Global J. Environ. Sci. Manage., 1(3): 245-254, Summer 2015
ISSN 2383 - 3572

Biochar impact on physiological and biochemical attributes of spinach *Spinacia oleracea* (L.) in nickel contaminated soil

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Received 16 December 2014; revised 21 February 2015; accepted 24 February 2015; available online 1 June 2015

ABSTRACT: Disastrous effect of nickel on spinach was discussed by number of authors but the effect of amendments like biochar with nickel on *Spinacea oleracea* L. is not still discussed by any author of the world because biochar was used as soil amendments which play a vital role in reducing mobilization and uptake of nickel by spinach plants. As nickel contaminated plants are very harmful for the consumption by living organisms. Nickel can be gathered in agronomic soils by anthropogenic actions such as Ni-Cd batteries. In this study, the growth, physiological, photosynthetic and biochemical responses of *Spinacia oleracea* grown in Ni-spiked soil (0, 25, 50 and 100 mg Ni/Kg soil) at three levels of cotton-sticks-derived biochar (0, 3 and 5 %) were evaluated. The results exposed significant decrease in growth, photosynthetic, physiological, and biochemical traits of *S. oleracea* when grown in Ni-polluted soil. However, this decrease was less pronounced in cotton-sticks-derived biochar amended soil. A steady rise in the MDA (0.66 µg/g to 2.08 µg /g), ascorbic acid (1.24 mg/g to 1.57 mg/g)and sugar concentrations (1.73 mg/g to 2.16 mg/g) was observed with increased concentration of Ni. The increasing percentages of cotton-sticks-derived biochar from 3 % to 5 % decreased Ni concentrations in root and shoot of experimental plant. Higher production of chlorophyll, amino acids and protein with cotton-sticks-derived biochar amendment looked like alleviation in Ni toxicity. Therefore, it is concluded that, Ni toxicity and availability to the plants can be reduced by cotton-sticks-derived biochar amendments.

Keywords: *Charcoal, cotton-sticks-derived biochar (CSB), Nickel toxicity, Pyrolysis, Spinacea oleracea*

INTRODUCTION

Spinach (*Spinacia oleracea*) is the important leafy vegetable that belongs to the family Amaranthaceae. Due to easy, pre-cleaned and pre-packaged units the demand of spinach among the peoples of world is continuously increasing (Lucier and Plummer, 2004). Having rich source beta carotene, folate, vitamin C, calcium, iron and highest ORAC (Oxygen Radical Absorbance Capacity) spinach is highly consumed all over the world (Dicoteau, 2000). The age related macular degeneration is also reduced due to the consumption of spinach as it is a rich source of lutein i.e. carotenoid (Morelock and Correll, 2008). The growth of spinach is observed best in sewage irrigated soil with broad leaves. As a result the quantity improved but the quality of spinach is destroyed due to hyper accumulation of heavy metals in its shoot and leaves (Wagner and Kaupenjohann, 2014).

The source of heavy metals are anthropogenic activities like mining and industrialization which disrupted the natural ecosystem (Shah and Nongkynrih, 2007; Younis *et al.*, 2015). Among these metals which are toxic for humans include Pb, As, Cd, Ni and mercury (Ahluwalia and Dinesh, 2007). Nickel is the 24th most plentiful element of the earth crust (Cempel and Nikel, 2006). Sewage sludge in high amounts adds large quantities of Ni in soils that are near to the industries (Salt and Krämer, 2000). In soil the concentration of Ni accumulation can

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Note. This manuscript was submitted on December 16, 2014; approved on February 24, 2015; published online on June 1, 2015. Discussion period open until October 1, 2015; discussion can be performed online on the Website "Show article" section for this article. This paper is part of the Global Journal of Environmental Science and Management (GJESM).

be varied from 3-100 ppm (Scott-Fordsmand, 1997). Ni exists in soil in several forms such as crystalline minerals (inorganic), on cations exchange surfaces which are inorganic, cations surfaces that are organic, as a free ion, water soluble and as a chelated compound (Scott-Fordsmand, 1997). Nickel is toxic to human health and causes cancer and other diseases on consumption of contaminated food.

