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Research article

Influence of rice straw biochar on growth, antioxidant capacity and copper uptake in ramie (*Boehmeria nivea* L.) grown as forage in aged copper-contaminated soil

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A B S T R A C T

Copper (Cu) contamination in agricultural soil poses severe threats to living organisms, and possible ecofriendly solutions need to be considered for Cu immobilization, such as using biochar. A pot study was conducted to examine the effectiveness of biochar derived from rice straw (RSB) at various application rates (0, 2.5, 5 and 10% w/w) to mitigate possible risks of Cu solubility and its uptake by ramie (*Boehmeria nivea* L.) as forage. The plant growth parameters as well as soil chemical properties (pH, electrical conductivity and cation exchange capacity) notably improved with the increasing RSB application. Moreover, prominent reduction was observed in soil bioavailable Cu concentration by 96% with RSB application of 10% relative to control. In addition, Cu content in *B. nivea* roots, leaves and stems decreased by 60, 28 and 22%, respectively, for 10% RSB application. It was noted that chlorophyll content and gas exchange parameters in leaves were significantly higher at 10% RSB application than in control. Furthermore, 10% RSB resulted in a greater reduction in oxidative stress from the Cu in soil. Thus, soil amendment with RSB demonstrated positive results for Cu stabilization in aged Cu-contaminated soil, thereby reducing its accumulation and translocation in *B. nivea* and mitigating livestock feed security risks.

1. Introduction

During recent decades, rapid increase in urbanization and industrialization has caused excessive release of heavy metals in farmlands with damaging effects on ecosystems (Lu et al., 2014). Release of heavy metals in farmlands from anthropogenic activities (e.g., wastewater irrigation, inadequate battery recycling practices and inappropriate use of pesticides and fertilizers) is a cause of global environmental pollution (Bashir et al., 2018b). Particularly, copper (Cu) is of important as a heavy metal pollutant but is also required as a micronutrient for crop production (Jiang et al., 2012a). However, high Cu concentrations in soils result in severe disorders in plant physiological and respiratory processes, which hinder plant growth (Chaffai et al., 2007; Zvezdanovic et al., 2007). The Cu toxicity level in plants is thought to be 20–30 mg kg^{-1} of dry mass (Marschner, 1995).

Ramie (*Boehmeria nivea*) is a fast growing perennial plant used in China as a source of bast fiber as well as nutritious green feed palatable to all classes of farm animals. Due to its high biomass production, high crude protein content and strong adaptability to different environments, cultivation of *B. nivea* could be an economically beneficial solution to fulfill the demand for fiber and feed in metal-contaminated areas. It is quite tolerant to Cu and can be grown as pioneer plant in mining areas to control heavy metal pollution (Min-fei et al., 2016). The stabilization of Cu in contaminated farmland will limit its buildup and translocation in aerial parts of *B. nivea* and so ensure animal food security but represents a big challenge. Several remediation techniques have been considered for Cu immobilization in farmland but *in situ* immobilization is the most important and economical approach

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available worldwide. Immobilization of heavy metals can be attained by transformation from soluble to residual form by means of adsorption, precipitation and complexation (Ahmad et al., 2014a). Various organic materials including biochar, plant residues and compost have been used as efficient soil amendments for metal stabilization and reducing metal toxicity in soil–plant systems (Bashir et al., 2018c).

Biochar is a carbon-rich organic byproduct, produced through pyrolysis of organic waste biomass (e.g. agricultural residues and animal manures) at high temperature with a limited oxygen supply (Ahmad et al., 2014b; Lu et al., 2014). Biochar is biochemically stable, resistant to decomposition, capable of enhancing the long-term soil organic carbon pool (Lehmann et al., 2011) and can improve soil properties and crop yield (Jeffery et al., 2011; Matovic, 2011). Various studies have reported the effectiveness of biochar in immobilizing different trace elements (e.g. Cu) as well as reducing phytotoxicity in polluted soils (Park et al., 2011; Luo et al., 2014). Biochar can minimize the detrimental effects of Cu in contaminated soils due to its surface and chemical properties (Park et al., 2011). According to Lu et al. (2014), applying biochar derived from rice straw (RSB) is more effective than bamboo biochar in immobilizing Cu, zinc (Zn) and lead (Pb) in soil and can reduce their bioavailability. Recently, Yang et al. (2016) concluded that RSB incorporation reduced the extractable Cu concentration by 97% in multi-contaminated paddy soil. Several studies revealed that application of various types of biochar in metal polluted soils have potential to minimize metal mobility and phytoavailability through adsorption and complexation (Park et al., 2011; Yuan et al., 2011; Jiang et al., 2012a; Bashir et al., 2018a). In this regard, different biochars have been examined for Cu remediation in soils polluted with heavy metals (Jiang et al., 2012a; Lu et al., 2014). However, insufficient information is available concerning the addition of RSB on Cu solubility and phytoavailability to *B. nivea* in soil highly contaminated by Cu.

The prime objectives of the present work were to (1) study the influence of RSB on *B. nivea* growth and antioxidative capacity under Cu stress, (2) identify the RSB-mediated reduction of Cu toxicity in *B. nivea* and (3) quantify the effect of RSB on post-harvest soil characteristics and extractable Cu.

