SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 34, No. 9, pp. 1962–1968, 2015

Printed in the USA

BIOCHAR AMENDMENT TO LEAD-CONTAMINATED SOIL: EFFECTS ON FLUORESCEIN DIACETATE HYDROLYTIC ACTIVITY AND PHYTOTOXICITY TO RICE

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(Submitted 23 December 2014; Returned for Revision 9 February 2015; Accepted 13 April 2015)

Abstract: The amendment effects of biochar on total microbial activity was measured by fluorescein diacetate (FDA) hydrolytic activity, and phytotoxicity in Pb(II)-contaminated soils was examined by the application of 4 different biochars to soil, with rice as a test plant. The FDA hydrolytic activities of biochar-amended soils were much higher than that of the control. The survival rate of rice in lead-contaminated biochar-amended soils showed significant improvement over the control, especially for bamboo biochar-amended soil (93.3%). In addition, rice grown in lead-contaminated control sediment displayed lower biomass production than that in biochar-amended soil. The immobilization of Pb(II) and the positive effects of biochar amendment on soil microorganisms may account for these effects. The results suggest that biochar may have an excellent ability to mitigate the toxic effects of Pb(II) on soil microorganisms and rice. *Environ Toxicol Chem* 2015;34:1962–1968. © 2015 SETAC

Keywords: Biochar Fluorescein diacetate hydrolytic activity Phytotoxicity Rice (*Oryza sativa L.*) Microbial activity Pb(II)-contaminated soil

INTRODUCTION

Lead (Pb) is a widespread contaminant in the environment and is highly toxic to biological systems [1]. Lead contamination in soils can cause profound toxic effects on soil microorganisms and their activities, resulting in soil fertility deterioration [2]. In addition, it can also directly induce phytotoxicity to rice (*Oryza sativa* L.), which leads to the inhibition of rice growth and yield [3]. Rice is a staple crop in China, contributing to approximately 40% of the national grain yield [4,5]. Previous studies have suggested that Pb might be accumulated in the tissues or grain of rice at considerable levels and then transferred to consumers at higher trophic levels, including humans [4,6]. Thus, long-term exposure to Pb through rice consumption may result in a critical health problem for China and the rest of the world.

The use of biochar has recently been proposed as a possible means of meeting soil remediation needs in the most cost-effective way [7–9]. Converting agricultural biomass and solid waste into biochar and applying it as an amendment for contaminated soil can mitigate pollutants and exert long-term beneficial effects for both agriculture and the environment. Researchers have reported that the addition of biochar to lead-contaminated soil can immobilize toxicants in soil [10–13], reduce bioavailability, and mitigate the phytotoxicity of lead to plants [11,14,15].

Interest has also been growing in the application of biochar to soil for managing soil microorganisms, which can have implications for soil structure and stability, nutrient cycles in soil, degradation of organic residues, synthesis of humic substances, carbon storage capacity, and pollutant degradation [16–18]. These effects of microorganisms on soil physicochemical properties may in turn influence plant growth. In most studies, microbial biomass and microbial activity have been found to increase as a result of biochar additions [19,20], which can serve as sensitive indicators of changes in soil fertility [21].

Microbial biomass and microbial activity have been determined in biochar-amended soil by various methods, such as total genomic DNA extraction, culturing and plate counting, and fumigation extraction [19,22,23]. To date, no studies have investigated the effects of biochar on the microorganisms in metal-contaminated soil using fluorescein diacetate (FDA). Hydrolysis by FDA is widely accepted as an accurate and simple method for measuring total microbial activity in soils [24–26], because FDA can be hydrolyzed by the enzymes involved in microbial activity (e.g., esterases, proteases, and lipases) [24–26]. In addition, FDA hydrolysis has been found to be significantly correlated with microbial biomass in soils [26].

In the present study, a sensitive and rapid method (FDA hydrolysis) was applied to evaluate the effects of biochar on total microbial activity of Pb(II)-contaminated soil. In addition, biochar effects on the bioavailability and phytotoxicity of Pb(II) was demonstrated by the application of 4 different biochars into soil, with rice as the test plant. The main goal of the present study was therefore to determine the effects of 4 different biochar amendments on FDA hydrolytic activity in Pb(II)-polluted soil and associated phytotoxicity to rice.