The edible part of many vegetables is the potential sink of Ni where it accumulates and becomes toxic for all the consumers (Satarug *et al.,* 2012). In plants the toxicity effects caused by Ni vary according to the concentration of Ni in the soil solution (Mizuno, 1968). The chlorosis and necrosis are usually observed in those plants that are suffering from high toxicity of Ni. Also it disturbs the natural mechanism of Iron (Fe) uptake and mitotic activity in the plants which adversely affect their growth (Madhava Rao and Sresty, 2000). Seed germination is also reduced in many plants when they are cultivated on sites contaminated with Ni (Madhava Rao and Sresty, 2000). In case of cereals especially wheat plants, the shoot growth becomes retarded due to toxicity of Ni which causes reduction in yield (Gajewska *et al.,* 2006; Tripathi and Guar, 2006). Therefore, there is a need to reclaim Ni in soil to reduce its hazardous impacts on plants and animals.

For the reclamation of heavy metals through process of immobilization in the soil mostly organic nature amendments are suggested. In these organic nature amendments biochar can prove very effective and can significantly enhance the metals immobilization in the soils through which metals bioavailability can be reduced (Verheijen *et al.,* 2010; Downie *et al.,* 2009). Recent studies show that the application of biochar in the soil enhances the soil's agronomic values as well as reclaims the organic and inorganic contamination through its high sorption capability (Asai *et al.,* 2009; Hossain *et al.,* 2010; Uchimiya *et al.,* 2012). The active sites on the biochar due to presence of its functional groups also make it an effective amendment for the reclamation of heavy metals in the soil. Heavy metals bind themselves with the active absorption sites of biochar which reduce their mobility in the soil (Machida *et al.,* 2005).

There is still great uncertainty among the scientists regarding the influence of the biochar on productivity of agricultural commodities (Jha *et al.,* 2010). In the present study, mobility and bioavailability of Ni by application of biochar and its subsequent effect on the growth of Spinach have been studied. It is also necessary to check the maximum rate of biochar application in the soil which would not make it a harmful degrading agent. Spinach is selected as an experimental plant due to its extensive consumption as a nutrient, capacity to uptake and store Ni ions.

MATERIALS AND METHODS

A pot experiment was conducted at the Botanical Garden of Bahauddin Zakariya University Multan, using a completely randomized factorial design involving spinach (*Spinacia oleracea*) and two treatment factors: biochar (0, 3, and 5 % w/w) and nickel additions (0, 25, 50 and 100 mg Ni/kg soil using $NiSO_4$). Each treatment was replicated four times. The source of cotton-sticks-derived biochar (CSB) (Table 1) was cotton-sticks treated at 450 °C in an especially designed pyrolysis reactor. The plants were grown in clay pots each filled with a mixture of 5 kg soil (Table 2) and the calculated amounts of respective treatment factors (CSB and Ni). Seeds of *Spinacia oleracea* were purchased from the local market and sowing was done in all the pots with their holes closed to prevent washing and leaching of Ni. At the seedling stage, 10 plants per pot were allowed to grow. The plants were irrigated on regular basis to maintain 50 % of water holding capacity. At maturity, the plants were harvested. Fresh and dry biomasses were determined. The concentrations of Ni in the plant samples were determined using di-acid digestion followed by using atomic absorption spectrophotometer equipped with graphite furnace and hydride/HydreA 134 technology, NovAA400, Analytic Jena (Rashid, 1986). Total soluble protein and amino acids were determined following the procedures of Bradford (1976). The concentration of ascorbic acid

Table 1: Physicochemical characteristics of biochar derived from cotton sticks

	pH	EC (dS/m)	Volatile matter (%)	Ash(%)	Fixed carbon (%)	Nitrogen (%)	Phosphorus (mg/kg)	Potassium (mg/kg)
Biochar	9.5	1.52	26	62	23	1.12	0.47	64

