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BIOCHAR-MEDIATED AMELIORATION OF NICKEL-INDUCED STRESS IN SPINACH (*SPINACIA OLERACEA* L.) PLANTS: A PHYTOREMEDIATION APPROACH

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Abstract

A study was conducted to explore the potential of biochar (BC) in mitigating nickel (Ni) toxicity and remediating soil conditions in two spinach varieties, Desi and Green Gold. We subjected these plants to two BC treatment levels (16.25 and 32.5 grams) in conjunction with a 5 mM Ni treatment. The results showed that the application of 32.5 g of BC per pot significantly improved the length, fresh and dry weight of both shoot and root components, while Ni stress had adverse effects on these growth parameters. Photosynthetic pigments, including chl *a*, chl *b*, total chl, and carotenoids, were found to increase markedly under Ni stress conditions when 32.5 g of BC was applied. Biochar also exhibited its ability to reduce the concentrations of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) at this treatment level. This reduction was attributed to the enhanced activity of enzymatic antioxidants like superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), while Ni stress led to elevated MDA and H_2O_2 levels. Regarding organic osmolytes, the 32.5 g BC treatment showed significant improvements in glycine betaine, total soluble proteins, and total soluble sugars. This mitigated the toxic effects of Ni stress on the plants. Furthermore, BC was found to enhance the uptake of beneficial mineral ions such as calcium (Ca^{2+}) and potassium (K^+), while reducing the concentration of harmful sodium (Na^+) under Ni stress conditions. In short, the application of 32.5 g of BC per pot had a multifaceted positive impact on the spinach plants. It improved their growth, photosynthetic pigments, organic osmolytes, antioxidants, and ionic content, while simultaneously reducing the oxidative stress induced by Ni. This comprehensive amelioration of Ni stress conditions highlights the potential of BC as an effective strategy for promoting plant health and remediating soil contaminated with Ni.

Key words: Antioxidants; Biochar; Nickel; Osmolytes; Photosynthesis

Introduction

Over time, the development of crops using treated wastewater results in a significant accumulation of nickel, which can cause toxicity in both humans and plants (Amjad *et al*., 2020a). Additionally, it has a detrimental impact on the biogeochemical cycles of the soil, such as carbon oxidation and the release of micronutrients (Gao *et al*., 2019; Xu *et al*., 2019). Nickel plays a role in plant enzymatic activity and functions as a stimulant of physiological processes, either directly or indirectly. However, at higher concentrations, this can impair the biochemical processes of plants (Rue *et al*., 2020). Nickel stress also reduces crop production, plant height, chlorophyll concentration, root length, and the levels of micronutrients and macronutrients, such as nitrogen, potassium, and phosphorus (Younis *et al*., 2016). To ensure the production of nutritious foods, it is necessary to limit the nickel content in plants cultivated in nickel-deposited soils.

In agricultural areas, BC has shown a positive effect on soil conditions and, ultimately, crop production. Due to its high porosity, it decreases soil bulk density and increases water retention capacity. The application of BC reduces soil acidity, enhances cation exchange capacity, and improves nitrogen retention, resulting in alterations to the physical, chemical, and biological properties of the soil (Joseph *et al*., 2021). Biochar also increases microbial activity and enhances mycorrhizal associations. Moreover, crop quality can be improved by applying BC under Ni stress conditions (Abdullahi, 2019).

In Pakistan, nearly 108,725 tonnes of spinach are produced on an area of 8,820 hectares, which is considerably lower than other major producers (Rashid *et al*., 2014). Spinach is rich in nutrients such as vitamins A, C, K, calcium, magnesium, iron, and manganese. Additionally, it possesses several biological characteristics that make it a valuable source of antioxidants, anti-inflammatory compounds, and chemotherapy agents (Ramaiyan *et al*., 2020). Spinach was chosen for this study due to its quick growth cycle, early spring growth, wide adaptability, ability to grow in wastewater, and tolerance to heavy metals.

Taking into account the aforementioned factors, this research makes a valuable contribution to the existing knowledge by providing a thorough assessment of how biochar (BC) plays a pivotal role in alleviating nickel (Ni) stress in spinach plants, effectively bridging the existing gaps in our understanding of this subject. It provides valuable insights for sustainable agriculture practices and offers a nuanced understanding of the specific mechanisms involved in improving plant resilience against heavy metal toxicity. It was hypothesized that the exogenous application of biochar might be helpful in amelioration of the adverse effects of the nickel toxicity in spinach. This study aimed to investigate the impact of BC on the growth

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and physiological attributes of spinach plants under Ni stress conditions and to assess the effectiveness of BC in reducing oxidative stress and enhancing the tolerance of spinach plants to Ni toxicity.

Material and Methods

Experimental design and treatments: A study involving potted plants was done at the Old Botanical Garden, University of Agriculture Faisalabad, to evaluate the effect of BC in alleviating Ni stress conditions.

The seeds of two types of spinach, namely Desi and Green Gold, were subjected to surface sterilization and then planted in pots containing 6.5 kilograms of soil. Thinning was performed one week after seed sowing, and five healthy and uniform seedlings were retained for the application of treatments. The plants were exposed to bright sunlight with an average day/night temperature of approximately 38.7/26.9±2°C, relative humidity of about 39/47±3%, and a photosynthetic photon flux density of 500-620 µmol/s.

Two weeks after germination, 1000 mL of Hoagland's nutrient solution was administered to each pot. Subsequently, every two weeks, 500 mL of nutrient solution was added to each pot. Nickel (5 mM) was introduced into the soil using Tween-20, two weeks after germination. Wood chips of BC (16.25 and 32.5 g), produced at a temperature of 500°C and consisting of crystallite phases such as calcite ($CaCO_3$) and quartz (SiO_2), were mixed into the soil in powder form, also two weeks after the Ni treatment. The plants were harvested 11 weeks after seed germination. To determine various parameters, fresh leaves were collected in plastic zipper bags and immediately stored in the freezer.

Determination of growth parameters: The measurement of shoot and root length was conducted using a measuring scale. The fresh weight of both the shoot and root was determined with a digital weighing balance right after collecting plant samples. To calculate the dry weight, the plants were placed in an oven at 62°C for two weeks and then weighed using a digital weighing balance. Leaf area was determined through the following formula:

$$\text{Leaf area} = \text{leaf length} \times \text{leaf width} \times \text{C. F}$$

Determination of photosynthetic pigments: The determination of photosynthetic pigments followed the procedure outlined by Arnon (1949). 100 mg of fresh leaf samples were finely chopped and put into small plastic containers. Next, 5 mL of an 80% acetone solution was introduced into each container. The sample containers were then left at room temperature (25°C) for a night, during which the color of the solution changed. The absorbance of these sample solutions was measured at 663, 645, and 480 nm using a spectrophotometer UV-1100, Hitachi 220 (Japan).

Chl. *a* (mg g^{-1} FW) = [12.7 (OD663) - 2.69 (OD645)] × V/1000 × W
Chl. *b* (mg g^{-1} FW) = [22.9 (OD645) - 4.68 (OD663)] × V/1000 × W
Carotenoids (mg g^{-1} FW) = Acar/ Em × 100
Acar =OD 480 + 0.114(OD 663) - 0.638 (OD 645) and Em = 2500
V= Volume of the extract (mL)
W= Weight of fresh leaf tissue (g)

Determination of enzymatic antioxidants: A fresh leaf sample, weighing 0.25 g, was ground in a mortar and pestle with the addition of 5 mL of cold potassium phosphate buffer. The resultant mixture was transferred to a 2 mL Eppendorf tube and centrifuged at 12,000 × g for 15 min. The solution, which was separated from contaminants during the centrifugation procedure, was stored at 20°C for future analysis.

Catalase (CAT): Catalase activity was assessed following the procedure outlined by Chance & Maehly (1955). A 1 mL extract from the plant sample was combined with 1.9 mL of cold potassium phosphate buffer and 1 mL of H_2O_2 in a cuvette. The absorbance was recorded at 240 nm at 0, 30, and 60 sec intervals using a spectrophotometer UV-1100, Hitachi 220 (Japan).

Superoxide dismutase (SOD): The measurement of SOD activity followed the procedure detailed by Oberley & Spitz (2001). To a 0.05 mL sample extract, the following solutions were added in a cuvette: 0.4 mL distilled water, 1 mL potassium phosphate buffer, 0.1 mL L-methionine, 0.1 mL Triton-X, 0.05 mL NBT, 0.05 mL sample extract, and 0.05 mL riboflavin. The cuvettes were then exposed to lamp light for 15-20 min, and the absorbance of the sample solutions at 560 nm was measured using a spectrophotometer UV-1100, Hitachi 220 (Japan).

Peroxidase (POD): Peroxidase activity was assessed using the method established by Chance & Maehly (1955). In cuvettes, the following solutions were added: 0.1 mL guaiacol, 0.1 mL H_2O_2, 50 µL of plant sample extract, and 750 µL of phosphate buffer. Absorbance readings were taken at 470 nm at 0, 30, 60, and 90-sec intervals using a spectrophotometer UV-1100, Hitachi 220 (Japan).

Determination of polyphenolic compounds

Flavonoids: Flavonoid determination was carried out in accordance with the method outlined by Marinova *et al*., (2005). In a flask, a mixture of plant sample extract (1 mL), distilled water (4 mL), and 5% $NaNO_2$ (0.3 mL) was prepared. After a 6 min interval, 1M NaOH (2 mL) was introduced into the solution. The volume was adjusted to 10 mL by the addition of distilled water. Absorbance measurements at 510 nm were conducted using spectrophotometer UV-1100, Hitachi 220 (Japan).

