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ORIGINAL PAPER

Differential effects of AM fungal isolates on *Medicago truncatula* growth and metal uptake in a multimetallic (Cd, Zn, Pb) contaminated agricultural soil

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Abstract Toxic metal accumulation in soils of agricultural interest is a serious problem needing more attention, and investigations on soil–plant metal transfer must be pursued to better understand the processes involved in metal uptake. Arbuscular mycorrhizal (AM) fungi are known to influence metal transfer in plants by increasing plant biomass and reducing metal toxicity to plants even if diverging results were reported. The effects of five AM fungi isolated from metal contaminated or non-contaminated soils on metal (Cd, Zn) uptake by plant and transfer to leachates was assessed with *Medicago truncatula* grown in a multimetallic contaminated agricultural soil. Fungi isolated from metal-contaminated soils were more effective to reduce shoot Cd concentration. Metal uptake capacity differed between AM fungi and depended on the origin of the isolate. Not only fungal tolerance and ability to reduce metal concentrations in plant but also interactions with rhizobacteria affected heavy metal transfer and plant growth. Indeed, thanks to association with nodulating rhizobacteria, one *Glomus intraradices* inoculum increased particularly plant biomass which allowed exporting twofold more Cd and Zn in shoots as compared to non-mycorrhizal treatment. Cd concentrations in leachates were variable among fungal treatments, but can be significantly influenced by AM inoculation. The differential strategies of AM fungal colonisation in metal stress conditions are also discussed.

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Introduction

Soil pollution by heavy metals disseminated from human activities is a major world concern because metals are very toxic and remain persistent in soils. Metals are not biodegradable and tend to accumulate in soils and organisms where anthropogenic impact is intense. Considered as a non-essential metal element, cadmium (Cd) was widely studied because of its acute toxicity. Even at low concentrations in the environment, Cd can accumulate in biological organisms and result in severe troubles (Wren et al. 1995). To prevent such risks, it is necessary to quantify Cd transfer to plants. Numerous studies investigated soil–plant metal transfer to find the most reliable methods for the prediction of heavy metal bioavailability and to understand the processes involved in the uptake of these toxic elements. In polluted soils, Cd is rarely present alone and is often associated to other heavy metals such as high levels of zinc (Zn). Cd and Zn behaviour in plants and soils are linked (Welch et al. 1999; Nan et al. 2002; Bradl 2004), but as an essential trace metal, Zn is of second concern because of its lower toxicity. Many soil parameters influence metal transfer to plants. The most important variables which control metal availability are: pH, redox potential, texture, organic matter, mineral composition, temperature and water regime (Kabata-Pendias 2004). Besides these abiotic factors, the biological components of soils (plant, microorganisms) play also a major role on metal fate. In particular, soil microorganisms affect root morphology, plant physiology and metal fractionation (Tao et al. 2005). The strong interactions of rhizospheric bacteria and fungi with roots thus

modify the capacity of metal pollutants to be absorbed by plants. Among soil microorganisms, arbuscular mycorrhizal (AM) fungi are widely studied because of their ability to improve plant fitness under stress conditions (Gildon and Tinker 1983; Leyval and Joner 2001; Khan 2005). Colonising most of the terrestrial plants, these ubiquitous symbiotic fungi provide essential nutrients to plants, such as low available P (Requena 2005), which allows increasing shoot biomass (Heggo et al. 1990). AM fungi increase nutrient acquisition by exploring a vast soil volume (Harley and Smith 1983) and can be beneficial to host plant growing in unfavourable soil conditions as in nutrient-deficient soils or in polluted areas. In heavy-metal-contaminated soils, AM fungi can also improve the growth of host plant by reducing metal stress (Rivera-Becerril et al. 2002) in addition to an enhancement of nutrient acquisition. The alleviation of stress may be partly due to the immobilisation of heavy metals in the mycorrhizosphere and to the decrease of metal concentration in mycorrhizal plants (Kapoor and Bhatnagar 2007). AM fungi are able to transport non-essential elements as Cd (Joner and Leyval 1997; Hutchinson et al. 2004) and to sequester them in roots (Galli et al. 1994; Janoušková et al. 2006). However, AM fungi have variable effects on metal uptake and growth of host plant (Hildebrandt et al. 2007), as in some cases, AM inoculation did not increase plant biomass (Killham and Firestone 1983; Weissenhorn and Leyval 1995). Many factors contribute to the divergences of AM fungal effects, among them the nature of the plant–fungus association, i.e. the plant and fungal genotypes. Metal-tolerant AM fungi isolated from contaminated sites are thought to have a better protecting effect towards plants than isolates from non-contaminated soils (Gildon and Tinker 1983; Hildebrandt et al. 1999). However, metal tolerance of AM fungi is not easy to assess since they cannot be grown without the plant. Their tolerance to heavy metals has thus been tested regarding spore germination (Weissenhorn et al. 1993) and root colonisation (Rydlová and Vosátka 2003) or considered for the AM isolates collected from contaminated soils without checking their effective tolerance.

