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INTERACTIVE POTENTIAL OF *BACILLUS MEGATERIUM* A12 AND BIOCHAR IN CHROMIUM STRESS MITIGATION IN *SPINACIA OLERACEA*: METHYLGLYOXAL DETOXIFICATION AND ACTIVATION OF ANTIOXIDANT ENZYMES

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Abstract

Metallic toxins are potential threats to human health and the achievement of optimum plant growth. In these toxins, chromium (Cr) is carcinogenic. Its higher plant accumulation caused oxidative damage by producing reactive oxygen species (ROS). However, antioxidant biosynthesis is a natural defensive mechanism that can alleviate Cr toxicity in plants. Furthermore, biochar (BC) addition in soil has been reported as an effective amendment for Cr immobilization. Inoculation of rhizobacteria is also well documented as an efficacious tool for improvement in plant growth under heavy metals stress. The present study was conducted to cover the knowledge gap of the combined use of *Bacillus megaterium* (A12) and BC for chromium (Cr) stress mitigation in *Spinacia oleracea*. There were eight treatments with five replications following a completely randomized design. Results showed that in Cr-contamination, A12 and BC decreased malondialdehyde (MDA) and electrolyte leakage (EL) in *S. oleracea* plants. A12 and BC significantly improved catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) activity. A significant increase in shoot dry weight (27%), chlorophyll a (31%), phenolic contents (59%) and total chlorophyll (32%) validated the efficacious role of BC+A12 over control. Furthermore, Cr stress alleviation was credited to the increment activity of antioxidative enzymes and the detoxification of methylglyoxal, besides increasing ascorbic acid and proline content. In conclusion, the synergistic interaction of BC and A12 is an efficacious approach to mitigating abiotic stresses in plants. More investigations are suggested at the field level to declare the best application rate of BC with A12 to alleviate Cr stress in different crops.

Key words: Activated carbon; Heavy metals; glyoxalase; Rhizobacteria; Spinach; Stress alleviation.

Introduction

Biochar is a high-carbon residue produced from organic material in anaerobic conditions by a thermo-chemical process known as pyrolysis, and it is widely applied as a soil amendment for the improvement of soil structure and fertility (Lehmann & Rondon, 2002). Biochar production from organic disposal is increasing to prevent harmful effects on plant growth (Lehmann *et al*., 2006). Biochar is used as fertilizer (Danish *et al*., 2015a; b). Wood, Crop debris, organic waste material and poultry litter can be used for biochar production (Sehrish *et al*., 2019). Biochar causes the immobilization of heavy metals and other unwanted materials in the soil (Nzediegwu *et al*., 2020). Biochar characteristic varies accordingly to the conditions in which it is produced and feedstock material (Khan *et al*., 2020). Applying biochar increases the plant's resistance to heavy metal stress (Danish *et al*., 2019).

On the other hand, plant-growth-promoting bacteria (PGPB) grow in the rhizospheric zone of the plant (Zafar-ul-Hye *et al*., 2018, 2020a;c; Ahmed *et al*., 2020; Danish *et al*., 2020a; Zafar-ul-Hye *et al*., 2020). Plant growth-promoting rhizobacteria are free-living bacteria that inhabit plant roots and flourish plant growth (Danish *et al*., 2015d; Zafar-ul-Hye *et al*., 2019; Zafar-ul-Hye *et al*., 2019). The classification of PGPR can be done based on its positive effects. Plant growth gets influenced by the degree of colonization of plants and bacteria (Grobelak *et al*., 2015). PGPR uses various direct and indirect mechanisms to improve plant growth (Kalam *et al*., 2017). Many species of bacteria and fungi form intricate associations with plants (Danish *et al*., 2020b; Wahid *et al*., 2020; Saboor *et al*., 2021). However, toxicity of heavy metals can decrease their proliferations and can induced toxicity in the cultivated crops.

It is well documented that metallic contaminants are released into the surrounding area through natural and human-induced activities. The incorporation of metal pollutants into the ecosystem is continuously increasing (Dotaniya *et al*., 2018). Heavy metals are prevalent hazardous wastes discharged into the environment, which have caused malfunctioning in plant metabolomics (Zaheer *et al*., 2020). Soil absorbs different heavy metals (Alia *et al*., 2015). Many heavy metals are non-

degradable and accumulate in vegetables grown in soil (Khan *et al*., 2020). The incorporation of heavy metals in the food chain can be destructive to human health (Eid *et al*., 2017). A higher amount of heavy metals is absorbed by green leafy vegetables. Contamination of the environment with heavy metals is a major global issue, and many researchers are trying to remove it from different contaminated areas (Dotaniya *et al*., 2018).

Chromium is also a heavy metal constantly discharged through artificial and natural means (Sehrish *et al*., 2019). Chromium is present in nature in two forms, i.e., CrIII and CrVI (Hamilton *et al*., 2020). Chromium in the soil is highly injurious to human health because it can enter the food web via polluted soil (Sehrish *et al*., 2019). Chromium moves inside humans' bodies through food polluted with Cr and can cause cancer, ulcer, and liver diseases (Zaheer *et al*., 2020). Chromium can be noxious and detrimental to growing plants (Sehrish *et al*., 2019). The harmfulness of chromium reduces the growth of crops by influencing chlorophyll biosynthesis (Danish *et al*., 2019).

Excessive Cr in plants produces reactive oxygen species (ROS). Higher levels of ROS reasons destructive effects to plant structure and appearance (Sehrish *et al*., 2019). The toxic effect of Cr can adversely affect nutrients and water uptake by plants (Dotaniya *et al*., 2018). Excessive absorption of Cr leads to poor seed germination (Danish *et al*., 2019). Severe Cr toxicity leads to affected plants' death (Zaheer *et al*., 2020). Chromium toxicity is a growing problem for numerous cultivated crops (Danish *et al*., 2019). Various methods have been developed to remove the toxic repercussions of Cr on plants (Sehrish *et al*., 2019). Cr aggregation in plants and food materials should be eliminated (Zaheer *et al*., 2020).

