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Preliminary study of Lead (Pb) immobilization by meat and bone meal combustion residues (MBMCR) in soil: Assessment of Pb toxicity (phytotoxicity and genotoxicity) using the tobacco model (*Nicotiana tabacum* var. *xanthi* Dulieu)

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Abstract Lead (Pb) is a major chemical pollutant in the environment. The present investigation evaluates the possible use of Meat and Bone Meal Combustion Residues (MBMCR), to sequester Pb from the soil compartment using the heterozygous tobacco model (*Nicotiana tabacum* var. *xanthi* Dulieu) characterized by the a_1^+/a_1 a_2^+/a_2 system. The toxic potential of Pb-contaminations (50, 100, 1,000, 2,000 and 10,000 mg Pb kg^{-1}) as $Pb(NO_3)$ in standard soil was investigated in lab conditions according to three endpoints: (i) acute toxicity of plants (mortality, height and surface area parameters), (ii) Pb-accumulation in roots, stems and leaves, and (iii) genetic effects as the expression of reversion in the leaf of plants. Moreover, chemical investigations of Pb interactions with soil were realized to complete the toxicity evaluation. The results demonstrated that: (i) MBMCR were not acutely toxic or genotoxic to tobacco plants, (ii) Pb is acutely toxic to tobacco plants at 10,000 mg Pb kg^{-1} of soil, (ii) but is not genotoxic, and (iii) Pb-bioaccumulation is significant in leaves, stems and roots (from 1,000, 2,000, and 50 mg Pb kg^{-1} of soil, respectively). In contrast, in the presence of MBMCR, the toxic impacts of Pb were inhibited and Pb-accumulation in tobacco plants was reduced. In complement, chemical analyses highlighted the high capacity of the standard soil to immobilize Pb. The results suggest that even if Pb is bioavailable from soils to plants, complex mechanisms could occur in plants protecting them from the toxic impact of Pb.

Keywords Lead · Meat and bone meal combustion residues · *Nicotiana tabacum* var. *xanthi* Dulieu · Genetic impact · Phytotoxicity

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Introduction

Lead (Pb) probably has the widest distribution in the human environment. Mining and smelting activities, sewage sludge usage in agriculture and contamination

from vehicle exhaust (although unleaded gasoline is now widely used) are the major sources of the large amount of spent lead into the environment. Pb is very stable and can therefore accumulate in soils, sediments and living organisms. Markus and McBratney (2001) review Pb-concentration data from both natural and contaminated soils on a range of scales in the world. For national surveys, Pb concentration ranges from 0.5 to 138 mg Pb kg^{-1} in Ireland (Dickson and Stevens 1983) and from 3 to 16,338 mg Pb kg^{-1} in England and Wales (McGrath and Loveland 1992). For urban and industrial surveys, it ranges from 6 to 650 mg Pb kg^{-1} in Poland (Czarnowska and Walczak 1988) and from 13 to 14,100 mg Pb kg^{-1} in garden soil in Great Britain (Culbard et al. 1988). In France, Pb in farming and foresters soils range from 1 to 7,000 mg Pb kg^{-1} (Baize 2000). The release of metals in biologically available forms, as a result of human activity, may damage or alter both natural and man-made ecosystems (Tyler et al. 1989). Pb is not essential for living organism metabolism and can become dangerous towards all of them, including plants. For years, most of the studies on its toxic evaluation have been carried out on animals whereas studies on higher plants are limited, especially regarding genetic impacts. Plant responses to Pb exposure include decrease in biomass and root elongation (Liu et al. 1994), inhibition of chlorophyll biosynthesis (Miranda and Ilangovan 1996) and various other effects on photosynthesis (Poskuta et al. 1988; Poskuta and Waclawczyk-Lach 1995): induction or inhibition of several enzymes (Van Assche and Clijsters 1990), transpiration (Rolfe and Bazzaz 1975) and seed germination effects (Wierzbicka and Obidzinska 1998) as well as DNA synthesis, mitotic activity and cell division disturbances (Gabara et al. 1992), or chromosomal lesions (Liu et al. 1994).

In the present work, the possible use of Meat and Bone Meal Combustion Residues (MBMCR) to sequester Pb from the soil compartment was investigated in order to open up the way towards Pb remediation strategies in the terrestrial compartment. MBMCR, which are natural, low-cost apatite-rich substances, were analyzed for their high Pb binding capacities (Deydier et al. 2003; Deydier et al. 2005).

The aim of the present work was thus to study the toxic effects of Pb in the presence and absence of MBMCR to evaluate the possible immobilization of Pb by MBMCR and to examine its possible use in the remediation of Pb-contaminated sites. This investigation was performed using the heterozygous tobacco (*Nicotiana tabacum* var. *xanthi* Dulieu) model characterized by the a_1^+/a_1 a_2^+/a_2 system (Dulieu 1975; Dulieu and Delabroux 1975; Fabries and Delpoux 1978) in lab conditions. Three different endpoints were investigated in tobacco plants cultured under Pb conditions with or without MBMCR present in the soil: (i) acute toxicity (mortality, height and surface area parameters), (ii) Pb accumulation in roots, stems and leaves, and (iii) genetic effects as the expression of reversion in the leaves of plants. This marker system is particularly appropriate for the detection of the genetic effects of low and very low doses of radiation (Fabries and Delpoux 1978), atmospheric pollutants (Devaud 1986), sludge-amended soils (Chenon et al. 2003) and soils polluted with heavy metals (Gichner et al. 2006). Lastly, chemical investigations of Pb interactions with the soil were done to complete the toxicity evaluation.

Materials and methods

Meat and Bone Meal Combustion Residues (MBMCR)

Low risk (Cat III) sterilized meat and bone meal (133°C/3 bars for 20 min to inactivate BSE's protein (Bovin Spongiform Encephalopathy), were provided by Fersobio (Agen, France). Meat and Bone Meal (MBM) were burned twice by calcinations in an electric furnace set to reach 850°C at 2°C/min. During combustion, MBM particles were melted and stuck together. Initial combustion gave a black carbon-rich residue which was mixed manually before a second combustion to complete decomposition and obtain clear ashes. Crude ashes were ground using a centrifugal mill of agate balls, and sieved at 250 µm. Chemical analysis shows that ashes are calcium (31.0% w/w) and phosphate (57.5% w/w) rich materials. Significant levels of sodium (2.4% w/w), potassium (1.5% w/w), chloride (2% w/w), magnesium (0.7% w/w) and silicon (0.7% w/w) were also observed. Structural analysis has been recently published (Deydier et al. 2006).