MATERIALS AND METHODS

Soil and biochars

The soil used in the present study was collected in Changsha, Hunan Province (28°10.91′N, 112°56.82′E) in China. The basic

All Supplemental Data may be found in the online version of this article.
* Address correspondence to hnuliuyunguo@gmail.com.
Published online 21 April 2015 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3023

properties are given in Table 1. The soil sample was air-dried and ground finely enough to pass through a 2-mm sieve.

Four feedstocks (bamboo, coconut shell, pine wood shavings, and sugarcane bagasse) were collected to produce biochar under an inert atmosphere. The temperature was programmed to increase to 450 °C at a rate of 7.1 °C min^{-1} and held at the peak temperature for 2 h. The resulting biochars were gently crushed and passed through a 60-mesh sieve. The biochars are referred to as bamboo biochar, coconut shell biochar, pine wood shavings biochar, and sugarcane bagasse biochar, respectively.

Properties of biochars

Moisture was determined by calculating weight loss after heating the biochars at 105 ± 5 °C for 4 h to a constant weight. Volatile matter was calculated as the weight loss after heating in a muffle furnace that had been previously heated to 850 ± 20 °C for 10 min. Ash content was also determined by measuring the residue weight after heating at 800 ± 20 °C for 4 h in an open-top crucible. The portion of fixed carbon in the biochar was calculated by subtracting the amount of moisture, ash, and volatile matter from the mass of dried biochar. Elemental analyses of C, H, and O contents of all biochars were carried out with an elemental analyzer (Vario EL III, Elementar). The pH of the biochar was measured in deionized water at a 1:10 w/w ratio. Each biochar sample was thoroughly mixed and allowed to equilibrate with deionized water for 1 h before the pH was measured with a meter.

Incubation of biochar-amended soil

The soil and biochar (2% biochar by weight) were mixed thoroughly in buckets and then wetted with Milli-Q water to 70% of field water holding capacity of the soil. Then the samples were incubated at a constant 25 ± 2 °C in the dark with constant moisture content for 360 d to stabilize the reaction between the soil and biochar, after which the samples were air-dried for the following experiments. The soils with different biochars added are referred to in the present study as bamboo biochar–soil, coconut shell biochar–soil, pine wood shavings biochar–soil, and sugarcane bagasse biochar–soil. The original soil without a biochar amendment is termed the control.

Triplicate samples of biochar-amended soil (50 g) were weighed into a Petri dish. Then 20 mL of the $Pb(NO_3)_2$ solution of varying concentrations (50 mg L^{-1}, 200 mg L^{-1}, and 500 mg L^{-1}) was added to each Petri dish (i.e., 20 mg kg^{-1}, 80 mg kg^{-1}, and 200 mg kg^{-1}). The samples were incubated as described above for 10 d. Then part of the soils was air-dried and ground sufficiently to pass through a 2-mm sieve for measurement. The morphological characteristics of biochar-amended soils after incubation were observed under a scanning electron microscope (TM3000; Hitachi).

Measurement of FDA hydrolysis in soil

The FDA hydrolysis was determined by modification of the procedure described in previous studies [18]. Samples of moist soil (equivalent to 1 g oven-dried soil) in triplicate were shaken with 15 mL of 0.2 M sodium phosphate buffer (pH 7.6) and 0.5 mL of FDA substrate solution (2 mg mL^{-1}) for 2 h at 30 °C. After the reaction was stopped with 8 mL of chloroform/methanol, FDA hydrolytic activity (μg FDA g^{-1} 2 h^{-1}) was calculated according to the absorbance of the supernatant solution measured using a spectrophotometer at 490 nm.

Sequential extraction

The Community Bureau of Reference sequential extraction procedure used in the present study was as previously described [27]. Four sequential extraction steps were conducted to give rise to 4 different fractions of Pb(II) in soil: 0.11 M acetic acid, 0.1 M hydroxylamine hydrochloride (pH 2), 8.8 M hydrogen peroxide (H_2O_2), and 1 M ammonium acetate (NH_4OAc; pH 2), and nitric acid–hydrofluoric acid–perchloric acid (HNO_3–HF–$HClO_4$) were applied to extract the acid-soluble fraction, reducible fraction, oxidizable fraction, and residual fraction of the metals, respectively. The extracted solutions were determined by atomic absorption spectroscopy (Analyst 700; PerkinElmer).