2. Materials and methods

2.1. Material collection and description

The soil was collected from a Cu mining area of Baisha village, DaYe County, Hubei, China (115.20′E, 29.85′N) at depth of 0–20 cm. The soil was thoroughly mixed, air dried under shade, ground and sieved through a 5-mm sieve before a pot experiment. The RSB (prepared at 300 °C) was obtained from an organic biochar manufacturer in Hubei, China. Following are the main agrochemical properties of the tested loam soil: pH 6.87, electrical conductivity (EC) of 269.0 μs cm^{-1}, cation exchange capacity (CEC) of 14.99 cmol kg^{-1}, organic matter of 3.96 g kg^{-1}, total nitrogen (N) of 0.16 g kg^{-1}, total phosphorus (P) of 1.97 g kg^{-1}, total potassium (K) of 12.25 g kg^{-1}, available Cu of 145.41 mg kg^{-1}, total Cu of 2221.15 mg kg^{-1}. Properties of the RSB were pH 9.42, total C of 47.14%, total N of 0.74%, available P of 262.2 g kg^{-1}, available K of 673 g kg^{-1} and carbon/N ratio of 63.54. The test plant was ramie cv Zhongsizhu No. 1, which was the first dedicated feed ramie variety released by the Ministry of Agriculture in 2012.

2.2. Pot experiment

The experiment was conducted in the green house at the College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, during spring 2018. Each plastic pot (height 25 cm and top and bottom diameter of 30 and 25 cm, respectively) was filled with 14 kg of aged Cu-contaminated soil on a dry weight basis. The soil was then homogeneously amended with RSB at 0, 2.5, 5 and 10% (w/w) on dry soil basis, and incubated for 60 days at 70% (w/v) soil water holding capacity. The experiment was arranged in a completely randomized design with three replications. Uniform sized (15 cm) segments of rhizome were obtained from ramie roots were planted as one plant in each pot. The moisture content of soil in pots was maintained at 70% soil water holding capacity, and weeds removed on a regular basis.

2.3. Sampling and data collection

A functional leaf (fifth from the top) in each treatment was picked at the rapid growth stage during 09:00–10:30 a.m. Fresh plant leaves were cleaned and immediately stored in liquid nitrogen and (−80 °C) for further analysis. Plants were harvested after 40 days of planting, by cutting stems at 5 cm above the soil surface. Before harvest, nine plants (three per replicate) per treatment were sampled randomly. The number of stems per plant was counted and plant height (cm) was measured from root neck to the top of stalk. Stem diameter (mm) at 15 cm above the root neck was measured using a digital Vernier caliper (ST22302, SG Tools, Hangzhou, China). Fresh shoot biomass was determined by weighing both leaves and stems, and then leaves were weighed separately after removing stems. After harvesting of plants the roots were uprooted and immersed in 20 mM Na_2EDTA for 15–20 min to remove Cu adhered to surface of roots. Then roots were washed thrice with distilled water, and finally once with de-ionized water. Roots, leaves and stems were then oven-dried to a constant weight at 70 °C for 72 h and weighed to determine their dry weights.

2.4. Chlorophyll content and gaseous exchange

Chlorophyll content from fresh plant leaves was determined according to Lichtenthaler (1987). Gaseous exchange parameters were measured using a portable photosynthesis system Li-6400 (Li-COR, Lincoln, NE, USA): net photosynthesis (*Pn*), transpiration rate (*Tr*), stomatal conductance (*Gs*) and intercellular CO_2 (C_i). These parameters were measured during 10:00–11:00 a.m. when all plant parts were fully functional.

2.5. Determination of Cu content in plants

The homogenized plant tissues were digested with a mixture of HNO_3:$HCLO_4$ (4:1) solution on a hot plate at 150–180 °C until clear liquid appeared. Finally, Cu contents were determined using an atomic absorption spectrophotometer (AAS) model Agilent 240FS-AA.

2.6. Soil sampling and analyses

After the crop harvest, soil was taken from each pot to measure physico-chemical properties. The soil pH and EC were determined at 1:2.5 and 1:5 (w/v) soil water suspensions, using automated pH and EC meters, respectively (Lu, 1999). Soil CEC was measured by ammonium acetate method at pH 7.0 (Lu, 1999). Bioavailable soil Cu was determined by extracting soil with 0.01 M $CaCl_2$ (2:20 w/v) mixture (Houben et al., 2013). After extraction and filtration, the supernatant was analyzed for Cu using AAS (Agilent 240FS-AA). Blanks and the certified reference material (GBW07405) Geophysical Geochemical Exploration, Geological Sciences Institute, Chinese Academy of Sciences, IGGE, Hebei, China were used in all analyses for quality control.

2.7. Determination of malondialdehyde (MDA) and proline content

Lipid peroxidation was assayed by determining the amount of MDA in the functional leaf according to Heath and Packer (1968). A leaf sample (0.5 g) was homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 × *g* for 5 min. In a glass tube, a 1-ml aliquot and 4 ml of 20% TCA (containing 0.5% thiobarbituric acid) were added and incubated at 95 °C for 30 min. The reaction was stopped by cooling in ice and mixture was centrifuged at 10,000 × *g* for 15 min. Absorbance was read at 532 and 600 nm using a microplate absorbance spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad, United States). The MDA contents were determined by the difference in absorption using 155 mM^{-1} cm^{-1} as the extinction coefficient. Proline contents were determined by the method of Bates et al. (1973) using a standard curve prepared with proline.

2.8. Analysis of antioxidant enzyme activities

The antioxidants superoxide dismutase (SOD) and peroxidase (POD) were extracted by crushing a 0.5-g fresh leaf sample using liquid nitrogen followed by homogenization in 5 ml of 50 mM sodium phosphate buffer (pH 7) containing 0.5 mM ethylenediaminetetraacetic acid and 0.15 M NaCl. The homogenate was then centrifuged at 12,000 × *g* for 10 min at 4 °C and supernatants were used for enzyme assays.

The SOD activity was assayed in 3 ml of reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 1.17 mM riboflavin, 10 mM methionine, 56 mM nitro-blue tetrazolium (NBT) and 100 µl of enzyme extract. Absorbance was recorded to measure inhibition of photochemical reduction of NBT at 560 nm using microplate absorbance spectrophotometer. The SOD activity was expressed as U g^{-1} fresh weight (FW). One unit of SOD was defined as "enzyme activity that reduced photoreduction of NBT to blue formazan by 50%" (Chen and Pan, 1996).

