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Research article

Phytotoxicity attenuation in *Vigna radiata* under heavy metal stress at the presence of biochar and N fixing bacteria

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A B S T R A C T

This study assesses the effect of N-fixing bacteria and biochar synergism on plant growth and development of *Vigna mungo* under heavy metal stress (HM). Heavy metal stress is a worldwide problem, which causes critical effects on plant life due to oxidative stress. Application of biochar is a recent biological remediation technique, which often leads to an immobilization of heavy metals in soil. . Synergism of bacteria and biochar is a novel aspect to enhance plant growth under heavy metal stress. Woody biochar a byproduct of a dendro power industry was added as 1, 2.5 and 5% amounts combination with *Bradyrhizobium japonicum*, where mung seedlings were planted in serpentine soil rich in Ni, Mn, Cr and Co. Pot experiments were conducted for 12 weeks. The plant height, heavy metal uptake by plants, soil bioavailable heavy metal contents, soil N and P and microbial biomass carbon (MBC) were measured. The plant growth was enhanced with biochar amendment but a retardation was observed with high biochar application (5%). The soil N and P increased with the increase of biochar addition percentage while soil MBC showed reductions at 5% biochar amendment. Both soil bioavailable fractions of HM and up take of HMs by plants were gradually reduced with increase in biochar content. Based on the results, 2.5% biochar synergism with bacteria was the best for plant growth and soil nutrition status. Despite the synergism, available N was negatively correlated with the decrease of bioavailable metal percentage in soil whereas it was conversely for P.

1. Introduction

The deleterious effect of environmental pollution on biotic factor is a strict concern in the recent decade. Due to the industrial revolution, excess usage of fertilizer and pesticides, wastewater discharge and use in agriculture, landfill leachates and mine tailings accumulates a number of heavy metals (HMs) in soil (Järup, 2003). The available fraction of HMs can easily be interact with soil fauna, soil microbes and vegetation (Giller et al., 1998). Via the production of reactive oxygen species or the attachment of the enzymes, heavy metals can hinder the cellular biochemical reactions (Gajewska and SkŁodowska, 2010). Oxidatively damaged lipids and proteins are the major indications in oxidative stressed cells. Peroxidation of lipids enhance the permeability of cell membranes and affects the physiological and biochemicals reactions (Stark, 2005). As a result of protein carbonylation ketone or aldehyde derivatives are formed and it alters the protein structure and leads to its activity inhibition. The irreversible carbonylation is considered as one of the major destructive modifications in cellular macromolecules (Møller et al., 2007).

Heavy metal contaminated soils may not necessarily be nutrient depleted but both phenomena can occur in parallel. Such extreme environments are neither suitable for plant nor soil microbial growth (Peralta et al., 2001). In recent years biochar (BC) has received significant attention as a biological soil amendment to improve soil fertility, crop production, and nutrient retention in soil (El-Naggar et al., 2015; Hussain et al., 2016; Zhang and Ok, 2014). They are resistant to degradation and are estimated to remain in

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soil for thousands of years (Lehmann et al., 2008). Chars are composed of numerous surface groups, such as carboxylic, phenolic, hydroxyl, carbonyl, and quinines (Cheng et al., 2006). Whereas its porous structure affects on soil cation exchange capacity (CEC), pH, and retention of water and nutrients (Glaser et al., 2002). The physical and chemical properties of chars vary significantly depending on the biomass source and pyrolyzing (Ahmad et al., 2014). In the recent past, the decrease of the bioavailable fraction of HMs and the increase in plant growth in contaminated soils have been reported in experiments conducted under greenhouse conditions (Houben et al., 2013a,b; Park et al., 2011).

Similar to the benefits, the limitations and drawbacks of the use of BC for the remediation of contaminated soils are also of particular concern specifically on the addition of BC to soil in excessive levels may cause detrimental impacts on soil structure, soil biota and pose serious consequences for the growth of crop plants. Yet, very little is known about the influence and mechanisms of biochar affects soil microbial communities. Many studies have indicated an increase or neutral action of BC on soil microbes (Lehmann et al., 2011; Xu et al., 2016), few studies have shown that high BC application may reduce or hinder soil microbial activities (Bandara et al., 2015; Dempster et al., 2012) however, those studies are limited and hence further research is essential. Application of promising soil microbe cultures may also improve soil health. Although different types of BCs exhibited successful results on reducing heavy metal stress in several crops under various conditions (Al-Wabel et al., 2015; Beesley et al., 2013; Herath et al., 2015), few studies have investigated the promising effect of combination of microbes and BCs (Bandara et al., 2015; Seneviratne et al., 2015). The ability of BCs to immobilize HMs in polluted soils may additionally lead to a favorable microenvironment for the inhabited organisms (Bandara et al., 2015).

