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Biochar particle size coupled with biofertilizer enhances soil carbon-nitrogen microbial pools and CO₂ sequestration in lentil

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Introduction: Biochar and biofertilizer have emerged as sustainable soil amendments to improve soil fertility and quality. However, it is necessary to determine how biochar's various particle sizes and biofertilizers affect soil microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), and CO₂ sequestration.

Methods: This research examined two rhizobia inoculum products—Biozote-N and Rhizogold—along with three biochar particle sizes (<2, 2–5, and 5–10 mm) under incubation intervals of 3, 6, and 10 days.

Results: The MBC ($\mu\text{g g}^{-1}$ soil) and MBN ($\mu\text{g g}^{-1}$ soil) increased significantly ($p < 0.05$), however, a significant ($p < 0.05$) decrease in the organic C and mineralizable C within the <2 mm biochar treatment was observed. With biochar size of 2 mm on day 3, the CO₂ evolution was 25% lower, but it increased 4%–19% from day 6–10 compared to the other biochar sizes and the biochar control. Sizes 2 and 2–5 mm were 43% lower in MBN, whereas 5–10 mm was 70% lower ($\mu\text{g g}^{-1}$ OC). Biozote-N outperformed Rhizogold and no-biofertilizer on days 6 and 10, registering 6% and 20% higher MBC (g g^{-1} soil/OC), respectively. No biofertilizer increased CO₂ production by 14% and 23% on days 3 and 6, respectively. On day 10, however, CO₂ production inside Biozote and Rhizogold plots increased by 67% and 45% relative to the control, respectively. Biofertilizers enhanced MBN ($\mu\text{g g}^{-1}$ OC) by 37%, with the contribution of Biozote-N being 18% more than that of Rhizogold. The Biozote-N and Rhizogold plots had a 13 and 28% increase in mineralizable N compared to the control. After the spring (lentil) crop, there was a 5%–18% higher MBC (g g^{-1} soil), 6%–17% higher MBC (g g^{-1} OC), 2%–10% greater CO₂ evolution, 23% higher MBN, and 77% higher mineralizable N than after the fall (maize) crop.

Discussion: The results demonstrated the usefulness of Biozote-N for enhanced MBC and MBN, as they decreased C mineralization at the small biochar size (2 mm) even after 1 year of their combined application to lentil crop, showing that biochar-Biozote-N were more successful at boosting soil organic fertility and C sequestration.

KEYWORDS

Biozote-N, biofertilizers, biochar, legumes, organic fertilizer, soil microbiology, soil fertility, soil ecology

1 Introduction

Because of the crucial structural, genetic, and functional contributions that nitrogen (N) makes to plant cells and the considerable growth and developmental processes necessary for increased yield, plants require enormous amounts of N (Bambara and Ndkidemi, 2010). Due to environmental concerns, the extensive use of N-based fertilizers in agriculture is under microscope. As a result, there has been an increased interest in environmentally friendly, sustainable agricultural methods, including inoculation with microorganisms (bio-fertilizers) to lessen the use of industrial fertilizer and its adverse environmental impacts (Lowe and Baldock, 2000). Rhizobia-associated lentils (*Lens culinaris* M.) boost the soil's organic fertility and N economy by fixing atmospheric nitrogen. Lentil can enhance yield by up to 2 t ha^{-1} and meet more than 85% of their nitrogen requirements through N fixation if infected with the suitable rhizobia (Bisen et al., 1980). Rhizobia association changes the rhizospheric environment to impact other nutrients available to the crop and fix the atmospheric N. Consequently, these bacteria are crucial living creatures in agriculture because they help flow plant nutrients, reducing the need for synthetic fertilizers (Mia and Shamsuddin, 2010).

Among different amendments, biochar is a potentially cost-effective carbon source with many agricultural and environmental applications. Biochar, a pyrolytic product of organic material (Lehmann and Joseph, 2009), is anticipated to hold soil carbon and significantly lower its losses. Application of carbon in the soil in the form of biochar (which contains carbon as nanoparticles in grounded form) (Nepal et al., 2023) or carbon nanoparticles (Nepal et al., 2022) offers the potential to improve soil fertility and quality. Each tonne of soil-applied biochar may absorb 1.4–2.9 tonnes of CO_2 and has several beneficial effects on soil characteristics and plant nutrition (Glaser et al., 2002; Kulyk, 2012). When used as a soil amendment, biochar improves the soil's physical and biochemical properties, which in turn increases soil fertility and productivity through increasing soil aggregation, water retention, pH, and microbial activities, thus, improving overall soil quality, potentially helping to reduce chemical fertilizer needs over time (Bhattarai et al., 2015; Nepal et al., 2023). In addition to increasing soil fertility, its ability to store carbon and nitrogen may lessen both immediate and long-term environmental deterioration and its harmful impacts on human and animal health (Verheijen et al., 2010). Biochar is an excellent adsorbent for nutrients and pollutants because of the various functional groups connected with it on its surface (Bilias et al., 2021). As a result, biochar improves the soil's ability to absorb nutrients and agricultural chemicals while lowering

their vaporization and leaching into the surface and groundwater (Nepal et al., 2023). Through weathering and decomposition, biochar may add many crucial nutrients to the soil. Biochar serves to lessen the bulk density of the soil, improves drainage, aeration, and root penetration, as well as the soil's ability to retain water and workability, thanks to its low density and up to 70% porous structure (Downie et al., 2009). According to Glaser et al. (2002), biochar is a product with a long-lasting impact on soil quality and carbon sequestration due to its greater C half-life (>1,000 years).

Due to its potential for reducing nutrient losses (Downie et al., 2009; Singh et al., 2010) and improving the fertilizer-use efficiency (Widowati et al., 2014), biochar has been embraced under research efforts for crop-nutrient management (Lehmann and Joseph, 2015). Its integration into poor soil enhances soil fertility, crop development, and productivity more than non-biochar soil (Steiner et al., 2007; Major et al., 2010; Zeeshan et al., 2020). However, all of these factors rely on the origin and characteristics of the applied biochar, soil characteristics, environmental plant responses, biochar-soil interactions, and its ability to absorb nutrients (Enders and Lehmann, 2012). It may have an impact on crop development both directly and indirectly depending on its natural nutritional content and capability for nutrient and water retention as well as the reduction of moisture stress (Lehmann et al., 2003; Karhu et al., 2011; Vaccari et al., 2011; Baidoo et al., 2016). In some cases, it is possible to anticipate poor crop performance for the first 30 days following cultivation on acidic soil (pH = 5.5), which may be caused by short-term N retention in N-deficient soil (Clough et al., 2013). According to Widowati et al. (2014), rice husk biochar caused significant N leaching, whereas wood biochar had the maximum water retention and minimized NO_3^{-1} leaching within the first 30 days following application. Jones et al. (2011) claim that biochar alters soil physical characteristics like bulk density, OM breakdown, and the organic carbon it liberates, suggesting a potential method for C sequestration. It effectively delivers nutrients into the soil while reducing nutrient leaching (Biederman and Harpole, 2012). After applying a biochar amendment to the soil, Laird et al. (2010) found a greater content of extractable nutrients, while Ali et al. (2015) reported an increase in soil fertility. Zeeshan et al. (2020) found that compared to the biochar control and the larger particle size fraction, the biochar amendment with small and medium particle size considerably decreased the bulk density of the soil and enhanced overall porosity, pH, and electrical conductivity. The tiny particle size fraction also greatly enhanced the soil's organic matter (OM) content and saturation.

