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Impact of biochar aging and crop rotation on soil physiochemical properties, enzyme activities, and productivity of medicinal plants

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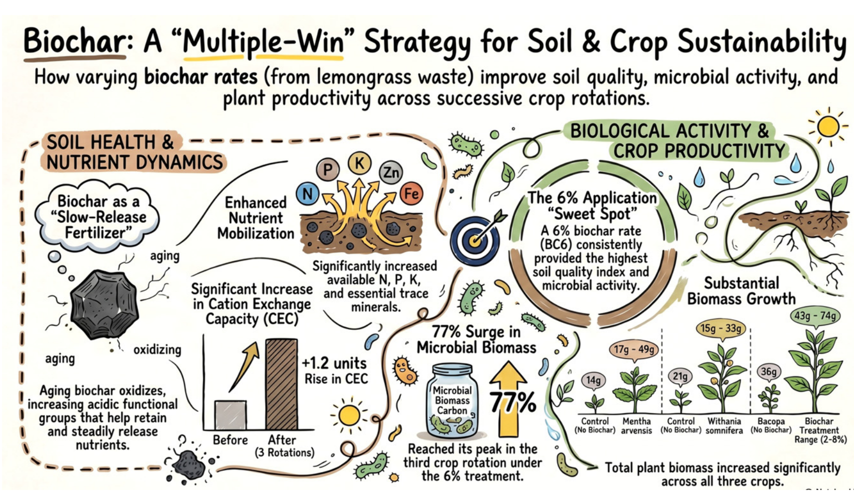
ABSTRACT

Biochar application is recognized for its potential to improve soil health, yet the interactive effects of biochar dosage and sequential crop rotation on soil physiochemical and biological properties remain complex. This study investigated the impact of varying biochar application rates (BC2 to BC8) across a rotation of three crops: *Mentha arvensis*, *Withania somnifera*, and *Bacopa monnieri*. Biochar application initially increased soil pH, but subsequent crop rotation led to a significant decrease in pH (0.2–0.4 units) due to surface oxidation and the formation of carboxylic (-COOH) and phenolic functional groups. Cation Exchange Capacity (CEC) and available nutrients (P, K, NH_4-N, and NO_3-N) increased significantly in a dose-response manner and further improved with crop rotation, suggesting a slow-release fertilizer effect. Soil microbial biomass carbon (SMBC) and enzyme activities (alkaline phosphatase, dehydrogenase, and urease) peaked at a 6% biochar application rate (BC6), which also yielded the highest Soil Quality Index (GMean). While plant biomass and protein content increased significantly under biochar treatments, a decline was observed at the highest application rate (8%). The study demonstrates that biochar aging through crop rotation enhances surface oxidation and nutrient retention. A 6% biochar amendment is recommended as the optimal rate for maximizing soil biological activity and crop productivity in sandy loamy soils.

Keywords: Biochar: Medicinal crop, Sustainability, Soil enzymes

GRAPHICAL ABSTRACT



Highlights

- The subsequent crop rotation triggered an aging of biochars increased cation exchange capacity.
- Biochar served as a slow-release fertilizer in a dose-responsive manner over time.
- The 6% application rate showed the highest enzymatic activities in soil.
- Applying biochar at 8% resulted in a decline in biomass and enzyme activities
- Biochar significantly enhanced the crop production in three rotation systems

INTRODUCTION

Produced through pyrolysis in low oxygen conditions, biochar is a nutrient-rich, carbon-based solid substance with high surface area and porosity. When added to soil, biochar can significantly enhance soil physical properties and promote plant growth, making it a popular choice for improving soil fertility and carbon sequestration (Yadav and Khare, 2020; Antonangelo *et al.,* 2025). Additionally, biochar is widely used as an adsorbent to remove organic and inorganic contaminants from water, reducing their bioavailability in soil (Braghiroli *et al.,* 2018; Hu *et al.,* 2020; Shetty and Prakash, 2020; Liang *et al.,* 2021). Using biochar application in soil can help remediate it by absorbing a pool of metals that are less available to plants, making them a viable option for reducing soil metals (Wang *et al.,* 2022). Additionally, biochar amendment can improve soil reaction (Purakayastha *et al.,* 2019), nutrient availability and levels (Sarfaraz *et al.,* 2020), reduce nutrient leaching (Alkharabsheh *et al.,* 2021), and increase microbial activity, resulting in better crop production (Yan *et al.,* 2021). Biochar has been suggested as a multiple-win strategy that increases soil organic carbon (Haris *et al.,* 2021), cation exchange capacity (Kharel *et al.,* 2019), immobilizes heavy metals (Irfan *et al.,* 2021), optimizes macro (N, P, and K) and micro-element concentrations (Prasad *et al.,* 2019), provides a habitat for symbiotic microorganisms (Tomczyk *et al.,* 2020; Dai *et al.,* 2021; Arshad, 2024), mitigates greenhouse effects, and facilitates long-term C sequestration (Yang *et al.,* 2025), which are all linked to soil health improvements. Biochar has been advocated as a multiple-win strategy which increases soil organic carbon, cation exchange capacity, immobilization of heavy metals, optimal concentration of macro (N, P, and K) and microelements, can provide habitats for symbiotic microorganisms (Tomczyk *et al.,* 2020), mitigate greenhouse effects and long-term C sequestration, which are linked to soil health improvements. Biochar has become a popular method for improving soil quality in various ways. It can increase nutrient availability for plants and microbes, stimulate biological and microbial activity, as well as interact with minerals and organic matter in the soil. Fresh biochar is particularly effective due to its unique structure, which provides a large surface area for interaction. Over time, biochar decomposes and mineralizes in the soil, which is influenced by both environmental factors and the type of soil and native microbes present. However, there is limited research on the aging processes of biochar-amended soil and how it affects mineralization and soil biology. As biochar particles age, they undergo oxidation processes that can alter their chemical and physical properties, potentially influencing the mineralization process and soil biology. Pore size distribution also plays a role in the effectiveness of biochar, with slow pyrolysis temperature and feedstock type being important factors.

MATERIALS AND METHODS

Material and Site Description

The biochar was prepared from distilled waste of *Cymbopogon flexuosus* (lemongrass) using method described previously (Ahsan *et al.,* 2025). The soil collected from the experimental farm of the CSIR-CIMAP, Lucknow (26°48′0″ N, 80°54′0″ E) and was classified as a sandy loam texture (Gupta *et al.,* 2017).

Experimental Design

The biochar was added in the soil with four different application rates (2%, 4%, 6% and 8% (w/w) and named as BC_2; BC_4; BC_6 and BC_8, respectively. The non-amended soil samples were taken as a control. All the experiments were performed in triplicates. In biochar treatments and control, three crops i.e. *Mentha arvensis* (Kosi); *Withania somnifera* (Poshita) and *Bacopa monnieri* were planted consecutively. Each crop was grown for the three months (*Mentha arvensis:* January to March; *Withania somnifera*: April to June and *Bacopa monnieri*: July to September) in same biochar treatments with control pot. The dry weight (DW) biomass was recorded after placing the plants in a hot air oven at 65°C for 48 h. The DW of each plant was used for major pharmacologically active compounds or secondary metabolites. The fresh leaves were taken before harvesting for protein, chlorophyll and carotenoid analysis. After the harvesting of each crop, soil samples were collected from each treatment near to rhizosphere zone of the plant.