Rice seed sprouting and growth

Triplicate soil samples (50 g) were placed in Petri dishes. Then 20 mL of the $Pb(NO_3)_2$ solution (500 mg L^{-1}) was added to each Petri dish (i.e., 200 mg kg^{-1}). The samples were incubated for 10 d as described above in *Incubation of biochar-amended soil*. The rice seeds were soaked in 2% H_2O_2 for 5 min, and rinsed with deionized water 5 times; then 20 seeds were placed evenly into each Petri dish. The dishes were incubated in an artificial climate box (26 ± 2 °C, illumination 4000 lx, humidity 70%) and then left for a 16:8-h light:dark photoperiod. The dishes were weighed every day, and water was added to maintain constant moisture content throughout the incubation period. After 3 d, 7 d, and 12 d of incubation, sprouting, and growth, the rice seeds were measured.

Statistical analyses

Differences among treatments were assessed by analysis of variance (ANOVA) using SPSS Ver 18. The results represent the average of 3 independent replicate treatments. The data are presented as means ± standard deviations (SDs).

RESULTS AND DISCUSSION

Biochar properties

The physicochemical characteristics of the 4 different biochars are shown in Table 2. Elemental analysis showed that all 4 biochars were carbon rich and contained 75.27% to 81.34% carbon. The oxygen and hydrogen contents of biochars, some of which may compose the surface functional groups [28], ranged from 11.30% to 18.21% and 4.19% to 4.88%, respectively. The coconut shell biochar contained the highest amount of carbon and the lowest amounts of oxygen and hydrogen, indicating a higher degree of carbonization. In contrast, pine wood shavings biochar had higher amounts of oxygen and hydrogen than other biochars. The H/C and O/C

Table 1. The basic properties of soil used in the present study

Organic carbon (g kg^{-1})	Total nitrogen (g kg^{-1})	Total phosphorus (g kg^{-1})	Total potassium (g kg^{-1})	Cation exchange capacity (cmol kg^{-1})	Lead (mg kg^{-1})
18.5	0.870	0.254	15.6	16.7	Undetected (< 0.4)

Table 2. Physicochemical characteristics of the 4 different biochars ($n = 4$)

		Elemental composition (%, mass based)									
Biochars	pH	C	H	O	O/C	H/C	Moisture (wt %)	Ash (wt %)	Volatile matter (wt %)	Fixed carbon (wt %)	Yield (%)
BB	10.92	77.01	4.19	16.67	0.16	0.65	2.22	3.20	19.78	74.80	35.97
CB	10.28	81.34	4.30	11.30	0.10	0.63	2.08	3.15	16.76	78.01	34.23
PB	9.77	75.27	4.88	18.21	0.18	0.78	2.44	5.69	17.43	74.44	37.36
SB	10.37	77.98	4.77	14.98	0.14	0.73	2.61	5.43	15.23	76.73	36.30

BB = bamboo biochar; CB = coconut shell biochar; PB = pine wood shavings biochar; SB = sugarcane bagasse biochar.

molar ratios changed from 0.63% to 0.78% and 0.10% to 0.18%, and increased in the order of coconut shell biochar < bamboo biochar < sugarcane bagasse biochar < pine wood shavings biochar, and coconut shell biochar < sugarcane bagasse biochar < bamboo biochar < pine wood shavings biochar, respectively. The degree of carbonization may be described by the H/C ratio [29,30], with a very low H/C ratio indicating a highly carbonized and aromatic structure [31]. A higher O/C ratio in a biochar material may indicate the presence of more functional groups (such as hydroxyl, carboxylate, and carbonyl) [7]. The pH values of all the biochars were alkaline (9.77–10.92), suggesting that they could be used as amendments to reduce soil acidity. Fixed carbon in the biochars changed from 74.44% to 78.01%, reflecting its stability and importance as a source of carbon sequestration in soil [32]. Ash content ranged from 3.15% to 5.69% in the different biochars, depending on their feedstock types.