Serpentine soil is considered as an extreme environment for plants and microbes, consists of extremely low levels of essential plant nutrients (e.g. N, P, Ca), enormously high levels of HMs, in particular Ni, Mn, Cr, and Co, and very poor water availability and retention (Vithanage et al., 2014). The crops that are grown in serpentine soil, exhibit phytotoxicity due to high content of HMs (Herath et al., 2014). The soil microbial pool may be low in serpentine soil, due to the heavy metal stress. Thereby, it hinders the effective nodulation and N fixation in legume plants (Bååth, 1989). To date, no studies have investigated the ability of N fixing bacterium and their interactions with BCs on plant growth and development. Nitrogen fixing bacteria such as *Bradyrhizobium japonicum* may provide N via nodulation where BCs offer more nutrients and immobilize heavy metals may enhance soil microbial activity and soil nutrient status in extreme soils. *Vigna radiate* is an annual legume, which is used as a food crop, especially in tropics. Under low soil N quantities legumes are able to fix atmospheric N (Rondon et al., 2007; Sawhney et al., 1990). Hence, this study assesses the synergistic effect of legume-root nodulating microbe, *Bradyrhizobium japonicum* with woody BC (produced by *Gliricidia sepium*) application on *Vigna radiate* in terms of enhancing nutrient availability, plant growth development and immobilizing heavy metals in serpentine as a model soil.

2. Materials and methods

2.1. Soil collection

Soil was obtained from Wasgamuwa (Latitude 7° 71′ 67″ N and longitude 80° 93′ 33″ E) serpentine area, Sri Lanka (Vithanage et al., 2014). Soil samples were collected within 10–15 cm from the surface after removing the surface litter layer from five random locations. The samples were sealed in polypropylene bags and transferred immediately to the laboratory and bulk soil was mixed together. The initial metal concentrations were reported in an earlier study as 6567, 2609, 14880 and 555 mg/kg of Ni, Mn, Cr and Co, respectively (Vithanage et al., 2014).

2.2. Microbial inoculums and biochar collection

A *Bradyrhizobium japonicum* suspension in yeast manitol broth of 0.573 at 600 nm Shimadzu UV-2450 was used as the microbial inoculums in our study. Biochar formed as a waste byproduct of a bioenergy industry (Dendro) (denotes as DBC) at Thiruppane, Anuradhapura district, Sri Lanka was used in the experiment. This DBC was produced by pyrolyzing the woody biomass (BM), *Gliricidia sepium* (Jacq.) Steud. in a closed reactor. The obtained BC was mechanically sieved to obtain <1 mm particles for ensure a homogeneous particle size. Dendro BC has been fully characterized and reported with high aromaticity with a high surface area of 714 m^2/g (Herath et al., 2015). The concentration of N seemed to be very low in DBC and has reported as 0.5% from the elemental analysis (Herath et al., 2015).

2.3. Pot experiment

The pot experiment was conducted under the green house condition for 3 months. Mung bean (*Vigna radiata*) was selected as experimental crop. Pots were filled with soil and BC depending on different treatment types. Untreated soil was used as a control for all treatments. Dendro BCs were mixed with 250 g of soil at a mass fraction of 1.0, 2.5, and 5.0% (w/w). All treated soils were thoroughly homogenized in large plastic containers and individually prepared prior to use. Plastic pots (11.5 cm in diameter and 10.5 cm in height) were filled with DBC amended soil. Pots were placed in a dark room to equilibrate soil mixture, over 2 weeks with 70% of water holding capacity to equilibrate the soil mixture. Surface sterilized Mung bean seeds were allowed to germinate on filter papers laid in petridishes after overnight soaking. Germinated seeds were transplanted in prepared pots as three seeds per pot. The bacterial inoculation treatment series were inoculated with *Bradyrhizobium japonicum* culture. After inoculation, pots were placed under greenhouse conditions and allowed the plant growth over 12 weeks. Each treatment was performed in triplicate. The soil was irrigated with equal amount of tap water (20 ml) once per day to maintain soil moisture at 70% of the water holding capacity.

The plant heights were measured weekly. At the end of the 12th week plants were obtained their post reproductive stage and at that stage plants were harvested. Meteorological parameters were collected daily by weather station, installed closed to the greenhouse. During the experiment, the minimum, maximum and mean air temperatures were 22, 30 and 28 °C respectively. The mean relative humidity and light intensity were observed 75% and 210-foot candle respectively.