Elemental Analysis

The total carbon (%), hydrogen and nitrogen (%) of biochar and soil sample were analyzed using elemental analyzer (EA-3000, Euro-Vector). The oxygen content was calculated on dry ash different basis.

The soil and leaf tissues were pre-digested in nitric acid overnight for elemental analysis. Then a mixture of nitric acid and perchloric acid $HClO_4$-HNO_3 (3:1, v/v) was added and digested using microwave digester (Anton Paar Multiwave Pro oven) at 120°C for 41 minutes. After that, the solution was filtered with Whatman filter paper and volume making up (Kumar *et al.,* 2020). The ICP-OES (Perkin Elmer Optima 5300V) was used for metal and elemental analysis.

Plant Analysis

The content of total chlorophyll in plant leaves was determined by using the spectrophotometry method (Hiscox and Israelstam, 1979). Protein content in plant leaves was estimated using standard method (Lowry *et al.,* 1951). The bacoside fractions in *B. monnieri* were performed using HPLC (Waters USA) system equipped with and autosampler (Waters 717 plus), a binary pump (1525 pump), with photodiode array detector (2996-PDA). The Empower® software from Waters (USA) was used for data collection and processing. Separation of each sample was achieved by isocratic elution using binary mobile phase (a mixture of water - Acetonitrile; 70: 30, v/v) at a flow rate of 0.8 ml min^{-1} for 30 min. Chromolith® HPLC column (Merck) RP-18e (100x 4.6mm) was used for the analysis with 20 μl injection volume and data was recorded at 205 nm.

For withanolide contents in *W. somnifera*, the separation was performed using a combination of 0.1% TFA (Trifluoro acetic acid) in water and acetonitrile (60:40,v/v) at 1 ml/minute flow rate for half an hour in isocratic condition. Waters Nova park, C18 column (150 × 3.9 mm i.d.; 4 μm) was taken for the analysis using 20 μL injection volume and finally, the data was taken at 220 nm.

Essential oil (EO) was extracted from the fresh herbs of *Mentha arvensis* by the hydro-distillation process in Clevenger's type apparatus. The fresh material was distilled for 2 hours at a boiling point up to the total extraction of essential oil. After extraction, the oil was segregated from the water phase, and then the oil yield (v/w) was calculated as ml of oil 100/g of fresh herb. The essential oil was then analyzed on a Gas Chromatograph (Agilent 4890D) fitted with a column of 30 m × 0.25 mm ID, film thickness 0.25 μm, Supelco Wax-10. The oven temperature was programmed to increase from 40–120°C at the rate of 3°C /min with a holding time of 9 min; then 120–250°C at the rate of 15°C min^{-1} with a detector temperature of 250°C, injector temperature of 250°C, split of 1:100. H_2 was used as carrier gas at 10 psi constant column head pressure. Identification of compounds was done using standards acquired from Sigma–Aldrich.

Soil Physicochemical and Biological Analysis

The functional group of biochar and soil was done using FT-IR spectroscopy Model 2000 (Perkin Elmer), respectively. The pH and electric conductivity (EC) (Mettler Seven Go DuoTM pH/Conductivity meter SG23), CEC (cation exchange capacity), water holding capacity (WHC), and bulk density (BD) were analyzed by using standard methods (Jeong *et al.,* 2016). Soil temperature in the pot was determined at a depth of 15 cm by using the probe (with ISO 9001:2000 sensor). Moisture content (%) of soil was determined by measuring fresh and dry weight after incubation at 105 °C for 24 h (Mazza *et al.,* 2019).

Soil organic carbon and microbial biomass carbon (MBC) in soil were measured according to the method described earlier (Yuan *et al.,* 2021). The spectrophotometric methods were used for the analysis of available phosphorous (AvP) (de Souza *et al.,* 2023), and nitrogen (indophenol method) (Sharmin *et al.,* 2020) in soil, while the turbidimetric method was used for inorganic sulfur (Subramanian *et al.,* 2022). Soil enzymes such as alkaline and acidic phosphatase, dehydrogenase, β-glucosidase, and urease were determined by previously used standard methods (Casida Jr *et al.,* 1964; Pancholy and Rice, 1973; Eivazi and Tabatabai, 1977; Eivazi and Tabatabai 1988; Acosta-Martinez and Tabatabai, 2000; Acosta-Martinez *et al.,* 2018). The geometric mean of all enzymes was calculated by the fusing formula given earlier (Sanchez-Hernandez, 2018).

Statistics

Analysis of variance (ANOVA) was performed among different treatments as control, to evaluate the significant variations in all the parameters among the treatments. One-way ANOVA was used for evaluation of the significant differences ($p < 0.05$) between all the treatments of all studies. Pearson's correlation was used for the evaluation of the correlation between all the chemical and biological parameters of the different treatments.

RESULTS

Soil Physiochemical Properties

The table 1 demonstrated the physicochemical properties of soil after the amendment of biochar and rotation of different crops. The application of biochar increased the soil pH in a dose-response manner, but the pH decreased significantly ($p<0.05$) with crop rotation. The decrease in soil pH of different biochar treatments from crop *M. arvensis* to *W. somnifera* was BC2: 0.2; BC4: 0.2; BC6: 0.3 and BC8: 0.3 units, and from *W. somnifera* to *B. monnieri* was BC2: 0.2; BC4: 0.3; BC6: 0.3 and BC8: 0.4 unit. The pH of control soil did not show any significant differences during crop rotation. The H/C and O/C of soil varied significantly with crop rotation in biochar treatments. The COOH and phenolic group in soil were higher in BC treatments as compared to control. The increase in COOH and phenolic group in soil was observed after crop rotation in BC treatments, while COOH and phenolic content showed no significant variations in control during crop rotation (Fig. 1).

The CEC values increased significantly ($p<0.05$) with both biochar application rate and crop rotation. In control, alteration in CEC values of soil was not significant. The increase in soil CEC of different biochar treatments from crop *M. arvensis* to *W. somnifera* was BC2: 0.3; BC4: 0.5; BC6: 0.6 and BC8: 0.8 unit and from *W. somnifera* to *B. monnieri* were BC2: 0.4; BC4: 0.7; BC6: 0.9 and BC8: 1.2 cmolc/kg. A significant increase in water holding capacity was observed in BC treatments (Table 1) ($p<0.05$).

