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Research article

Leonardite-aged biochar with soil inoculation of arbuscular mycorrhizal fungi enhances soil health, microbial activity, and lettuce antioxidant-biometric traits in sandy soil

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ABSTRACT

This study evaluated the potential of leonardite-aged biochar (ABC), applied alone or inoculated with arbuscular mycorrhizal fungi (AMF), to improve sandy soil health within a shorter time compared to fresh biochar (FBC). A pot experiment using lettuce as a test crop assessed changes in soil physicochemical properties, nutrient availability, microbial biomass, and cumulative respiration, enzymatic activities, and plant physiological and antioxidant responses under various treatments: control (CK), FBC, ABC, AMF, and ABC + AMF. Aging with leonardite significantly modified biochar surface chemistry, reduced alkalinity, and enhanced functional groups, resulting in improved interactions with soil physicochemical and biological processes. Among treatments, ABC + AMF at a 3% application rate produced pronounced benefits. It increased soil total organic carbon, microbial biomass carbon and nitrogen, soil respiration, and key soil enzymatic activities compared with CK, indicating enhanced microbial functioning and nutrient cycling. In contrast, FBC primarily influenced soil properties through alkalinity-driven effects, increasing soil pH, electrical conductivity, cation exchange capacity, and available phosphorus. Plant responses mirrored the improvements in soil, with ABC-3%+AMF markedly enhancing photosynthetic efficiency (NDVI, NDRE, SPAD), antioxidant capacity (TPC, TFC, CTC), and lettuce growth parameters. Multivariate analysis confirmed positive relationships between soil indicators and plant performance, demonstrating that biochar aging improves its agronomic effectiveness. Overall, the findings show that ABC, particularly inoculated with AMF, offers a practical strategy for restoring sandy soils by stimulating biological activity and nutrient availability while reducing reliance on chemical inputs. This integrated approach provides a sustainable tool for improving soil health and crop productivity in degraded sandy agroecosystems.

1. Introduction

Sandy soils are distributed globally and cover approximately 900 million hectares, particularly in arid and semiarid regions (Yost and Hartemink, 2019). The growing world population relies heavily on these soils for food, feed and fiber production, and other ecosystem services (Huang and Hartemink, 2020). Compared to other soils, sandy soils are generally more prone to degradation due to the low cohesiveness of soil particles, low content of soil organic matter (SOM), and low levels of biological activity (Yost and Hartemink, 2019). Inherent properties of sandy soils cause weak aggregation, poorly developed structure, poor water retention qualities, high permeability, high water infiltration, susceptibility to compaction, erosion, salinization, and low ability to retain and exchange nutrients (Bruand et al., 2005; Tye et al., 2013), causing low fertility, low crop yields, and food quality. Improvement of

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Abbreviations

Leonardite (L)
Biochar (BC)
Fresh biochar (FBC)
Leonardite-aged biochar (ABC)
Arbuscular mycorrhizal fungi (AMF)
Soil microbial biomass carbon (SMBC)
Soil microbial biomass nitrogen (SMBN)
Scanning electron microscopy (SEM)
Energy dispersive X-ray spectroscopy (EDX)
Diffuse Reflectance Infrared Fourier Transform spectroscopy (DRIFT)
Soil organic carbon (SOC)
Total nitrogen (TN)
Electrical conductivity value (EC)
Available phosphorus (P_{avail})
Normalized Difference Vegetation Index (NDVI)
Normalized Difference Red Edge Index (NDRE)
Total phenolic compounds (TPC)
Condensed tannin content (CTC)
Total flavonoid content (TFC)
principal component analysis (PCA)

sandy soil can increase the relevant ecosystem services such as carbon (C) sequestration, water availability, and biodiversity (Bendixen et al., 2021; Bonfante et al., 2020). The development of stress-tolerant plant varieties, the addition of organic matter to the soil, and the application of consortium-based biofertilizers are all investigated to sustain the quality of sandy soils and achieve these goals (Chen et al., 2011; Khan, 2022; O'Brien et al., 2021).

Biochar (BC), a byproduct of biomass pyrolysis, can enhance soil fertility when used as an amendment, particularly in low-fertility soils like sandy-textured ones (Bekchanova et al., 2024). BC increases soil C content and water retention, neutralizes acidic pH, reduces bulk density, and increases soil porosity, cation exchange capacity (CEC), and the content of primary plant nutrients (Zhang et al., 2024). Biochar-induced soil amelioration boosts crop productivity and microbial activity (Sharma et al., 2025). Most of the knowledge on the effects of BC on soil properties and biochemical activity has been obtained using fresh BC application in soils or post-production functionalization (Zhang et al., 2019), whereas less information is available on the impacts of aged BC. Studies show that BC aging occurs naturally in amended soils through the reduction of its aromaticity (Rombolà et al., 2023) and persistence of the inertinite after the degradation of labile BC fraction after 15 years of residence in soil (Chiaramonti et al., 2024). Biochar modification in soil occurs mainly through abiotic oxidation, disintegration due to the combined chemical solubilization of water, freeze-thaw and wet-dry cycles (Sigmund et al., 2023), C leaching, and microbial degradation (Mia et al., 2017). Effects of BC post-production aging by H_2O_2, HNO_3, KOH, $KMnO_4$, O_3, and $K_2Cr_2O_7$ on their potential for the remediation of polluted soils (Chen, 2020), whereas little research has been conducted on the effects of BC aging on the chemical and biochemical fertility of soils and growth of cultivated plants (Rahim et al., 2023, 2024).

Leonardite (L), an organic substance derived from the oxidation of lignite, has shown auxin-like properties (O'donnell, 1973) and the capability to enhance soil nutrient availability, plant uptake, root and shoot growth, soil aggregation, and microbial activity (Nardi et al., 2017; Wolny-Kołładka et al., 2022). It can increase crop yield by directly stimulating plant metabolic activity or indirectly by increasing microbe-mediated SOM decomposition and nutrient mineralization (Khan, 2022; Suzuki et al., 2005). Because of its positive effects on crop production, L is admitted as a plant bio-stimulant in the Regulation EU 2019/1009 on CE-labelled fertilizers (European Union, 2019). L has the potential to be used safely as the primary agent for aging biochars. The characteristics of L make it a naturally occurring agent rich in oxygenated functional groups, such as carboxyl, phenolic, and quinone; these active sites can bind onto the BC surface through adsorption, ion exchange, and/or mild oxidation reactions (Csicsor and Tombácz, 2022; Georgieva et al., 2017; Petrov et al., 2017; Stewart and Janin, 2014). These interactions may introduce additional polar functional groups onto BC, increase surface reactivity, and enhance cation exchange capacity, thereby mimicking natural soil aging processes (Ausavasukhi et al., 2016; Hammerschmiedt et al., 2021; Wang et al., 2020). Unlike chemical oxidants such as H_2O_2, HNO_3, and $KMnO_4$, commonly used for artificial aging, L-based aging provides a biologically relevant modification that simultaneously supplies labile organic compounds capable of stimulating soil microbial activity (Georgieva et al., 2017). Therefore, L-aged biochar (ABC) may represent a more agronomically realistic strategy for improving soil–biochar–microbe interactions than conventional chemical modifications. Nevertheless, little information is available on the effects of ABC on the biochemical fertility of sandy soils and on plant growth.

In addition to organic amendments, the use of effective microbes such as AMF also has the potential to enhance the productivity of degraded sandy soils (Fall et al., 2022). AMF form a symbiotic association with plant roots to help plants in nutrient uptake in the soil. They also assess the ability of plants to acquire water and withstand abiotic stressors (Chen et al., 2018). Their extensive hyphal networks contribute to soil aggregation and stabilization through the production of glomalin-related soil proteins and other binding agents (Morris et al., 2019). These functions are especially beneficial to sandy soils, which typically have a poorly developed soil structure, a low amount of organic matter, and a low level of potential nutrient retention (Fan et al., 2024). Recent studies suggest that BC can provide favorable habitats for AMF by increasing soil porosity, moisture retention, and nutrient availability, and by offering protected microsites for fungal hyphae and spores (Neuberger et al., 2024). Thus, combining AMF inoculation and BC may actually complement each other in enhancing the biochemical processes occurring in the soil and increasing overall plant performance (Ji et al., 2023). However, it remains largely unexplored whether such a combination can be further enhanced when AMF is specifically combined with aged biochar rather than fresh biochar. Understanding these combined treatment effects is essential for developing integrated soil restoration strategies for sandy agroecosystems.

Based on identified knowledge gaps, this study hypothesizes that aging with leonardite (L) can improve the surface functionality and reactivity of fresh biochar (FBC), which in turn would enhance soil nutrient availability and biochemical activity more significantly than FBC. The research also suggests that using L-aged BC (ABC) with soil inoculation of AMF could lead to combined treatment effects, resulting in improved fertility and health of sandy soil, as well as better physiological and antioxidant responses in lettuce. The objectives of the study was to (i) synthesize ABC via facile liquid-soaking technique, and characterize the elemental and surface functional groups modifications induced by L aging on FBC, (ii) to compare the effects of FBC, ABC, AMF inoculation, and their combination (ABC + AMF) on the physicochemical and biochemical fertility of a low-fertility sandy soil; and (iii) to evaluate how these treatments influence plant physiological status, antioxidant responses, and growth performance using lettuce as a model crop, chosen for its rapid growth cycle, quick physiological responses, and prevalence in sandy soil cultivation.