Table 1
Soil pH and EC in different treatments.

Treatment	pH	Electrical conductivity (dS/m)
S	5.26	0.126
S + B	5.32	0.129
S + 1%DBC	5.61	0.195
S + 1%DBC + B	5.74	0.158
S + 2.5%DBC	5.71	0.199
S + 2.5%DBC + B	5.85	0.122
S + 5%DBC	6.38	0.245
S + 5%DBC + B	6.81	0.228

2.4. Soil analysis

2.4.1. Analysis of bioavailable metal concentrations

The bioavailable metal (Ni, Mn, Cr and Co) contents were measured with 0.01 M $CaCl_2$ as extracting agent (Rajapaksha et al., 2012). Soil was mixed with 20 ml of calcium chloride solution (0.1 M $CaCl_2$, pH 7.0) at room temperature for 1 h with continuous agitation. The Ni, Mn, Cr and Co concentrations were measured with an atomic absorption spectrophotometer (GBC 933AA).

2.4.2. Soil parameters

Available phosphorous (P) was measured by the sodium bicarbonate extraction method (Olsen et al., 1954). Fresh soil (1.25 g) was mixed with 0.5 M sodium bicarbonate (25 ml) and shaken for 15 min at 180 rpm. The extract was filtered and mixed with ascorbic acid and molybdate reagent and incubated for 1 h and absorbance was read with a spectrophotometer (Shimadzu UV-2450) at 880 nm (Watanabe and Olsen, 1965). Available nitrogen (N) was measured using the colorimetric method by (Cataldo et al., 1975). Fresh soil (10 g) was mixed with K_2SO_4 and shaken at 60 rpm. A 0.5 ml of the extract was added into 1.0 ml of salicylic acid. After 10 min, sodium hydroxide (10 ml) was added and the mixture was incubated 1 h for colour development and absorbance was read using a spectrophotometer (Shimadzu UV-2450) at 410 nm. Microbial biomass carbon (MBC) was measured by chloroform fumigation method (Anderson and Ingram, 1989). Soil pH and electrical conductivity (EC) were measured using a 1:5 suspension of soil-to-water using with a glass electrode (702SM Titrino, Metrohm, Swiss) and using a digital electrical conductivity meter (Orion 5 star, Thermo Scientific) respectively.

2.5. Plant analysis

After 12 weeks Mung bean plants were uprooted and washed with deionized water. Length of shoots and roots, fresh weight of shoots, roots and pods, dry weight of shoot, root and pods (50 °C) were measured. Nickel, Mn, Cr and Co contents were measured with an atomic absorption spectrophotometer (AAS, GBC 933AA) after microwave digestion with HNO_3.

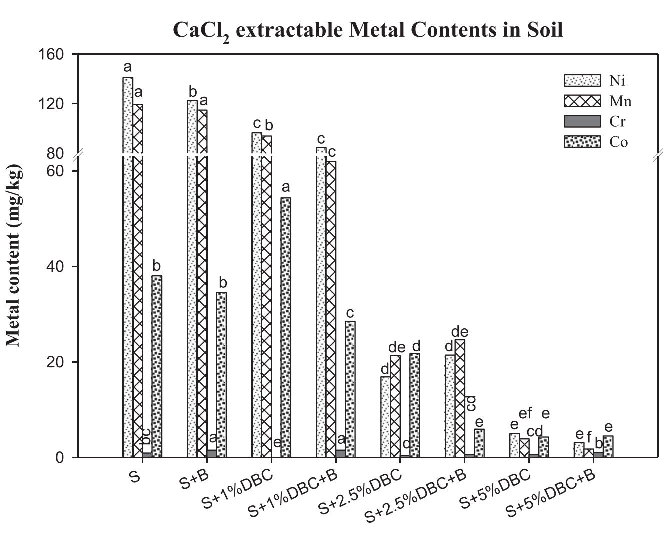

Fig. 1. The $MgCl_2$ extractable concentrations of Ni, Mn and Cr in BC-amended and co-inoculation of BC and bacteria amended serpentine soils.

2.6. Statistical data analysis

All results were expressed as the mean values. Data were analyzed by using a one-way analysis of variance (ANOVA). The Mean separation was done using Duncan's Multiple Range Test (at P = 0.05). All statistical analyses were carried out using Statistical software package (SAS 9.1).

