NARDI FUNDULEA, ROMANIA
www.incda-fundulea.ro; doi.org/10.59665/rar43103
ROMANIAN AGRICULTURAL RESEARCH, NO. 43, 2026
First Online: September, 2026. DII 2067-5720 RAR 2026-182

Harnessing Endophytic Fungi-Derived Biochar for Alleviating Chromium Toxicity in *Brassica rapa* L.

Maryam Bibi[1], Wasim Khan[1], Anwar Hussain[1*], Zakir Ullah[2], Heba I. Mohamed[3*], Haiam O. Elkatry[4], Khaled M.A. Ramadan[5], Ahmed Mahmoud Ismail[6], Mohamed El Oirdi[7], Abdelrahman R. Ahmed[4*]

[1]Department of Botany, Garden Campus, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa, Pakistan

[2]Department of Botany, Islamia College Peshawar, Peshawar, 25120, Khyber Pakhtunkhwa, Pakistan

[3]Department of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Cairo, 11341, Egypt

[4]Food and Nutrition Science Department, Agricultural Science and Food, King Faisal University, Al Ahsa 31982, Saudi Arabia

[5]Central Laboratories, Department of Chemistry, King Faisal University, Al-Ahsa 31982, Saudi Arabia

[6]Department of Arid Land Agriculture, College of Agricultural and Food Sciences, King Faisal University, Al-Ahsa 31982, Saudi Arabia

[7]Department of Life Sciences, College of Science, King Faisal University, Al Ahsa 31982, Saudi Arabia

[*]Corresponding authors. E-mail: hebaibrahim79@gmail.com; drhussain@awkum.edu.pk; arahmed@kfu.edu.sa

ABSTRACT

This study investigated the biochar derived from endophytic fungi as a sustainable soil amendment to mitigate the toxicity of hexavalent chromium [Cr(VI)] and to enhance the growth, physiological, and biochemical attributes of *Brassica rapa* L. seedlings exposed to chromium stress. Seedlings were subjected to Cr(VI) stress (300 µg mL^{-1}) with or without the application of fungal biochar at two different levels (1 g and 2 g). We assessed various growth parameters, including shoot and root length, fresh and dry biomass, chlorophyll content, levels of phytohormones (indole-3-acetic acid and salicylic acid), and biochemical metabolites (flavonoids, phenols, proteins, and soluble sugars) using standard spectrophotometric methods. The results showed that chromium stress significantly reduced plant growth, chlorophyll content, phytohormone levels, and metabolite concentrations compared to the control group ($p < 0.05$). However, the application of fungal biochar at both doses effectively reduced Cr-induced toxicity and enhanced growth and biochemical traits to levels that were comparable to or even higher than those observed in the control group. Under non-stressed conditions, the 2 g biochar treatment significantly improved shoot length, chlorophyll content, flavonoid accumulation, and soluble sugar content ($p < 0.05$). These findings suggest that fungal biochar represents a sustainable strategy for phytoremediation in chromium-contaminated soils.

Keywords: phytoremediation, plant growth, chlorophyll, phytohormones, antioxidants, flavonoids, phenolics, sustainable agriculture.

INTRODUCTION

Heavy metals are defined as elements with densities greater than 4 g/cm³ and pose significant environmental and health risks due to their toxicity, even at trace concentrations (Mohamed, 2011; Mohamed et al., 2025). This group includes transition metals such as chromium (Cr), lead (Pb), cadmium (Cd), copper (Cu), and zinc (Zn), among others. While certain metals, including Cr, Cu, and Zn, are essential micronutrients for plant growth at low concentrations, their accumulation beyond physiological thresholds can lead to toxicity. This toxicity can result in reduced biomass, impaired photosynthesis, altered enzyme activity, and increased production of reactive oxygen species (ROS) (Singh et al., 2013; Abu-Shahba et al., 2022). Experimental evidence has also shown that exposure to heavy metals significantly alters nutrient homeostasis and antioxidant metabolism in crop plants, leading to reductions in physiological performance and growth (Sahin et al., 2022). Non-essential metals like Pb,

Received 5 May 2026; accepted 2 September 2026.

Cd, and mercury provide no biological benefits and are particularly hazardous. They can contaminate air, soil, and water and ultimately enter the food chain and pose threats to human health (Asati et al., 2016). Anthropogenic activities, such as mining, fossil fuel combustion, industrial manufacturing, and agricultural practices, including the application of pesticides and fertilizers, have significantly elevated heavy metal concentrations in soils. Among these, chromium is a major concern due to its prevalence and toxicity, especially in its hexavalent form, Cr(VI) (Shahid et al., 2017).

Chromium (Cr) primarily exists in soil in two forms: trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)]. Among these, Cr(VI) is more soluble, mobile, and toxic. When taken up by plants, Cr(VI) disrupts the absorption of essential nutrients, damages cellular structures, and impairs growth, which can pose risks to human health through dietary exposure (Handa et al., 2018). Plants have developed various defense mechanisms to cope with heavy metal stress. These include sequestering metals into cell walls and vacuoles and complexing them with organic acids or proteins (Liu et al., 2018). However, these natural strategies often fall short in heavily contaminated soils, highlighting the need for innovative remediation approaches.

Bioremediation harnesses living organisms, such as plants and microorganisms, to provide an environmentally sustainable solution for detoxifying heavy metals (Ali et al., 2025). Among the various bioremediation techniques, biochar - a carbon-rich material produced through the pyrolysis of organic matter in low-oxygen conditions - has gained significant attention. Due to its high surface area, porous structure, and various functional groups, biochar can effectively bind heavy metals, thereby reducing their bioavailability (Tang et al., 2020).

Biochar is particularly effective in immobilizing Cr(VI), which decreases its uptake by plants and alleviates oxidative stress. Additionally, it enhances soil properties, including pH, nutrient retention, and cation exchange capacity (Ali et al., 2017, 2019; Abbas et al., 2018). Furthermore, biochar promotes plant growth by releasing essential nutrients like nitrogen and phosphorus while also fostering beneficial microbial communities. This may occur through mechanisms such as hormesis or by promoting the growth of plant growth-promoting rhizobacteria (Sofy et al., 2022).

Recent advancements in biochar applications involve using endophytic fungal biomass, which takes advantage of the symbiotic relationship between endophytic fungi and plants. These fungi help enhance plant resilience to heavy metal stress, thereby promoting growth in contaminated environments (El-Mahdy et al., 2021). Biochar produced from fungal biomass presents a promising and eco-friendly strategy for remediating chromium (Cr)-contaminated soils. This is particularly relevant for crops like *Brassica rapa*, a commonly cultivated vegetable in temperate regions that easily accumulate heavy metals, which poses health risks through the food chain.

This study investigated endophytic fungi-derived biochar as a sustainable soil amendment to mitigate hexavalent chromium [Cr(VI)] toxicity and improve the growth, physiological, and biochemical performance of *Brassica rapa* L.

MATERIAL AND METHODS

Requisition of plant growth-promoting endophytic fungi

Obtained plant growth-promoting endophytic fungus (*Aspergillus flavus* MH577051) from the microbial culture collection of the Plant Microbe Interaction (PMI) Lab of the Botany Department of Abdul Wali Khan University Mardan.

