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Synergistic effect of arbuscular mycorrhizal fungi, biochar and seaweed extract for improving the copper tolerance in hemp

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ABSTRACT

Contamination of soil with copper (Cu) is widespread because of industrialization, use of copper-based fertilizers and natural weathering of rocks. Copper (Cu) toxicity in agricultural lands is a growing global concern, but not a single study has been conducted till now on its alleviation by the synergistic effect of arbuscular mycorrhizal fungi (AMF), biochar (BC) and seaweed extract (SWE) in hemp. For this purpose, pot trial was conducted to evaluate various treatments i.e., BC addition [with BC and without BC] and soil application of AMF and various levels of SWE (T_0 = control, T_1 = AMF 10 g per pot, T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; $T_4 = T_1 + T_2$; $T_5 = T_1 + T_3$) on the hemp productivity and Cu accumulation grown under artificially spiked Cu stressed soil (300 mg kg^{-1}). Finding revealed that spiking of Cu significantly modulates the growth, biomass, photosynthetic and Cu accumulation attributes of hemp plant. A linear decrease in the leaves dry weight (45.50 %), stem diameter (21.70 %), total chlorophyll contents (54.33 %) while increase in the enzymatic antioxidants, oxidative stress and Cu accumulation attributes was noticed under Cu stressed soil. The simultaneous application of BC, AMF and SWE resulted in substantial enhancements in growth and biomass traits in comparison to untreated Cu-exposed plants. Synergistic approach of all the biostimulants application effectively reduced hydrogen per oxide (H_2O_2) production (50.83 %), proline accumulation (45.66 %) and generation of malondialdehyde (43.43 %), while increasing osmolytes accumulation i.e., soluble sugars (52.75 %) and soluble protein (87.15 %) in comparison with control. Furthermore, the synergistic effect of all biostimulants minimized the Cu accumulation in the hemp. Specifically, the cumulative impact Cu stress was mitigated by combined approach BC, AMF and SWE treatments, which increased growth, biomass, antioxidant enzyme activity, and osmoprotectants levels by limiting Cu accumulation. The combined novel approach of BC, AMF and SWE holds a practical approach for the alleviation of Cu stress for hemp production. This approach provides a comprehensive solution for successful hemp cultivation in Cu-contaminated soil, and thus, for remediating Cu contaminated soil.

1. Introduction

Copper (Cu) is a persistent and highly hazardous element found throughout the environment as a result of its utilization in several industry sectors (Izydorczyk et al., 2021). Copper addition to soil may originate from numerous sources, and the most common process involves anthropogenic inputs as products of mining, smelting and disposal of industrial wastes. Excessive utilization of Cu-based fungicides, pesticides and fertilizers used in farming systems also play a major role (Briffa et al., 2020; Cheng et al., 2024). The optimum level of Cu is highly essential for reproductive success from pollen formation and strength to the development of plant immunity against a range of fungal

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and bacterial pathogens. These roles explain why optimum level of Cu plays a vital role in enhancing of plant health and growth in the whole plant (Dey et al., 2024). Agricultural soil has been identified as a significant source of copper contamination due to its high toxicity to crops and consequent impact on human health. It can have a detrimental effect on plant growth and development throughout the life cycle, starting from germination to the harvesting stage (Okereafor et al., 2020; Mir et al., 2021; Srivastava et al., 2024). It has a physiological effect of limiting the activity of enzymes that are involved in important metabolic processes. It also decreases the amount of pigments, impairs the exchange of CO_2, and affects the uptake and accumulation of nutrients in plants (Rehman et al., 2019; Alshegaihi et al., 2024).

Hemp is an alternative crop that offers a more ecologically sustainable cultivation method compared to conventional crops (Deng et al., 2024; Ahmadi et al., 2024). Previous study has demonstrated that every part of hemp plants has high levels of heavy metals when cultivated in contaminated soil, which restricts their potential as commercially viable plant material (Peroni et al., 2024). Hemp would be deemed unsuitable for use in conventional industries such as food and textiles if it contains particularly excessive levels of heavy metals in its seeds, leaves, and fibres (Muedi et al., 2024). Even though fiber crops have developed multiple defensive mechanisms to alleviate metals stress (Singh et al., 2024). So, there is dire need to develop practical, sustainable and innovative techniques for the utilization of metals stressed soil and for alleviating the impact of metals stress on fiber crops.

Biostimulants are recognized as a viable method for achieving sustainable agricultural productivity and preserving soil health (Garg et al., 2024). These stimulants are primarily utilized for multiple uses in crop production, enhancing nutrient utilization efficiency, and promoting growth and development. Using growth stimulants as ecologically acceptable substances enhances flowering fruit growth, production of crops, and nutrient efficiency (Zulfiqar et al., 2024). Biochar has been extensively used as a cost-effective and ecologically sound method as soil amendment to address soil contamination by heavy metals. Biochar, developed through the process of pyrolysis, plays a significant role in the remediation of heavy metals in soil (Maqbool et al., 2024). For instance, the use of biochar increases the cation exchange capacity (CEC), which in turn enhances the immobilization of copper in soil, reducing its mobility and phytotoxicity. The use of biochar considerably improved the plant's nutritional status, biomass, and its ability to tolerate copper (Çelik et al., 2024). Another effective approach to enhance agricultural yields in the presence of abiotic stress involves the utilization of seaweed growth stimulants. These stimulants serve as economical and eco-friendly biofertilizers, and have demonstrated significant improvements in crop production in stressful conditions (Johnson et al., 2024). The SWE is composed of macronutrients and micronutrients, growth hormones, amino acids, vitamins, betaine, cytokinins, and sterols (Punitha et al., 2024). These compounds enhance the process of germination and enhance the growth of roots, increase the amount of chlorophyll in leaves, improve crop production, and enhance the physicochemical and biological properties of soil in the presence of metal stress (Thaimei et al., 2024). In addition, it has the ability to react with metal ions in soil and create protective colloids. It has a significant and indirect impact on the composition and functioning of soil microorganisms. It is also seen as a potential option for enhancing soil fertility in the presence of abiotic environmental factors (Kashyap et al., 2024). Arbuscular mycorrhizal (AM) fungal inoculation is a bioremediation technique used to address the issue of heavy metal (HM) pollution in soils (Seleiman et al., 2024). Most plants have associations with AM fungi located in the soil; moreover, their main role is in enhancing plant tolerance to abiotic stress (Herath et al., 2021; Guo et al., 2024). These fungi improve plant nutrient intake and physiological activity, leading to increased plant growth and a decrease in tissue Cu concentrations. The AM fungi bind copper in mycorrhizal structures, preventing its movement to plant roots (Dhalaria et al., 2020; Balestrini et al., 2024).

Multiple studies have proven that BC may influence AM fungus quantity and efficiency (Parihar et al., 2020; Wen et al., 2024). The combination of BC with AM fungi appears to offer significant potential for soil bioremediation applications (Riaz et al., 2021; Jia et al., 2024). Fayuan et al. (2022) demonstrated that the combination of AMF with three soil amendments, including biochar, improved phytostabilization of Cd, Pb, and Zn in sweet sorghum. Moreover, the combined utilization of biochar and AM fungi could also improve plant tolerance to a range of abiotic stress (Yan et al., 2021; Sousa et al., 2024). However, there is not a single study reported in which the synergistic effect of BC, AMF and SWE explained clearly under metal stressed soil in fiber crop.

Previous research mainly focuses on the individual applications of these components to mitigate metal stress, with no available literature on their cumulative effect in increasing physiological and antioxidant defence mechanisms during metal stress. Keeping in mind the importance of all the amendments, it was hypothesized that these amendments will improve hemp's growth, physiological performance, antioxidant defense mechanisms, and reduce oxidative damage caused by Cu stress. The specific objectives of the present study were to investigate: (a) how artificially spiked Cu metal affect plant growth, physiology, and antioxidative status; (b) how the biostimulants (BC, AMF and SWE) applied through soil and foliar method regulate the expressions of plant key processes to enhance plant Cu resistance independently or synergistically, (c) if the synergistic approach of BC, SWE and AM fungus alters Cu bioavailability, and alleviates Cu phytotoxicity. The experimental design, methodologies, and the outcomes extend current knowledge regarding the synergistic application of these bioremediation techniques in improving Cu stress tolerance in hemp plants. The findings would facilitate in depth understanding and explains the first ever reported synergistic effects of BC, AMF and SWE on hemp productivity and Cu stress tolerance, because this holistic strategy explores the combined effect of all the amendments for enhancing metal tolerance. Altogether, the study benefits the science by offering information on the possibilities of those treatments to restore the ability of the soil to support agriculture in Cu-contaminated soils, an area that remains underexplored.

2. Materials and methods

2.1. Study plan

A pot trial was conducted in green house at Yunnan University, Kunming City, Yunnan Province, China, from May to September 2022. Experiment was executed in artificially Cu spiked soil (300 mg kg^{-1}). The experimental treatments consisted of two factors, i.e., biochar (BC) treatments [with BC and without BC] and soil application of arbuscular mycorrhizal fungi (AMF) and various levels of seaweed extract (SWE) (T_0 = control, T_1 = AMF 10 g per pot, T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; $T_4 = T_1 + T_2$; $T_5 = T_1 + T_3$). The experiment was laid out in completely randomized design (CRD) under factorial arrangement and replicated thrice (each of the replications comprised 3 pots per treatment).

2.2. Soil preparation

Soil was collected from the Research station of institute at the depth of 0–15 cm. The initial physico-chemical properties of the soil were depicted in the Table 1. Initially the Cu contents in the soil were 13.21 mg kg^{-1}. The soil was artificially spiked with copper sulphate ($CuSO_4$. 5 H_2O) to make the final concentration of 300 mg kg^{-1} of the soil by following the protocol of Heile et al. (2021). Manual mixing was done on daily basis with light sprinkling of water for complete mixing of meta with the soil. Prior to starting the experiment, 10 kg of Cu spiked soil was mixed with biochar (50 g kg^{-1}) purchased from the Ingdingshan Green Source Activated Carbon Co., Ltd., China, which had dimensions of 40 cm × 30 cm. The mixture was then kept for 20 days to ensure complete homogeneity of the biochar. On daily basis the soil was sprinkled with water and manual hoeing was carried out for the

Table 1
Physico-chemical attributes of the soil used for experiment.