Morphological characteristics of biochar-amended soil

Scanning electron microscopy images of 4 biochars are shown in Figure 1. Four biochars exhibited irregular surfaces, with pores of different shapes and sizes. The bamboo biochar had larger pore sizes than the others, although obvious channel structures were observed in pine wood shavings biochar and sugarcane bagasse biochar. Surface properties are important for biochar's function in soil. After 360 d of incubation, biochars had been evenly combined with soil particles. The biochars were attached to the surface of the soil (Supplemental Data, Figure S1), so that pores between the biochar particles and soil aggregates were formed [33]. Improvements in soil pore structure, which can increase soil water and nutrient retention correspondingly [34], may be important for plant productivity as well as a reduction in irrigation frequency [35]. In addition, previous studies have suggested that the pore structure and the functional groups on the biochar can also improve the immobilization rates of soil pollutants [8,36].

Effect of biochar on FDA hydrolytic activity

To determine whether the biochar could be used as an amendment to mitigate toxic or harmful effects of Pb(II) on soil microorganisms, which negatively influence microbial activity [37], the biochar-amended soils were contaminated by Pb at

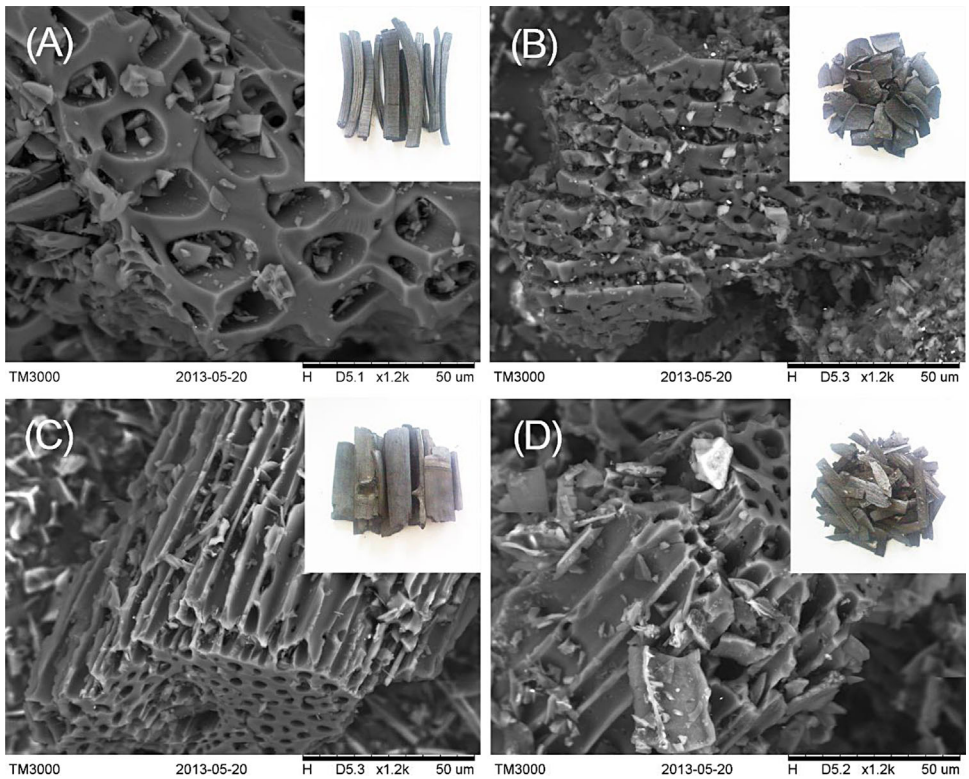

Figure 1. Scanning electron microscopy images of 4 biochars: (**A**) bamboo biochar, (**B**) coconut shell biochar, (**C**) pine wood shavings biochar, and (**D**) sugarcane bagasse biochar.