2. Materials and methods

2.1. Biochar (BC), leonardite (L), and arbuscular mycorrhiza fungi

Commercial BC of agronomic grade utilized in this work was obtained by Romagna Carbone s.n.c. (Italy). BC was produced from orchard pruning biomass by slow pyrolysis at 500 °C in a transportable ring kiln with a diameter of 2.2 m that could accommodate about 2 tons of feedstock. The produced BC was crushed into particles <5 cm in diameter. The main physicochemical characteristics of biochar were: pH value 9.8, CEC 101 cmol kg^{-1}, maximum water absorption capacity 4.53 g g^{-1} of d.m, BET 410 m^2 g^{-1}, and total porosity of 2722 mm^3 g^{-1} (Baronti et al., 2014). A commercially available Leonardite humic substance in solid form was provided by Demetra Italia srl (Italy). Arbuscular mycorrhizal fungi (AMF) were introduced using a commercial inoculum containing Glomus iranicum var. tenuihypharum (1% active ingredient; 120 propagules g^{-1}) together with beneficial rhizosphere bacteria (10^3 CFU g^{-1}) as declared by the supplier (Sigma-Aldrich, USA). The inoculum was thoroughly mixed into the root zone soil at planting according to the manufacturer's instructions to promote effective root colonization.

2.2. *Aging of biochar (BC) with leonardite (L) and characterization*

A facile liquid soaking procedure modified from Hammerschmiedt et al. (2021) was used to age BC with L. Briefly, 1 g of L and 5 g of biochar were mixed with 25 ml of ultrapure water (rate 1:5:25 w:w:v) in a 100 ml beaker. The mixture was vigorously shaken at room temperature (25 °C) for 2 h. After shaking, the suspension in the beaker was agitated, loosely covered with a thin film, and extensively aerated daily for 7 days to enhance the aging of biochar with L. The black content in the beaker was homogenized after the aging process was completed, and any excess water was carefully drained. The resulting black material was rinsed with ultrapure water three times and dried at 105 °C in an oven until a constant weight (Fig. 1) and finally stored in a Ziplock bag before use. The same scale and protocols were followed to prepare the L-aged biochar (ABC) in bulk for subsequent experiments with the sandy soil. The pH value of the resulting ABC was 7.8 compared to FBC (9.8). The total P content decreased from 2780 mg kg^{-1} to 1240 mg kg^{-1} after aging.

Scanning electron microscopy (SEM) was used to determine the surface morphology of L, FBC and ABC, using a Zeiss EVO 40 electron microscope (Zeiss, Oberkochen, Germany). Energy dispersive X-ray spectroscopy (EDX) was applied to know about the elemental composition on the surfaces of Leonardite, fresh and aged BC materials using Oxford EDS detector INCAx-act Model 51. Diffuse Reflectance Infrared Fourier Transform spectroscopy (DRIFT) was used to examine different functional groups on the surface of Leonardite, fresh, and aged BCs, using Bruker Vertex 70 V vacuum FTIR spectrometer.

2.3. *Experimental setup and preliminary soil analyses*

Sandy soil samples (0-20 cm) were collected from the agricultural farmlands located in the area surrounding the municipality of Copparo (Ferrara Province, N Italy, 44°53′18.6" N 12°08′50.6" E). Soil samples were mixed thoroughly, air-dried, and sieved (<2 mm), and their main physicochemical properties are shown in Table 1. The textural class of the surface horizon (0–20 cm depth) of soil was sandy-loam according to the USDA classification. Moreover, the physicochemical characteristics of the soil indicate a calcareous, low-organic-matter sandy soil with limited nutrient and water retention capacity. The pH value in H_2O and 1 N KCl (1:2.5 w:v) was measured with a pH meter (pH 211, Hanna Instruments). Soil organic carbon (SOC) and total nitrogen (TN) were determined by dry combustion method using an elemental analyzer (Thermo Soil NC–Flash EA1112, Waltham, MA, USA) (Stazi et al., 2018). The CEC was determined after extraction with 10% w/v $BaCl_2$ solution under pH 8.1 (Gillman, 1979), and the results were expressed as cmol kg^{-1} of soil. The electrical conductivity value (EC) was measured in a 1:5 w:v suspension using a conductivity meter (Orion, Germany), and total carbonates in soil were quantified by measuring the CO_2 released after

Table 1
Physicochemical characteristics of experimental soil.

Property	Unit	Value
pH (H_2O)	—	7.4 ± 0.008
pH (KCl)	—	6.5 ± 0.003
EC	μS cm^{-1}	136.1 ± 0.6
CEC	cmol kg^{-1}	14.2 ± 0.12
Carbonates	%	24 ± 0.33
Moisture content	%	3.2 ± 0.06
Total organic carbon	%	0.77 ± 0.0057
Total nitrogen	%	0.085 ± 0.00014
Available phosphorus	(μg g^{-1} fs)	35.8 ± 1.8

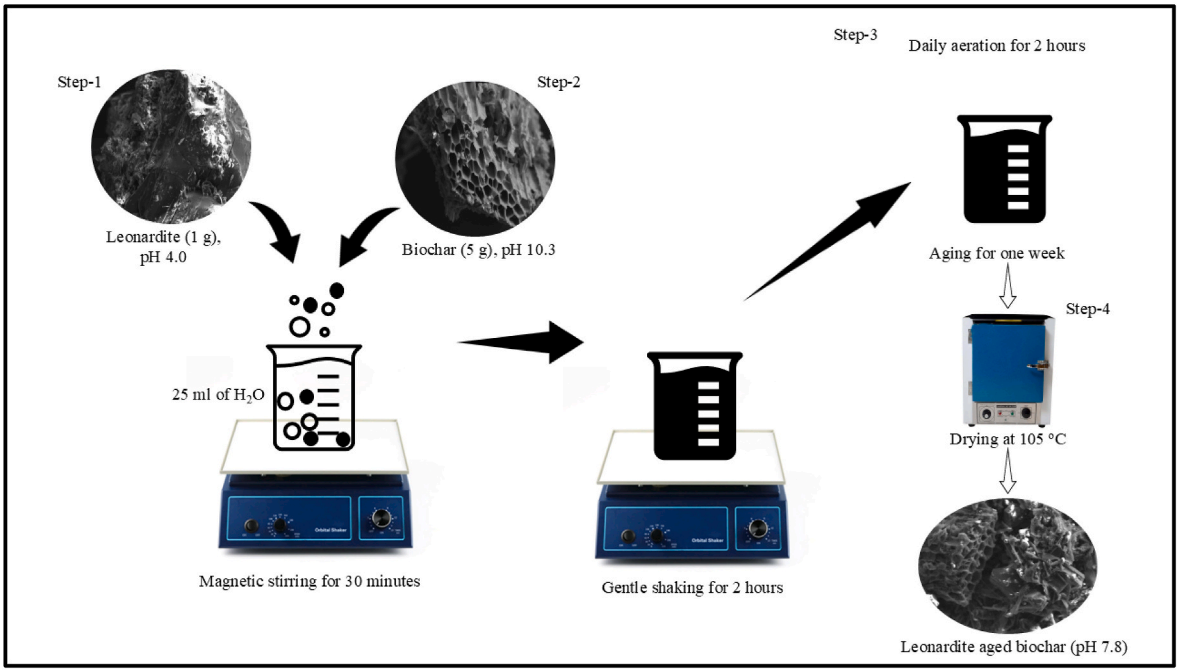


Fig. 1. Schematic representation of the leonardite aged biochar (ABC).

reaction with HCL (37%) following the official Italian calcimetric method (De Astis, 1999). Available phosphorus was quantified by acid extraction with 1 M NH_4F using method described by Bray and Kurtz (1945). Moisture content in soil samples was determined by gravimetric method of FAO (2023).

A pot experiment trial was conducted for 42 days (June 8th, 2023 - July 20th, 2023) in greenhouse facilities of the Institute of Higher Education "Vergani Navarra" in Ferrara (N. Italy), using the lettuce (*Lactuca Sativa* L., Canasta Verde variety) as a test crop. Plants received natural sunlight throughout the growing period, with an average daytime temperature of approximately 42 °C. Relative humidity was not monitored during the experiment; however, all treatments were maintained under identical environmental conditions. The following seven treatments were established: control (CK) with no substrate amendment, fresh biochar (FBC) at amendment rates of 1.5% and 3%, L-aged biochar (ABC) at amendment rates of 1.5% and 3%, sole arbuscular mycorrhizal fungi (AMF) at 2 g (equivalent to 1 g kg^{-1} of soil), and L-aged biochar at 3% with AMF (ABC-3%+AMF). Each treatment was replicated four times and arranged in a completely randomized design. Plastic pots of appropriate size were filled with 2 kg of soil based on dry soil weight which was previously thoroughly manually mixed with the different treatments and doses. Tap water was then added to each pot to adjust the moisture to 60% of the soil water-holding capacity, and soils were left to stabilize for one week. After the stabilization period, AMF was inoculated in the plant root zone, and one healthy lettuce seedling was transplanted into each pot. Pots were rotated weekly and manually irrigated as needed to maintain 60% of the water-holding capacity. No commercial fertilizers were applied for a better understanding of the efficacy of organic amendments.