Table 2: Physicochemical characteristics of experimental soil

Characteristic	Unit	Value
pH_s (saturated paste)	-	7.91
EC_e	dS/m	0.86
TSS	meq/L	8.6
Water Soluble Carbonates	meq/L	0
Water Soluble Bicarbonates	meq/L	4.77
Water Soluble Chlorite	meq/L	2.08
Water Soluble Sulphate	meq/L	1.75
Water Soluble Calcium + Magnesium	meq/L	4.62
Water Soluble Sodium	meq/L	3.98
Organic matter	%	0.42
Nickel	mg/Kg	1.09

was determined using the formula of Keller and Schwager, (1977). Plant malondialdihyde contents were assayed as thiobarbituric acid (TBA) method and soluble sugar was determined with the anthrone reagent (Cakmak and Horst, 1991). Gas exchange attributes (photosynthetic rate, transpiration rate (E) and sub-stomatal CO2 concentration (Ci)) were determined using an open system LCA-4 ADC portable infrared gas analyzer (Analytical Development Company, Hoddeson, England). The photosynthetic pigments were determined following the method of Arnon (1949).

Statistical analysis

The data were analyzed for statistical differences by performing analysis of variance (ANOVA) test using statistical software package SPSS version 18.0. The Tukey-HSD Test was used to find significant differences between various treatments.

RESULTS AND DISCUSSION

Growth

Fig. 1 is plotted by taking nickel concentrations (0 mg Ni/Kg, 25 mg Ni/Kg, 50 mg Ni/Kg, 100 mg Ni/Kg soil) on X-axis and fresh and dry biomass on Y-axis. It represented from the figure that the fresh-biomass of *S. oleracea* was greater at 3 % CSB application rate than at 5 % CSB and control treatments. The increase in Ni concentrations induced negative effect on fresh weight which decreased from 110.69 g (control) to 62.83 g (100 mg Ni /kg). However, the addition of CSB with increasing levels (3% and 5%) in the Ni-spiked soil significantly enhanced the fresh biomass and reduced the negative impact of Ni (Fig. 1). The dry biomass of experimental plants was also significantly ($P < 0.01$) decreased by the application of Ni levels from 25 mg Ni/kg to 100 mg Ni/kg (Fig. 1). However, the CSB application revealed significant increase of dry biomass at all levels of Ni. The dry biomass increased from 7.49 g (control + 100 mg Ni/kg) to 11.88 g with biochar application (3% CSB + 100 mg Ni/kg).

Spinach is a source of many vitamins and minerals like, A, B2, B6, C, E, K, folate, zinc, selenium, copper, magnesium, calcium, iron and potassium. It gives good supply of dietary fiber and omega-3 fatty acids. Pollution of agricultural industries is a main source of contamination in spinach. So, the foremost purpose of this study was to assess the role of biochar on spinach in Ni-contaminated soil. For this, *Spinacia oleracea* was selected due to its hyper accumulating ability of heavy metals. Similarly, biochar has the ability to adsorb Ni and reduced its toxicity. The Results of the study clearly indicated an increased in plant weight with increase of biochar application in controlled and Ni contaminated soil (Fig. 1). This increase was mainly due to biochar improving the soil's overall physicochemical properties. Moreover, biochar improves the ion uptake and water-holding capacity of soils, leading to increased water usage ability by the plants (Uzoma *et al.*, 2011). In our case, CSB reduced Ni stress, resulting in improved plant growth which is reduced in nickel stress. Madhava Rao and Sresty (2000), observed a negative impact of Ni on the fresh and dry biomass of roots and shoots of plants due to Ni storage in the plants body parts (Pandey and Sharma, 2002).