Artificial soil

Artificial soil was used as a medium substrate for plants. It was composed of nearly 70% w/w of quartz sand (fine sand dominant with more than 50% w/w of 0.05–0.2 mm size grain), 20% w/w of kaolin clay (kaolinite rate >30% w/w), 10% w/w dried matter of acid sphagnum peat, 0.6% w/w of calcium carbonate. As recommended by French standard criteria (AFNOR 1994a), water characteristics of standard soil were: 65.6% w/w of dried matter content—52.4% w/w of ponderal water—with a residual humidity rate of 34.4% w/w—pH 6.25 with KCl (AFNOR 1994b). Two soil controls were carried out: (i) artificial soil without Pb-contamination and without MBMCR, (ii) artificial soil with MBMCR and without Pb-contamination.

Pb-contamination of soils

Five concentrations of Pb alone and another five in the presence of MBMCR were tested. Contamination was achieved by spraying Pb (50, 100, 1,000, 2,000 and 10,000 mg Pb l^{-1} as lead nitrate $Pb(NO_3)_2$ in 3.4 l of an ultra-pure water solution, [10099-74-8] Sigma-Aldrich France, purity ≥99.0% w/w) into 10 polyethylene plates (40 × 40 × 5 cm) containing 3.4 kg of soil, or MBMCR-soil, per plate. MBMCR (the equivalent of 2 g of MBMCR per pot) were previously mixed with the soil using a mechanical stirrer (4 h, 300 rpm at room temperature). The control soils received the same ultra-pure water volume by spraying. Each one of the substrates was then dried in plastic plates at room temperature for 20 days and manually homogenized daily. Drying was completed in a furnace for 4 h (60°C). Each substrate was then sifted to 1 mm. For each condition, 20 plastic pots were then filled with 170 g of each substrate.

Tobacco test procedures

Tobacco plants (*Nicotiana tabacum* var. *xanthi* Dulieu) characterized by the a_1^+/a_1 a_2^+/a_2 system (Dulieu 1975; Dulieu and Delabroux 1975) were produced in vitro in the laboratory using culture medium 169 (Murashige and Skoog 1962) supplemented with EDTA-Fe and biotin at a pH of 5.5. At the 6–8 leaf stage, each plant was placed in a pot filled with (i) the soil control (noted 'C plants' for control plants), (ii) the soil control in presence of MBMCR (noted 'C_{MBMCR}'), (iii) the Pb-spiked soil (with and without MBMCR). To ensure tobacco's genetic response, a positive control condition (noted C^+, related to plants) was carried out by previous root immersion in ethyl methanesulfonate (EMS) solution (a well-known referenced mutagen) according to the Gichner and Plewa procedure (Gichner and Plewa 1998) to 1 g l^{-1} EMS dissolved in distilled water [62-50-0], Sigma, France) in darkness for 24-h at 20°C. After treatment, the plant roots were rinsed with ultra-pure water. The EMS-contaminated plants were finally transferred in control soil; the soil received the same ultra-pure water volume by spraying as the other conditions. Twenty plants were cultivated under each of 13 conditions. All pots were then maintained in a greenhouse supplied with filtered air (over a synthetic filter and activated charcoal) under constant overpressure, at 20–25°C with a 12-h per day photoperiod and a daily 1-min cycle of watering (40 ml per pot).

Phytotoxicity in tobacco cultured under the different conditions

Tobacco plants were cultivated for 7 weeks. The phytotoxic impact was assessed by (i) a weekly recording of the mortality of plants (ii) a weekly measuring of the height of each plant (H), and (iii) measuring the total leaf area (LA) of the 3 selected adult leaves at the end of the experimentation : the median, the highest and the lowest (Chenon et al. 2003). LA parameter was measured using MapInfo® software on the basis of digital photographs of the three leaves.

Genetic effects of the different culture conditions

At the end of the experiment, the genetic effects on the three selected leaves were assessed using a binocular magnifying glass (×7) to score and measure the reverted areas (RA) which appeared as a green spot due to partial chlorophyll deficiency. Indeed, the two loci a_1 and a_2 of the a_1^+/a_1 a_2^+/a_2 system are involved in chloroplast differentiation

without intercellular metabolic cooperation and the partially chlorophyll-deficient, double heterozygote a_1^+/a_1 a_2^+/a_2, has greenish-yellow leaves. Either spontaneously or under the action of chemical or physical mutagens, the genetic composition of the double heterozygous system can be modified, yielding mostly reverted green cells; the frequency of modifications to white and yellow cells is negligible and, hence, may be disregarded (Fabries and Delpoux 1978). As an individual grows, each reverted cell yields a clone that appears as a green spot in the palisade tissue of the greenish-yellow leaf.

Two parameters were then calculated: (i) the reversion ratio (RR = RA/LA × 1000) and the mean reversion rates per cell cycle (noted (p)) based on the number of reverted cells or, equally, on the reverted leaf areas. It has been shown (Dulieu and Dalebroux 1975; Fabries and Delpoux 1978) that for a given individual, a simple relationship exists between the total leaf area LA, the reverted area RA and the reversion rate p: $p = 1-[(LA-RA)/LA]^{1/t}$, where t is the number of cell cycles that occurs necessary from one single initial. Because spots smaller than 0.05 mm^2 cannot be detected by the observation method used (Fabries and Delpoux 1978), t is equal to $[\log N/\log 2]-7$. N is the total number of cells observed that corresponds to the total leaf area S, with cell density d (number of cells per unit area), so that $N = dS$. The cell density in the monolayer palissadic tissue was determined per transparency under a microscope (×1500), after leaf fragments were bleached. The bleaching was obtained after leaf fragments were immersed in water/ethanol (70% v/v).

Pb-concentrations in tobacco plants

At the end of the experimentation, above-ground biomass was cut at the collar. The roots, stems and leaves were analyzed separately. Roots were gently washed by immersing them in a bowl of ultra-pure water. Roots, stems and leaves were dried in quartz-glass beakers at 60°C in a furnace until constant mass (8 h). Dried materials (250 mg) were then digested with 10 ml of 66% nitric acid solution (HNO_3, analytical grade, Sigma France) for 30 min and placed on a hot block (60°C) for 3 h with 10 ml of hydrogen peroxide (H_2O_2, analytical grade, Sigma France). Three replicates per plant organ and per experimental condition were analyzed. Certified standards were obtained from LGC Promochem, France (Virginia tobacco leaves, trace elements). Pb was analyzed by atomic absorption spectrophotometry (AAS) equipped with a graphite furnace (Perkin Elmer SAMMA 6000). The detection limit was 0.2 mg Pb kg^{-1} ± 0.05. The results were expressed in average metal concentrations in µg Pb g^{-1} material (dry weight, d.w.) ± SD (standard deviation).

