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Effects of AM Inoculation and Organic Amendment, Alone or in Combination, on Growth, P Nutrition, and Heavy-Metal Uptake of Tobacco in Pb-Cd-Contaminated Soil

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Abstract As toxic pollutants commonly found in tobacco (*Nicotiana tabacum* L.) products, lead (Pb) and cadmium (Cd) can enter the human body via smoking and thus pose a potential health risk to smokers. We conducted a greenhouse experiment to study the effects of arbuscular mycorrhizal (AM) inoculation with *Glomus intraradices* BEG 141 and organic amendment with cattle manure, alone or in combination, on the growth, P nutrition, and heavy-metal uptake by tobacco plants grown in soil to which was added Pb-Cd at 0/0, 350/1, 500/10, and 1,000/100 mg kg^{-1}, respectively. In general, AM colonization and plant growth were greatly reduced by Pb-Cd contamination, whereas organic amendment alleviated Pb-Cd stress and showed some beneficial effects on AM symbiosis and some soil parameters. AM inoculation, alone or in combination with organic amendment, increased plant dry weights and improved P nutrition significantly at all Pb-Cd addition levels, and, in most cases, it decreased Pb and Cd concentrations in tobacco plants and DTPA-extractable concentrations in soil. AM inoculation increased total glomalin-related soil protein (GRSP) concentrations in soil to which Pb-Cd was added. The higher soil pH and GRSP contents and the lower DTPA-extractable Pb and Cd concentrations contributed by AM inoculation and/or organic amendment may be contributing factors that lead to higher growth promotion and lower metal toxicity and uptake by plants. Our findings suggest that AM inoculation in combination with organic manure may be a potential method for not only tobacco production but phytostabilization of Pb-Cd-contaminated soil.

Keywords Arbuscular mycorrhizal fungi · Heavy-metal pollution · Organic manure · Cadmium · Lead · Glomalin

Introduction

Lead (Pb) and cadmium (Cd) are considered the most toxic pollutants and nonessential elements without a known biological function. They easily accumulate in soil because they cannot be biodegraded and they may be taken up by plants grown in soil contaminated by them and enter human and animal bodies via the food chain. In general, excessive Pb and Cd could damage plants by interfering with biochemical and physiological processes. Plants grown in soil polluted with Pb and Cd usually have lower biomass and poorer mineral nutrition and quality.

Most nonaccumulating plants, including crops, bind mainly heavy metals in their roots. However, the tobacco plant is well known for its capacity to concentrate heavy metals from the environment in which it grows (Keller and others 2003) and accumulate high levels of heavy metals, particularly Cd, in leaves and shoots (Lugon-Moulin and others 2004, 2006). Tobacco grown in soil with higher available Cd and Pb levels correspondingly has higher levels of these metals in leaves (Adamu and others 1989; Lugon-Moulin and others

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2006). There have been numerous reports that cigarettes contain significant levels of various toxic metals such as Cd, Pb, and arsenic (As) (Stephens and Calder 2005; Pappas and others 2007; O'Connor and others 2010), resulting in greater exposure of smokers to toxic heavy metals and health risks.

Heavy-metal uptake by tobacco plants depends mainly on the concentrations of these metals in the soil and the factors influencing their bioavailability, such as soil amendments (Adamu and others 1989) and soil pH (Mulchi and others 1987). In addition, organic matter and soil microorganisms also influence metal bioavailability and hence metal content in tobacco. Many studies have shown that organic amendments with compost, farmyard manure, and biosolid compost reduced the availability of heavy metals in soil due to a high content of organic matter, phosphorus (P) and iron (Fe) (Brown and others 2003; Singh and others 2010), with the promising potential to decrease toxic metal content in tobacco plants (Lugon-Moulin and others 2004).

Soil microorganisms, notably arbuscular mycorrhizal (AM) fungi, are also able to alter heavy-metal bioavailability and the plant response to heavy metals. Numerous studies have demonstrated that AM symbioses contribute to a plant's metal tolerance, metal translocation, and uptake via diverse pathways, but the reported effects have not been consistent between different studies, and the mechanisms remain to be fully elucidated (González-Guerrero and others 2009). There have also been reports that AM inoculation decreased Cd and Pb concentrations in tobacco leaves (Janoušková and others 2005a, b, 2007; Sudová and others 2007), indicating that AM fungi may be used to decrease toxic metal residues in tobacco leaves, but the effects varied with AM fungal isolates, tobacco varieties, metal contamination levels and soil conditions. To our knowledge, little is known about the AM effects on heavy-metal uptake by tobacco under Pb-Cd combined contamination.

In most cases, organic manure and slow-release fertilizers do not suppress AM fungi and may even stimulate them (Gosling and others 2006). Many studies reported synergistic effects of AM fungi and organic amendments to improve soil quality and plant performance under stress conditions, in degraded or polluted soil (Medina and Azcón 2010; Alguacil and others 2009, 2011). However, whether AM inoculation and organic amendment can jointly function on heavy-metal uptake by plants remains to be elucidated. In this study we hypothesized that AM inoculation and organic amendments may play synergistic roles in growth promotion and reduction of metal content in tobacco. Thus, the objectives of the present study were (1) to evaluate the effect of the AM fungus *Glomus intraradices* BEG 141 and organic amendment with cattle manure on growth, P nutrition, heavy-metal concentrations in tobacco, and soil parameters (pH, heavy-metal availability, and glomalin contents) at different contamination levels of Pb-Cd, and (2) to identify whether there is a synergistic interaction between AM inoculation and organic manure.