2.4. *Photosynthetic pigments, morphology, and antioxidant compounds in lettuce leaves*

During the lettuce growing season, a Chlorophyll Concentration meter (MC-100) was used for the determination of the SPAD values of lettuce leaves to estimate the chlorophyll concentration. At the same time, a RapidScan CS-45 canopy sensor was used for estimating the Normalized Difference Vegetation Index (NDVI) and the Normalized Difference Red Edge Index (NDRE), respectively for monitoring the nutrient status of lettuce plants by the spectral analysis of the fully expanded leaf from the top of the plant. Plant morphological data were determined at harvested after 36 days of growth. The number of leaves was counted on each plant, plant shoots were cut at ground level, and the roots were gently removed from the soil and washed with tap water. Fresh shoot and root biomass weights were measured using a laboratory scale. The lettuce biomass was then dried in a forced-air oven at 60 °C until it was a constant weight to determine the dry weights of the shoots and roots.

Total phenolic content (TPC), total flavonoid content (TFC), and condensed tannin content (CTC) was determined in lettuce leaf extracts dried in the dark as described by Borella et al. (2023). Extraction involved immersing leaf material in 80% (v/v) methanol at a 1:10 (g: mL) ratio, shaking for 30 min, incubating in the dark at 4 °C for 48 h, and filtering; filtrates were used for TPC, TFC, and CTC assays. TPC were quantified using the Folin-Ciocalteu's reagent (Al-Duais et al., 2009). After reaction in the dark, Na_2CO_3 (7% w/v) and distilled water were added to samples, incubated for 90 min in the dark. Absorbance was read at 760 nm with an Agilent 8453 UV-Vis spectrophotometer (Santa Clara, CA, USA). Gallic acid (98%) was used as the standard (5–300 μg mL^{-1}), and results were expressed as gallic acid equivalent (GAE) mg g^{-1} DW of extract. TFC was quantified using the aluminum chloride colorimetric method (Chang et al., 2002). After reaction in the dark, samples were neutralized with 1 M NaOH and incubated in the dark for 15 min. Absorbance was measured at 415 nm with the Agilent 8453 spectrophotometer. Quercetin (≥95%) was the standard (12.5–150 μg mL^{-1}), and results were expressed as quercetin equivalent (QE) mg g^{-1} DW of extract. CTC was quantified using the acidified vanillin method (Broadhurst and Jones, 1978). After reaction in the dark, absorbance was read at 500 nm with the Agilent 8453 spectrophotometer. Tannic acid (ACS reagent) was the standard (12.5–900 μg ml^{-1}), and results were expressed as tannic acid equivalent (TAE) mg g^{-1} DW of extract.

2.5. *Post-harvest analysis of soil samples*

The soil samples from each pot were collected at the end of the experiment, air-dried, and sieved (<2 mm). Active and exchangeable acidity was measured with the same methods used for the original soil samples. Soil respiration was determined by the quantification of CO_2 evolved and trapped after 1, 3, 7, 10, 14, 17, 21, 24, 28, and 31 days of incubation in 4 ml of 1 N NaOH was determined by titration of the excess NaOH with 0.2 M HCl (Badalucco et al., 1992). Soil microbial biomass carbon (SMBC) and soil microbial biomass nitrogen (SMBN) were determined using the fumigation extraction method (Vance et al., 1987), and quantification of C and N in the extracts from fumigated and non-fumigated extracts by elemental analyzer (Elementar-Vario TOC Cube), using the conversion factors for biomass C and N as reported by Vance et al. (1987) and Brookes et al. (1985), respectively.

Concerning the soil enzyme activities, the alkaline and acid phosphomonoesterase activities were determined with the method of Tabatabai and Bremner (1969), the phosphodiesterase activity with the method of Browman and Tabatabai (1978), the arylesterase activity was measured with the method of Zornoza et al. (2009), the β-glucosidase activity was determined using the method of Eivazi and Tabatabai (1988), and the cellulase activity was measured with the method of Imparato et al. (2016). The phenol oxidase activity was determined using 2,2′azinobis-(-3ethylbenzothiazoline-6-sulfononic acid) diammonium salt (ABTS) as substrate (Floch et al., 2007), whereas the arylsulfatase activity was measured according to Tabatabai and Bremner (1970). The protease activity was measured using casein as substrate (Ladd and Butler, 1972), and the urease activity was determined according to Tabatabai and Bremner (1972). The concentration of 4-nitrophenol (p-NP) released by the acid and alkaline phosphomonoesterase, phosphodiesterase, arylesterase, β-glucosidase, and cellulase activities were measured with a UV/VIS spectrophotometer (Thermo) at wavelength of 400 nm, using a 0-180 mM p-NP calibration curve. The protease activity was determined by the colorimetric quantification of the tyrosine released by the casein, the urease activity was by the colorimetric quantification of the released NH_4^+-N using the Nessler reagent. The phenol oxidase activity was determined by spectrophotometric quantification of $ABTS^+$ released by the reaction at 420 nm, using a calibration curve performed to determine the coefficient of extinction of $ABTS^+$ from a purified laccase from *Marasmius quercophilus* (2000 U l^{-1}) after incubation at 30 °C for 24 h in the dark, in both the presence and absence of soil.

2.6. *Data analysis*

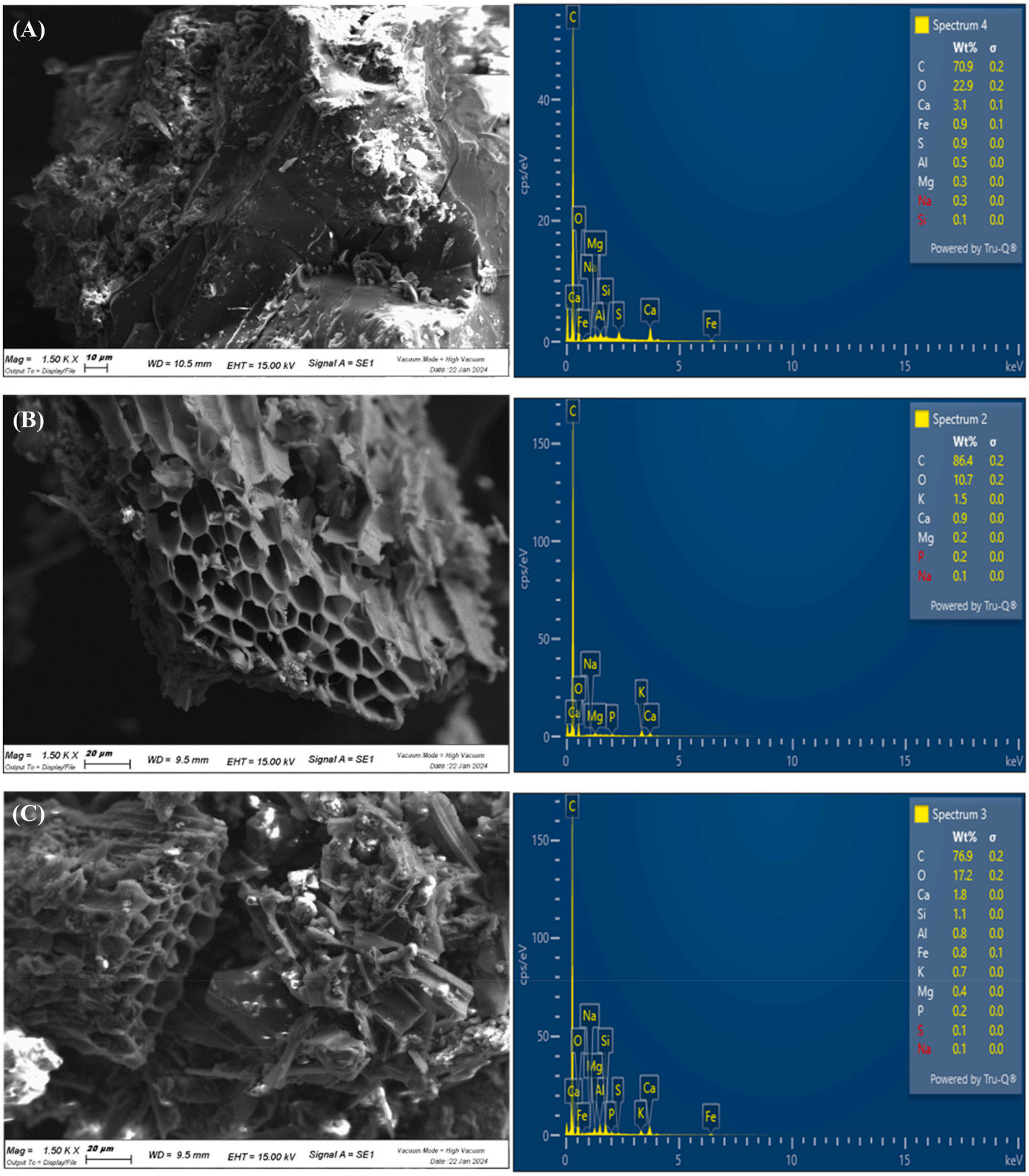
All the data collected on plant and soil parameters were analyzed using the linear model procedure within Statistix 8.1 software (Analytical Software Co., St. Paul, MN, USA). A one-way analysis of variance (ANOVA) with treatment as a single factor was conducted followed by multiple pairwise comparisons using the Tukey HSD test at a 95% confidence level to determine significant differences between mean values of different treatments. The GraphPad Prism-8 software was used to create the figures, whereas Pearson's correlation and principal component analysis (PCA) of the variables was performed using the Corrplot, Factoextra and FactoMineR packages in the R studio environment.