3. Results and discussion

The influence of BC and bacteria amendments on changes in several soil physiochemical parameters including pH, and EC of serpentine soils are summarized in Table 1. Control soil had a pH of 5.26 and it was the lowest among other samples. The pH values of all treatments were acidic and none were significant at a 5% probability level. The increase in the addition of BC percentage increased the soil pH and a further gradual increase was observed with the incorporation of bacteria. This is attributed as the alkali nature of BC (Chan and Xu, 2009). This alkali nature plays an important role in immobilization of heavy metals. Immobilization of Cd via alkalination of extra-radical mycelium has been recorded by (Janoušková and Pavlíková, 2010). Moreover, the drastic pH increment may attributed to reduce the water extractable fraction of HMs in contaminated soils (Hashimoto et al., 2009). The 5% BC treatment showed the highest EC value compared to the other treatments while soil amended with BC and bacteria showed a reduction in EC compared to BC alone.

3.1. Bioavailable heavy metal content

Toxicity of HMs in soils depends mainly on the bioavailable fractions (Rinklebe and Shaheen, 2014). When the bioavailable heavy metal content in rhizosphere is high, the uptake and the stress created by HMs also become high. Although the serpentine soil consist high contents of Ni, Cr, Mn, and Co with 6567, 2609, 14880 and 555 mg/kg of Ni, Mn, Cr and Co, respectively the $MgCl_2$ extractable exchangeable concentrations of Ni, Mn and Cr are low (Vithanage et al., 2014). Similarly, the bioavailable heavy metal concentrations of DBC-amended and synergism of DBC and bacteria amended serpentine soils are depicted in Fig. 1.

According to the results, amending with DBC showed a reduction in the bioavailable fractions of Ni, Cr, Mn and Co as the percentage of amendment increases. Similar data has been reported in the literature (Herath et al., 2015). Application of both chicken manure-derived BC and green waste BC on shooting range soil has significantly reduced Cd and Pb, however, BCs were not efficient in Cu immobilization (Park et al., 2011). In this study, both Ni and Mn shows a gradual reduction in bioavailability, while a significant reduction for Co was present only at 5% BC treatment. Electrostatic interactions between metal cations and carbon surface, ionic exchange between ionizable protons at the surface of a carbon and interaction at delocalized π electrons are the major mechanisms proposed (Ahmad et al., 2014). Moreover, a reduction in bioavailable metal contents has shown in the presence of bacteria. This can be explained as adsorption, ion exchange, chelation and heavy metal uptake by bacteria (Gavrilescu, 2004). Bacteria have the ability for both surface adsorption and uptake of HMs. The HMs that have entered to the cytoplasm can be immobilized as phytochelatins in cytoplasm and vacuole (Lehmann et al., 2011). Additionally, bacteria and BC combination has shown a further reduction of metal bioavailability. This attributes the synergistic effect of bacteria and BC combination, where BC has an extensive metal immobilization effect. However, beyond 1% BC addition there was no significant difference between BC alone and BC together with bacteria co-inoculations. This may be due to the increase in

