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Minimization of cadmium toxicity and improvement in growth and biochemical attributes of spinach by using acidified biochar

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Cadmium stress significantly affects plant growth by disrupting essential physiological and biochemical functions. It slows nutrient intake, causing slowed development and decreased biomass. Cd also produces reactive oxygen species, causing oxidative stress, which harms cell components like lipids, proteins, and DNA. This lowers chlorophyll levels, making photosynthesis difficult and stunting development. Cd's toxicity affects hormone balance, enzyme activity, and cell structural integrity, leading to poor plant growth and decreased agricultural output. Acidified biochar (BC) can effectively overcome this problem. Biochar features high cation exchange capacity (CEC) and oxygen-containing functional groups may aid in the immobilization of heavy metals in soil via surface complexation and precipitation. Cd immobilization can be increased by treating biochar with acid, which exposes additional adsorption sites. It can significantly enhance plant growth by improving soil structure, encouraging water retention, and improving microbial activity as a slow-release nutrient. This study investigates the effects of combining BC as amendments to spinach, both with Cd and without stress. Four treatments (control, 0.45BC, 0.90BC, and 1.20BC) were applied using a completely randomized design in four replications. Results showed that 1.20BC treatment showed a significant increase in shoot fresh weight (86.21%), root fresh weight (96.20%), shoot dry weight (223.24%), root dry weight (42.38%), total soluble sugar (16.05%), total soluble protein (54.70%), compared to the 0BC under 20 mg Cd/kg soil contamination. Additionally, there were notable improvements in chlorophyll a (121.26%), chlorophyll b (10.91%), and total chlorophyll (32.12%) above the control in Cd stress, also showing the potential of 1.20BC. A significant increase in N, P, and K concentrations of shoot and root of spinach was also noted, which validated the effectiveness of 1.20BC over 0BC under cadmium stress. It is concluded that applying 1.20BC can potentially alleviate the Cd-induced stress in spinach.

Keywords Chlorophyll contents, Activated carbon, Heavy metal, Growth attributes, Nutrients, Spinach

Cadmium (Cd) is a very poisonous element that harms humans, animals, and plants. Cd is transported into the environment (e.g., soils) through both natural and anthropogenic processes, and it is typically present at low levels[1]. Cd is generally scarce in the earth's crust as a pure metal, and it is mostly found in ferrous and nonferrous metal ores. During mining and milling processes, notably the extraction and concentration of ores and disposal of tailings, considerable amounts of Cd are released into the surface environment[2].

Due to its solubility in water, fluidity, and toxicity, Cd can easily be absorbed by plant roots, altering plant structural and functional features, inhibiting seed germination, and root elongation[3]. Cadmium stress significantly affects plant growth by disrupting essential physiological and biochemical functions[4,5]. It slows nutrient intake, causing slowed development and decreased biomass. Cd also produces reactive oxygen species, causing oxidative stress, which harms cell components like lipids, proteins, and DNA[6,7]. Cd inhibits Fe uptake and translocation to important organelles, including the chloroplast. The chloroplast synthesizes the iron-containing protein

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ferredoxin, which regulates photosystem I[8]. This lowers chlorophyll levels, making photosynthesis difficult and stunting development. Cd's toxicity affects hormone balance, enzyme activity, and cell structural integrity, leading to poor plant growth and decreased agricultural output[9,10]. First of all, cadmium promotes the generation of reactive oxygen species (ROS), including hydrogen peroxide and superoxide radicals, which can harm cellular components[11]. Second, cadmium interferes with the function of enzymes that scavenge and neutralize reactive oxygen species (ROS), responsible for the plant's antioxidant defense mechanism[12]. Heavy metals like zinc (Zn) and copper (Cu) play important roles in enzymes and biochemical processes, whereas lead (Pb) and cadmium (Cd) are not necessary for plant metabolism[13,14].Scientists have recently been interested in activated carbon biochar as a potential solution to this problem[15–17].Biochar is a carbon-rich material produced through the pyrolysis of organic biomass under limited oxygen conditions[18]. It has gained attention for its potential in improving soil health, enhancing crop productivity, and sequestering carbon to mitigate climate change. Biochar improves soil structure, water retention, and nutrient availability by increasing the cation exchange capacity, making it particularly beneficial for degraded or nutrient-poor soils[19,20]. It also aids in reducing the leaching of nutrients and heavy metals, helping to minimize environmental contamination[21,22]. Additionally, biochar serves as a habitat for beneficial microorganisms, promoting soil biodiversity and enhancing plant resilience against stress factors such as drought and heavy metal toxicity[23]. Biochar features such as high cation exchange capacity (CEC) and oxygen-containing functional groups may aid in the immobilization of heavy metals in soil via surface complexation and precipitation. Cd immobilization can be increased by treating biochar with acid, which exposes additional adsorption sites[24,25]. Based on available research, *Spinacia oleracea*is a commonly grown plant due to its high percentage of moisture, 91% protein, 0.4–0.6% fat content, and several necessary amino acids[26]. Moreover, this vegetable is highly sought after in the market, rendering it one of the most sought-after vegetables. In recent years, crop yield has been markedly decreased due to multiple environmental stresses, i.e., drought, heat, heavy metal toxicity, cold, flooding, and soil salinity[27]. Cadmium is of particular significance, as it can reduce global productivity[28].

Different substances, both synthetic and natural, are commonly employed to immobilize metals in polluted locations. Among these, biochar is regarded as an efficient soil amendment that is used to improve food safety by decreasing the bioavailability of heavy metals due to its ability to immobilize heavy metals. Pakistan is well known for its cotton production and textile exports. Cotton stalks are readily accessible at a low cost following the harvest.That's why in present study cotton stalks acidified biochar was used to find (a) the effects of acidified biochar (BC) for alleviating the morphological, physiological, and biochemical characteristics of spinach grown under cadmium stress. (b) to identify the optimal application proportion of acidified biochar to improve the growth of spinach under cadmium. (c) to check the immobilization of cadmium in soil for plant uptake under different concentrations of acidified biochar.

Material and methodology

Experimental site and design

A pot experiment was conducted using arid soil (USDA) from a depth of 0–6 inches from the top surface of the new departmental building at The Islamia University of Bahawalpur (IUB), Rahim Yar Khan campus (71°, 28.3 N; latitude: 70.43° E), Punjab, Pakistan. The experiment was conducted using a completely randomized design (CRD).

Soil characterization and pot preparation

The soil sample was sieved through a 5 mm sieve and artificially contaminated by using cadmium nitrate (CAS Number:10099-74-8; Batch Number: BCCJ5381; 3050 Spruce Street, Saint Louis, MO 63103, USA) salt to maintain 20 mg Cd per kg soil. Before contamination with cadmium, soil was analyzed for physio and biochemical parameters. The characteristics of the soil are provided in Table 1. The soil was filled into clay pots (12') at a rate of 5 kg pot^{-1} in three replicates.

Soil	Values	Biochar	Values	Irrigation	Values
pH	8.50	pH	6.45	pH	6.95
AK (µg/g)	146	EC*e* (dS/m)	3.05	EC (µS/cm)	805
SOC (%)	0.45	TP (%)	2.25	Bicarbonates (meq./L)	6.12
EC*e* (dS/m)	5.08	Volatile Matter (%)	35	Ca + Mg (meq./L)	5.19
EP (µg/g)	4.02	Ash Content (%)	30	Chloride (meq./L)	0.20
Silt (%)	20	TN (%)	0.15	Carbonates (meq./L)	0.00
Texture	Sandy Clay Loam	CEC (meq./100 g)	425	Sodium (mg/L)	663
Sand (%)	50	TK (%)	0.47	EP = Extractable Phosphorus EC = Electrical Conductivity AK = Available Potassium TN = Total Nitrogen CEC = Cation Exchange Capacity	
Clay (%)	30	Surface area (m^2/g)	340		
Fixed carbon (%)	35	TN (%)	0.022		

Table 1. Pre-experimental soil, biochar, and irrigation characteristics.

Treatment plan

As per experimental treatments, acidified biochar was added to the soil at four concentrations (0, 0.45, 0.90, and 1.20%; w/w). This acidified biochar was pre-mixed with essential nutrients per experimental treatments before sowing the seeds.