TABLE 1 Characteristics of wood biochar particle size fractions used in the experiment.

Particle size (mm)	Unit	<2 mm	2 -- 5 mm	5 -- 10 mm
Carbon	%	62.5 (±2.21)	62.2 (±1.67)	60.1 (±1.41)
Ash		25.3 (±1.02)	26.0 (±1.03)	27.5 (±1.13)
Moisture		1.50 (±0.043)	1.50 (±0.065)	1.92 (±0.08)
Volatile matter		10.7 (±0.31)	10.3 (±1.04)	10.5 (±0.71)
Total N	g kg ⁻¹	0.54 (±0.02)	0.49 (±0 ± 0.07)	0.48 (±0.02)
Total P		0.23 (±0.007)	0.22 (±0 ± .007)	0.23 (±0.07)
Total K		0.51 (±0.009)	0.48 (±0 ± .001)	0.45 (±0.34)
Total Na		0.04 (±0.0003)	0.036 (±0 ± .008)	0.03 (±0.002)
K/Na ratio	-	12.8 (±1.23)	13.3 (±1.01)	13.2 (±0.21)
pH	-	8.30 (±0.78)	8.22 (0 ± .98)	8.20 (±1.01)
EC	dS m ⁻¹	0.62 (±0.007)	0.59 (±0.003)	0.61 (±0.05)
Fe	mg kg ⁻¹	88.0 (±1.83)	86.2 (±2.42)	81.8 (±1.21)
Zn		39.6 (±1.04)	39.2 (0 ± .71)	41.01 (±2.32)
Mn		81.8 (±2.02)	74.1 (±1.21)	74.6 (±1.54)
Cu		18.2 (±0.67)	15.6 (±1.09)	17.6 (±1.14)

The biochar's ability for adsorption depends on its surface area (Uchimiya et al., 2010). When put into the soil, high surface area biochar with a high variable charge component will have a greater surface sorption capacity, affecting the soil's ability to retain water, nutrients, and organic molecules (Brown et al., 2006). The surface oxygenation of biochar, when it is applied to soil, changes the O₂-containing functional groups (carboxyl, OH, phenol, and carbonyl groups) on the vast interior surface area of the biochar, inducing a negative charge and raising the soil pH and cation exchange capacity (CEC) (Cheng et al., 2006; Lee et al., 2010). According to Chintala et al. (2014), biochar-related cation release into the soil reduces soil acidity and may significantly alter the soil's N (NH₄⁺ and NO₃⁻) and AB-DTPA extractable P and K contents (Zeeshan et al., 2020).

Due to its chemistry and properties, biochar can increase agricultural production, especially in soils with low fertility and soil degradation, where it can be especially beneficial to the world's poorest farmers. Biochar from biomass waste has been linked to better soil water-holding capacity and a decreased run-off of agricultural pesticides and fertilizers (Woolf et al., 2010). It has improved crop yield by stimulating beneficial soil microbes like mycorrhizal fungi (Warnock et al., 2007), increasing soil base saturation (Glaser et al., 2002; Major et al., 2010), increasing soil water holding capacity (Glaser et al., 2002), and retaining nutrients in the portion of the soil column containing roots and enhancing nutrient use efficiency (Chan et al., 2007; Steiner et al., 2008).

Biochar-treated soil may provide a better environment for beneficial soil bacteria to flourish and perform (Shenbagavalli and Mahimairaja, 2012). Inoculating lentils with rhizobia improved soil OM, micronutrient fertility (Fe, Zn, Mn, and Cu), and soil qualities (Ali et al., 2019). These advantages could change depending on the feedstock and pyrolysis temperature (Yao et al., 2012). The biochar particle size fraction may, however, significantly

influence soil properties. The influence of biochar particle size on changes in N and C mineralization and CO₂ evolution in a cereal-legume rotation was assessed in the current study. In a silt-loam (fine loamy mixed thermotypic haplustalf) soil, we expected that lowering wood biochar particle size would enhance its advantages in terms of growth, N and C mineralization. The results of our study will broaden and improve farmers' options for combining appropriate particle size of biochar with fitting rhizobia strains to increase N and C economy, thereby reducing CO₂ evolution from agricultural areas.

2 Materials and methods

2.1 Biochar production and soil sampling

Acacia tree branch clippings (3 inches long) were pyrolyzed at 450°C for 48 h in a stainless-steel kiln made locally by the Institute of Mechatronics, the University of Engineering and Technology, Peshawar, Pakistan (Arif et al., 2017). Through crushing and sieving, the final product was divided into fractions of 2 mm, 2–5 mm, and 5–10 mm particle size and was characterized as reported in Table 1.

Soil samples were collected from the experimental site located at the Research Farms of Amir Muhammad Khan (AMK) Campus Mardan, Pakistan (34°15'97"N and 72°05'88"E). The soil was silt loam in texture with a bulk density of 1.3 g cm⁻³, classified as fine loamy, mixed, thermic, typic haplustalfs. The soil's native moisture level and saturation water content were very low (4% and 8.3%, respectively). The soil was non-saline, alkaline in reaction, low in total N (0.01%) and mineral N (10.3 mg kg⁻¹), low in organic carbon (0.32%), high in C:N ratio (32:1) and moderate in Mehlich 3 extractable K, P, Fe, Cu, Zn, and Mn (81, 5.6, 4.9, 2.5, 1.45,

and 3.52 mg kg⁻¹, respectively). Further elaboration of extraction procedure have been reported previously in [Ali et al. \(2022\)](#).

