (0.28)	1.7 (0.30)
	BEG25	#1.9 (0.54)	0.8 (0.19)	2.5 (0.93)
	NM	*2.5 (0.35)*	*1.1 (0.18)*	*2.3 (0.47)*
Significance		**	*n.s.*	*n.s.*
F-value		*9.31*	*2.41*	*2.45*
TN90	BEG75	#2.2 (0.97)	0.7 (0.05)	3.0 (1.36)
	PH5	#2.1 (0.21)	0.6 (0.13)	3.3 (0.56)
	BEG25	#1.7 (0.74)	#0.6 (0.13)	2.9 (1.17)
	NM	*3.1 (0.26)*	*0.8 (0.11)*	*4.2 (0.93)*
Significance		**	*	*n.s.*
F-value		*5.84*	*3.55*	*2.04*

The values are given as means of 6 replicates (s.d.). Significant effects according to one-way ANOVA: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; n.s. non-significant effect. Means indicated by a cross are significantly lower than the corresponding NM treatment according to Dunnett's multiple range test at significance level $P < 0.05$. For abbreviations see Table 3.

Table 3: The dry weight (DW) of total shoot biomass, leaves and suckers and concentrations of the nutrients N, P and K in the middle leaves of three tobacco varieties (Basma BEK, K326 and TN90) as affected by inoculation with five AM fungal isolates. Suckers were formed only by the Basma BEK variety.

		BEG75	PH5	BEG25	Kb1	Madag	NM	F-value	Significance
Basma BEK	Shoot DW (g)	32 (2.6)	*36 (1.3)	*36 (1.8)	33 (1.7)	31 (1.7)	32 (2.1)	8.05	***
	Leaves DW (g)	12 (1.6)	12 (1.2)	13 (0.8)	13 (0.8)	13 (1.0)	13 (0.9)	1.82	n.s.
	Suckers DW (g)	0.4 (0.5)	*4.3 (2.3)	*2.7 (1.2)	*0.5 (0.3)	0.4 (0.7)	0.1 (0.1)	15.87	***
	N (%)	1.2 (0.1)	*2.1 (0.4)	*4.8 (0.3)	*1.6 (0.3)	0.9 (0.1)	0.9 (0.1)	266.79	***
	P (mg g^{-1})	*2.1 (0.5)	*3.4 (0.2)	*5.5 (0.6)	*2.3 (0.5)	1.4 (0.1)	1.3 (0.1)	90.75	***
	K (mg g^{-1})	*12.6 (1.7)	*19.0 (1.6)	*24.3 (1.0)	*14.2 (1.6)	9.5 (0.8)	9.0 (0.7)	124.27	***
K326	Shoot DW (g)	27 (2.8)	30 (1.4)	*33 (3.8)	29 (3.8)	30 (3.4)	26 (2.4)	3.66	*
	Leaves DW (g)	15 (2.4)	15 (1.3)	17 (2.0)	14 (2.3)	17 (3.3)	15 (2.3)	1.34	n.s.
	N (%)	0.8 (0.03)	1.1 (0.4)	*2.0 (1.2)	1.5 (0.4)	0.8 (0.05)	0.8 (0.1)	4.90	**
	P (mg g^{-1})	1.1 (0.2)	1.1 (0.6)	*2.7 (1.6)	0.9 (0.1)	1.3 (0.3)	1.2 (0.1)	6.37	***
	K (mg g^{-1})	15.0 (2.2)	17.4 (3.5)	19.8 (5.5)	18.8 (1.7)	15.0 (1.9)	15.5 (1.5)	2.74	*
TN90	Shoot DW (g)	26 (1.7)	25 (3.2)	29 (3.8)	28 (2.0)	#24 (1.3)	28 (1.9)	4.03	**
	Leaves DW (g)	15 (1.2)	14 (1.2)	15 (1.9)	16 (0.9)	#13 (0.7)	15 (0.5)	3.62	*
	N (%)	1.3 (0.1)	1.5 (0.1)	*3.4 (1.6)	1.3 (0.1)	1.6 (0.1)	1.4 (0.1)	8.12	***
	P (mg g^{-1})	1.4 (0.2)	1.3 (0.1)	*2.6 (1.0)	1.4 (0.2)	1.4 (0.1)	1.3 (0.2)	7.08	***
	K (mg g^{-1})	27.4 (2.8)	26.6 (4.5)	*37.2 (7.2)	26.1 (1.7)	28.3 (1.5)	26.7 (3.2)	6.73	***

The values are given as means of 6 replicates (s.d.). Significant effects according to one-way ANOVA: *** $P<0.001$, ** $P<0.01$, * $P<0.05$; n.s. non-significant effect. Means indicated by an asterisk are significantly higher than the corresponding NM treatment, means indicated by a cross are significantly lower than the corresponding NM treatment according to Dunnett's multiple range test at significance level $P<0.05$. Abbreviations: BEG75, PH5, BEG25, Kb1, Madag: treatments inoculated with the corresponding isolate; NM = non-mycorrhizal treatment

Figure 1: Root colonisation of three tobacco varieties (Basma BEK, K326 and TN90) by five AM fungal isolates (BE75, PH5, BEG25, Kb1, Madag) (a) and the ERM length of the isolates in substrate (b).