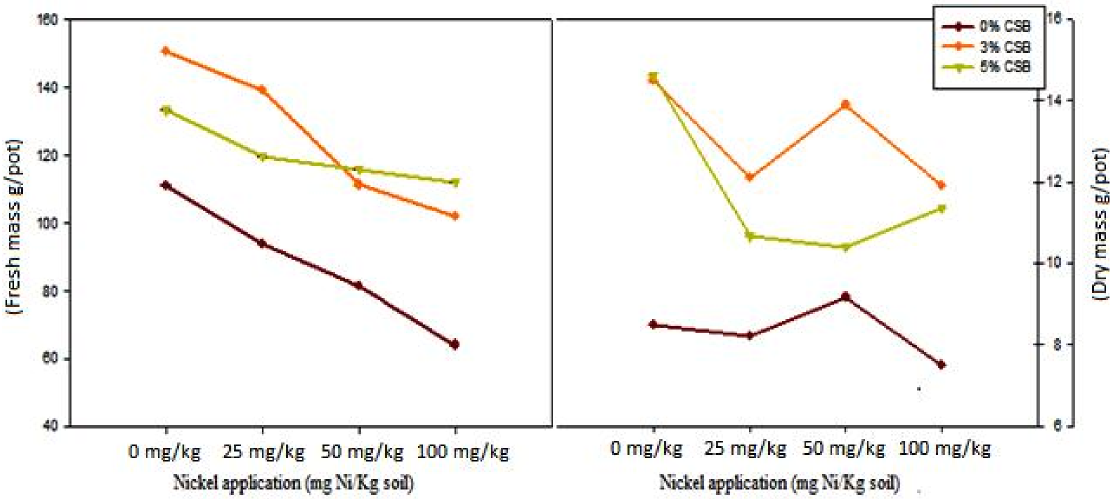

Fig. 1: Fresh and dry biomass of *S. oleracea* under different levels of nickel (0, 25, 50 and 100 mg Ni/kg soil) and cotton sticks biochar (CSB, 0 %, 3% and 5 %).

Physiological and photosynthetic

The physiological attributes of the plants were significantly influenced by the application of CSB and Ni (Fig. 2). The photosynthetic rate, transpiration rate and sub-stomatal CO_2 concentrations increased with increasing CSB percentages from 3% to 5%. By increasing the level of Ni (from 25 mg Ni/kg to 100 mg Ni/kg), physiological attributes decreased rapidly. However, this decrease was less pronounced in the medium containing CSB.

Similarly, Ni in the growth medium significantly reduced the chlorophyll a, b and total chlorophyll of *S. oleracea* (Table 3) expected for chlorophyll a at 25 mg Ni/kg (1.64 mg/g fresh weight) and for chlorophyll b (0.49 mg/g fresh weight) at 50 mg Ni/kg. There was a significant decrease in the concentrations of pigments with increasing Ni levels. Highest drop in total chlorophyll contents from 1.63 mg/g fresh weight (control) to 1.25 mg/g fresh weight were observed at 100 mg Ni/kg. It was observed that the CSB additions caused less decrease of these pigments. The carotenoid contents were also affected in a similar way like chlorophyll. Similarly, the concentrations of anthocyanin and lycopene gave treatment specific response at various levels of Ni and CSB.

The photosynthetic parameters serve as physiological indicators for determining the effects of Ni toxicity (Monni *et al.*, 2001). The substomatal CO_2 concentration, photosynthetic and transpiration rates decreased with increasing Ni levels but with the use of BC, this decrease was changed to an increase (Fig. 2). Similar results regarding Ni-induced reductions in photosynthetic and transpiration rates in various plants have been reported (Seregin and Ivanov, 2001). These decreases may be due to a decrease in chlorophyll contents, chlorophyll deprivation, and/or disintegration and destruction of Rubisco with stomata. It was observed that when plants were cultivated in the Ni toxic soils, the Ni treated plants showed the highest symptoms of chlorosis. These symptoms may have appeared due to the competition between the Ni with Ca and Mg intake in the plants. Less intake of Mg resulted in the deficiency of chlorophyll production in the plants and ultimately photosynthesis is reduced (Molas, 1997). Seregin and Ivanov (2001), noted that when plants uptake large amount of Ni and store it in the lamella regions, the PS II become disturbed due to which reduction in the photosynthesis takes place. It also changes the structure of electron carriers by changing the plastoquinone Q_A and Fe to plastoquinone Q_B (Krupa and Baszynski, 1995). An increase in chlorophyll contents was also observed with increased biochar percentages, as observed previously with Ni stress.