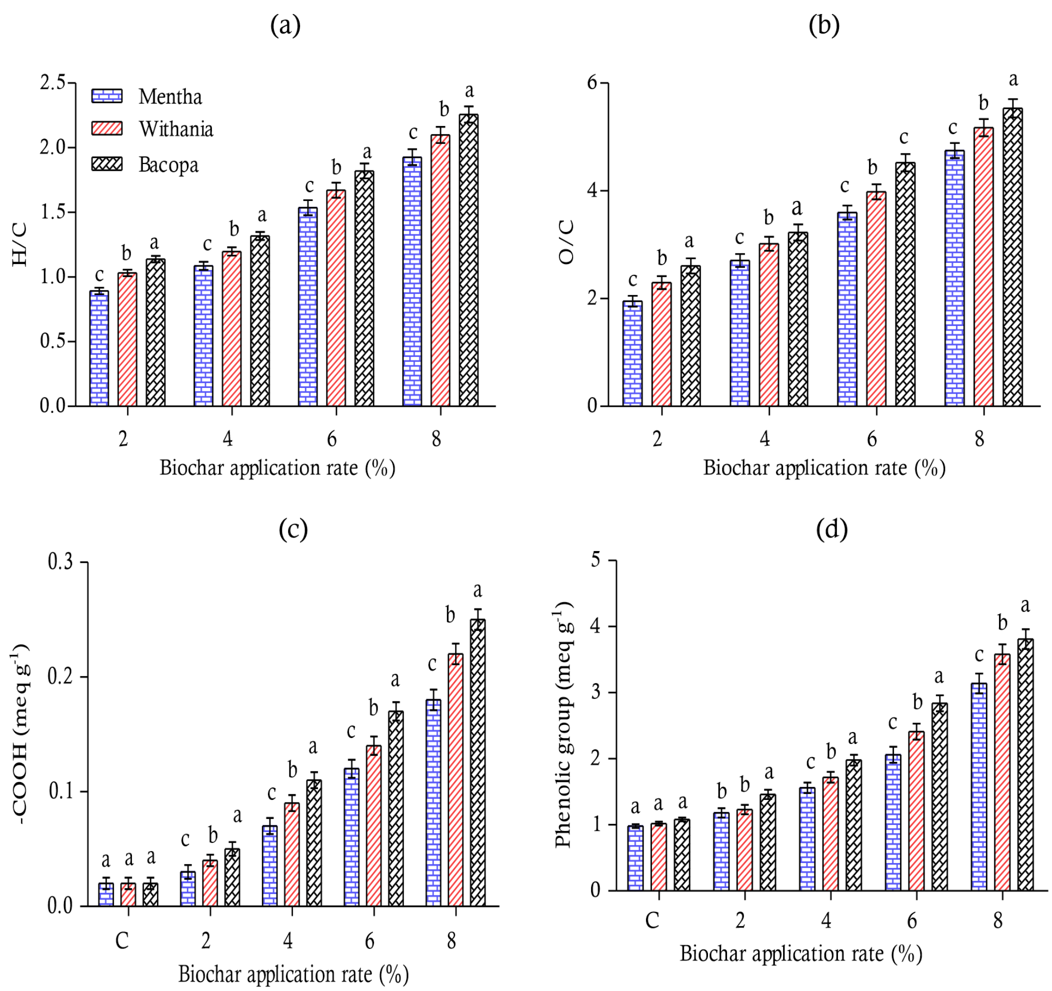


Fig. 1. Change in (a) H/C molar ratio, (b) O/C molar ratio, (c) –COOH functional group, (d) total phenolic functional group of soil after harvesting of the crop. [Different letters means significant differences from MAPs rotation within the same treatment (one way ANOVA, $p<0.05$)]

Table 1. Physico-chemical characteristics of soil after crop harvesting

	pH			WHC (%)			CEC ($cmol_c$/kg)		
	Mentha	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*
C	7.30±0.01^b	7.3±0.01^b	7.4±0.02^a	30±1^a	30±1^a	29±1^a	1.3±0.09^a	1.3±0.09^a	1.2±0.09^a
BC_2	8.16±0.02^a	7.9±0.02^b	7.7±0.02^c	35±2^a	32±2^b	31±2^b	1.5±0.12^c	1.8±0.12^b	2.2±0.12^a
BC_4	8.65±0.02^a	8.4±0.03^b	8.1±0.03^c	45±2^a	40±2^b	36±2^c	2.2±0.15^c	2.7±0.15^b	3.4±0.17^a
BC_6	9.25±0.04^a	8.9±0.04^b	8.4±0.05^c	51±1^a	49±1^b	45±1^c	3.6±0.17^c	4.3±0.17^b	5.2±0.18^a
BC_8	9.76±0.08^a	9.4±0.08^b	8.8±0.08^c	55±2^a	52±2^b	48±2^c	4.5±0.19^c	5.2±0.19^b	6.4±0.19^a

Note: [C= Control; BC_2, BC_4, BC_6, and BC_8 are the application rates of biochar at 2%, 4%, 6% and 8% respectively] Different letters mean significant differences from MAPs rotation within the same treatment (one way ANOVA, $p<0.05$).

The WHC decreased with crop rotation in BC treatments significantly ($p<0.05$). There was no significant change ($p<0.05$) in the control soil with crop rotation.

Soil Microbial Biomass Carbon and Enzymes

A dose response increase in SMBC was observed in BC treatments (Fig. 2). The highest microbial biomass carbon was observed at 6% application rate of biochar in all three crops. Increase in microbial biomass carbon in BC_6 treatment with respect to control was 59%, 70% and 77% for the crop *M. arvensis; W. somnifera* and *B. monnieri,* respectively.

The activities acidic (AciP) and alkaline (AlkP) phosphatases, dehydrogenase (DHA), β glucosidase (BGA) and urease (UR) are reported in Fig. 3. The AlkP, DHA and UR activities in soil were higher in biochar treatments as compared to control, however, their concentration in soil decreased with time. AciP and BGA activities were lower in BC treatments as compared to control and their concentration increased with time. The BC6 treatments demonstrated highest activities of AlkP, DHA and UR. Among all enzymes, maximum increase in urease activity was observed in the BC6 treatment, with a 52%, 48%, and 42% increase in *M. arvensis*, *W. somnifera*, and *B. monnieri*, respectively. The geometric mean an index of soil quality, with the highest value was higher in BC6 treatments as compared to other. The GMean value decreased with further increase in biochar application rate (BC8), but showed no significant difference in control soil or with different crop rotation.

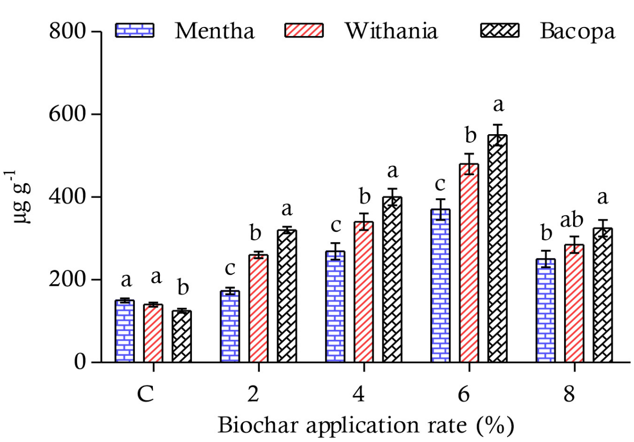


Fig. 2. Soil microbial biomass carbon

Available Nutrients

The available phosphorus, available potassium, NH_4-N and NO_3-N, and organic carbon were increased in biochar treatments as compared to control in dose response manner (Fig. 4). In regression analysis between concentration of these nutrients and time demonstrated an increase in both slope and intercept in biochar treatments with dose-response manner. The exchangeable nutrients (Ca, Mg, Fe, Cu, Mn, and Zn) in the soil after harvesting consecutive crops (*M. arvensis*, *W. somnifera*, and *B. monnieri*) demonstrated an increase in the biochar-added soil treatment (Table 2). However, there was no significant change in the nutritional value with crop rotation in the control soil.