3. Results

3.1. SEM-EDS and DRIFT characterization

The surface morphologies and elemental composition of L, FBC, and ABC characterized by SEM coupled with EDS are shown in Fig. 2. The L showed a rough, heterogeneous and rock-like surface (Fig. 2A), demonstrating a scaly and recessed surface dominated by C (71 wt%), O (23 wt%), and Ca (3.1 wt %), followed by other elements. The FBC showed a well-developed porous honeycomb-like structure, with a network of pores of various dimensions and shapes (Fig. 2B), highly enriched rich in C (86 wt%), O (11 wt%), and K potassium (1.5 wt%), followed by other elements. The ABC displayed higher roughness, porous structure, and twisted morphology, with sharper edges and corners than FBC (Fig. 2C), with elemental composition, indicating adsorption or impregnation of FBC by L moiety.

The DRIFT spectra of L showed an intense and broadband at around 3400 cm^{-1} that could be addressed to hydroxyl-groups (OH), followed by the weak peaks at 2915 cm^{-1} and 2845 cm^{-1} Fig. 3A) corresponding to the saturated C-H stretching vibrations (Xie et al., 2022). The peaks at lower wavelengths were due to the presence of alkenes or aromatic compounds (1942 cm^{-1}, 1620 cm^{-1}), carboxylic acids and derivatives (1710 cm^{-1}, 1220 cm^{-1}), amide (1515 cm^{-1}), and ketones (1710 cm^{-1}). The peak at 1050 cm^{-1} may be associated with the presence of aromatic C-O-C bonds (You et al., 2024) or sometimes Si–O–Si or Si–O–Al asymmetric stretching (Xie et al., 2022). The DRIFT spectrum of FBC (Fig. 3B) showed broadband at 3458 cm^{-1} due to the stretching vibration of O-H followed by the weak peaks at 2918 cm^{-1} and 2844 cm^{-1}



Spectrum 4

	Wt%	σ
C	70.9	0.2
O	22.9	0.2
Ca	3.1	0.1
Fe	0.9	0.1
S	0.9	0.0
Al	0.5	0.0
Mg	0.3	0.0
Na	0.3	0.0
Si	0.1	0.0

Spectrum 2

	Wt%	σ
C	86.4	0.2
O	10.7	0.2
K	1.5	0.0
Ca	0.9	0.0
Mg	0.2	0.0
P	0.2	0.0
Na	0.1	0.0

Spectrum 3

	Wt%	σ
C	76.9	0.2
O	17.2	0.2
Ca	1.8	0.0
Si	1.1	0.0
Al	0.8	0.0
Fe	0.8	0.1
K	0.7	0.0
Mg	0.4	0.0
P	0.2	0.0
S	0.1	0.0
Na	0.1	0.0

Fig. 2. SEM images coupled with EDX of L (A), FBC (B), and ABC (C).

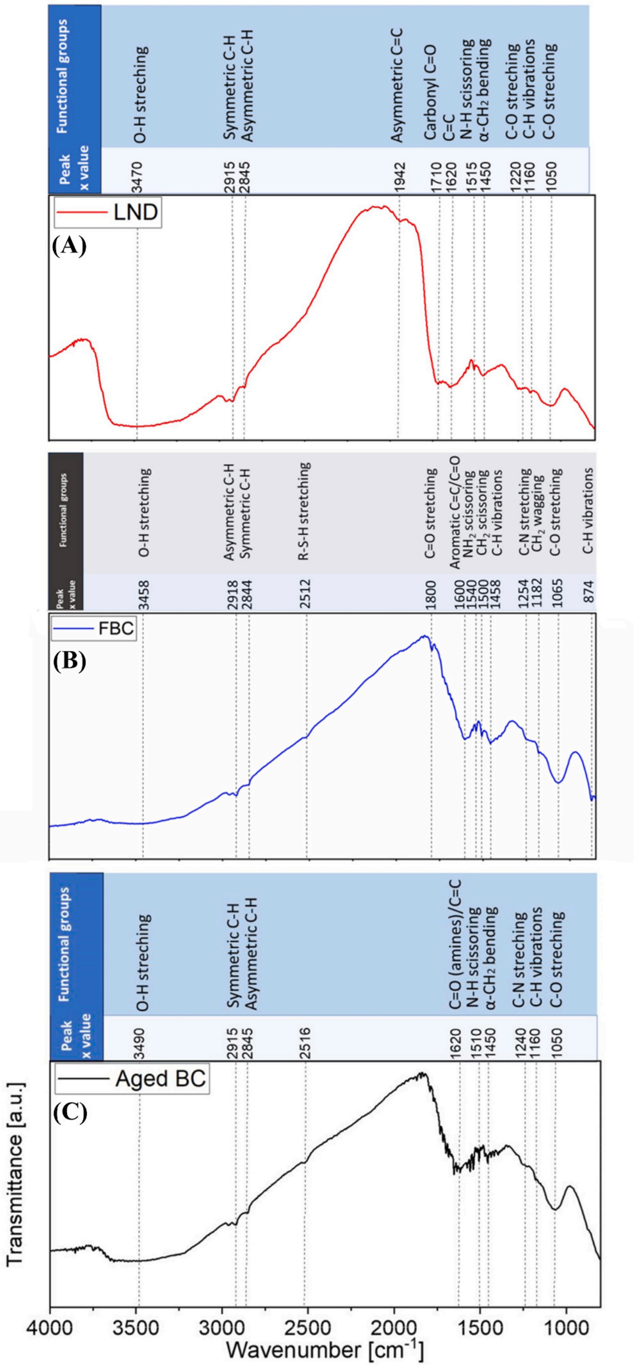


Fig. 3. DRIFT spectra of L (A), FBC (B), and ABC (C).

corresponding to the saturated C-H stretching vibration (Keiluweit et al., 2010). The peak at 2516 cm^{-1} did not match prior research on black carbon, but based on characteristic IR absorption peaks of functional groups it could be related to thiols (R-S-H stretch). Other peaks present in the FBC sample (Fig. 3B) were detected at: 1800 cm^{-1} attributable to C=O stretching of anhydrides (Do et al., 2022), 1600 cm^{-1} attributable to stretching vibration of aromatics C=C/C=O 1600 cm^{-1} (the stretching vibration of aromatic C=C/C=O) (Xie et al., 2022), 1540 cm^{-1} attributable to amines (Li et al., 2017), 1500 cm^{-1} attributable to methylene groups, 1458 cm^{-1} attributable to the bending vibration of C-H, 1254 cm^{-1} attributable to the stretching vibration of C-N of amines, 1182 cm^{-1} attributable to CH_2 wagging, 1054 cm^{-1} attributable to C-O stretching, and 874 cm^{-1} attributable to out-of-plane bending of the aromatic ring C-H bonds (Caporale et al., 2014; Keiluweit et al., 2010). The ABC DRIFT spectra (Fig. 3C) showed intense, and broadband around 3400 cm^{-1} that could be addressed to hydroxyl-groups (OH), followed by the weak peaks at 2915 cm^{-1} and 2845 cm^{-1} corresponding to the saturated C-H stretching vibration. The peak at 2516 cm^{-1} did not match prior research on black carbons, but based on characteristic IR absorption peaks of functional groups it could be related to thiols (R-S-H stretch). The peaks at lower wavelengths were attributed to the presence of aromatic compounds (1620 cm^{-1}) and amides or amines (1620 cm^{-1}, 1510 cm^{-1}, 1240 cm^{-1}). The peak at 1050 cm^{-1} could be attributed to the presence of aromatic C-O-C bonds (You et al., 2024), or to Si–O–Si or Si–O–Al (Xie et al., 2022). Narrow peaks in the ranges between 4000 and 3300 cm^{-1} and 2100–1300 cm^{-1} were related to water vapor inside the test chamber.

3.2. *Elemental composition of L, FBC, and ABC*

Compared to FBC, the ABC had significantly higher H (from 0.95% to 1.05%) and O (from 13% to 19%) contents and a slight increase in N (from 0.481% to 0.483%). However, there were no significant changes observed in the C content based on the ratio of C content to dry residues at 105 °C due to a higher moisture content of the ABC as compared to FBC, with dry residue decreasing from 87% in FBC to 58% in ABC (Table 2).

3.3. *Changes in physicochemical properties and nutrients dynamics of amended soils*

Changes in soil physicochemical properties and nutrients dynamics under different amendments are presented in Tables 3 and 4. FBC-3% produced the highest increases in soil pH and EC, reflecting the alkalinity of FBC, whereas ABC-3%+AMF resulted in only a moderate pH increase, indicating that L aging reduced the alkalizing effect. Sole AMF application had minimal influence on these parameters and slightly decreased EC. Soil CEC was most improved by ABC-3%+AMF (+28%), followed by FBC-3% (+15%), while ABC-3% alone slightly reduced CEC (−9%) relative to the CK. Total carbonate content increased in all biochar treatments, with maximum enhancements observed for FBC-3% (+55%) and ABC-3%+AMF (+54%). All biochar amendments markedly improved soil moisture retention, particularly ABC-3%+AMF and ABC-3%, FBC-3% compared with CK. Regarding nutrients, treatments significantly affected available phosphorus and TOC, but not total nitrogen. The highest increase in available phosphorus was obtained with FBC-3% (+93%), followed by ABC-3%+AMF (+72%). Soil TOC was most strongly enhanced by ABC-3%+AMF (+39%), exceeding both FBC-3% (+34%) and ABC-3% (+33%), while total nitrogen showed only small and non-significant increases. Overall, FBC mainly influenced soil chemical properties through pH and phosphorus availability, whereas ABC with soil inoculation of AMF exerted stronger combined effects on CEC, TOC, and moisture retention.