a)
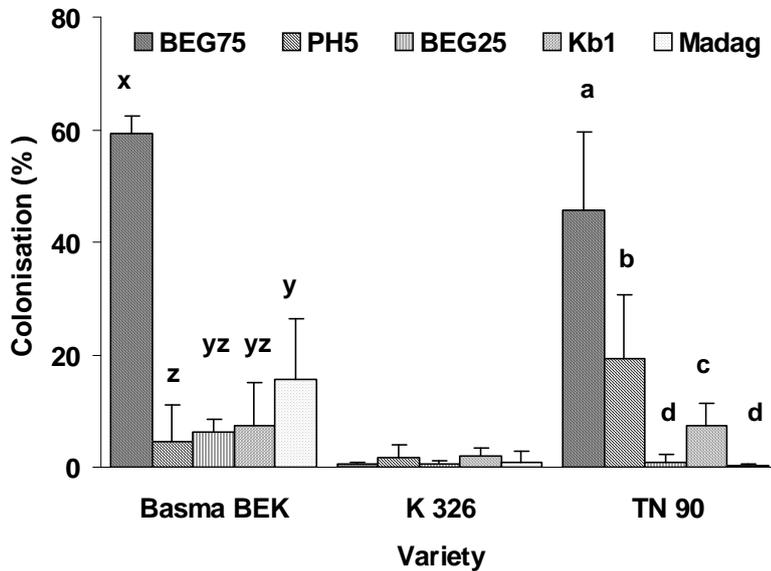
b)
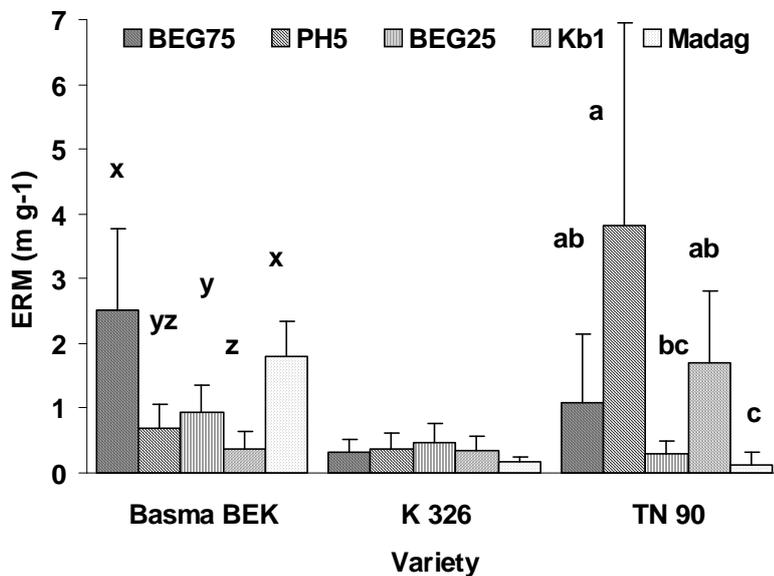
The values are given as means of 6 replicates (±s.e.). Letters indicate significant differences between the inoculation treatments within the Basma BEK and TN90 varieties according to one-way ANOVA and Tukey's HSD test at the level P<0.05. Columns indicated with the same letter are not significantly different.

Figure 2: Cadmium concentration in the middle leaves of three tobacco varieties (Basma BEK, K 326 and TN 90) as affected by inoculation with five AM fungal isolates.
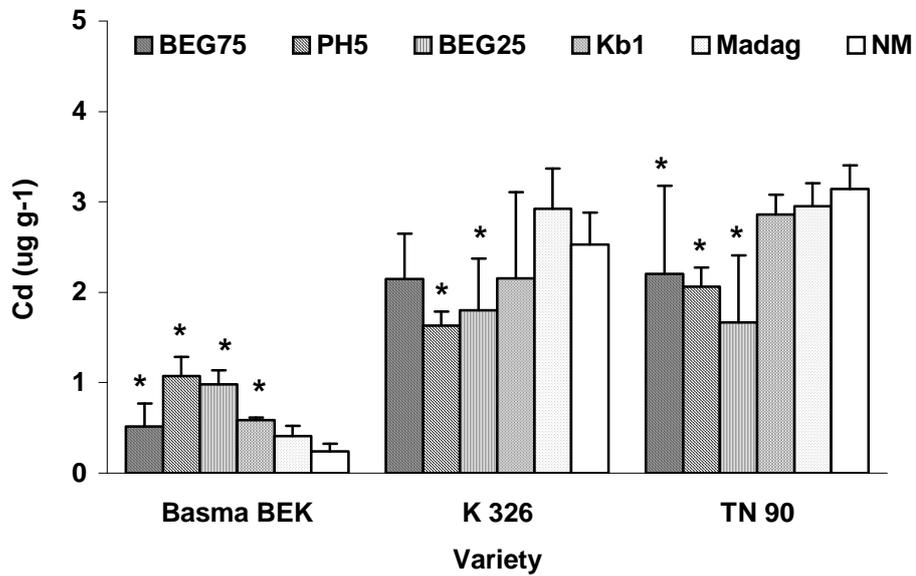
The values are given as means of 6 replicates (±s.e.). Columns indicated by an asterisk significantly differ from the NM treatment within tobacco variety according to Dunnett's multiple range test at the level P<0.05 (see Table 4). Abbreviations: BEG75, PH5, BEG25, Kb1, Madag: treatments inoculated with the corresponding isolate; NM = non-mycorrhizal treatment.

4.5   Further related publication: Mycorrhiza 25, 2005

Janoušková M, Vosátka M **(2005)** Response to cadmium of *Daucus carota* hairy roots dual cultures with *Glomus intraradices* or *Gigaspora margarita*. **Mycorrhiz**a 15: 217-224

Abstract

Ri T-DNA transformed carrot roots were cultivated in two experiments either non-inoculated or inoculated with *Glomus intraradices* or *Gigaspora margarita*. The influence of two concentrations of cadmium (Cd) in the medium (2 mg.l^{-1}, 4 mg.l^{-1}) on both root and mycelium growth was tested. Both parameters were estimated in 10 days intervals for 70 or 100 days for *G. intraradices* and *Gi. margarita*, respectively.

In the first experiment, *G. intraradices* showed a rapid spread of extraradical mycelium and reached average densities per treatment of about 90 cm.cm^{-2} of agar medium after 70 days. At the higher Cd level, the growth of extraradical mycelium was delayed in comparison to the treatment without Cd addition. Root growth was inhibited by both Cd levels, the inhibition was, however, significantly lower in the treatments inoculated with *G. intraradices* compared to the non-inoculated control. In the second experiment, the extraradical mycelium of *Gi. margarita* started to grow after a period of 50 days and reached average densities per treatment up to 27 cm.cm^{-2}, only, at the end of the cultivation. The growth of *Gi. margarita* mycelium was not inhibited by Cd. No differences in root growth were observed between the *Gi. margarita* inoculated and non-inoculated treatments. The inhibitory effect of Cd on root growth differed between the non-inoculated treatments of both experiments.

The study has shown that the AM fungus *Glomus intraradices* can alleviate Cd-induced growth inhibition to carrot hairy roots. Potential and limits of the monoxenic system in studying the interaction between arbuscular mycorrhizal fungi and heavy metals are discussed.

5   Synthesis of results and general discussion

5.1   Experimental systems

Cultivation experiments using sterilised (AM fungi free) substrate and comparing inoculated (mycorrhizal) and non-inoculated (non-mycorrhizal) plants are the main tool of mycorrhizal research. They are indispensable, enabling the identification of mycorrhizal effects on the host plant in the studied system.

Studies focusing on the AM-HM interaction usually work with a native soil from a contaminated site or with artificially contaminated substrate. The first approach is suitable for studies investigating particular situations e.g. the effect of AM on plant survival and growth on a specific site (Díaz and Honrubia 1995, Hildebrandt et al. 1999). However, it suffers an important drawback due to the impossibility to establish control treatments in the same soil without HM stress. AM effects on plant growth thus cannot be interpreted as contribution to HM tolerance as they cannot be related to AM effects in the absence of the HM stress. The second approach, cultivation in artificially contaminated substrate, has been therefore selected in this thesis for the experiments investigating the Cd-AM interactions at higher Cd levels (4.1, 4.2, 4.3). Native soils, however, were used in the case study exploring AM effects on Cd uptake by commercial tobacco varieties at Cd levels in soil within the background range (4.4). Tobacco was cultivated in soils originating from three tobacco plantations in order to reproduce as closely as possible the native conditions.