Plant Growth and Secondary Metabolite

The biomasses of *M. arvensis; W. somnifera and B. monnieri,* were 21±1 g, 14±1.03g and 36±1.5 g in control treatments, respectively and increased 27-49 g, 15-33 g and 43-74 g in biochars treatments, respectively (Fig. 5). However, the chlorophyll contents were increased in biochars treatment from 0.6 to 1.03, 0.97 to 1.57 and 1.18 to 1.39 *M. arvensis; W. somnifera and B. monnieri*, respectively. The protein content in plant leaves was also showed a significant increase in biochars treatments. The maximum increase was observed BC6 treatments. However, applying a higher rate of biochar (8%) led

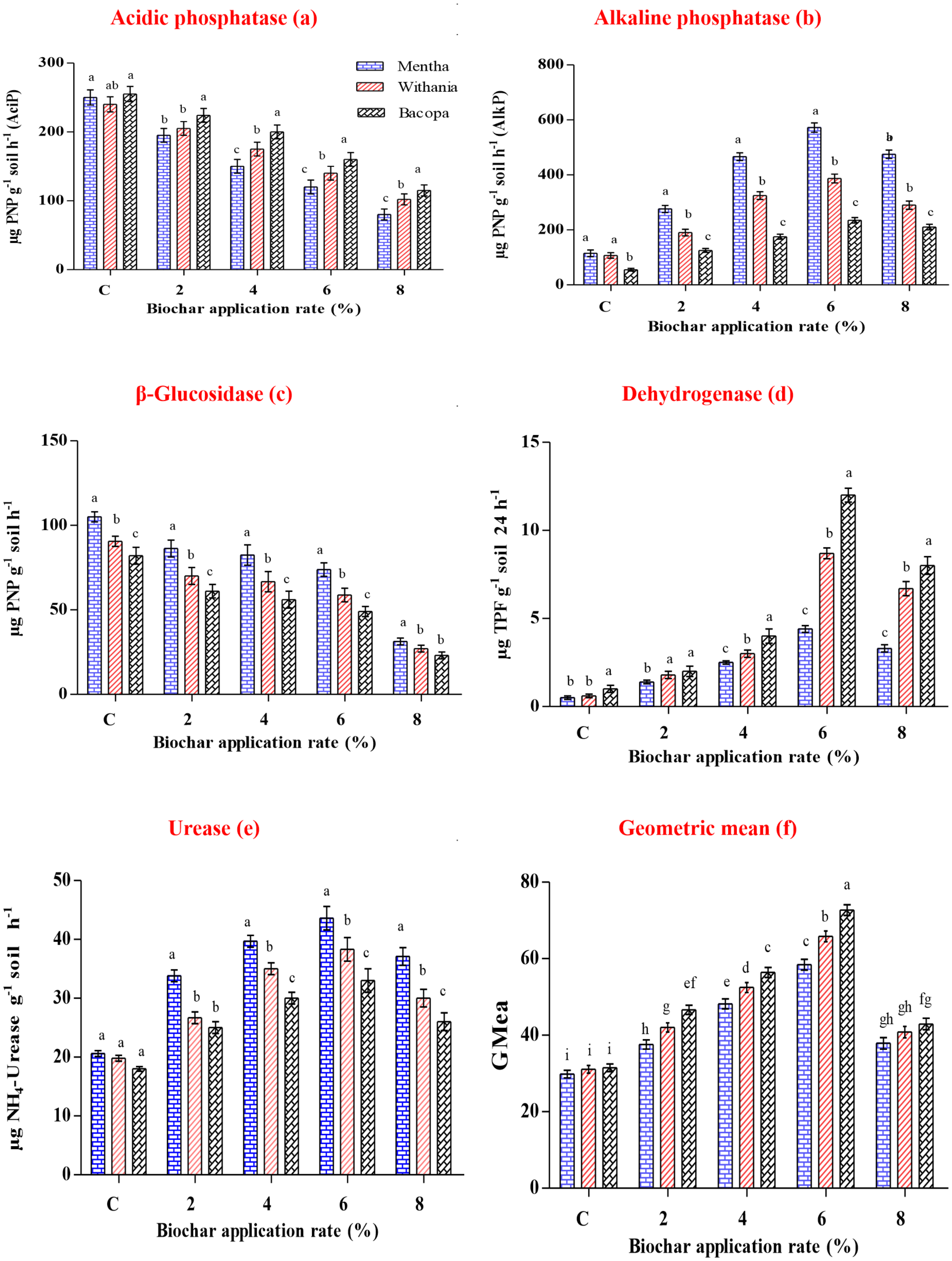


Fig. 3. Change in the soil enzyme activity and GMean after harvesting to the crop