The POD activity was determined by the increase in absorbance at 470 nm using microplate absorbance spectrophotometer and the method of Sakharov and Aridilla (1999). The reaction mixture comprised 0.1 ml of hydrogen peroxide (2%), 2.8 ml of guaiacol (3%) and 0.1 ml of enzyme extract. One unit of POD activity was defined as "increase in absorbance of 1.0 per min". The POD activity was expressed as U g^{-1} FW.

2.9. Statistical analysis

The data recorded were statistically analyzed using Statistix 8.1 (Analytical Software, Tallahassee, F.L., United States). Testing showed that all plant- or soil-related data were approximately normally distributed. Thus, the differences between treatments were determined using analysis of variance, and the least significant difference test ($P \leq 0.05$) used for multiple comparisons between treatment means. Pearson's correlation analysis was performed to quantify relationships between various analyzed variables. Graphical presentation was carried out using SigmaPlot 12.5. The R3.4.1 was used to calculate pearson's correlation.

3. Results

3.1. Plant growth and biomass

The effects of RSB and its application rates on *B. nivea* growth in Cu-contaminated soil are presented in Tables 1 and 2. Plant growth and biomass significantly increased with the increasing RSB rate within 0–10% (Table 1). The maximum shoot and leaf fresh and dry biomass, plant height, number of leaves and stems were determined for the RSB treatments relative to control. There were increases in shoot dry weight of 77, 133 and 224% when RSB was applied at 2.5, 5 and 10% rate relative to control, respectively, and correspondingly leaf dry weight increased by 86, 157 and 216%. Plant height and number of leaves also increased by 41% and 95% at 10% RSB rate relative to control, respectively (Table 2). Compared to control, the greatest increase in stem diameter was 53% for 10% RSB. Maximum numbers of stems per plant were produced at 10% RSB (3.33), and minimum (1.89) was for control.

3.2. Chlorophyll contents and gaseous exchange

Total chlorophyll contents in leaves increased relative to control (Table 2) by 35, 55 and 79% for RSB addition of 2.5, 5 and 10%, respectively. The *Pn* was increased with the progressive addition of RSB and maximum was observed 10% RSB. (Fig. 1A). There was no significant difference for *Tr* between 0 and 2.5% RSB treatments but *Tr* notably increased with the further increase in RSB. There was a large increase in *Tr* of 160% at 10% RSB compared to control (Fig. 1B). The *Gs* significantly increased by about 32, 47 and 86% at 2.5, 5 and 10% RSB addition compared to control, respectively (Fig. 1C). However, *Ci* did not significantly differ among treatments (Fig. 1D).

3.3. Distribution of Cu in plants

Addition of RSB in soil significantly reduced Cu accumulation in *B. nivea* (Fig. 2). The roots showed greater Cu accumulation in all treatments, followed by leaves and stem. The Cu contents were reduced in roots and leaves with RSB addition, the effect was more pronounced at 10% than 2.5 and 5% RSB rates. Addition of RSB at 2.5, 5 and 10% reduced the Cu concentration in roots (25, 48 and 60%, respectively) and leaves (13, 19 and 28%, respectively) compared to control (Fig. 2A and B). The Cu content in stems at 2.5% RSB was 36.3 mg kg^{-1} and did not significantly differ to control with 37.6 mg kg^{-1}. However, further increase in RSB at 5 and 10% resulted in reductions in Cu accumulation in stems by 9 and 22%, respectively (Fig. 2C).

3.4. Oxidative stress and antioxidant enzymes

Addition of RSB significantly affected the SOD and POD activities (Fig. 3). Addition of RSB significantly decreased SOD and POD activities in leaves compared to control. The greatest reductions in SOD and POD activities were by 38 and 63% after RSB incorporation at 10% relative to control, respectively (Fig. 3A and B). The RSB addition notably decreased the MDA and free proline contents in leaves compared with control – thus, RSB addition resulted in the reductions in oxidative stress. Application of RSB at 2.5, 5.0 and 10% significantly decreased MDA contents by 20, 40 and 57% relative to control, respectively (Fig. 3C). Similarly, the greatest reduction in free proline contents was 33% for 10% RSB relative to control (Fig. 3D).

3.5. Soil characteristics and extractable Cu concentration in soil

The physicochemical properties of post-harvest soil (pH, EC, CEC

Table 1
Changes in fresh and dry biomass of *B. nivea* under different concentrations of rice straw biochar in Cu polluted soil.

Rice biochar (W/W %)	Fresh biomass (g/plant)		Dry biomass (g/plant)	
	Shoot	Leaf	Shoot	Leaf
0	38.00 ± 2.79^{d}	28.04 ± 2.19^{d}	5.45 ± 0.18^{d}	3.36 ± 0.14^{d}
2.5	64.82 ± 2.83^{c}	43.69 ± 2.25^{c}	9.62 ± 0.94^{c}	6.24 ± 0.47^{c}
5	82.51 ± 4.99^{b}	52.60 ± 3.12^{b}	12.72 ± 1.03^{b}	8.63 ± 0.35^{b}
10	117.73 ± 4.66^{a}	77.89 ± 4.12^{a}	17.67 ± 0.86^{a}	10.60 ± 1.03^{a}

Values in the table are means ± SD (*n* = 3). Different letters in a column indicate significance of difference between treatments ($P \leq 0.05$).

Table 2
Changes in morphological traits of *B. nivea* under different concentrations of rice straw biochar in Cu polluted soil.