Materials and Methods

Mycorrhizal Inoculum, Cattle Manure, and Soil

Inoculum of the AM fungus *Glomus intraradices* BEG 141, kindly provided by China Agricultural University, was propagated on sudangrass [*Sorghum sudanese* (Piper) Stapf.] grown in autoclaved (121°C for 1 h on three successive days) sand for three successive propagation cycles, each 4 months long (Wang and others 2011). At the same time, the control nonmycorrhizal inoculum was also prepared with the same sterilized substratum on which sudangrass was cultivated. The inocula were air-dried and sieved (2 mm), and each consisted of a mixture of rhizospheric soil from pure pot culture containing spores, hyphae, and mycorrhizal root fragments (the control without AM propagules).

Cattle manure provided from the Institute of Soil Science, Chinese Academy of Sciences, was used to prepare the organic amendments for the contaminated soil. The air-dried cattle manure was sieved through a 2-mm mesh and stored at 4°C prior to use. The physicochemical properties were pH 6.89, total organic matter 169.3 g kg^{-1}, total nitrogen 15.10 g kg^{-1}, total phosphorus 3.71 g kg^{-1} and total potassium 4.92 g kg^{-1}.

The calcareous soil was collected from an experimental field (0–15 cm deep) at the Henan University of Science and Technology. After sifting through a 2-mm sieve, the soil was fumigated thoroughly with 10% formaldehyde under airtight plastic sheets for 5 days and the fumigant allowed to dissipate for 10 days. The soil was classified as Aquic Ustochrepts (US soil taxonomy) and soil texture was loamy, with a pH (1:2.5 soil/water) of 7.9, 1.71% organic matter, 50.0 mg kg^{-1} alkali-hydrolyzable N, 21.4 mg kg^{-1} Olsen P, 120.0 mg kg^{-1} 1 M NH_4OAc extractable K, 1.95 mg kg^{-1} diethylene triamine pentacetate acid (DTPA)-Pb, 11.15 mg kg^{-1} total Pb, 0.039 mg kg^{-1} DTPA-Cd, and 0.150 mg kg^{-1} total Cd.

Preparation of Mycorrhizal and Nonmycorrhizal Tobacco Seedlings

Seeds of tobacco (*Nicotiana tabacum* L. var. K326) were provided by Qingdao China Tobacco Seed Limited Liability Company. Uniform-sized seeds were surface-sterilized with 10% (v/v) H_2O_2 for 10 min and subsequently washed several times with distilled water. Then the seeds were sown in polystyrene trays (10 × 20 cells, each cell 2.4 × 5.0 cm) filled with AM inocula or equivalent nonmycorrhizal inocula in a greenhouse on May 23, 2010. One seedling was left after emergence (May 30, 2010) and fertilized with 1/4-strength Hoagland solution. Several seedlings were sampled 3–4 weeks after emergence and the root mycorrhizal colonization rate was determined by the acid fuchsin staining-grid intersect methods (Wang and others 2007). After the roots were colonized significantly (>30%), seedlings of uniform size were selected for the procedure discussed below.

Experimental Procedure

According to China Soil Environmental Quality Standards (GB15618–1995), soil containing 350 and 500 mg kg^{-1} Pb belongs to the second and third criteria, respectively, generally representing slight and moderate pollution. In this study, a pollution level of 1,000 mg kg^{-1} was designed to simulate heavy pollution. It is estimated that 1.3×10^4 ha of arable land has been polluted with Cd in China (Chen and others 1999; Zhao and others 2005). The Cd concentration in the polluted lands varied, ranging from less than 1 mg kg^{-1} to greater than 100 mg kg^{-1}. Considering the above background and our study's goals, four addition combinations of Pb-Cd (0/0, 350/1, 500/10, and 1,000/100 mg kg^{-1}, representing no, slight, moderate, and heavy pollution, respectively) were applied in an analytical grade $Pb(NO_3)_2$ and $Cd(NO_3)_2$ solution, mixed thoroughly with the soil. An appropriate amount of $Ca(NO_3)_2$ was added to the soil of the first three addition levels to keep the final NO_3^- contents in all treatments equal. The solutions were prepared in deionized water and mixed with the soil manually.

This experiment included four treatments, that is, the control (C), AM inoculation (M), and organic amendment (N) either alone or in combination (MN). For organic amendment treatment (N) and combination treatment (MN), 15 g of air-dried cattle manure was added to each plastic pot and mixed thoroughly with soil. Each pot (1.2-L volume) was filled with 1,500 g of air-dried soil (with or without cattle manure). The six-week-old nonmycorrhizal and mycorrhizal tobacco seedlings were transplanted in the pots of control and inoculated treatments, respectively, on July 20, 2010. Thus, there were 16 treatments in total with four replicates, giving a total of 64 pots in a randomized block design. The seedlings were arranged in a non-environment-controlled greenhouse and irrigated with tap water to maintain about 70% of field-water-holding capacity. Each pot was watered every 4 weeks with 100 ml of Hoagland solution after transplant.