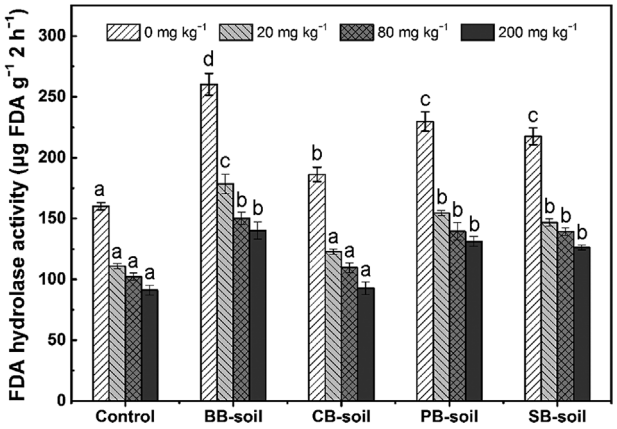

Figure 2. Effect of biochar on the fluorescein diacetate (FDA) hydrolytic activity of soil contaminated by different concentrations of Pb(II):0 mg kg^{-1}, 20 mg kg^{-1}, 80 mg kg^{-1}, and 200 mg kg^{-1}. The labeling (different letters) indicates significant differences across soils (the control and different biochar-amended soils) but within a given Pb(II) spiking level ($p < 0.05$). FDA = fluorescein diacetate; Control = nonamended soil; BB-soil = bamboo biochar-amended soil; CB-soil = coconut shell biochar-amended soil; PB-soil = pine wood shavings biochar-amended soil; SB-soil = sugarcane bagasse biochar-amended soil.

varying concentrations (0 mg kg^{-1}, 20 mg kg^{-1}, 80 mg kg^{-1}, and 200 mg kg^{-1}; Figure 2). The addition of different biochars (bamboo biochar, pine wood shavings biochar, and sugarcane bagasse biochar) led to a significant increase ($p < 0.05$) in FDA hydrolytic activity (217−260 μg FDA g^{-1} 2 h^{-1}), which was higher than that of the control (160 μg FDA g^{-1} 2 h^{-1}), without Pb(II) pollution (Figure 2). The increase in FDA hydrolytic activity suggested that biochar can increase the total microbial activity in soils [18,21]. This positive effect of biochar has been described previously [19,20].

When soil was contaminated by Pb(II), the FDA hydrolytic activity of all samples was decreased markedly, suggesting an inhibiting effect of Pb(II). The negative effect increased along with the increase in Pb(II) concentrations. The FDA hydrolytic activity of biochar-amended soils (129−178 μg FDA g^{-1} 2 h^{-1}) was still much higher ($p < 0.05$) than that of the control (91.1−110 μg FDA g^{-1} 2 h^{-1}), and followed the order of bamboo biochar–soil > pine wood shavings biochar–soil > sugarcane bagasse biochar–soil. Such activity was not significantly different ($p > 0.05$) from the control for coconut shell biochar–soil, however, suggesting that coconut shell biochar had a negligible effect on FDA hydrolytic activity. These data suggest that biochar can mitigate the toxic effects of Pb(II) on microorganisms and that this effect is different for different biochar feedstocks.

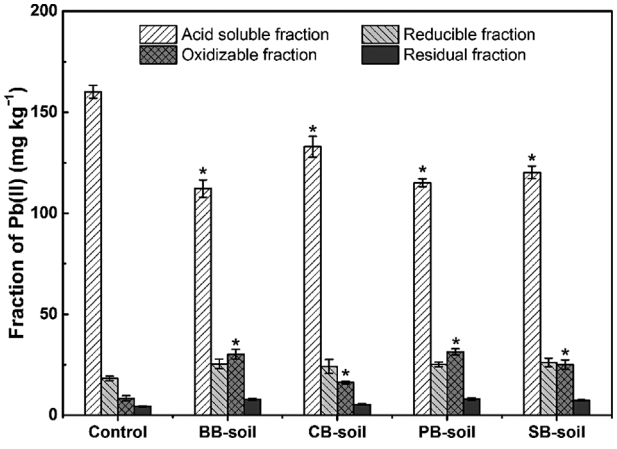

Figure 3. Fraction of Pb(II) determined by Community Bureau of Reference sequential extraction for the treatments with 200 mg kg^{-1} Pb(II). Values designated by an asterisk are significantly different from the control ($p < 0.05$). Control = nonamended soil; BB-soil = bamboo biochar-amended soil; CB-soil = coconut shell biochar-amended soil; PB-soil = pine wood shavings biochar-amended soil; SB-soil = sugarcane bagasse biochar-amended soil.