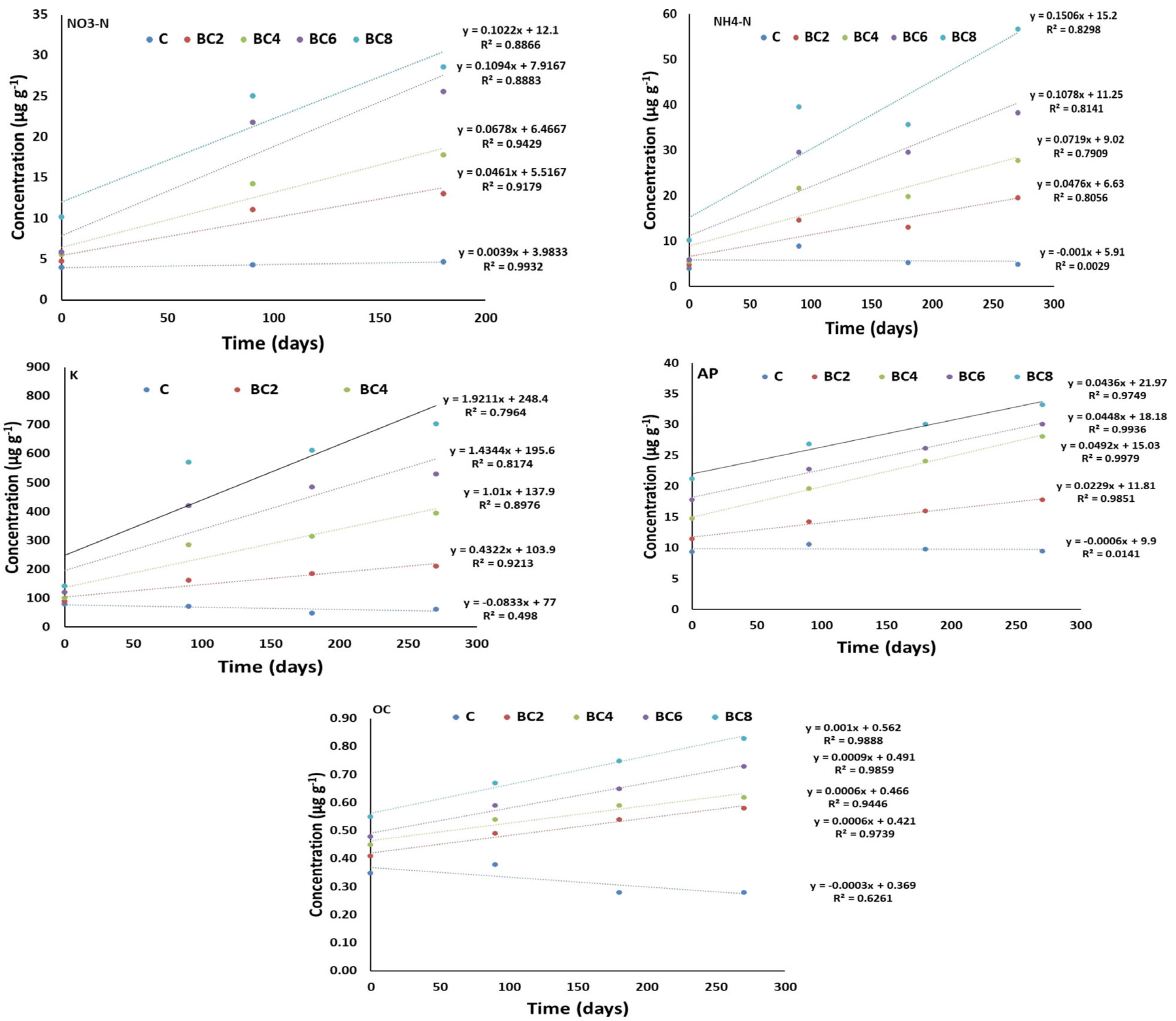


Fig. 4. Regression analysis between nutrients and organic carbon with different biochar dose: (a) NH_4-N, (b) NO_3-N, (c), Available Phosphorous (d) Organic carbon

to a decrease in biomass contents. The enhancement in oil content (in *M. arvensis*) and secondary metabolite content of *W. somnifera and B. monnieri* was also observed (Table 3). The Ca, Mg, Fe, Mn, Cu, and Zn nutrients in leave were also increased in BC treatments (Table 4).

Statistical Analysis

The correlation of plant biomass, Gmean and SMBC demonstrated that biochars application 2-6%, these parameters were positively correlated except in bacopa at 2% application rate. However, at higher rate (8%), the no significant relation was observed between these parameters (Fig. 5). The correspondence show two dimensions (D1 with 64% and D2: 22%). Among treatments three groups were formed 1 control group and associated with pH, WHC and Gmean 2 group containing 2%,4% and 6% biochars treatments in three crops associated with BM and SMBC 3 Group contained 8% biochars treatments in three crop associated with available nutrients and CEC (Fig. 6).

DISCUSSION

Alkalinization is a crucial biochar property as changes in pH have cascading impacts in different soil processes. The change in soil pH after biochar application in this study may be due to the presence of surface functional groups (as the conjugated base), inorganic alkalies (such as oxides, hydroxides,

Table 2. Extractable nutrients of soil and biochar treatments with different crops rotation

	Ca (g kg^{-1})			Mg (g kg^{-1})			Na (mg kg^{-1})			K (mg kg^{-1})		
	Mentha	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*
C	0.15±0.01^{a}	0.13±0.01^{a}	0.12±0.01^{a}	0.13±0.01^{a}	0.13±0.01^{a}	0.13±0.01^{a}	178±8^{a}	122±5^{b}	100±5^{c}	70±5^{a}	50±3^{b}	55±5^{b}
BC_2	0.80±0.02^{c}	1.10±0.02^{b}	1.56±0.02^{a}	0.26±0.02^{c}	0.33±0.02^{b}	0.37±0.02^{a}	145±10^{c}	163±12^{b}	200±13^{a}	152±10^{c}	179±12^{b}	205±15^{ab}
C_4	2.15±0.03^{c}	2.80±0.02^{b}	3.10±0.02^{a}	0.43±0.04^{c}	0.51±0.02^{b}	0.61±0.03^{a}	180±15^{a}	210±17^{b}	254±19^{c}	275±20^{c}	315±22^{b}	384±23^{a}
BC_6	3.10±0.03^{c}	3.70±0.03^{b}	4.15±0.03^{a}	0.52±0.04^{c}	0.60±0.02^{c}	0.68±0.04^{a}	243±20^{c}	310±21^{b}	375±23^{a}	412±25^{c}	485±25^{b}	521±26^{a}
BC_8	4.57±0.04^{c}	5.25±0.04^{b}	6.13±0.05^{a}	0.65±0.05^{c}	0.72±0.03^{b}	0.80±0.05^{a}	312±20^{c}	455±21^{b}	510±23^{a}	565±30^{c}	612±31^{b}	697±33^{a}

[C= Control, BC_2, BC_4, BC_6 and BC_8 are the application rate of biochar at 2%, 4%, 6% and 8% respectively]Different letters mean significant differences from MAPs rotation within the same treatment (one way ANOVA, p<0.05).

	Fe (g kg^{-1})			Mn (g kg^{-1})			Cu (mg kg^{-1})			Zn (mg kg^{-1})		
	Mentha	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*
C	0.17±0.01^{a}	0.18±0.01^{a}	0.18±0.01^{a}	0.16±0.01^{1}	0.16±0.01^{1}	0.26±0.01^{1}	2.31±0.01^{a}	2.31±0.01^{a}	2.26±0.02^{a}	1.5±0.01^{a}	1.4±0.01^{a}	1.3±0.01^{a}
BC_2	0.27±0.02^{c}	0.30±0.02^{b}	0.32±0.01^{a}	0.29±0.02^{c}	0.32±0.02^{b}	0.37±0.02^{a}	3.31±0.02^{c}	3.48±0.02^{b}	3.86±0.03^{a}	3.6±0.02^{c}	4.8±0.02^{b}	6.8±0.02^{a}
BC_4	0.35±0.02^{c}	0.39±0.02^{b}	0.43±0.02^{a}	0.37±0.03^{c}	0.41±0.03^{b}	0.46±0.02^{a}	3.29±0.03^{c}	4.80±0.03^{b}	4.96±0.04^{a}	8.0±0.04^{c}	10.5±0.03^{b}	18.0±0.03^{a}
BC_6	0.47±0.03^{c}	0.52±0.03^{b}	0.62±0.02^{a}	0.42±0.03^{c}	0.48±0.03^{b}	0.56±0.03^{a}	3.81±0.03^{c}	5.21±0.04^{b}	5.86±0.04^{a}	10.6±0.04^{c}	14.4±0.04^{b}	28.4±0.04^{a}
BC_8	0.77±0.04^{c}	0.86±0.04^{b}	0.96±0.02^{a}	0.48±0.03^{c}	0.61±0.04^{b}	0.75±0.04^{a}	4.81±0.04^{c}	5.32±0.04^{b}	5.93±0.04^{a}	17.5±0.08^{c}	19.5±0.08^{b}	32.4±0.08^{a}

[C= Control, BC_2, BC_4, BC_6 and BC_8 are the application rate of biochar at 2%, 4%, 6% and 8% respectively]Different letters means significant differences from MAPs rotation within the same treatment (one way ANOVA, p<0.05).