Statistical analysis

SigmaStat 3.1 was used for statistical analysis. Non-parametric tests were preferred because of (i) non normality, (ii) and /or non-homogeneity of variances and (iii) sample size ($n < 30$). Height variable (H) for each condition during the 7 weeks of exposure (paired data) was tested using the Wilcoxon test, followed by the Tukey test to isolate the group(s) that differ from the others using a multiple comparison procedure with paired data. E at a given date, LA, RR and p were tested using the Kruskall–Wallis test followed by Dunn's (same sample size) or Dunnet's (different sample size) test to isolate the group(s) that differ(s) using a multiple comparison procedure, with unpaired data, versus the negative control group. The significant difference can be positive or negative. «Dose-effect» and «MBMCR-effect» relationships were analyzed for E, LA, RR and p variables using the Mann–Whitney test (comparison on the basis of two and two experimental conditions).

Pb content in tobacco was tested using the Kruskall–Wallis test to compare all the conditions with the negative control. It was followed by Dunn's test for multiple comparisons versus a control group. T-test was used to analyze «dose-effect» and the «MBMCR-effect» relationships (comparison on the basis of two and two experimental conditions assuming normality and equal variance of data).

Soil analysis

Leaching experiments

These leaching tests were done on Pb-contaminated soils used for tobacco experiments (1,000, 2,000, 10,000 mg Pb kg^{-1} and 10,000 mg Pb kg^{-1} with

MBMCR) in order to evaluate the amount of Pb released by the soil. Five grams of artificial soil were stirred into 500 ml of deionised water for 90 min. During the experiment, 1 ml aliquots were collected at 2, 10 and 90 min, using a 2-ml propylene syringe equipped with a 0.2 μm nitrocellulose filter (Sigma, France). Samples were then acidified with a nitric acid HNO_3 solution (5% w/w) before analysis. Pb analysis was performed by atomic adsorption spectrophotometry (AAS) with a graphite furnace atomisation (Perkin Elmer SIMA 6000). Certified aqueous standards and matrix modifier ($Mg(NO_3)_2$ and $NH_4(H_2PO_4)$), were provided by Aldrich, France.

Capacity of Pb immobilisation by soil

About 2.5 g of soil, or its components (quartz sand, kaolin clay, sphagnum peat or calcium carbonate), were stirred into 500 ml of Pb^{2+} solutions ($Pb(NO_3)_2$ in deionised water) for 500 min. Concentration of Pb^{2+} solutions used were 25, 50, 75 and 100 mg Pb l^{-1}. Higher concentrations were necessary (500, 1,000 and 11,000 mg Pb l^{-1}) with $CaCO_3$. A tot al of 1 ml aliquots were collected regularly during the experiment, using a 2-ml propylene syringe equipped with a 0.2 μm filter, and samples were acidified with a HNO_3 solution (5% w/w) before analysis (see Figs. 3 and 4).

Pb analysis was performed by atomic adsorption spectrophotometry (AAS) with graphite furnace atomisation (Perkin Elmer SIMA 6000). Certified aqueous standards and matrix modifier ($Mg(NO_3)_2$ and $NH_4(H_2PO_4)$), were provided by Aldrich, France.

Results

Phytotoxicity of Pb with or without MBMCR

Mortality

Plant mortality was only observed at 10,000 mg Pb kg^{-1} without MBMCR (15%) and with MBMCR (10% mortality). The lowest Pb concentrations (50, 100, 1,000 and 2,000 mg Pb kg^{-1}) did not induce plant mortality, with or without MBMCR.

Height (H)

C and C_{MBMCR} plants showed significant H values from the third experimental week H3 (Fig. 1). Plants growing at 50, 100, 1,000 and 2,000 mg Pb kg^{-1}, with or without MBMCR, showed a significant height from the fourth week H4. A tot al of 10,000 mg Pb kg^{-1} induced also a significant growth from the fourth week H4 in the presence of MBMCR but from the fifth week H5 without MBMCR.

C^+ plants were significantly lower than C plants, at each experimental week (Fig. 1). In contrast, the C_{MBMCR} plants were significantly higher, compared to the C plants, from the third experimental week H3. Plants exposed to 10,000 mg Pb kg^{-1} without MBMCR, were the smallest, whatever the experimental week. The lowest Pb concentrations (50, 100, 1,000 and 2,000 mg Pb kg^{-1}) did not induce any significant difference in plant height, with or without MBMCR, irrespective of the experimental week.

The growth was dose dependent between two successive Pb-concentrations according to the experimental week and the Pb concentrations (Table 2). In contrast, with MBMCR, no "dose-effect" was observed on H parameter, between two successive Pb-concentrations, regardless of the experimental week.

Leaf areas (LA)

After 7 weeks of experimentation, C^+ plants showed significantly lower LA values, compared to C plants (Table 1). Pb conditions without MBMCR did not induce significant difference on LA parameter compared to C plants, except at 10,000 mg Pb kg^{-1}. However, none of the Pb concentrations with MBMCR induced a significant effect on LA parameter, compared to the C plants.

Pb bioaccumulation

No Pb accumulation was detected in C_{MBMCR} and C^+ plants (Table 2) compared to the C plants, while a significant Pb bioaccumulation was observed for plants exposed to Pb with and without MBMCR (i) in leaves (from 1,000 to 10,000 mg Pb kg^{-1}), (ii) in stems (to 2,000 and 10,000 mg Pb kg^{-1}), and (iii) in