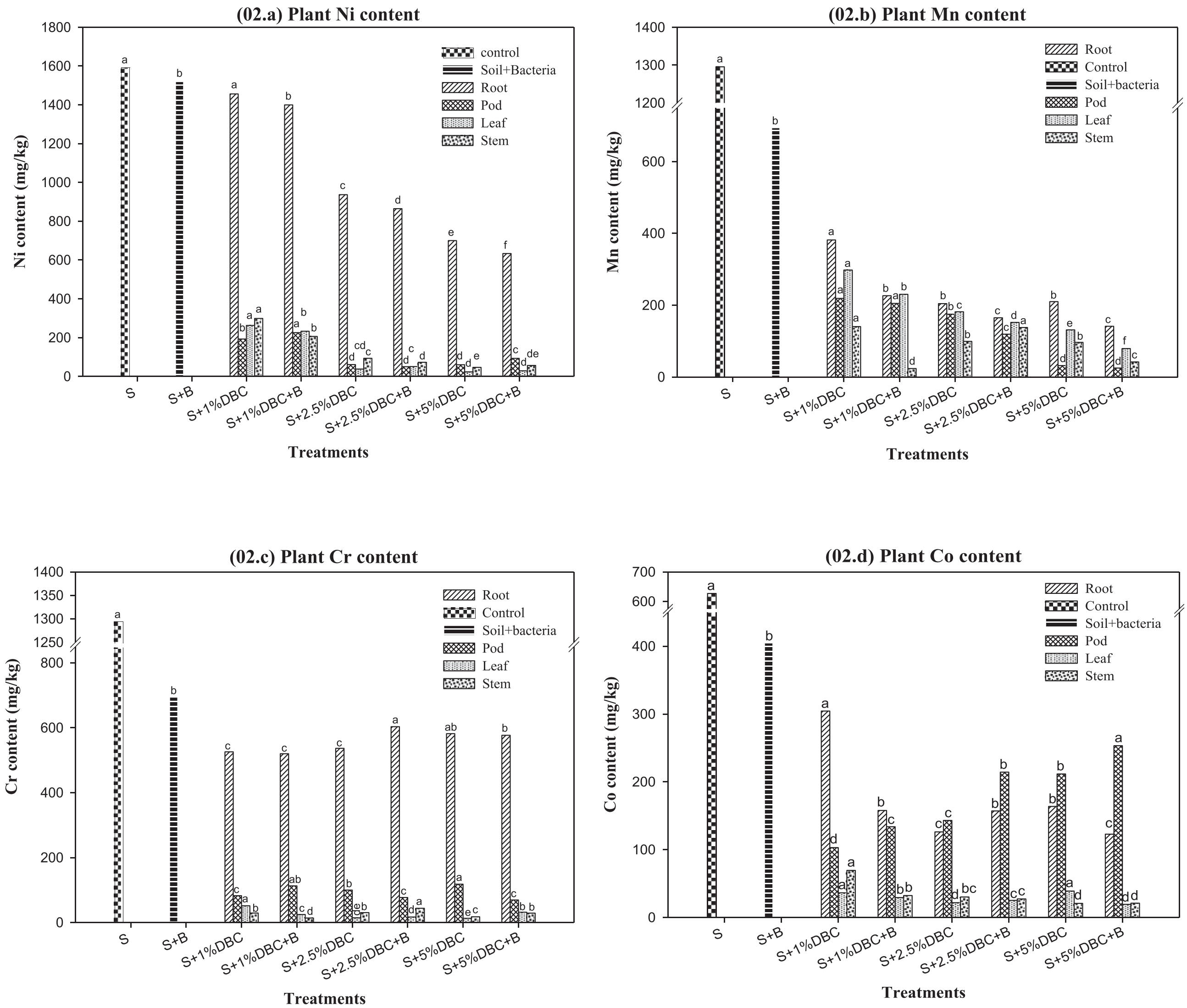

Fig. 2. The heavy metal uptake by different parts of the plants (a) Plant Ni content (b) Plant Mn content (c) Plant Cr content (d) Plant Co content.

sorption of signaling molecules and microbial enzymes by biochar. This may be due to the increase in sorption of signaling molecules and microbial enzymes by biochar. It reduces the interactions between microbes, which leads to a reduction in microbial sorption of HMs (DeLuca et al., 2009).

3.2. Plant heavy metal uptake

Since BC has a strong adsorption capacity with respect to positively charged ions, it has the ability to immobilize them in soil by reducing the stress situation for plants caused by HMs. The HM stress can cause retardation of plant growth caused by oxidative stress (Gill, 2014). In the present study, BC has shown a significant effect on HM induced stress retardation. Plant growth retardation, reduction of chlorophyll content, formation of necrotic lesions are the major phenotypic symptoms caused by HM stress (Bohra et al., 2015; Gill, 2014). Even though some of these act as micronutrients, under such extreme condition they are able to cause toxicity. The plants in control soil had a very short life span, which may reflect the toxicity caused by HMs in the model soil. Since BC can immobilize the bioavailable metals in soils, it reduces the plant uptake and thereby the HM stress.

Uptake by HMs, with respect to BC percentage varies depending on the metal and the plant part of accumulation (Fig. 2). We observed a significant reduction in plant uptake of Ni as the BC percentage increase from 1 to 2.5%. However, there is no reduction in Ni uptake as the amendment increases to 5%. More over the co-inoculation of bacteria and BC did not show any significant change to its parallel BC treatment. Chromium shows a significant reduction in the presence of BC irrespective to the amendment percentage. In the presence of BC Mn also showed a reduction bioavailable fraction. As the percentage increases from 1 to 2.5%, Mn showed a further reduction in bioavailability. However, beyond 2.5% there is no reduction in bioavailability. More over the accumulation in pods is greater than in roots. Whereas only in 1% BC and bacterial inoculation treatment shows a significant reduction of Mn in all plant parts. Similar to Mn, Co also reduced as the BC content increase from 1 to 2.5% and no change of Mn was observed with further increase. However, there is an increment in Mn accumulation in pods with the increase of BC percentage. The co-inoculation of BC and bacteria cause a reduction in Mn only in 1% BC treatment. Whereas, irrespective to the BC percentage the pod accumulation was increased with co-inoculation of bacteria and BC. Even though Co is not recorded as a potent toxic heavy metal, high levels of ingestion is toxic for human health (Barceloux and Barceloux, 1999).