Spinacia oleracea belongs to the order Amaranthaceae and consists of vegetables with wide, green foliage (Alia *et al*., 2015). It is among the globally cultivated vegetable because it is fast-growing and have high biofuel production and heavy metals usage (Zaheer *et al*., 2020). *S. oleracea* is also well known for nutritional homeostasis in organisms (Kumar *et al*., 2016). It is enriched with beta carotene, calcium, vitamin, phosphorous, carbon, potassium and iron (Danish *et al*., 2019). According to a recent estimate, the world's annual spinach production has reached about 24 million tons. Researchers widely select *S. oleracea* because of its fast growth rate and worldwide usage (Sardar *et al*., 2020). However, elevated concentration of Cr results in a disturbance in the growth of *S. oleracea* (Zaheer *et al*., 2020).

Many genera of bacteria are included in PGPR; some are *Pseudomonas*, *Bacillus*, *Acetobactor*, *Azotobactor*. PGPR is more important for nitrogen-deficient soil. Thus, bacteria efficiently promote plant growth by supplying the required elements (Grobelak *et al*., 2015). PGPR increases the growth of plants by using various methods like atmospheric nitrogen fixation and secretion of plant hormones (Gómez-Sagasti & Marino, 2015). They improve the plant's growth rate by enhancing the germination rate, amount of nitrogen, and crop yield (Zafar-ul-Hye *et al*., 2020b).

As PGPR inoculation has attained attention in stress scenarios, the current study was revealed to analyze the interactive perspective of *Bacillus megaterium* A12 and biochar in alleviating Cr toxicity in *S. oleracea.* The present study was conducted to cover the knowledge gap of the combined use of *Bacillus megaterium* (A12) and BC for chromium (Cr) stress mitigation in *Spinacia oleracea*. Moreover, this research work was performed to explore the influence of *B. megaterium* A12 alone or in combination with biochar on the growth and physiology of *S. oleracea*. It is hypothesized that *B. megaterium* A12 and biochar have the potential to alleviate Cr toxicity in *S. oleracea*.

Materials and Methods

Growth of *Spinacia oleracae*: *Spinacia oleracea* seeds attained from Punjab Seed Corporation Pakistan were decontaminated by dipping for 3 minutes in sodium hypochlorite solution and washed with distilled H_2O. Before sowing in plastic pots, seeds were air-dried for 24 hours at 25^oC by placing them over the blotting paper. After 52 days, the plants were harvested. The samples of plants collected from treated pots were rinsed with distilled H_2O and air-dried. Afterwards, fresh biomass of plant samples was noted. Then, plant samples were oven-dried for 72 hours at 65^oC to calculate the dry weight.

Soil analysis: The soil samples used to execute this experiment were taken from the Botany Department, University of Narowal, Pakistan. The soil used for the current research was acquired from a depth of 0-12 inches. The debris and mud particles were set apart using a 3mm sieve. Soil organic content was thoroughly analyzed using the described method. The soil components' dimensions were determined using a hydrometer (Bouyouces, 1962). Paper bags were used to store soil samples and carry out further analysis. The benchtop meter was utilized to measure pH (Page *et al*., 1982) and electrical conductivity (EC) (Rhoades, 1996) and the texture of the soil was measured with Mastersizer 2000.

Preparation of biochar: Bamboo was used for biochar production through pyrolysis at a temperature ranging from 300-600^oC. There are two primary reasons behind the selection of bamboo. The first is high lignocellulosic content; the second, bamboo is used in construction and paper. Before preceding pyrolysis, the biomass was ground and kiln-dried for 24 h at 700^oC. The pyrolysis of ground biomass was done in a steel crucible fitted with a Neylimited supply of oxygen. Slow pyrolysis was achieved by increasing temperature at the rate of tech Muffle Furnace with a 10^oC per minute and sustained at 300-600^oC (Qayyum *et al*., 2014).

Experimental setup: The research was executed in the research area of the Botany Department, University of Narowal, Pakistan, under a controlled set-up using pots. *S. oleracea* was selected as an experimental plant. The soil

samples used for the current study were dried in an oven for 2 hours. Dried soil was crushed with the help of Agate mortar and passed through a 2mm sieve. Under treatment, each pot was crowded with 4kg. Before putting in pots, spinach seeds were sterilized for 10 min using 30% H_2O_2 solution. The experimental study was designed in randomized block design having five replicates.

Assessment of chromium resistance: For the separation of Cr-resistant bacteria, the dilution plate method was selected utilizing Luria-Bertani (LB) agar medium surcharged with 50 mg L^{-1} of Cr as $K_2Cr_2O_7$. The plates were incubated for 3-4 days at 30 ± 2° C. The most noticeable separates of elected based on their physical factors were decontaminated through frequent splotching over the same particular means. The separates displaying maximum resistance to Cr were preferred for further studies (Bruno *et al*., 2020). *Bacillus megaterium* strain A12 showed maximum resistance upto 25 mg L^{-1} of Cr.

Determination of chlorophyll and carotenoid content: The fresh green leaves of spinach were examined to find the concentration of Chl by using the Arnon (1949) and Ravelo-Perez *et al*., (2008) methodologies. The leaf extract sample was incubated in an 80% acetone solution. The absorbance value on the spectrophotometer was calibrated to determine the concentration of Chl. *A*, Chl. *B*, and total Chl. Content. Quantification of carotenoids was carried out by adopting the methodology of Alba *et al*., (2005).