Rice biochar (W/W %)	Plant height (cm)	Stem diameter (mm)	No. of leaves ($plant^{-1}$)	No. of stems ($plant^{-1}$)	Chlorophyll ($mg\ g^{-1}FW$)
0	39.89 ± 2.83^{c}	4.15 ± 0.16^{c}	34.00 ± 2.00^{d}	1.89 ± 0.19^{c}	2.11 ± 0.04^{d}
2.5	45.56 ± 2.52^{b}	5.69 ± 0.39^{b}	40.33 ± 2.19^{c}	2.33 ± 0.33^{bc}	2.85 ± 0.09^{c}
5	49.78 ± 2.12^{b}	6.81 ± 0.44^{a}	51.00 ± 1.33^{b}	2.56 ± 0.19^{b}	3.27 ± 0.15^{b}
10	56.22 ± 3.42^{a}	6.35 ± 0.17^{a}	66.33 ± 2.08^{a}	3.33 ± 0.33^{a}	3.78 ± 0.09^{a}

Values in the table are means ± SD (n = 3). Different letters in a column indicate significance of difference between treatments ($P \leq 0.05$).

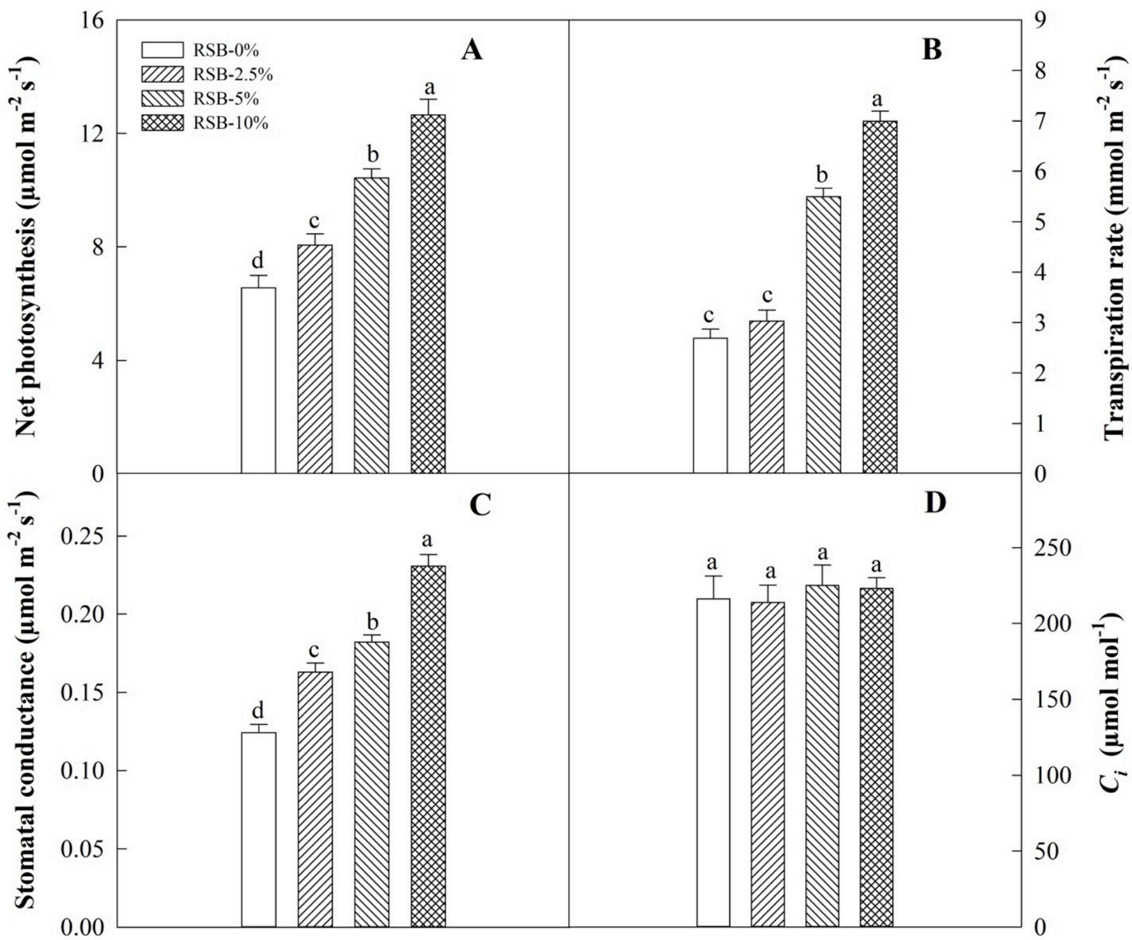

Fig. 1. Effects of increasing doses of biochar (0%, 2.5%, 5.0% and 10% w/w) on gas exchange parameters of *B. nivea* plants grown in Cu contaminated soil. Values represent mean ± SD (n = 3). Different letters indicate significant difference between treatments at $p \leq 0.05$.

and soluble Cu) in RSB-amended polluted soil are presented in Table 3. The soil chemical properties pH, EC and CEC showed prominent increments with RSB addition from 0 to 10%. The 10% RSB increased soil pH by 0.54 units compared to control. Similarly, the highest values for EC ($0.55\ dS\ m^{-1}$) and CEC ($18.38\ cmol\ kg^{-1}$) of post-harvest soil were for 10% RSB. Bioavailable Cu contents decreased prominently with increasing RSB dose. The maximum reduction of $CaCl_2$-extractable Cu (96%) in post-harvest soil was for 10% RSB compared to control.

3.6. Correlation analysis

Pearson's correlation analysis was used to quantify relationships between the measured variables. The amount of $CaCl_2$-extractable Cu in post-harvest soil was significantly negatively correlated with soil pH, EC and CEC (Fig. 4A). The *B. nivea* root Cu concentration was significantly positively correlated with Cu concentrations in leaves and stems. However, the root, leaf and stem Cu concentrations were significantly negatively correlated with shoot FW, shoot dry weight, leaf FW, leaf dry weight and number of leaves per plant (Fig. 4B).