Acidified biochar

The pyrolysis of crumpled cotton sticks was carried out in a pyrolyzer with no air supply. For two hours, the pyrolysis process occurred at a temperature of 723 K. The resultant char was then acidified by soaking it in H_2SO_4 for 12 h. For 48 h, the char was oven-dried at 65 °C to eliminate any residual moisture. The char was allowed to cool before being roughly chopped into 2 mm pieces and kept for later use. The char that was left over from the pyrolysis process was preserved for later use after being cooled and thoroughly chopped to a particle size of 2 mm. Several techniques were used to examine the composition of the biochar. Gravimetric analysis, as described by McLaughlin et al.[29], was used to identify the various components of biochar. The pH[30]and electrical conductivity (EC)[31] of the biochar were measured using aliquots of a 1:10 combination with distilled water.

Additionally, elemental analysis was done to find the biochar's total carbon content. The biochar model was dissolved and then distilled using Schouwvenberg's Kjeldahl distillation technique[32] to determine the concentration of nitrogen (N). The HNO_3-$HClO_4$technique was used to calculate the amounts of potassium (K+) and phosphorus (P) in the biochar[33]. The potassium content was ascertained using a flame photometer, and the phosphorus assessment was conducted using the Ammonium Vanadate-Ammonium Molybdate yellow color approach using a spectrophotometer[34]. The physicochemical characteristics of the biochar are provided in Table 1.

Seed collection, screening, and irrigation

The seeds of Savoy Spinach (*Spinacia oleracea*) were purchased from the local Khanpur market. Screening of seeds was done manually. Damaged and broken seeds were removed on visual observation. To maintain the soil's capacity to hold water, the weight loss in the pot was regularly measured, and the soil water content was maintained at 60%.

Sowing

On June 21, 2022, seeds were planted using a conventional planting method at a depth of 0.5 inches. On July 2, 2022, following a 12-day germination period, the seedlings were trimmed to three plants per pot. The pots were relocated to a semi-shaded area due to extreme weather conditions.

Harvesting and data collection

The spinach plants were harvested on 17-08-2022 after reaching maturity (58 days). Using an electronic scale, the fresh biomass of spinach roots and shoots was measured. The plant material was dried at 65 °C for 48 h to acquire the dry biomass of the sample.

Cd analysis

The plants were first allowed to air dry before being dried in an oven at 65 °C for one day. Subsequently, 10 ml of a di-acid combination (HNO_3:$HClO_4$) was added to 0.5 g of spinach sample that had been collected for digestion in a digestion flask[33]. The samples were placed on a hot plate with a temperature setting of 50 °C and heated gradually till 280 °C after being left overnight to allow the plant tissues to soften. After being taken off the electric hot plate, the samples were allowed to cool until thick white vapors began to exhaust. With the use of an atomic absorption spectrophotometer, the amount of Cd present in plant roots and shoots was assessed[35].

$$Metal\ uptake\ at\ root\ surface = \frac{Total\ plant\ metal\ content\ (mg)}{Total\ root\ dry\ weight\ (g)}$$

$$Metal\ translocation\ from\ root\ to\ shoot = \frac{Shoot\ metal\ content}{Root\ metal\ conent}$$

Chlorophyll contents, carotenoids, anthocyanin and lycopene

Arnon's method[36]were utilized to quantify the chlorophyll content in freshly harvested leaves. During the extraction process, an acetone solution of 80% was used. Absorbance readings were taken at various frequencies: 663 645, 480, 537, and 503 nm for the calculation of chlorophyll contents[36], carotenoids[37], anthocyanin[38]and lycopene[39].

Ascorbic acid

For the determination of ascorbate (AsA) content, an equivalent volume of 10% trichloroacetic acid was introduced to the homogenate, which was then subjected to centrifugation for 10 min at 12,000×g. The resulting supernatant was employed for the spectrophotometric determination of ASA at 525 nm, as described by[40].

$$Ascorbic\ acid\ \ (mg/g\ F.W) = [((Eo - Es - Et)\,V/100 \times\ W) \times\ 100]$$

Total soluble protein

The Biuret method[41] was used to measure total soluble protein levels. After the necessary chemical components were added and the tubes were thoroughly shaken, they were incubated for 25 min at room temperature. The

optical density was recorded utilizing a UV-spectrophotometer at a frequency of 545 nm. Bovine serum albumin was utilized to create a protein standard curve to measure the total protein content.

$$\textbf{Total}\ soluble\ protein\ \left(mg\ g^{-1}\ F.W\right) = Sample\ reading \times\ Sample\ volume \times \frac{Dilution\ factor}{W} \times\ 1000$$

Total amino acids

In a test tube, 1 mL of protein supernatant, 1 mL of 25% ninhydrin, and 1 mL of 10% pyridine were mixed. After that, the tubes were heated to between 80 and 90 degrees Celsius in a water bath for around half an hour. Following the completion of the reaction, the test tubes were diluted to a volume of 50 mL and refrigerated in an ice bath. This mixture's absorbance was measured at 570 nm, and[42] equations were used to calculate the total amino acid content.

$$Total\ Amino\ acids = sample\ reading \times\ sample\ volume \times\ dilution\ factor/W \times\ 100$$

Malondialdehyde (MDA)

The amount of malondialdehyde (MDA) in plant leaf tissue could be measured to evaluate the amount of cellular damage brought on by exposure to reactive oxygen species (ROS) as a result of Cd stress[43]. One gram of leaf tissue was homogenized in three milliliters of 0.1% (w/v) trichloroacetic acid (TCA) solution to conduct this assay. After centrifuging at 20,000 x g for 15 min, 0.5 mL of the supernatant was mixed with 0.5% (w/v) of thiobarbituric acid (TBA) dissolved in 20% TCA. The mixture was then heated in a water bath for 50 min at a temperature of around 95 °C. After being quickly chilled in an ice bath, the liquid was centrifuged at 10,000 x g for 10 min. Next, using a spectrophotometer, absorbance was measured at 532 and 600 nm, and the MDA concentration was computed using the following formula.

$$MDA\ level\ (nmol) = \frac{\Delta\ (A532nm - A600nm)}{1.56 \times\ 10.5}$$

MDA was determined by using an absorption coefficient of 156 $mmol^{-1}$ cm^{-1}

Soluble sugars

A sample of 0.1 g of leaves was extracted with 80% (C_2H_5OH) ethanol and incubated at 60 °C for 6 h. 1 ml of the extract was combined with 6 ml of enthrone reagent and heated in a boiling water bath for 10 min. Afterward, the test tubes were placed in an ice bath for 10 min and then incubated at 250 degrees Celsius for 20 min. The absorbance of soluble sugar was measured using a spectrophotometer at 625 nm. A standard curve was generated using this technique, and the soluble sugar content was calculated using this curve[44,45].

Analysis of total nitrogen (N) in spinach

The nitrogen content of spinach plants was calculated according to the method outlined by Black (1965) utilizing Kjeldhal's distillation apparatus. Following the digestion of the plant shoots and roots, Barton's reagent was added to 1 ml of the solution, and the final volume was reduced to 10 ml using deionized water. The spectrophotometer was calibrated using standards of (2, 4, 6, 8, 10, and 12 mg/L) at 470 nm wavelength. Subsequent readings of the samples were taken, and a standard curve was produced for reading correction[46]. The potassium content of digested spinach roots and shoots was calculated using a flame photometer[47].

Statistics analysis

Standard statistical techniques were used for the statistical analysis of collected data. Origin Pro software was used for statistical analysis and graph-making[48]. Fisher LSD was applied for the comparison of treatments at $p \leq 0.05$.

Results

Growth attributes

Under control conditions, adding 0.45BC, 0.90BC, and 1.20BC treatment showed an increase in shoot fresh weight (12.29%, 20.49%, and 23.42%), root fresh weight (5.35%, 11.55%, and 17.21%), shoot dry weight (4.82%, 10.24%, and 15.86%), and root dry weight (3.55%, 12.20%, and 21.51%) in comparison to the 0BC. Under 20 Cd stress, rise in shoot fresh weight (6.96%, 78.79%, and 86.21%), root fresh weight (64.66%, 76.09%, and 96.20%), shoot dry weight (119.02%, 191.55%, and 223.24%), and root dry weight (24.17%, 37.75%, and 42.38%) were recorded above 0BC with 0.45BC, 0.90BC, and 1.20BC (Fig. 1A, B, C, and D).