Generally, studies based on artificially HM contaminated substrates mostly use natural soils, where the HM is added before the start of the experiment in concentrations reflecting the immobilisation capacity of the soil for the given HM (e.g. Rivera-Becerril et al. 2002, Vivas et al. 2003b, Chen et al. 2004, Citterio et al. 2005). This approach assures equilibrium concentrations of nutrients and the HM for the whole cultivation period and simulates conditions of contaminated soils. In contrast, nutrients and HM have to be added continuously into sand culture due to the low sorption and buffering capacity of sand. This creates an artificial situation, where element concentrations in the soil solution vary. Nutrients as well as the tested HM may be subject to progressive accumulation when their supply is too high or can be depleted when the supply is too low. Cultivation in quartz sand may inhibit the development of the AM fungal symbiont with low levels of root colonisation as consequence

(unpublished results). Moreover, Weissenhorn and Leyval (1995) adverted that sand culture conditions may interact with the functional principle of AM symbiosis: A mycorrhizal benefit, which is usually related to an enhanced acquisition of immobile elements, can be assumed as less important in sand culture than in soil, because nutrients are supplied as soluble salts.

However, sand culture has some important advantages despite its artificial character and higher requirements on the optimisation of the cultivation system. These consist mainly in the easier isolation of roots and fungal material and in the simplicity of the cultivation system in comparison with soil culture. For this reason, sand culture has been first tested in one experiment in this study as described in 4.1. The experiment showed satisfactory development of the selected AM fungal symbiont in the given conditions and tobacco responded to AM symbiosis with enhanced growth and improved P nutrition. Experiments described in 4.3 then exploited two advantages of the sand culture system: The effects of AM symbiosis on Cd availability and soil pH could be determined in the sand, while they may have been masked by the buffering and sorption capacity of a soil. In combination with a separated hyphal compartment, sand cultivation enabled isolation of clean ERM of the AM fungus.

5.2   Mycorrhizal effects on the growth and Cd uptake of tobacco

The HM uptake by a plant (HM content per plant) results from the HM uptake per unit of biomass (HM concentration in the biomass) and the growth of the plant. The HM concentrations and contents in plant shoots, important from the points of view of plant-product safety or phytoextraction efficiency, are also determined by the root-to-shoot translocation of the HM within the plant. All three processes – plant growth, HM uptake and root-to-shoot translocation - can be influenced by AM symbiosis as reviewed e.g. by Leyval et al. (1997). The results of this thesis are therefore discussed separately as AM effects on tobacco growth, Cd concentration in roots, Cd translocation from roots to shoots and Cd concentration in shoots.

To the effects of inoculation with an AM fungus, it is referred as to "mycorrhizal" effects in this study. I am aware that this generalisation is problematic due to the known variability in the effects of AM fungal isolates on the nutrient uptake, growth, heavy metal uptake or root to shoot translocation of plants (e.g. Kaldorf et al. 1999, Tonin et al. 2001, Clark 2002, Liao et al. 2003, Wang et al. 2005). However, different AM fungal isolates only exceptionally have opposite effects on the above mentioned parameters, such as one isolate

increasing, another one decreasing HM concentrations in the plant. The differences between isolates mostly consist in the presence/absence of an effect e.g. on plant growth (Batkhuugyin et al. 2000) or in the extent of the effect, i.e. one isolate improves plant growth more than another (e.g. Rydlová and Vosátka 2003, Vivas et al. 2006). This was also indicated by those experiments within this thesis, where the model isolate *G. intraradices* PH5 was compared to other AM fungal isolates (4.2, 4.4): the isolates differed in their efficiency but did not have opposite effects on the determined plant parameters. It can be therefore assumed that inoculation with effective AM fungal isolates will principally have the described and discussed "mycorrhizal" effects, while not all AM fungal isolates will be effective.

5.2.1 Nutrient uptake and growth

Mycorrhiza consistently increased the P concentrations in the biomass of tobacco (4.1, 4.2, 4.4, unpublished data obtained within 4.3) in accordance with the main nutritional function of AM symbiosis (Clark and Zeto 2000). The experiment reported in 4.4 shows that mycorrhiza might also enhance the uptake of other macronutrients in tobacco. However, the enhanced P acquisition was not accompanied by consistent mycorrhizal growth response, which ranged from positive (4.1, 4.2, 4.3) over absent (4.2, 4.4) to negative (4.1, 4.3). Other authors have previously reported similar results; enhanced P nutrition of mycorrhizal plants and positive growth response to mycorrhiza are not always in close relationship (e.g. Zhu et al. 2001, Chen et al. 2004).

Interaction of Cd toxicity and mycorrhizal growth response, as observed in 4.1 and 4.2, suggests buffering of Cd-induced stress by mycorrhiza in accordance with previous reports on mycorrhizal effects on pea (Rivera-Becerril et al. 2002, Rivera-Becerril et al. 2005) or maize (Chen et al. 2004). The interaction was significant not only for shoot biomass, but also for root biomass. This indicates that mycorrhiza protected tobacco roots against Cd stress and alleviated Cd-induced growth inhibitions to non-mycorrhizal roots. A similar result was reported by Dueck et al. (1986) for Zn and is also in accordance with mycorrhizal effects on carrot roots grown under Cd stress in the monoxenic system (Janoušková and Vosátka 2005).

### 5.2.2 Cd concentrations in roots and translocation from roots to shoots

Cd concentrations in the roots of the Wisconsin 38 variety and mycorrhizal effects on this parameter were variable (Figure 1). They depended on cultivation substrate (4.1) and tobacco clone (4.2). When inoculation decreased the Cd root concentration, the effect was less pronounced than the effect on Cd shoot concentration (4.3). In some treatments, inoculation increased the Cd root concentration (4.2).

Nicotiana tabacum is a leaf accumulator of Cd (Lugon-Moulin et al. 2004) and this was also confirmed by the results of this study. The shoot/root concentration ratio decreased at high Cd availability in substrate, but still, Cd shoot concentrations were mostly higher than Cd root concentrations (Figure 2). Consequently, between 84 and 99% of the Cd taken up from soil was translocated into shoots in the Wisconsin 38 variety and similarly high rates can be assumed for the K326 and TN90 varieties based on the determined shoot/root concentration ratios. In comparison with other plant species, which are frequently used as model host plants in experiments with AM fungi, the translocation rates are unusually high: e.g. Cd shoot/root concentration ratios of only 0.02 and 0.30 were reported for maize and clover growing in Cd contaminated soil, respectively (Joner and Leyval 2001); pea transferred only 3-6 % of the Cd taken up into shoots (Rivera-Becerril et al. 2002), maize less than 5 % (Chen et al. 2004).