The decrease in photosynthetic pigments is due to the inhibition of the activities of enzyme that play roles in the synthesis of these pigments, such as δ-aminolevulinic acid dehydratase and proto-

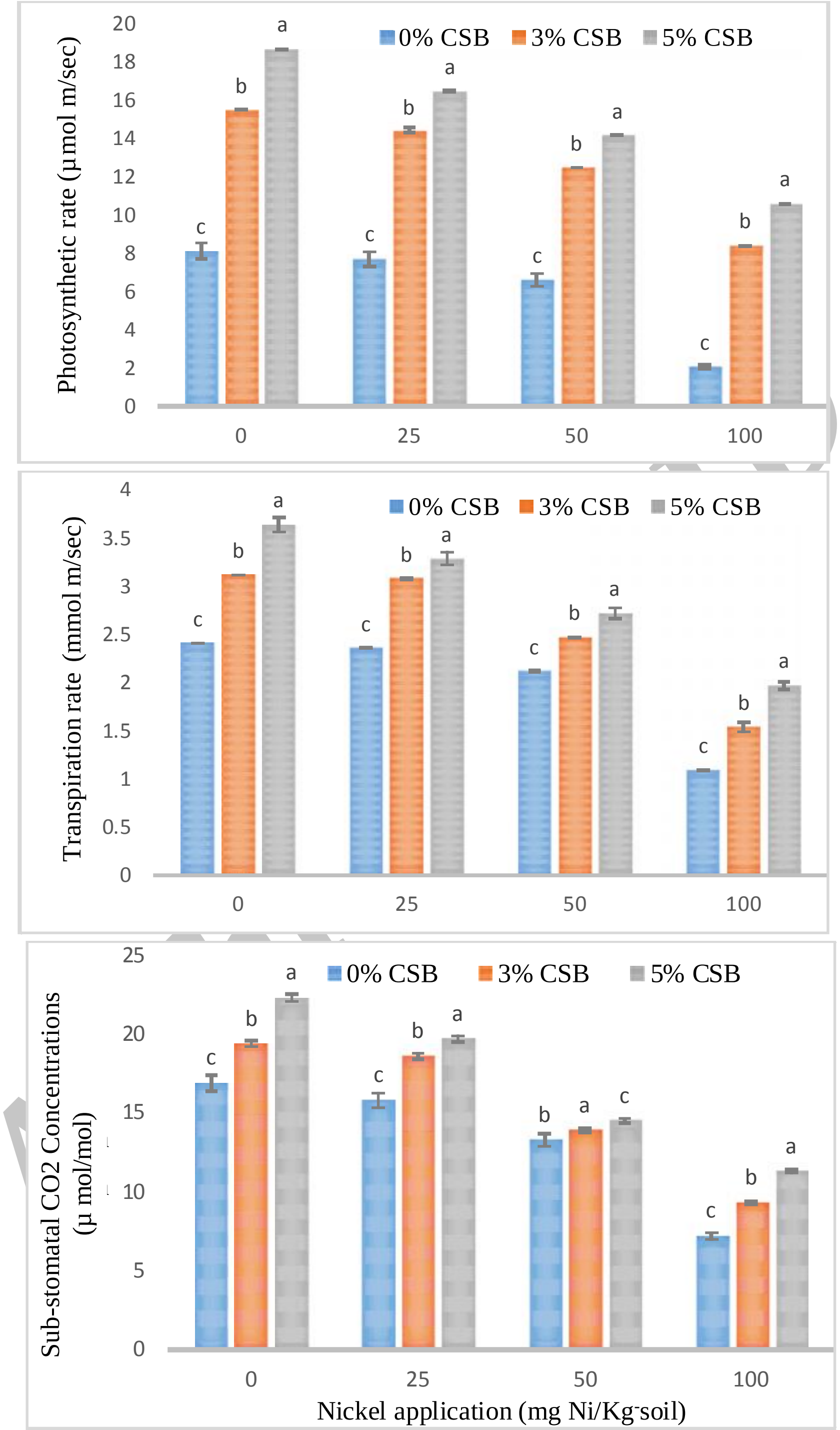

Fig. 2: Photosynthetic rate, transpiration rate and sub-stomatal CO_2 concentrations of *S. oleracea* under different levels of nickel (0, 25, 50 and 100 mg Ni/kg soil) and cotton sticks biochar (CSB, 0 %, 3% and 5 %) on. The different letters on bars show significant differences ($P < 0.01$) between biochar application levels within a nickel application.