Anthocyanin: The content of anthocyanin was determined following the procedure specified by Strack & Wray (1989). A fresh leaf (0.1 g) was taken in a test tube containing methanol (2 mL). The test tubes containing the plant sample were then heated in a water bath at 90°C for 1 h. The measurement of absorbance was conducted at 535 nm utilizing a spectrophotometer UV-1100, Hitachi 220 (Japan).

Determination of organic osmolytes

Glycine betaine: Glycine betaine was quantified using the method outlined by Grieve & Grattan (1993). Fresh spinach plant leaves (200 mg) were ground in distilled

water (5 mL), and the resulting extract was kept for centrifugation at 12,000 × g. To 1 mL of the plant extract, 2N H_2SO_4 was added, and this mixture (0.5 mL) was transferred to a test tube. To this mixture, potassium iodide (0.2 mL) solution was added and cooled. After a 90 min interval, 6 mL of dichloroethane and distilled water (2 mL) was mixed to the mixture. Two layers appeared in the test tube, and the sample was taken from the lower layer. The measurement of absorbance was conducted at 365 nm utilizing a spectrophotometer UV-1100, Hitachi 220 (Japan).

Total soluble sugars: The determination of the total soluble sugar content was carried out following the procedure outlined by Yoshida *et al*., (1976). Test tubes containing fresh leaf samples (100 mg) were filled with distilled water (10 mL). Subsequently, the test tubes were subjected to heating in a water bath at 90°C for duration of 1 h. Following the boiling step, distilled water (50 mL) was introduced into each test tube to dilute the samples. A volume of 1.5 mL was extracted from this mixture of plant samples and combined with Anthrone reagent (5 mL). Subsequently, the samples were incubated in a water bath at 90°C for duration of 20 min. Once cooled to 25°C, the absorbance was measured at 620 nm using a spectrophotometer UV-1100, Hitachi 220 (Japan).

Total soluble proteins: The determination of total soluble protein content was carried out according to the procedure outlined by Bradford (1976). Fresh leaf samples were ground in potassium phosphate buffer (5 mL), and the resulting extract was then centrifuged at 12,000 × g for 15 min to eliminate impurities. To plant extract (0.1 mL), Bradford reagent (5 mL) was added to test tubes and thoroughly mixed using a vortex. The absorbance was recorded at 595 nm using a spectrophotometer UV-1100, Hitachi 220 (Japan).

Determination of oxidative stress determinants

Hydrogen peroxide (H_2O_2): The concentration of H_2O_2 was assessed following the procedure outlined by Velikova *et al*., (2000). Fresh leaf samples (0.25 g) were pulverized in 0.5% trichloroacetic acid (TCA) solution (3 mL). Subsequently, the resulting solution was transferred to Eppendorf tubes and subjected to centrifugation. Following centrifugation, each test tube received 0.5 mL of the sample extract, 0.5 mL of potassium phosphate buffer, and 1 mL of KI, and the contents were mixed using a vortex. The absorbance was measured at 390 nm using a spectrophotometer UV-1100, Hitachi 220 (Japan).

Malondialdehyde (MDA): For the measurement of MDA content, the procedure outlined by Cakmak & Horst (1991) was followed. Fresh leaf samples (1 g) were ground in 2 mL of 0.1% trichloroacetic acid (TCA). The resulting solution was then centrifuged at 15,000 g for 10 min, and the supernatant was collected. To 0.5 mL of the supernatant, 1.5 mL of thiobarbituric acid (TBA) in 20% TCA was added. The mixture was shaken in a water bath for 20 min and then placed in an ice water bath. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer UV-1100, Hitachi 220 (Japan), and the value of non-specific absorption at 600 nm was subtracted.

Determination of inorganic osmolytes: The measurement of inorganic osmolytes was conducted following the method described by Allen *et al*., (1986). In digestion flasks, 0.1 g of dry plant material and 2 mL of sulfuric acid were added. The digestion flasks were covered with aluminum foil and left overnight. The following day, each digestion flask was placed on a hot plate heated to 200°C. Hydrogen peroxide was added drop by drop into the flasks until the solution became colorless. The digested solution was then filtered using filter paper, and the volume of the solution was adjusted to 50 mL by adding distilled water. The concentrations of Na^+, K^+, and Ca^{2+} ions were measured using a flame photometer.

Determination of Ni contents: The Ni content (mg/g dry weight) in the shoot and root of spinach plants was assessed using the Wolf (1982) method, involving the digestion of plant material in acid. The precise quantification of Ni was carried out using an Atomic Absorption Spectrophotometer (AAnalyst-300, Perkin Elmer, Germany) and is reported as mg/L in terms of the plant tissue's dry weight.

Analytical statistics

The experiment was carried out employing a completely randomized design, and three replicates were included. Analysis of variance was conducted utilizing Co-stat software version 6.303.

The least significant difference test was executed at a 5% significance level to detect distinctions among the means of different treatments (Steel *et al*., 1996). Graphical representation of the data was accomplished using Microsoft Excel.

Heatmap clustering and Principal Component Analysis (PCA) was conducted using RStudio (Version 1.1.463, RStudio, Inc.).

Results

Growth attributes: The results showed that Ni stress had a detrimental effect on all the studied growth attributes, including length, fresh and dry weights of shoot and root, and leaf area (Figs. 1, 2). However, BC supplementation mitigated the toxic effects of Ni stress by improving these attributes per plant. Both levels of BC (16.25 g and 32.5 g) were effective in reducing Ni toxicity, with the higher level (32.5 g) proving more effective. ANOVA revealed non-significant interactions among cultivar (C), Ni, and BC treatments (Table 1). Interestingly, both spinach cultivars (Desi and Green Gold) exhibited different responses to Ni application. Desi showed less reduction in growth attributes in comparison to the control under Ni toxicity. Overall, BC application improved Ni tolerance in both spinach cultivars by enhancing various studied growth attributes (Figs. 1, 2).

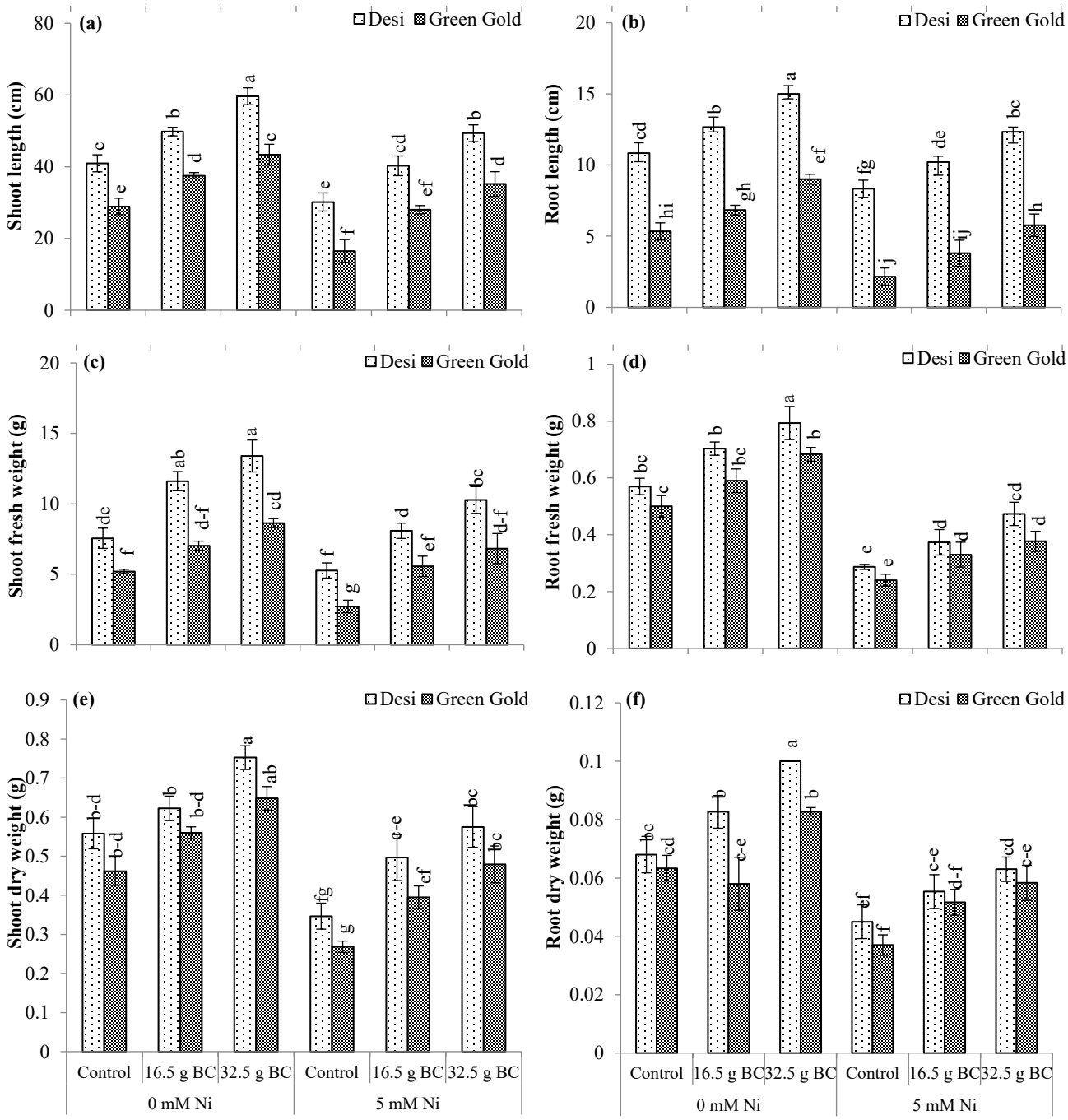

Fig. 1. Effect of different levels of BC on shoot length (cm) (a), root length (cm) (b), shoot fresh weight (g) (c), root fresh weight (g) (d), shoot dry weight (g) (e) and root dry weight (g) (f) of spinach (*Spinacea oleracea* L.) cultivars under Ni stress.