Plants were harvested after 10 weeks of growth on October 2, 2010. Shoots and roots were separated. Subsamples of fresh roots were taken to assess the root colonization rate using the acid fuchsin staining-grid intersect methods. The fresh weight of total roots and of subsamples was measured. Shoots and remaining roots were rinsed first with tap water and then with deionized water and wiped with tissue paper. They were subsequently weighed after oven drying at 70°C for 48 h and then ground to less than 0.25 mm in a stainless-steel mill. The percent of water content of the remaining roots and total root fresh weight was used to estimate total root dry weight. Soil from each pot was thoroughly mixed after root harvest and 100 g of fresh soil was sampled and air-dried for analysis.

Plant and Soil Analysis

The dried materials were wet-digested with a mixture of concentrated HNO_3 and $HClO_4$ (4:1 v/v, guaranteed reagent) mixed acid. The plant Pb and Cd concentrations, as well as soil DTPA-extractable concentrations, were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, Varian AA240). The concentration of P in the digested solution was measured using vanadium–molybdenum yellow colorimetry. Soil pH was determined in a 1:2.5 (w/v) soil/water suspension using an electrode pH meter. Total glomalin-related soil protein (GRSP) in soil after harvest was extracted and assayed from soils according to Wright and Upadhyaya (1996) and Wright and Jawson (2001). Briefly, 0.5 g of air-dried soil in 8 ml of 50 mM citric acid at pH 8.0 was autoclaved at 121°C for 60 min and then centrifuged at $10{,}000 \times g$ for 6 min to remove soil particles. The same soil sample was extracted three times using the same extraction process and all the supernatants were collected together. The GRSP concentration in the extracts was determined by the Bradford assay using bovine serum albumin as a standard.

Statistical Analysis

Data (mean ± SE, $n = 4$) were subjected to a one-way or three-way ANOVA using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA was used to compare the significance among all means in different treatments at all different addition levels of Pb-Cd, whereas three-way ANOVA was used to analyze the effect of interactions between organic amendment, AM inoculation, and Pb-Cd addition level at $p < 0.05$, 0.01, or 0.001.

Results

Mycorrhizal Colonization

Tobacco plants in the nonmycorrhizal controls and that underwent organic amendment treatments were not colonized. Root colonization rates of tobacco plants in MN treatment did not differ significantly between the four

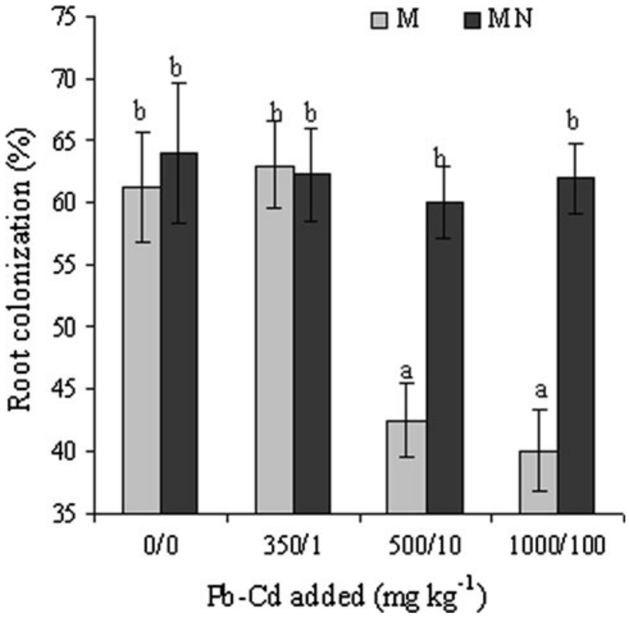

Fig. 1 Root mycorrhizal colonization (%) (mean ± SE, $n = 4$) of tobacco plants. M and MN represent inoculation with *G. intraradices* BEG 141 and combination treatment with AM inoculation and organic amendment, respectively. Different letters on the bar indicate significant differences among all means in different treatments at all Pb-Cd addition levels using Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA

addition levels, whereas those of plants in M treatment did decrease markedly at addition levels of 500/10 and 1,000/100 mg kg^{-1} (Fig. 1).

Plant Dry Weights and P Concentrations

Generally, shoot and root dry weights of the tobacco plants were significantly influenced by all factors (manure, inoculation, Pb-Cd addition), and all the interactions between them (except manure–mycorrhiza interaction) (Table 1). Comparing the *F* values of the factors, inoculation displayed the most significant effect among the three factors.

Shoot and root dry weights (except those in MN treatment) showed a decreasing trend with the increase of addition levels, and dramatically decreased at the 1,000/100-mg kg^{-1} addition level, especially for C and N treatments (Fig. 2). In MN treatment, plant dry weights were not influenced by Pb-Cd addition (except shoot dry weight at the 1,000/100-mg kg^{-1} addition level). Compared with the controls, N, M, and MN treatments all significantly increased plant dry weights at all four addition levels, with the most pronounced effect found with MN treatment (Fig. 2). The average shoot yields of tobacco in the N, M, and MN treatments were 1.9, 3.1, and 4.0 times of those of the controls, respectively. In general, the growth promotion effects of M and MN treatments were more significant when more Pb-Cd was added.