4. Discussion

4.1. Biochar effects on plant growth, chlorophyll contents and gas exchange parameters

Biochar incorporation as an amendment in soil contaminated by heavy metals is considered an emerging remediation option for soil restoration. Our results confirmed that RSB addition to Cu-contaminated soil improved *B. nivea* growth and biomass. As RSB percentage increased, plant FW and dry biomass, plant height and numbers of leaves and stems showed gradual increments (Tables 1 and 2) possibly due to significant reductions in Cu bioavailability in polluted soil. These findings were consistent with those Carter et al. (2013), who concluded that biochar incorporation in contaminated soil could increase plant dry biomass, plant height and number of leaves in lettuce and Chinese cabbage compared to non-biochar treatments. Such positive changes

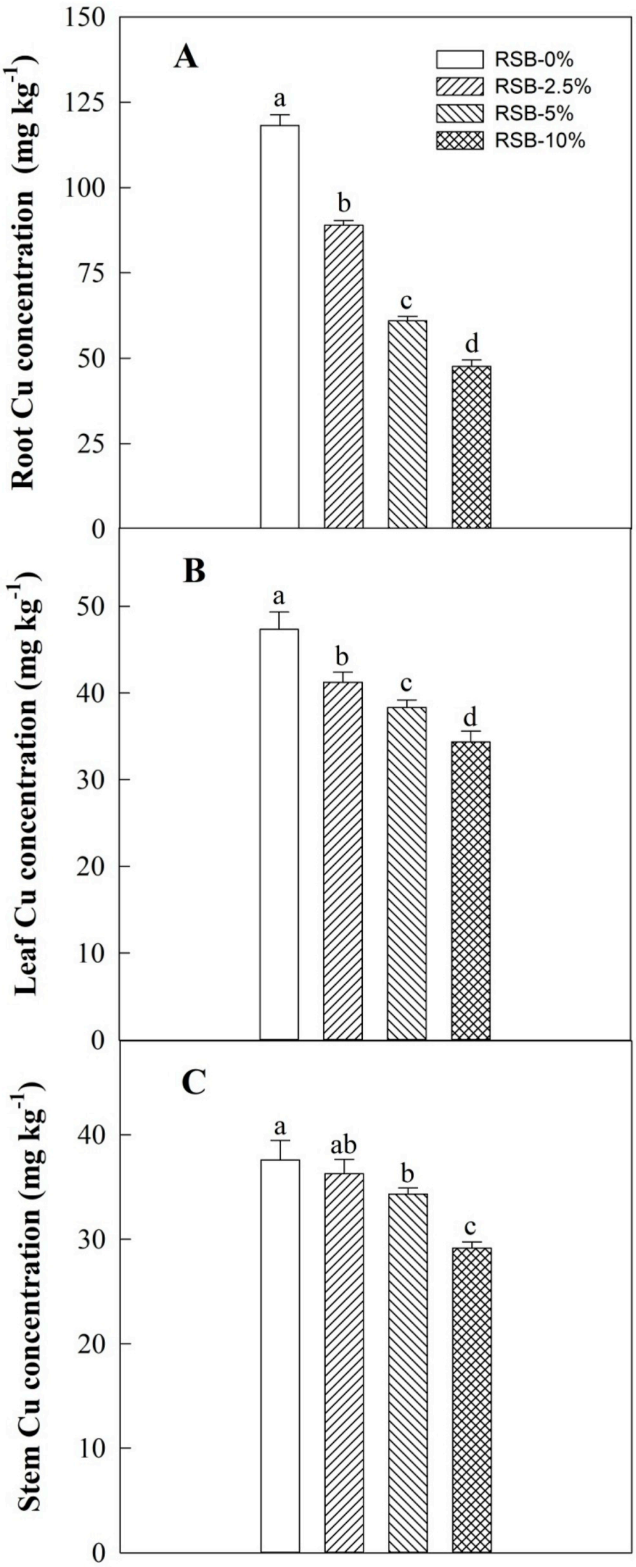

Fig. 2. Effects of increasing doses of biochar (0%, 2.5%, 5.0% and 10% w/w) on Cu concentration in roots, leaves and stems of *B. nivea* plants grown in Cu contaminated soil. Values represent mean ± SD (n = 3). Different letters indicate significant difference between treatments at $p \leq 0.05$.

are likely due to increases in soil pH and lower Cu solubility. We found that RSB addition significantly increased the photosynthetic activity of *B. nivea* relative to control (Fig. 1). Furthermore, this increase in photosynthesis may have contributed to increased dry biomass of *B. nivea* after RSB addition. In phyto-stabilization, significantly increased plant biomass is a key factor to judge growth. We observed that photosynthetic activity and chlorophyll contents played an important role in increasing plant dry biomass, consistent with results of Bashir et al. (2018), in which RSB incorporation increased dry biomass of Chinese cabbage shoot and root by 36.5 and 38.5% compared to control, respectively. Previous studies reported that biochar addition in heavy metal contaminated soil significantly increased plant growth and photosynthetic activity (Rehman et al., 2016; Rizwan et al., 2016b). Recent studies demonstrated that RSB incorporation in cadmium (Cd)-contaminated soil increased chlorophyll contents of wheat plants (Younis et al., 2016; Abbas et al., 2017). Elevated soil pH after biochar addition also contributed to increased production of maize, soybean and radish due to significant increments in soil available nutrients (Lehmann et al., 2003; Rondon et al., 2006; Chan et al., 2007; Chen et al., 2008). Application of biochar to heavy metal polluted land decreases the toxicity to plants, resulting in reduced metal accumulation in plant organs and improved growth (Park et al., 2011; Zhang et al., 2013b). Results of our study also indicated that RSB was a stable organic amendment and played significant role in alleviating Cu stress in *B. nivea*.