Parameters	Unit	Value
pH	-	8.06
Electrical conductivity	mS m^{-1}	24.3
Organic matter	g kg^{-1}	14.23
Total nitrogen	mg kg^{-1}	984.23
Total phosphorus	mg kg^{-1}	1023.21
Total potassium	%	1.67
Hydrolytic nitrogen	mg kg^{-1}	52.70
Available phosphorus	mg kg^{-1}	29.45
Quick available potassium	mg kg^{-1}	438.76
Cu contents	mg kg^{-1}	13.21

homogenization of experimental treatments with the Cu spiked soil. Before sowing of surface sterilized seeds of hemp cultivar (Yunma No.7) and the viable inoculum of AMF (*Glomus* spp.) at the rate of 10 g per pot was applied as per treatment plan obtained from Soil Microbiology Research Group in China.

2.3. *Crop management*

Initially 8–10 seeds were sown in the pots but after 15 days of sowing five healthy seedlings were kept in each pot. The nutritional requirement of the hemp crop was fulfilled by the exogenous added fertilizer in the form of urea (N-46 %), calcium superphosphate (P_2O_5-12 %) and potassium sulfate (K_2O-50 %) with dosage [N : 225 kg ha^{-1}; P: 75 kg ha^{-1}; and K = 200 kg ha^{-1} supplementation were applied as base fertilizer. For irrigation 500 ml of Cu free water was applied to each pot to ensure soil moisture requirement. The foliar application of seaweeds extract obtained from the Wuhan Yuanwen Biotechnology Co., Ltd. was applied after 45 days of sowing 500 ml per pot two times in week. Data pertaining to growth, biomass, and biochemical analysis was acquired 75 days after sowing.

2.4. *Data collection*

2.4.1. *Soil attributes*

From the depth of 0–15 cm the soil samples were collected. After collection of soil samples and dried at room temperature and stored in paper bags until further analysis. By following the protocol of Ryan et al. (2001) the water/soil suspension (2:1) the soil was utilized to measure electrical conductivity (EC) and pH of the soil samples. The GB9834–1988 method was used for the assessment of soil organic matter (OM) content of soil. According to the method GB 9836–1998, available and total potassium in the soil was determined using flame atomic absorption spectrophotometry. By using the protocols described in method of HJ/T 704–2014, using the sodium hydrogen carbonate solution-Mo-Sb anti spectrophotometric total and available phosphorus was determined. Nitrogen contents in the soil samples was determined by using the kjeldahl method. The Cu contents were measured by digesting a 0.1 g dried sample with a 4:1 mix of perchloric and nitric acid. With the help of an Atomic Absorption Spectrophotometer (Perkin-Elmer 3100) Cu levels in soil samples was measured. All the above-mentioned measurements were followed the standard protocols outlined by the Institute of Soil Science, Chinese Academy of Sciences (Nanjing, China).

2.4.2. *Growth attributes*

The growth traits of hemp plants were taken at harvesting stage. At this stage the leaf count was noted and the plant was segregated into roots and shoots and their respective length was measured with the help of measuring tape, while the stem diameter was computed using the vernier caliper (Deng et al., 2024).

2.4.3. *Biomass attributes*

After harvesting the plant was carefully uprooted from the growth medium a gentle stream of water was applied for removing the soil. After that roots were detached from the shoots and leaves. Fresh biomass of shoot, roots and leaves was measured using the analytical weighing balance for dry biomass the plant samples were dried for 48 hours at 65℃ until the weight was recorded to be constant (Zaman et al., 2024).

2.4.4. *Physiological attributes*

Fresh leaves samples (0.2 g) were collected from each treatment and after removing their veins crushed into small pieces. After that crushed leaves samples were homogenized in 10 ml of 80 % acetone solution. Following a 10-minute centrifugation at 4000 rpm, the leaf sample supernatant was transferred into a cuvette for analytical purposes. The absorbance values were measured at wavelengths of 665 nm, 649 nm, and 470 nm, respectively, with 80 % acetone serving as a control background. The concentrations of chlorophyll a, chlorophyll b and carotenoids contents were measured (Linger et al., 2005; Wang, and Huang, 2006). Using a Li-6400 portable photosynthesis system (Li-Cor Biosciences, USA), the net photosynthetic rate (*Pn*), stomatal conductance (*Gs*), intercellular carbon dioxide (CO_2) concentration (*Ci*), and transpiration rate (*Tr*) of fully expanded hemp leaves were recorded between 9:30 and 11:00.

2.4.5. *Lipid per oxidation and enzymatic antioxidants attributes*

The supernatant sample obtained from centrifuging a 1-gram leaf sample with a 50 mM phosphate buffer at 15,000 × g for 10 minutes was utilized to measure the activity of plant enzymes. The enzymatic activities superoxide dismutase activity (SOD), peroxidase activity (POD), ascorbate peroxidase (APX) and catalase activity (CAT), and lipid peroxidation attributes (MDA contents) were determined using assay kits (A064, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the procedures provided by the manufacturer.

2.4.6. *Osmolytes and oxidative stress related attributes*

A fresh leaf sample weighing 0.5 g taken 20 days after drought stress was grounded with a buffer (pH 7.2). The pH of the saline buffer solution was adjusted using hydrochloric acid (HCl), followed by autoclaving. The extract was then centrifuged at 12,000 × g for 5 min for the separation of supernatant. Proline content was determined following the method outlined by Chance and Maehly and Chance., (1954). The soluble sugars and soluble protein contents were assessed using the techniques reported by Giannakoula et al. (2008) and the Bradford., (1976) method, respectively. After reaction with titanium tetrachloride ($TiCl_4$) and the absorbance was taken at 410 nm, the concentration of hydrogen per oxide (H_2O_2) was measured. The final value of H_2O_2 was calculated by using a standard curve (Hsu and Kao, 2007).

2.4.7. *Copper contents in the plant*

After digesting a 0.1 g dried plant sample with a 4:1 mix of nitric and perchloric acid Cu contents were measured. An Atomic Absorption Spectrophotometer (Perkin-Elmer 3100) was used to measure Cu levels in plants samples.

2.5. *Statistical analysis*

A statistical analysis of variance (ANOVA) technique (two-way) was performed on the dataset to investigate any significant differences and prevailing patterns among the treatments that were applied. Tukey's Honest Significant Difference (HSD) test was used to determine which specific group means are different after a significant ANOVA result. Pearson's correlation was used to explore the connections and associations between the variables. Data was analyzed using the statistical software package Statistics 8.01. The statistical and visualization tool of R-Studio software was used to compute principal component analysis, correlation, and chord analysis.

3. Results

3.1. Growth attributes

The findings of this study based on the data analysis showed that artificially spiked Cu stressed soil affected the growth attributes of hemp plants. A linear decrease was noticed in all the growth attributes by the Cu stress. However, the addition biochar (BC) significantly ($p \leq 0.05$) contributed to the improvement of the growth traits of the hemp plants under control and Cu-stressed conditions (Fig. 1). Addition of BC improved the root length (59.07 %), shoot length (24.05 %), number of leaves (7.06 %) and stem diameter (25.61 %) as compared with those where no biochar was used. Similarly, the AMF and SWE treatments enhanced the growth attributes of hemp plants. Sole application of AMF improved the root length (20.76 %), shoot length (11.81 %), number of leaves (28.83 %) and stem diameter (13.67 %) as compared to control. Similarly, the application of SWE at the rate 3 g L^{-1} proved best response in improving the growth attributes of the hemp plants as compared to those pots where SWE was applied at the rate of 1 g L^{-1}. In comparison with the SWE (3 g L^{-1}) and AMF treatments, both treatments represent statistically similar results for all the growth attributes. The treatment T_4 (AMF 10 g pot^{-1} + SWE 1 g L^{-1}) improved the growth attributes of the hemp plants. This treatment improved the root length (32.68 %), shoot length (20.82 %), number of leaves (44.14 %) and stem diameter (18.02 %) as compared to control. The treatment T_5 (AMF 10 g pot^{-1} + SWE 3 g L^{-1}) proved the best in improving the growth attributes of the hemp plants. This treatment improved the root length (44.91 %), shoot length (27.95 %), number of leaves (58.56 %) and stem diameter (21.76 %) as compared to control where no AMF and SWE extract was applied. The decreasing pattern in terms of improved growth attributes hemp plants grown under Cu stressed soil for the BC treatments were with biochar > without biochar and for the AMF and SWE treatments were $T_5 > T_4 > T_3 > T_1 > T_2 > T_0$.