In a previous work, we pointed out the effect of an AM fungus isolated from a heavy-metal-contaminated soil on Cd transfer to plants growing in soils with different availabilities and different kinds of pollution (Redon et al. 2008). AM inoculation with *Glomus intraradices* increased *Medicago truncatula* growth, decreased Cd concentration in plant shoots and Cd content in leachates (−30%), although the total metal content in shoots was increased twofold. The objective of the present study was to compare the effects of five *Glomus* strains isolated from contaminated and non-contaminated soils on Cd and Zn uptake by *M. truncatula* and on metal concentration in leachates in a multimetallic contaminated agricultural soil.

Materials and methods

Soil and inoculum

The agricultural soil was sampled near a former smelter (MetalEurope, North of France). It contained high levels of heavy metals (Pb, Zn and Cd) due to historical atmospheric deposition from the smelter. This silty-loamy soil, ground and 4-mm-sieved, had the following characteristics: pH_{H2O} 7.7; 15.3 $cmol^{+}\ kg^{-1}$ $CEC_{cobaltihexammine}$; 16.6 g kg^{-1} organic C; 1.2 g kg^{-1} total N; 148 mg kg^{-1} P_{Olsen}; 7.87 mg kg^{-1} total Cd (0.8 mg kg^{-1} NH_4NO_3 extractable Cd); 627 mg kg^{-1} total Zn; 444 mg kg^{-1} total Pb. Metal pollutants had a relatively low availability because of a slightly alkaline pH and an aged pollution. The soil was sterilised by γ-irradiation at 25 kGray to eliminate all indigenous fungi and 10 mL of a 5-μm filtrated unsterilised soil suspension (15 g soil in 150 mL NaCl 0.8%) was added to re-inoculate the indigenous microflora of the soil free of AM fungal spores.

Different mycorrhizal treatments were performed (three replicates per treatment) with five AM fungi and a non-mycorrhizal control. The origins of the fungal isolates are shown in Table 1. Two AM fungi were isolated from heavy-metal-contaminated soils, one from a soil with a low Cd content due to sludge amendment and two from non contaminated soils. Metal tolerance of all the isolates was not quantified as such. However, except *Glomus* sp., the isolates were shown to be able to colonise plants growing in contaminated soil or substrates with Cd concentrations in the same order of magnitude or higher as in the present study (Redon et al. 2008 for *G. intraradices* no. 1; Rivera-Becerril et al. 2002 for *G. intraradices* no. 2; Weissenhorn and Leyval 1995 for *Glomus mosseae* P2 and BEG 12). Using spore germination tests, *G. mosseae* P2 (BEG 69) was shown to be more tolerant to Cd than *G. mosseae* BEG 12 (Weissenhorn et al. 1993): The mean effective Cd concentration at which 50% of the spores failed to germinate (EC50) was 0.8 mg L^{-1} for *G. mosseae* BEG 12 and 7 mg L^{-1} for *G. mosseae* P2. *G. mosseae* P2 was the only isolate of the study propagated in a metal-containing medium. Both *G. intraradices* isolates were inoculated using 100 g of inoculum substrate added to 900 g of soil, whilst the *G. mosseae* and *Glomus* sp. were added as 200 spores per pot. Non-mycorrhizal control, *G. mosseae* and *Glomus* sp. treatments also received 100 g of autoclaved and mixed G.i. no. 1 (35%) and no. 2 (65%) inoculums. Mycorrhizal and non-mycorrhizal plants were grown for 45 days. Three additional non-mycorrhizal (NM) and

Table 1 Isolates of arbuscular mycorrhizal fungi used in the study

Treatment name	Species	No. of BEG	Origin	Inoculum source	Reference
Gi no. 1	*Glomus intraradices*	None	Heavy-metal-rich soil ("Breinigerberg" soil)	Institüt für Pflanzenkultur (Solkau, Germany)	Hildebrandt et al. 1999
Gi no. 2	*Glomus intraradices*	BEG 141	Not polluted soil	V. Gianinazzi-Pearson	Rivera-Becerril et al. 2002
GmP2	*Glomus mosseae* P2	BEG 69	Metal-polluted soil (atmospheric deposition from smelter)	Leek culture on the original soil at LIMOS	Weissenhorn et al. 1993
Gm	*Glomus mosseae*	BEG 12	Not polluted soil	Leek culture on non-contaminated expanded clay at LIMOS	Weissenhorn et al. 1993
Gsp	*Glomus* sp.	None	Soil amended with slightly metal-contaminated sludge	Leek culture on non-contaminated expanded clay at LIMOS	Joner et al. 2000