Table 3: Photosynthetic attributes of *S. oleracea* under different levels of nickel (0, 25, 50 and 100 mg Ni/Kg soil) and cotton sticks biochar (CSB, 0 %, 3% and 5 %). The different letters within column show significant differences (P < 0.01) between biochar application levels within a nickel application.

Ni Treatments	Biochar application (%)	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoids	Lycopene mg/g	Anthocyanin (umol/ml)
Control	0% CSB	1.63 c	0.47 c	2.10 c	0.55 c	0.10 c	0.10 b
	3% CSB	2.23 b	0.74 b	2.98 b	0.68 b	0.11 b	0.14 ab
	5% CSB	2.61 a	1.08 a	3.68 a	0.71 a	0.13 a	0.16 a
25 mg Ni/Kg	0% CSB	1.64 c	0.44 c	2.09 c	0.46 c	0.09 c	0.12 b
	3% CSB	2.05 a	0.76 b	2.81 a	0.68 a	0.11 b	0.13 a
	5% CSB	1.80 b	0.91 a	2.71 b	0.65	0.12 a	0.12 b
50 mg Ni/Kg	0% CSB	1.51 c	0.49 b	2.00 c	0.50 a	0.09 b	0.09 b
	3% CSB	1.63 b	0.63 a	2.27 b	0.50 a	0.08 b	0.11 a
	5% CSB	2.20 a	0.42 c	2.63 a	0.51 a	0.10 a	0.09 b
100 mg Ni/Kg	0% CSB	1.25 c	0.31 b	1.57 c	0.41 a	0.07 b	0.08 a
	3% CSB	1.49 b	0.48 a	1.97 b	0.38 b	0.09 a	0.08 a
	5% CSB	1.82 a	0.34 b	2.17 a	0.34 b	0.09 a	0.07 b

chlorophylli-dereductase. It controls the bio manufacturing of pigments and Calvin cycle. Similarly, Somashekaraiah *et al.*, (1992) suggested that the decrease in the chl.a and chl.b are resulted due to damaging of chloroplast by Ni. The increase in carotenoid contents in the present study is consistent with the findings of Chaneva *et al.*, (2009) who reported increased carotenoid contents in maize under heavy metal stress.

Biochemical attributes

The concentration of soluble proteins in the leaves was significantly affected by the application of Ni and CSB (Fig. 3). The decrease in protein concentration (15.37 mg/g to 6.46 mg/g) was noted with increasing levels of Ni irrespective of CSB application. Similar results were observed for total soluble amino-acids. Lowest concentration (0.33 mg/g) of total soluble amino acids was noted at 100 mg Ni/kg. The MDA contents were also significantly (P < 0.01) influenced by Ni treatment along with CSB. By increasing Ni concentrations the MDA contents also increased from 0.67 µg/g (control) to 2.07 µg/g (100 mg Ni/kg). However, by the addition of CSB, this increase was changed into decrease at various levels of Ni (2.07 µg/g to 1.14 µg/g at 100 mg Ni/Kg with 5% biochar). A similar pattern for the sugar and ascorbic acid concentrations was observed at different levels of Ni and CSB. However, sugar concentration increased more rapidly at 100 mg Ni/kg as compared to MDA and ascorbic acid.

Ni stress was also noticed in the form of decreased soluble proteins in the plants. This decrease in protein content might have been due to the reduction of *de novo* synthesis of enzymes (Fig. 3). High amount of Ni intake reduces the production of proteins in the sunflower, maize as well as in soybean (Sharma and Dhiman, 2013). In the forms of enzymes many cell functions are performed by the proteins in the body of plants. The increase in the concentrations of metal-binding complexes (phytochelatins and metallothioneins) with heavy metal pollution was also demonstrated by Inouhe, (2005). The increase in proteins with biochar application under Ni stress might have resulted from the same reason. Amino acids are critically important for metabolism of plants as these are a connection between carbon and nitrogen metabolism. The synthesis of amino acids by plants is an environmentally controlled factor; thus, plants produce high quantities of amino acids under limited stress conditions (Pant *et al.*, 2011). Similar results were observed in our study. The amount of amino acids increased at 25 mg Ni/Kg soil. This increase was more visible at 5% biochar application with Ni stress.