3.4. *Effects of amendments on soil microbial activity*

Soil microbial activity responded markedly to different amendments

Table 2
Concentration of macro-elemental composition of L (leonardite), FBC, and ABC.

Parameters	Unit	L	FBC	ABC
Dry residues (105 °C)	(%)	81.1 ± 5.7	87.4 ± 6.1	58.2 ± 4.1
Nitrogen	(%)	1.3 ± 0.15	0.48 ± 0.06	0.483 ± 0.058
Carbon	(%)	40.8 ± 4.9	50.0 ± 6.0	25.1 ± 0.0
Hydrogen	(%)	2.6 ± 0.31	0.9 ± 0.11	1.05 ± 0.13
Oxygen	(%)	21.8 ± 3.3	13.1 ± 2.0	18.8 ± 2.8
Phosphorus	mg kg^{-1} (s.s)	39.8 ± 6.0	2780 ± 420	1240 ± 190

Table 3
Effect of experimental treatments on the physicochemical properties of sandy soil.

Treatments	pH (H_2O)	pH (KCl)	EC (µS cm^{-1})	CEC (cmol + kg^{-1})	Carbonates (%)	MC (%)
CK	7.5 ± 0.05b	6.6 ± 0.01d	136.5 ± 1.19d	15.6 ± 0.39b	25.0 ± 0.71c	3.3 ± 0.333d
FBC-1.5%	7.6 ± 0.08b	6.8 ± 0.009bc	137.5 ± 1.32d	14.3 ± 0.74b	35.3 ± 0.85a	8.2 ± 1.084b
FBC-3%	7.8 ± 0.13a	7.2 ± 0.04a	159.3 ± 0.63a	17.9 ± 0.89ab	38.7 ± 0.63a	10.1 ± 0.381ab
ABC-1.5%	7.6 ± 0.08ab	6.8 ± 0.05c	142.3 ± 0.95c	16.8 ± 1.152ab	30.5 ± 1.44b	7.8 ± 0.714bc
ABC-3%	7.5 ± 0.03ab	6.9 ± 0.01b	146.0 ± 1.08bc	14.2 ± 0.76b	35.0 ± 0.41a	11.0 ± 0.41a
AMF	7.5 ± 0.07b	6.7 ± 0.012cd	136.0 ± 0.71d	16.8 ± 0.534ab	27.5 ± 1.32bc	5.3 ± 0.144cd
ABC-3%+AMF	7.6 ± 0.04ab	6.7 ± 0.023cd	149.8 ± 0.25b	20.0 ± 1.032a	38.5 ± 0.65a	11.2 ± 0.63a
Significance	**	***	***	***	***	***

The values are the means of four replicates and include standard errors of the means ($n = 4$). Different letters within each column indicate significant differences between values at $p < 0.001$***, $p < 0.01$**, and $p < 0.05$*. MC; moisture content.

Table 4
Effect of experimental treatments on the physicochemical properties of sandy soil.

Treatments	Total organic carbon (%)	Available phosphorous (µg g^{-1} fs)	Total nitrogen (%)
CK	0.97 ± 0.099c	35.40 ± 0.035d	0.0845 ± 0.0031
FBC-1.5%	1.11 ± 0.072abc	50.05 ± 0.050bc	0.0868 ± 0.0014
FBC-3%	1.29 ± 0.013ab	68.19 ± 0.068a	0.0883 ± 0.0019
ABC-1.5%	1.17 ± 0.040abc	55.38 ± 0.055abc	0.0865 ± 0.0055
ABC-3%	1.30 ± 0.006ab	56.04 ± 0.056abc	0.0887 ± 0.0027
AMF	1.01 ± 0.105bc	45.25 ± 0.045cd	0.0858 ± 0.0019
ABC-3%+ AMF	1.35 ± 0.011a	60.88 ± 0.060ab	0.0913 ± 0.0032
Significance	**	***	*ns*

The values are the means of four replicates and include standard errors of the means ($n = 4$). Different letters within each column indicate significant differences between values at $p < 0.001$***, $p < 0.01$**, and $p < 0.05$*. *ns; non-significant.*

(Figs. 4 and 5). Cumulative soil respiration was highest under ABC-3%+AMF, ABC-1.5%, and FBC-3%, indicating enhanced microbial activity compared with CK. Microbial biomass carbon and nitrogen were most strongly stimulated by ABC-3%+AMF, showing increases of 33% and 16%, respectively, relative to CK. Soil enzyme activities exhibited treatment-specific responses: alkaline phosphatase was notably enhanced by ABC-3%+AMF (+48%) and ABC-3% (+38%), while phosphodiesterase activity increased mainly with ABC-3%+AMF (+38%) and ABC-3% (+33%). Cellulase, arylesterase, protease, and urease activities were generally stimulated across most biochar treatments, with particularly strong effects observed for ABC-3%+AMF and FBC-3%. In contrast, β-glucosidase showed limited response, increasing only under AMF alone (+12%). Acid phosphatase and phenol oxidase activities displayed more variable patterns depending on treatment, with both increases and decreases observed. Overall, the combined application of ABC with soil inoculation of AMF consistently produced the most pronounced improvements in soil microbial biomass and several key soil enzyme activities, highlighting a combined treatment effects on soil biochemical functioning.

3.5. *Changes in photosynthetic pigments, content of antioxidant compounds, and morphology of lettuce plants*

Soil amendments significantly improved the physiological status, antioxidant content, and growth of lettuce plants (Fig. 6). The ABC-3%+AMF treatment consistently produced maximum values for photosynthetic performance, with NDVI (+44%), NDRE (+83%), and SPAD (+37%) exceeding all other treatments. Antioxidant compounds, including TPC, TFC, and CTC contents, were also maximized under ABC-3%+AMF (+29%, +26%, and +56%, respectively), while FBC-3% and ABC-3% alone produced moderate increases and lower rates were less effective. Morphologically, ABC-3%+AMF stimulated the maximum enhancements in plant height (+34%), number of leaves (+49%), shoot fresh (+100%) and dry biomass (+103%), and root fresh (+53%) and dry biomass (+89%) compared with CK, whereas other treatments showed smaller improvements.

3.6. *Multivariate analysis of soil and plant responses*

To obtain an integrated understanding of treatment effects, Pearson correlation analysis and principal component analysis (PCA) were used to evaluate relationships among soil physicochemical, biochemical, and plant variables. Correlation analysis demonstrated predominantly positive relationships between soil physicochemical and biochemical parameters (Fig. 7A). Increases in soil TOC, CEC, available P, and moisture content were strongly associated with higher soil microbial biomass and enhanced soil enzyme activities, confirming that biochar amendments, particularly the combined effect of ABC-3%+AMF, improved the biological functioning of sandy soil. Similarly, positive correlations were observed between soil properties and plant physiological indicators, including NDVI, NDRE, and SPAD values (Fig. 7B). These associations indicate that improvements in soil fertility and biochemical activity promoted greater nutrient availability and photosynthetic efficiency in lettuce plants. Correlation analysis between soil properties and plant morphological traits (Fig. 7C) further supported this trend, showing that enhanced soil water retention, nutrient status, and enzymatic activity translated into improved plant height, leaf number, and biomass. Strong positive relationships were also found between plant photosynthetic pigments and morphological attributes (Fig. 7D), highlighting that physiological improvements were directly linked to better plant growth performance.

The PCA provided complementary insights into the overall treatment effects (Fig. 8). The first principal component (PC1) accounted for 67.6% of the total variance and was mainly driven by soil fertility indicators (TOC, available P, total N), soil microbial biomass (SMBC and SMBN), and most soil enzyme activities. These variables were positively associated with plant photosynthetic pigments and morphological parameters, indicating that soil biochemical improvements were the primary drivers of enhanced plant performance. In contrast, ACP and β-glucosidase showed weaker or negative associations, suggesting enzyme-specific responses to amendments. Treatments involving ABC-3%+AMF and FBC-3% clustered closely with key soil and plant variables, confirming their higher performance compared to other treatments.