Photosynthetic pigments: The results indicated that Ni stress led to a decrease in all studied photosynthetic pigments (chl *a*, chl *b*, chl *a*/chl *b*, total chl, and carotenoids) in both spinach cultivars (Fig. 3). However, the application of BC mitigated the effects of Ni stress by enhancing photosynthetic pigments. Both levels of BC application (16.25 g and 32.5 g) improved photosynthetic pigments by reducing the toxic effects of Ni stress in both cultivars. The higher BC level (32.5 g) was more effective in improving photosynthetic pigments. The response of both cultivars exhibited remarkable differences, except for carotenoids under stress application. Desi showed a higher concentration of photosynthetic pigments compared to Green Gold under Ni stress. Statistical analysis showed non-significant interactions among C, BC, and Ni, except for chl *b*, which exhibited a significant C × BC interaction (Table 1). In summary, the BC supplementation at 32.5 g improved photosynthetic pigments in both studied spinach cultivars under Ni stress conditions (Fig. 3).

Enzymatic antioxidants: Nickel toxicity highly increased the activity of enzymatic antioxidants (SOD, POD, and CAT) at the 5 mM Ni level in both cultivars (Fig. 4). The application of BC also improved the activity of antioxidants at both applied levels (16.25 g and 32.5 g per pot) even under Ni stress conditions. The maximum improvement in antioxidant activity was observed at the 32.5 g BC level. Both cultivars responded differently to BC application under Ni stress conditions, with Desi showing higher antioxidant activity compared to Green

Gold (Fig. 4). Statistical analysis revealed that SOD exhibited a significant C × Ni × BC interaction, while POD and CAT showed non-significant interactions (Table 2). The activity of antioxidants was enhanced under Ni toxicity, and the BC supplementation at 32.5 g showed a synergistic response in improving antioxidants in both studied spinach cultivars (Fig. 4).

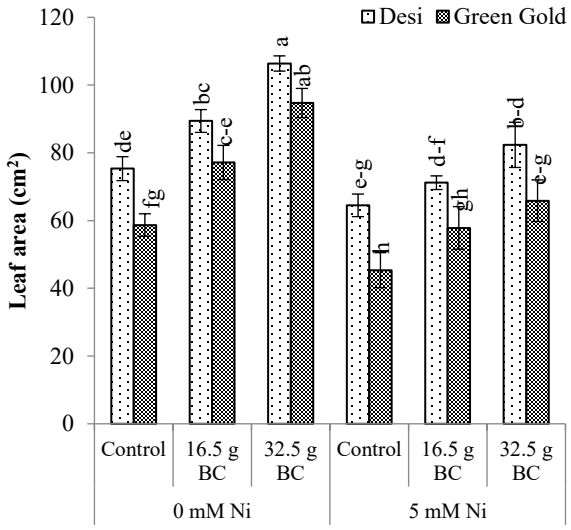

Fig. 2. Effect of different levels of BC on leaf area (cm^2) of spinach (*Spinacea oleracea* L.) cultivars under Ni stress.

Table 1. Mean squares from analysis of variance (ANOVA) of the data for growth parameters and photosynthetic attributes of spinach subjected to different levels of biochar under Ni stress conditions.

SoV	df	SL	RL	SFW
Cultivar (C)	1	1628.79 ***	332.45 ***	102.26 ***
Nickel (Ni)	1	921.62 ***	72.81 ***	54.01 ***
Biochar (BC)	2	946.56 ***	44.85 ***	64.79 ***
C × Ni	1	0.07 ns	0.81 ns	2.50 ns
C × BC	2	7.38 ns	0.15 ns	2.11 ns
Ni × BC	2	4.99 ns	0.03 ns	0.009 ns
C × Ni × BC	2	2.67 ns	0.002 ns	0.99 ns
Error	24	17.43	1.11	1.45
SoV		**RFW**	**SDW**	**RDW**
Cultivar (C)	1	0.05 ***	0.07 ***	9.92 **
Nickel (Ni)	1	0.77 ***	0.27 ***	0.005 ***
Biochar (BC)	2	0.10 ***	0.12 ***	0.0015 ***
C × Ni	1	0.002 ns	3.80 ns	2.30 ns
C × BC	2	0.001 ns	2.51 ns	5.65 ns
Ni × BC	2	0.0013 ns	0.002 ns	1.44 ns
C × Ni × BC	2	6.86 ns	7.03 ns	1.14 ns
Error	24	0.0039	0.0041	8.15
SoV		**LA**	**Chl *a***	**Chl *b***
Cultivar (C)	1	2003.38 ***	0.29 ***	2.44 ***
Nickel (Ni)	1	3282.43 ***	0.16 ***	1.41 ***
Biochar (BC)	2	2084.35 ***	0.10 ***	0.42 ***
C × Ni	1	17.73 ns	5.48 ns	0.008 ns
C × BC	2	21.11 ns	4.44 ns	0.037 *
Ni × BC	2	154.77 ns	0.004 ns	0.0043 ns
C × Ni × BC	2	2.65 ns	0.002 ns	0.0028 ns
Error	24	61.62	0.0049	0.0077
SoV		**Total Chl**	**Chl Chl *a*/*b***	**Carotenoids**
Cultivar (C)	1	2.27 ***	0.059 **	0.72 ***
Nickel (Ni)	1	2.54 ***	0.017 ns	1.307 ***
Biochar (BC)	2	0.96 ***	6.06 ns	0.36 ***
C × Ni	1	0.13 ns	1.602 ns	0.0079 ns
C × BC	2	0.045 ns	0.004 ns	2.58 ns
Ni × BC	2	0.014 ns	0.0014 ns	0.012 ns
C × Ni × BC	2	0.0071 ns	0.0029 ns	0.0071 ns
Error	24	0.014	0.0045	0.0062

*, **, *** = Significant at 0.05, 0.01 and 0.001 levels, respectively
ns = Non-significant

Polyphenolic compounds: The results showed that Ni stress significantly increased flavonoid contents in both spinach cultivars (Fig. 5). The application of BC worked synergistically to increase flavonoid contents, with the maximum improvement observed at 32.5 g BC in both cultivars. However, all interactions among BC, C, and Ni application were found to be non-significant (Table 2). Moreover, Desi exhibited a higher improvement in flavonoid content compared to Green Gold under Ni stress conditions. In summary, the application of BC reduced the toxic effects of Ni stress by increasing flavonoid contents in both spinach cultivars (Fig. 5). The results revealed that the imposition of Ni stress significantly decreased anthocyanin content in both spinach cultivars (Fig. 5). Biochar application improved the anthocyanin concentration and significantly reduced the toxic effects of Ni stress. Both levels of BC (16.25 g and 32.5 g) were effective in reducing Ni toxicity, with the higher BC level (32.5 g) being the most effective. In comparison between both cultivars (Desi and Green Gold), Green Gold exhibited a lower concentration of anthocyanins compared to Desi under Ni stress conditions (Fig. 5).

Organic osmolytes: Nickel stress significantly enhanced glycine betaine, total soluble protein, and total soluble sugar content in both studied spinach cultivars (Fig. 6). A significant C × Ni interaction was observed for glycine betaine and total soluble protein, while total soluble sugar showed a non-significant C × Ni interaction (Table 2). The application of BC improved the glycine betaine, total soluble protein, and total soluble sugar content under Ni toxicity in both studied cultivars. This was supported by a significant C × BC interaction in glycine betaine and a significant interaction in Ni × BC for total soluble sugar (Table 2). Both studied BC levels (16.25 g and 32.5 g) improved the concentration of all studied organic osmolytes, with the 32.5 g BC level being more efficient in improving osmolytes and reducing Ni stress in spinach cultivars. Among both cultivars, Desi accumulated a higher concentration of glycine betaine, total soluble proteins, and total soluble sugar. Among both cultivars (Desi and Green Gold), Desi exhibited higher total soluble sugar and protein contents compared to Green Gold. Biochar at 32.5 g showed a significant effect in reducing Ni stress by enhancing the concentration of organic osmolytes in both cultivars (Fig. 6).

Oxidative stress determinants: The results showed that Ni stress significantly increased reactive oxygen species (H_2O_2 and MDA) content in both studied spinach cultivars (Fig. 7). However, the application of BC lowered the concentration of H_2O_2 and MDA at both studied levels (16.25 g and 32.5 g). The maximum reduction in ROS content was found at 32.5 g BC. The cultivar Desi showed higher H_2O_2 and MDA content compared to Green Gold under Ni toxicity. In short, the amendment of BC reduced the MDA concentration more in Green Gold compared to Desi. The accumulation of oxidative stress determinants was greater in both varieties, Green Gold as well as Desi (Fig. 7).