Community Bureau of Reference fractions of Pb(II)-contaminated soil

The acid-soluble fraction of Pb(II), primarily composed of soluble, exchangeable, surface-adsorbed, and carbonate combined heavy metals, is considered the primary active and bioavailable fraction [10,27]. As shown in Figure 3, the acid-soluble fraction of Pb(II) significantly decreased with the addition of all 4 biochars ($p < 0.05$). When 2% bamboo biochar, coconut shell biochar, pine wood shavings biochar, and sugarcane bagasse biochar were added, the amount of acid-soluble Pb(II) decreased from 160 mg kg^{-1} to 112 mg kg^{-1}, 133 mg kg^{-1}, 115 mg kg^{-1}, and 120 mg kg^{-1}, respectively. This decrease in acid-soluble Pb(II) suggested that the incorporation of biochar significantly decreased the bioavailability and activity of Pb(II) in biochar-amended soil. Correspondingly, oxidizable Pb(II) increased ($p < 0.05$), which was attributed to the formation of complexes of Pb(II) with organic functional groups on the biochars [38]. However, the reducible and residual Pb(II) content only changed a little. In the biochar-amended soil, carbonates and phosphates in the biochar may inhibit the formation of Pb complexation with Fe and Mn (oxides) because of their competitive effect [7], resulting in the small effect on the reducible fraction. In addition, the short contact time of incubation and manual addition of the biochars might have resulted in the small effect on the residual fraction of Pb [10], which was usually comprised of the refractory mineral components.

Effect of biochar on the phytotoxicity of Pb(II)-contaminated soil to rice

In addition to the effects of biochar on microbial activity, we observed a considerable change in the phytotoxicity of Pb(II)-contaminated soil to rice (Figure 4A). As shown in Figure 4B, after 3 d of incubation, the survival rate of rice seedlings was only slightly enhanced in biochar-amended soil (93.3−98.3%) compared with control (93.3%); 7 d later, however, the survival rate of rice seedlings in biochar-amended soil (85.0−93.3%) was higher than that of the control (78.3%). This rate became gradually stable with longer incubation time and decreased relatively little after 7 d. Seedlings in all biochar-amended soils had significantly higher survival ($p < 0.05$) than the control, especially in bamboo biochar–soil (93.3%).

The results for rice seedlings grown in the original soil samples (without Pb(II) pollution) for 12 d suggest that the 4 biochars improved seedling growth differently (Figure 4C and D). The bamboo biochar had an obvious effect ($p < 0.05$) on growth, whereas growth was only slightly improved for coconut shell biochar, sugarcane bagasse biochar, and pine wood shavings biochar ($p > 0.05$). In terms of the contaminated soils, in rice grown in the control soil, lower biomass production (a common symptom of lead phytotoxity) was seen than in biochar-amended soils (Figure 4C and D) [39,40]. Root and shoot lengths of rice seedlings were considerably greater in