4. Discussion

4.1. *Soil physicochemical properties and nutrients dynamics*

The present study demonstrates that both FBC and ABC significantly

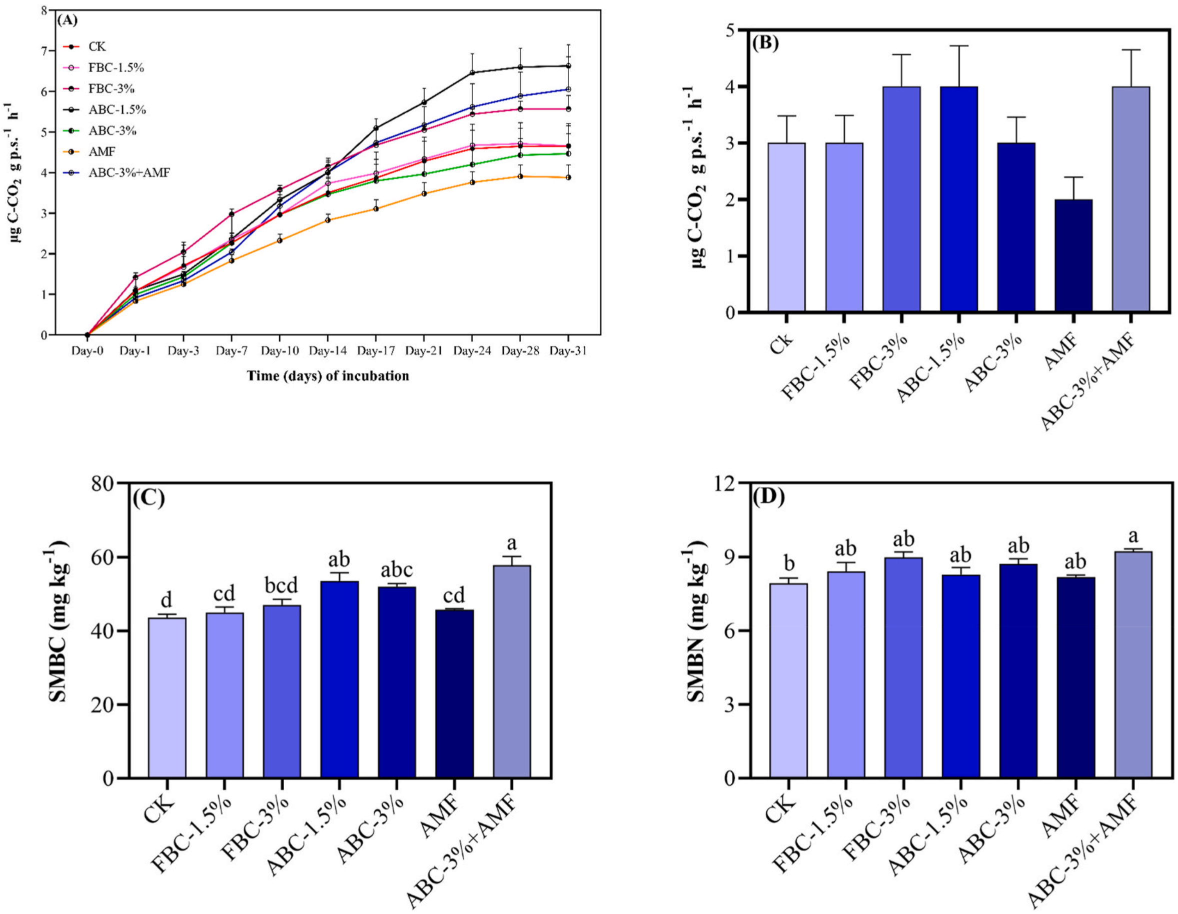


Fig. 4. Changes in cumulative soil respiration during 31 days of incubation (A), total respiration during 31 days of incubation (B), microbial biomass carbon (C), and microbial biomass nitrogen (D) under different treatments. The values represent the mean value ($n = 4$), and the error bar indicates the standard error of the means. Different letters indicate significant differences at ($p < 0.05$).

improved the physicochemical properties and nutrients dynamics of sandy soil (Tables 3 and 4), but through distinct mechanisms. FBC primarily acted as a liming and mineral amendment, whereas ABC, particularly when inoculated with AMF, operated through more complex biochemical interactions. The increase in soil pH following FBC application can be attributed to the intrinsic alkalinity of biochar, which contains inorganic carbonates, oxides, and alkaline surface functional groups capable of neutralizing soil acidity (Fidel et al., 2017). In contrast, the comparatively smaller pH increase observed with ABC and ABC-3%+AMF treatments suggests that the aging process with leonardite introduced additional acidic functional groups, which partially neutralized the alkaline components of FBC (Pastorelli et al., 1999). This moderation effect is particularly relevant in sandy soils, which are characterized by low buffering capacity and are highly sensitive to pH fluctuations (Huang and Hartemink, 2020). Similar pH increases after fresh biochar application have been widely reported in previous studies (de Sousa Lima et al., 2018; Wu et al., 2020). The significant improvement in CEC in amended soils reflects the high surface area and negative charge density provided by biochar and leonardite-derived organic matter (Liang et al., 2006). The maximum CEC enhancement in the ABC-3%+AMF treatment indicates a combined effect among biochar surfaces, humic substances, soil colloids, and AMF-associated root exudates. Such interactions likely promoted the formation of stable organo-mineral complexes, improving nutrient retention in this inherently low-fertility sandy soil (Hailegnaw et al., 2019; Yan et al., 2023). EC increased in all biochar-amended soils due to the release of soluble cations from biochar and leonardite as well as the ion-complexing capacity of their functional groups (Alotaibi and Schoenau, 2019; Chintala et al., 2014). Interestingly, sole AMF application slightly reduced EC, possibly because actively growing fungal hyphae immobilized base cations and decreased ion solubility (Giri et al., 2003). This supports the hypothesis that AMF can regulate nutrient dynamics through biological uptake rather than chemical addition. The marked increase in TOC in ABC-3%+AMF, FBC-3%, and ABC-3% treatments (Table 4) align with previous findings that biochar amendments enhance soil organic carbon pools and stimulate microbial activity (Huang et al., 2023; Zhang et al., 2024). The particularly strong combined treatment effect of ABC-3%+AMF suggests that AMF may have facilitated the stabilization and incorporation of biochar- and leonardite-derived carbon into soil aggregates (Jiang et al., 2023). Increased water retention induced by these amendments may have further promoted carbon accumulation in sandy soils (Amoakwah et al., 2022). Consistent with earlier studies, both biochar and leonardite improved P phytoavailability (Karimi et al., 2020). However, none of the treatments significantly altered total soil N, confirming that these amendments mainly influence N transformations rather than directly supplying nitrogen.

4.2. Soil microbial activity

Soil respiration and microbial biomass are sensitive indicators of biological activity and organic matter turnover. The higher cumulative respiration observed in ABC-3%+AMF, ABC, and FBC treatments (Fig. 4A and B) can be attributed to increased availability of labile organic carbon and enhanced microbial metabolism. These findings help reconcile conflicting reports in the literature, where biochar has been shown to increase (Watzinger et al., 2014), decrease (Kuzyakov et al.,

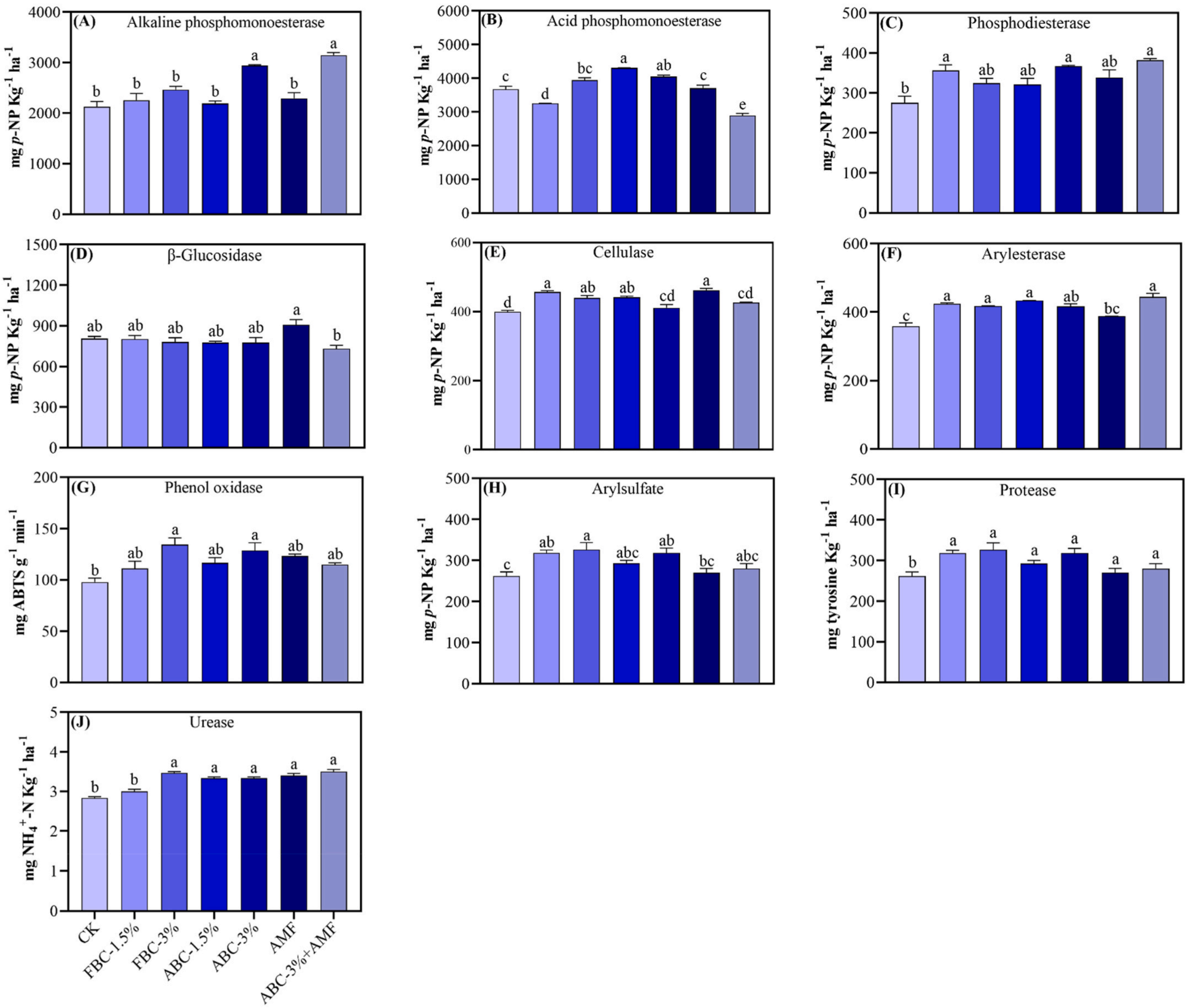


Fig. 5. Effect of treatments on soil enzymes activity. The values represent the mean value ($n = 4$), and the error bar indicates the standard error of the means. Different letters indicate significant differences at ($p < 0.05$).