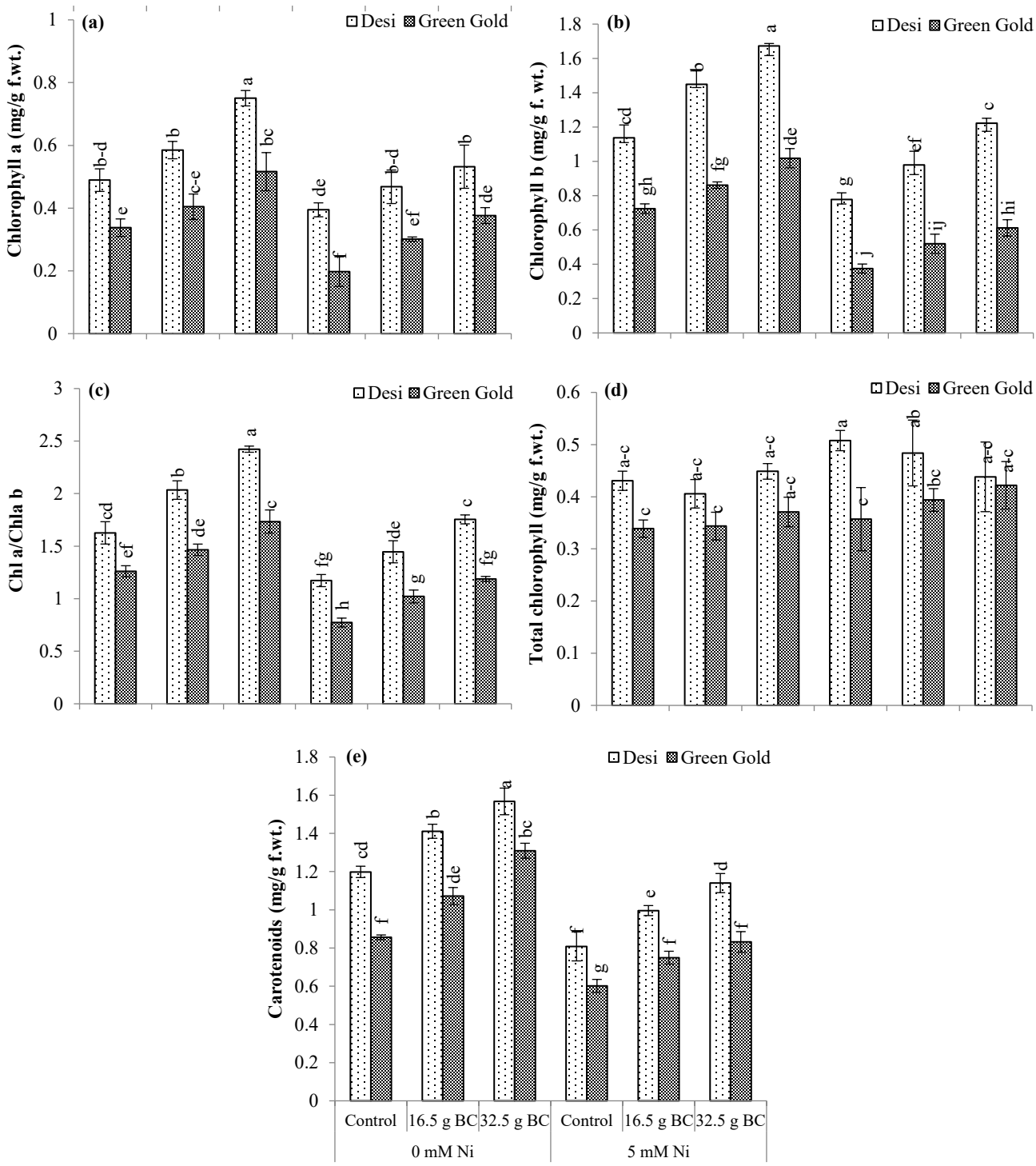

Fig. 3. Effect of different levels of BC on chl *a* (mg/g fresh weight) (a) chl *b* (mg/g fresh weight) (b) chl*a*/chlb (c), total chl (mg/g fresh weight) (d) and carotenoids (mg/g fresh weight) (e) of spinach (*Spinacea oleracea* L.) cultivars under Ni stress.

Inorganic osmolytes: Nickel application on spinach significantly increased the Na^+ content in the shoot and root of both spinach cultivars (Fig. 8). This was supported by a statistically significant C × Ni interaction in both cultivars (Table 3). Biochar supplementation lowered the Na^+ concentration in all studied parts of spinach, with the maximum reduction observed at 32.5 g BC. The cultivar Green Gold accumulated a higher Na^+ content compared to Desi (Fig. 8). Moreover, Ni stress also reduced the K^+ and Ca^{2+} content in both studied spinach cultivars. This was revealed by a statistically significant C × Ni interaction for Ca^{2+} content in the shoot of spinach cultivars. However, the root showed a non-significant C × Ni interaction for Ca^{2+} content in the plants (Table 3). Biochar supplementation at 32.5 g highly lowered Ni stress by increasing the Ca^{2+} content in the spinach shoot. A statistically significant BC × Ni interaction for Ca^{2+} content of the shoot highlighted this fact. However, all interaction terms were found non-significant for K^+ content in the spinach plants. Among both studied spinach cultivars, Desi exhibited increased Ca^{2+} and K^+ content compared to Green Gold under Ni stress conditions (Fig. 8).

Table 2. Mean squares from analysis of variance (ANOVA) of the data for antioxidants, polyphenolic compounds, organic osmolytes and oxidative stress determinants of spinach subjected to different levels of biochar under Ni stress conditions.

SoV	df	SOD	POD	CAT	Flavonoid	Anthocyanin
Cultivar (C)	1	3.61 ***	3817502.4 ***	7588832.3 ***	0.042 **	0.18 ***
Nickel (Ni)	1	0.0014 ***	5423940.3 ***	7698933.6 ***	0.03 ***	0.19 ***
Biochar (BC)	2	3.05 ***	1680477.6 ***	1575475.5 ***	0.01 ***	0.07 ***
C × Ni	1	1.97 ns	16009.09 ns	1366.3848 ns	4.91 ns	9.61 ns
C × BC	2	6.42 ns	11140.206 ns	27654.74 ns	1.45 ns	7.78 ns
Ni × BC	2	3.51 ns	5269.847 ns	9676.25 ns	2.17 ns	1.52 ns
C × Ni × BC	2	2.41 **	102472.8 ns	9189.17 ns	1.05 ns	5.60 ns
Error	24	4.21	50941.33	34583.68	2.48	0.0031
SoV		**GB**	**TSP**	**TSS**	**H_2O_2**	**MDA**
Cultivar (C)	1	1.43 ***	26.18 ***	0.007 ***	4.96 ***	57.33 ***
Nickel (Ni)	1	0.16 ***	1.29 *	4.87 *	2.14 ***	37.14 ***
Biochar (BC)	2	0.31 ***	8.85 ***	0.001 ***	2.55 ***	36.68 ***
C × Ni	1	0.57 ***	2.016 *	7.65 ns	1.09 ns	0.24 ns
C × BC	2	0.044 *	0.26 ns	3.41 ns	1.22 ns	0.65 ns
Ni × BC	2	0.0025 ns	0.12 ns	3.077 *	8.48 ns	0.38 ns
C × Ni × BC	2	0.0011 ns	0.10 ns	2.62 ns	2.31 ns	0.056 ns
Error	24	0.009	0.25	8.77	7.58	1.26

*, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant

Abbreviations: Exponent (e), Superoxide Dismutase (SOD), Peroxidase (POD), Catalase (CAT), Glycine Betains (GB), Total Soluble Proteins (TSP), Total Solubble Sugars (TSS), Hydrogen Peroxide (H_2O_2), Malondialdehyde (MDA)

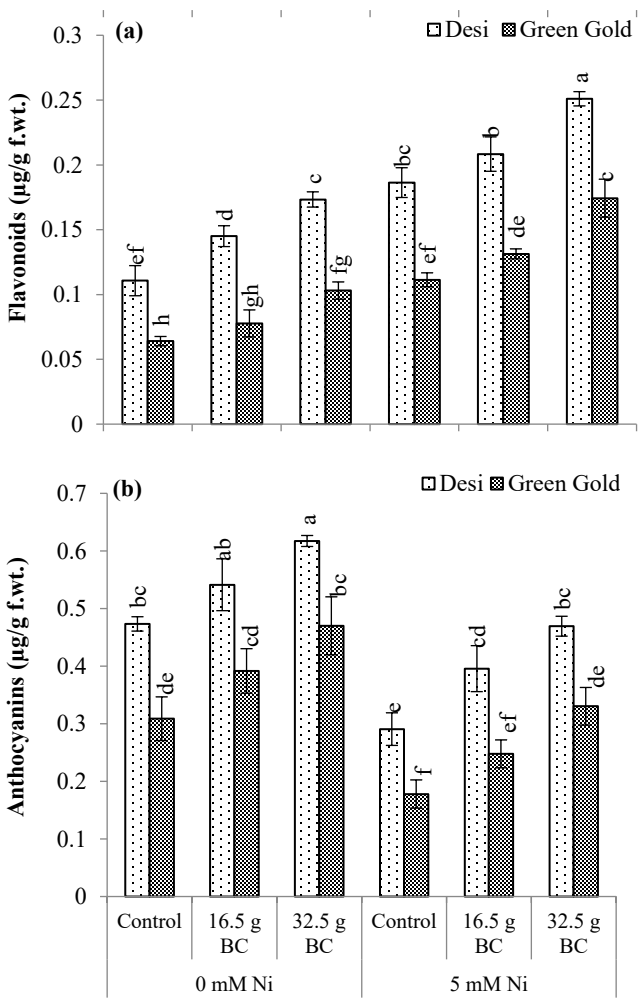

Fig. 5. Effect of different levels of BC on flavonoids (μg/g fresh weight) (a) and anthocyanins (μg/g fresh weight) (b) of spinach (*Spinacea oleracea* L.) cultivars under Ni stress.