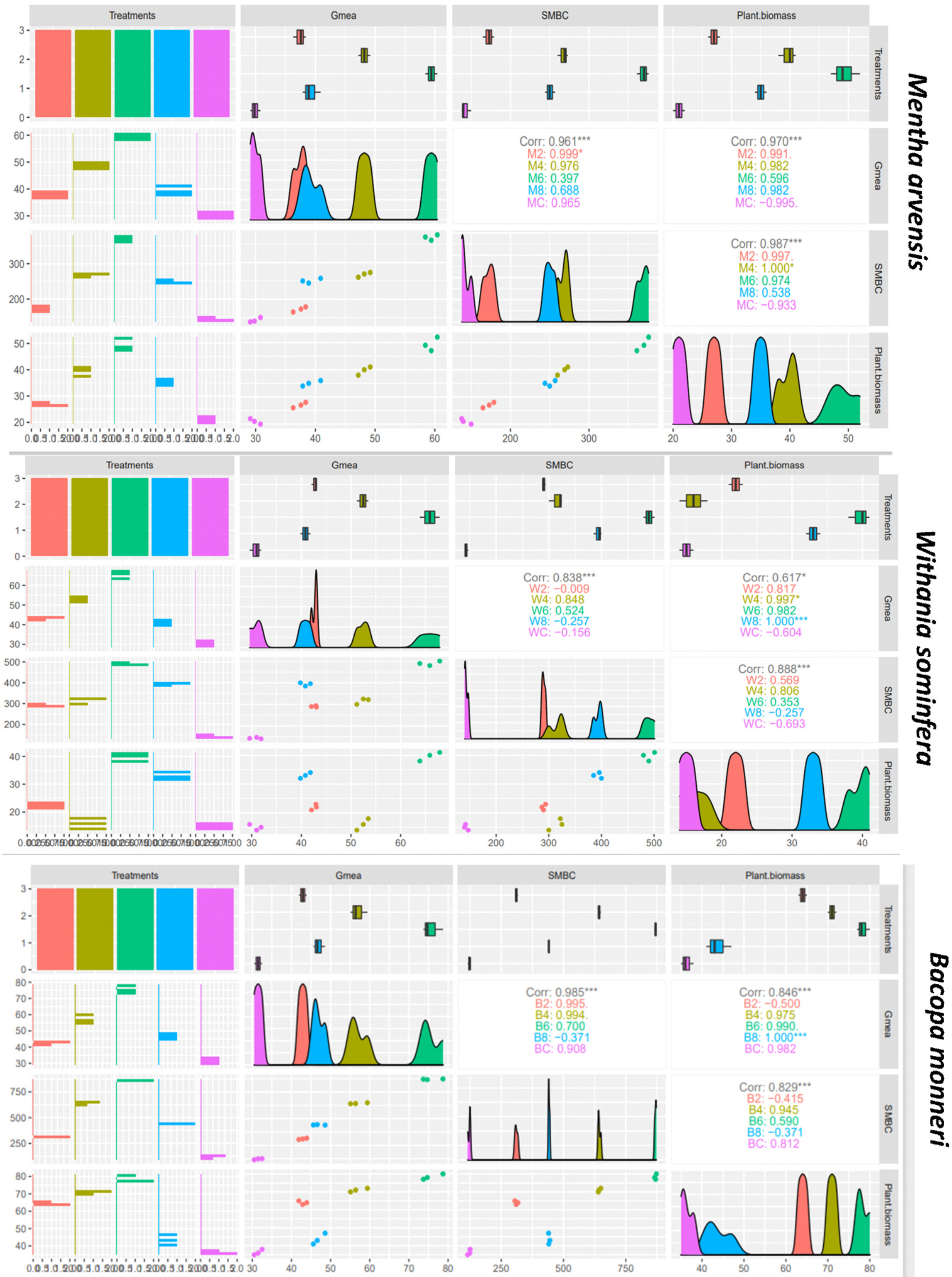


Fig. 5. (a) Plant biomass b Protein C chlorophyll contents [Different letters means significant differences from MAPs rotation within the same treatment (one way ANOVA, p<0.05)]

Table 3. Secondary metabolite in cultivated MAPs during the experiment

	Mentha	*Withania*		*Bacopa*
	Oil Yield	Withaferin-A	Withanolide-A	Total Bacosides
C	0.75±0.01[a]	0.58±0.01[a]	0.070±0.01[c]	0.52±0.01[a]
BC_2	0.75±0.01[a]	0.58±0.01[a]	0.070±0.01[c]	0.52±0.01[a]
BC_4	0.76±0.02[a]	0.59±0.01[a]	0.073±0.01[b]	0.53±0.01[a]
BC_6	0.76±0.02[a]	0.59±0.01[a]	0.075±0.01[a]	0.54±0.01[a]
BC_8	0.75±0.02[a]	0.61±0.01[a]	0.074±0.01[b]	0.53±0.01[a]

[C= Control, BC_2, BC_4, BC_6 and BC_8 are the application rate of biochar at 2%, 4%, 6% and 8% respectively]. Different letters means significant differences with different biochar treatment (one way ANOVA, $p<0.05$)].

sulfates, and ortho-phosphate), and soluble organic compounds (also conjugated bases of weak acids). Variations in pH values, CEC, and WHC during crop rotation may be due to the aging process, which leads to oxidation of surface, absorption capacity, nutrient retention, and carbon mineralization of biochar. The increase in H/C (Fig.4.8-a) and O/C (Fig.4.8-b) ratios of biochar amended as compared to control were due to the surface oxidation of biochar during crop rotation. The correlation analysis shows significant negative correlations between pH and the H/C (-0.99 to -0.90; $p<0.05$) and O/C (-0.99 to -0.94; $p<0.05$) molar ratios.

The findings of our study are in line with Spokas research from 2014, which showed a pH decrease of 1.0 unit after three years of 1% biochar aging in soil. The elemental composition and surface functional groups of biochar can change due to crop rotation or aging in soil. Our study also confirms the pH decrease with biochar aging, as previously reported by Rechberger *et al.,* (2017), and Apostoloviæ *et al.,* (2024). We observed an increase in functional groups, such as -COOH and phenolic, which suggests that oxidation of biochar may enhance surface activities responsible for surface oxidation. Wang *et al.,* (2017) also reported that natural