AM symbiosis tends to decrease the translocation of HMs from roots to shoots in plants growing in HM contaminated soils (e.g. Loth and Höfner 1995, Ricken and Höfner 1996, Joner and Leyval 1997, Tonin et al. 2001, Christie et al. 2004, Chen et al. 2004), though opposite effects of AM symbiosis have been occasionally reported as well (e.g. Citterio et al. 2005). In this study, AM symbiosis decreased Cd translocation from roots to shoots in one experiment (4.2), while only non-significant trends to lower translocation rates in mycorrhizal plants were observed in other experiments (4.1, 4.4). The variability of mycorrhizal effects on Cd translocation to shoots was related to the variability of mycorrhizal effects on Cd root concentrations.

It can be concluded that AM symbiosis may decrease the high Cd shoot/root concentration ratio of tobacco, but not substantially. On the other hand, it is not probable that mycorrhiza could even further enhance Cd translocation from roots to shoots in tobacco.

Figure 1: Mycorrhizal effects on Cd root concentrations were variable.
Effects of AM symbiosis on Cd concentrations in tobacco roots in the
experiments presented in 4.1, 4.2 and 4.4 (including unpublished data from
control treatments with Cd availability in soil within the background range).
Mycorrhizal effects (y-axis) are presented in relation to Cd availability in
soil, expressed as root concentrations of the corresponding control non-
mycorrhizal (NM) plants (x-axis). Symbols represent mean values per
treatment (experiment x inoculation x Cd level in soil). Red line: no
influence of mycorrhiza on Cd root concentrations.

Figure 2: Cd translocation from roots to shoots is unusually high in tobacco. Cd shoot/root concentration ratios of tobacco in the experiments presented in 4.1, 4.2 and 4.4 (including unpublished data from control treatments with Cd availability in soil within the background range). The Cd shoot/root concentration ratio (y-axis) is presented in relation to Cd availability in soil, expressed as root concentrations of the corresponding control non-mycorrhizal (NM) plants (x-axis). Symbols represent mean values per treatment (experiment x inoculation x Cd level in soil), ratios of non-mycorrhizal and mycorrhizal plants are not differentiated by distinct symbols.
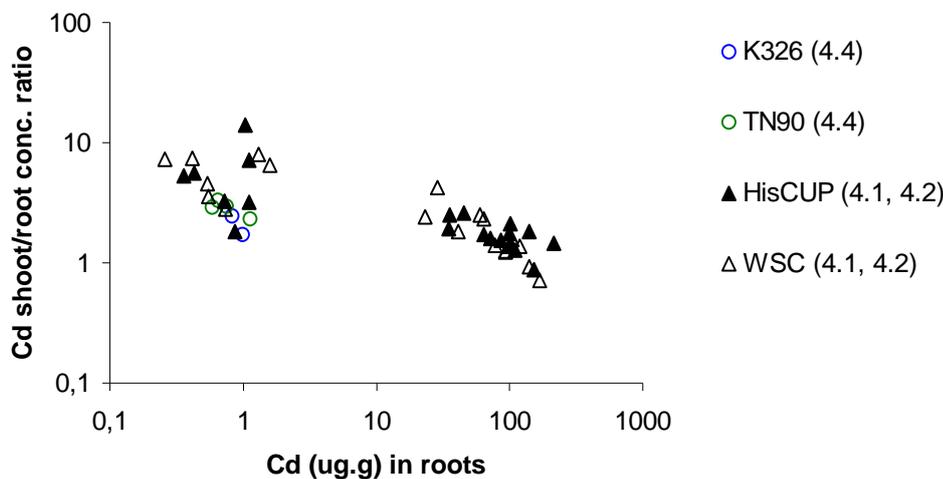
### 5.2.3 Cd concentrations in shoots

At Cd availability in soil simulating Cd contamination, AM symbiosis predominantly decreased the Cd shoot concentrations of the Wisconsin 38 variety (4.1, 4.2, 4.3). This result was consistent among four experiments with variable cultivation conditions such as cultivation substrate, pot size, nutrient availability and light intensity - parameters that had substantially influenced the effect of mycorrhiza on Cd uptake by maize (Weissenhorn et al. 1995a, Weissenhorn and Leyval 1995, Joner and Leyval 2001). However, AM symbiosis failed to decrease the Cd concentration in the biomass of the WSC clone at Cd concentrations in soil, which were highly toxic to the non-mycorrhizal plants (Exp. 1 in 4.1, 4.2). Interestingly, mycorrhiza was effective in the more tolerant HisCUP clone at the same Cd soil concentrations. This indicates that the mechanisms decreasing Cd uptake in mycorrhizal

tobacco are effective only when the viability of the plant is not severely limited by Cd toxicity.

However, the experiment described in 4.4 shows that AM symbiosis may also increase the Cd concentration in tobacco shoots, when Cd availability in soil is very low. This result is in accordance with the study of Heggo et al. (1990), who demonstrated enhanced uptake of Cd and other HMs in mycorrhizal soybean plants in soils with low concentrations of these elements, but opposite effects of mycorrhiza when the HM concentrations were high. AM symbiosis most effectively enhances the host plant's uptake of those nutrients, which are relatively immobile or deficient in soil (Smith and Read 1997). This may similarly apply to the non-essential element Cd, which is also transported into plant roots by the ERM of AM fungi (Guo et al. 1996, Joner and Leyval 1997). The higher shoot Cd concentrations in mycorrhizal tobacco may be therefore explained by Cd uptake via AM fungal hyphae, which is highly effective in comparison with root uptake at low Cd availability in soil. In contrast, hyphal uptake will contribute less to the total uptake by the plant at higher Cd availability in soil. Mechanisms decreasing the Cd uptake per unit of biomass in mycorrhizal plants, as discussed in 5.3, will be more effective and dominate over the contribution of mycorrhiza to the Cd uptake of the plant.

The suggested relation of mycorrhizal effects on Cd concentration in tobacco shoots with Cd availability in soil is illustrated in Figure 3. It summarises data from the conducted experiments including unpublished data from control treatments, where the Wisconsin 38 variety was grown in substrates with Cd concentrations within the background range. At Cd availability in soil corresponding to Cd shoot concentrations between 1 and 12 μg.g^{-1}, inoculation mostly decreased the Cd concentration in shoots though the effect was less consistent than in the treatments with simulated Cd contamination (Cd shoot concentrations >100 μg.g^{-1}). The critical Cd level below which mycorrhiza increases the Cd concentrations in tobacco shoots is therefore lower than 1 μg Cd g^{-1} of shoot dry weight. The critical level concept has been introduced by Chen et al. (2003) and Christie et al. (2004) for mycorrhizal effects on the Zn uptake by plants and based on Zn concentrations in soil. The presented results show that it may be also useful for Cd and that the critical level can be related to the HM concentration in plant shoots.