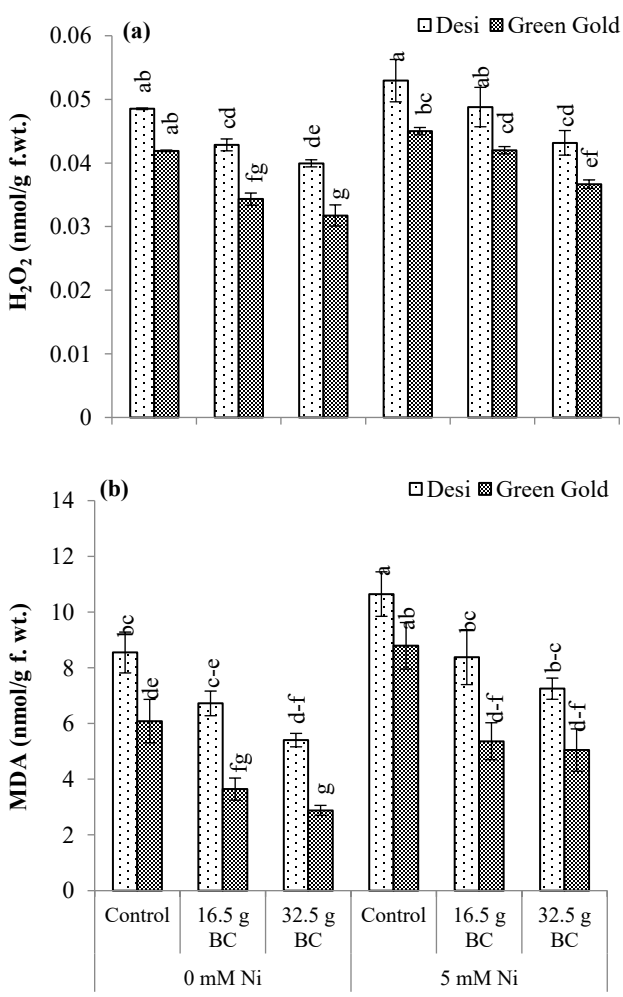

Fig. 7. Effect of different levels of BC on malondialdehyde (μg/g fresh weight) (a) and H_2O_2 (μg/g fresh weight) (b) of spinach (*Spinacea olearacea* L.) cultivars under Ni strees.

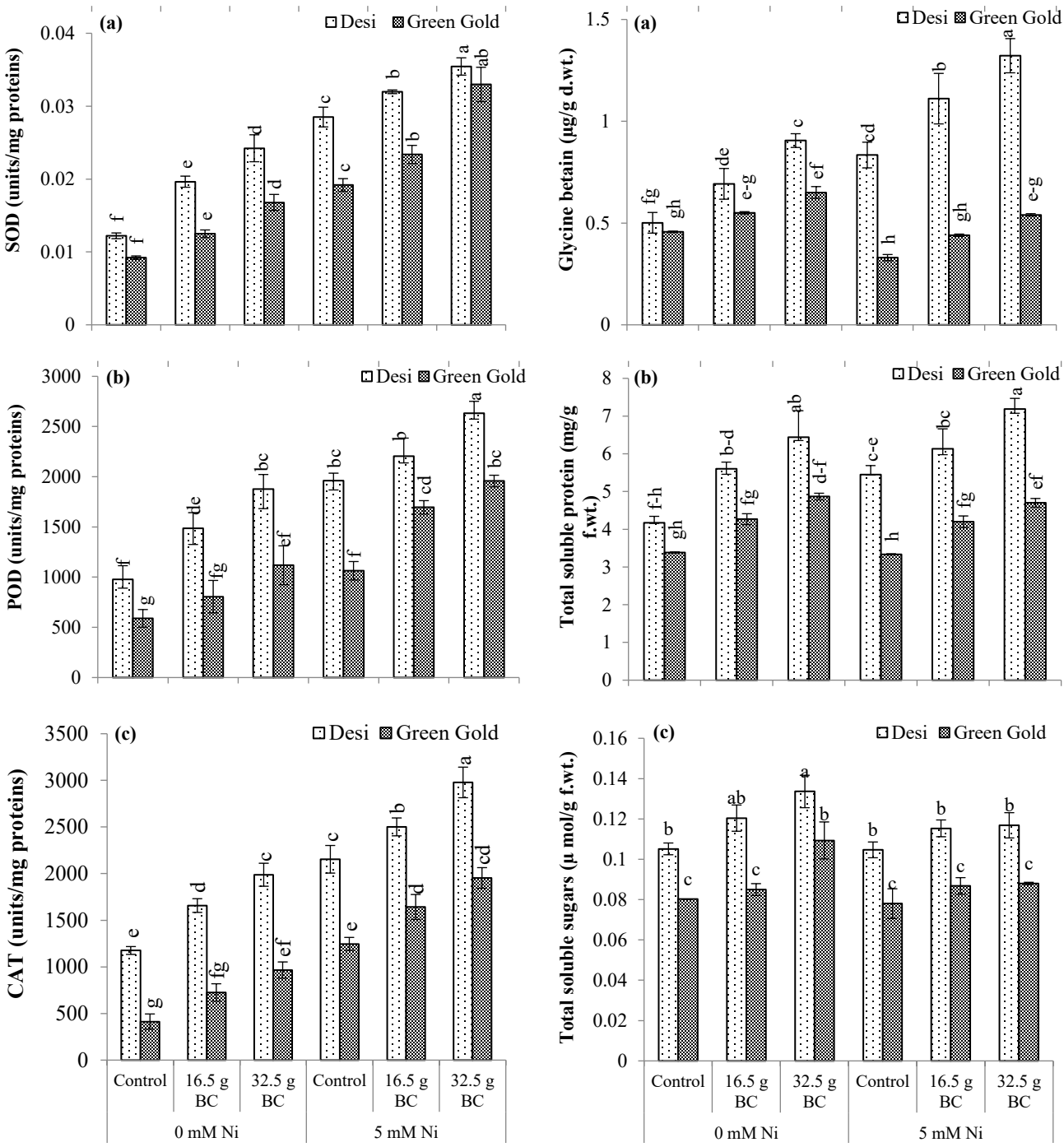

Fig. 4. Effect of different levels of BC on superoxide dismutase (SOD) (units/mg proteins) (a), peroxidase (POD) (units/mg proteins) (b) and catalase (CAT) (units/mg proteins) (c) of spinach (*Spinacea oleracea* L.) cultivars under Ni stress.

Fig. 6. Effect of different levels of BC on glycine betaine (μg/g dry weight) (a), total soluble protein (mg/g fresh weight) (b) and total soluble sugars (μmol/g fresh weight) (c) of spinach (*Spinacea oleracea* L.) cultivars under Ni stress.

Table 3. Mean squares from analysis of variance (ANOVA) of the data for inorganic osmolytes of spinach subjected to different levels of biochar under Ni stress conditions.

SoV	df	Na (S)	Na (R)	K (S)	K(R)	Ca (S)	Ca (R)
Cultivar (C)	1	3338.91 ***	15334.69 ***	312.11 ***	17.57 ****	4.69 ns	49.02 ***
Nickel (Ni)	1	1427.58 ***	747.11 ***	210.25***	51.97***	160.45 ***	18.78 ***
Biochar (BC)	2	175.57 ***	167.64 ***	120.58***	13.98***	76.55 ***	2.55 **
C × Ni	1	27.21 *	245.44 ***	9 ns	0.44 ns	20.25 **	1.37 ns
C × BC	2	1.34 ns	3.006 ns	1.37 ns	0.28 ns	0.43 ns	0.15 ns
Ni × BC	2	0.65 ns	4.21 ns	9.25 ns	0.11 ns	8.55 *	0.010 ns
C × Ni × BC	2	0.84 ns	0.29 ns	1 ns	0.15 ns	0.81 ns	0.049 ns
Error	24	6.25	6.02	5.72	0.56	1.53	0.33

*, **, *** = Significant at 0.05, 0.01 and 0.001 levels, respectively; ns = Non-significant