Assessment of chromium content: To determine the amount of Cr on atomic absorption spectrophotometer, the samples of leaves and roots were dissolved utilizing a mixture of acids (HNO_3; $HClO_4$) (Chapman & Pratt, 1961).

Assessment of electrolyte leakage (EL): Electrolyte leakage analysis was done using the technique of Lutts *et al*., (1996). Firstly, spinach foliage samples were rinsed with distilled H_2O and then, using a steel cylinder, discs of 1cm diameter were prepared. 1g discs of equal size were dipped in about 20 ml distilled H_2O and then incubated for a day at 25°C. In the next step, determine the first electrical conductivity (EC1), while determining the second electrical conductivity (EC2), the test tubes were heated in a water bath for 20 min at 120°C (Lutts *et al*., 1996). The final electrical leakage calculation was done using the following formula:

$$EL\ (\%) = EC1 / EC2 \times 100$$

Determination of MDA and H_2O_2 content: Lipoxidation value was assessed by quantification of MDA content which was estimated through the thiobarbituric acid (TBA) reactions (Cakmak & Horst, 1991). Foliage samples (0.5 g) were vortexed in 0.1 % tricarboxylic acid (10 ml) and subjected to centrifugation at 12,000 × g for 5 min. The supernatant (1 ml) was added to 0.5% TBA (4 ml). Afterwards, the solution obtained was placed at 95°C for 30 minutes, cooled and subjected to centrifugation at 12,000 rmp for 5 min. The absorbance value of the supernatant was calibrated using UV-VIS spectrophotometer. For the evaluation of MDA content, an extinction coefficient 155 mM^{-1} cm^{-1} was used.

Okuda *et al*., (1991) technique was used to estimate hydrogen peroxide content. 250 mg leaf sample was grounded in ice with the help of perchloric acid (200 mM). The sample was centrifuged at 1200 rpm for ten minutes. The supernatant obtained was mixed with 4 M KOH. Insoluble $KClO_4$ was removed through centrifugation at 500 rpm for 3 min. afterwards, peroxidase was added, and the spectrophotometric value was measured at 590 nm.

Quantification of proline and ascorbate: Bates *et al*., (1973) method was employed for assessing proline content. Fresh leaves (600 mg) were mixed with 6 mL of 3% sulfosalicylic acid. The obtained filtrate was thoroughly mixed with 1 ml glacial acetic acid and ninhydrin for 60 min. A solution containing a test tube was kept in the water bath at a temperature of 100°C. The reaction was completed by keeping the test tube on ice. Toluene was used for extraction, and the absorbance value was calibrated at 520 nm.

To quantify ascorbate, Keller and Schwanger (Keller & Schwager, 1977) protocol was used that includes 2,6-dichlorophenol-Indophenol's dye procedure. The foliage sample (1000 mg) was mixed in the ice bath using extracting solution (40 ml). The homogeneous mixture was obtained was subjected to centrifugation at 6000 rpm for 15 min. The supernatant (2 ml) obtained was homogenized with 2, 6-dichlorophenol-Indophenol solution (5 ml). Afterwards, the colourimetric value of the mixture was measured at 520 nm.

Assessment of methylglyoxal level: Assessment of methylglyoxal value was carried out as per the methodology given by Wild *et al*., (2012). A homogenous mixture was obtained using perchloric acid (5 %) and later centrifuged at 11,000 rpm for 10 min. Charcoal was added for the decolourization of the supernatant. A sodium carbonate solution was added to neutralize the decolorized supernatant. Afterwards, this neutralized supernatant was used to measure MG by adding sodium N-acetyl-Lcysteine and dihydrogen phosphate to make a final volume of 1 mL. Following ten minutes, N-α-acetyl-S-(1-hydroxy-2-oxoprop-1-yl) cysteine formation was calibrated at 288 nm. Mathyglyoxal level was evaluated by comparing it with a standard curve of known concentration.

Determination of phenolic content and flavonoids: Folin Ciocalteu procedure was adopted for the quantification of total phenolic content (Kaur & Kapoor, 2002). About 200L of extract in crude form (1 mg/ml) was prepared by dissolution of 3 mL distilled H_2O with 0.5 mL of Folin Ciocalteu reagent for three minutes, and finally, 2 mL sodium carbonate (80 % v/w) was mixed. The prepared solution was placed in the absence of light for 1 hr. Subsequently, the absorbance value was calibrated at 650 nm. The phenolic amount was estimated by employing a calibration curve.

The total flavonoid value was quantified by using the $AlCl_3$ colourimetric method (Chang *et al*., 2002). Methanolic extract (1 mg/ mL) was prepared up to 1 mL through dissolution with methanol (1 mL), distilled water (4 mL), $NaNO_2$ solution and $AlCl_3$ solution (10 %), which were added following 5 min of incubation. After 6 minutes of the standing mixture, 2 mL NaOH solution (1 mol/L) was mixed to obtain a final volume of 10 mL by using double-distilled H_2O. Following 15 minutes in which the mixture was allowed to stand, the absorbance value was calibrated at 510 nm. The total flavonoid value was quantified using the standard curve.

Estimation of antioxidative enzymes: The 0.5 g leaf sample was subjected to homogenization in pre-chilled phosphate buffer (50 mM) at pH 7.0, having AsA (1 mM), KCl (100 mM), β-mercaptoethanol (5 mM) and glycerol (10%, w/v) with the help of pre-chilled mortar and pestle. The homogenate obtained was centrifuged at 11,500 rpm for 10 min, and the supernatant was used to determine the activity of antioxidative enzymes. A temperature range of 0–4°C was retained to determine the activity of all antioxidant enzymes. Lipoxygenase activity was assessed per the method narrated by Doderer *et al*., (1992). The spectrophotometric value was analyzed at 234 nm using linolenic acid as substrate. The activity of the antioxidant enzyme was estimated using an extinction coefficient of 25 mM^{-1} cm^{-1}. Superoxide dismutase activity was evaluated as per the method narrated by El-Shabrawi *et al*., (2010). For this, the xanthine-xanthine oxidase system was used. The reaction mixture included catalase (0.1 units), xanthine (2.36 mM), 2.24 mM NBT, 50 mM K-P buffer, xanthine oxidase (0.1 units) and enzyme extract. The absorbance value was calculated at 560 nm.