It is mentioned whether the fungi were isolated for the first time in a metal-polluted or not polluted soil

G. intraradices no. 1 inoculated pots were added to collect data at 30 days of culture.

Pot experiment

Seeds of barrel medics (*M. truncatula* Gaertn. line J5) were surface-sterilised (ethanol 50% for 5 min, calcium hypochlorite 20% for 30 min, chloramine T 2% for 10 min, streptomycin 2%+ one drop of TWEEN 80 for 10 min, seeds were rinsed two times with distilled water after each bath) before germination on autoclaved vermiculite. Five 5-day seedlings were planted per pot and grown under controlled conditions (16-h photoperiod, 22°C/18°C day/night, 400 μmol photons per square metre per second, 70% relative humidity). The culture was thinned at three seedlings per pot after 1 week. Plants were watered daily with distilled water to maintain 70% of water holding capacity (WHC). Every week after 2 weeks of culture, plants were watered with a P-deficient nutritive solution [1 mM NH_4NO_3; 1 mM $Ca(NO_3)_2$,$4H_2O$; 0.1 mM Na_2HPO_4,$2H_2O$; 1 mM K_2SO_4; 0.75 mM $MgSO_4 \cdot 7H_2O$; 12.5 μM H_3BO_3; 2.5 μM $MnSO_4 \cdot 4H_20$; 0.3 μM $CuSO_4 \cdot 5H_2O$; 1 μM $ZnSO_4 \cdot 7H_2O$; 0.05 μM $Na_2MoO_4 \cdot 2H_2O$; 0.2 μM $CoCl_2 \cdot 6H_2O$; 0.2 μM $CoSO_4 \cdot 7H_2O$; 20 μM Fe-EDTA) instead of distilled water. Every 15 days, abundant rainfall was simulated by watering pots at 100% of WHC and the volume of leachates was measured. An aliquot of leaching water was filtered at 45 μm where pH was measured and metal concentrations were determined by graphite furnace atomic absorption spectrometry.

Harvest and analyses

After 30 days or 45 days of culture, shoots were cut and roots were separated from the soil with forceps and by sieving. Roots were rinsed with tap water and then rinsed with ultrapure water. Soils were oven-dried at 60°C. Roots were cut into 1-cm-long segments. Mycorrhizal colonisation of roots (0.7 g of fresh clean roots) was determined by Trypan blue staining as described by Koske and Gemma (1989), and notation was performed using Trouvelot et al. (1986) method. Roots and shoots were oven-dried at 60°C and ground in liquid nitrogen. Root and shoot dry matter was weighed. Dry plant samples (0.2 g) were digested in HNO_3 65% (4 mL) and H_2O_2 30% (2 mL) at high temperature and pressure (170°C, 20 bars) using special Teflon® pressure vessels in a microwave digesting system (MARS 5). The digests were filtered and made up to 50 mL with ultrapure water. Concentrations of P, Pb, Zn and Cd were measured by inductively coupled plasma atomic emission spectroscopy or graphite furnace atomic absorption spectrometry for Cd concentration in shoots.

Extraradical hyphae were extracted from 1 g of rhizospheric soil using the aqueous membrane filtration technique (Jakobsen et al. 1992; Boddington et al. 1999) and hyphal length was estimated after Trypan blue staining by the grid-line intersect method (Jakobsen et al. 1992) over 25 fields of view at 100× magnification. The hyphal background found in non-mycorrhizal pots was subtracted from the values measured in the different mycorrhizal treatments. Presence and approximate number of root nodules were counted at harvest. Presence of nodulating rhizobacteria in AM inoculated or not inoculated roots at harvest and in *G. intraradices* no. 1 inoculum was also assessed by polymerase chain reaction (PCR) amplification of *nodC* gene using nodCF and nodCI primers as described by Laguerre et al. (2001).

Statistical analysis

Analyses of variance between AM treatments were performed with SigmaStat 3.11 using the Tukey's test for mean comparison.