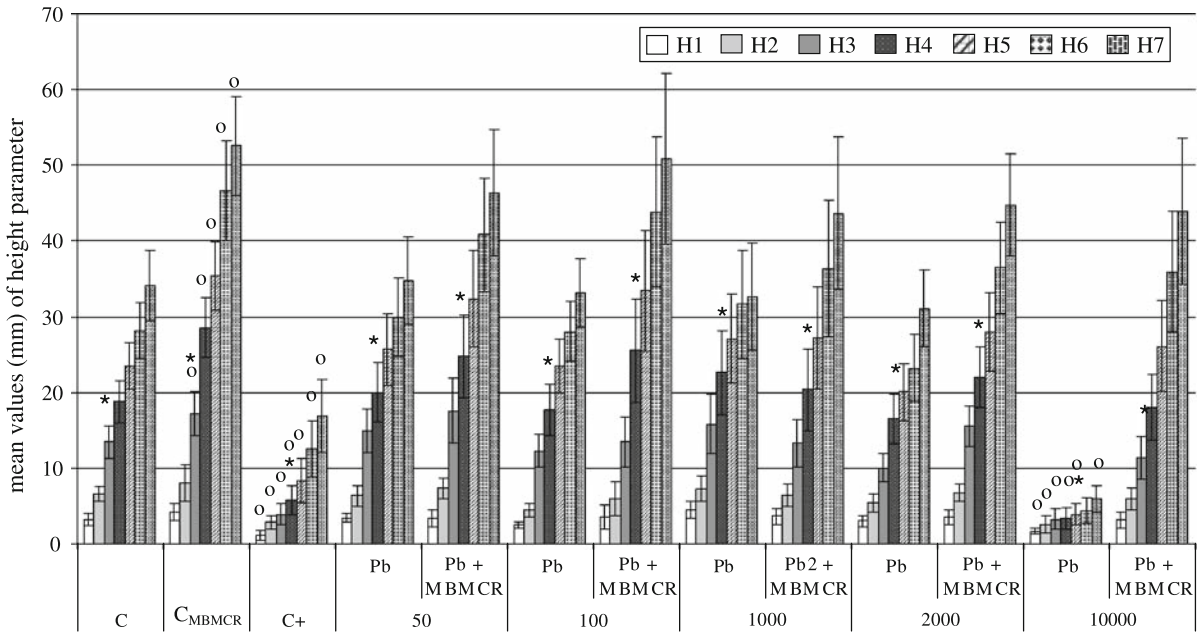

Fig. 1 Height (H) of tobacco plants during 7 weeks of culture on Pb-contaminated soil, with (C_{MBMCR} plants) or without MBMCR (C plants). For 7 weeks (from H1 to H7), for each condition and expressed in mm (mean values followed by the Mean Confidence Interval CI). Pb-concentrations are expressed in mg Pb kg^{-1}. C: control; C_{MBMCR}: control with MBMCR (Meat and Bone Meal Combustion Residues). C^+: Positive control (EMS, 1 g l^{-1}); "*" indicates the first significant height, compared to E1 (height values at the first experimental week) according to the Wilcoxon test ($P < 0.01$). "o" indicates a significant (negative or positive) height compared to C plants (Kruskall–Wallis followed by Dunn's or Dunnet's test, $P < 0.05$)

roots (whatever the Pb concentration). In Pb conditions without MBMCR, a significant Pb accumulation was observed ranging from (i) 506.67 ± 0.01 to 4,620 ± 616.15 µg Pb g^{-1} d.w. (dry weight) in plant leaves exposed from 1,000 up to 10,000 mg Pb kg^{-1}, (ii) 175.10 ± 11.19 to 3,220 ± 305 µg Pb g^{-1} d.w. in plant stems exposed to 2,000 and 10,000 mg Pb kg^{-1}, and (iii) 88.00 ± 5.29 to 19332.69 ± 3138.95 µg Pb g^{-1} d.w. in plant roots exposed from 50 up to 10,000 mg Pb kg^{-1}. In the presence of MBMCR, a significant Pb accumulation ranged from (i) 460 ± 1.15 to 1620 ± 47.26 µg Pb g^{-1} d.w. in plant leaves exposed from 1,000 up to 10,000 mg Pb kg^{-1}, (ii) 88.55 ± 4.75 to 1053.33 ± 11.63 µg Pb g^{-1} d.w. in plant stems exposed to 2,000 and 10,000 mg Pb kg^{-1}, and (iii) 25.62 ± 4.50 to 5585.89 ± 578.81 µg Pb g^{-1} d.w. in plant roots exposed from 50 up to 10,000 mg Pb kg^{-1}. The same statistical difference was found compared to the C_{MBMCR}. In both conditions (with or without MBMCR), Pb content was higher in roots than in leaves and the lowest Pb contents were measured in stems.

Genetic effects of Pb with or without MBMCR: Reversion ratios (RR) and mean reversion rates per cell cycle (p)

After 7 weeks of experimentation, the two genetic parameters relative to the C^+ plants showed significantly higher values compared to the C plants (Table 3). In contrast, RR and p parameters of C_{MBMCR} and C plants were not significantly different. Pb concentrations, with or without MBMCR, induced no significant difference on the two genetic parameters compared to the C plants.

"Dose"-effects of Pb and Pb MBMCR mixtures on toxic and genetic parameters in tobacco plants (Table 3)

The H and LA parameters and genetic ones (RR, *p*) were dose dependent between two successive Pb-concentrations, according to the experimental week for H parameter, and according to the Pb concentrations,

Table 1 Results of toxic (leaf areas, LA) and genetic effects (reversion ratios, RR) and mean reversion rates per cell cycle (p) after 7 weeks of tobacco culture on Pb-contaminated soil, with or without MBMCR

		LA		RR		p	
	C	2603.67 ± 278.67		0.22 ± 0.16		1.67 ± 1.22	
	C_{MBMCR}	2807.58 ± 214.07		0.07 ± 0.04		0.52 ± 0.12	
	C^+	1882.78 ± 316.16*		0.55 ± 0.12*		4.35 ± 1.43*	
		Pb^{2+}	Pb^{2+} + MBMCR	Pb^{2+}	Pb^{2+} + MBMCR	Pb^{2+}	Pb^{2+} + MBMCR
Pb-concentrations (mg Pb kg^{-1})	50	2060.25 ± 281.61	2456.29 ± 266.40	0.14 ± 0.21	0.12 ± 0.06	1.08 ± 1.59	0.88 ± 0.45
	100	2345.76 ± 408.72	2773.29 ± 302.23	0.26 ± 0.23	0.22 ± 0.17	2.03 ± 1.77	1.74 ± 1.36
	1,000	2632.79 ± 277.94	3018.37 ± 390.06	0.10 ± 0.07	0.18 ± 0.19	0.81 ± 0.60	1.41 ± 1.47
	2,000	2204.23 ± 248.18	2736.72 ± 264.88	0.17 ± 0.11	0.10 ± 0.05	1.36 ± 0.89	0.78 ± 0.41
	10,000	954.53 ± 139.46*	3055.74 ± 414.79	0.47 ± 0.43	0.08 ± 0.06	4.24 ± 3.91	0.68 ± 0.52