The reduction in spectrophotometric value due to the decomposition of H_2O_2 was measured at 240 nm. The reaction mixture contained 15 mM H_2O_2, 50 mM K-P buffer at pH 7.0 and enzymatic solution, making volume up to 700 µL. For the estimation of catalase activity, the extinction coefficient 39.4 M^{-1} cm^{-1} was used (Chance & Maehly, 1955). To estimate ascorbate peroxidase (APX) activity, the volume of the reaction mixture composed of 0.1 mM EDTA, 50 mM phosphate buffer at pH 7.0, 0.1 mM H_2O_2 was made 700 µL. The activity was measured in terms of decreased absorbance at 290 nm with the help of an extinction coefficient 2.8 mM^{-1} cm^{-1} (Sevilla, 1998).

Statistical analysis

The SPSS was employed to examine the data. One-way ANOVA was performed on the collected value. MS-Excel was used to calculate the mean, standard deviation (SD) and coefficient of correlation (r-value) of soil and parameters of the crop with the concentration of sludge (ver. 2003 Microsoft Redmond Campus, Redmond, WA), and a sigma plot was used to create a graph (ver.12.3, Systat Software, Inc., Chicago, IL) (Steel *et al*., 1997).

Results

Effect of biochar and PGPB on growth parameters: Chromium stress hampered root and shoot length, leaf area, fresh and dry weight of root, and shoot of *S. oleracea* plants compared with CK treatment. Individual application of biochar and combined treatment of biochar and PGPB enhanced the growth of *S. oleracea* grown in normal and Cr-polluted soil. Combined treatment of biochar and PGPB enhanced root length, shoot length, leaf area, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight by 17.45%, 26.31%, 16.03%, 17.51%, 25.95%, 18.25% and 26.31% respectively in *S. oleracea* seedlings grown in Cr-toxic soil, as compared to Cr-only treatment (Table 1).

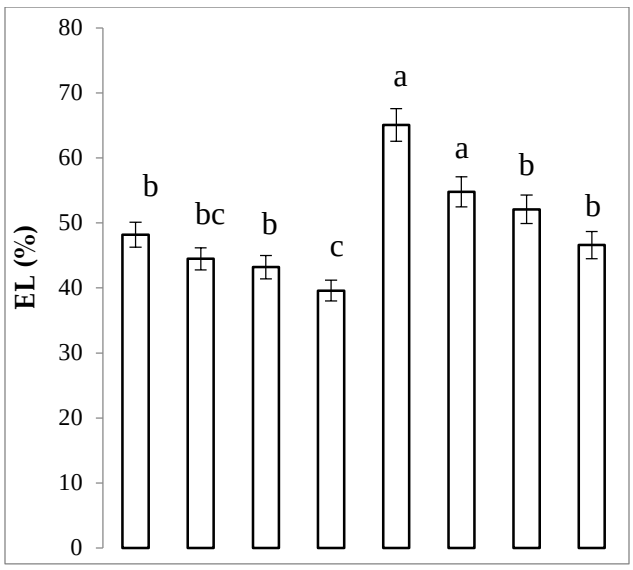
Effect of biochar and PGPB on tolerance index (TI), bioconcentration factor (BCF) and translocation factor (TF): In the case of stress response in plants, BCF and TF are key attributes in the feasibility study of heavy metals plant remediation potential (Usman *et al*., 2019). Table 3 shows the approximate BCF, TF, and TI values for biochar and the A1 Cr strain. When plants were inoculated with *Bacillus megaterium* A12 and biochar, the BCF, TF, and TI values were reduced compared to the non-inoculated control.

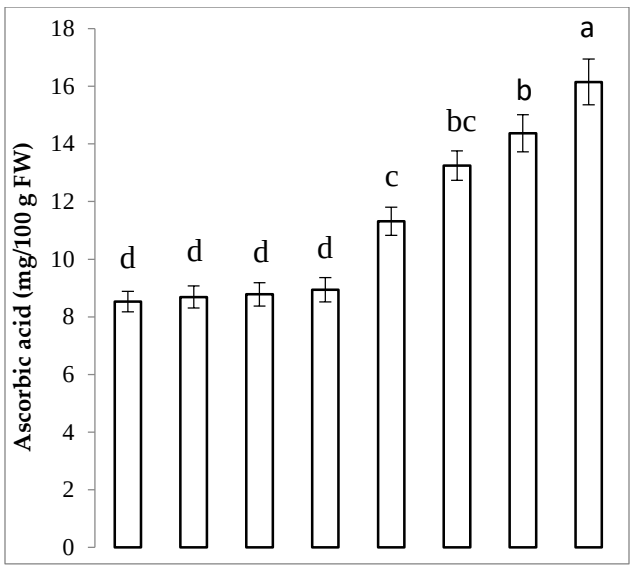
The BCF is commonly used to measure a plant's ability to remove metals from soils (Zayed *et al*., 1998). The BCF values for Cr were in order Cr (0.67) > Cr+BC (0.58) > Cr+A12 (0.48) > Cr+ BC+ A12 (0.45). TF values for Cr were in order Cr (0.51) > Cr+BC (0.49) > Cr+A12 (0.48) > Cr+ BC+ A12 (0.46). Ti was analyzed to determine the potential of plants subjected to metal stress. The Ti values for Cr were in order Cr+BC (1.12) > Cr+A12 (1.18) > Cr+ BC+ A12 (1.30).