4.2. Biochar effect on Cu distribution in plants

The RSB incorporation in Cu-polluted soil showed significant reductions in Cu uptake by plant tissues, confirming the low Cu bioavailability in contaminated soil with increasing biochar rates. The minimum absorption of Cu by plant roots and shoots could be due to low Cu solubility and leachability in soil, which was directly related to the increase of soil pH in RSB-amended soil.

Biochar is an effective soil amendment for reducing pore-water Cu concentrations (Karami et al., 2011) and increasing Cu binding with soil organic matter, and resulted in reduced Cu contents in different *B. nivea* tissues compared with control (Fig. 2). Addition of biochar can reduce the availability of Cu due to sorption, complexation and precipitation (Beesley et al., 2010; Zhang et al., 2013a). This suggested that RSB addition could increase Cu adsorption in soil, which might be one reason for the reduced Cu phytoavailability to *B. nivea* relative to control. Recent studies reported minimum uptake of Cu by ryegrass and *Brassica juncea* upon addition of biochar to soil (Karami et al., 2011; Park et al., 2011). Significant decreases in Cu, Zn, Pb and Cd concentrations in plant shoots after biochar addition were due to precipitation or co-precipitation of metals following significant increases in soil pH (Ahmad et al., 2012) or adsorption of metals on the surface of added biochar (Namgay et al., 2010). In our study, Cu mainly accumulated in roots followed by leaves and stems (Fig. 2), consistent with results of Rehman et al. (2019) for Cu accumulation in the same *B. nivea* tissues. Accumulation of metals largely in roots showed that plants may reduce metal translocation to shoots by localization in plant tissues (Rizwan et al., 2012). Thus, significant decreases in Cu uptake with increasing RSB rates may have positively influenced plant growth. Addition of biochar in soil can increase available P concentration (Chintala et al., 2014), which might contribute to Cu immobilization through precipitation in contaminated soil and thereby reduce its phytoavailability.

4.3. Biochar effect on oxidative stress and antioxidant enzymes

Stress conditions can disturb the dynamic equilibrium of reactive oxygen species (ROS) production and elimination in plants during normal growth (Rizwan et al., 2016a) which promotes ROS accumulation, membrane lipid peroxidation and disrupts the structure and function of the cell membrane system (Sgherri et al., 2007; Quartacci et al., 2000). In our study, 0% RSB treatment indicated that excess Cu uptake by plants could induce oxidative stress in *B. nivea*, whereas RSB application resulted in significant reduction in antioxidant enzymes activities (SOD and POD) as well as concentrations of MDA and proline