Screening of growth-promoting endophytic fungi for chromate resistance

To assess the resistance of growth-promoting fungal strains to hexavalent chromium, they were cultivated on Czapek medium with hexavalent chromium concentrations of 100 µg/ml and 300 µg/ml. The Czapek growth medium was prepared following a standard protocol described

earlier. The prepared medium was divided into 250 ml flasks, with each flask containing 50 mL of Czapek media. Hexavalent chromium was sourced from potassium chromate (K_2CrO_4). To create a stock solution, one gram of this salt was dissolved in one liter of autoclaved distilled water. To achieve the desired hexavalent chromium stress levels of 100 µg/mL and 300 µg/mL in the growth medium, 1.5 mL of the 1 M stock solution was added to each flask containing 50 mL of Czapek media. The flasks were then wrapped in aluminum foil and sterilized using standard autoclave techniques, as previously described. After sterilizing, spores from each isolated strain were injected into their respective labeled flasks in triplicate. These inoculated flasks were shaken for 6 days at 27°C at a speed of 120 rpm in a shaking incubator.

Biochemical analysis of fungal culture filtrate

Phenolic compounds, flavonoids, salicylic acid, indole-3-acetic acid (IAA), soluble sugars, and total protein levels were tested for the fungal culture filtrates through colorimetric assays. The level of phenolics was obtained by mixing 0.5 mL of culture filtrate in 3 mL of distilled water before adding 0.5 mL of Folin reagent (diluted 1:1 v/v) and 2 mL of 20% sodium carbonate, then heating to 70°C, cooling, and reading the absorbance at 650 nm against a reagent blank (Chun et al., 2003). The flavonoids were determined from the mixture of 0.5 mL of filtrate, 4.3 mL of 80% methanol, 0.1 mL of 10% aluminum chloride solution, and 0.1 mL of 10% potassium acetate at 415-420 nm wavelength (Brighente et al., 2007). Measurement of salicylic acid level was carried out by mixing 0.1 mL of filtrate with 2.9 mL of 0.1% $FeCl_3$ solution (Warrier et al., 2013).

IAA concentration was quantified by the addition of 2 mL Salkowski reagent (containing $FeCl_3$ and perchloric acid) to 1 mL filtrate, followed by dark incubation for 30 min, and absorbance was measured at a 540 nm wavelength (Gordon and Weber, 1951). Quantification of soluble sugars was performed by combining 0.1 mL filtrate with 1 mL 80% phenol solution, followed by the addition of 5 mL concentrated sulfuric acid, incubating for 1 h, and recording the absorbance at 485 nm wavelength (Rover et al., 2013). Total protein concentration was estimated through an improved Lowry procedure in which 0.1 mL of filtrate was made up to 1 mL with distilled water, reacted with alkali copper reagent (sodium carbonate, sodium potassium tartrate, and copper sulfate solution), added to Folin reagent, incubated for 30 min, and absorbance recorded at 650 nm (Zhou and Regenstein, 2006).

Production of biomass of growth-promoting endophytic fungi

Cultivation of the chosen endophytes was carried out on Czapek medium to produce biomass by inoculating 100 mL of medium in 250 mL flasks. Czapek medium was prepared by adding 10 g glucose, 15 g peptone, 0.5 g magnesium sulfate ($MgSO_4$), 0.5 g potassium chloride (KCl), and 0.02 g iron sulfate in 1000 mL distilled water. The mixture was then sterilized in an autoclave. Cultured flasks were incubated at 28°C and shaken at 120 rpm for 7 days. Filtration using Whatman No. 1 filter paper was used to separate the biomass.

Biochar preparation

The preparation of biochar was done by producing 500 g of fungal biomass in Czapek broth, harvesting the biomass, and oven drying it at 80°C for 20 minutes before carrying out pyrolysis. The dried fungal biomass was kept in airtight containers until the next step was to be performed. The pyrolysis of the fungal biomass was carried out using previously described procedures by Jack et al. (2019), wherein the biomass was placed in an air-tight crucible to reduce its exposure to oxygen and subsequently heated in a furnace in an oxygen-limited atmosphere. The heating was performed at a rate of 10°C/min and held for 60 minutes in the selected heating rate and residence time. These parameters were chosen following

previous studies since slower heating leads to enhanced volatilization and thus greater microporosity of biochar (Gu et al., 2023).

Experiment design

The seeds of *B. rapa* were germinated, and healthy seedlings were transferred into garden soil-filled plastic pots. These pots were divided into different treatment series as follows:

T1 = Control
T2 = 300 µg/mL of Cr
T3 = 1 g biochar
T4 = 2 g biochar
T5 = 1 g biochar + 300 µg/mL of Cr
T6 = 2 g biochar + 300 µg/mL of Cr

All the sets were grown under identical conditions for 21 days. The seedlings were harvested and immediately frozen for further study.

Extraction and estimation of chlorophyll

Chlorophyll content was determined using the method described by Maclachlan and Zalik (1963). Fresh leaf samples weighing 0.5 grams were finely powdered in 80% acetone. The mixture was then centrifuged at 10,000 rpm to separate the components. After centrifugation, the volume of the sample was adjusted to 7 ml by adding more acetone. Optical density (OD) measurements were taken at specific wavelengths to quantify chlorophyll a, chlorophyll b, and carotenoids. The wavelengths used for these measurements were 480 nm, 645 nm, and 663 nm, respectively.

Determination of indole acetic acid (IAA)

Total indole-acetic acid (IAA) was measured in the leaves of young plants after various treatments. A 0.3-gram leaf sample was coarsely pulverized in 4 milliliters of distilled water, then homogenized and centrifuged at 10,000 rpm for under 2 minutes. One milliliter of the supernatant was added to a test tube with 2 milliliters of Salkowski reagent, prepared by mixing 0.5 M ferric chloride ($FeCl_3$) with 35% perchloric acid and allowing it to sit in darkness for 30 minutes. The solution turned yellowish blue, indicating the presence of IAA, and the optical density (OD) was recorded at 540 nm against a blank reagent.

Determination of salicylic acid

The sample volume was reduced to 0.1 mL. Next, 2.9 mL of a 0.1% ferric chloride ($FeCl_3$) solution was added, following a modified version of the procedure described by Warrier et al. (2013). This resulted in a violet color upon mixing. The reaction was assessed by measuring its optical density (OD) at 540 nm, in accordance with the revised protocol.

Determination of total phenolic content

To determine the total phenolic content of leaves from seedlings under various treatments, we followed these steps: We ground 0.5 grams of fresh leaf sample in 10 mL of distilled water (dH_2O) and centrifuged the mixture at 3000 rpm for 10 minutes. We transferred 0.5 mL of the supernatant to a test tube and adjusted the volume to 3 mL with dH_2O. Then, we added 0.5 mL of diluted Folin reagent and 2 mL of a 20% sodium carbonate solution, noting a blue hue indicating phenols. After heating the mixture to 70 degrees Celsius and cooling it to room temperature, we measured the optical density (OD) at 650 nm, as per Chun et al. (2003), using a reagent blank for comparison.

Determination of flavonoids

Flavonoid content was assessed in seedling leaves subjected to various treatments. A 0.5-gram leaf sample was finely ground in 5 mL of distilled water (dH_2O) and then centrifuged at 3000 rpm for 10 minutes. A 0.5 mL aliquot of the supernatant was transferred to a new test tube, and 0.1 mL each of 10% aluminum chloride ($AlCl_3$) and 10% acetic acid was added, followed by 4.3 mL of 80% methanol to reach a final volume of 5 mL. The solution was vortexed, and the reagent blank was measured at 415 nm, with optical density (OD) calculated according to Brighente et al. (2007).