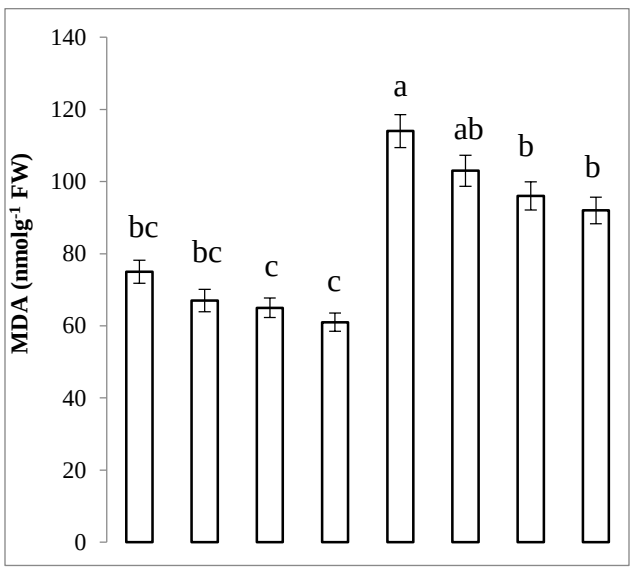
Effect of biochar and PGPB on malondialdehyde (MDA), electrolyte leakage (EL) and hydrogen peroxide (H_2O_2) levels: Chromium toxicity significantly enhanced EL, MDA and H_2O_2 levels in *S. oleracea* plants compared to CK-treatment. The treatment, including PGPB and biochar, alone or in interaction, reduced EL, MDA and H_2O_2 levels in *S. oleracea* plants raised in normal and Cr-toxic potted soil (Fig. 1).

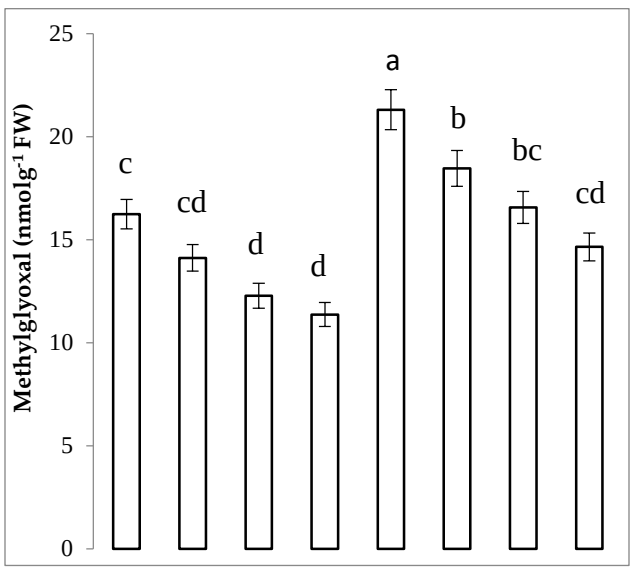
Effect of biochar and PGPB on Ascorbic acid, methylglyoxal and proline contents: Inoculation of plants under all treatments Cr+BC, Cr+ A1, Cr+BC+ A12 resulted in a substantial increase in proline and ascorbic acid. At the same time, the treatment of Cr+BC resulted in an elevated MG content compared to Cr+ A1, Cr+BC+ A12 (Fig. 2).

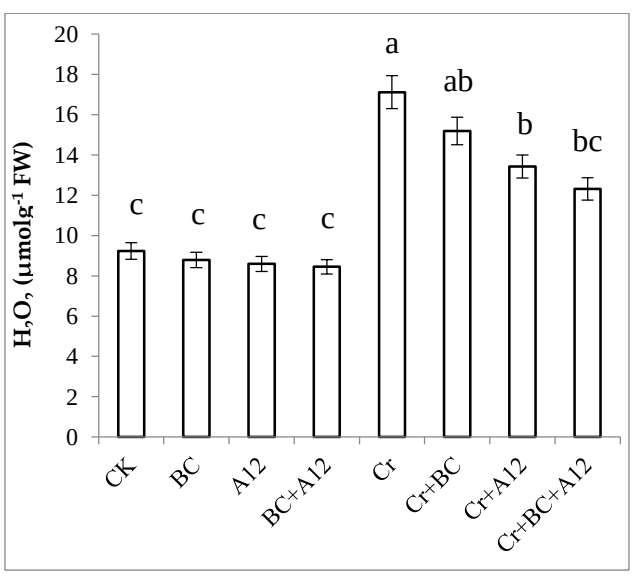
Effect of biochar and PGPB on antioxidant enzymes: Higher Cr concentration in soil elevated the activity of antioxidative enzymes compared to CK-treatment. The combined treatment of biochar and PGPB significantly improved the activity of SOD, CAT and APX in *S. oleracea* seedlings growing in Cr-spiked soil, as compared to the Cr-only treatment (Fig. 3).











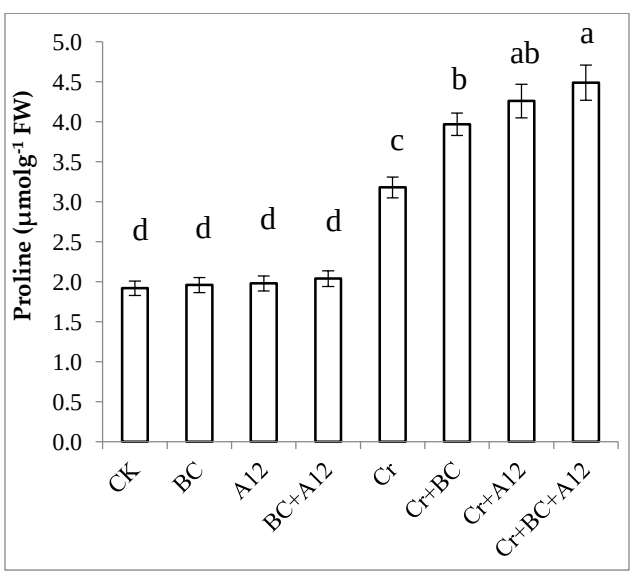


Fig. 1. Impression of *Bacillus megaterium* A12 and biochar on electrolyte leakage (EL), malondialdehyde (MDA), hydrogen peroxide (H_2O_2) levels of *Spinacia oleracea* grown in Cr-contaminated soil. CK: Control, BC: Biochar, A12: B. megaterium A12, Cr: Cr amendment at the rate of 10mg Cr kg^{-1} soil.