2.2 Experimental design

The study comprised two crops grown in the winter and summer of the same year. The experiment started in November 2019 by growing lentil and using a randomized complete block split-plot design (RCBD split plot) replicated three times. The treatments included two factor; biochar particle size (control, 2 mm, 2–5 mm, and 5–10 mm) applied to main plots whereas second factor, rhizobia inoculum products (Bizote-N and Rhizogold) were arranged in subplot. Biochar was applied at the rate of 1% on soil mass basis and the seeds of lentil were treated with respective biofertilizers at a rate of 0.5%. The recommended basal doses of N (30 kg ha⁻¹) and P₂O₅ (60 kg ha⁻¹) for the lentil crop were added to all experimental units (4 m * 3 m) uniformly. During field preparation for lentil, soil in the prescribed sub-plots was added and well mixed with biochar particle size fractions (2 mm, 2–5 mm, and 5–10 mm) while keeping one biochar control. Two rhizobia inoculum products, Bizote-N and Rhizogold, were procured from the National Agricultural Research Center (NARC), Islamabad, and the University of Agriculture, Faisalabad (Pakistan), respectively, and used to inoculate lentil seeds before being compared to one biofertilizer control in main plots. The lentil crop was planted on 5 November 2019, and harvested on 5 May 2020 (data not presented in this paper). At the time of lentil harvest, soil samples from all treatment plots were taken at depths ranging from 0 to 30 cm to analyze the soil's microbial biomass, C and N mineralization, and CO₂ evolution. The same experimental setup was used for the subsequent maize crop grown in the summer season, however the soil was supplemented with N, P₂O₅, and K₂O (120, 90, and 60 kg ha⁻¹), respectively, based on maize crop fertilizer recommendations. On 20 June 2020, maize was planted and harvested on 15 October 2020 (data not shown in this paper). To evaluate the residual impact of different biochar particle sizes applied and rhizobia biofertilizer products inoculated to the previous lentil crop on soil microbial biomass C and N, C and N mineralization and CO₂ evolution, soil samples from all treatment plots were taken at a depth of 0–30 cm.

2.3 Determination of C, N, and soil microbial biomass

To determine biomass C and N, chloroform fumigation and subsequent incubation techniques were utilized ([Brookes et al., 1985](#); [Vance et al., 1987](#); [Horwath and Paul, 1994](#)). Chloroform fumigation was used to kill the microorganisms in 20 g of fresh soil. After that, the soil was inoculated with 1 g of the same fresh soil that had not been fumigated, and it was then incubated for 3, 6, and 10 days intervals with 0.3M NaOH in vials suspended in incubated flasks for CO₂ trapping. The same procedure was repeated, simultaneously, for unfumigated soil. The solution was titrated against 0.1N HCl until the pink hue faded after adding 10 mL of 1M BaCl₂ solution and 4–5 drops of phenolphthalein. On day 10, after recording the CO₂ evolution, total mineral N was examined in both the fumigated

and unfumigated samples. Fc = Fumigated sample CO₂ and Ufc = Un-fumigated sample CO₂ were used to compute the microbial biomass C (Fc-Ufc)/Kc and biomass-N (Fn-Ufn)/Kn ([Jenkinson, 1981](#)), where Fn stands for mineral-N from fumigated soil, Ufn for mineral-N from non-fumigated soil, and Kn for 0.54 ([Jenkinson, 1988](#)).

For the computation of the quantity of mineralizable C based on 44 g of CO₂ containing 12 g of C, the whole amount of CO₂ from an unfumigated sample over 10 days of incubation was collected. Before and following the incubation (day 0 and day 10) using the method described above, mineral N in samples was estimated wherein difference at days 0 and 10 represents mineralizable N.

2.4 Statistical analysis

All the data were submitted to an analysis of variance (ANOVA) using the statistical software STATISTIX 8.1. The Least Significant Difference (LSD) test was used to determine if the treatment means for each parameter were significantly different ([Steel and Torrie, 1980](#)). The MS Excel program was used to create graphs showing the interaction data between biochar particle size and biofertilizers, while the R software (version 3.43.) was used to create the Principal Component Analysis (PCA) graphs and correlation matrix. All the statistical significance level was maintained at $p < 0.05$, unless otherwise specified.

3 Results

3.1 Microbial biomass carbon

As measured at 3, 6, and 10 days of incubation intervals, microbial biomass carbon (MBC, µg g⁻¹ soil day⁻¹) was substantially ($p < 0.05$) greater in soil treated with biochar than in soil with no biochar (control). At day 3, MBC with biochar increased 126%, 108%, and 72% over the no biochar treatment. On day 6, the rise was 50%, 24%, and 40%; on day 10, it increased by 29%, 26%, and 25% ([Table 2](#)). The order of increased MBC with respect to incubation time was day 3 > day 6 > day 10, and the efficiency of biochar particle size to raise MBC was; 2 mm > 2–5 mm > 5–10 mm. On days 3 and 6, the microbial biomass C in the treatment with less than 2 mm of biochar was 20% and 7% greater than in the treatment with 2–5 and 5–10 mm of biochar, respectively. On day 10, biochar sizes did not appear to have any impact on MBC; however, when biochar sizes of less than 2 mm were used, MBC increased by 2% and 3% in comparison to treatments using biochar sizes of 2–5, 5–10 mm, respectively.

On day 3, there was little difference in microbial biomass C (µg g⁻¹ organic carbon (OC)) between the treatments for biochar size ([Table 2](#)); however, compared to the biochar control, MBC (µg g⁻¹ OC) was 37%, 24%, and 31% lower on day 6 with <2 mm, 2–5 mm, and 5–10 mm particle size of biochar and on day 10, the respective reduction was 69%, 45%, and 58%. Compared to Rhizogold, Bizote-N observed MBC values were 19% higher for µg g⁻¹ soil day⁻¹ and 18% higher for µg g⁻¹ OC on day 6, as well as a 25% increase in each case over the biofertilizer control value ([Table 2](#)). On day 10, the MBC in the Bizote-N treatment concerning the

TABLE 2 Soil microbial biomass C (MBC) at different intervals, biochar particle size, and biofertilizers

Treatments	Microbial biomass C (MBC)					
 $\mu\text{g g}^{-1}$ soil day ⁻¹ $\mu\text{g g}^{-1}$ organic C		
Study interval	3 rd day	6 th day	10 th day	3 rd day	6 th day	10 th day
Biochar Sizes						
<2 mm	109.2 a	86.0 a	64.6 a	30.4	48.7 b	59.3 c
2–5 mm	100.8 ab	71.4 b	63.5 a	34.0	54.9 b	73.6 b
5–10 mm	83.5 b	80.5 a	62.8 a	26.9	51.5 b	66.4 bc
Control	48.4 c	57.4 c	50.2 b	26.4	66.7 a	100.5 a
LSD(p < 0.05)	19.4	10.27	3.3	Ns	10.6	13.1
Biofertilizer products						
Biozote-N	92.8	86.9 a	64.2 a	29.9	62.8 a	80.7 a
Rhizogold	80.9	72.9 b	60.2 b	27.7	53.4 ab	67.0 b
Control	82.7	69.2 b	56.5 c	30.5	50.1 b	77.2 a
LSD(p < 0.05)	Ns	7.11	2.61	Ns	9.72	7.62
Seasons						
Spring (Lentils)	86.2 a	78.2 a	63.5 a	30.2 a	57.7 a	80.8 a
fall (Maize)	84.7 b	74.4 b	57.1 b	28.5 b	53.2 b	69.1 b
LSD(p < 0.05)	1.37	0.10	1.1	1.61	1.85	1.9

BC, biochar; BF, biofertilizer. Means with similar letters are statistically similar at $p=0.05$. Ns: non-significant.