Ascorbic acid, total amino acid, total soluble sugar, and total soluble protein

In no stress (Control), applying 0.45BC, 0.90BC, and 1.20BC treatments caused a decrease in ascorbic acid (−5.33%, −16.30%, and − 40.44%), and an increase in total amino acid (9.30%, 36.04%, and 81.39%), total soluble sugar (7.47%, 27.32% and 36.98%), and total soluble protein (7.32%, 15.96%, and 22.21%) in comparison to the 0BC. Under 20Cd stress, a significant decrease in ascorbic acid (−13.63%, −23.52%, and − 27.47%), and an increase in total amino acid (12.25%, 40.82%, and 59.19%), total soluble sugar (5.42%, 11.50%, and 16.05%), and total soluble protein (20.63%, 41.11%, and 54.70%) were recorded above 0BC (Fig. 2A, B, C, and D).

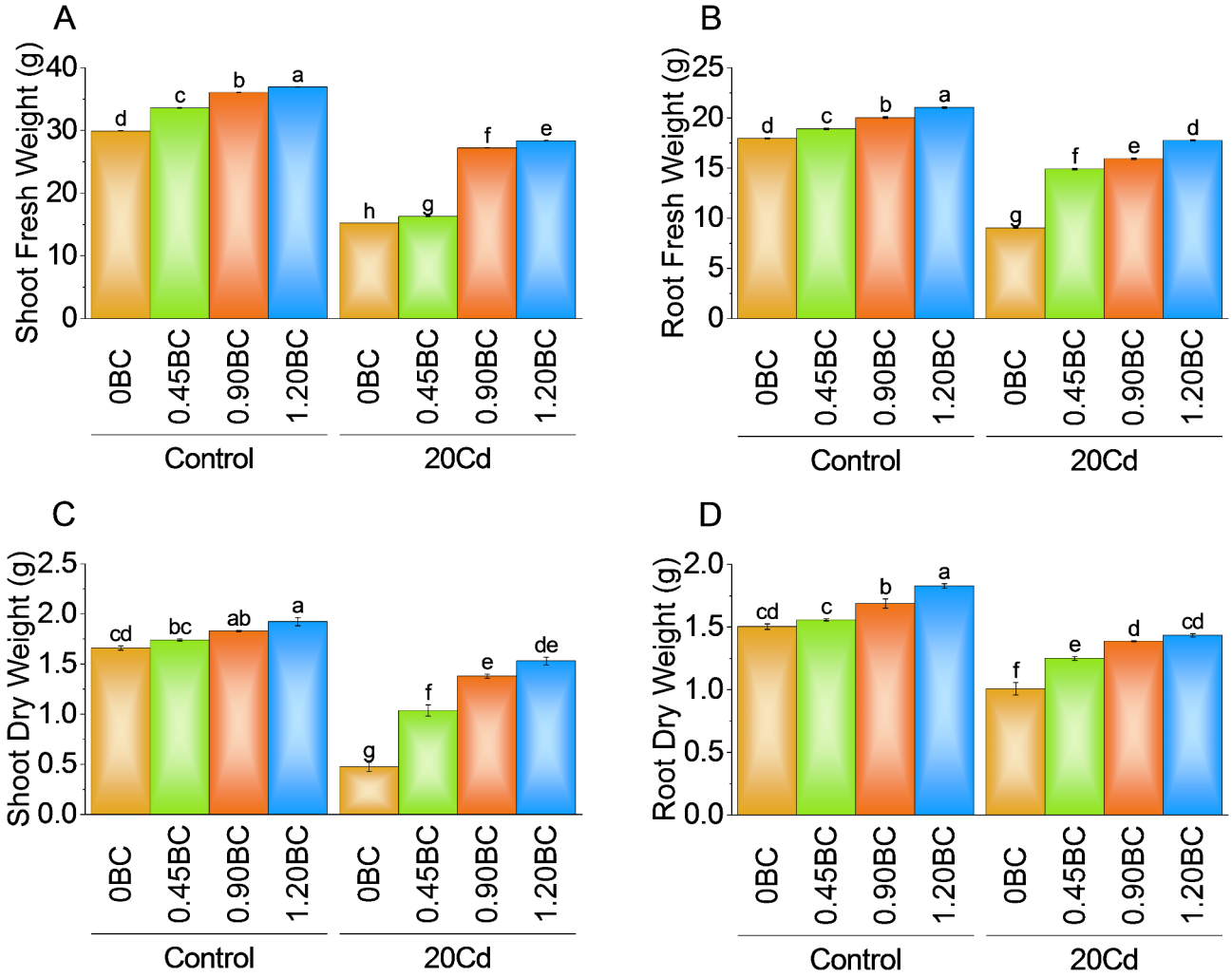

Fig. 1. Impact of different application rates of biochar on *Spinania oleracea* shoot fresh weight (**A**), root fresh weight (**B**), shoot dry weight (**B**), and root dry weight (**D**) cultivated in control and 20Cd stress. The bars represent the means of four replicates with standard error. The Fisher LSD test revealed significant changes at $p < 0.05$, shown by the different letters on the bars.

Chlorophyll contents and carotenoids

A significant 3.78%, 6.32%, and 8.60% increase in chlorophyll a, 3.77%, 17.97%, and 48.20% rise in chlorophyll b, 22.24%, 25.74%, and 28.21% in total chlorophyll, and 2.13%, 27.20%, and 38.05% in carotenoids were observed with 0.45BC, 0.90BC, and 1.20BC treatments under control than the 0BC. Applying 0.45BC, 0.90BC, and 1.20BC treatments exhibit a 39.40%, 98.58%, and 121.26% increase in chlorophyll a, 6.08%, 8.28%, and 10.91% in chlorophyll b, 17.16%, 24.55%, and 32.12% in total chlorophyll, and 2.10%, 5.03%, and 7.63% in carotenoids under 20Cd stress compared to the 0BC (Fig. 3A, B, C, and D).

MDA and anthocyanin

Applying 0.45BC, 0.90BC, and 1.20BC led to −19.42%, −40.50%, and − 67.36% decrease in MDA under control (no Stress) and caused a 9.26%, 13.26%, and 26.50% increase in anthocyanin above 0BC. The 0.45BC, 0.90BC, and 1.20BC treatments under 20Cd stress showed a −13.68%, −25.75%, and − 36.62% decrease in MDA and 16.31%, 19.68%, and 30.05% increase in anthocyanin (Fig. 4A and B).

Shoot N, P K, and shoot Cd uptake

Under control conditions, adding 0.45BC, 0.90BC, and 1.20BC treatment showed an increase in shoot N (7.11%, 18.78%, and 23.86%), shoot P (11.77%, 17.65%, and 38.24%), shoot K (21.34%, 27.44%, and 44.51%), and caused a decrease in shoot Cd uptake (17.04%, 35.69%, and 59.49%) in comparison to the 0BC. Under 20 Cd stress, a significant rise in shoot N (23.27%, 38.61%, and 55.45%), shoot P (24.99%, 34.99%, and 49.99%), shoot K (50.00%, 78.79%, and 100.00%), and caused a decrease in shoot Cd uptake (16.98%, 19.08%, and 32.70%) was recorded above 0BC with 0.45BC, 0.90BC, and 1.20BC (Fig. 5A, B, C, and D).