Table 2 Mycorrhiza frequency (*F*%), intensity of colonization (*M*%), hyphal length (m g^{-1}) and number of nodules per pot at harvest (after 30 or 45 days) in the different fungal inoculation treatments

Treatment name	Days of culture at harvest	*F*%	*M*%	Hyphal length (m g^{-1})	Number of nodules
Gi no. 1	45	90±0	44±4	0.82±0.39	117±15
Gi no. 2	45	100±0	63±6	0.80±0.57	0±0
GmP2	45	19±5	3±2	0.28±0.25	0±0
Gm	45	8±13	1±2	0.11±0.16	0.3±0.6
Gsp	45	13±18	3±4	0.32±0.11	0±0
NM	45	0	0	0	0.3±0.6
Gi no. 1	30	42±5	12±1	ND	90±10
NM	30	0	0	ND	5±8

Values are means of three replicates ± standard deviation
ND not determined

Results

Mycorrhiza frequency (*F*%) and intensity of mycorrhizal colonisation (*M*%) were very high in pots inoculated with both *G. intraradices*, much lower in pots inoculated with *G. mosseae* P2 and *Glomus* sp. and very low in *G. mosseae* BEG 12 (Table 2). Non-inoculated plants were not mycorrhizal. The growth of external mycelia in soil was consistent with root colonisation, as hyphal length increased with the mycorrhiza frequency (Table 2). Plant biomass was differently influenced by the fungal strains (Fig. 1). After 45 days of culture, *G. intraradices* no. 1 strongly increased shoot (+216%) and root (+48%) biomass as compared to NM treatment. Both *G. mosseae* and *Glomus* sp. increased shoot biomass to a lesser extent (about +20%). On the contrary, *G. intraradices* no. 2 did not change shoot biomass and decreased root dry weight (−23%). Numerous nodules in pots inoculated with *G. intraradices* no. 1 were observed, whilst very few nodules were observed with the other fungi and in NM treatments (Table 2). Nodulating rhizobacteria were detected in roots, AM inoculated or not, where nodules were observed, but not in *G. intraradices* no. 1 inoculum (data not shown). The different inoculations also showed various effects on P and metal plant contents (Table 3). Strains isolated from metal-polluted sites (*G. intraradices* no. 1 and *G. mosseae* P2) significantly ($P<0.05$) decreased P and Cd and tended to decrease Zn shoot concentrations as compared to NM and other mycorrhizal treatments (Table 3). Pb contents in shoots were below detection limit. Both isolates of *G. intraradices* had opposite effects on P root concentration (Table 3). It was significantly decreased in *G. intraradices* no. 1 pots, whilst it was significantly increased in *G. intraradices* no. 2 pots as compared to NM pots. *G. intraradices* no. 2 tended to reduce non-essential metals (Cd and Pb) absorption by roots, but significantly increased Zn concentration in shoots. *G. mosseae* BEG 12 and *Glomus* sp. had no significant effects on measured elements in plant.

The total Zn plant content was about tenfold higher than the total Cd plant content. The total Cd and Zn transferred to shoots and roots in the different fungal treatments had almost the same pattern; therefore, results are shown for Cd only (Fig. 2). *G. intraradices* no. 1 increased by 2–2.5 times the total Cd (Fig. 2) and Zn (data not shown) contents in shoots and roots. *G. intraradices* no. 2 decreased total Cd and Zn root contents, whilst the other isolates did not modify the metal content in plant as compared to NM treatment, except in *G. mosseae* treatment where the total Cd root content was increased. *G. intraradices* no. 1 and NM treatments were compared 30 and 45 days after planting. Forty-five-day-old mycorrhizal medics (8.37 g shoot dry weight, dw) were fourfold bigger than 30-day-old mycorrhizal medics (2.07 g shoot dw), whilst non-mycorrhizal medics were 1.6-fold bigger after 45 days (2.65 g shoot dw) than after 30 days (1.6 g shoot dw). After 30 days, decreased shoot metal concentrations (−23% Cd and −20% Zn) were observed in mycorrhizal pots as compared to NM pots, but the total Cd (and Zn) shoot and root contents were not significantly different between *G. intraradices* no. 1 and NM (Fig. 2). Aboveground parts of *G. intraradices* no. 1 inoculated medics accumulated 4.2-fold less Cd after 30 days than after 45 days.