3.3. Plant growth

The plant growth enhancement in the presence of BC has been recorded under recent research (Chan and Xu, 2009). Our results also provide supportive information on plant growth in the presence of BC. As the BC percentage increases, the plant height showed a gradual increment (Fig. S1) which could be due to the increase in HM stress reduction and mobilization of nutrients such as C, N and P. The increment of soil CEC with addition of BC also provides a favorable environment for plant growth. A significant increase in soil nutrients with increasing BC application rates may have positively influenced the plant growth. Interestingly in the presence of BC the soil Ca level has increased, which is a limiting factor for plant life in serpentine soil (Rajakaruna et al., 2012) where, Ca plays a crucial role in plant stress tolerance (Anil and Rao, 2001; Minorsky, 1985; Mittler et al., 2004).

Even though BC gives favorable effects on plant growth, higher percentages of BC is unfavorable for plant growth. A retardation of plant height in 5% BC was observed compared to 2.5% BC application (Fig. S1). Since the porous nature is increased with the increase of BC amendment, it can sorb low molecular weight substances such as plant growth regulators, which are essential for plant growth and development. Synergistic effect is prominent as the BC percentage increases with the presence of bacteria. However, high BC concentrations did not show an enhancement in plant growth with bacteria combination. This could be attributed to the sorption of bacterial signaling molecules by BC, which declines the positive plant bacterial interactions. Moreover 2.5% BC amendment in combination with bacteria showed the most encouraging effect on plant growth.

Heavy metals showed negative influence on roots as well. The root inhibition under HM stress has been reported in numerous studies (Peralta et al., 2001). Since the root system is the basis of plant water and nutrient uptake, the affects of root system causes negative effects on each plant physiological and biochemical activity. Interestingly, it was observed that the root length of *Vigna radiate* has increased with BC addition irrespective with the percentage (Fig. S2) which might specifically due to reduction of oxidative stress caused by HMs.

3.4. Microbial biomass carbon

Microbial biomass carbon is a measure of soil microbial biomass in soils. According to our results the inoculation of bacteria leads to an increase of soil microbial biomass (Fig. S2). Interestingly it has