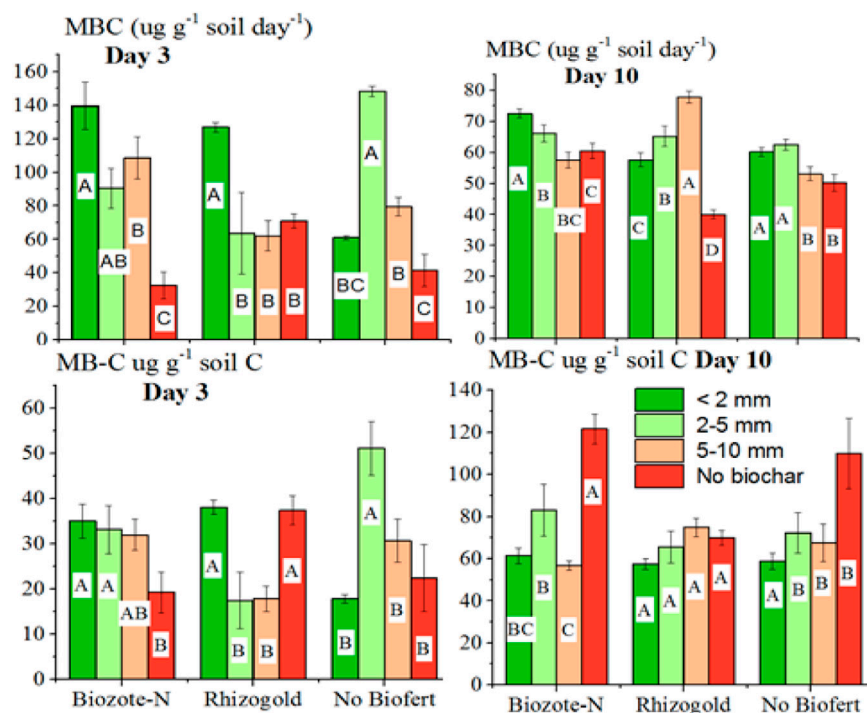


FIGURE 1 Microbial biomass C ($\mu\text{g g}^{-1}$ soil day⁻¹ and $\mu\text{g g}^{-1}$ soil OC) at day 3 and 10 as a result of the interaction between biochar particle size and biofertilizers.

TABLE 3 CO₂ evolution, mineralizable C and microbial biomass N (MBN) under different biochar size and biofertilizer treatments.

Treatments	CO ₂ evolution			Microbial biomass N		Minbl C	Minbl N
 μg g ⁻¹ soil ⁻¹ day ⁻¹			μg g ⁻¹ soil	μg g ⁻¹ OC μg g ⁻¹ soil	
	3 rd day	6 th day	10 th day				
Biochar Sizes							
<2 mm	35.0 b	23.5 a	7.8 a	41.8 a	3.9 b	56.1 b	19.4 b
2–5 mm	46.5 a	21.6 ab	6.9 c	35.0 b	3.9 b	64.3 a	20.6 b
5–10 mm	44.6 a	19.7 b	7.7 ab	30.7 bc	3.3 b	61.7 a	22.5 b
Control	44.4 a	22.7 ab	7.3 b	28.0 c	5.6 a	64.5 a	31.0 a
LSD(p < 0.05)	2.85	3.16	0.44	6.61	0.95	3.5	3.9
Biofertilizer products							
Biozote-N	42.9 b	19.0 b	9.2 a	37.3 a	4.5 a	62.9	23.2 b
Rhizogold	37.6 c	24.0 a	7.5 b	37.0 a	4.2 ab	60.2	26.3 a
Control	47.3 a	22.6 ab	5.5 c	27.4 b	3.8 b	62.1	20.6 c
LSD(p < 0.05)	3.21	4.07	0.55	3.02	0.51	ns	2.23
Seasons							
Spring (Lentils)	43.0 a	22.4 a	7.8 a	37.4 a	4.7 a	62.3	29.9 a
fall (Maize)	42.2 b	21.4 b	7.1 b	30.3 b	3.7 b	61.1	16.9 b
LSD(p < 0.05)	0.25	0.24	0.20	1.51	0.86	ns	0.59

BC, biochar; BF, biofertilizer; Minbl, mineralizable; OC, organic carbon. Means followed by similar letters are statistically at par with each other at 5% level of probability.