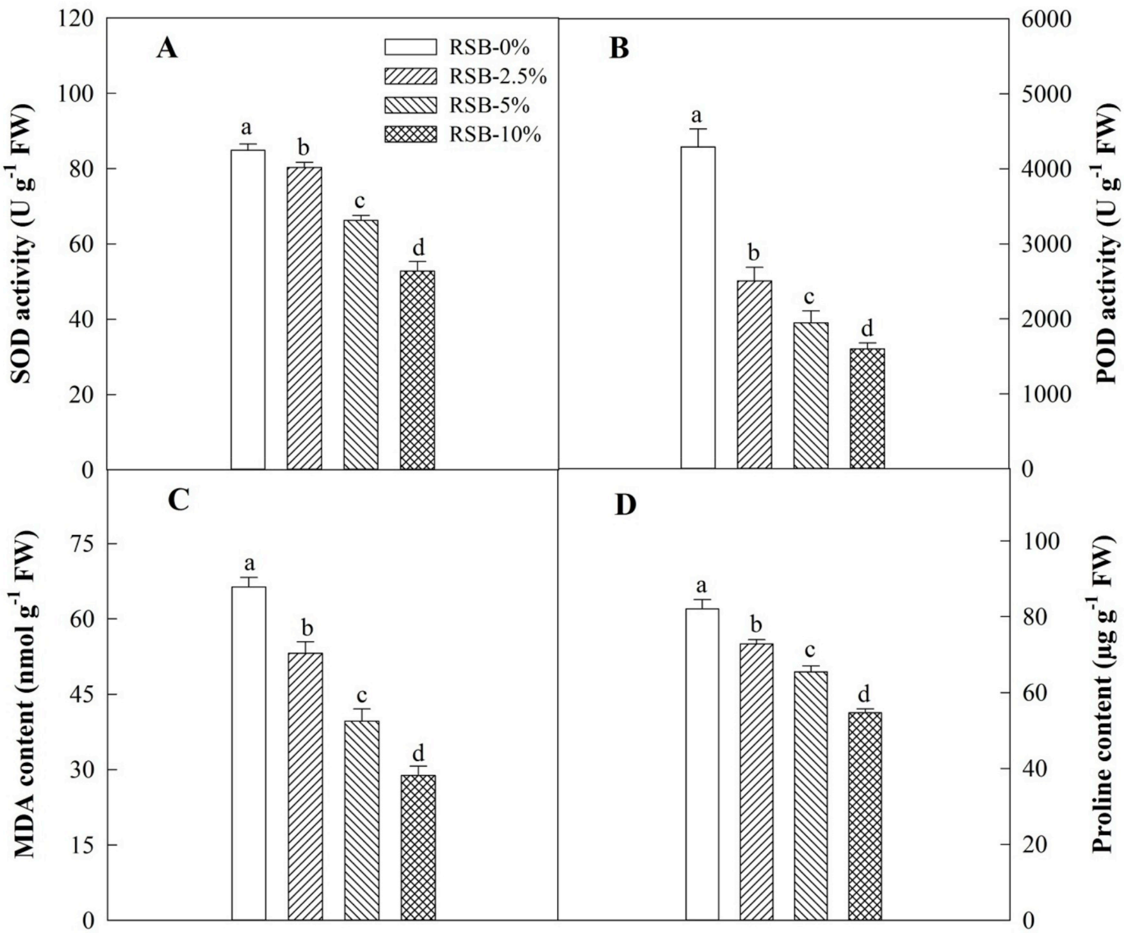

Fig. 3. Effects of increasing doses of biochar (0%, 2.5%, 5.0% and 10% w/w) on superoxide dismutase (SOD), peroxidase (POD) activities and malondialdehyde (MDA), and proline concentrations in leaves of *B. nivea* plants grown in Cu contaminated soil. Values represent mean ± SD (n = 3). Different letters indicate significant difference between treatments at $p \le 0.05$.

Table 3
Soil pHs, electrical conductivity (EC), cation exchange capacity (CEC) and extractable copper (Cu) concentrations measured after crop harvest in a naturally Cu contaminated soil sown with *B. nivea* plants and treated with increasing doses of rice straw biochar.

Rice biochar (w/w %)	pH	EC (dS m^{-1})	CEC (cmol kg^{-1})	Cu (mg kg^{-1})
0	6.89 ± 0.09^{c}	0.27 ± 0.02^{c}	15.64 ± 0.18^{c}	16.98 ± 0.45^{a}
2.5	7.03 ± 0.06^{c}	0.44 ± 0.05^{b}	16.23 ± 0.38^{b}	7.33 ± 0.44^{b}
5	7.22 ± 0.06^{b}	0.49 ± 0.03^{ab}	16.61 ± 0.19^{b}	1.69 ± 0.14^{c}
10	7.43 ± 0.11^{a}	0.55 ± 0.06^{a}	18.38 ± 0.09^{a}	0.68 ± 0.05^{d}

Values in the table are means ± SD ($n = 3$). Different letters in a column indicate significance of difference between treatments ($P \le 0.05$).

(Fig. 3). Moreover, the reduction in oxidative stress might be due to the capacity of biochar to bind free Cu^{2+} ions in soil, thus preventing their entry into plant cells (Quartacci et al., 2015). The reduction in SOD activity might be due to the low uptake of Cu by *B. nivea* and showed self-adjustment adjacent to Cu stress, after RSB incorporation. Our results showed that RSB addition to Cu-contaminated soil significantly decreased Cu accumulation in plant tissue and thereby reduced POD activity, possibly due to Cu immobilization in contaminated soil. Similarly, Li et al. (2016) found that POD activity significantly decreased with biochar addition. Pei et al. (2010) also reported that biochar addition significantly decreased POD activity in plant leaves and shoots, as a result of higher ROS and hydrogen peroxide as products of dismutating more superoxide by SOD.

These results suggested that RSB was an efficient organic amendment to alleviate Cu-induced oxidative stress on *B. nivea*. These findings were in accordance with those of Younis et al. (2015) that biochar incorporation in metal-polluted soils notably reduced oxidative stress in plants. Recently, Bashir et al. (2018c) reported that SOD and POD activities in water spinach (*Ipomoea aquatica*) decreased with a 3% RSB application rate. Zhang et al. (2014) also observed reduced activities of protective enzymes upon biochar addition to a metal-contaminated soil, with a constructive effect on relieving metal stress in rice.

4.4. Biochar effects on soil characteristics and extractable Cu in soil

The significant changes in soil chemical properties after RSB incorporation may have influenced the soil Cu immobilization. The Cu stabilization after RSB incorporation probably depends on changes in surface properties and pH. Several recent studies reported that different biochars have potential to increase heavy metal immobilization in contaminated soils due to their porous structure, high pH, high CEC and the presence of wide range of oxygen- and N-containing functional groups (Bashir et al., 2018a, 2018b). The profound increment in soil pH after RSB addition might have greatly contributed to minimizing Cu extractability by providing new adsorption sites in RSB-amended soils.

Most biochars have a liming effect (Beesley and Marmiroli, 2011), which may contribute to increasing soil pH – a key factor controlling the bioavailable concentration of heavy metals in soils (Rieuwerts et al., 2006). Our results showed significant increases in pH of post-harvest