This table presents the mean values of the leaf area (LA) expressed in mm^2, the reversion ratio (RR) and the mean reversion rates per cell cycle (p), followed by the Mean Confidence Interval, for tobacco plants after 7 weeks of Pb and Pb/MBMCR mixture treatment. C: control. C_{MBMCR}: control with MBMCR (Meat and Bone Meal Combustion Residues). C^+: positive control. LA is expressed in mm^2. RR corresponds to (LA/RA) × 1000. * Significant negative LA or RR compared to C (Kruskall–Wallis followed by Dunn's or Dunnet's test, $P < 0.05$)

Table 2 Accumulation in leaves, stems and roots of tobacco plants exposed to Pb, with or without MBMCR

	µg g^{-1} (d.w.)					
	Leaves		Stems		Roots	
	Pb^{2+}	Pb^{2+} + MBMCR	Pb^{2+}	Pb^{2+} + MBMCR	Pb^{2+}	Pb^{2+} + MBMCR
C	2.00 ± 0.00		3.33 ± 1.15		1.99 ± 0.01	
C_{MBMCR}	0.67 ± 0.15		2.00 ± 0.01		1.33 ± 1.15	
C^+	2.00 ± 0.09		2.00 ± 0.00		1.98 ± 0.03	
50	2.67 ± 1.15	2.67 ± 1.15	2.64 ± 1.15	0.67 ± 0.15	98.00 ± 14*[a]	25.62 ± 4.50*[a]
100	3.33 ± 1.15	3.33 ± 1.15	2.61 ± 1.10	1.38 ± 2.74	88.00 ± 5.29*[a]	78.67 ± 1.15*[a]
1000	506.67 ± 0.01*[a]	460.00 ± 1.15*[a]	3.96 ± 5.88	2.60 ± 2.74	1698.34 ± 124.51*[a]	966.05 ± 87.14*[a]
2000	693.34 ± 14*[a]	513.34 ± 39.46*[a]	175.10 ± 11.19*[a]	86.55 ± 4.75*[a]	2383.85 ± 125.68*[a]	1486.67 ± 254.82*[a]
10,000	462000 ± 616.15*[a]	1620.00 ± 47.26*[a]	3220.00 ± 305*[a]	1053.33 ± 11.63*[a]	19 332.69 ± 3138.95*[a]	5585.89 ± 578.81*[a]

C: control, C_{MBMCR}: control with MBMCR (Meat and Bone Meal Combustion Residues, C^+: positive control (EMS, 1 g l^{-1}), MBMCR: meat and bone meal combustion residues control. Pb-accumulation is expressed as the mean values ($n = 3$ replicates) ± SD (Standard deviation). * Significant bioaccumulation of Pb in tobacco plants compared to C plants (Kruskall–Wallis followed by Dunn's test, $P < 0.05$). [a] significant bioaccumulation of Pb in tobacco plants compared to NC_{MBMCR} (Kruskall–Wallis followed by Dunn's test, $P < 0.05$)

Table 3 Dose-effects of Pb-treatment, with and without MBMCR, on phytotoxic (E, LA, Pb accumulation) and genetic (RR, p) parameters in tobacco plants

		0–50		50–100		100–1,000		1,000–2,000		2,000–10,000	
		Pb^{2+}	Pb^{2+} + MBMCR	Pb^{2+}	Pb^{2+} + MBMCR	Pb^{2+}	Pb^{2+} + MBMCR	Pb^{2+}	Pb^{2+} + MBMCR	Pb^{2+}	Pb^{2+} + MBMCR
H	H1	–	–	SD↓	–	SD↑	–	SD↓	–	SD↓	–
	H2	–	–	–	–	SD↑	–	–	–	SD↓	–
	H3	–	–	–	–	–	–	SD↓	–	SD↓	–
	H4	–	–	–	–	–	–	–	–	SD↓	–
	H5	–	–	–	–	–	–	–	–	SD↓	–
	H6	–	–	–	–	–	–	SD↓	–	SD↓	–
	H7	–	–	–	–	–	–	–	–	SD↓	–
LA	H7	SD↓	–	–	–	–	–	SD↓	–	SD↓	–
RR	H7	SD↓	–	SD↑	–	–	–	–	–	–	–
(p)	H7	SD↓	–	SD↑	–	–	–	SD↓	–	SD↓	–
Pb-bioaccumulation	Leaves	–	–	–	–	SD↑	SD↑	SD↑	SD↑	SD↑	SD↑
	Stems	–	–	–	–	–	–	SD↑	SD↑	SD↑	SD↑
	Roots	–	–	–	SD↑	SD↑	SD↑	SD↑	SD↑	SD↑	SD↑

SD: significant difference (Mann–Whitney, $P < 0.01$) between two successive Pb-concentrations, with or without MBMCR. "–": no significant difference. ↑: Beneficial dose-effect between two successive concentrations and ↓: negative dose-effect between two concentrations. MBMCR: meat and bone meal combustion residues. LA: leaf area. RR: reversion ratio. (p): mean reversion rates per cell cycle. (Beneficial effects for H and LA correspond to higher values compared to C plants and lower ones for RR, *p* and accumulation values.)

whatever the parameter was (Table 3). In contrast, with MBMCR, no "dose-effect" was observed on the H parameter, between two successive Pb-concentrations, (and whatever the experimental week for H parameter). Pb concentrations in plants were also dose dependent in leaves, stems and roots according to Pb concentrations, with or without MBMCR.

Effects of MBMCR on physiological and genetic parameters of tobacco plants in culture on Pb-supplied soils (Table 4)

MBMCR promoted the growth of plants cultivated at 50, 100, 2,000 and 10,000 mg Pb kg^{-1} from H6, H4, H3 and H1 respectively. MBMCR with 50, 100, 200 and 10,000 mg Pb kg^{-1} soils also promoted leaf growth (LA). In contrast, MBMCR with 1,000 mg Pb kg^{-1} did not induce a significant effect on LA parameter. RR and p values of plants cultured on 50 and 10,000 mg Pb kg^{-1} supplied soil were positively affected by the presence of MBMCR. Pb bioaccumulation was significantly reduced (i) in leaves of plants cultured on 1,000, 2,000 and 10,000 mg Pb kg^{-1}, (ii) in stems of plants cultured on 2,000 and 10,000 mg Pb kg^{-1} and (iii) in roots whatever the Pb concentration.