As seen in Table 1, both shoot and root P concentrations were significantly influenced by Pb-Cd addition and inoculation but not significantly by manure. There was a significant interaction effect on shoot P concentration between manure and inoculation. One-way ANOVA results showed that addition of 1,000/100 mg kg^{-1} Pb-Cd decreased shoot and root P concentrations (except those in M treatment) (Table 2). Compared with the controls, organic amendment alone did not improve shoot or root P concentrations. In most cases, MN treatment improved plant P nutrition, whereas the effect of M depended on Pb-Cd addition levels and was positive only at 1,000/100 mg kg^{-1}.

Table 1 Significance levels (*F* values) of organic amendment, AM inoculation, Pb-Cd addition level, and their interactions on measured variables on a three-way ANOVA analysis

Variables	N	M	H	N×M	N×H	M×H	N×M×H
Shoot DW	317.0***	1,694.9***	213.8***	0.0 ns	12.2***	57.4***	14.0***
Root DW	144.4***	1,496.9***	141.8***	3.3 ns	17.6***	32.1***	9.3***
Shoot P conc.	1.7 ns	65.5***	15.9***	5.8*	0.8 ns	0.9 ns	0.4 ns
Root P conc.	0.6 ns	26.1***	34.1***	0.4 ns	1.5 ns	3.3*	4.1*
Shoot Pb conc.	39.6***	470.2***	1,509.1***	51.5***	8.0***	313.0***	9.0***
Root Pb conc.	78.6***	99.0***	1,085.5***	0.7 ns	13.6***	49.6***	7.0***
Shoot Cd conc.	6.5*	182.3***	1,846.3***	0.0 ns	16.7***	43.1***	7.0***
Root Cd conc.	1.1 ns	81.3***	1,309.7***	0.93 ns	0.8 ns	32.8***	1.2 ns
DTPA-Pb conc.	24.4***	22.4***	810.2***	0.0 ns	1.1 ns	2.1 ns	9.1***
DTPA-Cd conc.	4.5*	19.3***	5,951.7***	9.0**	0.9 ns	4.0*	2.9*
Total GRSP	71.3***	29.2***	0.4 ns	3.6*	0.0 ns	0.3 ns	0.1 ns
Soil pH	59.5***	402.5***	56.3***	4.6*	4.3**	27.2***	0.6 ns

N, M, and H represent organic amendment with cattle manure, inoculation with *G. intraradices* BEG 141, and Pb-Cd addition level, respectively

Significant effects: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; *ns* nonsignificant effect

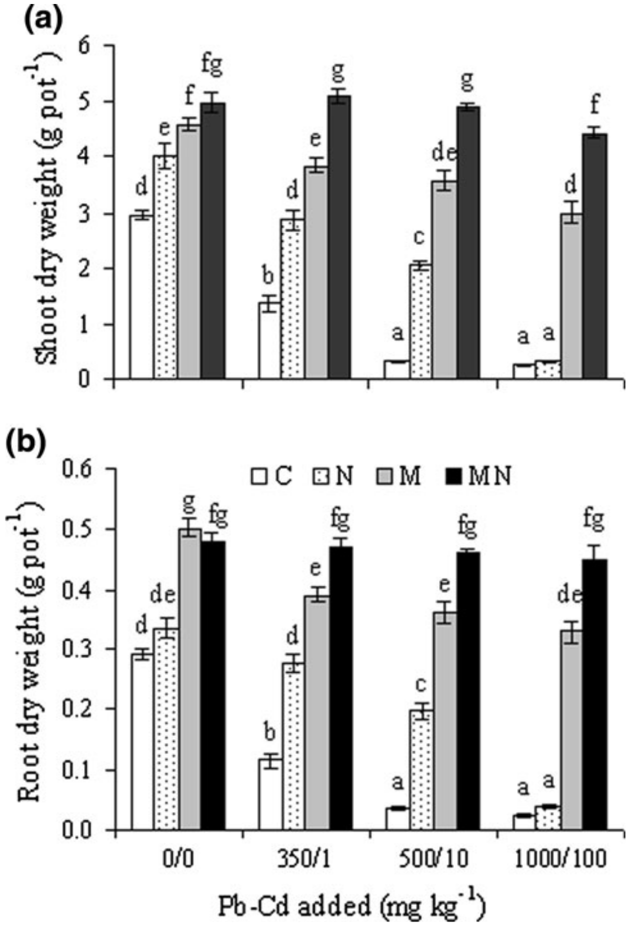

Fig. 2 Shoot (**a**) and root (**b**) dry weights (mean ± SE, $n = 4$) of tobacco plants. C, N, M, and MN represent the control treatment, organic amendment with cattle manure, inoculation with *G. intraradices* BEG 141, and combination treatment with AM inoculation and organic amendment, respectively. Different letters on the bar indicate significant differences among all means in different treatments at all Pb-Cd addition levels using Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA

Pb and Cd Concentrations in Tobacco Plants

On the whole, shoot Pb concentrations were significantly influenced by each of the three factors separately and all their interactions (Table 1). The concentrations of Pb in the root and Cd in the shoot were also significantly influenced by the three factors and the interactions between them (except manure–inoculation interaction), whereas Cd concentrations in the root were significantly influenced only by inoculation, Pb-Cd addition, and their interactions. *F* values showed that Pb-Cd addition levels had the most significant effects on both Pb and Cd concentrations in the plants.