Figure 3: Mycorrhiza decreased Cd shoot concentrations at high Cd availability in soil, but increased them at low Cd availability. *Effects of AM symbiosis on Cd concentrations in tobacco shoots in the experiments presented in 4.1, 4.2 and 4.4 (including unpublished data from control treatments with Cd availability in soil within the background range). Mycorrhizal effects (y-axis) are presented in relation to Cd availability in soil, expressed as shoot concentrations of the corresponding control non-mycorrhizal (NM) plants (x-axis). Symbols represent mean values per treatment (experiment x inoculation x Cd level in soil). Red line: no influence of mycorrhiza on Cd shoot concentrations.*
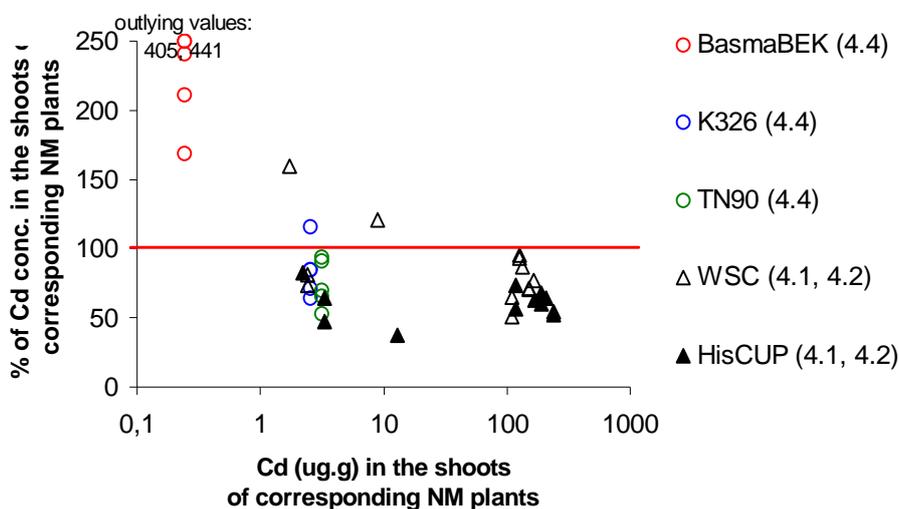
5.2.4 Cadmium content per plant

Cd contents in tobacco shoots (x) and the total Cd uptake by tobacco (Cd contents in shoots + roots = y) were almost identical (y = 0.99x, R^2 = 0.98; regression based on average values per treatment in 4.1, 4.2 and 4.3). This is explained by the high shoot/root biomass ratio and high Cd translocation rates from roots to shoots,

Mycorrhizal effects on the Cd contents in tobacco shoots depended on the growth response to mycorrhiza. Mycorrhiza decreased the Cd shoot concentration of tobacco, and thus, the total Cd content of mycorrhizal plants was lower when mycorrhiza had no effect on tobacco growth (4.1 Exp. 1, some treatments in 4.2). When mycorrhiza slightly improved tobacco growth, mycorrhizal and non-mycorrhizal plants contained the same amount of Cd (4.1 Exp. 2, 4.3). This was the case in treatments with Cd soil concentrations that did not

inhibit the growth of tobacco. Only at high Cd concentrations in soil inducing severe growth inhibition in non-mycorrhizal tobacco, alleviation of Cd stress to the tobacco plants by mycorrhiza, accompanied by growth improvements in the range of 150-200 %, lead to higher Cd contents in the shoots of mycorrhizal tobacco (4.3 WSC plants).

Chen et al. (2005) and Wang et al. (2005) suggested that mycorrhiza could enhance the accumulation of Pb and other HMs in plant shoots based on the combination of higher HM uptake per unit of biomass and growth improvements. Citterio et al. (2005) determined a similar potential of mycorrhiza in hemp due to enhanced Cr and Ni translocation from roots to shoots in mycorrhizal plants. The results of this thesis do not support the idea for tobacco and Cd. Mycorrhizal growth effects did not compensate for the lower shoot Cd concentrations in mycorrhizal tobacco except in extremely Cd contaminated soils where the growth of non-mycorrhizal tobacco was severely inhibited.

5.3   Mechanisms decreasing Cd concentrations in mycorrhizal tobacco

Mycorrhiza mostly decreased the Cd concentrations in tobacco shoots as summarised and discussed in 5.2.3. While higher Cd uptake of mycorrhizal plants can be well explained by Cd uptake and transport by the extraradical hyphae of AM fungi (see 3.2.1.2), the mechanisms of lower Cd uptake are not clear. The inconsistency of reported effects (as reviewed by Leyval et al. 1997 and Meharg and Cairney 1999) indicates that several mechanisms may contribute with varying relevance and efficiency depending on plant species, HM concentration in substrate or plant growth conditions. Apart from direct interactions of fungal structures with HMs in soil or roots, the nutritional effects of AM symbiosis must be also taken into account. The different mechanisms, which may have been responsible, are discussed in the following subchapters.

5.3.1   Biomass dilution

Better growth of mycorrhizal plants was suggested to "dilute" excess HMs in the biomass of plants growing in contaminated soils (Kucey and Janzen 1987, Weissenhorn et al. 1995a). Typically, this explanation may be considered for results such as in Exp. 2 of 4.1 and Exp. 2 of 4.3, where mycorrhizal plants had lower Cd shoot concentrations but the same Cd shoot contents as non-mycorrhizal plants due to higher shoot biomass.

Weissenhorn et al. (1995a) showed that positive mycorrhizal growth response can be accompanied by biomass dilution of HMs and P and suggested other than nutritional benefits

of mycorrhiza in their study. In that rather untypical case, the capacity to acquire nutrients (and other elements) from soil seemed lower in mycorrhizal than in non-mycorrhizal plants. Dilution of some elements in the larger biomass of mycorrhizal plants, however, is usually accompanied by improved P status (e.g. Syvertsen and Graham 1999). Positive mycorrhizal growth response accompanied by improved P acquisition, as observed in some experiments of this thesis, does therefore not exclude biomass dilution of other nutrients.