Whilst pHs of leachates were not different after 11 days and 24 days of culture, they were variable between fungal treatments after 39 days (Table 4). At this time of growth, *G. intraradices* no. 1 significantly decreased pH of leachates as compared to other fungi and NM pots. Cd concentrations in leachates were higher after 39 days than

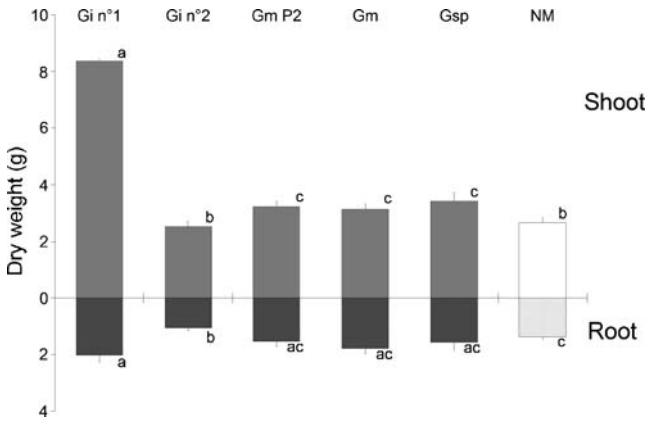

Fig. 1 Shoot and root dry weights (g) of 45-day-old *M. truncatula* inoculated with different AM fungal isolates (*Gi Glomus intraradices*, *Gm Glomus mosseae*, *Gsp Glomus* sp.) or not inoculated (*NM*). Values are means of three replicates. *Error bars* are standard deviations. *Bars with the same letter* are not significantly different at the 5% level according to the Tukey's test

Table 3 Concentrations of metals (Cd, Zn, Pb) and phosphorus in shoots and roots of 45-day-old *M. truncatula* inoculated with different AM fungal isolates or not inoculated (NM)

Inoculated fungus	Cd (μg g^{-1})	Zn (μg g^{-1})	Pb (μg g^{-1})	P (mg g^{-1})
Shoots				
Glomus intraradices no. 1	1.99±0.18 a	36.7±1.9 b	<d.l.	2.75±0.29 a
Glomus intraradices no. 2	3.08±0.22 bc	57.9±1.9 a	<d.l.	3.36±0.07 bd
Glomus mosseae P2	2.55±0.23 b	38.8±1.3 b	<d.l.	3.12±0.18 ad
Glomus mosseae	3.14±0.23 c	48.8±4.3 c	<d.l.	3.64±0.20 bc
Glomus sp.	2.95±0.20 bc	43.3±1.4 c	<d.l.	3.60±0.06 c
NM	3.29±0.39 c	45.8±7.1 bc	<d.l.	3.78±0.36 bc
Roots				
Glomus intraradices no. 1	50.4±1.9 a	486±26 a	116±22 a	3.24±0.16 a
Glomus intraradices no. 2	34.6±1.4 b	363±21 b	53±6 b	8.72±0.09 b
Glomus mosseae P2	42.5±3.8 c	322±20 b	75±9 c	4.67±0.64 c
Glomus mosseae	44.6±6.4 abc	327±23 b	121±31 ac	5.40±0.48 c
Glomus sp.	37.5±2.2 bc	320±43 b	123±47 abc	5.26±0.38 c
NM	43.8±6.6 abc	323±45 b	153±69 abc	5.50±0.17 c

Values are means of three replicates ± standard deviation. Means in the same column followed by the same letter are not significantly different at the 5% level according to the Tukey's test
<*d.l.* under detection limit (<2 μg g^{-1})

after 11 and 24 days. Cd concentrations after 39 days of culture ranged from 19.2 to 54.5 μg L^{-1} and were significantly higher with *Glomus* sp. and *G. mosseae* than with both *G. intraradices* isolates and NM treatment. Zn concentrations in leachates were about tenfold higher than Cd concentrations, but were not presented because they were of the same order of magnitude as additional Zn brought by the nutrient solution.

Discussion

Many agricultural soils are contaminated by heavy metals and such situations need careful attention. Whilst the studied soil was not extremely contaminated (as compared to industrial waste lands), the NH_4NO_3 extractable Cd concentration was higher than the German limitation of 0.1 mg kg^{-1} (BbodSchV 1999), and Cd concentrations measured in shoots (a minimum of 0.3 mg kg^{-1} fresh weight was observed in *G. intraradices* no. 1 treatment) were above the maximum levels in vegetables and cereals (0.05 to 0.2 mg kg^{-1} fresh weight depending on the category of foodstuff) defined by the European Commission no. 466/2001 for human consumption of foodstuffs. Zn and Pb total soil concentrations are above the precaution values of metals given by the German Federal Soil Protection (BbodSchV 1999), and Zn root concentration in *M. truncatula* was in the range of chronic toxicity (NOEC, 300 μg g^{-1}) reported for *Medicago sativa* (Boawn and Rasmussen 1971). Furthermore, in a previous study, the growth of non-mycorrhizal medics was shown to be lower in this soil than in artificially Cd-contaminated soils