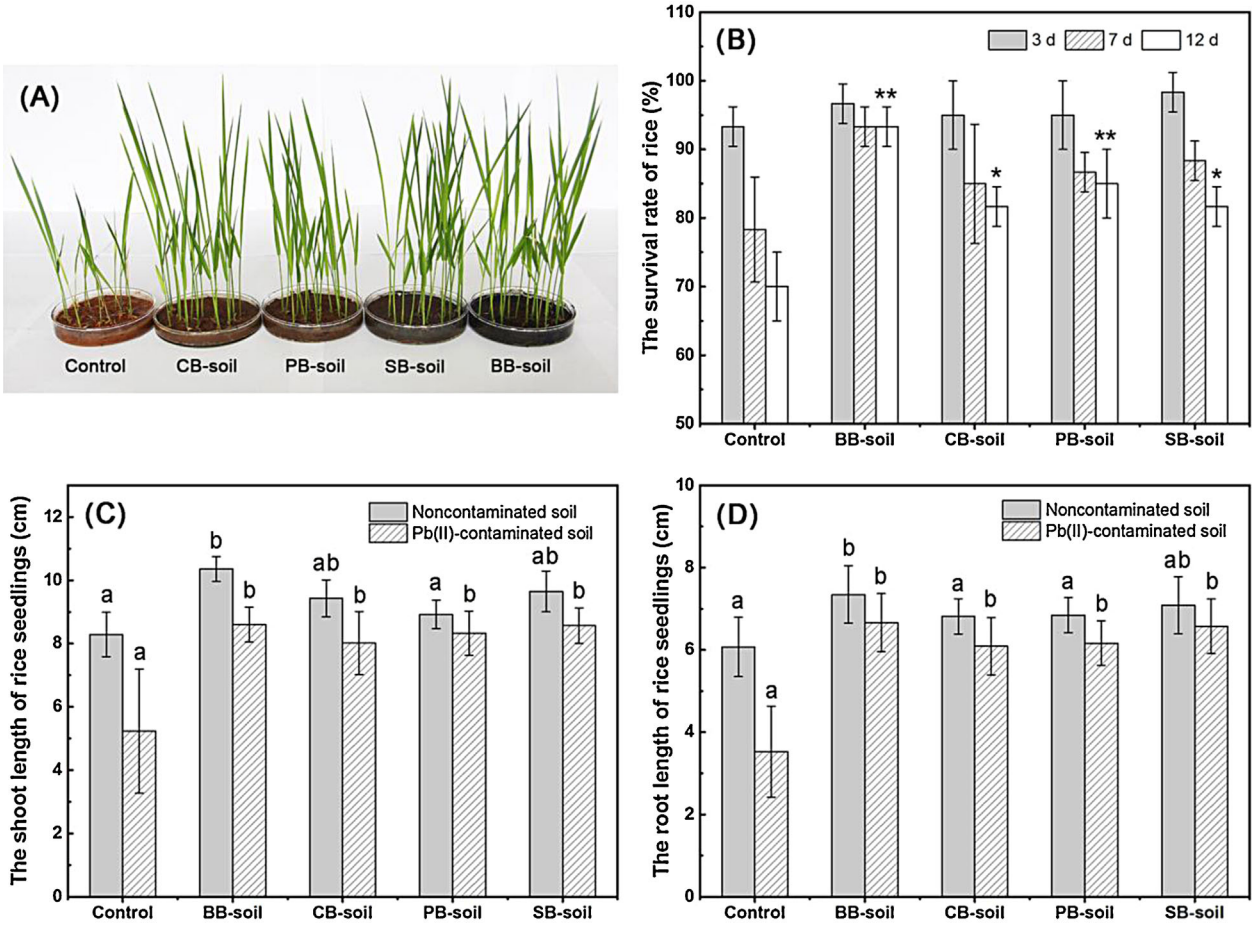

Figure 4. Effect of biochar on the phytotoxicity of Pb(II)-contaminated soil to rice: (**A**) rice grown in different biochar-amended soils after 12 d; (**B**) survival rate of rice (%); (**C**) shoot lengths of rice seedlings (cm); and (**D**) root lengths of rice seedlings (cm). Values designated by an asterisk are significantly different from the control after 12 d ($^{*}p < 0.05$; $^{**}p < 0.01$). The labeling (different letters) indicates significant differences across soils (the control and different biochar-amended soils) but within the uncontaminated soil and within the Pb-contaminated treatments ($p < 0.05$). Control = nonamended soil; BB-soil = bamboo biochar-amended soil; CB-soil = coconut shell biochar-amended soil; PB-soil = pine wood shavings biochar-amended soil; SB-soil = sugarcane bagasse biochar-amended soil.

biochar-amended soil compared with the control ($p < 0.05$). These results suggest that biochar can significantly mitigate the phytotoxicity of Pb(II)-contaminated soil to rice.