Determination of soluble sugar content

To measure total soluble sugar in seedling leaves, 0.5 grams of fresh leaf material were ground with 10 milliliters of distilled water, then centrifuged at 3000 rpm for 10 minutes. Approximately 0.1 milliliters of the supernatant were transferred to a new test tube, and 1

milliliter of 80% phenol was added. After sitting for 10 minutes, the mixture was incubated for one hour with concentrated sulfuric acid (H_2SO_4), and the optical density (OD) was measured at 485 nm against a reagent blank (Rover et al., 2013).

Determination of total protein

Protein concentrations were quantified in extracts from leaves based on the method reported by Zhou and Regenstein (2006). Leaf samples (0.1 g) were ground in 1 mL phosphate buffer and centrifuged at 3000 rpm for 10 minutes. The supernatant (0.1 mL) was diluted to 1 mL with distilled water, and then 1 mL of reagent C (reagent A: reagent B, 50:1) was added. Following a 10-minute incubation period, 0.1 mL of diluted Folin reagent (1:1, v/v) was added and further incubated for 30 minutes, after which absorbance was read at 650 nm against a blank.

Determination of heavy metals via atomic absorption spectroscopy

The concentration of chromium will be determined in both plant samples and biochar using atomic absorption spectrometry (Ali et al., 2018). The samples were analyzed using a flame atomic absorption spectrophotometer, specifically the Perkin Elmer A Analyst 700 from the United States, to determine their chromium concentration. An acetylene/air flame was used for the readings, and the operating settings for the analytical components were adjusted according to the manufacturer's specifications.

Statistical analysis

The results of the study were subjected to analysis of variance (ANOVA) in order to assess the impact of chromium stress, biochar amendment, and the interaction between the two on various aspects of plant physiology. The experimental design used was a completely randomized design with three replications for each treatment. Comparison of means was done using Tukey's HSD test at $p \leq 0.05$ level.

RESULTS AND DISCUSSION

The levels of phytohormones and metabolites in *A. flavus* MH577051

The levels of phytohormones and metabolites in selected endophytic fungi varied markedly between control and chromate-stressed conditions (Figure 1). Under normal conditions, culture filtrates showed appreciable concentrations of key compounds, including salicylic acid (SA; 56.22 µg/mL) and indole-3-acetic acid (IAA; 60.33 µg/mL). Among secondary metabolites, total phenols (79.32 µg/mL) and soluble sugars (80.92 µg/mL) were abundant, followed by proteins (45.13 µg/mL) and flavonoids (18.56 µg/mL). Exposure to chromate stress (100 ppm) induced significant alterations in metabolite profiles. SA levels declined markedly to 37.43 µg/mL, while IAA remained unchanged at 60.33 µg/mL, indicating its stability under stress. A substantial increase in flavonoid content was observed, rising to 79.32 µg/mL, suggesting activation of antioxidant defense mechanisms. In contrast, total phenols decreased sharply to 27.52 µg/mL. Protein content showed a negligible reduction (45.10 µg/mL), whereas soluble sugars decreased to 69.85 µg/mL under stress conditions. Overall, chromate exposure triggered differential modulation of fungal metabolites, with a notable shift toward flavonoid accumulation and reduced phenolic and sugar contents, reflecting adaptive biochemical responses of endophytic fungi to heavy metal stress.

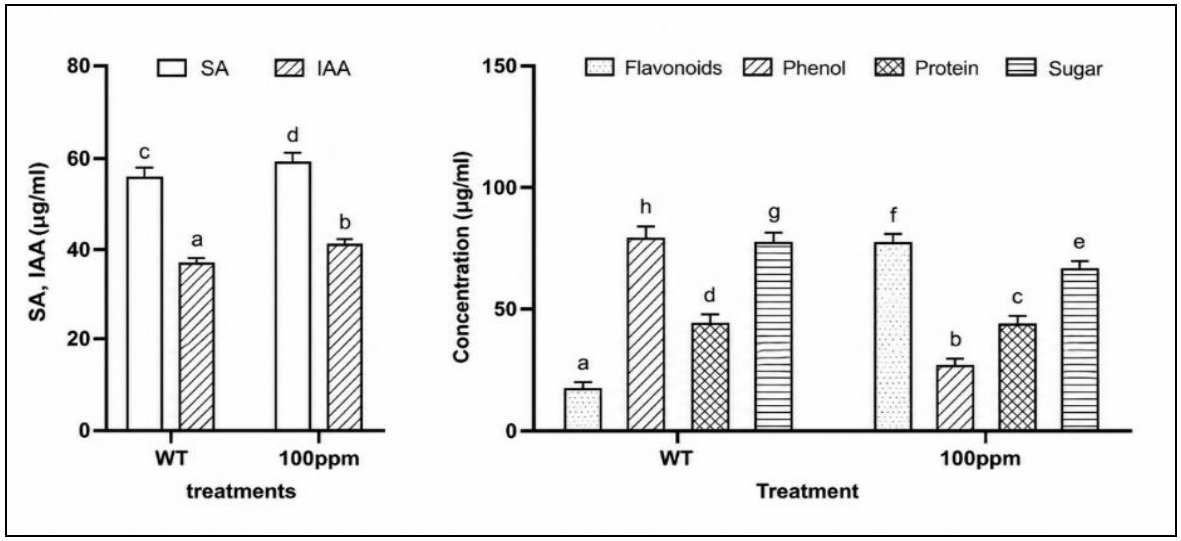

Figure 1. Impact of chromate stress on flavonoids, phenols, proteins, sugars, SA, and IAA contents in fungal filtrate. Data are expressed as mean ± standard deviation (n = 3). Different letters indicate significant differences among treatments at $p \leq 0.05$, according to Tukey's HSD test.

Impact of biochar on *B. rapa* growth under chromium stress

The effects of biochar application on the root and shoot length of *B. rapa* under hexavalent chromium (Cr) stress (300 µg/mL) were evaluated. In comparison to the control group, shoot length was significantly reduced under Cr stress (Figures 2 and 3). However, the application of 1 g of biochar in the presence of 300 µg/mL of Cr restored shoot length to levels similar to those of the control group, with additional increases observed in the absence of Cr stress. Similarly, the addition of 2 g of biochar under 300 µg/mL of Cr stress not only restored shoot length but also significantly enhanced it compared to the control ($p < 0.05$). Root length exhibited a similar trend, showing a significant reduction under Cr stress compared to the control. Nevertheless, the application of biochar (1 g and 2 g) mitigated this effect, restoring and promoting significant increases in root length, particularly with 2 g of biochar in the absence of Cr stress.

The most pronounced effects of Cr stress were observed on the fresh and dry weight of *B. rapa* seedlings, both of which were significantly reduced compared to the control ($p < 0.05$). The application of biochar (1 g and 2 g) significantly alleviated this reduction, with both the fresh and dry weights showing marked increases, especially with the 2 g treatment, under both Cr-stressed and non-stressed conditions ($p < 0.05$). These findings highlight the potential of biochar to mitigate Cr-induced stress and enhance the growth parameters of *B. rapa.*

The presence of heavy metal toxins poses a significant threat to plant survival. This primarily occurs because these toxins lead to the production of reactive species, which disrupt the plant's internal balance and impede its normal metabolic processes (El-Mahdy et al., 2021). Many studies have investigated the impact of biochar in alleviating abiotic stressors in plants (Haiying et al., 2022). Biochar has been documented to minimize chromium bioavailability and enhance the growth of *Brassica napus* (Naveed et al., 2021). It achieves this by absorbing Cr on its surface through various mechanisms, including electrostatic contact, cation exchange, and precipitation, which can modulate Cr bioavailability (Naveed et al., 2021).