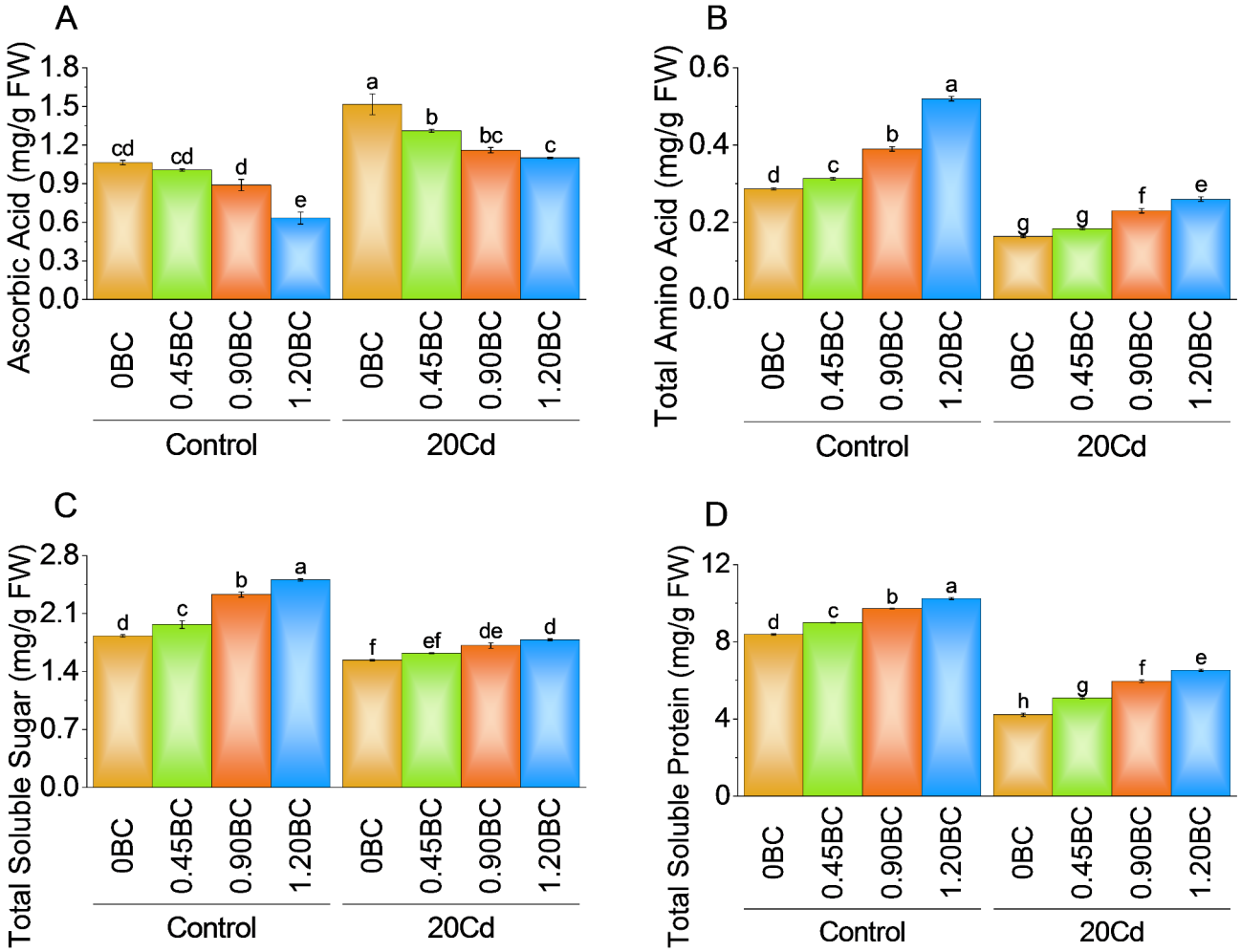

Fig. 2. Impact of different application rates of biochar on *Spinania oleracea* ascorbic acid (**A**), total amino acid (**B**), total soluble sugar (**C**), and total soluble protein (**D**) cultivated in control and 20Cd stress. The bars represent the means of four replicates with standard error. The Fisher LSD test revealed significant changes at $p < 0.05$, shown by the different letters on the bars.

Root N, P K, and root cd uptake

Under control conditions, adding 0.45BC, 0.90BC, and 1.20BC treatment showed an increase in root N (3.56%, 6.83%, and 12.46%), root P (14.71%, 41.18%, and 205.89%), root K (10.09%, 13.76%, and 40.37%), and caused a decrease in root Cd uptake (11.57%, 29.89%, and 29.89%) in comparison to the 0BC. Under 20 Cd stress, a significant rise in root N (14.50%, 16.73%, and 21.93%), root P (4.76%, 19.04%, and 71.43%), root K (7.61%, 15.22%, and 19.57%), and caused a decrease in root Cd uptake (6.43%, 22.47%, and 33.33%) was recorded above 0BC with 0.45BC, 0.90BC, and 1.20BC (Fig. 6A, B, C, and D).

Pearson correlation analysis

Notably, Cd treatment exhibits a strong negative correlation with shoot fresh weight ($r = -0.7967$, $p < 0.001$), root fresh weight ($r = -0.7199$, $p < 0.001$), shoot dry weight ($r = -0.75195$, $p < 0.001$), and root dry weight ($r = -0.77778$, $p < 0.001$), indicating a detrimental effect on plant growth. Conversely, Biochar shows positive correlations with these attributes, with coefficients ranging from 0.53222 to 0.59477 ($p < 0.001$), suggesting a beneficial impact on plant development. Furthermore, biochemical parameters such as ascorbic acid and MDA exhibit negative correlations with Cd treatment. Ascorbic acid shows a strong negative correlation ($r = -0.73571$, $p < 0.001$), while MDA exhibits a very strong negative correlation ($r = -0.87653$, $p < 0.001$), indicating a potential reduction in oxidative stress under Biochar treatment. The study also reveals positive correlations between Biochar and attributes such as total soluble sugar, protein, chlorophyll content, carotenoids, and anthocyanins. For example, total soluble sugar shows a strong positive correlation with Biochar ($r = 0.76836$, $p < 0.001$), indicating increased sugar content in plants treated with Biochar. Similarly, chlorophyll a ($r = 0.73981$, $p < 0.001$) and chlorophyll b ($r = 0.65738$, $p < 0.001$) show moderate positive correlations, suggesting enhanced photosynthetic activity. Additionally, strong positive correlations are observed between Cd concentration and shoot and root cadmium levels ($r = 0.95018$ and $r = 0.85373$, respectively, $p < 0.001$), indicating the accumulation of Cd in plant tissues. These correlations highlight the potential phytotoxicity of Cd and its uptake by plants (Fig. 7).