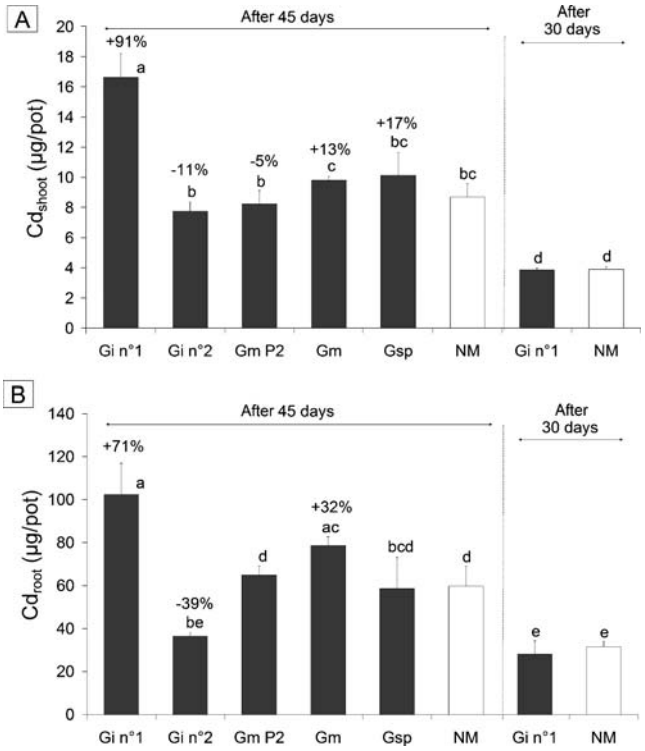

Fig. 2 Total Cd content (microgram per pot) in shoots (Cd_{shoot}) (**a**) and roots (Cd_{root}) (**b**) of *M. truncatula* inoculated with different AM fungal isolates (*Gi Glomus intraradices*, *Gm Glomus mosseae*, *Gsp Glomus* sp.) or not inoculated (*NM*) after 45 days or 30 days. Values are means of three replicates. *Error bars* are standard deviations. *Bars with the same letter* are not significantly different at the 5% level according to the Tukey's test

Table 4 pH and Cd concentrations of leachates after 11, 24 and 39 days of culture

	$pH_{leachates}$			$[Cd]_{leachates}$ (μg L^{-1})		
	11 days	24 days	39 days	11 days	24 days	39 days
Glomus intraradices no. 1	7.65±0.19 a	7.54±0.07 ab	7.08±0.15 c	7.6±2.3 ab	11.3±2.0 abe	19.2±8.4 de
Glomus intraradices no. 2	7.36±0.03 b	7.47±0.04 ab	7.65±0.09 a	8.1±3.2 ab	11.3±3.5 abe	29.6±11.4 cd
Glomus mosseae P2	7.39±0.07 b	7.43±0.07 ab	7.34±0.06 b	6.2±1.4 ab	10.2±2.9 abe	36.5±2.9 cd
Glomus mosseae	7.48±0.09 ab	7.50±0.04 ab	7.41±0.07 ab	7.4±0.8 ab	12.3±1.7 be	45.8±13.2 c
Glomus sp.	7.51±0.06 ab	7.53±0.02 ab	7.54±0.03 ab	8.9±1.9 ab	11.7±1.6 abe	54.5±3.8 c
NM	7.56±0.08 ab	7.46±0.04 ab	7.49±0.11 ab	5.7±2.2 a	11.2±1.4 abe	31.0±5.5 cd
Analysis of variance	*F* ratios/Significance					
Fungus	3.50*			3.69**		
Days of culture	4.42*			166.89***		
Fungus × days of culture	9.24***			2.09 n.s.		

Values are means of three replicates ± standard deviation. Means followed by the same letter are not significantly different at the 5% level according to the Tukey's test. Data were log-transformed for the analysis of variance of Cd concentrations in leachates
n.s. not significantly different
*$p<0.05$; **$p<0.01$; ***$p<0.001$

with the same concentration range (Redon et al. 2008), suggesting a relative increased toxicity due to the multi-pollution with Cd, Zn and Pb in the soil.