Leaching experiments

Figure 2 presents Pb release (mg Pb g^{-1} soil) in the four studied Pb-spiked soils under leaching test conditions. Soils spiked at 1,000 and 2,000 mg Pb kg^{-1} did not show any Pb^{2+}-ion release. Incubation of the spiked soil at 10,000 mg Pb kg^{-1} in deionised water induced a rapid increase of Pb^{2+} ion concentration up to nearly 4.5 mg l^{-1} of solution after a few minutes, indicating that the soil releases nearly 0.45 mg Pb g^{-1} of soil. Ash-amended soil contaminated at 10,000 mg Pb kg^{-1} (Fig. 2) did not release any Pb^{2+} ions.

Capacity of Pb immobilisation in soil

Figure 3 presents Pb uptake (mg Pb g^{-1} soil) of the standard soil versus time for initial solutions at 25, 50, 75 and 100 mg Pb l^{-1}. With 25 mg Pb l^{-1} solution, 100% of Pb is immobilized within 60 min and the residual Pb concentration is lower than 0.1 mg Pb l^{-1}. After 0.2 μm filtration, this Pb-contaminated soil was added to 500 ml of deionised water and even with a liquid-to-solid ratio equal to 200, no Pb release was observed (detection limit around 0.1 μg l^{-1}). Experiments with higher

Table 4 Effects of MBMCR on tobacco plants cultured on Pb-contaminated soil, with or without MBMCR

		Pb (mg Pb kg^{-1}) conditions in the presence of MBMCR				
		50	100	1,000	2,000	10,000
H	H1	–	–	–	–	*↑
	H2	–	–	–		*↑
	H3	–	–	–	*↑	*↑
	H4	–	*↑	–	*↑	*↑
	H5	–	*↑	–	*↑	*↑
	H6	*↑	*↑	–	*↑	*↑
	H7	*↑	*↑	–	*↑	*↑
LA	H7	*↑	*↑	–	*↑	*↑
RR	H7	*↑	–	–	–	*↑
(p)	H7	*↑	–	–	–	*↑
Pb-bioaccumulation	H7 leaves	–	–	*↓	*↓	*↓
	H7 stems	–	–	–	*↓	*↓
	H7 roots	*↓	*↓	*↓	*↓	*↓

Comparison of phytotoxicity and genotoxicity. *: significant effect of MBMCR for a given Pb-concentration and a given parameter. ↑: Beneficial and ↓ negative effects of MBMCR (Beneficial effects for H and LA correspond to higher values compared to C plants and lower ones for RR, *p* and accumulation values. "–": no significant effect of MBMCR. MBMCR: meat and bone meal combustion residues. LA: leaf area. RR: reversion ratio. (p): mean reversion rates per cell cycle

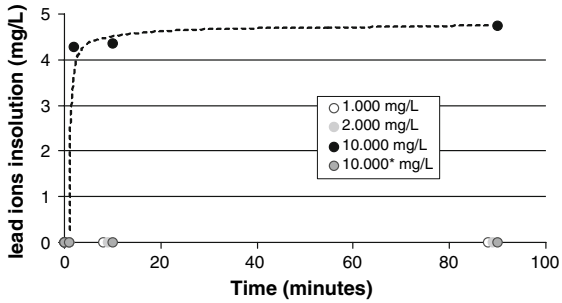

Fig. 2 Quantity of Pb released (mg Pb l^{-1}) versus time by four differents soils (previously contaminated with 1,000, 2,000 and 10,000 mg Pb l^{-1} and dry) during leaching experiment. The * indicates that the soil contains MBMCR

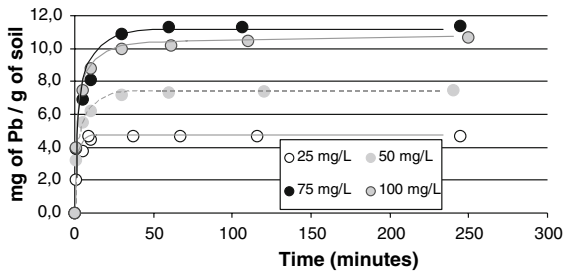

Fig. 3 Rate of Pb-immobilization in soil for initial solutions at 25, 50, 75 and 100 mg Pb l^{-1}

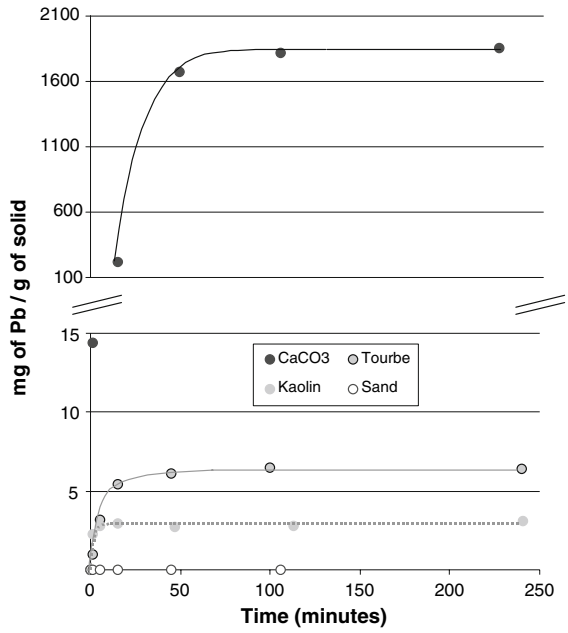

Fig. 4 Pb extraction quantity (mg Pb g^{-1}solid), by the different constituents of the artificial soil versus time

concentrations (75 and 100 mg Pb l^{-1}) made it possible to evaluate a maximum capacity of nearly 11 mg of Pb g^{-1} of soil.

In order to identify which component is involved in Pb immobilisation by the standard soil, similar experiments (Fig. 4) were performed with sand, kaolin clay, sphagnum peat (with Pb solutions at 100 mg Pb l^{-1}) and calcium carbonate (with Pb solutions at 11,000 mg Pb l^{-1}). These experiments allowed us to evaluate different capacities: nearly 3 mg of Pb^{2+} g^{-1} of kaolin, 6 mg of Pb^{2+} g^{-1} of peat and 1,800 mg Pb^{2+} g^{-1} of $CaCO_3$ (and no adsorption of Pb^{2+} by sand).