In many plants the oxidation of polyunsaturated fatty acids named as lipids is also observed when

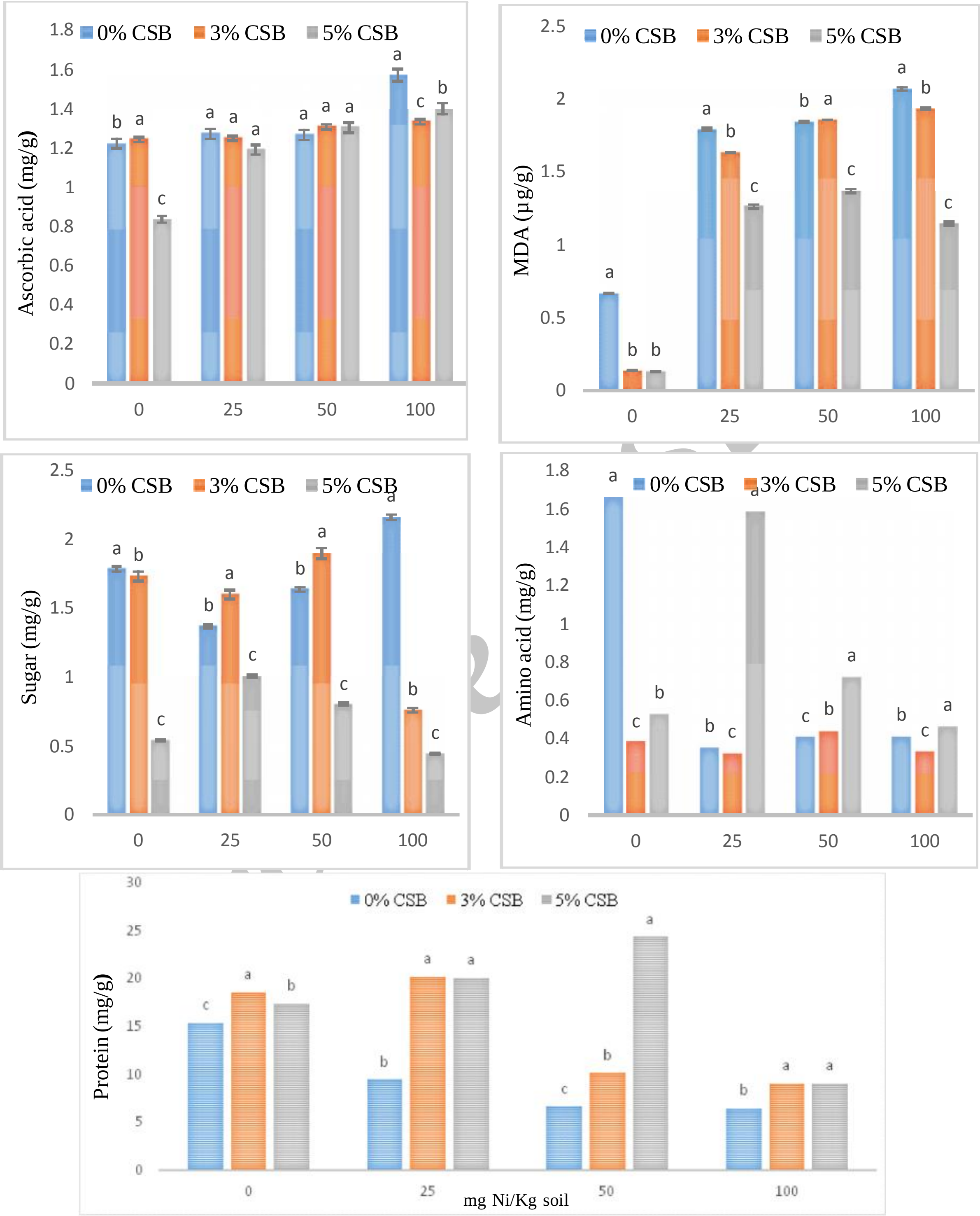

Fig. 3: Biochemical attributes (Ascorbic acid, MDA, sugar, amino acids and protein) of *S. oleracea* under different levels of nickel (0, 25, 50 and 100 mg Ni/kg soil) and cotton sticks biochar (CSB, 0 %, 3% and 5 %). The different letters on bars show significant differences ($P < 0.01$) between biochar application levels within a nickel application.