Within these soil conditions, differential behaviour of AM isolates in response to abiotic stress were observed, leading to different impact on plant tolerance to metal contamination. Growth of the model legume *M. truncatula* was improved by four of the five inoculated fungi, although P was not limiting, as illustrated by no higher P concentrations in mycorrhizal plants. Indeed, agricultural soils are often overfertilised, especially in the case of soils with high levels of heavy metals where farmers increase pH to reduce metal bioavailability. However, origin and stress adaptation of fungi may have played a key role on metal transfer and root colonisation by fungi. On one hand, mycorrhizal plants inoculated with isolates from soils containing high levels of metals (i.e. *G. intraradices* no. 1 and *G. mosseae* P2) showed lower Cd and Zn concentrations in shoots than plants inoculated with isolates from not contaminated soils. This is consistent with previous observations like results obtained by Diaz et al. (1996) on Zn and Pb uptake by *Lygeum spartum* and *Anthyllis cytisoides*. Fungal isolates from contaminated areas seemed to have developed a strategy adapted to metal stress. The high metal-binding capacity of *G. mosseae* P2 external hyphae (Joner et al. 2000) may be one mechanism allowing the decrease of metal shoot concentrations. Nevertheless, the influence of this metal-tolerant fungus on plant fitness and Cd transfer seemed to vary according to the host plant and growth substrate. Weissenhorn and Leyval (1995) reported no significant effects of *G. mosseae* P2 on maize grown in sand contaminated with less than 2 mg L^{-1} of Cd as compared to non-mycorrhizal treatment. The same fungus had also no significant effect and even tended to decrease shoot biomass of *Trifolium subterraneum* grown in a soil contaminated with 200 mg Zn kg^{-1} and 2 mg Cd kg^{-1} (Tonin et al. 2001). Orlowska et al. (2005) pointed out the better ability of AM fungi from metal-contaminated soils to colonise roots in heavy-metal-polluted soils and insisted upon the importance of selection of the most efficient inoculum. Our data confirmed previously shown results that fungi isolated from metal contaminated soils increase metal tolerance of plants growing in polluted soils by decreasing plant concentration. This was true for *G. intraradices* no. 1 and *G. mosseae* P2.

However, for *G. intraradices* no. 1, the lower Cd and slightly lower Zn concentrations in shoots might be at least partly the result of a dilution effect due to the higher growth rate. The higher biomass of *G. intraradices* no. 1 inoculated plants was associated to the presence of numerous root nodules. The PCR amplification of the *nodC* gene indicated that nodulating bacteria were not brought by *G. intraradices* no. 1 inoculum. Root nodulation by indigenous bacteria could thus have been stimulated by this fungal isolate, whilst the other fungal isolates did not. Interactions between bacteria and fungi were often studied (Artursson et al. 2006) and may play a crucial role in crop system. Association with plant growth promoting rhizobacteria (PGPR) was also shown to give beneficial effects to mycorrhizal plant tolerance to Cd contamination (Vivas et al. 2003). Thus, not only fungal tolerance and ability to reduce metal concentrations in plants but also AM interactions with PGPR might contribute to the improved plant biomass and reduced metal concentration in *M. truncatula* by *G. intraradices* no. 1. *Rhizobium* are sensitive to Cd (EC_{50} range from 1.5 to 9.5 μM; Neumann et al. 1998), but metal-tolerant isolates of *Rhizobium* were also reported (Lakzian et al. 2002). This major plant growth effect due to AM inoculation might be

due to a N response, even if the total N content in soil was not low (typical of agricultural soils) and was enriched by weekly fertilisation during the experiment, but could also be intensified by a fungal response to heavy metal stress. Both nutritional and metal response mechanisms could thus have occurred.

On the other hand, when fungal strains are not metal-tolerant, they may have some difficulties to colonise roots because metal toxicity reduces spore germination of non-tolerant isolates (Leyval et al. 1997). It may be a reason why inoculation with spores of *G. mosseae* BEG 12, which was not previously exposed to high levels of heavy metals and seemed to have a low tolerance (Weissenhorn et al. 1993), led to very low levels of colonisation. Metal toxicity can affect AM fungi at different life stages, from spore germination to hyphal elongation and root colonisation; tolerance can thus occur at different levels and is difficult to define. Fungal isolates can also tolerate high levels of metals without being natives of metal polluted areas and strongly colonise plants growing on a contaminated medium. Indeed, inoculation with *G. intraradices* no. 2 (BEG 141) led to very high frequency of mycorrhization (*F*%=100%), whilst it was not previously exposed to metals. Such a high level of infection tended to affect plant development, as plant biomass was reduced by the presence of *G. intraradices* no. 2 as compared to other treatments. Previous studies showed different effects of *G. intraradices* BEG 141 (*G. intraradices* no. 2) colonising *Pisum sativum* in a 100 mg Cd kg^{-1} contaminated soil/sand mix. The total amount of Cd transferred to shoots was enhanced in mycorrhizal pots, but it depended on the pea genotype (Rivera-Becerril et al. 2002). The comparison between *G. intraradices* no. 1 and no. 2, which had both well-colonised roots and soil, showed that the two isolates had different effects on root Cd and Zn concentrations. The fungus isolated from a metal-contaminated soil reduced Cd concentration in shoots and enhanced Zn concentration in roots, whilst the other did not significantly affect Cd plant uptake and increased Zn concentration in shoots. It thus seems that even though both fungi showed a high colonisation rate and should be considered as tolerant to Cd and Zn concentrations present in the soil, metal uptake capacity differed between them and depended on the origin of the fungal isolates. Finally, the fungus isolated from a soil containing low concentrations of metals, *Glomus* sp., colonised roots at the same rate as *G. mosseae* P2, but although it slightly increased plant growth, this fungus had no significant effect on plant metal uptake. This could be due to the lower capacity for Cd sorption by *Glomus* sp. hyphae than *G. mosseae* P2, as previously shown by Joner et al. (2000).