Mechanisms of biochar effects on the Pb(II)-contaminated soil

We can see from Figure 2 that FDA hydrolytic activity of the control (i.e., soil without biochar amendment) decreased significantly with the addition of $Pb(NO_3)_2$. Even though the added nitrate from $Pb(NO_3)_2$ may have a positive effect on FDA hydrolytic activity, it cannot overcome the toxic effect of Pb. The biochar-amended soil, however, showed much higher FDA hydrolytic activity than nonamended soil with or without Pb pollution (except for coconut shell biochar–soil), which suggested that biochar played a major role in FDA hydrolytic activity. Similar results can also be found in the effects of biochar on rice seedling germination rate and root and shoot length.

Biochar could influence soil microbial activity in several ways. It may improve nutrient and carbon availability, influence soil pH, provide a habitat for microorganisms, protect them from other biota, and serve as a substrate [17,41]. In addition, biochar may also serve as an adsorbent to immobilize toxicant in Pb(II)-contaminated soil [17]. Biochars lead to the redistribution of Pb to soil fractions that are less bioavailable, resulting in

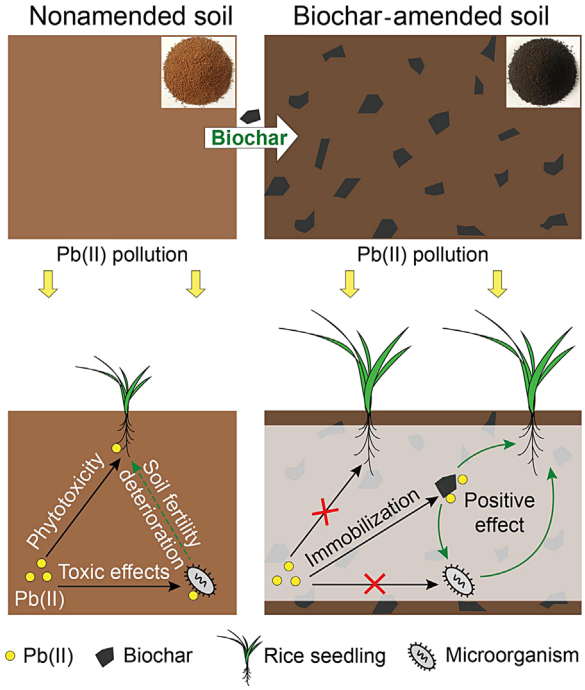

Figure 5. Mechanisms of amendment effects of biochars on the Pb(II)-contaminated soil.

less direct phytotoxicity, less impact of Pb on microbial activity, and therefore a lower indirect effect of Pb on the microbial population (Figure 5).

Biochar can also mitigate the phytotoxicity of Pb(II) through an increase in nutrient availability and direct removal of Pb(II) [42,43]. The effect of biochar on rice growth in the contaminated soil was somewhat in line with its effect on the FDA hydrolytic activity. This may be attributed to the fact that biochar has positive effects on soil microorganisms, and in turn soil microorganisms will improve soil function and ecosystem services (e.g., soil structure and stability, nutrient cycling) [17], which can have implications for plant growth.

CONCLUSIONS

In summary, our results from the present study suggest that biochar amendment has significant effects on soil properties and that biochar-amended soil can effectively resist the toxic effects of future lead contamination. Biochar can mitigate the toxic effects of Pb(II) to the soil microorganisms and the phytotoxicity of Pb(II) to rice. This positive effect may be attributed to the influence of biochar on the physicochemical properties of soil and the immobilization of Pb(II). Thus, biochar may provide a possible cost-effective solution to the long-term health threats of Pb through rice consumption; however, future field trials are needed to confirm whether biochar can provide long-term soil effects. Furthermore, the effects of biochar on the geochemical distribution of native Pb contamination should also be studied.

SUPPLEMENTAL DATA

Figure S1. (278 KB DOC).

Acknowledgment—The authors are grateful for financial support from the National Natural Science Foundation of China (grant 41271332), the Science and Technology Planning Project of Hunan Province, China (grant 2012SK2021), and the Hunan Provincial Innovation Foundation for Postgraduates (grant CX2012B138).

Data availability—All relevant data are available in the manuscript; for additional data requests, contact the authors (hnuliuyunguo@gmail.com).

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