3.2. Biomass attributes

Data on the biomass attributes of hemp plant was in Fig. 2 exhibited that Cu stress decreased the biomass (fresh and dry) attributes of the hemp plant. The Cu stressed soil decreased biomass attributes of hemp plant as compared with other treatments were BC, AMF and foliar application of SWE was applied in the individual and combined from. Soil applied BC improved the root fresh biomass (107.10 %), stem fresh biomass (30.14 %), leaf fresh biomass (50.04 %), root dry biomass (122.51 %), stem dry biomass (40.06 %) and leaf dry biomass (45.43 %) in comparison with control where no BC was applied. Individual application of AMF improved the root fresh biomass (34.90 %), stem fresh biomass (24.13 %), leaf fresh biomass (26.93 %), root dry biomass (36.99 %), stem dry biomass (25.14 %) and leaf dry biomass (24.67 %) as compared to control. Similarly, the sole application of SWE at the rate 3 g L^{-1} proved best response in improving the biomass attributes of the hemp plants as compared to those pots where SWE was applied at the rate of 1 g L^{-1}. In comparison with the SWE (3 g L^{-1}) and AMF treatments, both treatments represent statistically similar results for all the biomass attributes. The treatment T_4 (AMF 10 g pot^{-1} + SWE 1 g L^{-1}) improved the biomass attributes of the hemp plants. This treatment improved the root fresh biomass (58.71 %), root dry biomass (62.19 %), stem fresh biomass (35.53 %), stem dry biomass (36.90 %), leaf fresh biomass (39.00 %), and leaf dry biomass (36.39 %) as compared to control. Best treatments of AMF and SWE (3 g L^{-1}) improved the root fresh biomass (76.05 %), root dry biomass (79.21 %), stem fresh biomass (46.52 %), stem dry biomass (49.25 %), leaf fresh biomass (48.18 %), and leaf dry biomass (45.43 %) in comparison with control

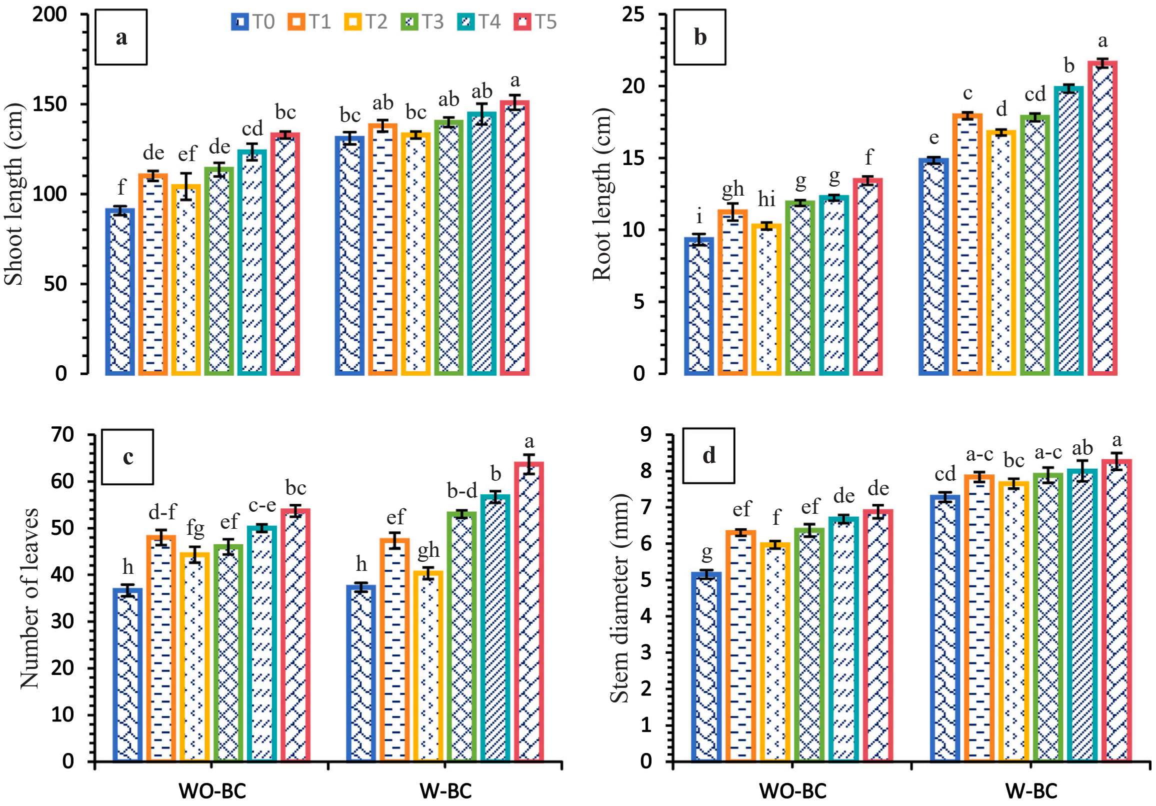

Fig. 1. Synergistic effect of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) on the growth attributes hemp plants under copper (Cu) stressed soil. Small letters on bars indicate a statistically significant difference between means ($p \leq 0.05$) based on a two-way ANOVA (Tukey's-HSD test). Capped lines denote the standard deviation of three replicates; WO-BC = without biochar; W-BC = with biochar; T_0 = control; T_1 = AMF 10 g pot^{-1}; T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; $T_4 = T_1 + T_2$; $T_5 = T_2 + T_4$; a) shoot length; b) root length; c) number of leaves; d) stem diameter.

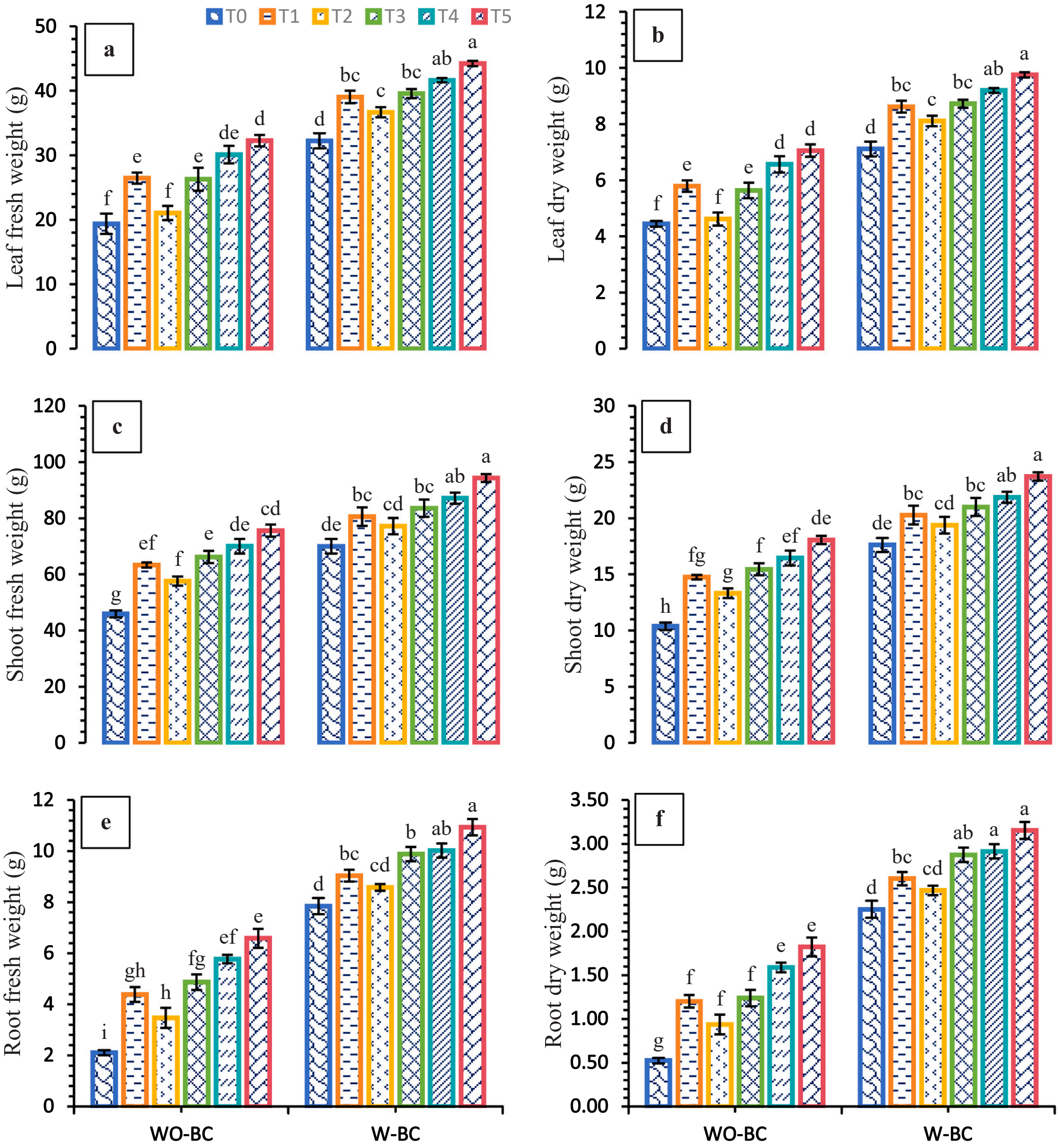

Fig. 2. Synergistic effect of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) on the biomass (fresh and dry) attributes various parts of hemp plants under copper (Cu) stressed soil. Small letters on bars indicate a statistically significant difference between means ($p \leq 0.05$) based on a two-way ANOVA (Tukey's-HSD test). Capped lines denote the standard deviation of three replicates; WO-BC = without biochar; W-BC = with biochar; T_0 = control; T_1 = AMF 10 g pot^{-1}; T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; $T_4 = T_1 + T_2$; $T_5 = T_2 + T_4$; a) leaf fresh weight; b) leaf dry weight; c) shoot fresh weight; d) shoot dry weight; e) root fresh weight; f) root dry weight.

where no application of AMF and SWE was done. Similarly, the treatment T_4 (AMF 10 g pot^{-1} + SWE 1 g L^{-1}) improved the root fresh biomass (58.71 %), stem fresh biomass (35.53 %), leaf fresh biomass (39.00 %), root dry biomass (62.19 %), stem dry biomass (36.90 %) and leaf dry biomass (36.39 %) in comparison with control.