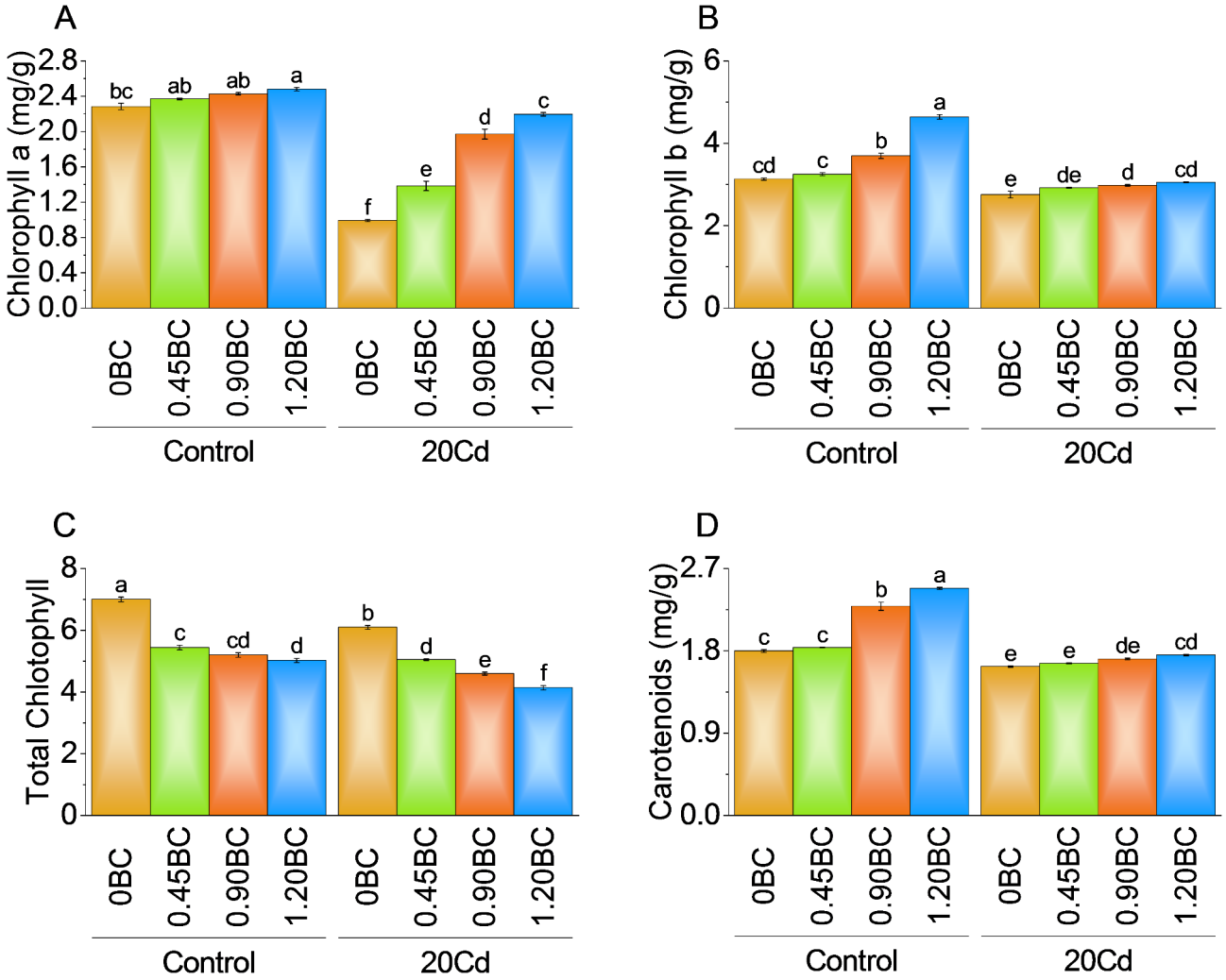

Fig. 3. Impact of different application rates of biochar on *Spinania oleracea* chlorophyll a (**A**), chlorophyll b (**B**), total chlorophyll (**C**), and carotenoids (**D**) cultivated in control and 20Cd stress. The bars represent the means of four replicates with standard error. The Fisher LSD test revealed significant changes at $p < 0.05$, shown by the different letters on the bars.

Principal component analysis and convex hull

The results presented in Table 2; Fig. 8 provide information on the values of Biochar and Cd (Cadmium) for convex hull cluster plot and principal component analysis loading for the studied attributes. The first column represents the Biochar treatment, while the second column represents the Cd treatment. The subsequent columns display the scores and loadings for the principal components (PC) 1 and 2. The percentages below the PC values indicate the variance explained by each principal component. For the attribute "Shoot Fresh Weight (g)," the scores for Biochar treatment 0BC and Cd treatment Control were 0.45 and − 1.57, respectively. The loadings for PC 1 and PC 2 were 0.21295 and − 0.17118. For the attribute Root Fresh Weight, the scores for Biochar treatment 0BC and Cd treatment Control were 0.93 and − 1.54, with loadings of 0.21024 and − 0.1683 for PC 1 and PC 2. For Shoot Dry Weight, Biochar treatment 0BC and Cd treatment Control scored 1.09 and − 1.63, with PC 1 and PC 2 loadings of 0.21035 and − 0.22795. Regarding Root Dry Weight, under Biochar treatment 0.45BC and Cd treatment Control, scores were 1.91 and − 1.34, and loadings for PC 1 and PC 2 were 0.22202 and − 0.03333. The Ascorbic Acid attribute showed scores of 2.24 and − 1.33 for Biochar treatment 0.45BC and Cd treatment Control, and the loadings for PC 1 and PC 2 were − 0.22016 and − 0.06605. For MDA, Biochar treatment 0.45BC and Cd treatment Control scored 2.58 and − 1.24, with PC 1 and PC 2 loadings of −0.22344 and 0.06017.

Total Amino Acid had scores of 3.76 and − 0.75 for Biochar treatment 0.90BC and Cd treatment Control, with loadings for PC 1 and PC 2 being 0.21676 and 0.20147. Total Soluble Sugar scores for Biochar treatment 0.90BC and Cd treatment Control were 4.14 and − 0.55, with PC 1 and PC 2 loadings of 0.21385 and 0.17745. The scores for Total Soluble Protein were 4.72 and − 0.37 for Biochar treatment 0.90BC and Cd treatment Control, and the loadings for PC 1 and PC 2 were 0.21961 and − 0.11488.

For Cd treatment 20Cd, Carotenoids under Biochar treatment 0BC had scores of −7.83 and 1.11, with PC 1 and PC 2 loadings of 0.20056 and 0.28909. Lycopene under the same conditions scored − 7.03 and 1.19, with

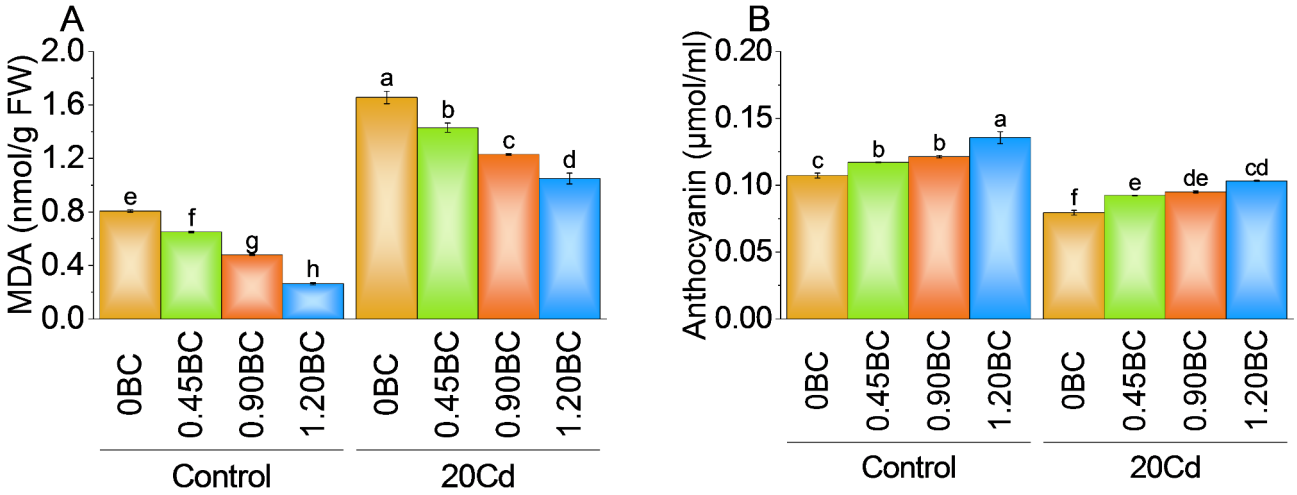

Fig. 4. The impact of different biochar application rates on *Spinania oleracea* MDA (**A**) and anthocyanin (**B**) cultivated in control and 20Cd stress. The bars represent the means of four replicates with standard error. The Fisher LSD test revealed significant changes at $p < 0.05$, shown by the different letters on the bars.

loadings of −0.21573 and 0.20624 for PC 1 and PC 2. For Anthocyanin, Biochar treatment 0BC and Cd treatment 20Cd had scores of −6.49 and 1.19, and the PC 1 and PC 2 loadings were 0.22247 and 0.03879.

Discussion

The present study examined the effect of acidified biochar (BC) on *Spinacia oleracea* L. grown in cadmium-contaminated soil. Cadmium stress significantly affects plant growth by disrupting essential physiological and biochemical functions. It slows nutrient intake, causing slowed development and decreased biomass. Cd also produces reactive oxygen species, causing oxidative stress, which harms cell components like lipids, proteins, and DNA. This lowers chlorophyll levels, making photosynthesis difficult and stunting development. Cd's toxicity affects hormone balance, enzyme activity, and cell structural integrity, leading to poor plant growth and decreased agricultural output. This is important as heavy metal contamination is primary threat to global food security.

The concentration of chlorophyll was established as a key indicator to assess the deleterious effects of cadmium concentrations on plants[11,49]. Decreases in plant biomass and chlorophyll content may be attributed to reduced nutrient uptake because of cadmium toxicity in the control pots. Similar decreases in chlorophyll content and fresh and dry weight of plants were reported previously in maize and chickpeas[50,51]. Applying acidified biochar (BC) at concentrations of 0.45% and 1.20% to alkaline and cadmium-contaminated soils showed a gradual increase in spinach plants' biomass and chlorophyll content. The 1.20% AB treatment yielded significantly superior results for biomass (fresh and dry weight), chlorophyll a, b, and total content[52]. reported that the highest improvement in spinach crop yield (9.51%) was observed when AB was applied to cadmium-contaminated soils.