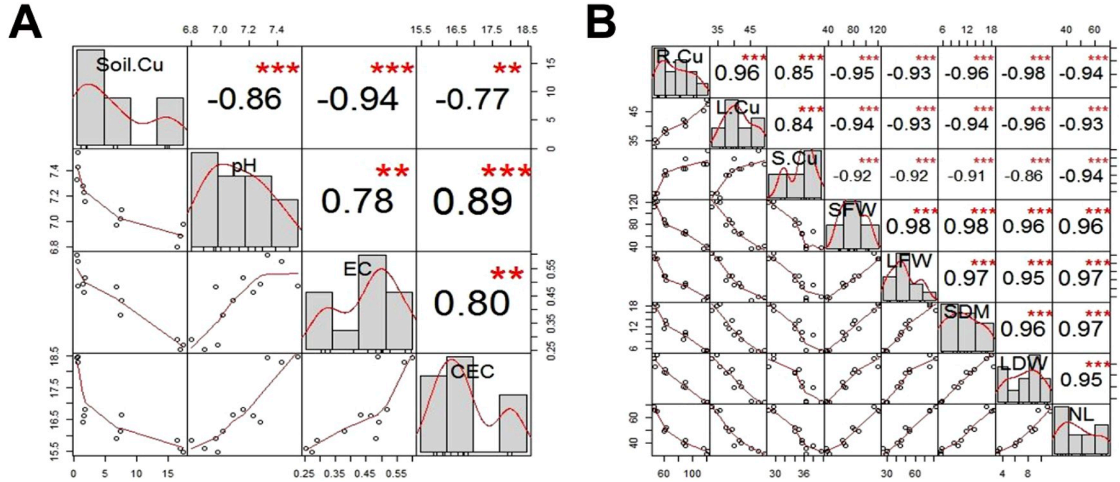

Fig. 4. (A) Correlation between soil physicochemical properties (pH, EC and CEC) and concentrations of $CaCl_2$-extractable Cu . (B) Correlation between Cu contents in plants parts and plant fresh and dry biomass and number of leaves. (R.Cu: Cu contents in root, L.Cu: Cu contents in leaf, S.Cu: Cu contents in stem, SFW: Shoot fresh weight, LFW: Leaf fresh weight, SDM: Shoot dry weight, LDW: Leaf dry weight, NL: Number of leaves per plant).

soil with the progressive addition of RSB (Table 3). This increase in soil pH might be due to biochar characteristics such as high pH, ash content, surface alkalinity and base cations (Yang et al., 2016) or presence of surface functional groups (Uchimiya et al., 2011).

A number of possible mechanisms may affect the increased Cu immobilization when RSB was added to Cu-contaminated soil: (1) high alkalinity increasing soil pH, (2) porous structure and surface functional groups of biochar may promote complexation and adsorption and (3) different structural and chemical composition of biochar induced from various feedstocks. The prominent increase in soil pH can enhance stabilization of heavy metals through adsorption and precipitation, thus decreasing their bioavailability (Zhang et al., 2013a), which may lead to reduced metal uptake by plant tissues. Lu et al. (2014) concluded that addition of bamboo-derived biochar could promote Cu stabilization through precipitation as $Cu(OH)_2$. Our progressive increase in RSB treatment increased the CEC, and previous studies also revealed greater CEC in metal-contaminated soils following biochar incorporation (Beesley et al., 2010; Yuan et al., 2011; Ahmad et al., 2012; Zhang et al., 2013a; Almaroai et al., 2014). We found that the increment in soil pH, EC and CEC provided a favorable environment for *B. nivea* growth in Cu-contaminated soil. The increased pH might have contributed to immobilization of $CaCl_2$-extractable Cu in RSB-amended soil. Similarly, Yang et al. (2016) observed a significant reduction in $CaCl_2$-extractable Cu and Pb concentrations with progressive addition of both RSB and bamboo biochar. Our results are consistent with those of Uchimiya et al. (2010) in which incorporation of biochar increased soil pH and cation exchange and resulted in immobilization of metals in soil. Lu et al. (2014) found that RSB had significant effects on metals extractable by the toxicity characteristic leaching procedure. Jiang et al. (2012b) incorporated RSB in three types of soils at different doses and observed a dose-dependent reduction of available Pb with RSB addition to soil. However, biochar addition was more effective for immobilization of Cu and Pb than for Cd (Jiang et al., 2012a).

4.5. Correlation analysis

Correlations between different studied parameters were investigated (Fig. 4). There was a strong and significant negative correlation between $CaCl_2$-extractable Cu concentration and soil pH, indicating the importance of pH in reducing extractable Cu in soil. Due to its alkalinity, biochar exerts a liming effect when added to soil (Ahmad et al., 2014b). We also found significant negative correlations for $CaCl_2$-extractable Cu contents with soil EC and CEC. Oxygen-containing functional groups (Ahmad et al., 2014a, 2014b), high surface alkalinity and silicon content of biochar makes RSB more effective for immobilization of heavy metals (Lu et al., 2014). The Cu concentration in roots was significantly positively correlated with Cu concentration in leaves and stems; however, root, leaf and stem Cu concentrations were significantly negatively correlated with plant fresh and dry biomass and number of leaves per plant. These correlations reflected a close connection between Cu uptake and growth in *B. nivea* plants.

Several studies have been conducted on various plant species using soil artificially spiked with different Cu salts which may interfere with present results due to changes in Cu bioavailability, pH of soil and aging when soil is not equilibrated with Cu. In our study, *B. nivea* grew on naturally Cu-contaminated soil. Although the Cu content in soil before our experiment was rather high, there were significant reductions in $CaCl_2$-extractable Cu in soil and accumulation of Cu by *B. nivea* with addition of RSB. Overall, amending soil with RSB resulted in reduced uptake of Cu by plants and extractable Cu contents in soil. In future, field studies in Cu-contaminated areas are required to validate these results.

5. Conclusions and future perspectives

The addition of RSB significantly improved growth performance, chlorophyll content and photosynthesis in *B. nivea* grown as forage in an aged Cu-contaminated soil and has capacity for in-situ remediation by immobilizing or decreasing Cu uptake. The RSB addition, particularly at 10% rate, markedly reduced the activities of antioxidant enzymes as well as contents of MDA and free proline in plants. Thus, RSB has potential to protect *B. nivea* against Cu by alleviating Cu-induced oxidative stress. Increased soil pH and CEC in biochar treatments may have contributed to the reduced Cu toxicity, thus increasing *B. nivea* growth. This study provides a direction for future research and may support the use of RSB in increasing *B. nivea* production in terms of bast fiber or animal feed. However, future research is needed on the effects of biochar on quality of both fiber and fodder from *B. nivea*. Moreover, potential for biochar in remediation of soils polluted with heavy metals should be tested under field conditions.

Conflicts of interest

The authors declare that they have no competing interests.

Contribution

Lijun Liu, Kadambot HM Siddique and Dingxiang Peng supervised and designed the project. Muzammal Rehman performed the experiment and collected data. Saqib Bashir, Muhammad Hamza Saleem and Chen Chen helped in data analysis and preparation of manuscript.

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