3.3. Photosynthetic and gas exchange attributes

Soil-applied BC, AMF addition and various levels of foliar applied SWE in individual and combined form caused significant change ($p \leq 0.05$) in photosynthetic and gas exchange attributes of the hemp plants (Fig. 3). The linear change in the form of decreasing trend was noticed in the gas exchange and photosynthetic attributes of the hemp plants. The addition of biochar proved maximum of gas exchange and photosynthetic attributes of the hemp plants as compared to those pots where no biochar was applied. The addition of BC improved the photosynthetic and gas exchange attributes of the hemp plants. This treatment improved the chlorophyll a (33.36 %), chlorophyll *b* (59.50 %), total chlorophyll (42.59 %), carotenoids contents (106.10 %), photosynthetic rate (73.32 %), transpiration rate (82.08 %), stomatal conductance (66.94 %) and intercellular CO_2 concentration (22.93 %) as compared to control. The sole application of SWE at the rate 3 g L^{-1} proved best response in improving the photosynthetic and gas exchange attributes of the hemp plants as compared to those pots where SWE was applied at the rate of 1 g L^{-1} irrespective of biochar application in contaminated soil. The treatment T_4 (AMF 10 g pot^{-1} + SWE 1 g L^{-1}) improved the photosynthetic and gas exchange attributes of the hemp plants. This treatment improved the chlorophyll a (33.55 %), chlorophyll *b* (49.44 %), total chlorophyll (38.79 %), carotenoids contents (54.78 %), photosynthetic rate (39.78 %), transpiration

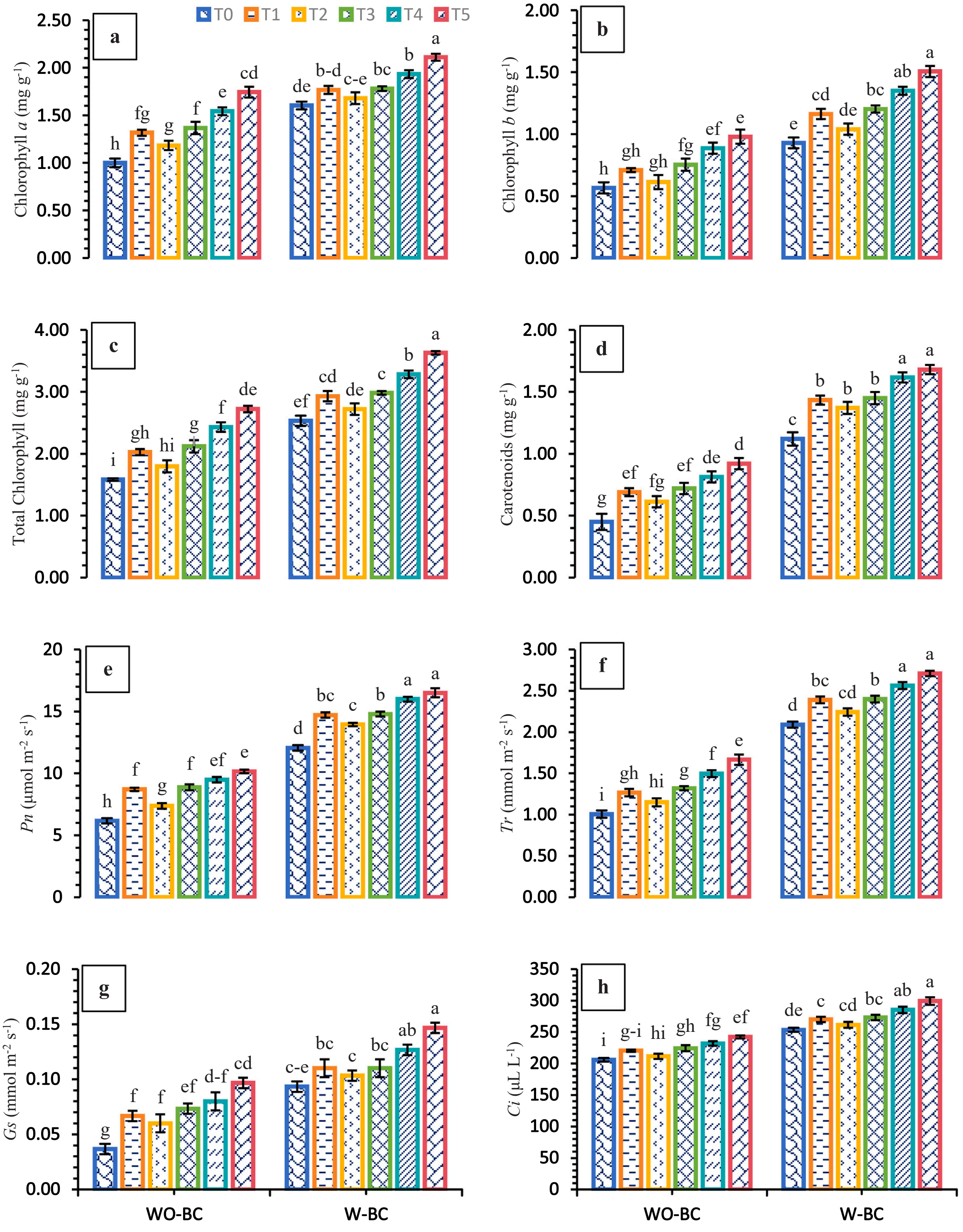

Fig. 3. Synergistic effect of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) on the physiological attributes of hemp plants under copper (Cu) stressed soil. Small letters on bars indicate a statistically significant difference between means ($p \leq 0.05$) based on a two-way ANOVA (Tukey's-HSD test). Capped lines denote the standard deviation of three replicates; WO-BC = without biochar; W-BC = with biochar; T_0 = control; T_1 = AMF 10 g pot^{-1}; T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; $T_4 = T_1 + T_2$; $T_5 = T_2 + T_4$; a) chlorophyll *a*; b) *chlorophyll b*; c) total chlorophyll; d) carotenoids; e) photosynthetic rate; f) transpiration rate; g) stomatal conductance; h) intercellular CO_2 concentration.

rate (31.11 %), stomatal conductance (58.97 %) and intercellular CO_2 concentration (12.54 %) as compared to control. In comparison with the SWE (3 g L^{-1}) and AMF treatments, both treatments represent statistically similar results for all the photosynthetic and gas exchange attributes. Combined application of BC, AMF and SWE increased the chlorophyll a (48.02 %), chlorophyll *b* (66.15 %), total chlorophyll (54.33 %), carotenoids contents (65.61 %), photosynthetic rate (46.30 %), transpiration rate (41.33 %), stomatal conductance (87.18 %) and intercellular CO_2 concentration (17.86 %) in comparison with control. The decreasing pattern in terms of gas exchange and photosynthetic attributes of hemp plants grown under Cu stressed soil for the for the AMF and SWE treatments were $T_5 > T_4 > T_3 > T_1 > T_2$

> T_0.

3.4. *Enzymatic antioxidants and oxidative stress attributes*

Data depicted in Table 2 represented the impact of soil applied BC, AMF addition and various levels of foliar applied SWE on the enzymatic antioxidant and oxidative stress attributes of hemp grown under Cu stressed soil under greenhouse conditions. The Cu soil significantly increased the enzymatic antioxidants and oxidative stress parameters of the hemp plants. Addition of BC decreased catalase activity (36.55 %), superoxide dismutase activity (24.82 %), peroxidase activity (53.45 %), APX activity (48.55 %) and H_2O_2 contents (39.93 %) in comparison with control. However, soil applied BC and AMF and foliar applied SWE significantly modulate the activities of enzymatic antioxidants and the hydrogen per oxide in comparison with control. The addition of BC decreased the enzymatic antioxidant and H_2O_2 contents in the hemp leaves under Cu stressed conditions. Sole application of AMF decreased the catalase activity (12.53 %), superoxide dismutase activity (15.08 %), peroxidase activity (22.19 %), APX activity (18.70 %) and H_2O_2 contents (24.79 %) in comparison with control. The application of SWE at the rate 3 g L^{-1} proved best response enzymatic antioxidants and oxidative stress attributes of the hemp plants as compared to those pots where SWE was applied at the rate of 1 g L^{-1} under both conditions of biochar. In comparison with the SWE (3 g L^{-1}) and AMF treatments, both treatments represent statistically similar results for all the enzymatic antioxidants and oxidative stress attributes. The best treatment of AMF and SWE (AMF 10 g pot^{-1} + SWE 3 g L^{-1}) decreased the catalase activity (27.34 %), superoxide dismutase activity (34.16 %), peroxidase activity (43.56 %), APX activity (45.96 %) and H_2O_2 contents (50.83 %) in comparison with control.

Table 2

Synergistic effect of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) on the enzymatic antioxidants and oxidative stress attributes of hemp plants under copper (Cu) stressed soil.

Treatments		Enzymatic Antioxidants and Oxidative Stress Attributes				
		SOD (U g^{-1})	POD (U g^{-1})	CAT (U g^{-1})	APX (U g^{-1})	H_2O_2 (nmol mg^{-1})
WO-BC	**T_0**	174.26 ± 3.27 a	30361.12 ± 296.86 a	127.32 ± 2.55 a	1.29 ± 0.05 a	0.95 ± 0.03 a
	T_1	147.59 ± 3.67 b	25363.27 ± 297.78c	116.65 ± 2.85 bc	1.10 ± 0.02 bc	0.72 ± 0.02c
	T_2	162.66 ± 2.70 a	27108.85 ± 334.79 b	124.56 ± 2.12 ab	1.19 ± 0.03 ab	0.85 ± 0.03 ab
	T_3	145.78 ± 2.41 b	24704.55 ± 408.57c	120.59 ± 2.11 bc	1.08 ± 0.03 bc	0.74 ± 0.03 bc
	T_4	132.15 ± 3.74c	22771.02 ± 426.66 d	118.21 ± 2.32 bc	0.99 ± 0.04 cd	0.65 ± 0.03 cd
	T_5	110.42 ± 2.86 d	19787.85 ± 303.30 e	114.43 ± 3.05c	0.88 ± 0.03 de	0.55 ± 0.03 de
W-BC	**T_0**	130.73 ± 3.68c	17027.95 ± 455.37 f	93.45 ± 2.45 d	0.81 ± 0.03 ef	0.65 ± 0.04 cd
	T_1	111.41 ± 3.40 d	11509.81 ± 486.68 h	77.89 ± 1.57 e	0.61 ± 0.04 gh	0.48 ± 0.02 ef
	T_2	117.20 ± 3.81 d	14013.54 ± 366.24 g	82.34 ± 3.43 e	0.71 ± 0.03 fg	0.54 ± 0.04 d-f
	T_3	111.09 ± 3.22 d	11409.67 ± 693.60 h	80.43 ± 2.90 e	0.56 ± 0.02 h	0.43 ± 0.03 fg
	T_4	95.38 ± 3.61 e	8956.97 ± 374.55 i	69.43 ± 3.46 f	0.41 ± 0.03 i	0.34 ± 0.03 gh
	T_5	90.37 ± 2.86 e	6957.24 ± 237.78 j	49.54 ± 2.90 g	0.26 ± 0.05 j	0.23 ± 0.03 h