Chromium, especially in its hexavalent form (Cr VI), interferes with cellular division, elongation, and nutrient absorption, resulting in a decrease in biomass. The observed alleviation of chromium-induced stress when using biochar can likely be attributed to a reduction in chromium toxicity and improved nutrient availability, as evidenced by the enhanced growth parameters (Yue et al. 2025).

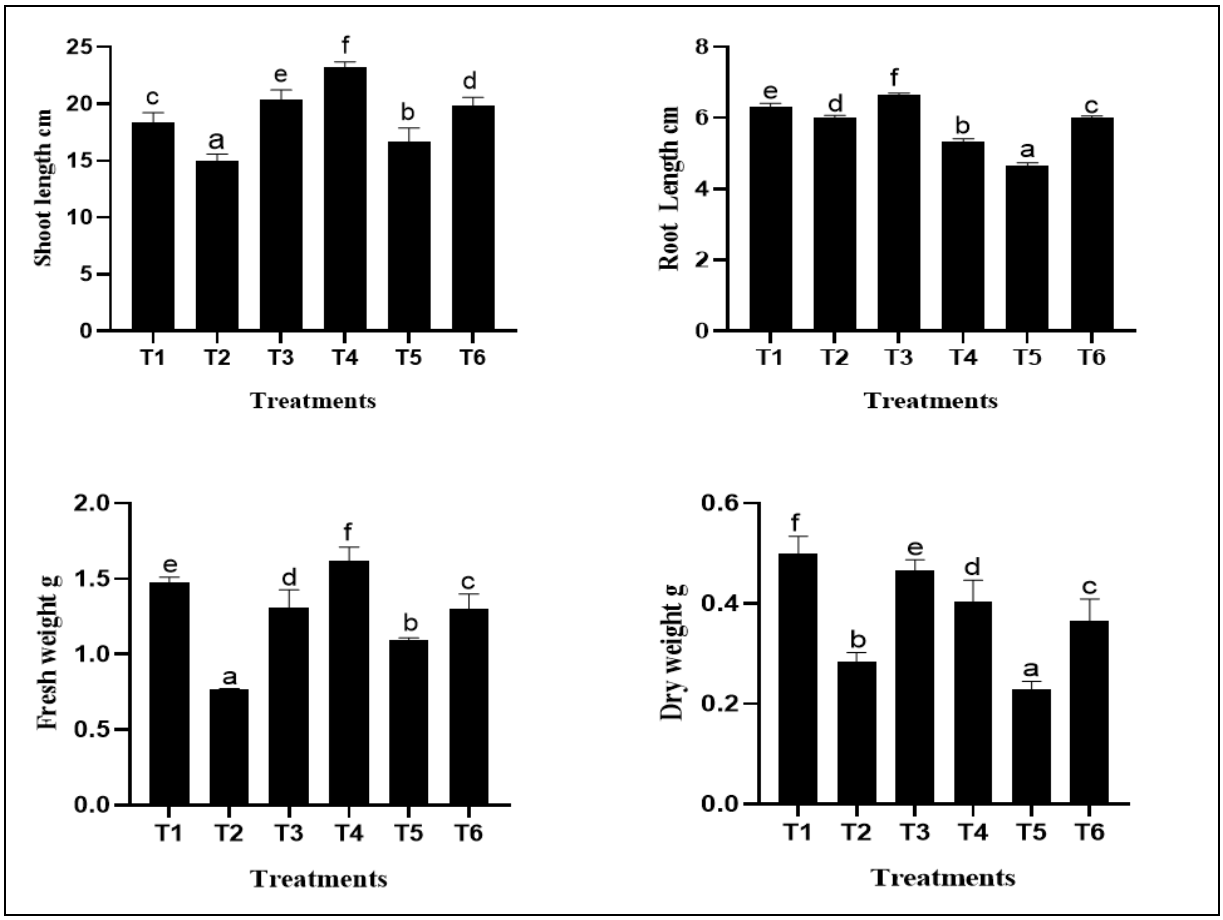

Figure 2. Effect of chromium and biochar on the growth of root and shoot length and fresh dry weight of *B. rapa* L. seedlings. T1 = Control, T2 = Cr. 300 µg mL^{-1}, T3 = 1g Biochar, T4 = 2g Biochar, T5 = 1g Biochar + Cr. 300 µg mL^{-1}, and T6 = 2g Biochar + Cr. 300 µg mL^{-1}. Data are expressed as mean ± standard deviation (n = 3). Different letters indicate significant differences among treatments at $p \leq 0.05$, according to Tukey's HSD test.

Effects of biochar on chlorophyll content in *B. rapa* under chromium stress

The total chlorophyll content in *B. rapa* L. seedlings was measured under hexavalent chromium (Cr) stress (300 µg mL^{-1}), both with and without the application of biochar. Compared to the control seedlings, the chlorophyll content was significantly lower under Cr stress (Figure 3). However, the addition of biochar (1 g and 2 g) under Cr stress significantly restored chlorophyll levels, bringing them closer to those of the control group ($p < 0.05$). Additionally, applying 1 g and 2 g of biochar in the absence of Cr stress led to an increase in chlorophyll production beyond the control levels ($p < 0.05$), with the 2 g biochar treatment showing the most substantial enhancement. These results demonstrate that biochar effectively reduces the negative impact of Cr on photosynthetic pigments and promotes increased chlorophyll synthesis in *B. rapa* seedlings.

The Cr treatment reduced chlorophyll a, chlorophyll b, and total chlorophyll content, which reflects inhibited photosynthesis. This decline is attributed to the overproduction of reactive oxygen species (ROS) induced by Cr, disrupting chlorophyll biosynthesis, degrading photosynthetic enzymes, and damaging chloroplast ultrastructure (Yue et al. 2025). In addition, biochar reduces chromium bioavailability through adsorption, thereby mitigating oxidative stress and supporting pigment synthesis. The significant effect observed in biochar likely reflects improved root health, enhanced nutrient uptake, and increased antioxidant capacity, which collectively minimize ROS damage and boost photosynthetic efficiency (Rabiya et al., 2022; Yue et al., 2025).