The current study observed that an increase in acidified biochar concentration enhanced the nutrient availability to the plant, which consequently induced an increase in plant biomass, which agrees with the findings of[53]. Applying 2% and 3%, biochar substantially raised the chlorophyll levels in basil leaves. Similarly, in the present study, an increase in the carotenoid, lycopene, and anthocyanin content of tissues was noticed with a higher percentage of acidified biochar. The current study demonstrated that lycopene content in spinach tissues increased when grown in 0% AB. This reduction in lycopene synthesis due to Cd exposure has been previously documented in many plants[7,54].

Additionally, Cd-induced anthocyanin production has been reported in several species[55]. It was also observed in the present study that the lycopene and anthocyanin content of spinach leaves were significantly reduced when grown in 1.20% AB in cadmium and alkaline soil. Applying biochar increased spinach's anthocyanin content in cadmium-exposed alkaline soil, indicating that plants could somewhat withstand salinity and metal stress[56]. However, no significant enhancement in anthocyanin production was observed with biochar amendment in alkaline and cadmium-contaminated soil, which might be attributable to the activation of additional defense mechanisms that reduced Cd stress on the plant, thereby preventing the need for increased anthocyanin production. This finding is consistent with the findings. Previous studies have indicated that Cd stress can.

increase soluble protein content in plants[57].

Moreover, it documented those certain proteins, such as antioxidants and stress-related proteins, were augmented by cadmium stress. Additionally, the present study revealed an increase in spinach's protein and amino acid content. The results of this study suggest that Cadmium toxicity in combination with salinity has a more pronounced effect on protein and amino content in the absence of biochar[56], with the highest levels of amino acids and protein observed in 1.20% treatment of AB in Cadmium-affected soil and demonstrated that alterations in protein and amino acid content under stress conditions are likely to be modulated by the plant genotype. Furthermore, proteolytic enzyme (anti-stressor) activation due to the presence of cadmium in

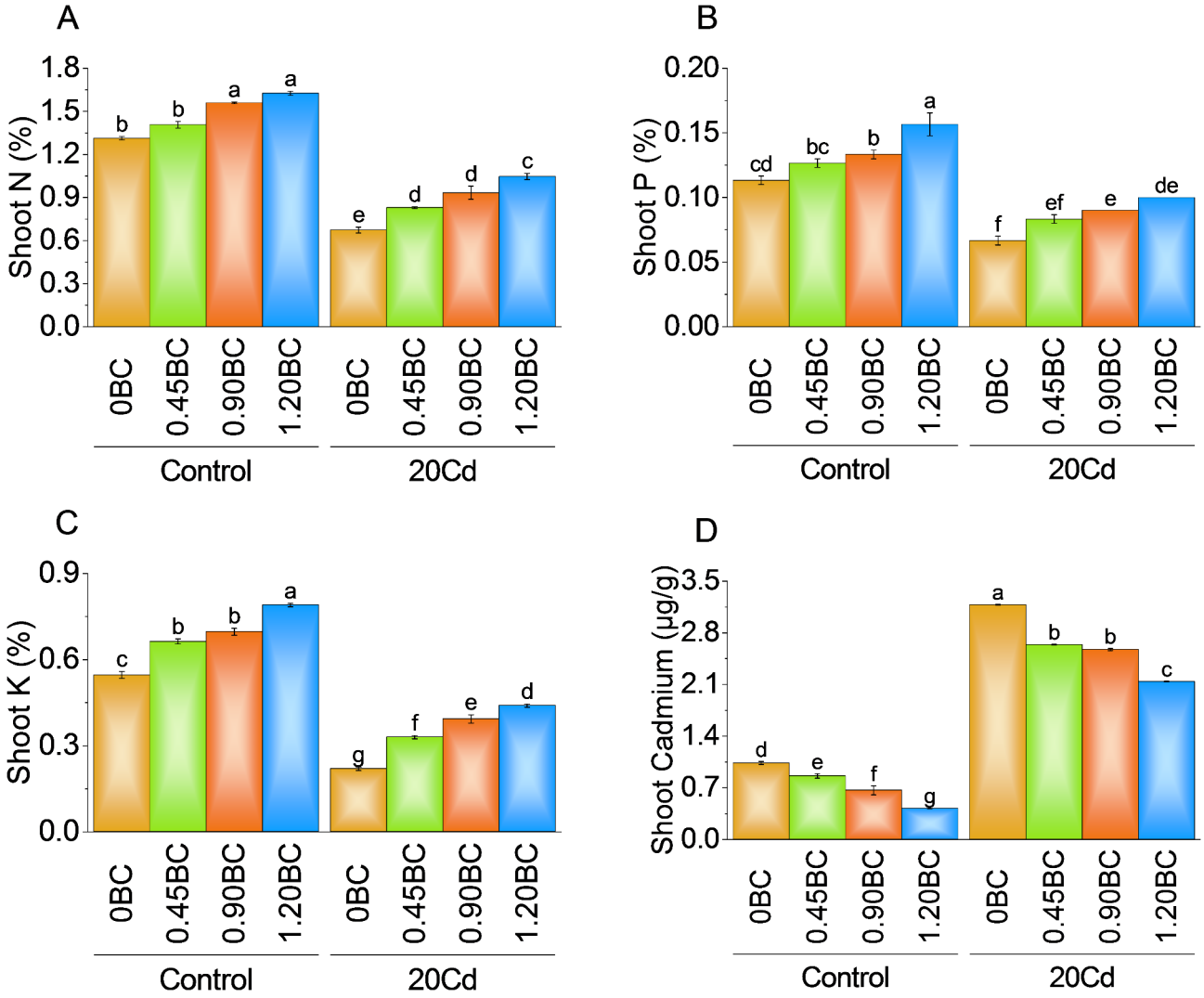

Fig. 5. Impact of different application rates of biochar on *Spinania oleracea* shoot N (**A**), shoot P (**B**), shoot K (**C**), and shoot Cd (**D**) cultivated in control and 20Cd stress. The bars represent the means of four replicates with standard error. The Fisher LSD test revealed significant changes at $p < 0.05$, shown by the different letters on the bars.

the soil may also be associated with this phenomenon. Subsequent studies by[58] corroborated the considerable enhancement of Zea mays' protein and amino acid content.

The present investigation demonstrated that cadmium toxicity led to a decrease in the soluble sugar content of spinach in the control. However, after treatment with acidified biochar, the sugar content was significantly diminished in both soils. Subsequently, it was established that 1.2BC was the most effective in augmenting the sugar content of spinach[59]. A congruent trend of enhancing the total soluble sugar content of mango was reported by Under abiotic stress conditions, Reactive Oxygen Species (ROS) have been shown to increase levels of oxidative stress in plants. This was evidenced by a decrease in Ascorbic acid and MDA contents of spinach under alkaline and cadmium-contaminated alkaline soil treatments compared to 0BC treatment, with higher levels seen in cadmium-treated pots. The lowest concentrations of ascorbic acid and MDA were observed in 1.20BC (acidified biochar) in both cadmium-free and cadmium-tinted alkaline soils ($p \leq 0.05$)[60].

This ascorbic acid and MDA levels response has been previously reported in tomato plants under cadmium toxicity and super japonica rice grown under cadmium stress.

This study demonstrated that applying biochar to soil significantly increased the total N+, P, and K + concentrations in spinach (roots and shoots) under heavy metal stress. It was hypothesized that the addition of acidified biochar (AB) to soil had the effect of increasing bioavailable P content[61]. The high cation exchange capacity of biochar may also have increased the soil's N retention capacity (NH_4^+), thus limiting the loss of nitrogen through the oxidation of ammonia ions and denitrification and making N more accessible to plants Organic Matter (OM) has been demonstrated to have an impact on the bioavailability of potassium in soil, both directly and indirectly[53]. This is due to organic functional groups acting as negative charge-carrying sites, which attract cations and enhance the nutrient-holding capacity of the soil. Recent studies have shown that plants