2009; Paz-Ferreiro et al., 2012), or have neutral effects on soil respiration (Wu et al., 2013). Our results indicate that the presence of leonardite and AMF provided additional metabolizable substrates, leading to a net stimulatory effect. The significant increases in microbial biomass C and N under ABC-3%+AMF (Fig. 4C and D) confirm previous observations that biochar combined with humic substances can enhance microbial proliferation (Holatko et al., 2020; Liu et al., 2024). The present study extends these findings by demonstrating, for the first time, that ABC with soil inoculation of AMF provides a particularly favorable environment for microbial biomass development. Enzyme activity patterns revealed important functional shifts in soil microbial metabolism. The increase in phenol oxidase and arylesterase activities, coupled with reductions in β-glucosidase and cellulase activities (Fig. 5), suggests a transition from utilization of simple carbohydrates toward decomposition of more aromatic and complex substrates. This shift likely reflects the selective adsorption of labile sugars onto biochar surfaces and the preferential microbial use of aromatic dissolved organic matter (Giagnoni and Renella, 2022). The increase in alkaline phosphatase and phosphodiesterase activities and the reduction in acid phosphatase is consistent with the rise in soil pH, which favors enzymes with alkaline optima (Herbien and Neal, 1990). Enhanced arylsulfatase activity indicates greater potential for S mineralization, a response commonly associated with higher microbial biomass and respiration (Castellano and Dick, 1991; Klose et al., 2011). Despite the absence of significant changes in total soil N, the stimulation of protease and urease activities demonstrates that biochar and AMF enhanced N cycling processes. These responses are particularly important for sandy soils, which are typically N-deficient (Yost and Hartemink, 2019). Similar biochar-induced stimulation of N-transforming enzymes has been previously reported (Zhang et al., 2022). The contribution of AMF to improved N uptake and enzyme activity is also well documented (Bahadur et al., 2019; Veresoglou et al., 2012). Overall, enzyme and respiration data indicate that combined effect of ABC-3%+AMF did not merely increase microbial abundance but promoted a functional reorganization of the soil microbial community toward more efficient nutrient cycling.

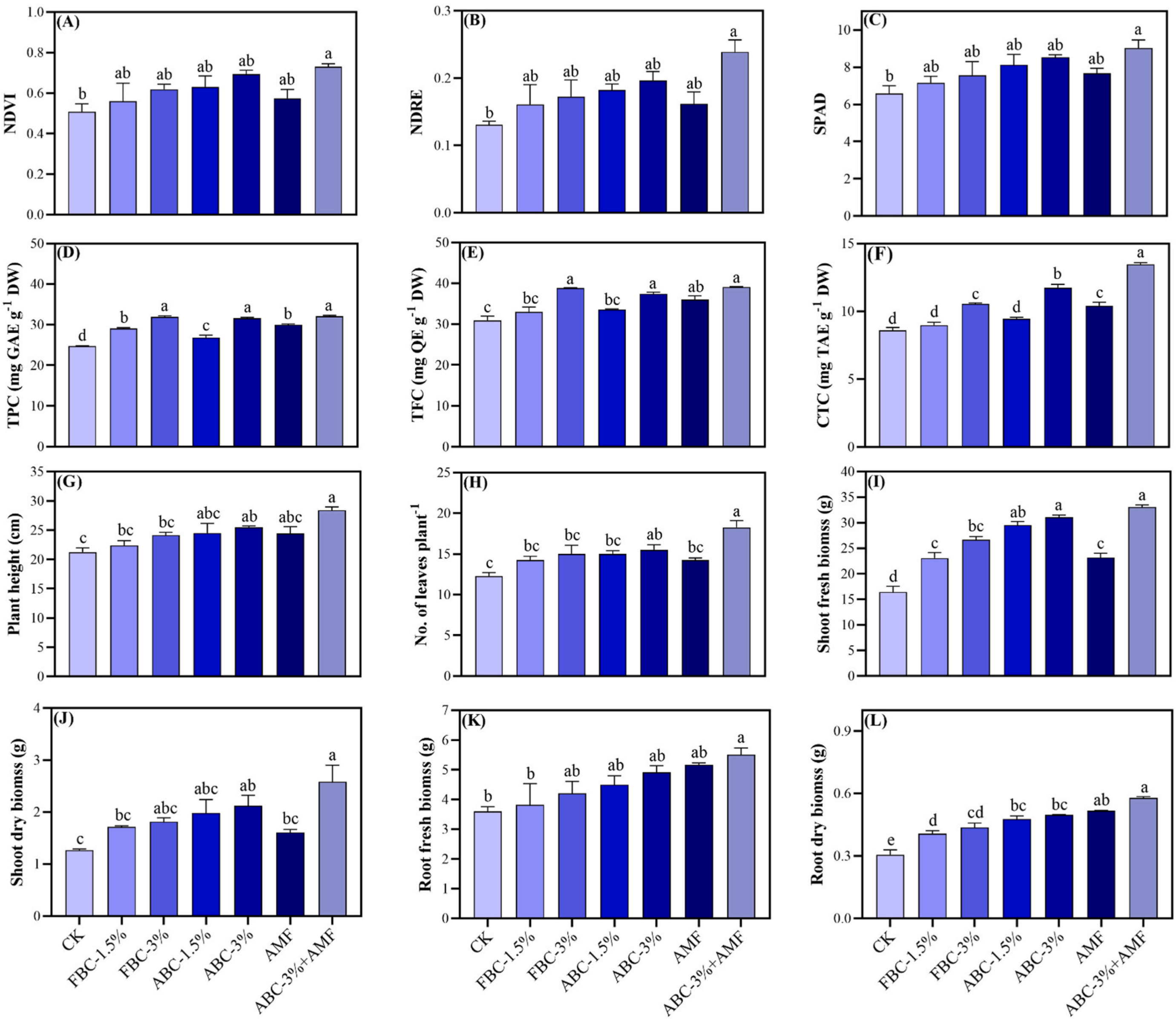


Fig. 6. Effects of treatments on NDVI (A), NDRE (B), SPAD (C), TPC (D), TFC (E), CTC (F), plant height (G), no. of leaves plant^{-1} (H), shoot fresh and dry biomass (I&J), and root fresh and dry biomass (K&L). The values represent the mean value ($n = 4$), and the error bar indicates the standard error of the means. Different letters indicate significant differences at ($p < 0.05$).

4.3. Photosynthetic pigments, antioxidants compounds, and morphology of lettuce plants

The improvements observed in plant physiological and morphological traits can be mechanistically linked to the soil changes induced by the amendments. Biochar's porous structure enhances soil water retention, aeration, and nutrient availability in sandy soils (Abel et al., 2013; Xu et al., 2013). It also modifies soil biochemical characteristics (Pimenta et al., 2019; Pukalchik et al., 2017) and can improve nutrient sensing and uptake (An et al., 2022). Similarly, humic substances released from leonardite improve soil aggregation and root growth by regulating micro- and macropore distribution (Pukalchik et al., 2017). AMF further enhances nutrient acquisition through symbiotic associations with plant roots (Jabborova et al., 2021). The higher NDVI, NDRE, and SPAD values observed in ABC-3%+AMF treatments (Fig. 6A–C) therefore reflect integrated improvements in soil structure, nutrient supply, and root functionality. These physiological enhancements translated into higher chlorophyll content and better photosynthetic performance. Increases in TPC and TFC (Fig. 6D and E) can be attributed to improved nutrient availability and reduced physiological stress. Enhanced microbial activity and better soil moisture conditions favor nutrient uptake, which in turn stimulates secondary metabolite production (Baiamonte et al., 2019; Gul et al., 2015). Humic substances are also known to act as plant biostimulants, promoting antioxidant pathways (Canellas and Olivares, 2014; de Santiago et al., 2008). Previous studies have similarly reported increases in TPC and TFC in lettuce grown with humic amendments and AMF inoculation (Avio et al., 2017; Borguini et al., 2013; Gholami et al., 2018). Morphological improvements in lettuce, particularly under combined effect of ABC-3%+AMF, reflect the alleviation of key limitations of sandy soils. Enhanced moisture retention, P availability, and microbial activity contributed to greater shoot and root biomass (Fig. 6G–L). Humic-based products have been widely shown to stimulate plant growth (Ampong et al., 2022; Tiwari et al., 2023), while AMF symbiosis improves nutrient uptake and biomass accumulation (Amendola et al., 2017; Jabborova et al., 2021). These results confirm that biochar improves plant growth through its