However, the lower Cd concentrations in the shoots of mycorrhizal tobacco were not always related to improved growth (4.1, 4.2, 4.4). Biomass dilution may have contributed to the decreased Cd concentrations in the treatments with improved growth, but other mechanisms must have restricted the Cd uptake of tobacco per unit of biomass in other treatments. While the biomass dilution effect cannot be excluded in some experiments, it cannot explain lower Cd shoot concentrations of mycorrhizal tobacco in other experiments.

5.3.2 Sequestration in intraradical AM fungal structures

Lower shoot/root concentration ratios of HMs in mycorrhizal plants, as observed in 4.2, can be explained by HM sequestration in the intraradical AM fungal structures, as suggested e.g. by Loth and Höfner (1995), Ricken and Höfner (1996), Joner and Leyval (1997) or Hutchinson et al. (2004). Turnau et al. (1993) brought direct evidence for this mechanism reporting higher Cd concentrations in the intraradical fungal cells than in the root cells of mycorrhizal *Pteridium acquilinum*. Similarly, Kaldorf et al. (1999) observed selective enrichment of Fe, Ni and Zn in the inner cortical cell region of mycorrhizal maize roots containing fungal structures.

However, it is not clear whether HM sequestration in intraradical AM fungal structures may be responsible for lower HM concentrations in the shoots of mycorrhizal plants in comparison with non-mycorrhizal plants. Joner and Leyval (1997) distinguished Cd uptake via fungal hyphae and via roots in mycorrhizal clover growing in pots with compartments accessed only by AM fungal ERM. Their results indicate that only the fraction of Cd that has been transported into roots by AM fungal hyphae is sequestered in mycorrhizal roots, presumably in the fungal structures. This assumption is also supported by the fact that lower shoot/root translocation ratios of mycorrhizal plants were often based on higher Cd concentrations in roots but unchanged Cd concentrations in shoots (Ricken and Höfner 1996, Tonin et al. 2001). HM sequestration in the intraradical structures of AM fungi seems to constitute mainly a barrier against enhanced HM assimilation in mycorrhizal plants supplied

with additional HM by the extraradical hyphae of AM fungi. It can be even hypothesised that mycorrhiza significantly contributed to Cd assimilation in tobacco in those experiments, where the Cd translocation from roots to shoots was similar in mycorrhizal and non-mycorrhizal plants.

5.3.3 Enhanced Cd immobilisation in the rhizosphere of mycorrhizal plants

Mycorrhiza decreased not only the Cd concentrations but also the total Cd uptake of tobacco in some treatments (see 4.1 and 4.2), despite similar or even better growth of the mycorrhizal tobacco. This indicates that Cd bioavailability may have been lower in the rhizosphere of mycorrhizal than of non-mycorrhizal tobacco. One of the two experiments presented in 4.3 confirmed that substrate colonised by mycorrhizal tobacco had higher capacity to immobilise Cd than substrate colonised by non-mycorrhizal tobacco. This is in accordance with the suggestion of Huang et al. (2005) that Zn and Cu may be less bioavailable in the rhizosphere of mycorrhizal plants, and the result of Shen et al. (2006), who determined lower soil solution concentrations of Zn and Cd in the rhizosphere of mycorrhizal plants.

It can be therefore assumed that mycorrhiza decreases bioavailability of Cd in the rhizosphere of tobacco, though it is not entirely clear, how this effect was related to the lower Cd uptake of tobacco in 4.1 and 4.2. The results presented in 4.3 suggest more effective immobilisation of Cd in mycorrhizal rhizosphere especially at high Cd concentrations in substrate, but mycorrhiza effectively decreased Cd uptake by tobacco also at lower Cd concentrations. The results, however, do not exclude lower Cd bioavailability in mycorrhizal rhizosphere over the whole range of concentrations tested, and more sensitive indicators than Cd toxicity should be utilised in future studies. The observed effects can be attributed to Cd immobilisation by the ERM of AM fungi or to plant-mediated effects of AM symbiosis, such as changes in the root-exudation of mycorrhizal plants (see 4.3 for the discussion of this point). In Exp. 2 of 4.3, ERM was shown to possess high Cd binding capacity in accordance with previous results (Joner et al. 2000). However, the effects of different AM fungal isolates on the Cd uptake by tobacco were not related to their ERM density in soil (4.4). Inoculation with the PH5 isolate decreased Cd concentrations in the HisCUP clone of Wisconsin 38 tobacco, but had no effect on Cd uptake by the WSC clone despite similar ERM density in both treatments (4.2). The results of the conducted experiments therefore do not bring evidence for a dominant role of Cd immobilisation by sorption on ERM.

The assumption of plant-mediated effects of mycorrhiza on rhizospheric properties, which may influence HM availability, is attractive, because this mechanism would be complex enough to explain the variability in reported mycorrhizal effects on HM uptake by plants. In this thesis, the idea is supported by indirect evidence. Mycorrhizal effects on the host plant's physiology are influenced by a large variety of factors, e.g. plant species, fungal symbiont, nutrient availability, light intensity or water supply (Smith and Read 1997). Published studies give indications of possible rhizospheric changes resulting from the mycorrhizal effects on plant physiology. Mycorrhizal plants may have higher rhizosphere pH in comparison with non-mycorrhizal plants (Marschner and Baumann 2003, Chen et al. 2004, Shen et al. 2006, 4.3 of this thesis). The pH differences between mycorrhizal and non-mycorrhizal rhizosphere seem to be plant-mediated, but not related to the P nutrition of plants (Marschner and Baumann 2003). However, acidification is more pronounced in the rhizosphere of P-deficient plants (see Hinsinger et al. 2003), so that a role of the higher P status of mycorrhizal plants cannot be excluded. Mycorrhiza may also reduce the carbon release by plant roots (Mada and Bagyaraj 1993, Marschner et al. 1997) and change the composition of root exudates (Mada and Bagyaraj 1993). Both soil pH and root exudates can affect Cd availability in soil as discussed in 4.3. Moreover, the composition of soil bacterial communities is modified by AM of plants (Andrade et al. 1997), pH changes in mycorrhizal rhizosphere (Marschner and Baumann 2003) and changes in root exudation patterns (Marschner et al. 1997). This factor was not considered in this thesis, but synergic effects of mycorrhiza and bacteria have been shown to decrease Cd bioavailability in soil to *Trifolium* plants (Vivas et al. 2003a).

The results of this thesis show that more attention should be paid to differences in Cd bioavailability between the rhizosphere of mycorrhizal and non-mycorrhizal plants. In tobacco rhizosphere, Cd bioavailability seems to be decreased by mycorrhiza, and involvement of plant-mediated effects is suggested.