At the zero addition level, Pb was not detected in tobacco shoots from the four treatments and in roots of M and MN treatments. With the increase of addition levels, root Pb concentrations significantly increased, whereas shoot Pb concentrations increased only in C and N treatments and changed slightly in M and MN treatments (Table 3). Pb concentrations were much higher in the roots than in the shoots. Compared with the controls, N and MN treatments significantly decreased shoot and root Pb concentrations when Pb-Cd was added (except N treatment at 1,000/100 mg kg^{-1}). M treatment decreased shoot Pb concentrations but showed significant effects on root Pb only at the 1,000/100-mg kg^{-1} addition level.

Cd was detected in the shoots and roots even without Pb-Cd addition. Cd concentrations were much higher in roots than in shoots at the 1,000/100-mg kg^{-1} addition level, whereas the opposite result was observed at other addition levels (Table 4). Compared with the controls, shoot Cd concentrations markedly decreased in M and MN treatments, whereas in N treatment, they decreased only at the 500/10-mg kg^{-1} addition level and did not change at other addition levels. When Pb-Cd was added, all treatments with N, M, and MN significantly decreased root Cd concentrations (except N treatment at 1,000/100 mg kg^{-1}).

Table 2 Shoot and root P concentrations (mg kg^{-1}) of tobacco plants

		Pb-Cd added (mg kg^{-1})			
		0/0	350/1	500/10	1000/100
Shoot	C	417.4 (24.0)bc	351.7 (29.1)b	343.2 (32.8)b	220.7 (20.0)a
	N	387.3 (45.8)b	396.3 (17.9)bc	393.8 (24.2)bc	244.3 (21.4)a
	M	448.2 (25.8)c	451.4 (23.0)c	417.7 (33.3)bc	370.9 (18.5)bc
	MN	545.4 (23.0)d	557.9 (23.0)d	548.8 (21.2)d	425.7 (8.1)c
Root	C	540.6 (39.9)d	460.8 (10.1)c	385.1 (38.1)bc	309.4 (37.1)a
	N	468.0 (10.9)c	410.4 (21.1)bc	403.1 (39.9)bc	362.1 (34.8)ab
	M	507.3 (25.2)cd	470.1 (25.1)c	454.4 (20.9)c	435.0 (29.1)c
	MN	541.6 (13.2)d	462.2 (32.1)c	425.3 (16.6)c	432.2 (11.4)c

Values are mean (SE) ($n = 4$)

C, N, M, and MN represent the control treatment, organic amendment with cattle manure, inoculation with *G. intraradices* BEG 141, and combination treatment with AM inoculation and organic amendment, respectively. Different letters indicate significant differences among all means in different treatments at all Pb-Cd addition levels using Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA

Table 3 Shoot and root Pb concentrations (mg kg^{-1}) of tobacco plants

		Pb-Cd added (mg kg^{-1})			
		0/0	350/1	500/10	1000/100
Shoot	C	nd	11.42 (0.64)b	16.57 (1.43)c	27.21 (3.44)d
	N	nd	8.15 (0.52)a	11.18 (0.48)b	25.00 (3.10)d
	M	nd	9.31 (0.35)a	11.98 (0.48)b	10.66 (0.30)ab
	MN	nd	8.62 (0.54)a	12.49 (0.87)b	11.55 (0.26)b
Root	C	0.68 (0.09)a	116.74 (11.57)c	172.16 (16.56)d	444.13 (37.24)g
	N	0.25 (0.05)a	82.57 (4.78)b	100.20 (6.38)bc	404.33 (32.22)fg
	M	nd	91.77 (7.91)bc	133.97 (13.29)cd	341.74 (10.07)f
	MN	nd	69.27 (4.75)b	95.86 (4.89)bc	224.92 (12.67)e

Values are mean (SE) ($n = 4$); *nd* not detected

C, N, M, and MN represent the control treatment, organic amendment with cattle manure, inoculation with *G. intraradices* BEG 141, and combination treatment with AM inoculation and organic amendment, respectively. Different letters indicate significant differences among all means in different treatments at all Pb-Cd addition levels using Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA

Table 4 Shoot and root Cd concentrations (mg kg^{-1}) of tobacco plants

		Pb-Cd added (mg kg^{-1})			
		0/0	350/1	500/10	1000/100
Shoot	C	5.38 (0.25)c	26.25 (2.04)e	187.04 (11.63)i	298.47 (14.44)k
	N	5.67 (0.33)c	24.58 (1.17)e	125.10 (6.69)h	330.00 (38.70)k
	M	2.63 (0.06)a	15.68 (1.99)d	93.01 (1.19)g	245.22 (3.98)j
	MN	3.83 (0.33)b	14.15 (0.93)d	70.84 (5.06)f	240.18 (8.65)j
Root	C	1.86 (0.38)a	10.45 (1.18)c	111.07 (12.75)f	500.80 (47.88)h
	N	0.62 (0.26)a	5.69 (0.69)b	65.55 (1.04)e	506.24 (48.81)h
	M	0.62 (0.54)a	4.05 (0.32)b	25.05 (3.73)d	365.49 (22.54)g
	MN	1.00 (0.23)a	4.71 (0.98)b	27.32 (2.48)d	360.54 (17.97)g