they are cultivated in the Ni toxic soils. At the highest levels of nickel application, the concentrations of MDA, sugars, and ascorbic acid were increased (Fig. 3). The increases in these concentrations, especially MDA and ascorbic acid, play protective roles against Ni stress. The increase in MDA (polyunsaturated fatty acids) has also been observed in different plants with Ni treatment (Demiral and Türkan, 2005). Similarly the high concentration of Ni in the wheat plants also increase the synthesis of MDA as found by many scientists (Gonnelli *et al.*, 2001). The increased in ascorbic acid under Ni stress was also observed by Mishra and Choudhuri (1999), who suggested the defensive role of ascorbic acid in Ni stress. However, these increases were much less in BC-treated soils, suggesting a role for BC in reducing the toxicity of Ni by sorption it in soils.

Nickel concentration in plants

The concentration of Ni in root and shoot increased significantly ($P<0.01$) with increasing Ni application levels (Table 4), though, this increase was more pronounced at rhizosphere level. Maximum value of Ni concentration in root (6.652 ppm) was noted at 100 mg Ni/kg followed by 50 mg Ni/kg (7.026 ppm), and the minimum (0.159 ppm) was under 5% CSB. However the addition of CSB in Ni applied pots significantly reduced the Ni concentrations in the roots and shoots of respective plants.

Table 4: Effect of various application levels of nickel (0, 25, 50 and 100 mg Ni/kg soil) and cotton sticks biochar (CSB, 0 %, 3% and 5 %) on nickel concentration in *S. oleracea*. The different letters within column show significant differences ($P < 0.01$) between biochar application levels within a nickel application.

Ni Treatments	Biochar % age	Nickel concentrations (mg Ni/Kg dry mass)	
		Root	Shoot
Control	0% CSB	0.199 a	0.099 a
	3% CSB	0.166 b	0.096 b
	5% CSB	0.159 c	0.093 c
25 mg Ni/Kg	0% CSB	6.723 a	5.254 a
	3% CSB	6.716 a	4.662 b
	5% CSB	6.360 b	4.629 b
50 mg Ni/Kg	0% CSB	7.026 a	6.060 a
	3% CSB	6.054 b	5.115 b
	5% CSB	5.032 c	5.065 c
100 mg Ni/Kg	0% CSB	7.652 a	7.546 a
	3% CSB	7.492 b	5.364 b
	5% CSB	7.459 c	5.298 b

CONCLUSION

The results of the present study clearly indicated that different levels of nickel had negative effects on growth, chlorophyll and physiological attributes of spinach plants. Furthermore, the increased in production of ascorbic acid, sugar and MDA indicate the damage impose by nickel on plants. Though, the cotton sticks biochar had revealed the capability to sorb metal in the soil and delimited transfer of nickel ions to the aerial tissue. Similarly, biochar application also enhanced the growth and photosynthetic attributes of spinach under nickel stress. Therefore, biochar can be chose as an amendment in conditions where irrigation water is impure with significant quantity of nickel as well as for the operation of unrestrained soils polluted with this metal.

ACKNOWLEDGEMENTS

This research constitutes a part of Ph.D. thesis of the first author. The authors acknowledge the Higher Education Commission Pakistan for financial support for this research work and Nouman Mirza of University of Melbourne, Australia for critically reviewing the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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How to cite this article: (Harvard style)
Younis, U.; Athar, M.; Malik, S.A.; Raza Shah, M.H.; Mahmood, S., (2015). Biochar impact on physiological and biochemical attributes of spinach Spinacia oleracea (L.) in nickel contaminated soil, Global J. Environ. Sci. Manage., 1(3): 245-254.