5.4 <u>Mechanisms of the buffering of cadmium induced stress by mycorrhiza</u>

Meharg and Cairney (1999) concluded in their review that there is no systematic evidence that AM symbiosis protects plants from HM toxicity by reducing their HM assimilation. This is in accordance with the results of this thesis: mycorrhiza predominantly reduced Cd assimilation in tobacco, the buffering of Cd induced stress in mycorrhizal tobacco

was not always related to lower Cd concentrations in tobacco shoots (see 4.2, WSC clone at the highest Cd level in soil).

Mycorrhiza alleviated the Cd induced growth inhibition already on the root level (4.1, 4.2). A similar effect of mycorrhiza in monoxenic cultures of Ri T-DNA transformed carrot roots indicated that the higher tolerance of mycorrhizal roots is not shoot-mediated (Janoušková and Vosátka 2005).

Cd interferes in plants with the uptake and metabolism of nutrients (Seregin and Ivanov 2001). Nutrient deficiency in Cd-stressed plants can be also a result of the high sensitivity of root growth, which is inhibited already by low Cd concentrations (Vázquez et al. 1992). Symbiosis with less Cd-sensitive AM fungi could help the plant to overcome nutritional disorders by additional nutrient supply via AM fungal hyphae. Shetty et al. (1995) suggested that improved P nutrition increased the Zn tolerance of *Andropogon gerardii*. Similarly, Vivas et al. (2006) related the improved growth of mycorrhizal *Trifolium repens* in Zn contaminated soil to its higher nutritional status. In both studies, mycorrhizal plants had higher tissue concentrations of Zn than non-mycorrhizal plants so that the mycorrhizal effects could not be explained by Zn exclusion. However, the nutritional effects of mycorrhiza in the Zn contaminated soils were not experimentally related to mycorrhizal effects in the absence of Zn stress by these authors.

In this thesis, mycorrhiza improved the P status of Cd stressed tobacco more substantially than that of tobacco growing without Cd stress (4.1, 4.2). In some inoculated treatments, this was also true for the specific absorption rate of P per unit of root biomass (SAR_P), a parameter introduced by Gray and Schlesinger (1983) (Figure 4). The P status of Cd-stressed plants was therefore improved not only by the alleviation of Cd-induced root growth inhibition, but also by higher P acquisition per unit of root biomass. Figure 4 also shows that Cd stress did not dramatically decrease the SAR_P in non-mycorrhizal plants, the interaction of the factors mycorrhiza and Cd consisted in higher SAR_P of mycorrhizal plants at higher Cd availability. The data do therefore not support the idea of a simple relationship that mycorrhiza compensates for a less effective P acquisition by Cd-stressed roots. A more thorough inside into this topic would require analysis of more nutrients as well as inclusion of root-length and architecture data of mycorrhizal and non-mycorrhizal plants, growing with or without Cd stress.

The inconsistency between the nutritional and Cd-tolerance effects of two AM fungal isolates, observed in 4.2, leaves space for speculations on further mechanisms, by which mycorrhiza may protect plants against HM stress. However, exploring the molecular basis of

the Cd x mycorrhiza interaction is at its beginning and gives still little indication to potential mechanisms (see 3.2.3.3).

Figure 4: P absorption per unit of root biomass was increased under Cd stress in mycorrhizal plants. *Specific absorption rate of P per unit of root biomass (SAR$_P$) in mycorrhizal and non-mycorrhizal tobacco, grown with or without Cd stress: a) Exp. 1 in 4.1; b) 4.2. Significant effects of factors and their interactions are presented according to three-way ANOVA: ***P<0.001, **P<0.01, *P<0.05; non-significant interactions are omitted; # significantly different from corresponding Cd 0 according to Dunnett's test (P<0.05).*

a)
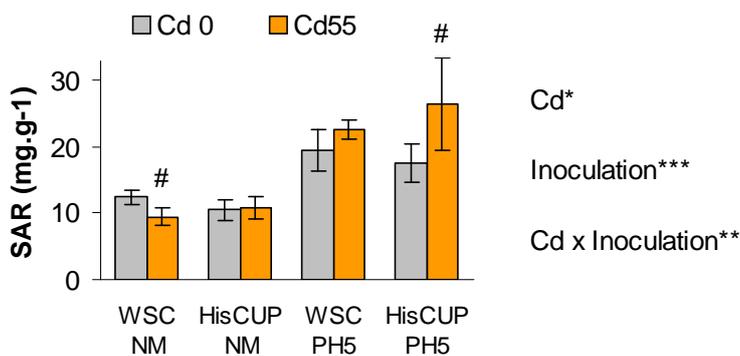
b)
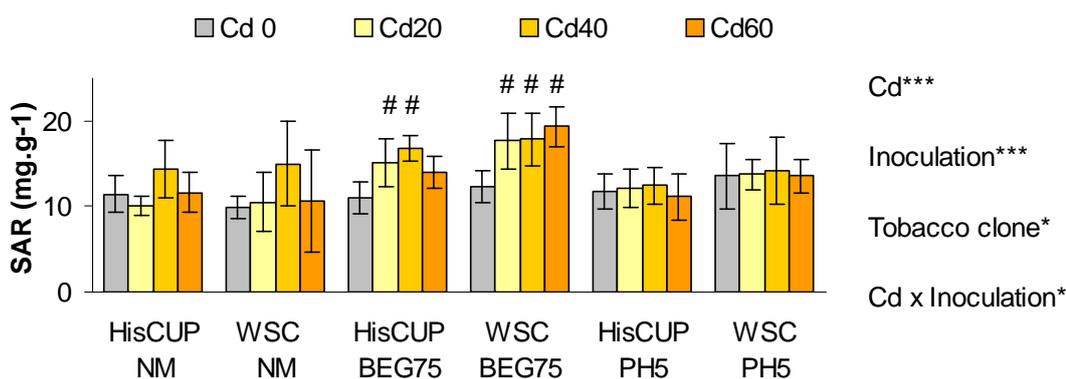
Abbreviations: NM = non-mycorrhizal; BEG75 = mycorrhizal with the G. intraradices isolate BEG75; PH5 = mycorrhizal with the G. intraradices isolate BEG75; Cd number = Cd addition to soil (mg.kg^{-1}).

5.5   Interaction of mycorrhiza and the introduced HisCUP construct

Only few reports are available on the performance of transgenic plants with modified HM accumulation in soil-based cultivation or in field conditions (Krämer and Chardonnens 2001, Pilon-Smits and Pilon 2002). For this reason, probably, interactions of the effects of introduced transgenes with the effects of soil microorganisms have not been described yet.