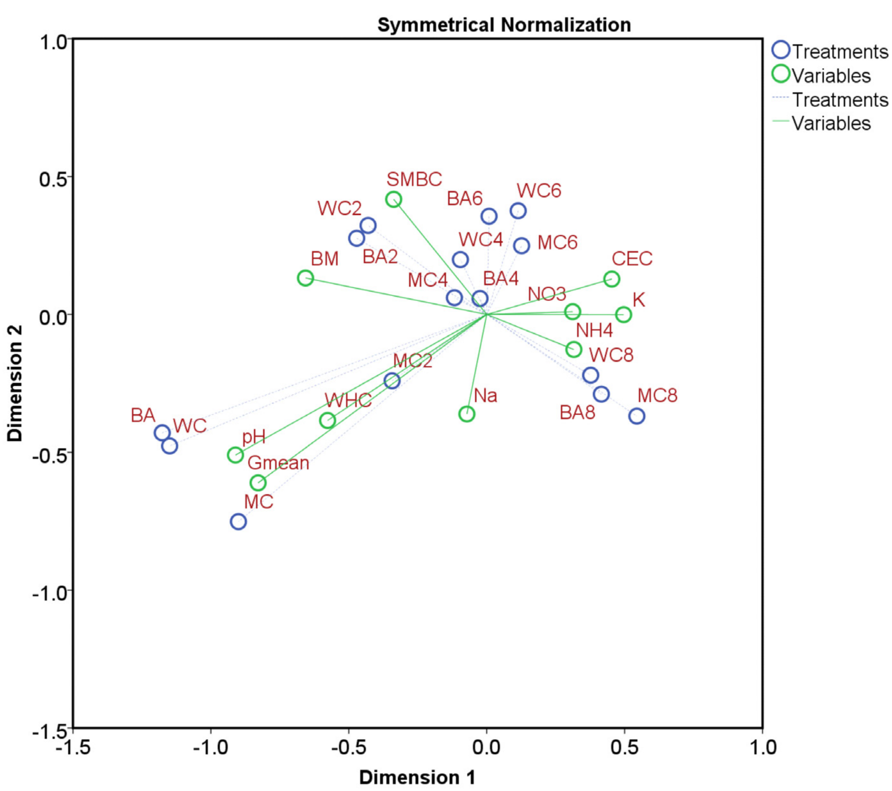


Fig. 6. Principal Component analysis

Table 4. Relative nutrients content in plant

	Ca			Mg		
	Mentha	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*
BC_2	1.10 ± 0.1^{c}	1.18 ± 0.2^{b}	1.28 ± 0.1^{a}	1.01 ± 0.1^{j}	1.11 ± 0.1^{h}	1.31 ± 0.1^{i}
BC_4	1.30 ± 0.1^{c}	1.36 ± 0.2^{b}	1.47 ± 0.2^{a}	1.27 ± 0.1^{c}	1.48 ± 0.2^{b}	1.56 ± 0.2^{a}
BC_6	1.61 ± 0.2^{c}	1.74 ± 0.3^{b}	1.94 ± 0.3^{a}	1.62 ± 0.2^{c}	1.74 ± 0.2^{b}	1.75 ± 0.2^{a}
BC_8	1.50 ± 0.2^{c}	1.46 ± 0.3^{b}	1.76 ± 0.3^{a}	1.40 ± 0.2^{c}	1.68 ± 0.2^{b}	$1.46\pm0.0.2^{a}$

Note: Relative = (Treatment/Control) plant nutrients
[BC_2, BC_4, BC_6 and BC_8 are the application rates of biochar at 2%, 4%, 6% and 8% respectively]
Different letters mean significant differences from MAPs rotation within the same treatment (one way ANOVA, $p<0.05$).

	Fe			Mn			Cu			Zn		
	Mentha	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*	*Mentha*	*Withania*	*Bacopa*
BC_2	1.02 ± 0.1^{b}	1.09 ± 0.1^{b}	1.30 ± 0.1^{a}	1.05 ± 0.1^{c}	1.10 ± 0.1^{b}	1.29 ± 0.1^{a}	1.07 ± 0.1^{c}	1.21 ± 0.1^{b}	1.58 ± 0.2^{a}	1.06 ± 0.1^{b}	1.09 ± 0.1^{b}	1.13 ± 0.1^{a}
BC_4	1.16 ± 0.1^{c}	1.22 ± 0.1^{b}	1.36 ± 0.1^{a}	1.13 ± 0.1^{c}	1.24 ± 0.1^{b}	1.54 ± 0.2^{a}	1.22±0.1c	1.68 ± 0.2^{b}	2.33 ± 0.3^{a}	1.15 ± 0.1^{b}	1.20 ± 0.1^{b}	1.30 ± 0.2^{a}
BC_6	1.42 ± 0.2^{c}	1.48 ± 02^{b}	1.64 ± 0.2^{a}	1.23 ± 0.2^{c}	1.57 ± 0.2^{b}	2.33 ± 0.3^{a}	1.41 ± 0.2^{c}	2.49 ± 0.3^{b}	3.83 ± 0.3^{a}	1.33 ± 0.2^{c}	1.40 ± 0.2^{b}	1.48 ± 0.2^{a}
BC_8	1.22 ± 0.2^{c}	1.33 ± 0.2^{b}	1.37 ± 0.1^{a}	1.04 ± 0.1^{c}	1.46 ± 0.2^{b}	1.73 ± 0.2^{a}	1.33 ± 0.2^{c}	2.07 ± 0.2^{b}	3.25 ± 0.2^{a}	1.21 ± 02^{c}	1.21 ± 0.1^{b}	1.43 ± 0.2^{a}

Note: Relative (Treatment/Control)
[BC_2, BC_4, BC_6 and BC_8 are the application rates of biochar at 2%, 4%, 6% and 8% respectively]
Different letters mean significant differences from MAPs rotation within the same treatment (one way ANOVA, $p<0.05$).

oxidation of biochar increases carboxylic and phenolic functional groups and negative charges on the surface while decreasing the surface positive charge. Other reports show an increase in CEC with biochar application and/or crop rotation, which can be explained by the addition, conversion, or precipitation of oxygen-containing functional groups or cationic nutrients. Biochar aging or crop rotation can enhance labile carbon and carbon derivatives components, increasing the availability of substrate for microbes and water-holding capacity (WHC) of soil. Biochar also acts as a source of nutrients and absorbent for nutrients in the soil, with nutrient mineralization enhancing microbial activity. Extractable nutrients in the soil increase faster in some crops than others, and biochar amendments lead to improved nutrient retention and exchange capacity during crop rotation. These findings suggest that biochar amendments can have positive effects on soil properties and crop growth.

Increased mineralized nutrient (Available phosphorous, NH_4-N, and NO_3-N) availability suggests that nutrient is mobilized more rapidly with the crop rotation in biochar amendments (Zhang *et al.,* 2020). It is possible that with different crop rotation biochar used in the present study would act as a "slow release fertilizer". In addition, biochar efficiently releases a steady stream of nutrients after the most or all the negative charges and pores are saturated with nutrients by adsorption process (Luo *et al.,* 2023). The increasing trend of available phosphorus over time could be attributed to the release of phosphorus with time. It may also be possible that crop rotation provides the habitat to the microorganism responsible for the conversation of total phosphorus (Fig. 4) to available phosphorus. This is consistent with enhancement in the alkaline phosphatase enzyme results with the biochar application with the rotation of the different crop. Further, availability of phosphorus was likely coupled to positively charged sites on biochar surface that improve soil fertility to improve and exchange phosphate ions (Shi *et al.,* 2023). In soil, precipitation of complexes such as Ca^{2+}, Al^{3+}, and $Fe^{2+/3+}$ depends on pH. Alteration of the pH may affect its bonding as well as sorption or absorption during crop rotation. Further, increase in the phosphorous availability with crop rotation suggests enhanced contact formation between soil particles in biochar amended soils (Aller *et al.,* 2017).

In our study, the increased in nitrogen mineralization increases with different crop rotation is consistent with other studies (Aller *et al.,* 2017). Crop rotation causes aging of biochar, which carries an increase in surface acidic functional groups (Rechberger *et al.,* 2017). Biochar could retain NH_4^+–N in soils through acid functional groups (e.g. carboxyl and hydroxyl) on its surface via cation exchange (Esfandbod *et al.,* 2017).