Values are mean (SE) ($n = 4$)

C, N, M, and MN represent the control treatment, organic amendment with cattle manure, inoculation with *G. intraradices* BEG 141, and combination treatment with AM inoculation and organic amendment, respectively. Different letters indicate significant differences among all means in different treatments at all Pb-Cd addition levels using Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA

DTPA-extractable Pb and Cd Concentrations in Soil after Harvest

Overall, DTPA-extractable Pb concentrations in soil after harvest were significantly influenced by each of the three factors separately and the triple interaction between them, and DTPA-extractable Cd concentrations were significantly influenced by each of the three factors and all the interactions between them except the one between manure and Pb-Cd addition (Table 1).

Both DTPA-extractable Pb and Cd concentrations in soil increased as the addition levels increased (Table 5). Compared with the controls, soil DTPA-extractable Pb and Cd concentrations in N, M, and MN treatments did not change at the zero addition level, whereas they decreased at the 500/10-mg kg^{-1} addition level, and at the 350/1-mg kg^{-1} addition level only Pb concentrations decreased (Table 5). At the 1,000/100-mg kg^{-1} addition level, MN treatment decreased both DTPA-extractable Pb and Cd concentrations, whereas N or M singly decreased Cd concentrations significantly but did not affect Pb.

Total GRSP in Soil after Harvest

Total GRSP concentrations in soil after harvest were significantly influenced by manure and inoculation and their interactions, but not by Pb-Cd addition or the interactions between Pb-Cd addition and other factors (Table 1). One-way ANOVA analysis also showed that there were no significant differences in total GRSP concentrations between Pb-Cd addition levels (Fig. 3). Compared with the controls, treatments with N, M, and MN all increased total GRSP concentrations with an order of MN>M>N>C.

Table 5 Soil DTPA-extractable Pb and Cd concentrations (mg kg^{-1}) after tobacco harvest

		Pb-Cd added (mg kg^{-1})			
		0/0	350/1	500/10	1000/100
Pb	C	1.58 (0.15)a	3.71 (0.16)c	5.59 (0.09)e	6.69 (0.24)f
	N	1.49 (0.13)a	2.67 (0.17)b	5.02 (0.17)d	6.88 (0.07)f
	M	1.59 (0.13)a	2.72 (0.27)b	4.90 (0.20)d	6.90 (0.14)f
	MN	1.39 (0.07)a	2.73 (0.13)b	4.57 (0.20)d	5.84 (0.20)e
Cd	C	0.07 (0.002)a	0.22 (0.008)b	1.01 (0.049)d	3.29 (0.065)f
	N	0.07 (0.002)a	0.20 (0.004)b	0.81 (0.050)cd	3.08 (0.060)e
	M	0.07 (0.001)a	0.18 (0.003)b	0.74 (0.030)c	3.00 (0.072)e
	MN	0.07 (0.001)a	0.17 (0.005)b	0.76 (0.015)c	3.05 (0.084)e

C, N, M, and MN represent the control treatment, organic amendment with cattle manure, inoculation with *G. intraradices* BEG 141, and combination treatment with AM inoculation and organic amendment, respectively. Different letters indicate significant differences among all means in different treatments at all Pb-Cd addition levels using Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA

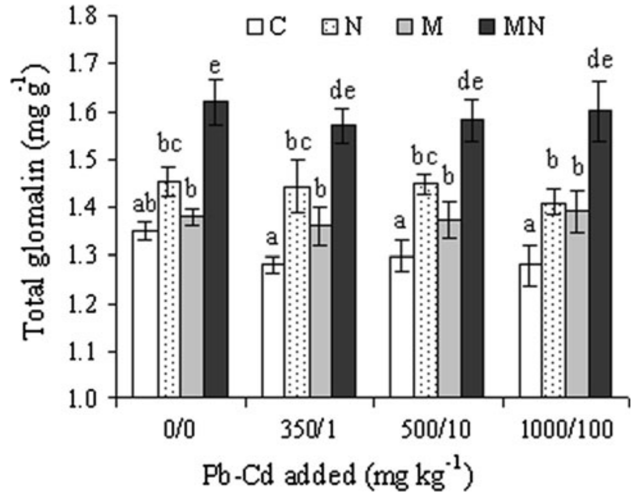

Fig. 3 Total GRSP concentrations (mean ± SE, $n = 4$) in soil after tobacco harvest. C, N, M, and MN represent the control treatment, organic amendment with cattle manure, inoculation with *G. intraradices* BEG 141, and combination treatment with AM inoculation and organic amendment, respectively. Different letters on the bar indicate significant differences among all means in different treatments at all Pb-Cd addition levels using Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA

Soil pH after Harvest

Soil pH after harvest was significantly influenced by each of the three factors separately and all the interactions between them, except the triple interaction (Table 1). Soil pH after harvest decreased gradually with more added Pb-Cd in both the control and N treatments but not in M and MN treatments (Fig. 4). Compared with the controls, treatments with N, M, and MN did not affect soil pH without Pb-Cd addition, but they significantly increased it when Pb-Cd was added, and MN treatment showed the most positive effect (Fig. 4).