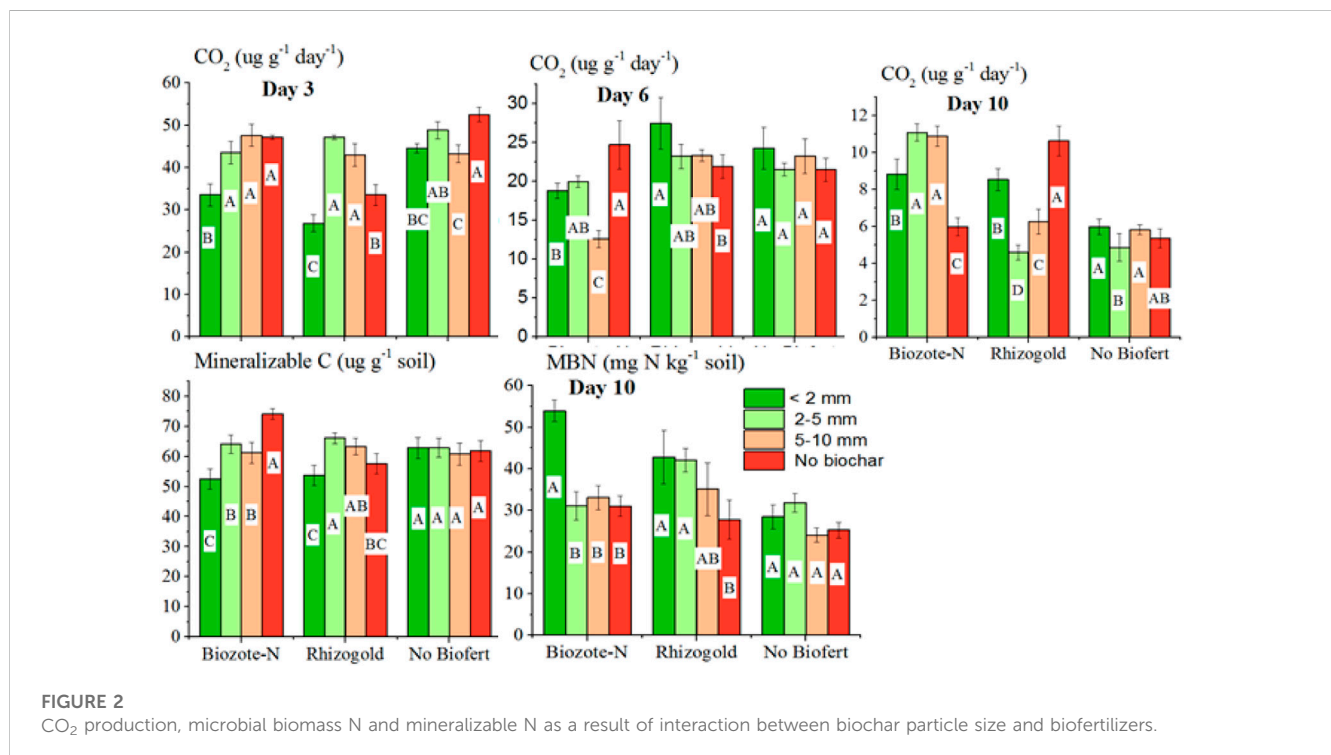


FIGURE 2 CO₂ production, microbial biomass N and mineralizable N as a result of interaction between biochar particle size and biofertilizers.

units mentioned above increased by 6% and 20% over the Rhizogold treatment and by 13% and 5% over the biofertilizer control, respectively. When examined for 3, 6, and 10 days of incubation, MBC ($\mu\text{g g}^{-1}$ soil day⁻¹) was 18%, 5%, and 11% greater after lentil than the next maize crop, and MBC ($\mu\text{g g}^{-1}$ OC) was 6%, 8%, and 17% higher (Table 2). In the interaction (Figure 1), Rhizogold recorded increased MBC ($\mu\text{g g}^{-1}$ OC) at the smaller particle size fraction at day 3 only, but the Biozote-N considerably enhanced the MBC ($\mu\text{g g}^{-1}$ soil day⁻¹ and $\mu\text{g g}^{-1}$ OC) both at days 3 and 10.

3.2 Carbon dioxide evolution

The varying particle size fraction of biochar had a substantial ($p < 0.05$) impact on the CO₂ evolution measured at various time intervals (3, 6, and 10 days) (Table 3). At day 3, biochar size of <2 mm showed significantly low CO₂ evolution (by 25%) compared to the no biochar treatment. However, 2–5 mm and 5–10 mm biochar sizes were statistically similar to the no biochar treatment. On day 6, the CO₂ evolution with the smallest particle size was substantially greater (by 19%) than the evolution with 5–10 mm biochar size, and it was 9% and 4% higher than the evolution with 2–5 mm biochar size and no biochar, respectively. Similar to day 6, CO₂ evolution with the biochar size of <2 mm at day 10 was substantially greater (by 13% and 7%, respectively) than that with 2–5 mm particle size and no biochar. At the beginning (day 3), no-biofertilizer considerably outproduced CO₂ production in Biozote-N and Rhizogold plots (14% and 23%, respectively). At day 6, midway through incubation, Rhizogold had a 5% greater CO₂ evolution than the biofertilizer control, whereas Biozote-N had a 21% lower evolution. Biozote and Rhizogold had considerably greater CO₂ evolution at day 10 (67% and 45%, respectively) than the biofertilizer control.

At all three incubation intervals (day 3, day 6, and day 10), CO₂ evolution was strongly influenced by the interaction between biochar size and bio-fertilizer (Figure 2). At the three and 6-day incubations, Biozote and Rhizogold were either at par with or below the bio-fertilizer control; however, at the tenth-day of incubation, Biozote-N was at par with Rhizogold at <2 mm while both significantly higher than the bio-fertilizer control. At the 2–5 and 5–10 mm particles, Biozote was higher in CO₂ evolution than Rhizogold and the biofertilizer control. At day 3, day 6, and day 10 of incubation, CO₂ evolution was substantially greater in spring after the lentil crop than in fall after the maize crop (by 2, 5, and 10%, respectively).

3.3 Microbial biomass nitrogen

Compared to the Microbial biomass N (MBN) in the 2–5 mm, 5–10 mm biochar size treatments and biochar control, the MBN in the <2 mm biochar size treatment was significantly ($p < 0.05$) higher (by 19%, 36%, and 49%, respectively). While the 5–10 mm biochar particle size induced rise in MBN remained statistically identical with the biochar control, the 2–5 mm biochar particle size treatment also showed a significant edge of a 10% increase in MBN over the biochar control (Table 3). However, MBN ($\mu\text{g g}^{-1}$ OC) in biochar treatments was significantly ($p < 0.05$) lower in <2 mm, 2–5 mm (by