For each parameter, small letters in columns indicate a statistically significant difference between means ($p \leq 0.05$) based on a two-way ANOVA (Tukey's-HSD test). Values represent the treatment means and standard deviation of three replicates; WO-BC = without biochar; W-BC = with biochar; T_0 = control; T_1 = AMF 10 g pot^{-1}; T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; T_4 = T_1 + T_2; T_5 = T_2 + T_4

3.5. *Osmolytes and lipid per oxidation attributes*

The hemp plants subjected to Cu stressed conditions showed a linear decrease in the osmolytes attributes while increase in the lipid per oxidation attributes (Table 3). More accumulation of osmolyte attributes was noticed where biochar addition was done in Cu stressed soils. Soil applied BC decreased the soluble sugar (50.88 %), soluble protein (35.92 %), proline accumulation (51.87 %) and MDA contents (41.81 %) in comparison with control where no BC was applied. This osmolyte accumulation is further anticipated to exhibit a negative interaction with lipid peroxidation since osmolytes prevent cellular membranes from oxidation mediated by copper stress-induced ROS accumulation. The treatment T_4 (AMF 10 g pot^{-1} + SWE 1 g L^{-1}) improved soluble sugar (40.04 %), soluble protein (70.77 %), and decreased the proline accumulation (34.24 %) and MDA contents (37.00 %) in comparison with control. The synergistic effect of BC, AMF and SWE decreased the malonaldehyde contents and proline accumulation and improved the soluble sugars and soluble protein in the hemp plants under stressed and non-stressed conditions.

3.6. *Copper accumulation in the plant parts*

The addition of soil-applied BC combined with the AMF and SWE caused significant change ($p \leq 0.05$) in Cu accumulation attributes in various parts of the hemp plants (Table 4). Change in the Cu accumulation was noticed by the application of BC, AMF and SWE treatments in comparison with the control. Sole application of AMF decreased the root Cu (8.26 %), stem Cu (6.15 %) and leaf Cu (11.01 %) in comparison

Table 3

Synergistic effect of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) on the osmolytes and lipid per oxidation attributes of hemp plants under copper (Cu) stressed soil.

Treatments		Osmolytes and Lipid per Oxidation Attributes			
		MDA (nmol g^{-1})	Soluble sugars (mg g^{-1})	Soluble protein (mg g^{-1})	Proline (µmol g^{-1})
WO-BC	**T_0**	65.35 ± 0.78 a	8.04 ± 0.19 d	36.43 ± 1.66 fg	0.84 ± 0.03 a
	T_1	51.15 ± 1.66c	9.41 ± 0.14 bc	46.51 ± 2.33 cd	0.74 ± 0.02 bc
	T_2	59.37 ± 1.57 b	8.78 ± 0.26 cd	42.28 ± 1.99 de	0.80 ± 0.01 ab
	T_3	50.25 ± 2.36c	9.54 ± 0.22 bc	48.98 ± 1.64 bc	0.72 ± 0.02 c
	T_4	44.53 ± 1.17 d	10.15 ± 0.24 ab	52.35 ± 1.17 ab	0.62 ± 0.02 d
	T_5	40.58 ± 1.57 d	10.94 ± 0.14 a	57.58 ± 1.42 a	0.55 ± 0.01 de
W-BC	**T_0**	42.65 ± 0.83 d	3.05 ± 0.29 h	16.78 ± 0.89 j	0.50 ± 0.02 ef
	T_1	30.21 ± 1.24 e	4.68 ± 0.29 fg	29.96 ± 1.68 h	0.35 ± 0.02 g
	T_2	34.86 ± 2.00 e	4.06 ± 0.25 g	22.61 ± 0.80 i	0.44 ± 0.01 f
	T_3	29.38 ± 1.73 e	4.76 ± 0.26 fg	32.22 ± 1.00 gh	0.33 ± 0.02 gh
	T_4	23.50 ± 1.67 f	5.38 ± 0.18 ef	38.51 ± 0.99 ef	0.26 ± 0.01 h
	T_5	20.51 ± 0.91 f	6.00 ± 0.23 f	42.00 ± 1.39 de	0.18 ± 0.02 i

For each parameter, small letters in columns indicate a statistically significant difference between means ($p \leq 0.05$) based on a two-way ANOVA (Tukey's-HSD test). Values represent the treatment means and standard deviation of three replicates; WO-BC = without biochar; W-BC = with biochar; T_0 = control; T_1 = AMF 10 g pot^{-1}; T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; T_4 = T_1 + T_2; T_5 = T_2 + T_4

Table 4
Synergistic effect of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) on the Cu accumulation in various parts of hemp plants under copper (Cu) stressed soil.

Treatments		Cu Accumulation		
		Root Cu (mg kg^{-1})	Shoot Cu (mg kg^{-1})	Leaf Cu (mg kg^{-1})
WO-BC	T_0	169.76 ± 3.38 a	128.55 ± 2.07 a	95.59 ± 1.76 a
	T_1	162.99 ± 1.29 a-c	122.85 ± 2.40 a	87.18 ± 1.71 bc
	T_2	166.06 ± 2.38 ab	123.79 ± 2.45 a	92.01 ± 2.11 ab
	T_3	159.10 ± 3.25 bc	121.22 ± 2.54 ab	85.10 ± 1.37 bc
	T_4	154.80 ± 3.38 cd	115.11 ± 2.60 bc	82.29 ± 1.72 cd
	T_5	145.84 ± 3.39 de	109.82 ± 2.02 c	72.22 ± 2.09 ef
W-BC	T_0	139.80 ± 2.27 e	99.10 ± 1.15 d	75.44 ± 1.78 de
	T_1	120.99 ± 2.16 f	90.81 ± 2.62 ef	65.02 ± 3.38 fg
	T_2	129.73 ± 2.80 f	94.61 ± 1.60 de	70.92 ± 2.25 ef
	T_3	111.12 ± 2.02 g	86.58 ± 2.02 fg	63.21 ± 0.80 gh
	T_4	105.03 ± 2.47 g	79.02 ± 1.92 g	57.18 ± 2.05 h
	T_5	94.44 ± 2.47 h	69.03 ± 1.21 h	48.21 ± 2.29 i

For each parameter, small letters in columns indicate a statistically significant difference between means ($p \leq 0.05$) based on a two-way ANOVA (Tukey's-HSD test). Values represent the treatment means and standard deviation of three replicates; WO-BC = without biochar; W-BC = with biochar; T_0 = control; T_1 = AMF 10 g pot^{-1}; T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; T_4 = T_1 + T_2; T_5 = T_2 + T_4

with control. Maximum accumulation of Cu in hemp plant parts was noticed where no BC, AMF and SWE was applied. The treatment T_4 (AMF 10 g pot^{-1} + SWE 1 g L^{-1}) decreased root Cu (16.07 %), stem Cu (14.72 %) and leaf Cu (18.45 %) in comparison with control. Combined approach of BC, AMF and SWE decreased the root Cu (22.38 %), stem Cu (21.44 %) and leaf Cu (29.59 %) in comparison with control.

3.7. *Principal component analysis*

Principal component analysis (PCA) plot indicating groups based on associations of measured parameters by transforming the data set of variables into a smaller one while containing most of the information in the large set (Fig. 4). Two principal components accounted for 96.9 % variability. One of the major clusters consist of carotenoids, shoot dry weight, transpiration rate, photosynthetic rate, total chlorophyll contents and several other parameters that is plotted in the positive PC1 axis. This group is dominated by biochar (W-BC) application as part of the hemp plants treatment. The other major cluster consist of malonaldehyde contents, proline accumulation, hydrogen per oxide, catalase activity, super oxide dismutase activity, and other parameters that plotted in the negative direction of the PC1 axis. This cluster of parameters is dominated by the applied treatment without biochar (WO-BC). Such trends are grouping is an indicative of addition or absence of applied biochar to hemp plants. The other measured parameters comprising the soluble protein, soluble sugars and number of leaves plotted away from the major clusters indicating the lack of association with the applied treatments and induced stressed conditions of plants.