However, anionic surface charge sites on biochar will not able to retain NO_3-N on the surface of biochar. Hence, no large variation was observed in NO_3-N on the surface of biochar during crop rotation. Biochar addition has been shown to have mostly positive effects on microbial biomass, largely due to its ability to provide a suitable habitat and substrate for soil microbial communities (Palansooriya *et al.,* 2019; Giagnoni and Renella, 2022). The porous structure of biochar may be responsible for increasing soil microbes by providing a highly suitable habitat for microorganisms (Yang *et al.,* 2024). Biochar's condensed aromatic structure, along with non-aromatic carbon, may also make it more susceptible to serving as a substrate for microbes (Ye *et al.,* 2017; Palansooriya *et al.,* 2019). Microbial biomass in soil is influenced by soil pH, with lower pH values leading to a decrease in microbial biomass (Le Guen *et al.,* 2017; Liang *et al.,* 2024). Biochar contains large macro and micro-pores that increase oxygen content in the soil, leading to more decomposition of organic carbon and thus more substrate available for microbes (Aziz *et al.,* 2024). A high C/N ratio tends to immobilize soil microbial nitrogen, leading to low microbe-associated activities (Cheng *et al.,* 2017; Sun *et al.,* 2022). When the C:N ratio is greater than 32, microbes will immobilize nitrogen, and both NH_4-N and NO_3-N can be immobilized (Cao *et al.,* 2021). Additionally, a high C/N ratio reduces microbial biomass activity in soil due to the limited availability of substrate for microbes (Liang *et al.,* 2017). Research by Bera *et al.,* (2016) found that adding biochar to dairy effluent resulted in a 36% decrease in AciP enzyme activity and a 27% increase in AlkP enzyme activity. These enzymes play a crucial role in converting phosphorous from organic to inorganic form, which is essential for microorganisms and plants. Crop rotation may lead to a decrease in AlkP activity due to the activation of phosphorous, evidenced by the increase in available phosphorus content and changes in pH and substrate (Pingree, 2017). Inorganic phosphorous species form stable complexes with Ca^{2+}/Mg^{2+} and other anions at higher pH, leading to a decrease in AciP activity

(Penn and Camberato, 2019). The increase in microbial biomass carbon with crop rotation and aging of biochar in soil is consistent with the findings of (Nyambo *et al.,* 2021), who observed an increase in SMBC by applying biochar at different rates. The C/N ratio plays a vital role in microbial activity and decomposition rate in soil, with higher C/N ratios leading to a decrease in microbial biomass activity (Xie *et al.,* 2022). The persistence of nitrogen in microbial breakdown depends on the C/N ratio of the decomposing organic matter (Averill and Waring, 2018).

Fig. 4.10c and 4.10d show the effects of crop rotation on β-Glucosidase (β-Glu) and dehydrogenase enzymes with the addition of biochar to the soil. The activity of β-Glu decreased significantly due to crop rotation, but increased with an increase in biochar application rate (Birol and Günal, 2024). On the other hand, dehydrogenase activity in the biochar-added soil treatment increased with MAPs rotation, and the highest activity was observed in the BC6 treatment. The activity of β-Glu is significant in the global carbon cycle as it hydrolyzes cellulose by converting the disaccharide cellobiose to glucose molecules (Kaur and Singh, 2021). The reduction of β-Glu activity in biochar-amended soil with crop rotation could be due to a lower need for glucose and cellulose. These findings are similar to (Chen *et al.,* 2017) observations of decreased β-Glu activity with biochar incorporation during a nine-month incubation study, which was attributed to the high specific charge characteristic on biochar surface that more readily transfers organic matter to the soil. Changes in the β-Glu activity in the soil could reflect a change in the potential of the soil microbial community for the degradation of lignocellulose in the soil organic matter (Zhang *et al.,* 2024). Crop rotation-induced decrease in soil pH may enhance the degradability of organic matter in soil components due to the formation of acidic functional groups (Duan *et al.,* 2026). A significant ($p < 0.05$) negative correlation was observed between β-Glu and soil pH, suggesting that improved labile organic matter solubility could improve the accessibility of lignocellulose in the soil matrix for β-Glu enzymes.

This study revealed that biochar amendments in sandy loam soil provided a readily available carbon source to soil microorganisms, enhancing their proliferation and increasing dehydrogenase activity (Igalavithana *et al.,* 2017). The positive correlation between dehydrogenase activity and organic carbon content ($r = 0.81$ to 0.77, $p < 0.05$) supports this theory. Similarly, Irfan *et al.* (2019) reported that increased dehydrogenase activity with biochar addition may be due to the supplemental labile carbon to microbes by biochar. The increase in urease enzyme activity in biochar-added soil may be attributed to the increase in soil labile organic matter and numbers of microorganisms in soil with crop rotation (Khan *et al.,* 2024). Several studies have confirmed that biochar amendments alter soil nitrogen dynamics (Gupta *et al., 2024*, Tang, 2025), and this study demonstrates that crop rotation significantly influences nitrogen dynamics. It is possible that the enhancement in the C/N ratio with crop rotation increases the urease activity in biochar-amended soil. High C/N ratio immobilizes nitrogen in the soil, making it unavailable to microbes and plants (Koç *et al.,* 2025), resulting in an increase in the urease enzyme activity in the soil. The increase in GMean index with different crop rotation in the biochar added soil treatment suggests that crop rotation in soil-biochar mixture provides a suitable habitat for soil microorganisms, and the pore of biochar could be a dispersion medium for soil enzymes (Khan *et al.,* 2024). Overall, the results indicate that the best application rate for improving soil quality in terms of GMean is 6% biochar amendment (Fig. 4.10f). The addition of biochar alters the availability of nutrients such as Ca, Mg, Fe, Mn, Cu, and Zn, soil enzyme activities, and plant-soil water relation, which may lead to increased plant productivity. Earlier studies have shown that enhanced plant growth is significantly associated with the positive influence of biochar on soil properties (Ahsan *et al.,* 2025). The decrease in pH or increase in CEC consistently observed over time with biochar may be related to longer exposure of biochar particles in the soil to crop rotation or both. Prolonged exposure of biochar particles in the soil may lead to the oxidation of the surface and core of biochar particles (Wang *et al.,* 2020).

CONCLUSION

This study concludes that biochar application significantly transforms the physiochemical and biological landscape of sandy loamy soil, with its efficacy heavily influenced by the aging process inherent in crop rotation. While biochar initially acts as a liming agent, the subsequent decrease in pH and increase in O/C and H/C ratios over time indicate natural oxidation, a process that enhances surface functional groups specifically carboxylic and phenolic groups—leading to a significant rise in Cation Exchange Capacity (CEC) and improved nutrient retention. Furthermore, biochar serves as a functional "slow-release fertilizer," where the steady increase in available phosphorus and nitrogen mineralization across consecutive harvests suggests that biochar pores and surface charges facilitate a sustained supply of nutrients to plants. Biological optimization of soil health, measured via microbial biomass carbon and enzyme activities such as alkaline phosphatase, dehydrogenase, and urease, peaked at a 6% application rate; however, the decline in these activities and the GMean index at the 8% threshold suggests that excessive biochar may lead to nutrient immobilization due to an unfavorable C/N ratio. Agricultural productivity in the rotation of *M. arvensis*, *W. somnifera*, and *B. monnieri* benefited from these changes through increased biomass and protein content. Ultimately, biochar is not a static amendment, and its integration into crop rotation systems promotes long-term soil fertility and microbial synergy, with a 6% amendment identified as the most effective dosage for sustainable yields.

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