The different behaviour of fungal isolates also raises the question of the choice of plant–fungus associations to study gene expression during mycorrhizal symbiosis in metal stress conditions. The response of a studied plant–fungus combination to metal stress may be different from one another because of the differential effects of fungal strains and also because plant genotypes differ in their ability to be colonised by AM fungi (Lingua et al. 2008).

Not only metal concentrations but also the total mass of metals transferred from soil to plant is important to consider when evaluating heavy metal partitioning in soil–plant systems. The total shoot metal content depends on plant biomass, and the ability of mycorrhizal inoculation to affect plant growth seems to be the most important parameter concerning metal accumulation in plant. In previous studies, when plant biomass was sufficiently enhanced by fungal inoculation, mycorrhizae contributed to increase the total amount of Cd transferred to plants (Heggo et al. 1990; Lee and George 2005; Redon et al. 2008). In the present experiment, the total Cd and Zn contents in the above-ground biomass were significantly increased only with *G. intraradices* no. 1 where plant biomass was strongly enhanced. Plant age at harvest is also important to consider when metal uptake experiments are performed. Even if plant biomass was increased and shoot Cd and Zn concentrations were reduced in *G. intraradices* no. 1 treatment as compared to NM treatment after both 30 and 45 days, the quantities of Cd and Zn exported to shoots were significantly higher only after 45 days. When plants were younger, the AM fungus did not improve plant biomass sufficiently to increase metal accumulation in shoots.

Although the quality of groundwater is very important for human consumption, very little information is available about AM fungal influence on metal transfer in leaching water. We recently showed that fungal inoculation with *G. intradices* no. 1 reduced Cd concentration in leachates (Redon et al. 2008). In pots inoculated with fungi isolated from contaminated soils, pH of leachates was slightly decreased, which may have modified the behaviour of ionic elements. Such pH decrease should increase the availability of Cd in soil (Krishnamurti and Naidu 2003), as well as Zn, which may increase the risk of transfer to leachates and plant. However, inoculation with *G. intraradices* no. 1 did not increase, even tended to decrease, Cd concentration in leachates as compared to NM pots because it increased plant biomass and Cd uptake. Glomalin, a glycoprotein released by AM fungi (Wright and Upadhyaya 1998), may also have played a role in Cd sequestration (González-Chávez et al. 2004) and reduction of Cd transfer in leachates. Cd concentrations in leachates were very variable among and within fungal treatments after 39 days of culture. Differences among treatments were observed after 39 days of culture, but not after 11 and 24 days. Changes in pH and Cd concentrations of leachates can be due to the development of the root system and plant growth that may be influenced by mycorrhizal colonisation. Cd is toxic at

low concentrations (Wren et al. 1995), and limitation in water for human consumption is 5.0 μg L^{-1} as defined by the European directive 98/83/CE. As a result, a few dozen micrograms per litre modification of Cd concentrations in leachates by AM fungal inoculation may be determinant for water quality. Zinc concentrations in leachates were below the guideline value of 3 mg L^{-1} in drinking water acceptable to consumers as proposed by the World Health Organization (2003) based on taste considerations; toxic concentrations are by far higher than this value.

In conclusion, contribution of AM fungi on Cd and Zn transfer to the model legume *M. truncatula* varied according to the fungal isolate used as inoculum. Fungi isolated from metal-polluted soils were more effective to reduce metal stress by decreasing shoot metal concentrations. This was not due to a higher ability of these fungi to colonise roots and to form external hyphae. However, the large improvement of shoot biomass by one isolate led to an increased plant metal uptake (+91% Cd to shoots and +71% Cd to roots with *G. intraradices* no. 1 which increased by three the shoot biomass). Such improvement of plant biomass can be due to a close relationship between AM fungi and N-fixing nodulating bacteria. Finally, Cd concentrations in leachates were variable among fungal treatments but can be significantly affected by AM inoculation. Soil modification after fungal colonisation of the root system may certainly have impact on water quality.

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