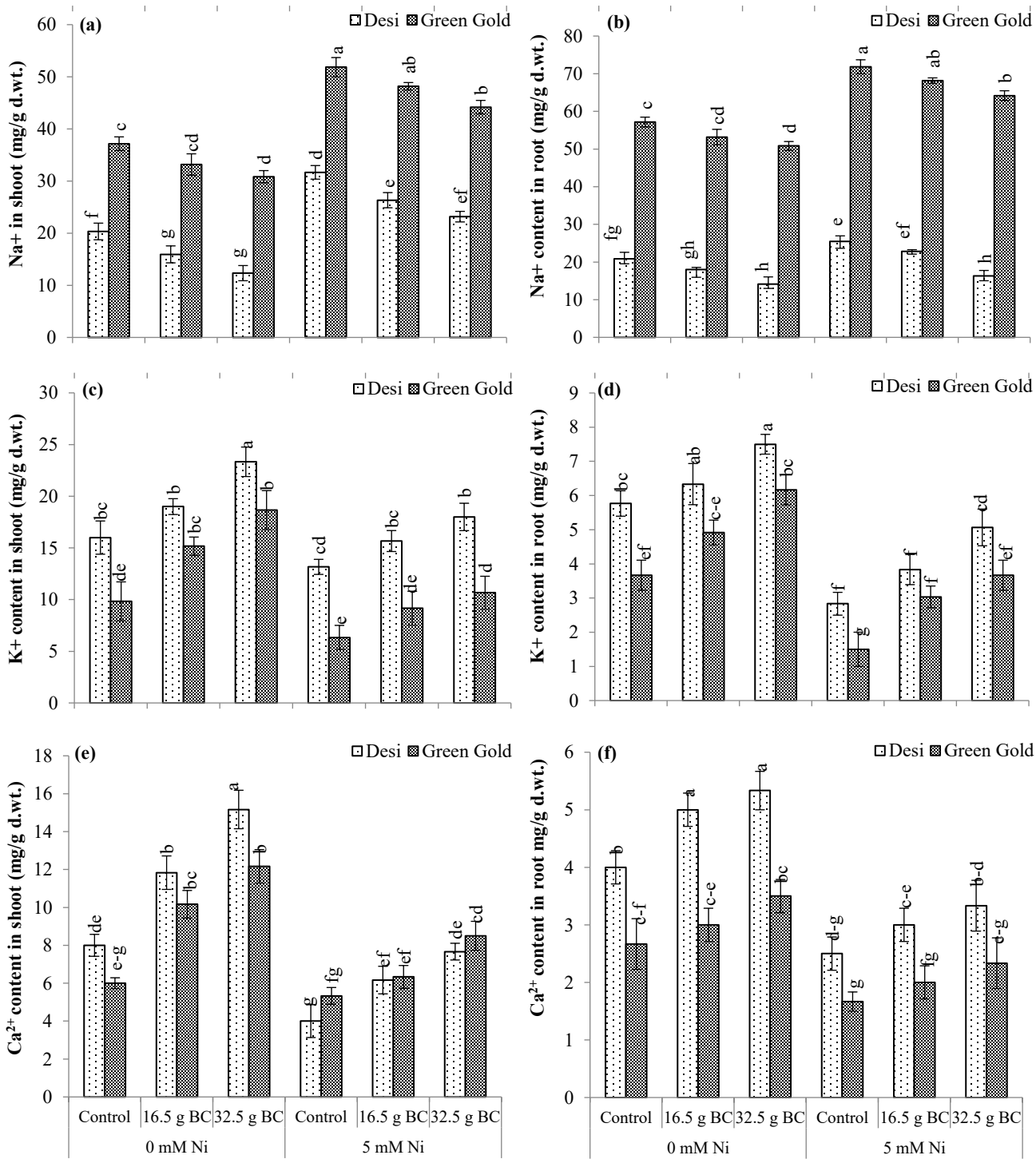

Fig. 8. Effect of different levels of BC on sodium (Na^+) content in shoot (mg/g dry weight) (a) sodium (Na^+) content in root (mg/g dry weight) (b), potassium (K^+) content in shoot (mg/g dry weight) (c), potassium (K^+) content in root (mg/g dry weight) (d), calcium (Ca^{2+}) content in root (mg/g dry weight) (g) (e) and calcium (Ca^{2+}) content in root (f) of spinach (*Spinacea oleracea* L.) cultivars under Ni stress.

Determination of Ni contents: The Ni concentration in both the shoot and root of spinach plants showed a significant elevation under nickel toxicity. Application of BC significantly lowered the Ni stress in both studied spinach cultivars. Notably, Green Gold accumulated a greater amount of Ni in all the plant organs under investigation compared to Desi (Fig. 9).

Heatmap: To observe the impact of BC on various paramenters of spinach in Ni stress conditions, a two-way clustered heatmap was drawn. The parameters were grouped according to their similarity under a particular treatment, and the correlation was shown in colored squares. Black color indicated positive correlation, while light grey indicated negative correlation of various attributes affected by BC under Ni stress conditions. The heatmap clustered all studied parameters into five groups. In the first group, organic osmolytes (TSS, GB, TSP) and root length were clustered. Root length (RL) and total soluble protein (TSP) showed strong positive correlation with the control in Desi and weak correlation in Green Gold at 32.5 g BC without Ni application. However, under

Ni stress conditions, glycine betaine (GB) and total soluble protein (TSP) showed strong positive correlation in Desi and weak correlation in Green Gold at 32.5 g BC. All the parameters in this group showed weak correlation in Desi and a negative correlation in Green Gold at the 5 mM Ni application level without BC application, indicating that BC helped reduce Ni toxicity by improving organic osmolyte content and root length to supply nutrient contents to support adverse Ni stress conditions. Desi showed higher Ni tolerance compared to Green Gold in this group**.** The second group was the largest and contained various parameters, including growth attributes (shoot length (SL), fresh and dry weight of shoot and root (SFW, RFW, SDW, and RDW)), photosynthetic attributes (chl *a*, chl *b*, total chl, carotenoids (car)), nutrient contents (calcium and potassium content in shoot and root), and anthocyanins (Anth). All these parameters showed strong positive correlation with the control at 32.5 g BC application level in Desi and weak correlation in Green Gold without Ni application. However, under Ni stress conditions, Desi exhibited weak positive correlation, and Green Gold showed negative correlation without BC application. The application of 32.5 g BC showed positive correlation in Desi and weak positive correlation in Green Gold under Ni stress conditions. These findings indicated that BC application helped improve growth attributes, photosynthetic contents, and ionic contents of spinach plants in both studied cultivars. In the third group, antioxidants, chl*a*/*b*, and flavonoids were clustered. Antioxidants and flavonoids showed strong positive correlation at 32.5 g under Ni toxicity in Desi, while in Green Gold, these attributes showed weak positive correlation with the control. This indicated that BC helped increase antioxidant activity under Ni stress conditions in both cultivars (Fig. 10). The fourth group clustered oxidative stress determinants. These attributes showed strong positive correlation at the 5 mM Ni level in Desi and weak positive correlation in Green Gold. Under 32.5 g BC application, these attributes exhibited a positive correlation in Desi and a weak positive correlation in Green Gold. This showed that the application of BC helped reduce H_2O_2 and MDA content under Ni stress conditions in both cultivars. The sodium contents in the shoot and root of spinach plants were clustered in the fifth group, showing strong positive correlation with the control under Ni stress conditions without BC application (Fig. 10).

Principal component analysis (PCA): Principal component analysis was conducted to assess the impact of biochar (BC) on spinach plants, specifically Desi and Green Gold varieties, under nickel stress conditions. The PCA plot revealed that 64.9% of the variability was explained by PC1, and 24.6% was explained by PC2. Most parameters were plotted closer to cultivar Desi, indicating a strong relationship of growth parameters in this cultivar under Ni stress conditions. MDA, H_2O_2, and Na^+ contents in the shoot and root of spinach plants were shown to be more related to Green Gold. Shoot and root dry weight, chlorophyll contents, and Ca^{2+} and K^+ contents were plotted closer to the 16.25 g/pot BC level, indicating that the application of BC supported plant growth by improving chlorophyll contents, anthocyanin contents, and balancing nutrient uptake (Na^+, K^+). The 32.5 g/pot BC improved root and shoot length, organic osmolytes, antioxidants, and phenolic components while reducing oxidative stress determinants in Desi. This indicated that BC improved Ni tolerance in Desi compared to Green Gold (Fig. 11).

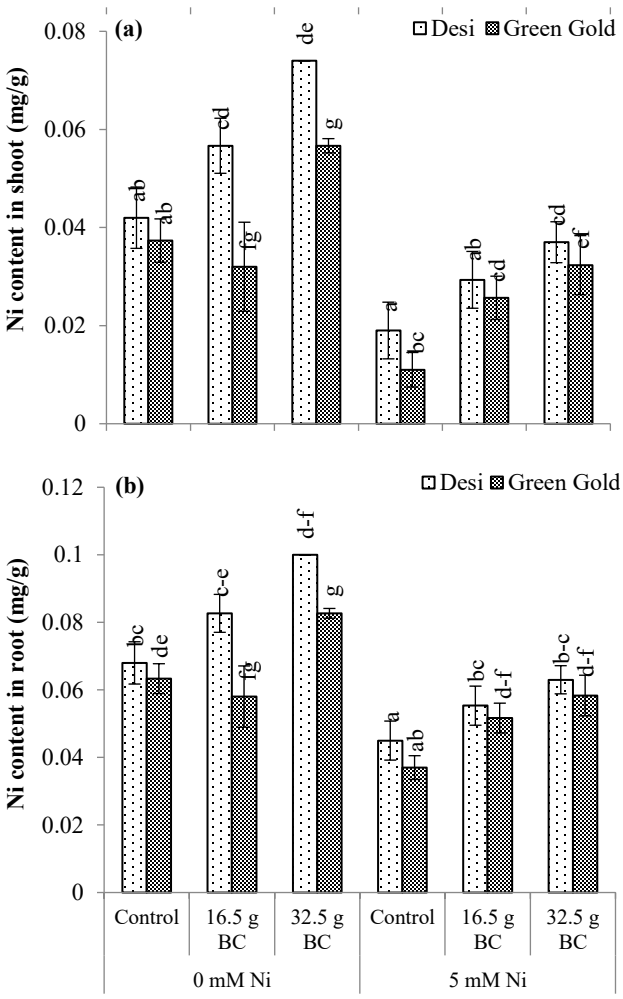

Fig. 9. Effect of different levels of BC on Ni contents in shoot (mg/g) (a) and root (mg/g) of spinach (*Spinacea oleracea* L.) cultivars under Ni stress.

Discussion

Nickel contamination has become widespread in soil and water which has reduced sustainable plant productivity worldwide (Panagopoulos *et al*., 2015). Among various remedial methods, the use of BC is a novel approach for addressing Ni contamination in soil. Biochar has the potential to immobilize Ni through ion exchange, physical adsorption, and surface area attraction (El-Naggar *et al*., 2021). It has exhibited high efficiency in immobilizing Ni in both soil (Munir *et al*., 2020) and aquatic systems.