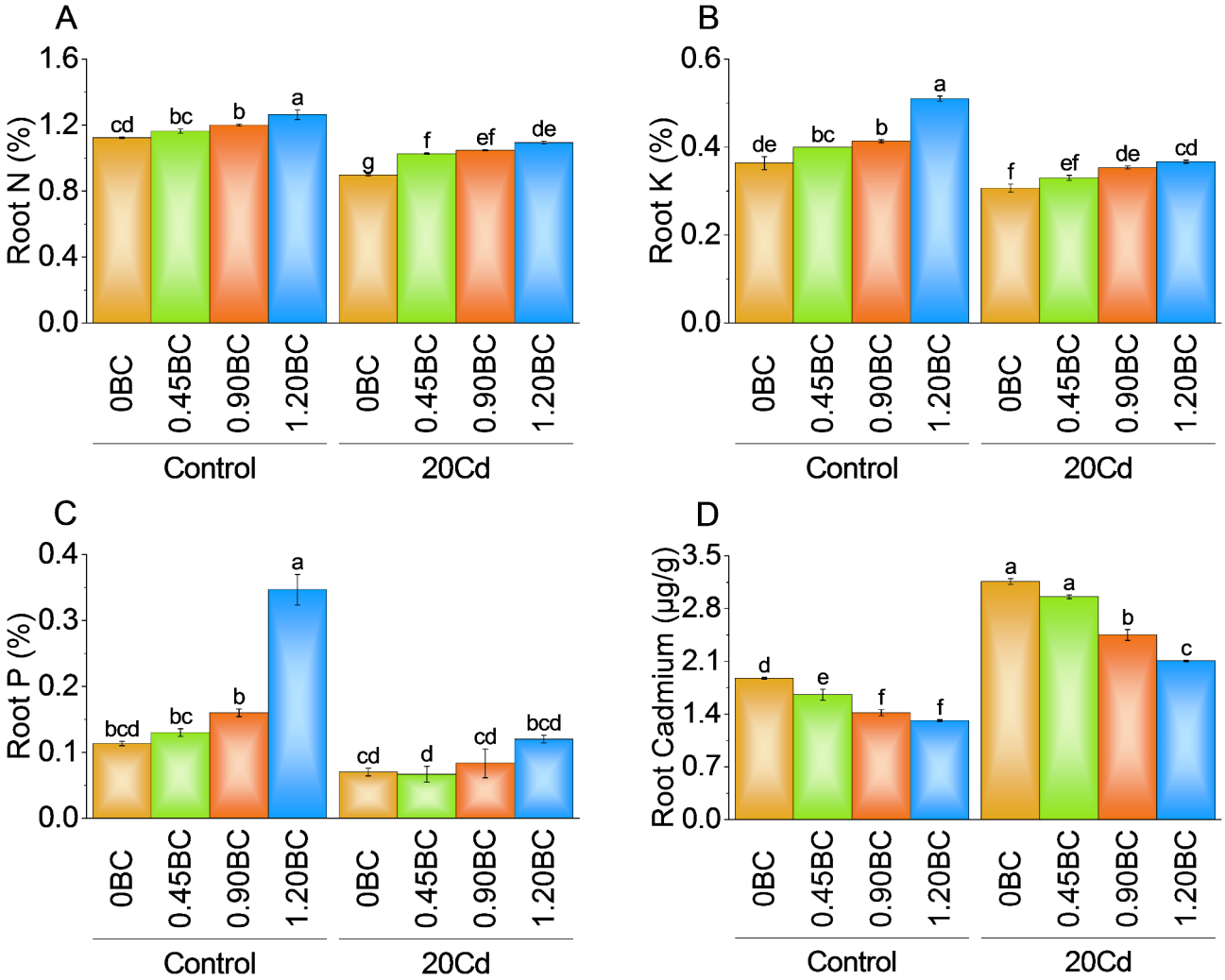

Fig. 6. Impact of different application rates of biochar on *Spinania oleracea* root N (**A**), root P (**B**), root K (**C**), and root Cd (**D**) cultivated in control and 20Cd stress. The bars represent the means of four replicates with standard error. The Fisher LSD test revealed significant changes at $p < 0.05$, shown by the different letters on the bars.

growing on soils amended with biochar have higher absorption of nitrogen, phosphorus, and potassium from soil, with the highest nitrogen, potassium, and phosphorus content in the shoots being obtained in treatments with 0.9 and 1.2 acidified biochar, respectively[62,63].

The translocation coefficient was utilized to elucidate the capacity of heavy metals (HM) to move from soil to the edible section of plants[64]. Experiments in acidified char in alkaline and Cd-contaminated alkaline soil revealed significantly reduced concentrations of Cd absorption in root and shoot, likely due to the lower availability of Cd in the soil. These results indicated a notable decrease in Cd translocation to shoot and root with 0.45, 0.90, and 1.20BC treatments in alkaline and Cd-polluted[65] alkaline soil, with the greatest effect observed in the 1.20BC treatment. It was thus determined that 1.20BC applied to alkaline soil and cadmium soil provided the most effective remediation of HM-contaminated soil.

Conclusion

It is concluded that the 1.20% acidified biochar is more useful in alleviating the cadmium stress in spinach plants. Treatment1.20% acidified biochar has more potential to increase growth and biochemical attributes (soluble protein, soluble sugar, chlorophyll contents, and N, P, K) under 20 mg Cd/kg soil stress. Less Cd uptake in root and shoot along with low level of MDA in leaves of spinach plants under 20 mg Cd/kg soil stress also confirmed the effectiveness of 1.20% biochar. More investigations are suggested at the field level under different climates to declare 1.20% acidified biochar as the best amendment for different crop cultivation under Cd toxicity. For future research, the long-term effects of acidified biochar on soil health and cadmium bioavailability, testing a wider range of cadmium concentrations, and exploring its impact on different soil types, climates, and plant species. Further studies are recommended to address potential environmental risks, such as changes in soil pH and leaching of residues from biochar, to better understand its sustainability and broader applicability.

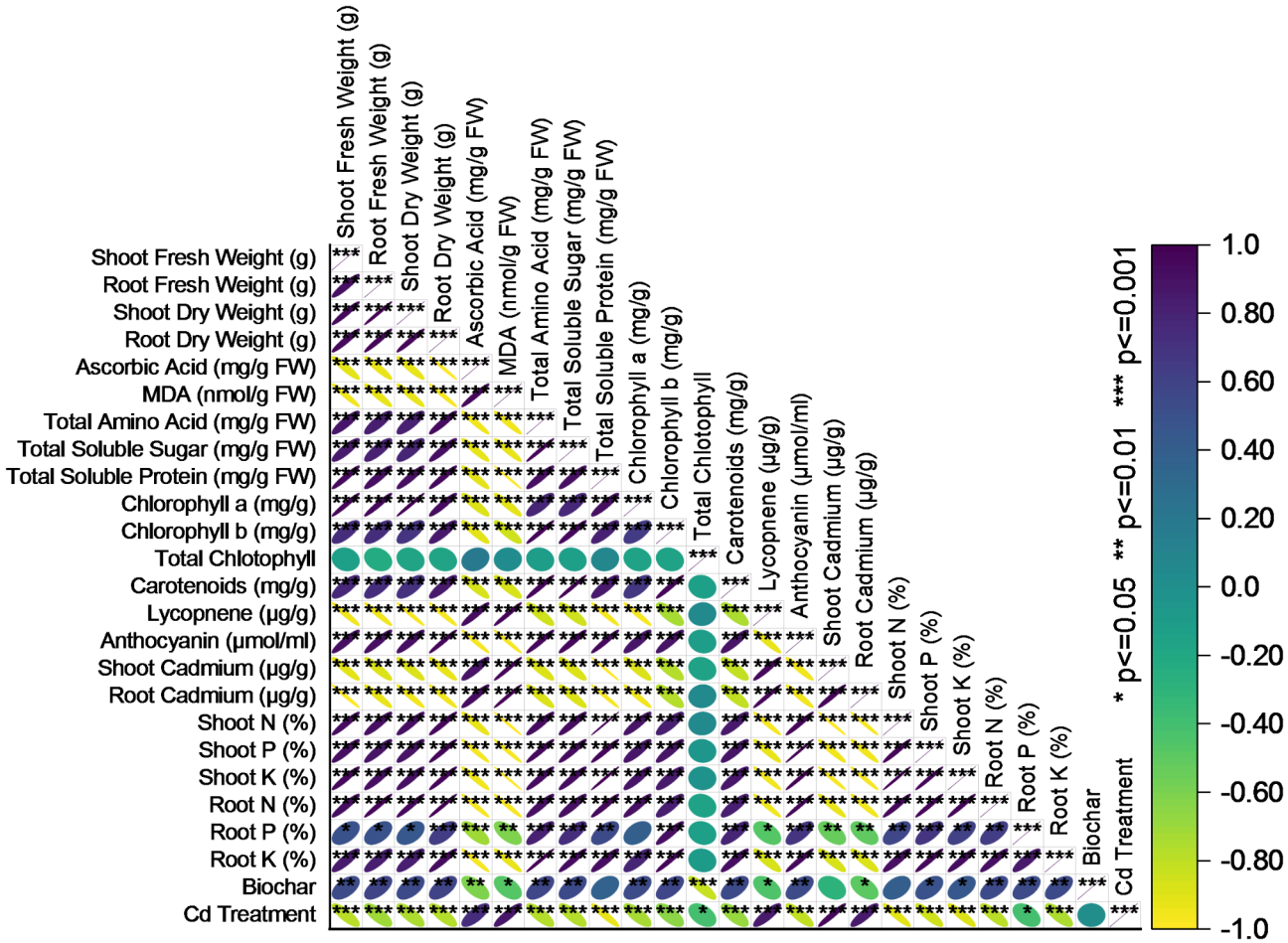

Fig. 7. Pearson correlation for the studied attributes.