Fig. 2. Effect of *Bacillus megaterium* A12 and biochar on ascorbic acid, methylglyoxal and proline contents of *Spinacia oleracea* grown in Cr-contaminated soil. CK: Control, BC: Biochar, A12: B. megaterium A12, Cr: Cr amendment at the rate of 10mg Cr kg^{-1} soil.

Table 1. Effect of *Bacillus megaterium* A12 and biochar on growth traits of *S. oleracea* grown in Cr-contaminated soil.

Treatments	Root L (cm)	Shoot L (cm)	Leaf area (cm^2)	Shoot FW (g)	Root FW (g)	Shoot DW (g)	Root DW (g)
CK	9.2±0.41b	23±1.12b	0.16±.0075ab	6.52±0.29ab	1.83±0.08b	1.85±0.09b	0.56±0.023ab
BC	10.5±0.47ab	27±1.24ab	0.18±.0078a	7.69±0.32a	2.12±0.09ab	2.16±0.11ab	0.64±0.027a
A12	11.2±0.52a	28±1.35a	0.19±0.0082a	7.98±0.34a	2.28±0.094a	2.24±0.12a	0.68±0.031a
BC+A12	12.6±0.57a	31±1.37a	0.21±0.0085a	8.31±0.38a	2.37±0.11a	2.35±0.13a	0.71±0.032a
Cr	7.1±0.28c	14±0.65c	0.11±.0046b	5.32±0.21b	1.31±0.06c	1.26±0.05c	0.38±0.018b
Cr+BC	7.8±0.29c	16±0.72c	0.119±.0037b	6.05±0.25b	1.46±0.07bc	1.38±0.06c	0.41±0.019b
Cr+A12	8.1±0.34bc	17±0.74bc	0.124±0.0053b	6.27±0.27ab	1.57±0.073bc	1.43±0.07bc	0.45±0.021b
Cr+BC+A12	8.6±0.35bc	19±0.81bc	0.131±0.0048ab	6.45±0.31ab	1.65±0.08b	1.49±0.074bc	0.48±0.023ab

CK: Control, BC: Biochar, A12: *Bacillus megaterium* A12, Cr: Cr amendment at the rate of 10mg Cr kg^{-1} soil

3.2. Effect of PGPB and biochar on photosynthetic pigmentation

Regarding the effects of biochar and the A12 strain on plants, inoculated plants possessed significantly higher levels of anthocyanins, carotenoids, and flavonoids than the control group. The treatment resulted in a slight increase in both Chl a (Fig. 1c) and Chl b (Fig. 1d), but a slight decrease equated to the control. The Chl a/b ratio was maintained (Table 2).

Table 2. Impact of *Bacillus megaterium* A12 and biochar on chlorophyll, carotenoids, phenolic and flavonoid contents of *S. oleracea* grown in Cr-contaminated soil.

Treatments	Chlorophyll *a*	Chlorophyll *b*	Total Chl.	Carotenoids	Phenolic contents	Flavonoids
	mg g^{-1} FW	mg g^{-1} FW	mg g^{-1} FW	mg g^{-1} DW	mg(GAE)/g FW	mg (QE)/g FW
CK	2.41 ± 0.11b	0.73 ± 0.031ab	3.14 ± 0.13b	1.83 ± 0.08ab	0.86 ± 0.04b	19.82 ± 0.85c
BC	2.86 ± 0.12ab	0.91 ± 0.037a	3.77 ± 0.14ab	2.29 ± 0.09a	1.12 ± 0.05b	24.16 ± 0.91bc
A12	3.02 ± 0.14a	0.94 ± 0.038a	3.96 ± 0.16a	2.38 ± 0.11a	1.28 ± 0.05ab	27.24 ± 1.15bc
BC+A12	3.16 ± 0.15a	0.97 ± 0.041a	4.13 ± 0.19a	2.51 ± 0.12a	1.37 ± 0.06a	28.35 ± 1.26bc
Cr	1.68 ± 0.07c	0.52 ± 0.021b	2.20 ± 0.09c	1.12 ± 0.05b	0.95 ± 0.04b	32.26 ± 1.38b
Cr+BC	1.82 ± 0.08c	0.58 ± 0.023b	3.40 ± 0.15b	1.25 ± 0.05b	1.46 ± 0.06a	38.38 ± 1.69ab
Cr+A12	1.94 ± 0.09bc	0.62 ± 0.024ab	2.56 ± 0.11bc	1.38 ± 0.06b	1.57 ± 0.07a	42.43 ± 1.82a
Cr+BC+A12	1.98 ± 0.09bc	0.64 ± 0.025ab	2.62 ± 0.12bc	1.42 ± 0.06ab	1.65 ± 0.07a	45.49 ± 1.87a

CK: Control, BC: Biochar, A12: *B. megaterium* A12, Cr: Cr amendment at the rate of 10mg Cr kg^{-1} soil

Table 3. Influence of *Bacillus megaterium* A12 and biochar on metal uptake, bio-concentration factor (BCF), translocation factor (TF) and tolerance index (TI) of *S. oleracea* grown in Cr contaminated soil.