In this study, a high reduction in shoot and root length of spinach plants was found under Ni stress conditions. This finding is consistent with previous studies. For instance, Parlak (2016) reported a significant reduction in root and shoot length in wheat as Ni concentration increased. Comparable findings have been documented in rice, Indian mustard, and pea plants, all of which showed reduced plant length in response to increased Ni stress (Singh *et al*., 2004; Kaur *et al*., 2019). This reduction in plant length can be attributed to the unavailability of

essential nutrients under Ni stress conditions, which, in turn, reduces nutrient uptake and overall plant length (Shahbaz *et al*., 2019). Additionally, shoot and root fresh and dry weights and leaf area of spinach were also reduced under Ni stress conditions, aligning with previous studies on spinach under Ni toxicity (Amjad *et al*., 2020a; Helaoui *et al*., 2020; Altaf *et al*., 2021; Aqeel *et al*., 2021). Higher uptake or tissue accumulation of heavy metals can generally reduce mitotic cell division in root meristematic tissues. In particular, Ni can become a part of root cells after crossing the endodermal barrier and becomes deposited in the pericycle, thereby reducing plant fresh and dry biomass (Yang *et al*., 2017; Pipalde *et al*., 2018: Shahzad *et al*., 2023). Nickel stress can also block proton pumps, which reduces the rate of cell division and cell elongation, ultimately resulting in reduced leaf area (Eltahawy *et al*., 2022). Nevertheless, the utilization of BC mitigated the adverse impacts of Ni stress by enhancing shoot and root length, as well as improving fresh and dry weights and leaf area under Ni stress conditions. This improvement is attributed to the fact that BC increases the availability of phosphorus (P), nitrogen (N), and exchangeable potassium (K) in the soil, which, in turn, enhances plant growth attributes and cell division, leading to an increase in leaf area per plant (Tahir *et al*., 2018).

The imposition of Ni stress led to a notable decrease in the concentration of photosynthetic pigments in spinach plants. This phenomenon can be ascribed to the point that Ni stress hinders the absorption of essential elements such as magnesium (Mg) and iron (Fe), which are critical for various stages of chl synthesis. This interference results in a decrease in chlorophyll content in stressed plants (Boostani *et al*., 2019). Moreover, Ni stress impairs membrane permeability, reduces carbon dioxide (CO_2) fixation, and affects the electron transport chain, which collectively lead to a reduced rate of photosynthesis (Abedini & Mohammadian, 2018).

In this study, the utilization of biochar resulted in an enhancement of chl *a*, chl *b*, total chl, and carotenoid concentrations. Comparable findings have been documented by He *et al*., (2020) who found that BC application significantly increased the photosynthetic rate and chl concentration by 27.1% and 16.1%, respectively. The improvement in chl content in leaves can be attributed to BC's ability to increase the availability of nitrogen (N) in the soil thereby improving N concentration in plant leaves (Bai *et al*., 2015).

In the presence of Ni stress, there was an elevation in the activity of enzymatic antioxidants. Previous studies have reported an elevation in catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) activity under Ni toxicity in various crops, including maize, rice, and wheat (Amjad *et al*., 2020a; Amjad *et al*., 2020b). Exposure to heavy metal stress induces the production of reactive oxygen species (ROS) in plants, leading the plant to enhance the activity of antioxidant enzymes within cytosolic cells to neutralize the generated ROS and fortify its defense mechanism (Shahbaz *et al*., 2019; Najm-ul-seher *et al*., 2021). Biochar supplementation further increased the production of antioxidants in spinach leaves. This can be explained by BC's ability to increase antioxidant production, effectively scavenging ROS (Mehmood *et al*., 2020; Sofy *et al*., 2022) and enhancing oxidative stress tolerance in plants (Hasanuzzaman *et al*., 2021).

Under Ni stress conditions, flavonoid content increased. Flavonoids function as a protective mechanism and increase the activities of enzymes that scavenge free radicals produced during Ni toxicity (Kumar *et al*., 2020). Moreover, flavonoids can chelate metal compounds and reduce Ni toxicity (Jahan *et al*., 2020). Strejckova *et al*., (2019) also reported an increase in flavonoids under Ni stress conditions. However, anthocyanin content was reduced under Ni stress. Anthocyanins have powerful antioxidant properties, as they scavenge reactive oxygen species and chelate metal ions under heavy metal stress (Dai *et al*., 2012). The application of BC in the soil significantly enhanced the availability of flavonoids in spinach plants. Similar findings have been reported in spinach, where BC application improved flavonoid activity by reducing the adverse effects of free radicals and ROS (Prasetya *et al*., 2021). In the current study, BC (at 32.5 g/pot) improved anthocyanin production in spinach, consistent with previous investigations. Quartacci *et al*., (2017) found that BC restored and increased anthocyanin contents under heavy metal stress conditions.

The study revealed that Ni stress elevated the glycine betaine content in spinach plants. This aligns with findings from earlier research. Ali *et al*., (2020) found that glycine betaine is a crucial osmoregulator that becomes enhanced under Ni stress. Glycine betaine activates several antioxidants that scavenge the reactive oxygen species produced under heavy metal stress, thereby improving plant growth, the photosynthetic process, and yield attributes (Ali *et al*., 2020).

In this study, Ni stress increased the total soluble protein content in spinach plants, consistent with earlier studies. Kumar (2020) found that the total soluble protein content increased with Ni treatment. Yilmaz & Parlak (2011) and Helaoui *et al*., (2020) reported that the protein content exhibited an increase during Ni toxicity. The total soluble sugars content was also increased under Ni stress, which is consistent with earlier research. Kumar *et al*., (2022) found that soluble sugar contents increased by 72.1% under Ni stress in sweet potatoes. Application of BC improved glycine betaine, total soluble protein, and total soluble sugar content. Lalay *et al*., (2022) found that the application of BC improved glycine betaine content, which protects the plants against ROS. This improvement is explained by BC's ability to increase soil water-holding capacity, which enhances water movement to the leaves through the xylem, thus improving the transport of nutrients that trigger plant metabolism, including the production of starch, protein, sugar, and amino acids (Turan, 2020; Khan *et al*., 2020).

Ni stress increased H_2O_2 and MDA content in spinach plants. Previous studies supported these findings, as an increase in MDA and H_2O_2 content under Ni stress has been reported in various crops such as *Vicia sativa*, tomatoes, and rice plants (Ivanishchev & Abramova, 2015; Altaf *et al*., 2021). However, the application of BC effectively sequestered reactive oxygen species and induced Ni tolerance in spinach plants. This can be attributed to the enhanced activity of antioxidant enzymes in the presence of BC, which detoxify the generated ROS and improve plant stress tolerance (Mehmood *et al*., 2020; Naeem *et al*., 2020).

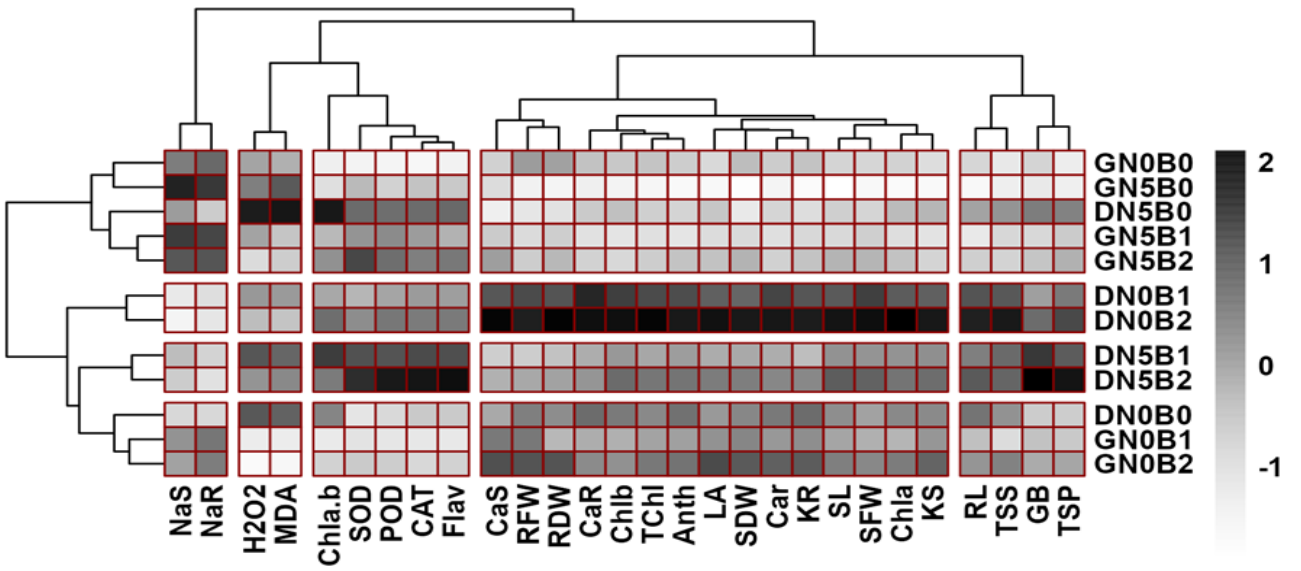

Fig. 10. The heatmap showing various attributes of spinach cultivars affected by BC under Ni stress conditions. The legends on right showed cultivar, Ni level and BC level. The black colour indicates strong positive correlation, light grey shows negative correlation. The dendrogram on top and left shows parameters grouped in similar clusters.