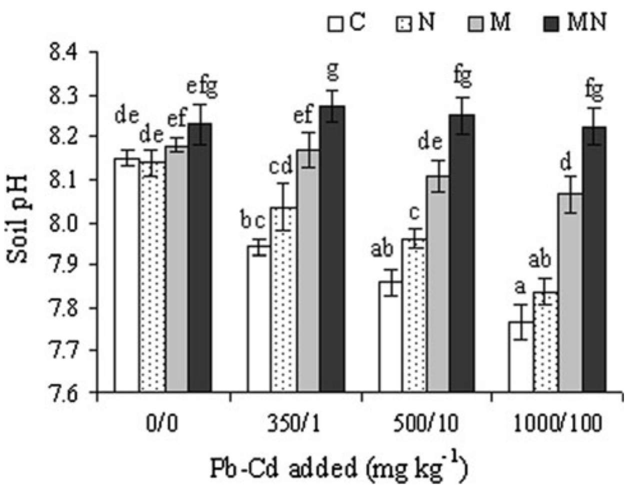

Fig. 4 Soil pH (mean ± SE, $n = 4$) after tobacco harvest. C, N, M and MN represent the control treatment, organic amendment with cattle manure, inoculation with *G. intraradices* BEG 141, and combination treatment with AM inoculation and organic amendment, respectively. Different letters on the bar indicate significant differences among all means in different treatments at all Pb-Cd addition levels using Duncan's multiple-range test ($p < 0.05$) following one-way ANOVA

Discussion

Pb and Cd are toxic nonessential elements to plants. The combination of Pb and Cd significantly decreased tobacco biomass (particularly for plants in C and N treatments), even at a slight contamination level, and when presented at a heavy contamination level, the control plants hardly grew at all. This growth reduction might be due to the competition for the same uptake systems that occurs between Cd, Pb, and other divalent ions (such as Ca^{2+}, Fe^{2+}) required for plant development (Alcantara and others 1994; Lozano-Rodriguez and others 1997). Additionally, the higher shoot Cd concentration compared to Pb suggests that Cd may contribute more to growth depression. Cd accumulation by tobacco was lower in roots than in shoots at slight and moderate pollution levels, confirming that transport of Cd to shoots by tobacco was easier compared to Pb. In general, Cd was more toxic for plants than Pb, and the strong affinity of Cd ions for sulfhydryl groups of several compounds and phosphate groups involved in plant metabolism might explain the greater toxicity (Påhlsson 1989). Compared to Cd, the phytotoxicity of Pb to plants is relatively low due to low bioavailability and the very limited uptake of Pb from soil and translocation from roots to aerial shoots (Påhlsson 1989).

There have been several reports that AM fungi can confer different degrees of Pb and Cd tolerance to their host plants (de Andrade and others 2008; de Souza and others 2012; Garg and Aggarwal 2011, 2012). Tobacco is a crop with high mycorrhizal dependency. Our present findings confirm that AM inoculation can alleviate the phytotoxicity of Pb-Cd and their negative effects on host plants. One possible mechanism may be the dilution effect of the toxic elements linked to increased growth due to improved mineral nutrition (particularly P), which may partly explain the lower Pb and Cd concentrations in mycorrhizal plants. However, the dilution effect appears unlikely to be the only explanation, because mycorrhizal roots had higher biomass and larger surface area, and if they have the same or higher capacity for metal uptake, the Pb and Cd concentrations in their tissues should not be lower than in the nonmycorrhizal controls. One more common mechanism is the chelation/immobilization of metals by fungal structures, especially extraradical mycelium (González-Guerrero and others 2009). It has been shown that extraradical mycelia could accumulate 10–20 times more Cd per unit of biomass than tobacco roots (Janoušková and others 2006). Extraradical mycelia also can induce alkalinization in substrates and contribute to lower Cd toxicity, indicating that they may enhance Cd immobilization in soil not only because of their high Cd sorption capacity but also their activity (Janoušková and Pavlíková 2010). In addition, AM fungal spores and vesicles of the intraradical mycelia could also accumulate more heavy metal (González-Guerrero and others 2009).

GRSP (formerly called glomalin), a glycoprotein produced by AM fungi, has shown a potential role in heavy-metal immobilization (González-Chávez and others 2004; Vodnik and others 2008). In soils, GRSP has been proven to bind up to 0.08 mg g^{-1} Cd and 1.12 mg g^{-1} Pb (González-Chávez and others 2004). It also binds Fe, manganese (Mn), and copper (Cu) but with lower affinity (Chern and others 2007). We also found that the contents of the total GRSP in soil after harvest were all increased by AM inoculation, and further analysis showed they contained high amounts of Pb and Cd (data not shown). This might be one reason why DTPA-extractable Pb and Cd concentrations were lower in the M treatment than in the controls. Additionally, we observed higher total GRSP contents in organic amended soil than in the controls. Obviously, this is a false-positive result due to the protein contained in cattle manure. Nevertheless, when applied in combination with AM inoculation, cattle manure may increase GRSP production via its fertilization and protection on mycorrhizal plants.