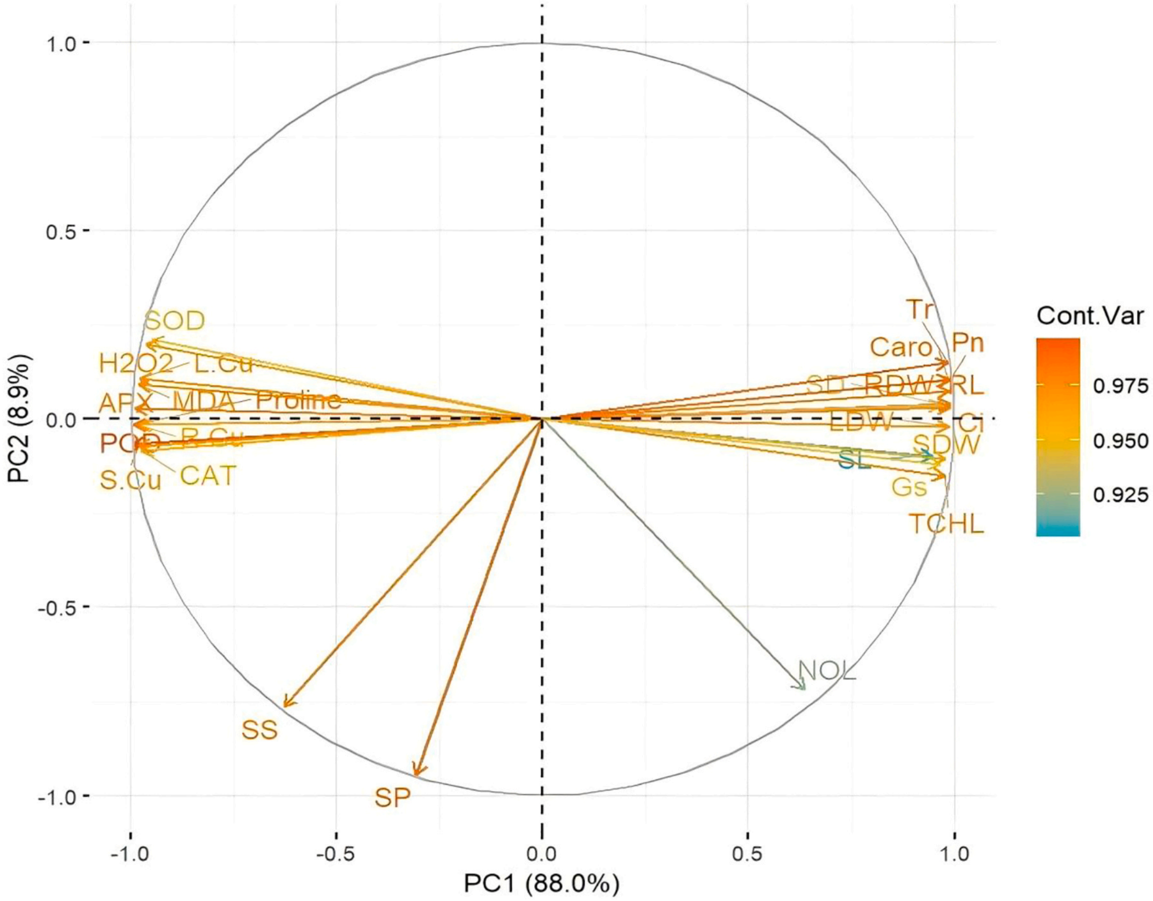

Fig. 4. The principal component analysis (PCA) plot with reduced dimensionality of data sets of measured parameters of hemp plants. SL = shoot length; RL = root length; NOL = number of leaves; RDW = root dry weight; SDW = shoot dry weight; LDW = leaf dry weight; Tr = transpiration rate; Pn = photosynthetic rate; SD = stem diameter; Gs = stomatal conductance; TCHL = total chlorophyll contents; Ci = intercellular CO_2 concentration; SOD = superoxide dismutase activity; CAT = catalase activity; POD = peroxidase activity; MDA = malonaldehyde contents; APX = Ascorbate activity; H_2O_2 = hydrogen per oxide activity; S.Cu = shoot Cu contents; R.Cu = Root Cu contents; L.Cu = leaf Cu contents; SS = soluble sugars; SP = soluble proteins.

The soluble proteins and soluble sugars contents are related to the applied treatments without the biochar application.

3.8. Correlation matrix

A clear association was evident among all growth, photosynthetic, biochemical, lipid peroxidation, enzymatic, and osmolytes related variables of hemp plants grown under Cu stressed soil. Total chlorophyll and all the gas exchange variables exhibited negative correlations with enzymatic activities and MDA. The enzymatic, ROS related, lipid peroxidation, and Cu contents in plant parts, as well as the osmolyte attributes, showed significant negative correlations with stem diameter, dry biomass of leaf, stem, and roots of the hemp plant. Furthermore, an expected positive relationship between lipid peroxidation and Cu is expected as high Cu concentrations causes increased generation of oxidative stress and, thus, leads to enhanced membrane degradation. Similarly, there were strong positive correlations observed between the biomass, photosynthetic, gas exchange and growth-related attributes of the hemp plant. High Cu accumulation may impair biomass under high stress, the positive effect of AMF, BC and SWE could reduce or even neutralize the effect of Cu through a reverse correlation of biomass and Cu accumulation (Fig. 5).

3.9. Chord analysis

A chord diagram depicts the graphical presentation of the determined data of hemp plants and display the inter-relationships between entities in a matrix (Fig. 6). The flows or connections among several entities are shown by chords indicating direction and degree of association. The Peroxidase (POD) dominated among the measured variable of hemp plants as shown on the outer part of the circular layout and the connection to experimental conditions of with or without biochar applications. All treatments applied to hemp plants are displayed on the lower half of the circular diagram. The POD is very effective in oxidizing various substrates and prevents builds up of H_2O_2 in plants generated under stress conditions.

3.10. Redar analysis

The radar chart displaying the changes in measured variables of hemp plants subjected under various treatments of BC, AMF and SWE. This chart uses a radial format, with axes radiating from a central point like spokes on a wheel. Each axis corresponds to a specific parameter, and the data points plotted along these axes form polygons, highlighting variations among the treatments. The radial display presents unique quantitative values along with axes emerging like spokes. Parameters measured under the condition of without biochar (WO-BC) registered

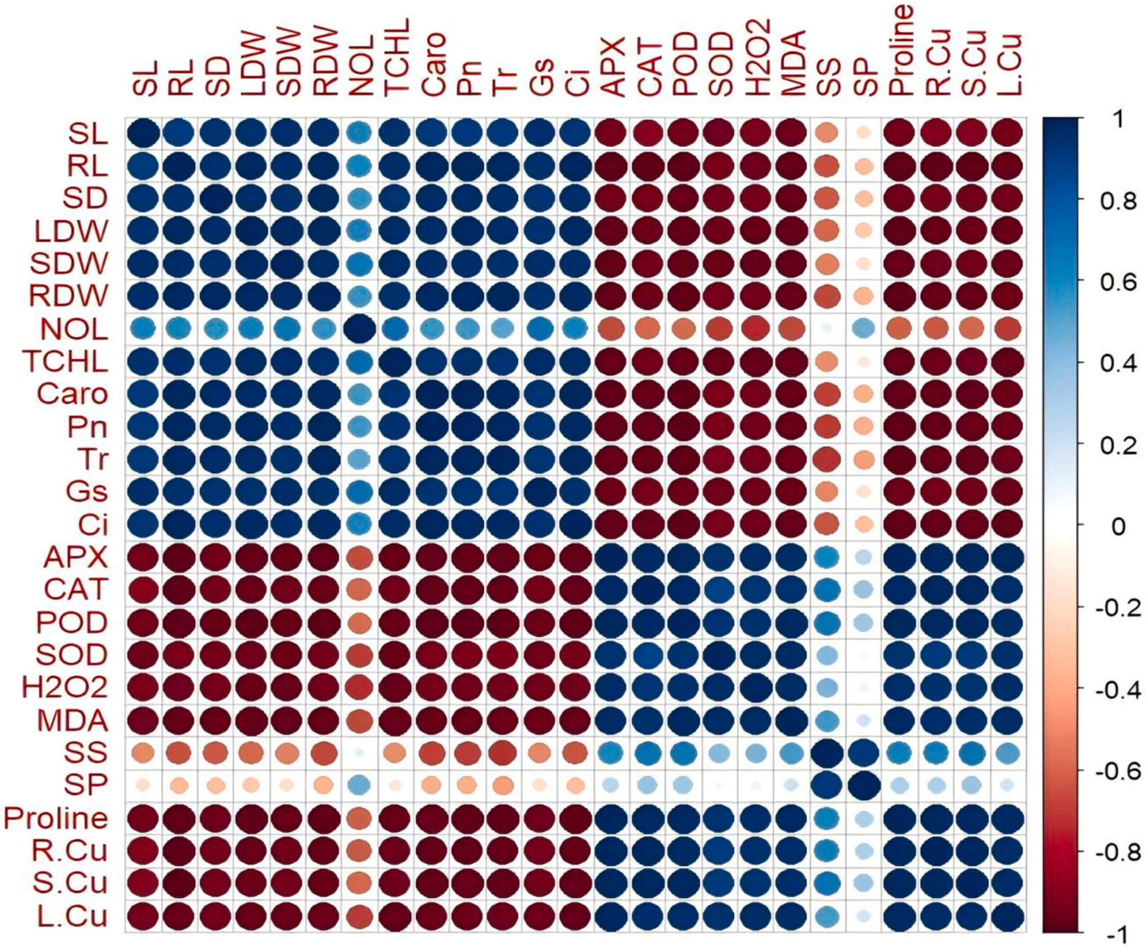

Fig. 5. The correlation matrix of various attributes of hemp plant by combined application of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) grown under copper (Cu) stressed soil; SL = shoot length; RL = root length; NOL = number of leaves; RDW = root dry weight; SDW = shoot dry weight; LDW = leaf dry weight; Tr = transpiration rate; Pn = photosynthetic rate; SD = stem diameter; Gs = stomatal conductance; TCHL = total chlorophyll contents; Ci = intercellular CO_2 concentration; SOD = superoxide dismutase activity; CAT = catalase activity; POD = peroxidase activity; MDA = malonaldehyde contents; APX = Ascorbate activity; H_2O_2 = hydrogen per oxide activity; S.Cu = shoot Cu contents; R.Cu = Root Cu contents; L.Cu = leaf Cu contents; SS = soluble sugars; SP = soluble proteins.