Treatments	Cr Root µg Cr g^{-1}	Cr Shoot µg Cr g^{-1}	BCF	TF	TI (%)
CK	ND	ND	-	-	-
BC	ND	ND	-	-	1.16 ± 0.047b
A12	ND	ND	-	-	1.21 ± 0.049ab
BC+A12	ND	ND	-	-	1.35 ± 0.053a
Cr	13.15 ± 0.51a	6.75 ± 0.28a	0.67 ± 0.024a	0.51 ± 0.023a	-
Cr+BC	11.82 ± 0.47ab	5.84 ± 0.23ab	0.58 ± 0.023ab	0.49 ± 0.021a	1.12 ± 0.048b
Cr+A12	10.16 ± 0.45ab	4.89 ± 0.21ab	0.48 ± 0.021ab	0.48 ± 0.019a	1.18 ± 0.051ab
Cr+BC+A12	9.85 ± 0.39b	4.56 ± 0.22b	0.45 ± 0.019b	0.46 ± 0.018a	1.30 ± 0.054a

CK: Control, BC: Biochar, A12: *B. megaterium* A12, Cr: Cr amendment at the rate of 10mg Cr kg^{-1} soil

Discussion

Heavy metal toxicants are one of the serious issues to plant survival due to the synthesis of reactive species. These ROS disturb the internal homeostatic state, hindering normal metabolomics in plants (Zengin & Munzuroglu, 2005; Farid *et al*., 2013). Many researchers have reported biochar's role in reducing abiotic stresses in plants (Medyńska-Juraszek *et al*., 2020). Progressive utilization of untreated agrochemicals waste results in the accretion of toxic metals in soil. Higher levels of metal in soil have been identified in many areas of Pakistan, leading to higher levels in edible plant parts (Nazli *et al*., 2020). Various bacterial strains have been identified to induce metal resistance in plants due to phytohormones' production. Crop plants can be inoculated with these microbes to help them thrive in stressful conditions. An effective strategy for coping with metal pollution and improving agricultural productivity is applying phytohormone-synthesizing, metal-tolerant PGPB (Nemat *et al*., 2020).

Like other heavy metals, Chromium stress had a detrimental effect on growth parameters. The effect of PGPB and biochar produced by Bamboo (*Bambusa vulgaris*), which belongs to the Bambusoideae subfamily of the grass family Poaceae, on different growth parameters of the genus *Spinacia oleracea* of the Amaranthaceae family, is reported in our study. Biochar and PGPB amendment substantially increased biomass, accordant with previous research demonstrating the benefits of biochar applications (Dai *et al*., 2020).

Biochar and PGPB enhanced the fresh and dry weight of *S. oleracea* seedlings grown in potting soil. Our results are inconsistent with Trupiano *et al*., (2017), who demonstrated that biochar augments soil fertility,

vegetative growth and biomass production in *Lactuca sativa*. Photosynthetic activity influences growth and development (Niinemets & Tenhunen, 1997). Plant photosynthetic pigments are reduced when they are exposed to heavy metals (Yang *et al*., 2020). Exogenous Cr significantly declined carotenoids, Chl *a* and Chl *b* levels in this study (Table 2). Our findings revealed a progressive association among pigment content and biochar and PGPB treatments, which is consistent with the findings of Akram *et al*., (2019), who found that a *B. megaterium* strain increased the levels of carotenoids, Chl *a* and Chl *b* in tomato plants. When Shah *et al*., (2020) inoculated tomato plants with four different *Bacillus* isolates, similar results were reported (Zhu *et al*., 2020).

Free radicals are scavenged by phenolic contents, which have reducing properties (Omena *et al*., 2012). The inoculant favoured the accumulation of these compounds in terms of total phenolic content (TPC). These findings are consistent with Abd-Allah *et al*., (2018), who found that a *B. subtilis* isolate increased TPC in chickpea plants. Also, the inoculation of biochar and PGPB significantly enhanced the quantity of flavonoid compared to control and Cr. stress. Correspondingly, intensifications in total flavonoid levels through *B. licheniformis* inoculation have been observed in tomatoes (Ochoa-Velasco *et al*., 2016; Chandrasekaran *et al*., 2019).

Ascorbic acid is required for chlorophyll formation and serves as a substrate for the ascorbate peroxidase enzyme. Ascorbic acid content is crucial for Cr stress tolerance in tomato roots (Al-Huqail *et al*., 2020). This study showed that when treated with BC and PGRB, there was a considerable escalation in ascorbic acid at maximum Cr compared to the control level.

Proline and ascorbic acid content are pivotal in the plant's defence mechanism against heavy metals stress (Ashraf & Foolad, 2007). *S. oleracea* showed a significant rise in proline synthesis under Cr regimes compared to the control. When inoculated with biochar and PGRB, proline, which is thought to be a protective mechanism contrary to metal stress, increased significantly.

Compared to non-Cr conditions, Cr stress amplified the activity of CAT, POD, and SOD. According to the findings, stimulating plant tolerance machinery may be responsible for increased antioxidant enzymes (Wang *et al*., 2016). In our finding, biochar perhaps regulated plants' synthesis of antioxidative enzymes and improved Cr tolerance (Thomas *et al*., 2013). Limited research has shown the effect of biochar on oxidative stress and antioxidative enzyme activity in plants exposed to heavy metal toxicants.

Exogenous Cr reduced SOD and CAT activity in roots and leaves while increasing EL and MDA content. On the other hand, using biochar improved antioxidant enzyme activity and the antioxidative reactions in plants to heavy metal toxicity. Biochar augmented SOD and CAT activity and decreased MDA content and electrolyte leakage rate in this study, effectively mitigating the hazardous effects of Cr in *S. oleracea* (Figs. 2, 3). This is because CAT activity is known for detoxifying peroxides in plant cells, while SOD is the essential enzyme in converting superoxide radicals into H_2O_2.