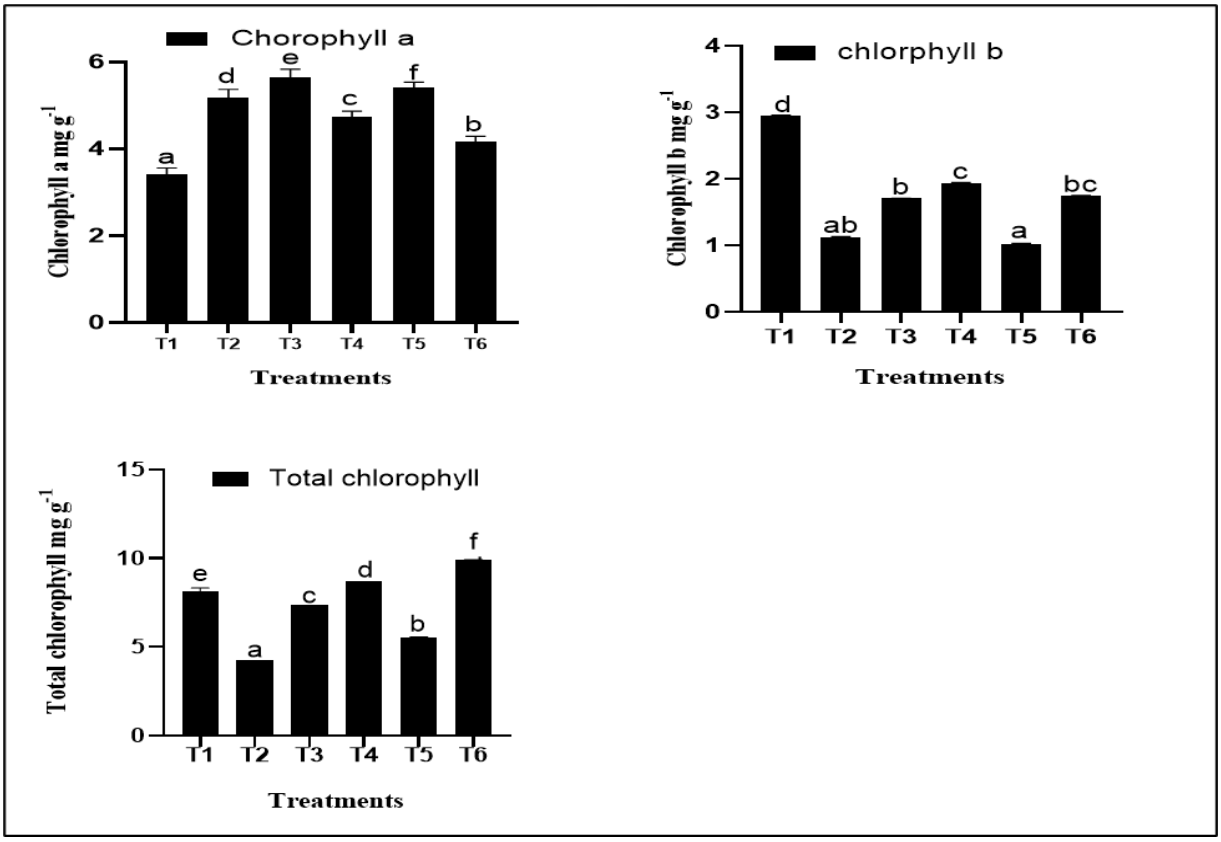

Figure 3. Impact of chromium stress and biochar on the level of chlorophyll a, chlorophyll b, and total chlorophyll of *B. rapa* seedlings. T1 = Control, T2 = Cr. 300 µg mL^{-1}, T3 = 1g Biochar, T4 = 2g Biochar, T5 = 1g Biochar + Cr. 300 µg mL^{-1} and T6 = 2g Biochar + Cr. 300 µg mL^{-1}. Data are expressed as mean ± standard deviation (n = 3). Different letters indicate significant differences among treatments at $p \leq 0.05$, according to Tukey's HSD test.

Effects of biochar on phytohormone content in *B. rapa* under chromium stress

Indole-3-acetic acid (IAA) concentrations in Brassica seedlings were measured under chromium stress, both with and without the addition of biochar. The inclusion of 2 grams of biochar significantly increased IAA levels compared to both the control group and the chromium stress group (Figure 4). Similarly, salicylic acid (SA) concentrations were evaluated under the same conditions. The application of 1 gram and 2 grams of biochar significantly enhanced SA levels compared to the control and the chromium stress treatments. In contrast, chromium stress alone led to a reduction in both IAA and SA concentrations. However, when biochar was combined with chromium stress, the levels of IAA and SA were restored to levels that were comparable to or even exceeded those of the control group.

Phytohormones like IAA and SA act as key components in regulating plant growth and responses to stress conditions. During Cr stress conditions, there is a considerable reduction in the levels of IAA and SA mainly because of interference in their metabolic processes. There are recent findings that show that the presence of toxic metals like Cr leads to imbalance in hormones and growth regulation in plants. The results obtained indicate an effective alleviation of the toxic effects induced by chromium using biochar at 2 g. The reasons for such alleviation could be attributed to biochar's role in immobilizing chromium, which decreases its availability in soil and improves physical and chemical characteristics of the soil and microbial activities (Liu et al., 2022). It is important to note that SA serves as a signaling molecule responsible for improving antioxidant defense mechanisms and controlling stress-responsive pathways under abiotic stresses (Emamverdian et al., 2020). Likewise, IAA plays an indispensable role in cell division and elongation processes.

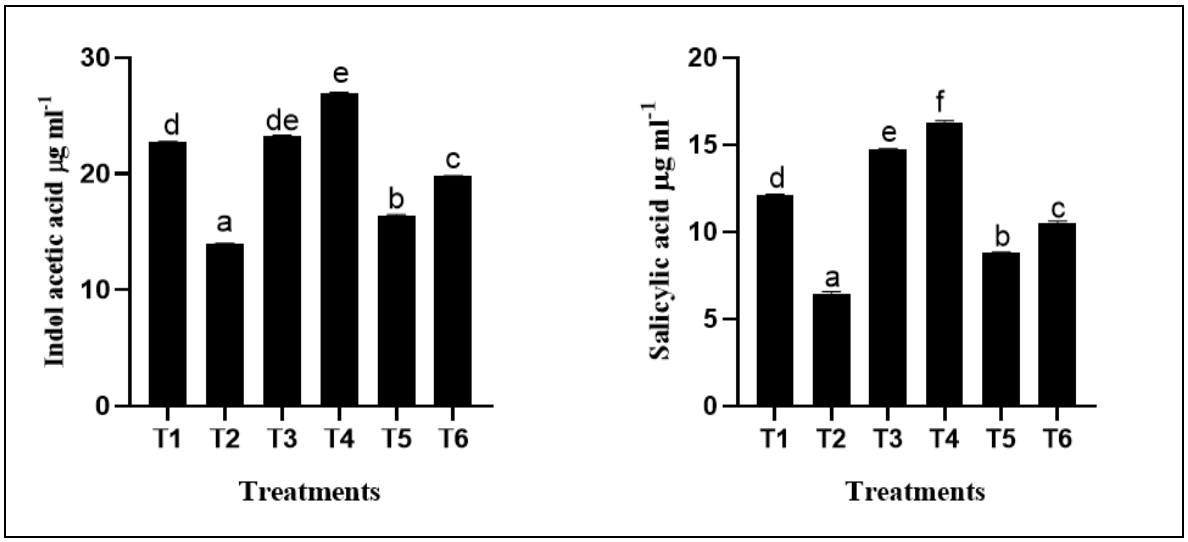

Figure 4. Impact of chromate stress and fungal biochar on the amount of IAA and SA in the leaves of *B. rapa* seedlings. T1 = Control, T2 = Cr. 300 µg mL^{-1}, T3 = 1g Biochar, T4 = 2g Biochar, T5 = 1g Biochar + Cr. 300 µg mL^{-1}, and T6 = 2g Biochar + Cr. 300 µg mL^{-1}. Data are expressed as mean ± standard deviation (n = 3). Different letters indicate significant differences among treatments at $p \leq 0.05$, according to Tukey's HSD test.