Abbreviations: Green gold (G), Desi (D), Nickel (N), Biochar (B), Shoot length (SL), Length (RL), Shoot fresh weight (SFW), Root fresh weight (RFW), Shoot dry weight (SDW), Root dry weight (RDW), Leaf area (LA), Chlorophyll (Chl), Superoxide dismutase (SOD), Peroxidase (POD), Catalase (CAT), Glycine betains (GB), Total soluble proteins (TSP), Total soluble sugars (TSS), Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA), Sodium (Na), Potassium (K), Calcium (Ca), S (Shoot), R (Root), Flavonoids (Flav); Anthocyanins

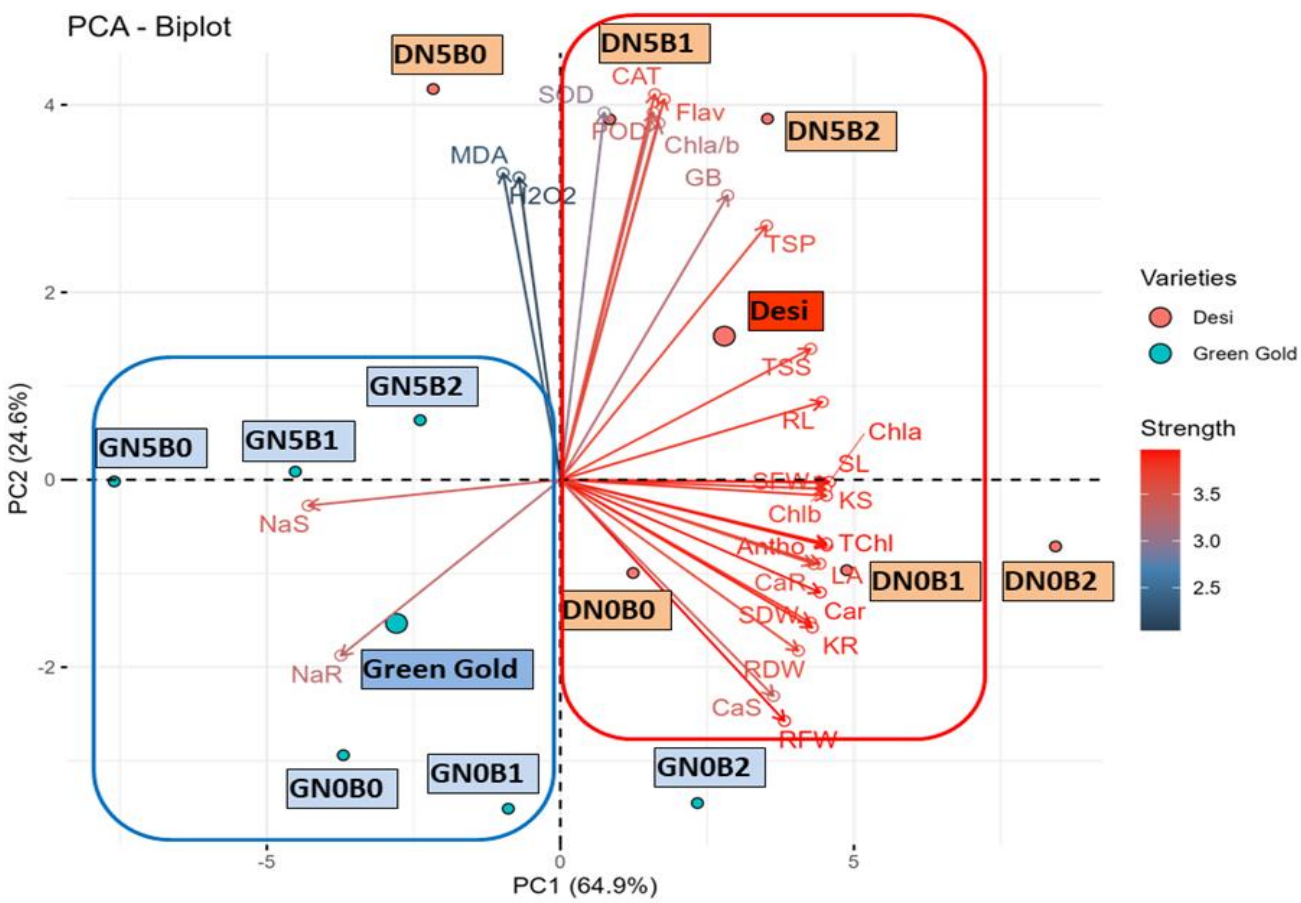

Fig. 11. Principal component analysis (PCA) plot for different vegetative, physiological and biochemical attributes of the two spinach cultivars. Ellipses are drawn to categorize various parameters with spinach cultivars, where most of vegetative, physiological and biochemical attributes are shown towards 32.5g/pot in Desi.

Abbreviations: Shoot length (SL), Root length (RL), Shoot fresh weight (SFW), Root fresh weight (RFW), Shoot dry weight (SDW), Root dry weight (RDW), Leaf area (LA), Chlorophyll (Chl), Superoxide dismutase (SOD), Peroxidase (POD), Catalase (CAT), Glycine betains (GB), Total soluble proteins (TSP), Total soluble sugars (TSS), Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA), Sodium (Na), Potassium (K), Calcium (Ca), S (Shoot), R (Root), Flavonoids (Flav); Anthocyanins

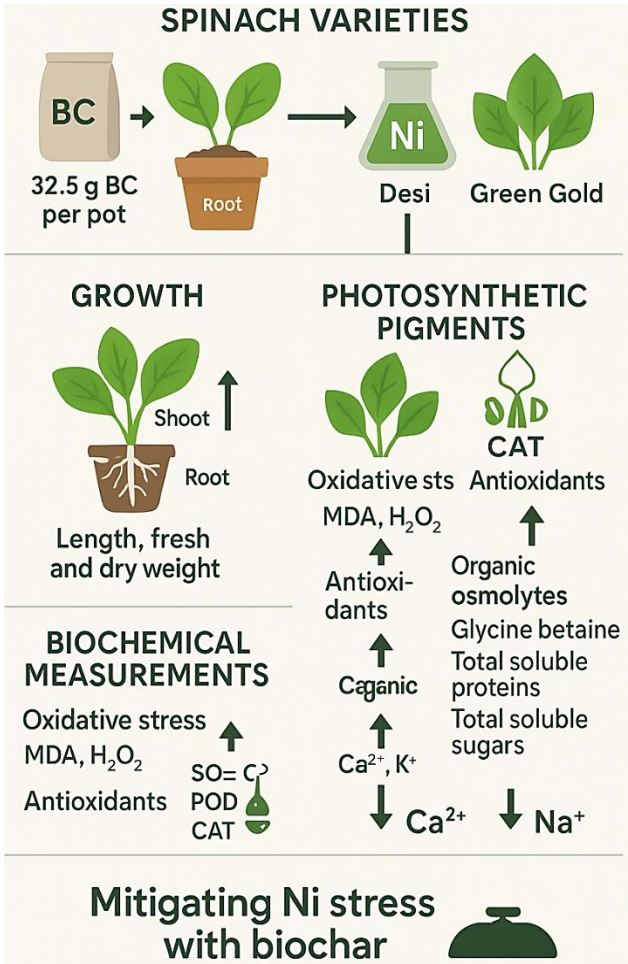

Fig. 12. Proposed mechanism of growth enhancement in nickel stressed spinach cultivars under biochar application.

The results showed that Ni stress increased Na^+ content while reducing K^+ and Ca^{2+} content in spinach plants. Earlier studies have supported these findings. It has been explained that the presence of BC in the soil promotes the availability and adsorption of nutrients, while also reducing the leaching of macronutrients from the root zone. This improved nutrient levels in spinach plants (Jabborova *et al*., 2021). Ain *et al*., (2016) found that 20 mg/kg Ni improved K^+ content while reducing Na^+ content, but 40 mg/kg concentration, there was a reduction in K^+ and an increase in Na^+ content in wheat. Dotaniya *et al*., (2019) also reported significant decreases in macronutrient concentrations, such as phosphorus (P), K, and sulfur (S), in spinach plant sections under heavy metal stress.

A heatmap was used to cluster the effect of BC in reducing Ni toxicity. It revealed that BC at the 32.5 g/pot level exhibited positive correlations with various growth, photosynthetic, and ionic contents, while lower levels showed a weak positive correlation with the control. This was supported by previous studies (Simiele *et al*., 2022). The results were further confirmed by a PCA plot. In the PCA plot, all antioxidants, secondary metabolites, oxidative stress determinants, phenolic components, and some growth parameters were plotted with 32.5 g BC level during Ni toxicity in cultivar Desi. Some growth parameters, photosynthetic pigments, and ionic contents were drawn at 16.25 g BC without Ni stress conditions. This indicates that BC regulates early plant growth at 16.25 g, while under Ni stress conditions, the 32.5 g level of BC is effective. Earlier studies have supported these findings (Martínez-Gómez *et al*., 2022; Agarwal *et al*., 2022).

Conclusion and recommendations: Ni-induced stress in spinach plants resulted in reduced growth, photosynthetic pigments, antioxidants, osmolytes, and ionic contents. However, the application of medium levels of biochar (32.5 g/pot BC) significantly improved these metabolic parameters. Biochar's ability to immobilize Ni, scavenge free radicals, and reduce oxidative stress determinants proved effective in enhancing Ni tolerance in spinach plants. Furthermore, it lowered the concentration of Na^+ ions, indicating its role in bolstering the plant's immunity against Ni stress (Fig. 12). These findings offer practical implications for cultivating spinach in Ni-contaminated soil, providing a strategy to improve plant health and productivity in challenging environments.

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