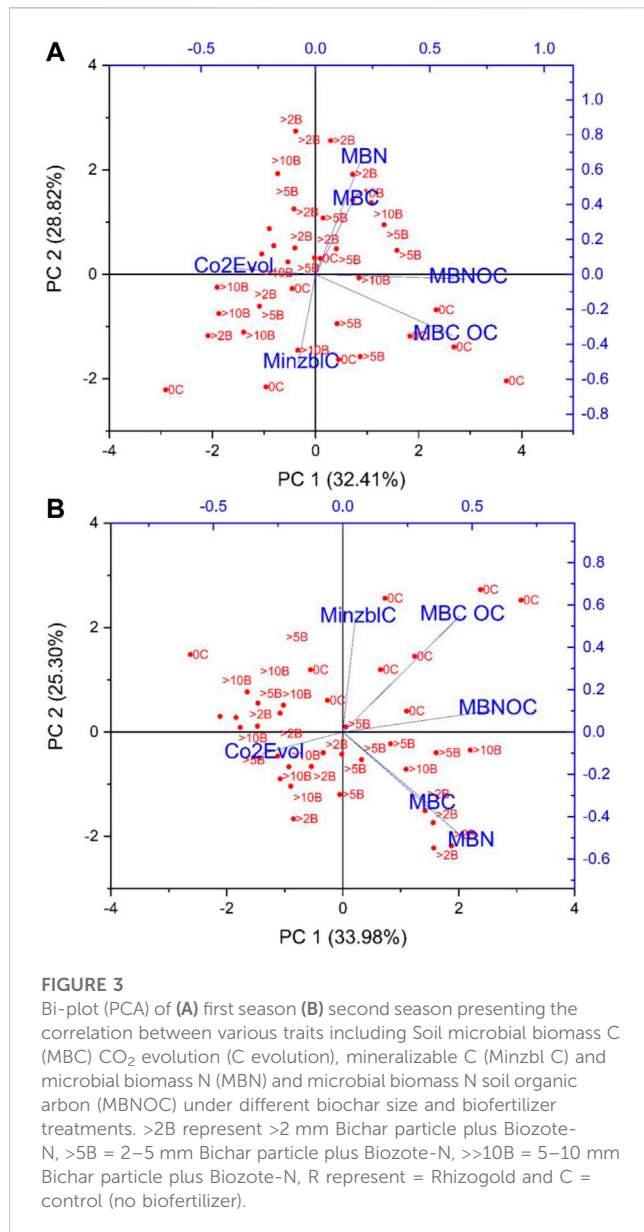
43% each), and 5–10 mm (by 70%) than the no biochar plot accounting for the majority of the OC added by biochar and having a more wide-ranging C:N ratio (Table 1). All biochar particle size treatments had identical statistical results for MBN ($\mu\text{g g}^{-1}$ OC). While both biofertilizers were statistically similar, MBN in the biofertilizer treatments (Biozote N and Rhizogold) was considerably ($p < 0.05$) greater (by 37% and 36%, respectively) than MBN in control (no biofertilizer) treatment (Table 3). The amount of MBN ($\mu\text{g g}^{-1}$ OC) in the Biozote-N was significantly higher (by 18%) than that of the no-biofertilizer and registered a non-significant increase over that of the Rhizogold. Furthermore, the no-biofertilizer plot and Rhizogold were statistically comparable in MBN ($\mu\text{g g}^{-1}$ OC).

Seasons with various crops in the cropping pattern differed noticeably in MBN ($\mu\text{g g}^{-1}$ soil and $\mu\text{g g}^{-1}$ OC). A lentil crop in the Spring resulted in 23% higher MBN ($\mu\text{g g}^{-1}$ soil and $\mu\text{g g}^{-1}$ OC) measurements than a maize crop in the fall. According to a significant interaction, Biozote-N treatment at the <2 mm size biochar was significantly ($p < 0.05$) higher in MBN ($\mu\text{g g}^{-1}$ soil) than the Rhizogold and biofertilizer control at either of the biochar sizes and no biochar plot. Rhizogold's performance at particle sizes of <2 mm and 2–5 mm was comparable to and much better ($p < 0.05$) than at particle sizes of 5–10 mm biochar and without biochar.

3.4 Mineralizable carbon

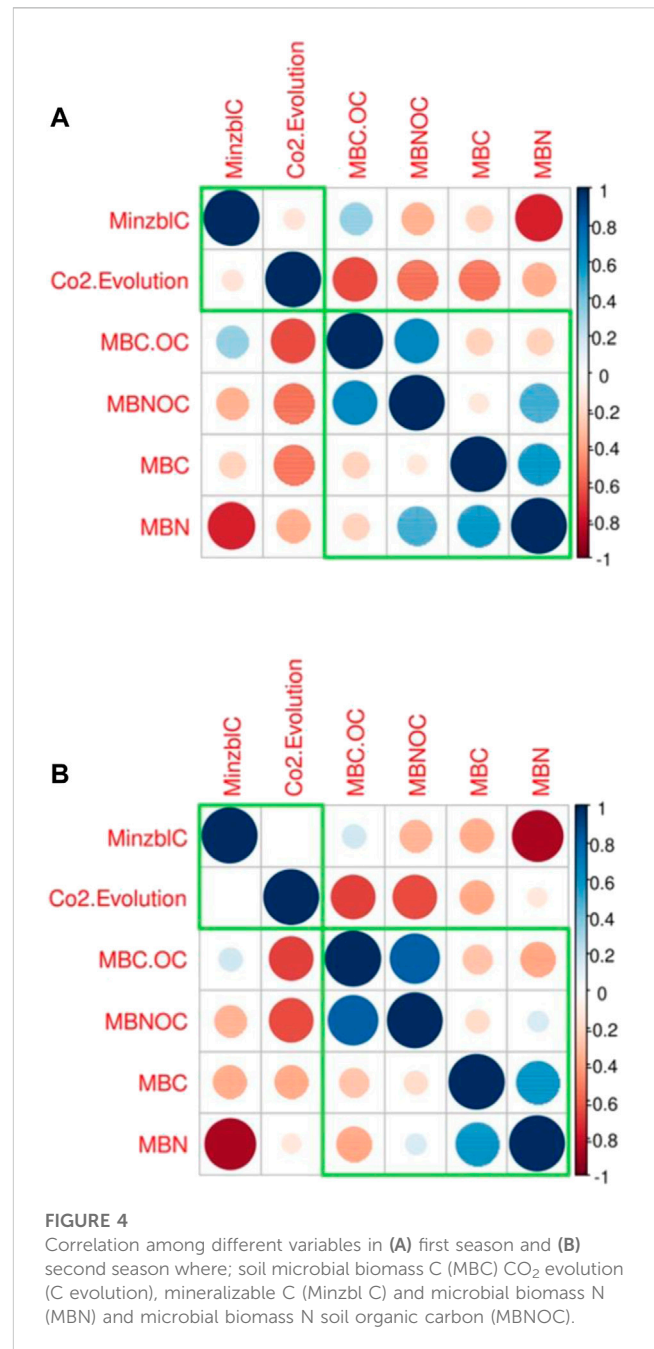
Mineralizable C ($\mu\text{g g}^{-1}$ soil) at the <2 mm biochar particle size was significantly ($p < 0.05$) lower (by 15, 10, and 15%) than mineralizable C in 2–5, 5–10 mm size biochar and no biochar treatments, respectively (Table 3). There was no appreciable difference in mineralizable C ($\mu\text{g g}^{-1}$ soil) between the biofertilizer and no-biofertilizer treatments. Additionally, despite 2% greater mineralizable C ($\mu\text{g g}^{-1}$ soil) after legumes harvest in the Spring than maize in the fall, mineralizable C remained statistically constant between crop seasons. Mineralizable C was significantly impacted by the interactions between biochar particle size treatments and biofertilizers (Figure 2). Both biofertilizer treatments applied with the smallest size (<2 mm) biochar size had lower mineralizable C than the no-biofertilizer treatment applied with the same size biochar. However, compared to Rhizogold and no-biofertilizer, Biozote had considerably ($p < 0.05$) greater levels of mineralizable C in the no-biochar treatment. The effects of both biofertilizer products and no biofertilizer for the mineralizable C content of the soil were statistically comparable for the other sizes of biochar additions (2–5 mm and 5–10 mm).