Effects of biochar on flavonoids, phenol, protein, and sugar contents in *B. rapa* under chromium stress

The concentrations of flavonoids, phenols, proteins, and sugars in Brassica seedlings were measured under chromium stress, both with and without the addition of biochar (Figure 5). Chromium stress significantly reduced flavonoid concentrations compared to control and other treatment groups. However, the application of biochar alongside chromium stress helped to restore flavonoid levels, with both 1 g and 2 g additions leading to significant increases in flavonoid concentrations. Similarly, phenol content decreased under chromium stress but was restored when biochar was applied. The addition of 1 g and 2 g of biochar further enhanced phenol concentrations compared to the control.

Protein and sugar contents were also examined under these conditions. Alone, chromium stress reduced protein levels, but adding 1 g and 2 g of biochar significantly increased protein concentrations compared to both the control and the stress treatment. Likewise, sugar concentrations dropped under chromium stress, but the introduction of 1 g and 2 g of biochar significantly boosted sugar levels, surpassing both the control and the chromium stress conditions.

In addition to this, it has been established that soil enriched with biochar is favorable for the growth of microbes. These microbes have the ability to produce phytohormones, including auxins, which further enhance the concentration of IAA present within the plant, thus bringing about a balance in the hormones (Ramzan et al., 2023). Moreover, it has been recently reported that the use of biochar helps in enhancing antioxidant activity and stress resistance in the presence of heavy metals like chromium.

In our study, we observed that when *B. rapa* was exposed to chromium stress, the concentrations of flavonoids decreased. This decline aligns with earlier research, suggesting that heavy metal stressors can hinder the formation of secondary metabolites in plants, such as flavonoids. Flavonoids play a critical role in plant defense systems and antioxidant activities, so a decrease in their levels indicates a compromised oxidative stress defense system. Interestingly, the addition of biochar derived from endophytic fungi helped restore flavonoid concentrations. The notable increase in flavonoid and phenol contents observed with the application of biochar under non-stressed conditions may suggest a stimulating effect on the phenylpropanoid pathways, as reported by Franić et al. (2025).

Additionally, the enhanced levels of protein and sugar resulting from biochar application indicate improved nitrogen metabolism and photosynthetic efficiency, particularly under chromium (Cr) stress. These findings align with those of Younis et al. (2015), who noted that biochar promotes carbohydrate accumulation by enhancing soil water retention and reducing metal toxicity. This finding is significant because it suggests that biochar may act as a buffer against the detrimental effects of chromium stress (Naveed et al., 2021). Biochar from endophytic fungi might be particularly effective in enhancing flavonoid production, potentially by improving nutrient availability or altering microbial populations.

Additionally, our study showed a decrease in phenol concentrations under chromium stress, which is consistent with previous findings indicating that heavy metal stressors often lead to reduced phenol synthesis. Phenols serve as antioxidants and contribute to the plant's defense mechanisms against various abiotic stresses (Kumar et al., 2023). However, when biochar - especially that derived from endophytic fungi - was applied, phenol concentrations increased. This suggests that the presence of biochar triggers a response in the plant that promotes increased phenol synthesis (Ullah et al., 2026).

Biochar has been shown to influence soil microbe populations and enhance nutrient availability, which appears to be linked to increased phenol synthesis. Additionally, when measuring sugar content, it was found to be lower in subjects under stress. Seedlings grown with biochar produced more sugar than those grown under control conditions or chromium stress. The significant improvement observed in the biochar treatment is linked to enhanced photosynthetic capacity and decreased chromium uptake, which together promote sugar synthesis and accumulation (Christou et al., 2022). Ullah et al. (2026) observed that biochar also elevated the levels of various chemicals, including IAA, proteins, phenols, and other metabolites.

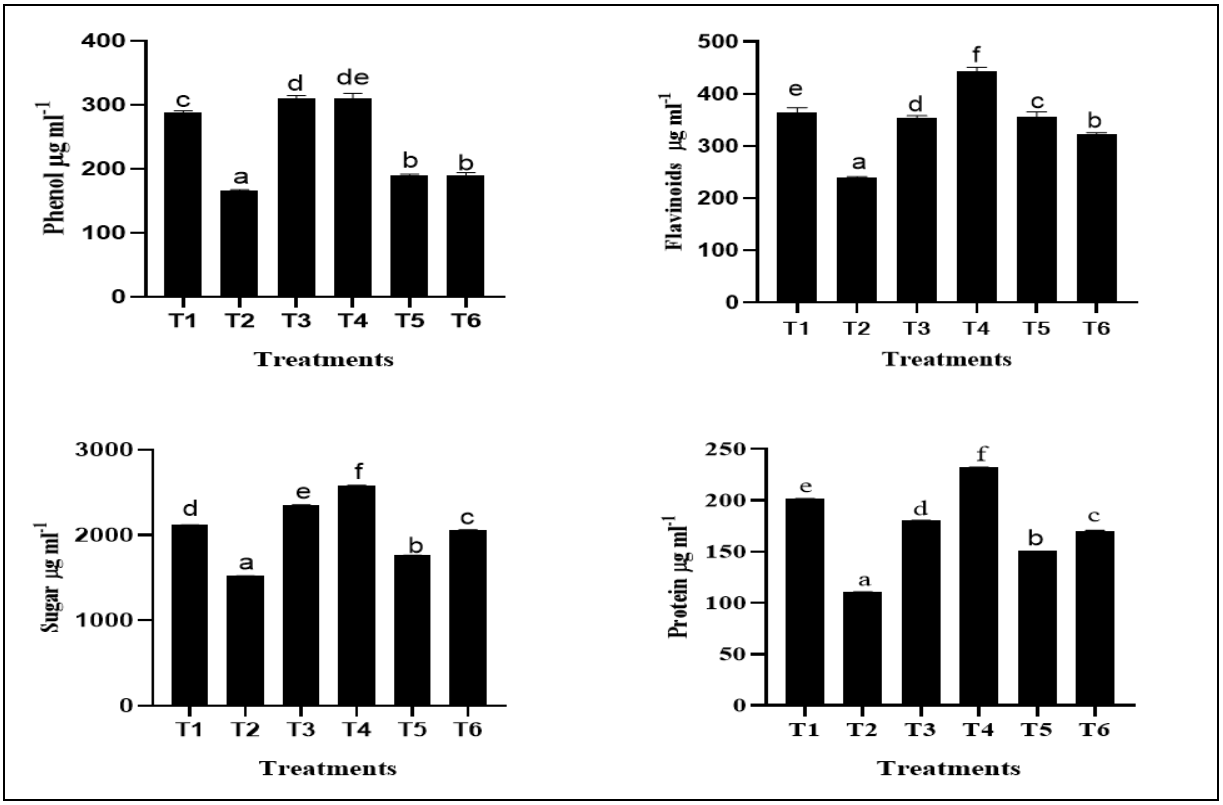

Figure 5. Impact of chromate and fungal biochar on the concentration of phenols, flavonoids, sugars, and protein of *B. rapa* seedlings. T1 = Control, T2 = Cr. 300 µg mL^{-1}, T3 = 1g Biochar, T4 = 2g Biochar, T5 = 1g Biochar + Cr. 300 µg mL^{-1} and T6 = 2g Biochar + Cr. 300 µg mL^{-1}. Data are expressed as mean ± standard deviation (n = 3). Different letters indicate significant differences among treatments at $p \leq 0.05$, according to Tukey's HSD test.

Effect of biochar on root chromium accumulation

The concentration of chromium (Cr) in the roots of *B. rapa* seedlings showed significant variation among the different treatments (Figure 6). The highest accumulation of Cr was observed in plants exposed to chromium stress alone (T2), indicating maximum uptake without the presence of biochar. In contrast, the control group (T1) exhibited relatively lower levels of Cr.