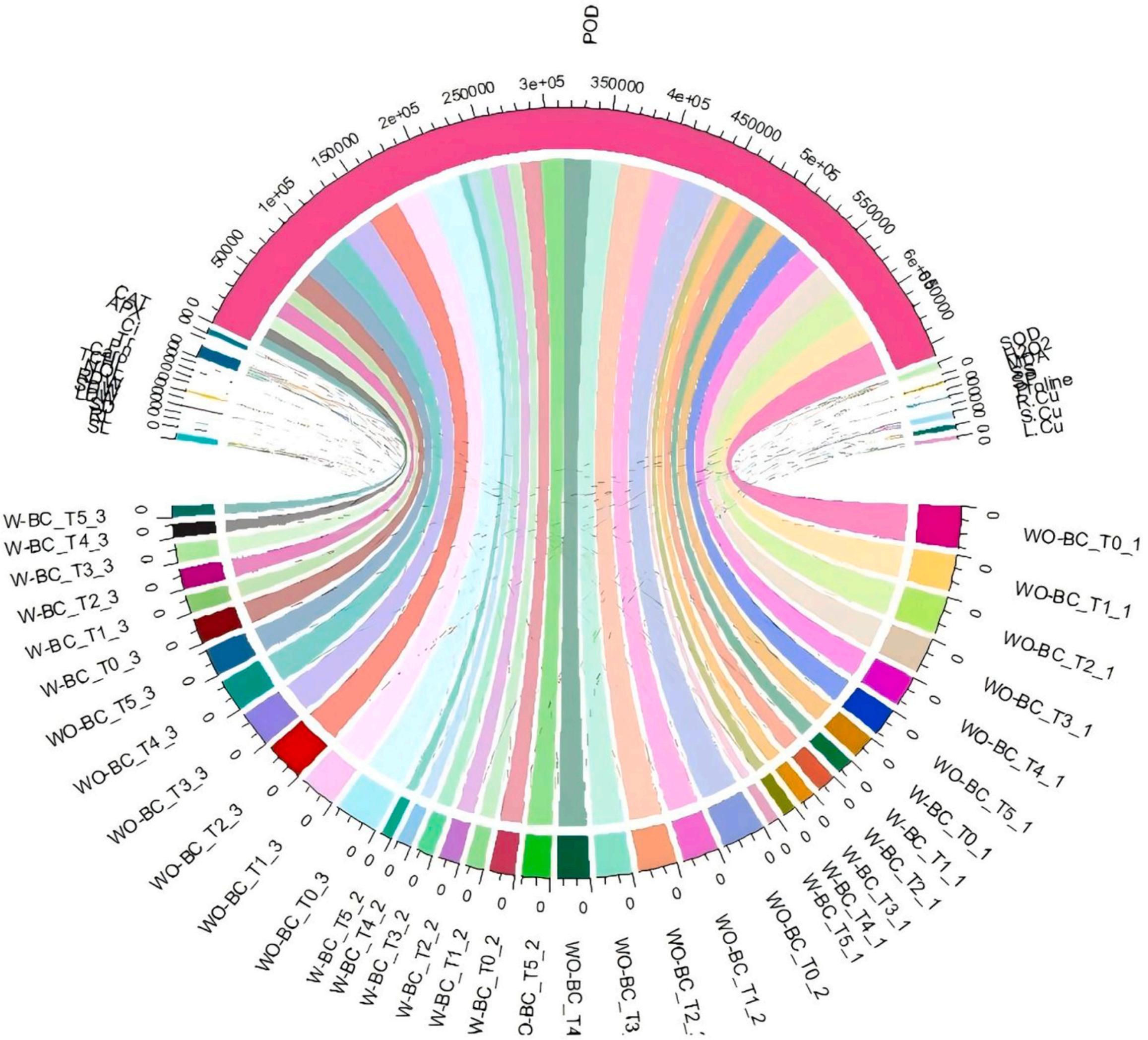

Fig. 6. Chord diagram depiction of relationships among various entities of hemp plant by combined application of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) grown under copper (Cu) stressed soil; WO-BC = without biochar; W-BC = with biochar; T_0 = control; T_1 = AMF 10 g pot^{-1}; T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; $T_4 = T_1 + T_2$; $T_5 = T_2 + T_4$; SL = shoot length; RL = root length; NOL = number of leaves; RDW = root dry weight; SDW = shoot dry weight; LDW = leaf dry weight; Tr = transpiration rate; Pn = photosynthetic rate; SD = stem diameter; Gs = stomatal conductance; TCHL = total chlorophyll contents; Ci = intercellular CO_2 concentration; SOD = superoxide dismutase activity; CAT = catalase activity; POD = peroxidase activity; MDA = malonaldehyde contents; APX = Ascorbate activity; H_2O_2 = hydrogen per oxide activity; S.Cu = shoot Cu contents; R.Cu = Root Cu contents; L.Cu = leaf Cu contents; SS = soluble sugars; SP = soluble proteins.

slightly lower levels as compared to the biochar applied (W-BC) experimental conditions. With biochar application under the applied treatment (e.g., W-BC_T5) dominated in levels from Ci to SL (clockwise) in hemp plants. This indicates the enhancing effect of biochar on the performance of these parameters in hemp plants. Radial display also indicated five minor gaps where none of the measured parameters showed high levels (Fig. 7).

4. Discussion

Soils contaminated with Cu and other heavy metals have negative effects on the growth and development of crops used for food and fiber (Munir et al., 2021). This study examines the possibility of using soil contaminated with Cu for growing hemp crops without affecting their quality. It also offers the potential of hemp to endure and mitigate Cu-contaminated soils, with the goal of offering a new approach to mitigate the impacts of soil pollution and promote sustainable crop cultivation. The findings of the current investigation demonstrated that exposure to Cu stress significantly influenced the growth and biomass characteristics of the hemp crop. The utilization of BC, AMF, and SWE, either alone or in combination, improved the growth and development of the hemp crop (Figs. 1, 2). The reduction in growth and biomass traits is highly associated with the stress caused by Cu, which is probably caused by the toxic effect of Cu on cell division and elongation, resulting in decreased plant growth and biomass accumulation (Franić and Galić., 2019; Mir et al., 2021). The presence of Cu in plants interferes with the production of plant hormones, specifically auxins, which play a crucial role in cell elongation and division (Rahman et al., 2023). This disruption has a direct impact on the growth and biomass properties of the plants (Zhang et al., 2024). It was noticed in the current study that Cu caused a linear decrease in leaf count, stem diameter, and plant biomass (Figs. 1, 2). This is primarily caused by the suppression of cellular division and proliferation (Napieraj et al., 2023). The various part of the hemp plant exhibited a decrease in their biomass that might be due to the degradation of the cell membrane. In order to mitigate the cellular damage that leads to reduced growth and development, the combined use of biostimulants serves as a stress-reducing agent (De Diego and Spíchal, 2020). This aids in mitigating the detrimental impacts of Cu

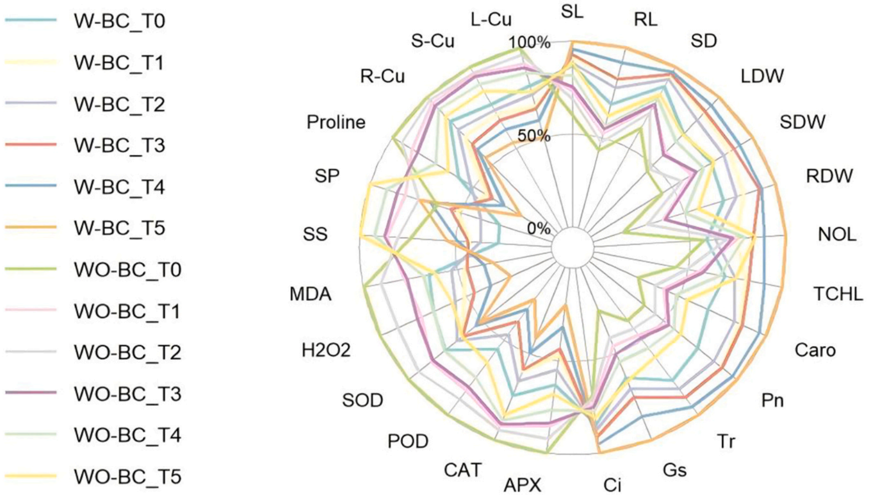

Fig. 7. Radar chart presenting 24 variables contribution and variation in percentage of the hemp plants' response to combined application of biochar (BC), arbuscular mycorrhizal fungi (AMF) and various levels of foliar applied seaweed extract (SWE) grown under copper (Cu) stressed soil; WO-BC = without biochar; W-BC = with biochar; T_0 = control; T_1 = AMF 10 g pot^{-1}; T_2 = SWE 1 g L^{-1}; T_3 = SWE 3 g L^{-1}; $T_4 = T_1 + T_2$; $T_5 = T_2 + T_4$; SL = shoot length; RL = root length; NOL = number of leaves; RDW = root dry weight; SDW = shoot dry weight; LDW = leaf dry weight; Tr = transpiration rate; Pn = photosynthetic rate; SD = stem diameter; Gs = stomatal conductance; TCHL = total chlorophyll contents; Ci = intercellular CO2concentration; SOD = superoxide dismutase activity; CAT = catalase activity; POD = peroxidase activity; MDA = malonaldehyde contents; APX = Ascorbate activity; H_2O_2 = hydrogen per oxide activity; S.Cu = shoot Cu contents; R.Cu = Root Cu contents; L.Cu = leaf Cu contents; SS = soluble sugars; SP = soluble proteins.

stress by modulating physiological and metabolic systems. Soil applied BC and AMF involves promoting the development of roots, managing the osmotic pressure within cells, and ensuring an appropriate balance between antioxidant enzymes and reactive oxygen species, all while maintaining a stable equilibrium (Aziz et al., 2023). The improve growth due to the use of AMF is might be due to the use of AMF acquires photosynthetic carbohydrates/carbon from the roots of host plants, while reciprocally supplying nutrients and water from the soil to the host plants via their extensive hyphal network (Basiru and Hijri, 2024). In addition, SWE provides numerous vital nutrients for growth, including polysaccharides, amino acids, vitamins, and other active compounds (Subbiah et al., 2023). SWE is a source of phytohormones, micronutrients, and biostimulants that improve abiotic stress tolerance by upmodulating stress- responsive genes involved in photosynthesis, water use efficiency, and hormone signaling pathways (Aziz et al., 2024). These nutrients not only promote the development of a strong root system in plants, but also enhance the absorption of water and nutrients (SujataGoyal et al., 2023). The co-effects of these amendments may have enhanced stress tolerance mechanisms resulting to improved physiological and morphological responses in hemp under Cu-stressed environment. That might be the reason of improved growth and biomass of hemp plants under Cu stressed soil. In general, the AMF and SWE has demonstrated growth-promoting effects in plants. Therefore, our study has shown that the combination of BC, AMF and SWE can potentially have a synergistic effect in increasing the resistance of hemp plants to metals.