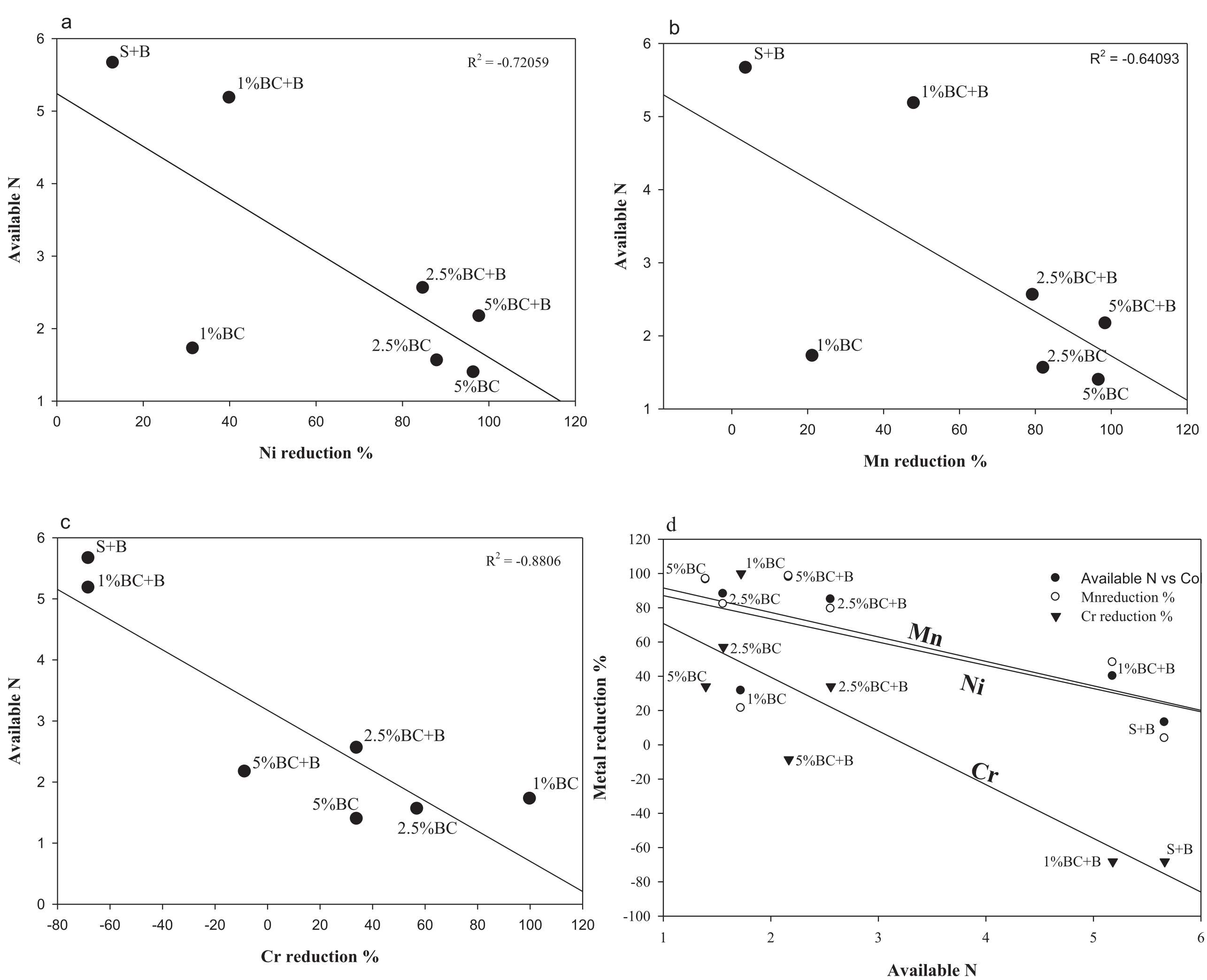

Fig. 3. Correlation of bioavailable metal reduction percentages vs. available N. Available N reported here is in mg/kg.