When fungal biochar was applied on its own (T3 and T4), there was a slight reduction in Cr concentration compared to the Cr-treated plants. However, when biochar was combined with chromium stress (T5 and T6), a significant decrease in Cr accumulation was noted. The lowest Cr concentration was recorded in treatment T6 (2 g of biochar + Cr), indicating the most effective mitigation of chromium uptake. The statistical analysis, shown by different letters, confirms significant differences among all treatments ($p < 0.05$), demonstrating the efficacy of biochar in reducing chromium uptake in plant roots.

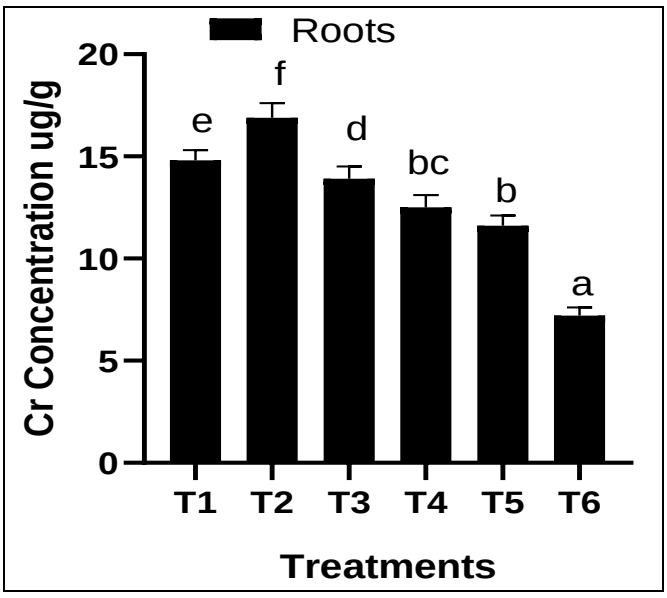

Figure 6. Effect of chromium stress and fungal biochar on chromium (Cr) concentration (µg g^{-1}) in the roots of *B. rapa* seedlings. T1 = Control, T2 = Cr. 300 µg mL^{-1}, T3 = 1g Biochar, T4 = 2g Biochar, T5 = 1g Biochar + Cr. 300 µg mL^{-1} and T6 = 2g Biochar + Cr. 300 µg mL-1. Data are expressed as mean ± standard deviation (n = 3). Different letters indicate significant differences among treatments at $p \leq 0.05$, according to Tukey's HSD test.

CONCLUSIONS

Application of fungal-derived biochar at 1 g and 2 g effectively alleviated chromium (Cr)-induced phytotoxicity in *Brassica rapa* L. by improving plant growth, photosynthetic pigment content, and the accumulation of phytohormones and antioxidant metabolites. The beneficial effects were more pronounced at the higher biochar dose, indicating a dose-dependent response. These findings highlight the potential of fungal biochar as a sustainable and eco-friendly strategy for alleviating heavy metal stress in crops, thereby contributing to improved plant productivity and the remediation of chromium-contaminated soils.

FUNDING

Supported by the Deanship of Scientific Research and Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (KFU264131).

ACKNOWLEDGEMENTS

We thank the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal

University, Saudi Arabia (KFU264131), for supporting this research work. We thank the Department of Botany, Abdul Wali Khan University, Mardan, which provided the facilities and support to do this research.

REFERENCES

Abbas, T., Rizwan, M., Ali, S., Adrees, M., Zia-ur-Rehman, M., Qayyum, M.F., Ok, Y.S., Murtaza, G., 2018. *Effect of biochar on alleviation of cadmium toxicity in wheat (Triticum aestivum L.) grown on Cd-contaminated saline soil.* Environmental Science and Pollution Research, 27(12): 13430-13441.

Abu-Shahba, M.S., Mansour, M.M., Mohamed, H.I., Sofy, M.R., 2022. *Effect of biosorptive removal of cadmium ions from hydroponic solution containing indigenous garlic peel and mercerized garlic peel on lettuce productivity.* Scientia Horticulturae, 293: 110727.

Ali, S., Rizwan, M., Qayyum, M.F., Ok, Y.S., Ibrahim, M., Riaz, M., Arif, M.S., Hafeez, F., Al-Wabel, M.I., Shahzad, A.N., 2017. *Biochar soil amendment on alleviation of drought and salt stress in plants: a critical review.* Environmental Science and Pollution Research, 24(14): 12700-12712.

Ali, S., Rizwan, M., Bano, R., Bharwana, S.A., Rehman, M.Z.U., Hussain, M.B., Al-Wabel, M.I., 2018. *Effects of biochar on growth, photosynthesis, and chromium (Cr) uptake in Brassica rapa L. under Cr stress.* Arabian Journal of Geosciences, 11(17): 507.

Ali, S., Rizwan, M., Noureen, S., Anwar, S., Ali, B., Naveed, M., Abd_Allah, E.F., Alqarawi, A.A., Ahmad, P., 2019. *Combined use of biochar and zinc oxide nanoparticle foliar spray alleviated Cd toxicity in wheat.* Environmental Science and Pollution Research, 25(36): 36491-36501.

Ali, M.H., Abdelkarim, M.S., Mousa, I.E., Belal, D.M., 2025. *Extracted microbial exopolysaccharides: a sustainable approach to bioremediation of nickel, cobalt and copper ions; equilibrium, kinetics and thermodynamics studies.* Chemistry and Ecology, 41(3): 375-395.

Asati, A., Pichhode, M., Nikhil, K., 2016. *Effect of heavy metals on plants: an overview*. International Journal of Application or Innovation in Engineering & Management, 5(3): 56-66.

Brighente, I.M.C., Dias, M., Verdi, L.G., Pizzolatti, M.G., 2007. *Antioxidant activity and total phenolic content of some Brazilian species.* Pharmaceutical Biology, 45(2): 156-161.

Christou, A., Stylianou, M., Georgiadou, E.C., Gedeon, S., Ioannou, A., Michael, C., Papanastasiou, P., Fotopoulos, V., Fatta-Kassinos, D., 2022. *Effects of biochar derived from the pyrolysis of either biosolids, manure or spent coffee grounds on the growth, physiology and quality attributes of field-grown lettuce plants.* Environmental Technology & Innovation, 26: 102263.

Chun, O.K., Kim, D.O., Lee, C.Y., 2003. *Superoxide radical scavenging activity of the major polyphenols in fresh plums.* Journal of Agricultural and Food Chemistry, 51(27): 8067-8072.

El-Mahdy, O.M., Mohamed, H.I., Mogazy, A.M., 2021. *Biosorption effect of Aspergillus niger and Penicillium chrysosporium for Cd-and Pb-contaminated soil and their physiological effects on Vicia faba L.* Environmental Science and Pollution Research, 28(47): 67608-67631.

Emamverdian, A., Ding, Y., Mokhberdoran, F., 2020. *The role of salicylic acid and gibberellin signaling in plant responses to abiotic stress with an emphasis on heavy metals.* Plant Signaling Behavior, 15(7): 1777372.

Franić, M., Palčić, I., Marcelić, Š., Major, N., Ban, D., Kovačević, T.K., Anđelini, D., Prelac, M., Cvitan, D., Goreta Ban, S., Užila, Z., 2025. *Modulating Secondary Metabolite Content in Olive Leaves Through Foliar Application of Biochar and Olive Leaf-Based Phenolic Extracts.* Sustainability, 17(24): 11290.