All biochar size (<2 mm, 2–5 mm, and 5–10 mm) treatments' mineralizable N values were statistically equivalent but significantly ($p < 0.05$; by 60%, 50%, and 38%) lower than the control values, respectively (Table 3). When compared to the no biofertilizer treatment, mineralizable N increased significantly ($p < 0.05$) in both Biozote-N and Rhizogold by 13% and 28%, respectively (Table 3). Rhizogold, however, was also significantly ($p < 0.05$) higher than Biozote-N (by 14%). The results also revealed significant ($p < 0.05$) seasonal differences (Table 3), with the mineralizable nitrogen content of the soil in the Spring after legume harvest being 77% greater than that of the soil in the fall when maize was grown there.



3.5 Principal component analysis

PCA loading plots were prepared to measure the effects of biochar particle size and bio-fertilizer application on Soil microbial biomass C (MBC), CO₂ evolution (C evolution), mineralizable C (Minzbl C), and microbial biomass N (MBN) and microbial biomass N soil organic carbon (MBNOC) (Figure 1, Figure 2). The responses of the different parameters influenced by different biochar sizes and biofertilizer treatments were visualized as PC1 (principal component 1) and PC2 (principal component 2). Figure 3 revealed that PC1 and PC2 explained 64.1% and 22.6% of the total variance. The relation among the above mentioned parameters were further validated using a correlation matrix (Fig: 5) for the spring (a:post-lentil harvest) and fall (b:post-maize harvest) seasons. The PCA results indicated a clear separation among the studied traits under biochar size and biofertilizer treatments. PC1 was positively correlated with mineralizable C



and MBCOC and MBNOC under smaller biochar particles with no biofertilizer application, however, MBC and MBN were positively correlated under smaller size of biochar with Rhizogold. Carbon mineralization was negatively associated with MBN, C evolution and MBC. The Minerlized carbon was greater in large particle size with no biofertilization. PC1 was positively correlated with mineralizable C and MBCOC and MBNOC under smaller biochar particles with no biofertilizer application; however, MBC and MBN were positively correlated under smaller size of biochar with Rhizogold. Moreover, PC2 was positively correlated with CO₂ evolution under large particles combined with Rhizogold. Figure 4. Represent biplot for 2nd season revealed that PC1 was positively correlated with MBN and MBC under

smaller biochar particles with Rhizogold whereas it was negatively correlated under PC2 under large particle size with no biofertilizer application. PC1 was positively correlated with MBN and MBC under smaller particles of biochar with Rhizogold whereas PC2 was negatively correlated with C evolution, minzblC under large particle size with no biofertilizer application. Moreover, C evolution was negatively associated with MBCOC and MBNOC. It was also noted that MBC and MBN were closely associated and were substantially influenced by smaller particle sizes with Biozote.

4 Discussion

Significantly higher microbial biomass carbon (MBC, g g^{-1} soil day^{-1}) in the small size (<2 mm) biochar treatment appears to be due to the lowest size fraction having the largest surface area and absorbing most of the nutrients, resulting in increased microbial activity and higher microbial biomass carbon (Ahmad et al., 2014). Similarly, early incubation (day 3) appears to have higher nutritional content than later incubation intervals, resulting in higher MBC. Ascough et al. (2010) noticed a significant increase in microbial population after applying biochar to the soil. Since nutrients are a source of energy for microorganisms (Goyal and Welch, 2006), a decrease in MBC as incubation progresses may be due to nutrient depletion. This demonstrated a greater potential for tiny biochar sizes to sustain MBC for longer than its uses for larger particle-size biochar.

In the present study, lower microbial biomass C ($\mu\text{g g}^{-1}$ OC) within the <2 mm size biochar is anticipated due to its more consistent mixing with soil matrix and providing more organic carbon to the soil, resulting in an MBC that is lower per Gram of OC in comparison to larger biochar particles. Also, it was noted that as the difference in the MBC ($\mu\text{g g}^{-1}$ OC) between the different particle size treatments grew, so did the difference in the incubation time. On day 3, the difference was not substantial, however, on days 6 and 10, the <2 mm biochar size had the most significant decrease in MBC ($\mu\text{g g}^{-1}$ OC) over the no biochar plot, followed by the 2–5 mm and 5–10 mm biochar sizes. The nutrients, energy sources for bacteria (Goyal and Welch, 2006), would be less available for microbial growth over time in the free-soil solution in the <2 mm biochar treatment because they would stick to more exposed surfaces than on the bigger particles or in the control. This data showing a decrease in MBC ($\mu\text{g g}^{-1}$ OC) revealed that the microbial community has less effect on the turnover of the biochar C after the easily degradable free C has been used up. This is more noticeable later in the incubation process than at the beginning. Even biochar has been shown to reduce FYM carbon mineralization and CO_2 release (Vasu, 2015), which is very similar to what we found. Our results also showed that the ability of biochar to keep soil C safe increased as the particle size fraction decreased which showed the biggest difference between the MBC ($\mu\text{g g}^{-1}$ OC) and the plot with no biochar (Ali et al., 2022).

The ability of microorganisms to recycle plant nutrients is crucial. In this study, only the Bizote-N showed a significant difference in MBC ($\mu\text{g g}^{-1}$ soil day^{-1} and $\mu\text{g g}^{-1}$ OC) on days 6 and 10. Biozote-N appeared to have adequate activity through atmospheric N fixation and increased substrate for greater microbial growth than Rhizogold and the biofertilizer control.