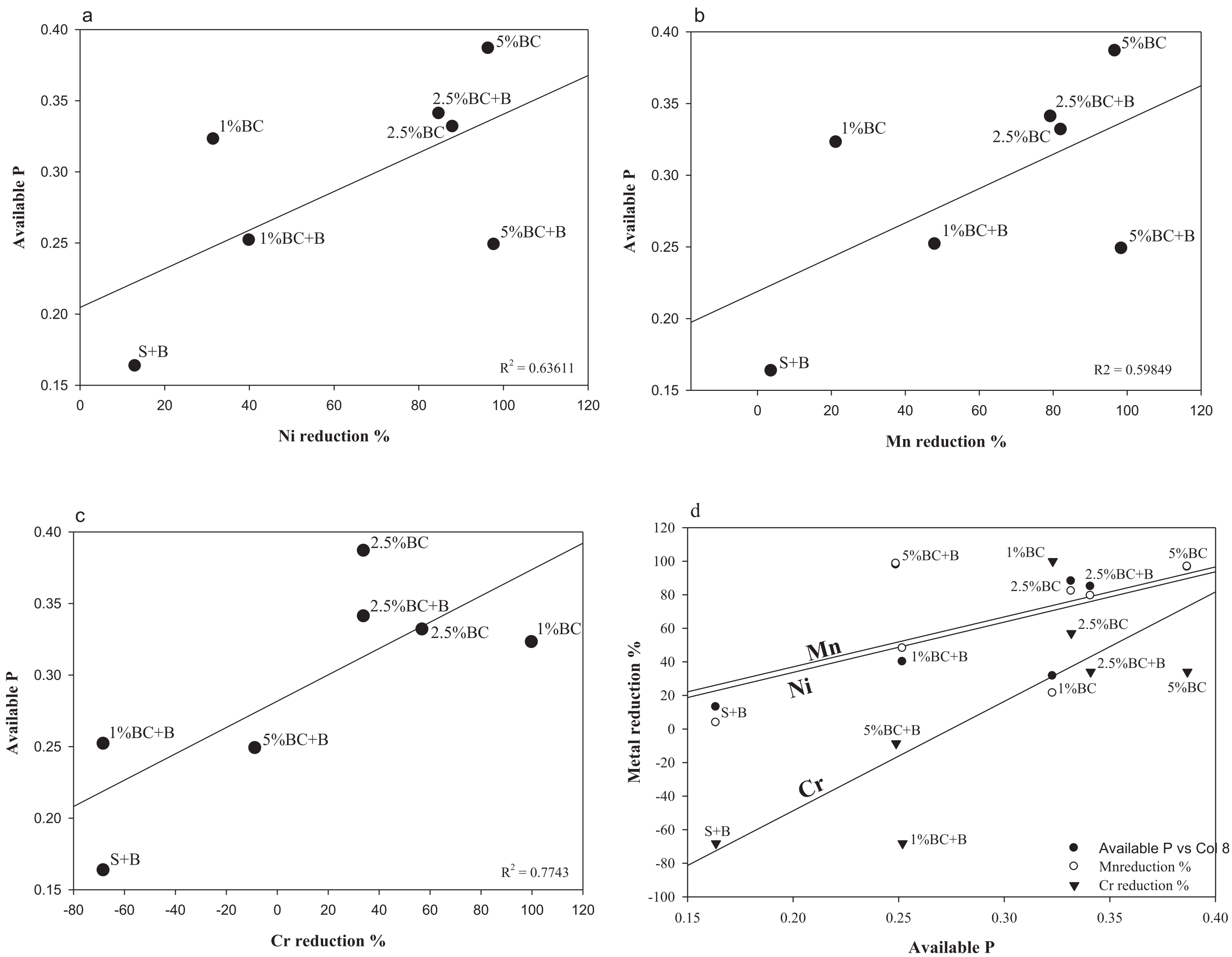

Fig. 4. Correlation of bioavailable metal reduction percentages vs. available P. Available P reported here is in mg/kg.

shown that the addition of BC has contributed to further enhancement of the microbial development. Moreover the quantity of BC leads to a great effect on soil microbial biomass carbon. The 2.5% was the most effective percentage that gave a 40% increment of soil microbial biomass carbon. This may be due to the increase of soil nutrients (N, P) and water retention capacity of soil. Even though the C in BC might be highly unavailable to soil microbes, the changes in soil physicochemical properties and the introduction of metabolically available labile-C compounds associated with the BC could shift the soil microbial community structure (Anderson et al., 2011). Moreover as percentage of BC become greater than 2.5% there was a reduction in quantity of soil microbialbiomass, even with the inoculation of bacteria. This could be attributed again by signal molecules sorption by BC (Warnock et al., 2007).

3.5. *Soil N and P contents*

Soil available N and P are key factors in soil nutrition. Since this a degraded soil both N and P contents are very low for a successful plant life (Rajakaruna and Bohm, 2002). This study showed an increase in soil available N content with treatments (Fig. S3). Soil N was significantly improved with BC addition which may be due to the N species in BC (Mukherjee and Zimmerman, 2013). Inoculation of *Bradyrhizobium japonicum* has shown a significant increment in soil available N content and the maximum was observed with the control soil without BC. However, there was a gradual reduction of soil available N with the increase of BC percentage. This could be attributed as the sorption of rhizospheric microbial signaling molecules and enzymes and allelopathic effects associated with hydrocarbons or toxic levels of HMs present in BC (Rondon et al., 2007). The pH increase with BC additions may also contribute to increase the soil available nitrogen content. However, compared with the HM reduction percentage in the bioavailable fraction of soil, the treatments showed negative relationship with N irrespective of the synergism (Fig. 3). Not only on N, the addition of BC has influenced soil P as well. We observed an increment in soil available P content with regard to BC amendment, irrespectively to the percentage amendment (Fig. S3) which might have caused by a release of nutrients by BC (Mukherjee and Zimmerman, 2013). Reduction of bioavailable metal percentages in treatments was positively correlated with the available P content in soil (Fig. 4). That can be ascribed to the immobilization of metals via metal-phosphate complexes with phosphates released from BC.

4. Conclusions

The biochar was able to reduce the bioavailability of HMs with the increase of biochar application percentage which could be attributed to the increase of pore surface area and the increase of pH with the presence of biochar. The inoculation of *Bradyrhizobium*

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japonicum was able to increase the soil available N content reflecting its N fixation ability even under HM stress. Soil microbial biomass carbon exhibited an increment with biochar application percentage however, with high quantity of biochar, the microbial biomass carbon has reduced even under bacterial inoculation. This might be due to the sorption of signaling molecules by biochar. The phosphorous content increased with biochar addition due to the presence of P in biochar itself. A positive correlation was observed with available P and metal reduction percentage in the bioavailable fraction of soil whereas it was vice versa for N. Interestingly, biochar up to 2.5% has shown a favorable effect on plant growth, however, a retardation of plant growth was observed at 5% which may be reasoned as the sorption of plant growth regulators. Hence, this study conclude that, even high amount of biochar can reduce the HM stress, plant growth promoting bacteria can increase the plant growth, 2.5% BC addition and bacteria synergism gives the maximum favorability on plant growth. Molecular level visualization studies may be necessary to reveal the actual effect of biochar on soil microbiota.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvman.2016.07.024.

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