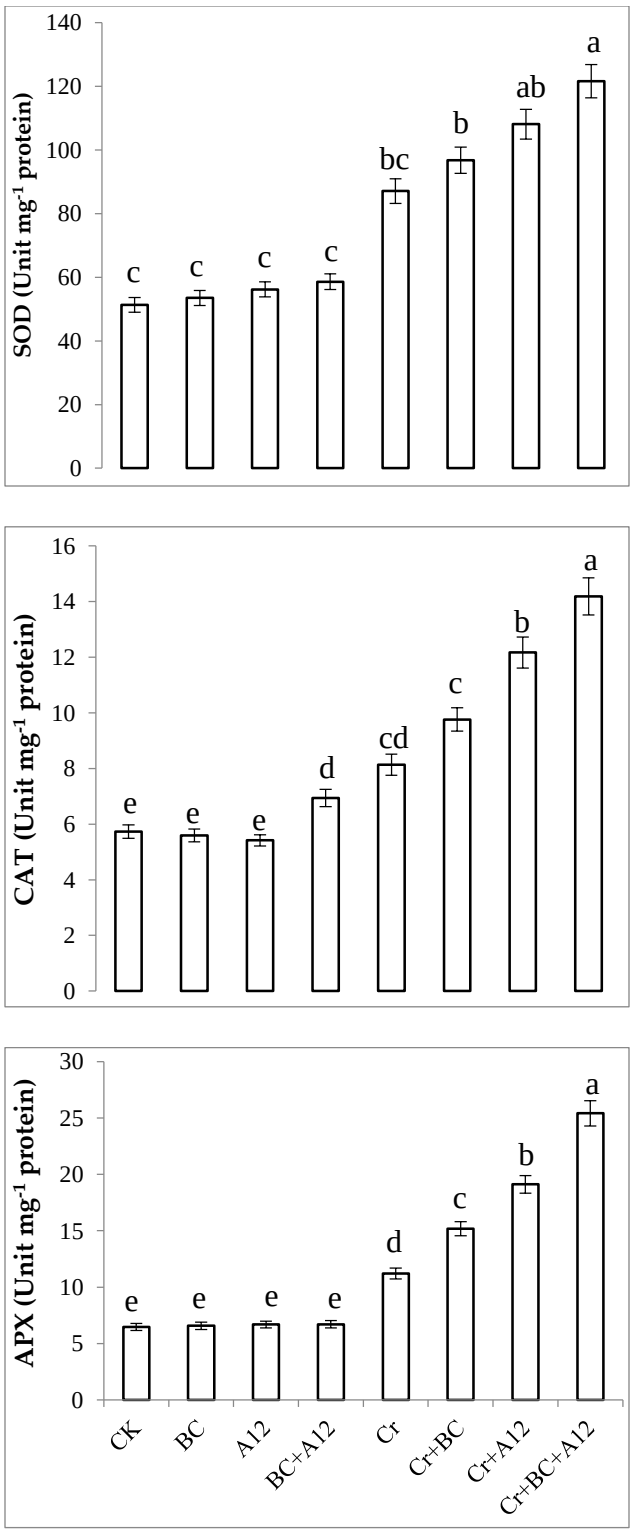


Fig. 3. Role of *Bacillus megaterium* A12 and biochar in modulation of antioxidant enzymes (SOD, CAT & APX) in *Spinacia oleracea* grown in Cr-contaminated soil. CK: Control, BC: Biochar, A12: B. megaterium A12, Cr: Cr amendment at the rate of 10mg Cr kg^{-1} soil.

The bioconcentration factor indicates a plant's capability to absorb metal pollutants in its tissues from the soil. Our findings on improving TI in inoculated *S. oleracea* plants under metal stress agree with Chauhan & Rai (2009). Biochar and PGPB increased the heavy metal tolerance index (MTI) in inoculated *Sedum alfredii*,

according to Li *et al*., (2007). The heavy MTI has been reported as one of the most critical factors to consider when choosing a plant for phytoremediation. Plant species highly resistant to heavy metals can be used in contaminated soil phytoextraction (Wu *et al*., 2011). Due to their growth-promoting biochemicals, which promote nutrient uptake and allow plants to withstand stress, some metal-resistant PGPR can improve plant growth and phytoremediation at the same time (Rajkumar *et al*., 2009).

Our findings show that *S. oleracea* inoculated with biochar and PGPR had higher BCF and TF during all treatments than uninoculated plants. Our findings on increased BCF, TF, and Cr extraction in bio-inoculated *S. oleracea* plants are consistent with Ahmad *et al*., (2016) findings. The translocation factor is a parameter that assesses a phytoextractor's ability to transport absorbed heavy metals from root to shoot tissues. On the other hand, the bioconcentration element refers to the transference of metals from the rhizosphere to aerial plant sections (Shi *et al*., 2017). Plant bioaccumulation and TF aid in the determination of metal content adsorption, translocation, and phytoextraction. Plants with higher TF and BCF are preferred for the remediation of metallic-polluted areas, according to Yoon *et al*., (2006).

Conclusions

Chromium stress reduced growth aspects of *S. oleracea*. Combined treatment of *B. megaterium* A12 and biochar reduced MDA content and EL in *S. oleracea* plants raised in Cr-polluted soil. The combined application of biochar and *B. megaterium* A12 enhanced the activity of antioxidative enzymes and elevated the amount of ascorbic acid and proline. Growers can use *B. megaterium* A12 and biochar in combination to mitigate the Cr stress in the *S. oleracea*. Collective application of biochar and PGPB strains can be helpful in alleviation of abiotic stresses.

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