The findings of our study showed the application of BC, AMF, and SWE in Cu stressed soil led to a substantial enhancement of photosynthetic and gas exchange parameters. Simultaneous applying all the biostimulants resulted in a significant enhancement of the photosynthetic and gas exchange parameters, as shown in Fig. 3. The artificially spiked Cu stress negatively affects the enzyme Rubisco, which leads to a decrease in carbon fixation during the Calvin cycle (Amaral et al., 2024). Similarly, the presence of Cu stress leads to the closure of stomata, resulting in a decrease in stomatal conductance and transpiration rate (Martins et al., 2024). This reduction in stomatal activity restricts the intake of carbon dioxide (CO_2). As a result, the rate of photosynthesis decreases similar observation were noticed in the current study. An observed outcome of using biostimulants is the enhancement of photosynthetic pigments, which have a crucial function in the production of chlorophyll (Punitha et al., 2024). The increased level of photosynthetic pigments is due to a high content of free amino acids, including alanine, aspartate, asparagine and glutamate in the biostimulant (Lardos et al., 2024). The observed enhancement in photosynthetic pigments and gaseous exchange parameters in hemp plants subjected to BC, AMF, and SWE treatment may be attributed to the promotion of root growth and improved nutrient absorption that was clearly depicted from the correlation analysis (Fig. 5). The outcomes of our investigation align with the findings obtained from prior research of Rasoli et al. (2023). Application of SWE can enhance the overall photosynthetic rate, stomatal conductance, and water usage efficiency (Ahmed et al., 2024). AMF plays a role in improving the plants nutrient acquisition mainly phosphorus, which works directly in the photosynthesis process by synthesizing ATP and the formation of chlorophyll (Bhantana et al., 2021). In addition, it was found that oxidative damage to chloroplasts was reduced due to AMF-exercised antioxidant activity. SWE contains phytohormones and biostimulants which reactive stomata responsibly and increase WUE, contributing to the efficient gases exchange under stress (Aziz et al., 2024). Furthermore, SWE is rich in cytokinin, a compound that plays a protective action on chloroplasts and stimulates the production of endogenous cytokinin in plants that are treated with it (Ali et al., 2024). The results of our study indicate that the combined application of BC, SWE, and AMF enhances photosynthetic parameters and pigments content. The combined application probably provides an added advantage, decreasing metal-induced stress while enhancing physiological processes that support and boost the photosynthetic efficiency and gas exchange of hemp in Cu-contaminated soils. In general, the results may be attributed to the application of SWE, which enhanced the total chlorophyll content in the leaves. This improvement in chlorophyll content positively affected the capacity and efficiency of photosynthesis, leading to enhanced vegetative growth in plants (Figs. 4, 5).

The presence of soil polluted with Cu resulted in significant changes in the levels of enzymatic antioxidants, lipid peroxidation, oxidative stress, and osmolytes parameters in hemp leaves (Tables 2, 3). The BC addition enhances antioxidants by modulating plant metabolic processes and cell growth, hence decreasing the generation of reactive oxygen species (ROS) and promoting improved interactions among soil, plants, and water (Zhu et al., 2023). Using BC results in an increased concentration of oxygen as a functional group and porous structure, hence enhancing the antioxidant defence system against ROS (Deng et al., 2024). Biochar enhances plants' ability to mitigate the adverse impacts of Cu-induced ROS via enhancing the antioxidant and glyoxalase systems (Khan et al., 2023). The study revealed that hemp plants subjected to Cu stress and not treated with BC, AMF, and SWE exhibited a higher concentration of osmolytes (Table 4). The results of our research were consistent with previous studies that demonstrated the reduction of abiotic stresses by using biostimulants. The current study highly recommends osmoprotectants to modify osmolytes, turgor pressure and water content of the cell with a view of acquiring resistance to Cu. Insufficient soil moisture for plants results in elevated MDA accumulation when subjected to Cu stress (Ameen et al., 2023). Consequently, this leads to a decrease in the efficiency of photosynthesis as it stimulates the degradation of chlorophyll and the oxidation of lipids (Yusefi-Tanha et al., 2024). The findings suggest that the combined application of BC, AMF, and SWE resulted in higher chlorophyll levels in stressed hemp plants compared to the control plants (Fig. 3). Moreover, the utilization of biostimulants is anticipated to alleviate the negative effects of osmotic stress by maintaining physiological function (Rakkammal et al., 2023). The AMF and SWE exhibit a beneficial influence in mitigating damage inflicted on cell membranes due to osmotic stress (Pal et al., 2024). SWE encourages building up osmoprotectants like proline and soluble sugars essential for stabilizing proteins and membrane in giving cellular homeostasis when faced with stress (Kumari et al., 2022). The simultaneous use of these amendments appears to have a synergistic interaction: in addition to decreasing Cu-triggered oxidative damage, as demonstrated by the decrease in lipid peroxidation content, these amendments also help enhance the plants' antioxidant defence capacity and osmoprotectants accumulation, allowing hemp plants to better endure Cu stress. The study's findings indicate that the application of BC and AMF initially enhances the properties of the soil, followed by the subsequent stimulation of antioxidant enzyme activity in plants by SWE as depicted from the chord and radar analysis (Figs. 6, 7).

The investigation revealed a substantial accumulation of Cu in the plant tissues of hemp leaves (Table 4). By efficiently using BC, AMF, and SWE, the chlorophyll levels in the plant leaves were elevated, leading to improved growth and biomass. The utilization of this ideally blended mixture of biostimulants also resulted in a reduction in the buildup of Cu in the hemp tissues, hence enhancing the overall growth of the plant and its capacity to endure stress caused by Cu. Our investigation revealed that the utilization of BC, AMF, and SWE influenced the amounts of Cu in the plants. Therefore, our results align with prior research that suggests that Cu toxicity significantly affects the incorporation of BC and AMF, as well as the application of SWE in various plant parts of maize (Kotby et al., 2023). BC, in turn, decreases bioavailability of Cu in the soil, but increases the adsorption and slow release of available nutrient concentration for proper regulation of copper absorption (Zaman et al., 2024). Applying BC and AMF to Cu-contaminated soils immobilizes Cu ions, hence restricting their absorption by plants (Wang et al., 2023). Simultaneously, the addition of SWE enhances plant growth, which is essential for multiple physiological processes such as osmoregulation, enzyme activation, and stomatal function (Bahmani Jafarlou et al., 2023). AMF colonize with plant roots to facilitate the movement of metals; they store Cu within the hyphal or vesicular structures, thereby avoiding toxic concentrations while promoting the slow release of Cu to tissues (Zhang et al., 2024). SWE, contained high levels of biostimulants, stimulates the intake of nutrients and improves the root activity and the metal transporters genes. The application of SWE helps alleviate the oxidative stress induced by Cu stress by boosting the plant's antioxidant defence mechanism which ultimately results in improved growth that clearly depicted the lower accumulation of Cu ions in the hemp plant. It could be suggested that such treatments provide an overall homeostasis between the Cu uptake and detoxification in the plant as it is used for the metabolic process and not as a toxic element; which could lead to an improved physiological plant performance even under stress. The combined action of BC, AMF, and SWE not only mitigates the negative impacts of Cu-induced phytotoxicity but also enhances plant growth, resulting in improved overall plant health and increased resistance to heavy metal stress.

5. Conclusion

The present study clearly shown that the addition of Cu into the soil significantly influenced the key physiological and biochemical traits of the hemp plant. This study is the first to investigate the combined impacts of BC, AMF, and SWE on the growth, physiological, and biochemical attributes of hemp. The study indicated that the application of BC, AMF, and 3 g L^{-1} of SWE had a positive effect on the physiological responses of the hemp plants, particularly when applied simultaneously. The combined treatment of BC, AMF, and SWE significantly improved growth, photosynthetic pigments, and gaseous exchange attributes. The application of biostimulants in combination effectively mitigated oxidative stress induced by Cu-contaminated soil by reducing levels of H_2O_2 (50.83 %) and MDA (43.43 %). Combined approach of BC, AMF and SWE decreased the root Cu (22.38 %), stem Cu (21.44 %) and leaf Cu (29.59 %) in comparison with control. In summary, the concurrent application of BC, AMF, and SWE has the potential to enhance hemp growth in soil with high Cu stress, hence offering a sustainable and effective biofertilization approach. Biostimulants may serve as an approach for optimizing conventional agricultural techniques by enhancing crop growth and production. Additionally, they have the ability to promote crop yield in the presence of abiotic stress, whether applied alone or in combination. In addition, future research should priorities enhancing the phytoremediation potential of hemp by utilizing soil conditioners and non-toxic chelating agents to improve the mobilization of metals.

CRediT authorship contribution statement

Xia Cheng: Validation, Software, Methodology, Formal analysis. **Kamran Ashraf:** Writing – original draft, Methodology. **Gang Deng:** Writing – original draft, Visualization, Validation, Supervision, Methodology, Conceptualization. **Qamar uz Zaman:** Resources, Methodology, Investigation, Conceptualization. **Lihong Guo:** Project administration, Methodology, Funding acquisition, Formal analysis. **Xiaorong He:** Visualization, Validation, Methodology. **Yan Luo:** Software, Data curation. **Chen Liu:** Software, Funding acquisition, Data curation. **Ghulam Murtaza:** Software, Methodology, Data curation. **Khawar Sultan:** Visualization, Validation, Project administration, Data curation. **Shah Fahad:** Writing – review & editing, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Data availability

Data will be made available on request.

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