We also observed the growth promotion effects of organic amendments, although their effects were not so significant compared to AM inoculation. Obviously, both organic manure and AM fungi can improve soil fertility and structure and provide essential nutrients for plants directly or indirectly, thus leading to improved plant growth and development. Under heavy-metal stress, organic amendments (especially organic matter) generally reduce the bioavailability of metals through their effects on adsorption, complexation, precipitation, and other reactions of metals (Park and others 2011). The organic amendment-induced immobilization of heavy metals is often attributed to an increase in surface charge and the presence of metal-binding compounds. Many studies have shown that organic amendments transform soluble/exchangeable forms to organic-bound fractions and consequently decrease bioavailability and phytotoxicity of metals and uptake by plants (Walker and others 2004; Liu and others 2009; Chen and others 2010). Humic substances, as a major part of organic matter of compost, can reduce metal solubility by formation of stable metal chelates (Walker and others 2003, 2004; Janoš and others 2010). In addition, phosphate compounds are generally considered the most common amendment to precipitate heavy metals effectively in contaminated soils or water (Park and others 2011). The high P content in cattle manure could form precipitates with Pb and Cd and thus reduce metal solubility and toxicity.

Soil pH is usually the most important factor that controls the bioavailability of heavy metals and hence uptake by plants. Therefore, to raise soil pH and reduce metal toxicity, amendments such as lime and manure are often used for remediation of heavy-metal pollution (Clemente and others 2003; Walker and others 2004; Kirkham 2006). The decreased effects of organic amendments on metal bioavailability appeared to be related to an increase of soil pH due to inhibition of sulfide oxidation/hydrolysis. The present results show that there is a significant positive correlation between plant growth and soil pH (data not shown), and organic amendment with cattle manure increased soil pH, which may be the major contributing factor resulting in a lower bioavailability of heavy metals in soil and increased plant growth.

Similar to organic amendments, AM inoculation also increased soil pH and decreased DTPA-extractable Pb and Cd concentrations. These data are in accordance with those of other reports that showed that the lower soil solution concentrations of heavy metals in the mycorrhizosphere were often associated with higher pH (Bi and others 2003; Li and Christie 2001; Wang and others 2007). This indicates that AM symbioses can modify heavy-metal speciation in the mycorrhizosphere via altering soil chemical characteristics, and thus they may be able to survive in heavy-metal stress conditions.

Generally, organic manure with slow-release nutrients is beneficial to AM fungi (Gosling and others 2006; Gryndler and others 2006; Alguacil and others 2009). Several studies have demonstrated the beneficial effects of various organic amendments on the proliferation and development of natural AM fungal populations in crop systems (Gryndler and others 2006), degraded soils (Alguacil and others 2009), or heavy-metal-polluted soil (Alguacil and others 2011). We also observed a protective effect from organic manure on AM colonization against metal toxicity, and an additive effect and/or a synergistic interaction between AM inoculation and organic amendment on plant growth, nutrition, metal uptake, or some soil parameters. Under moderate and heavy contamination levels, AM colonization rates decreased when inoculated alone, but remained at a high level when applied in combination with cattle manure, indicating a protective effect from metal toxicity on AM fungi and symbionts via the mechanisms mentioned above. On the other hand, AM symbiosis can both enhance decomposition of and increase N capture from organic material (Hodge and Fitter 2010; Hodge and others 2001), and mycorrhizal hyphae produce phosphatase to hydrolyze organic P compounds (Koide and Kabir 2000; Feng and others 2003). Therefore, not surprisingly, AM symbiosis may extract the nutrients from organic manure and help transform slow-release nutrients in organic forms to bioavailable fractions and hence contribute to plant nutrition and growth. Particularly at heavy contamination levels, tobacco plants hardly grew with cattle manure alone, but they produced a greater biomass with the combination treatment. Our findings suggest that there may be a synergistic positive effect between AM fungi and organic manure on improving plant nutrition and growth, decreasing Pb and Cd phytotoxicity and immobilizing heavy metals in soil, thus preventing them from leaching into other environmental compartments. Thus, they are beneficial for not only tobacco production but also phytostabilization of Pb-Cd-contaminated soil.

Conclusion

AM inoculation, alone or in combination with organic manure, improved tobacco growth, P nutrition, and tolerance to Pb-Cd stress and decreased Pb and Cd concentrations in tobacco plants and DTPA-extractable concentrations in soil. Generally, organic amendment showed additive effects and/or synergistic interaction with AM inoculation on plant growth, nutrition, metal uptake, or some soil parameters, particularly when more Pb-Cd was added. Various mechanisms involved in plant growth promotion and alleviation of Pb-Cd stress may include improved plant mineral nutrition, higher soil pH, and binding/immobilization of heavy metals by GRSP. These findings suggest that AM inoculation in combination with organic manure may be considered an effective and low-cost method for reducing the levels of toxic metals in tobacco plants as well as for phytostabilization of Pb-Cd-contaminated soil.

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