Biochar	Cd	PC 1	PC 2		PC 1	PC 2
		85.87%	6.54%		85.87%	6.54%
Scores	Scores	Scores	Scores	Loadings	Scores	Scores
0BC	Control	0.45	−1.57	Shoot Fresh Weight (g)	0.21295	−0.17118
0BC	Control	0.93	−1.54	Root Fresh Weight (g)	0.21024	−0.1683
0BC	Control	1.09	−1.63	Shoot Dry Weight (g)	0.21035	−0.22795
0.45BC	Control	1.91	−1.34	Root Dry Weight (g)	0.22202	−0.03333
0.45BC	Control	2.24	−1.33	Ascorbic Acid (mg/g FW)	−0.22016	−0.06605
0.45BC	Control	2.58	−1.24	MDA (nmol/g FW)	−0.22344	0.06017
0.90BC	Control	3.76	−0.75	Total Amino Acid (mg/g FW)	0.21676	0.20147
0.90BC	Control	4.14	−0.55	Total Soluble Sugar (mg/g FW)	0.21385	0.17745
0.90BC	Control	4.72	−0.37	Total Soluble Protein (mg/g FW)	0.21961	−0.11488
1.20BC	Control	6.50	2.04	Chlorophyll a (mg/g)	0.20468	−0.27445
1.20BC	Control	7.29	2.30	Chlorophyll b (mg/g)	0.19984	0.36869
1.20BC	Control	7.84	2.46	Total Chlorophyll	−0.02207	−0.21396
0BC	20Cd	−7.83	1.11	Carotenoids (mg/g)	0.20056	0.28909
0BC	20Cd	−7.03	1.19	Lycopene (µg/g)	−0.21573	0.20624
0BC	20Cd	−6.49	1.19	Anthocyanin (µmol/ml)	0.22247	0.03879
0.45BC	20Cd	−4.68	0.62	Shoot Cadmium (µg/g)	−0.21465	0.14774
0.45BC	20Cd	−4.30	0.53	Root Cadmium (µg/g)	−0.21896	0.152
0.45BC	20Cd	−3.92	0.43	Shoot N (%)	0.22018	−0.09994
0.90BC	20Cd	−2.65	−0.09	Shoot P (%)	0.2214	0.03196
0.90BC	20Cd	−2.25	−0.15	Shoot K (%)	0.22228	−0.05178
0.90BC	20Cd	−1.87	−0.16	Root N (%)	0.22075	−0.04868
1.20BC	20Cd	−1.10	−0.37	Root P (%)	0.15135	0.5569
1.20BC	20Cd	−0.85	−0.36	Root K (%)	0.21549	0.20067
1.20BC	20Cd	−0.47	−0.44			

Table 2. Values of Biochar and Cd for convex hull cluster plot and principal component analysis loading for studied attributes.

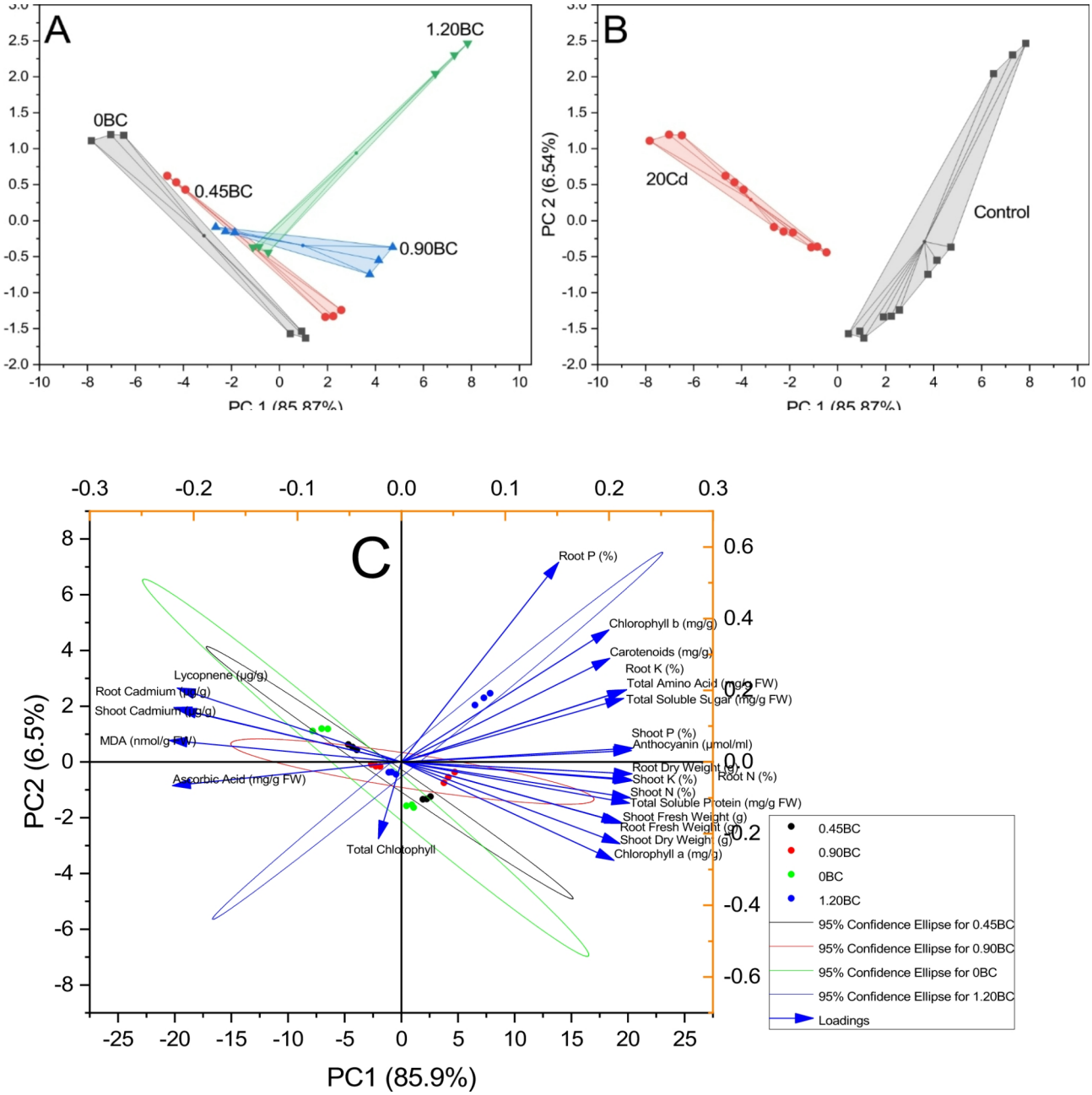

Fig. 8. Cluster plot convex hull for biochar levels (**A**), Cd levels (**B**), and principal component analysis for studied attributes.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

Conceptualization; L.S.; N.N.; U.Y.; Conducted experiment; T.S.; N.N.; U.Y.; Formal analysis; S.O.; A.A.A.; S.S.A.; Methodology; S.N.; T.N.; Writing—original draft; L.S.; N.N.; U.Y.; Writing—review & editing; M.J.A.; S.O.; A.A.A.; S.S.A.;

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

We all declare that manuscript reporting studies do not involve any human participants, human data, or human tissue. So, it is not applicable.

Study protocol must comply with relevant institutional, national, and international guidelines and legislation

Our experiment follows the with relevant institutional, national, and international guidelines and legislation.

Additional information

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