Discussion

Phytotoxic and genetic impact of Pb

In our experiment, 10,000 mg Pb kg^{-1} of soil induced lethal toxicity in tobacco plants. Plants cultured on Pb conditions showed delayed growth, compared to the C plants. The same tendency was obtained in *Nicotania glauca* (Barazani et al. 2004), in *Nicotiana tabacum var. xanthi* (Gichner et al. 2006), and in *Pisum sativum* (Rooney et al. 1999) cultured in metal-contaminated soil, and in *Vigna radiata* cultured in Pb conditions (Singh et al. 2003). The delay in plant growth may be due to interference with enzymes essential for normal metabolism and development (Van Assche and Clijsters 1990), photosynthetic processes (Fodor et al. 1996), water and mineral nutrient absorption (Burzynski 1987).

No phytotoxic Pb effects were observed in tobacco plants until 10,000 mg Pb kg^{-1}. Neither were genetic effects observed whatever the Pb concentration. Nevertheless, Pb accumulation was observed in roots from the lowest Pb concentration tested (50 mg Pb kg^{-1}), in leaves (from 1,000 mg Pb kg^{-1}), and in stems (from 2,000 mg Pb kg^{-1}).

The amount of metal absorbed by a plant, potentially phytotoxic, depends on several factors: soil adsorption, concentration and speciation of the metal in the soil medium, movement of the metal from bulk soils to the root surface, transport of the metal from the root surface into the root, and translocation of the metal from the root to the shoot.

The first barrier that Pb must pass through to enter the plant's organism is, obviously, the soil. Pb-phytotoxicity depends on soil characteristics such as pH, organic matter content and nature, mineral composition and status, etc. (Foy et al. 1978).

Once the soil barrier is "broken", the toxic metal ions can enter the cell through the same processes occurring for essential micronutrient metal ion uptake. The translocation of the metal ions in plants is, however, limited and most of the Pb bounds remain located at the root (IPCS 1989). Hence, in most experimental studies on Pb toxicity, high Pb concentrations in soils (ranging from 100 to 1,000 mg Pb kg^{-1}) are needed to cause toxic effects on photosynthesis, growth, and other parameters (IPCS 1989). Pb-excess content in plants could inhibit various physiological processes. Hewitt (1966) hypothesized that elements with similar physical and chemical properties would act antagonistically to each other in the organism. Similar elements compete for the same transport and binding sites in the cell, displacing each other from reactive enzymatic and receptor proteins. Calcium (Ca), magnesium (Mg) and phosphorus (P) have been reported to play a protective role against the toxic effects of metals for many years (Foy et al. 1978; Garland and Wilkins 1981; Rashid and Popovic 1990; Sela et al. 1990). Antosiewicz (2005) suggested that Ca^{2+} could play a role in the regulation of Pb-detoxification by influencing the formation of Pb-precipitates in cell walls. Around 90% of the Pb taken up by the plants is sequestered in the cell walls, and its subsequent deposit is considered as an important defense mechanism against its toxic effects (Ernst 1998).

In order to survive, plants growing on metal-contaminated sites need to develop a high level of tolerance to metal toxicity. Ca is probably but one of the factors involved. Literature reports such mechanisms in numerous plant varieties (Patra et al. 2004). Although Pb accumulation in tobacco plants has been highlighted, the lack of physiological and genetic Pb impact suggests that such defense mechanisms are probably involved in tobacco growth.

Metals, especially Pb, are known to induce clastogenic and mutagenic effects in higher plants (Patra et al. 2004). The genotoxic effects depend on the oxidation state of the metal, its concentration and the duration of exposure time. In general, effects are more pronounced at higher concentrations and with longer exposure (Bhowmik 2000; Patra 1999). Pb nitrates are less clastogenic than Pb chloride according to Mukherji and Maitra (1976) and Wierzbicka (1998). Moreover, plant species respond differently to exposure to the same metal depending on the number of diploid chromosomes, the total length of the diploid complement and the number of metacentric chromosomes (Ma et al. 1995b).

Significant Pb accumulation in tobacco plants was recorded in the present work. This result is in agreement with those obtained by Gichner et al. (2006) suggesting that the *Nicotiana* plant is capable of Pb storage. Pb content was higher in roots than in leaves and the lowest Pb content was measured in stems. The preferential accumulation of Pb in the root system is commonly reported in the literature (Alloway 1990; Antosiewicz 1993; Kabata-Pendias et Pendias 2000; Patra et al. 2004; Ramos et al. 2002; Wierzbicka and Antosiewicz 1993; Wierzbicka 1999). The roots act as a barrier to the uptake and translocation of metals (Vassilev et al. 1998). The root distribution in tobacco plants indicates that Pb is not very mobile from the roots to the leaves, due to the plant tolerance mechanisms (Sunkar et al. 2000; Arazi et al. 2000; Eapen and Souza 2005).

Nevertheless, Pb accumulation was also significantly recorded in leaves and in stems although with lower levels than in roots. This result suggests that Pb was mobile enough to move partially from the roots towards upper biomass. However, the backward release of Pb into the soil cannot be excluded since Pb content was not measured in the soil during the experiment. Pb accumulation was lower in stems suggesting that tobacco stems constitute organs of Pb transfer, contrary to storage organs such as roots and leaves.

Phytotoxicity and genetic impact of MBMCR

The C_{MBMCR} plants were significantly higher than C plants. Even if LA and genetic values relative to MBMCR were not significant compared to the C plants, results tend to exhibit the highest values for LA and the lowest for RR and p. These results suggest the beneficial impact of MBMCR amendment on tobacco plant growth. MBMCR seem to exhibit a fertilizing potential, obviously related to their high phosphate composition.