Gordon, S.A., and Weber, R.P., 1951. *Colorimetric estimation of indoleacetic acid.* Plant Physiology, 26: 192-195.

Gu, R., Fu, D., Jin, Y., Jia, M., Nie, K., 2023. *Aspergillus niger fermentation residues application to produce biochar for the anode of lithium-ion batteries.* Journal of Environmental Management, 346: 118985.

Haiying, T.A.N.G., Shubin, W.A.N.G., Ying, L.I.U., Ying, S.O.N.G., Yasser, S.M., 2022. *Biochar: A promising soil amendment to mitigate heavy metals toxicity in plants.* Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 50(3): 12778-12778.

Handa, N., Kohli, S.K., Kaur, R., Sharma, A., Kumar, V., Thukral, A.K., Arora, S., Bhardwaj, R., 2018. *Role of compatible solutes in enhancing antioxidative defense in plants exposed to metal toxicity*. Plants under metal and metalloid stress: responses, tolerance and remediation, Singapore, Springer Singapore: 207-228.

Jack, J., Huggins, T.M., Huang, Y., Fang, Y., Ren, Z.J., 2019. *Production of magnetic biochar from waste-derived fungal biomass for phosphorus removal and recovery.* Journal of Cleaner Production, 224: 100-106.

Kumar, K., Debnath, P., Singh, S., Kumar, N., 2023. *An overview of plant phenolics and their involvement in abiotic stress tolerance.* Stresses, 3(3): 570-585.

Liu, J., Qiu, G., Liu, C., Li, H., Chen, X., Fu, Q., Lin, Y., Guo, B., 2022. *Salicylic Acid, a Multifaceted Hormone, Combats Abiotic Stresses in Plants.* Life (Basel), 12(6): 886.
doi: 10.3390/life12060886

Liu, L., Li, J., Yue, F., Yan, X., Wang, F., Bloszies, S., Wang, Y., 2018. *Effects of arbuscular mycorrhizal inoculation and biochar amendment on maize*

growth, cadmium uptake and soil cadmium speciation in Cd-contaminated soil. Chemosphere, 194: 495-503.

Maclachlan, S., and Zalik, S., 1963. *Plastid structure, chlorophyll concentration, and free amino acid composition of a chlorophyll mutant of barley.* Canadian Journal of Botany, 41(7): 1053-1062.

Mohamed, H.I., 2011. *Molecular and biochemical studies on the effect of gamma rays on lead toxicity in cowpea (Vigna sinensis) plants*. Biological Trace Element Research, 144(1): 1205-1218.

Mohamed, H.I., Ullah, I., Toor, M.D., Tanveer, N.A., Din, M.M.U., Basit, A., Sultan, Y., Muhammad, M., Rehman, M.U., 2025. *Heavy metals toxicity in plants: Understanding mechanisms and developing coping strategies for remediation: A review.* Bioresources and Bioprocessing, 12(1), 95.

Naveed, M., Tanvir, B., Xiukang, W., Brtnicky, M., Ditta, A., Kucerik, J., Subhani, Z., Nazir, M.Z., Radziemska, M., Saeed, Q., Mustafa, A., 2021. *Co-composted biochar enhances growth, physiological, and phytostabilization efficiency of Brassica napus and reduces associated health risks under chromium stress.* Frontiers in Plant Science, 12: 775785.

Rabiya, U.E., Ali, M., Farooq, M.A., Siddiq, Z., Alamri, S.A., Siddiqui, M.H., Khan, W.U.D., 2022. *Comparative efficiency of silica gel, biochar, and plant growth promoting bacteria on Cr and Pb availability to Solanum melongena L. in contaminated soil irrigated with wastewater.* Frontiers in Plant Science, 13: 950362.

Ramzan, M., Jamshaid, T., Ali, L., Dawar, K., Saba, R., Jamshaid, U., Fahad, S., Salmen, S.H., Ansari, M.J., Danish, S., Hareem, M., 2023. *Modulation of sunflower growth via regulation of antioxidants, oil content and gas exchange by arbuscular mycorrhizal fungi and quantum dot biochar under chromium stress.* BMC Plant Biology, 23(1), 629.

Rover, M.R., Johnston, P.A., Lamsal, B.P., Brown, R.C., 2013. *Total water-soluble sugars quantification in bio-oil using the phenol-sulfuric acid assay.* Journal of Analytical and Applied Pyrolysis, 104: 194-201.

Sahin, S., Kısa, D., Ataklı, S.B., 2022. *Determination of changes in nutritional elements and antioxidant amounts in the plant by application of heavy metals (Cu, Pb, Cd) to corn plant (Zea mays L.).* Romanian Agricultural Research, 39: 57-66. https://doi.org/10.59665/rar3906

Shahid, M., Shamshad, S., Rafiq, M., Khalid, S., Baffoe, B., Dumat, C., 2017. *Chromium speciation, bioavailability, uptake, toxicity and detoxification in soil-plant system: A review.* Chemosphere, 178: 513-533.

Singh, H.P., Mahajan, P., Kaur, S., Batish, D.R., Kohli, R.K., 2013. *Chromium toxicity and tolerance in plants.* Environmental Chemistry Letters, 11(3): 229-254.

Sofy, M., Mohamed, H., Dawood, M., Abu-Elsaoud, A., Soliman, M., 2022. *Integrated usage of Trichoderma harzianum and biochar to ameliorate salt stress on spinach plants.* Archives of Agronomy and Soil Science, 68(14): 2005-2026.

Tang, J., Zhang, L., Zhang, J., Ren, L., Zhou, Y., Zheng, Y., Luo, L., Yang, Y., Huang, H., Chen, A., 2020. *Physicochemical features, metal availability and enzyme activity in heavy metal-polluted soil remediated by biochar and compost.* Science of the Total Environment, 701: 134751.

Ullah, Z., Khan, W., Shah, N., Ahmad, A., Mohamed, H.I., Ahmed, A.R., Elkatry, H.O., Ismail, A.M., El-Mogy, M.M., Elmenofy, W., 2026. *Synergistic role of biochar and beneficial Aspergillus fumigatus (P2P) in improving growth, physiological performance, and antioxidant activity of garlic (Allium sativum L.).* Journal of Soil Science and Plant Nutrition, 26(2): 4856-4869.

Warrier, R.R., Paul, M., Vineetha, M.V., 2013. *Estimation of salicylic acid in Eucalyptus leaves using spectrophotometric methods.* Genetics and Plant Physiology, 3(1-2): 90-97.

Younis, U., Qayyum, M.F., Shah, M.H.R., Danish, S., Shahzad, A.N., Malik, S.A., Mahmood, S., 2015. *Growth, survival, and heavy metal (Cd and Ni) uptake of spinach (Spinacia oleracea) and fenugreek (Trigonella corniculata) in a biochar-amended sewage-irrigated contaminated soil.* Journal of Plant Nutrition and Soil Science, 178(2): 209-217.

Yue, S., Li, W., Qin, F., Dong, M., Weng, G., Ali, H.M., Weng, J., Mehmood, S., 2025. *Assessment of biochar filter application in improving chromium stress tolerance and plant physiology in Chinese cabbage (Brassica rapa) under a flow-through water setup.* BMC Biotechnology, 25, 74.

Zhou, P., and Regenstein, J.M., 2006. *Determination of total protein content in gelatin solutions with the Lowry or Biuret assay.* Journal of Food Science, 71(8): C474-C479.