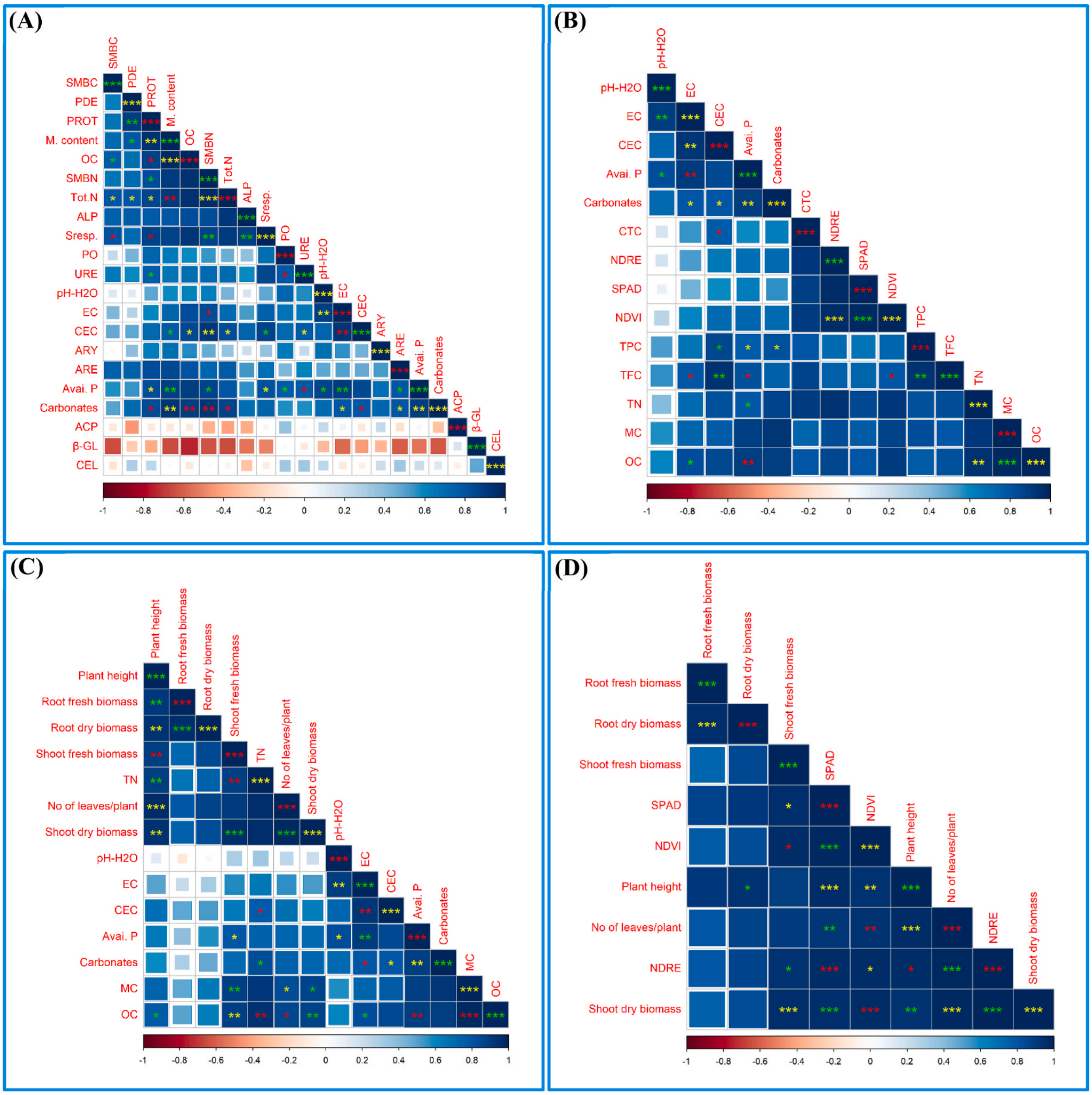


Fig. 7. Pearson's correlation matrix showing relationships between: (A) soil physicochemical and soil biochemical properties, (B) soil physicochemical properties and plant photosynthetic pigments, (C) soil physicochemical properties and plant growth attributes, and (D) plant photosynthetic pigments and plant growth attributes. Significance levels are indicated as $p < 0.001^{(***)}$, $p < 0.01^{(**)}$, and $p < 0.05^{(*)}$. Alkaline phosphatase (ALP), acid phosphatase (ACP), phosphodiesterase (PDE), β-glucosidase (β-GL), cellulase (CEL), arylesterase (ARE), phenol oxidase (PO), arylsulfatase (ARY), protease (PROT), urease (URE), moisture content (MC), soil microbial biomass carbon (SMBC), soil microbial biomass nitrogen (SMBN), soil respiration (S.resp.), organic carbon (OC), total nitrogen (TN), available phosphorous (Avai. P), cation exchange capacity (CEC), electrical conductivity (EC), normalized difference vegetation index (NDVI), normalized difference red edge index (NDRE), total phenolic compounds (TPC), condensed tannin content (CTC), total flavonoid content (TFC).

multifunctional surface properties and indirect biological effects (Jiang et al., 2020). It is important to highlight that this study was conducted under greenhouse conditions, with high daytime temperatures (~42 °C). Relative humidity was not continuously monitored. All treatments were subjected to the same experimental conditions, allowing valid comparisons. However, temperature and humidity may have influenced plant performance. Use caution when applying these findings to field conditions. Further studies in open-field environments are recommended.

5. Conclusions

This study demonstrated that aging fresh biochar with leonardite (L) using a liquid-soaking technique significantly enhanced its capacity to improve the fertility of agronomic sandy soil. The improved performance was attributed to L-induced modifications in biochar morphology, elemental composition, and surface functional groups, which positively influenced key soil quality indicators such as moisture retention, cation exchange capacity, nutrient availability, microbial biomass, and enzymatic activity. Among the tested treatments, the 3% application rate exhibited the most consistent and beneficial effects on soil health and lettuce performance. These findings highlight the potential of ABC as an effective, low-cost, and environmentally sustainable strategy for restoring degraded sandy soils and enhancing crop productivity. The study provides a mechanistic basis for integrating ABC and AMF inoculation into soil management practices, contributing to more resilient and resource-efficient agricultural systems.

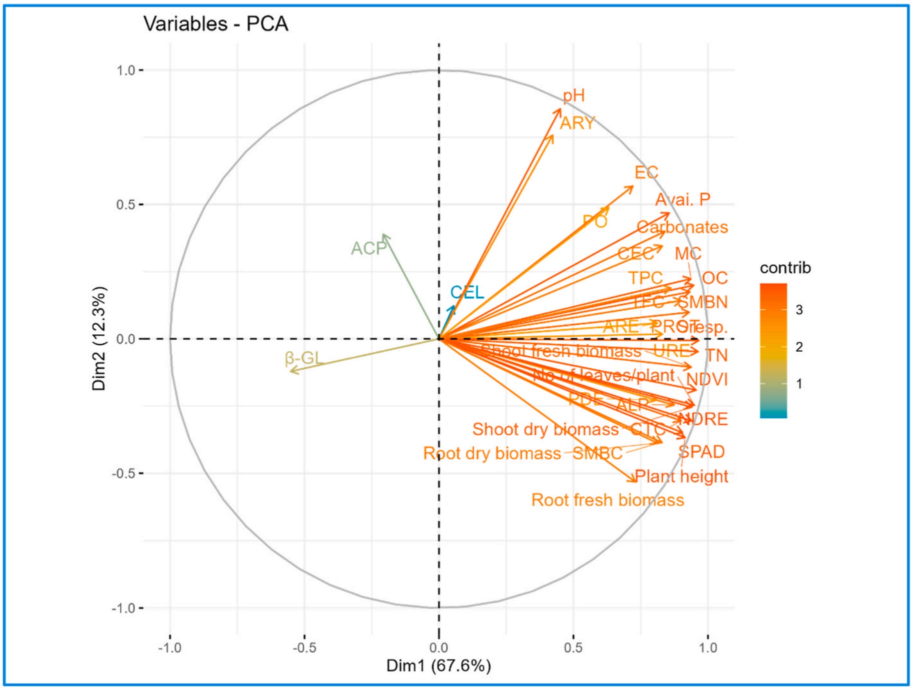


Fig. 8. The variable correlation PCA plot illustrates the relationships between soil quality and plant health variables. Alkaline phosphatase (ALP), acid phosphatase (ACP), phosphodiesterase (PDE), β-glucosidase (β-GL), cellulase (CEL), arylesterase (ARE), phenol oxidase (PO), arylsulfatase (ARY), protease (PROT), urease (URE), moisture content (MC), soil microbial biomass carbon (SMBC), soil microbial biomass nitrogen (SMBN), organic carbon (OC), total nitrogen (TN), available phosphorous (Avai. P), cation exchange capacity (CEC), electrical conductivity (EC), normalized difference vegetation index (NDVI), normalized difference red edge index (NDRE), total phenolic compounds (TPC), condensed tannin content (CTC), total flavonoid content (TFC).

CRediT authorship contribution statement

Hafeez Ur Rahim: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Maria Ianiri:** Validation, Investigation. **Mortadha Ben Hassine:** Validation, Investigation. **Arianna Rossi:** Validation, Investigation. **Elena Spagnoli:** Validation, Investigation. **Emanuele Radicetti:** Writing – review & editing, Visualization, Validation, Resources. **Giancarlo Renella:** Writing – review & editing, Validation, Investigation. **Silvia Celletti:** Validation, Investigation. **Riccardo Fedeli:** Validation, Investigation. **Enrica Allevato:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Conceptualization. **Francesco Primo Vaccari:** Writing – review & editing, Visualization, Validation, Resources. **Silvia Rita Stazi:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

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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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