Phytotoxicity and genetic impact of Pb in presence of MBMCR

Lethal toxicity appeared at 10,000 mg Pb kg^{-1} of soil in presence of MBMCR as well as without. Moreover, survival plants in presence of MBMCR grew sooner than plants without MBMCR. Even if no significant phytotoxic effects were observed at lower Pb concentrations and no significant genetic effects regardless of Pb concentration, beneficial effects of MBMCR on tobacco plants were observed depending on Pb concentrations (increasing of E and LA values, and reducing of RR and *p* values) (Table 5). Moreover, even if Pb accumulation is still significant in presence of MBMCR depending on Pb concentrations, the values of the measured or calculated parameters were systematically reduced in presence of MBMCR for a given Pb concentration. Significant MBMCR effects were observed from 1,000 mg Pb kg^{-1} in leaves, from 2,000 mg Pb kg^{-1} in stems and from the lowest Pb concentration in roots suggesting that the MBMCR effect is first the action of Pb-binding in soil. Thus, it can be suggested that MBMCR would have a beneficial effect by limiting Pb transfer from soil to plants, in relation to the soil's chemical composition. Its high content in phosphate (PO_4) may decrease Pb phytotoxicity. Indeed, Pb–PO_4 minerals are the most insoluble and stable forms of Pb in soils (Cao et al. 2003). In the present work, MBMCR concentration was first chosen on the basis of its Pb-binding capacity in aqueous solutions and of its realistic soil amendment conditions on Pb-contaminated soils. This bioinorganic, natural, apatite-rich material has already been investigated and compared to those of synthetic apatite (Deydier et al. 2003). Deydier et al. (2003) showed that MBMCR can remove large quantities of Pb from solutions in just a few minutes to reach a capacity of nearly 275 mg g^{-1} of MBMCR. However, this proportion of uptake remains lower than the theoretical maximum capacity (if Ca had been entirely substituted by Pb). The leaching experiments highlight that (i) MBMCR immobilized Pb in soil (comparison between 10,000 mg Pb kg^{-1} with and without MBMCR (Fig. 2)), (ii) the soil itself immobilized Pb (experiments without MBMCR at lower concentrations) and (iii) soil immobilisation capacity can be evaluated at nearly 10 mg of Pb g^{-1} of soil (0.45 mg Pb g^{-1} of soil were liberated and are equivalent to 450 mg of lost Pb per kg of soil). At 10,000 mg Pb kg^{-1} of soil, 9,550 mg Pb were immobilized (10,000–450) per kg of soil, i.e. a capacity of 9.55 mg Pb g^{-1}). Capacity of soil (Table 5, 11 mg Pb g^{-1} of soil) is similar to results obtained from leaching tests. Moreover, this value is in agreement with the calculated value (12 mg Pb g^{-1} of soil) considering its composition and the capacity of its different constituents. $CaCO_3$ capacity is nearly 90% of soil capacity (nearly 11 mg Pb g^{-1} of CaCO3). The slight difference between measured and calculated capacity could then be explained by solid non-homogeneity of sample used for experiment (2.5–5 g) as $CaCO_3$ concentration in soil is very low (nearly 0.6% w/w).

The beneficial effect of MBMCR was already demonstrated in a previous work highlighting inhibition or reduction of the toxicity and genotoxicity of Pb towards amphibian larvae in water (Mouchet et al. 2006). *A fortiori*, the present approach using plants is of great ecotoxicological interest and an innovative line of research since, to our knowledge, only three studies on the effects of MBMCR (or similar materials) using bacterial bioassays (Gulyurtlu et al. 2005; Hodson et al. 2001) and superior organisms

Table 5 Pb-immobilisation capacity of soil and its constituents

Soil		Pb-immobilisation capacity (mg Pb g^{-1} solid)			
		Soil components		Soil	
Composition	% (weight)	Alone (measured)	In soil (calculated)	Calculated	Measured
Sand	69.4	0	0		
Kaolin clay	20.0	3	0.6		
Sphagnum peat	10.0	6	0.6	12	11
Calcium carbonate	0.6	1,800	11		

(Mouchet et al. 2006) have been published to this date. Moreover, the contamination of soils with toxic metals is a major issue that requires immediate action either by removal or immobilization. Currently, conventional processes, including soil excavation and ex situ landfill of the upper contaminated soil layer are often expensive because of the high cost involved in the disposal of contaminated soil and in the transportation and backfill of the original site with clean soil. More recently, phytoremediation has been widely considered as a cost-effective approach to remediate metal-contaminated soils but the process is slow, reducing its application. In situ chemical immobilization is a promising technique and allows a decrease in the mobility of contaminants in the ecosystems (Zhu et al. 2004). The use of phosphate as a stabilizing agent (leading to metal phosphate) for in situ remediation processes is particularly attractive (Cao et al. 2003; Cotter-Howells and Caporn 1996; Hettiarachchi et al. 2000; Laperche et al. 1997), especially when the key contaminant is Pb, based on the low Pb-phosphate solubility (Boisson et al. 1999; Cotter-Howells 1996; Cotter-Howells and Caporn 1996; Hettiarachchi et al. 2000 and 2001; Laperche et al. 1997; Ma et al. 1994; Ma et al. 1995a; Ma and Rao 1997; Rabinowitz 1993; Theodorantos et al. 2002). The choice of phosphate is based on both empirical observations and thermodynamic considerations, and supported by several geochemical studies, indicating that Pb phosphate minerals are very stable under a wide range of environmental conditions (Theodoratos et al. 2002). Cotter-Howells (1996) suggested that the formation of pyromorphite in urban soils could lead to reduction in the human bioavailability of Pb as the solubility of pyromorphite is low. Ruby et al. (1994) suggested that in situ immobilization of Pb by phosphate amendment would provide a cost-effective method to reduce the leaching, migration and bioavailability of Pb from soils. In any case, the addition of phosphate is a common agricultural practice. The present study innovates in this research area since bioinorganic natural apatite-rich materials such as MBMCR are low-cost materials and are stockpiled in large amounts, especially in Europe, waiting to be recovered. Indeed, since MBM were banned from animal foodstuffs in 2000 due to the risks of bovine spongiform encephalopathy, most of them are incinerated (MAF 2000). In this economical context, the possible use of MBMCR as adsorbents could be technically analyzed in view of remediation studies in two ways: (i) with other metals in lab conditions and (ii) implementation of this technology in field conditions at heavily-contaminated sites, even if more mechanistic and environmental investigations are needed, especially in public health.

Conclusion

This study demonstrates that Pb induced few phytotoxic impacts even at high Pb concentration (10,000 mg Pb kg^{-1}) and no genetic effects on tobacco plants whatever the Pb concentrations, probably in relation with (i) the high Pb storage capacity and Pb tolerance processes of tobacco and (ii) the high capacity of Pb immobilization of the standard soil. Added to soil, MBMCR were neither toxic nor genotoxic towards tobacco plants and enhance a physiological benefit suggesting that MBMCR would be considered as a valuable source of plant-available nutrient and organic matter and could be recycled as fertilizer on agricultural land. Moreover, MBMCR reduces the expression of phytotoxicity at toxic Pb concentrations. Pb accumulation in tobacco cultured with MBMCR in soil was lower than Pb accumulation in tobacco cultured without MBMCR. In agreement with previous conclusions reporting the possible use of MBMCR for aquatic Pb remediation (Mouchet et al. 2006), the present results suggest that MBMCR may be a key material in the investigation of metal remediation in contaminated soils and thus encourage further studies.

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