The HisCUP clone used in this study had been previously shown to take up more Cd per unit of biomass and to be more tolerant to high Cd concentrations in substrate than the control WSC clone (Macek et al. 2002, Pavlíková et al. 2004). The difference in Cd uptake between the HisCUP and control tobacco had been confirmed both in sand culture and in soil, for broad range of Cd availability (Pavlíková et al. 2004). However, the experiments conducted within this thesis (4.1, 4.2, unpublished data from 4.3) did not confirm consistently higher Cd uptake of the HisCUP clone. The reasons for this have not been investigated, but it seems that the HisCUP clone, when non-mycorrhizal, accumulated more Cd per unit of biomass only at growth-inhibitory Cd levels in soils. Thus, the HisCUP construct and mycorrhiza were effective at different Cd soil concentrations: mycorrhiza tended to be most effective to the WSC clone at elevated, but not growth-inhibitory Cd levels.

In the first two conducted experiments (4.1), mycorrhiza altered the Cd accumulation of the HisCUP clone relatively to the WSC clone, which was confirmed also in the experiment described in 4.2. This was based mainly on the differential effects of mycorrhiza on Cd uptake by the WSC and HisCUP clone: mycorrhiza either did not decrease Cd concentrations in the WSC clone at all or decreased them to a lesser extent than in the HisCUP clone. The interaction was effective also at the root level (see Figure 2), which shows that it was not related to Cd translocation from roots to shoots. The mechanism of the interaction is unknown, but it always lowered the Cd accumulation of the HisCUP clone relatively to the WSC clone. This shows that mycorrhiza may modify the performance of transgenic plants designed to accumulate more HMs.

5.6   Concluding remarks

In commercially produced tobacco, reduction of Cd concentrations in leaves is an important aim due to the health risks connected to Cd in tobacco smoke. On the other hand, preferential Cd accumulation in the leaves of tobacco and the relatively high biomass production of this crop make it a potential candidate plant for Cd phytoextraction (Lugon-Moulin et al. 2004).

Inoculation of suitable plants with mycorrhizal fungi has been suggested to enhance the potential of the phytoextraction technique (Ernst 2000, Khan et al. 2000), which could become a clean-up procedure especially for slightly HM contaminated soils (Ernst 1996). Based on the presented results, however, enhanced Cd accumulation by mycorrhizal tobacco in comparison with non-mycorrhizal tobacco can be excluded in slightly contaminated soils. In contrast, mycorrhiza should be expected to decrease Cd concentrations in tobacco shoots even if based on low root colonisation levels. The results suggest application of AM symbiosis rather in decreasing Cd concentrations in the leaves of commercially produced tobacco when grown on soils with higher Cd availability. Furthermore, the enhanced Cd immobilisation in the rhizosphere of mycorrhizal tobacco indicates that mycorrhiza could rather contribute to the stabilisation of HMs in contaminated soils in accordance with previous suggestions (Leyval et al. 2002, Jeffries et al. 2003, Gaur and Adholeya 2004). However, this effect of mycorrhiza must be verified with suitable candidate plants, as it was at least partly plant-mediated. Nevertheless, it has been demonstrated that AM symbiosis must be taken into account when the phytoextraction potential of mycotrophic plants is assessed.

The use of AM fungal isolates not originating from Cd contaminated soils in this thesis indicates that previous adaptations of an isolate to a certain type of contamination or soil conditions are not a prerequisite for the isolate to survive and confer benefits to the host plant. Without underestimating the importance of AM fungal adaptation to HM contamination, it is suggested that certain AM fungal isolates may be successfully applied in a wider range of conditions. In contrast, if the effects of AM symbiosis on the HM uptake of plants are largely plant-mediated, plant species and growth conditions are important factors, which have to be taken into account prior any practical application of mycorrhiza.

6 <u>Conclusions</u>

- **Mycorrhizal effects on Cd concentrations in tobacco shoots depended on Cd availability in soil**. Mycorrhiza decreased the Cd shoot concentrations of tobacco over broad range of Cd availabilities in soil, but had no effect at highly toxic Cd levels. In contrast, when Cd availability in soil was very low, mycorrhiza increased Cd concentrations in the shoots of tobacco.

- Mycorrhiza consistently improved the P nutrition of tobacco, but mycorrhizal growth response ranged from positive over neutral to negative. **Mycorrhiza alleviated Cd-induced growth-inhibition** in tobacco **both at shoot and root level**.

- The effect of mycorrhiza on the total Cd uptake by tobacco on contaminated soils depended on the mycorrhizal growth response of the plants. Based on the collected evidence, **enhanced Cd accumulation in the shoots of mycorrhizal tobacco does not occur at Cd levels in soil that do not inhibit tobacco growth.** In these conditions, even positive mycorrhizal growth response of tobacco did not compensate for the decreased Cd concentrations in mycorrhizal tobacco shoots. **Mycorrhiza can increase the total Cd uptake by tobacco only at Cd concentrations in soil inducing severe growth inhibition in the non-mycorrhizal tobacco** by alleviating this growth inhibition.

- Lower Cd concentrations in the shoots of mycorrhizal tobacco were not consistently associated with positive mycorrhizal growth response or lower root to shoot translocation of Cd as compared with non-mycorrhizal plants. Thus, **the biomass dilution effect or enhanced Cd retention in mycorrhizal roots did not explain the lower Cd concentrations in the shoots of mycorrhizal tobacco**, though a contribution of both effects cannot be excluded in some experiments.

- **Enhanced immobilisation of Cd was confirmed for the rhizosphere of mycorrhizal tobacco** in comparison with non-mycorrhizal tobacco. The ERM of the AM fungal isolate *G. intraradices* PH5 was shown to accumulate high Cd concentrations when exposed to Cd in vivo. However, the collected evidence suggests that **plant-mediated effects of mycorrhiza on rhizospheric properties were responsible** for the enhanced Cd immobilisation in mycorrhizal rhizosphere **rather than Cd sorption by AM fungal ERM.**

- **The buffering of Cd-induced stress in tobacco by mycorrhiza**, demonstrated as alleviation of Cd-induced growth inhibition, **could not be attributed to lower Cd concentrations in mycorrhizal plants only**. **Improved P acquisition was involved as well** as mycorrhiza improved the P status in Cd stressed tobacco more substantially than in tobacco growing without Cd stress.

- **Mycorrhiza decreased Cd accumulation by the transgenic HisCUP clone of tobacco relatively to the wild type clone**. This was based on a more pronounced effect of mycorrhiza on Cd shoot concentrations in the transgenic than in the wild type tobacco. Thus, the study has shown that mycorrhiza may modify the performance of transgenic plants designed to accumulate higher concentrations of HMs.

- It is suggested that **mycorrhiza could be useful in decreasing Cd concentrations in the leaves of commercially produced tobacco**. In contrast, **application of mycorrhiza to enhance Cd accumulation in tobacco shoots** and thus to support Cd phytoextraction **on slightly contaminated soils can be excluded** based on the results of this thesis.

7 <u>References in Chapters 3 and 5</u>

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