On day 10, Rhizogold exceeded the biofertilizer control in MBC ($\mu\text{g g}^{-1}$ soil day^{-1}) by 7%, indicating that its onset of N fixation is delayed in relation to the production of N fixation substrates in Biozote-N plots for increased microbial growth. This is supported by Billah et al. (2019), who demonstrated statistically comparable grain and straw nitrogen levels in lentils infected with Rhizogold and Biozote-N, respectively. Rhizogold's significantly lower MBC ($\mu\text{g g}^{-1}$ OC) (by 19%) over the biofertilizer control is due to the higher OC in the Rhizogold treatment compared to the biofertilizer control. It is possible that the increased MBC in Rhizogold-treated plots is not proportional to the increased OC. According to Ahmad et al. (2014), MBC values were higher 10 days after legume exposure. Greater microbial biomass carbon (MBC) after lentil than after maize confirms leguminous N-rich residues and a greater contribution to soil organic matter than cereals. In cropping patterns, legumes have a significantly higher concentration of microbial biomass C (Ahmad et al., 2014). In the present study, increased MBC in both Biozote-N and Rhizogold at the smallest biochar particle size fraction is indicative of its uniform distribution in soil and improved soil physical environment (Ahmad et al., 2014) for favorable microbial inoculation performance and growth are reasons why the smaller biochar particle size is essential for enhancing microbial properties.

At the initial incubation, CO_2 evolution with <2 mm biochar size was significantly lower, however, at the mid and final incubation, the CO_2 evolution with the smallest particle size was substantially greater (by 19%) than larger biochar size and no biochar. The soil exchanges its microorganisms with biochar once it is put into the soil since the intrinsic microbial community of biochar is often relatively low (Jones et al., 2011). Thus, the organic matter in native soil is negatively primed by biochar (Jones et al., 2011). Since soil matrix, including native organic matter, is anticipated to mix well with <2 mm particles, a more uniform negative priming impact is expected. As a result, the initial rate of CO_2 evolution (day 3) was lowered due to poor intrinsic microbial population and a negative priming impact of <2 mm size on soil organic matter. Smaller particles blend well with soil, creating intra-aggregates and particle bridges that increase porosity (Keech et al., 2005) and lower bulk density (Billah et al., 2019). This results in a better soil environment, which increases the aeration of native soil OM and increases CO_2 evolution. Since, it would probably take at least 3–6 days to complete, more CO_2 liberation was seen on days 6 and 10 rather than at initial interval of day 3. The lower CO_2 evolution in our study (Table 3) is consistent with the findings of Jones et al. (2011), who found that a significant portion of the CO_2 release from biochar-amended soil comes from a dissolved inorganic portion of the biochar and that the release is, therefore, more abiotically mediated and not related to the extent of microbial activity. This is true even though day 3 (Table 2) showed higher microbial activity. The higher CO_2 release at the middle (day 6) and later (day 10) incubation stages (Table 3) seem to be more biotically mediated than abiotic, according to (Jones et al., 2011), who noted that depending on the type of feedstock and temperature of pyrolysis, dissolved organic carbon is approximately equal to dissolved inorganic carbon and their enhancement in CO_2 flux is also approximately equal.

At the beginning (day 3), higher CO₂ production in biofertilizer control (Table 3) shows the breakdown of native soil organic matter. Compared to Rhizogold and the Control, the soil organic matter level in the Biozote-N plot treated with the previous lentil crop was considerably greater (Ali et al., 2019). The fresh addition of soil organic matter (OM) from legume crops also negatively primes the soil organic matter. Here again, higher microbial activity in the Biozote-N plot than in Rhizogold and biofertilizer control (Table 2) did not increase CO₂, indicating that most initial CO₂ release is abiotic. Midway through incubation, greater CO₂ evolution within Rhizogold and within the Biozote-N and Rhizogold at day 10 (Table 3) are consistent with Table 2's findings that biofertilizer plots had greater levels of microbial activity than biofertilizer controls seemingly the CO₂ release at this stage was biotically driven. Treatments with biofertilizers could need some time before organic matter breaks down. The prolonged availability of organic carbon for decomposition in bio-fertilizer inoculated plots, particularly Biozote-N, is potentially the cause of the extended CO₂ evolution duration, whereas, in the no-biofertilizer plot, the same has been maximally exhausted by mid-incubation stage.

The CO₂ evolution within Biozote and Rhizogold amended to <2 mm biochar size plots were either at par with or below the bio-fertilizer control at the initial and mid-incubation but outperformed it at the final incubation; however, Biozote was significantly higher than both Rhizogold and the biofertilizer control. Since lentils commonly lose their leaves and leave a thin coating of the litter on the soil surface, this may be due to greater soil organic matter content following lentils than cereal crops (Ahmad et al., 2014). Due to the increased N content of lentil organic matter compared to cereal organic matter (Giller, 2001), which better stimulates microbial activity (Table 2), spring had more CO₂ evolution than the fall maize crop (Table 3).

Enhanced microbial biomass N (MBN) inside the <2 mm biochar size followed by the 2–5 mm biochar particle size treatment (Table 3) indicated that biochar had sequestered soil nitrogen by allowing it to be consumed by microorganisms and converted to MBN. According to Table 2, the smallest biochar size had the highest MBC (μg g⁻¹ soil), which denotes a greater microbial population. Therefore, the greatest amount of microbial protein may have been present, which would account for the treatment's significantly greater MBN. Significantly ($p < 0.05$) lower MBN (μg g⁻¹ OC) within biochar treatments accounting for most of the OC added by biochar and having a wider C:N ratio. Therefore, it is important to note that biochar may affect nutrient cycles and organo-chemical properties in addition to changing the physical properties of the soil (Billah et al., 2019) and increasing soil microbial biomass (Table 2) (Jin, 2010; Liang et al., 2010; Nguyen et al., 2010; Lehmann et al., 2011).

Biofertilizers are organic products that include live cells of various microorganism types that may biologically change inaccessible forms of nutritionally important nutrients into available forms (Vessey, 2003). An increase in MBN (μg g⁻¹ soil and μg g⁻¹ OC) with biofertilizer inoculation is a testimonial of the N fixation compared to no biofertilizer plot because microorganisms being significant features in agriculture to improve the circulation of plant nutrients and minimize the demand for chemical fertilizers (Pouryoucef et al., 2007). The

higher MBN (μg g⁻¹ OC) in the Biozote-N indicated the highest potential for N fixation by the Biozote-N and consuming it in their body proteins. Biozote-N provides similar support with a higher MBC than Rhizogold (Table 3). The biofertilizer significantly aided the availability of nitrogen for the crop and the provision of a favorable environment for plant growth. Additionally, nutrient digestion inside microbial biomass improves the nutrients' retention and recycling. In addition, as a micro-organism dies and decays, it becomes an energy source for more species in the soil food web (Miller et al., 2009).

Season having lentil was significantly higher in MBN than season having maize crop. As was previously indicated, grain crops give less organic matter to soil than legumes. Thus, increased microbial activity and increased N availability from N-rich organic matter may be used to explain greater MBN following legumes. Following the maize harvest in the fall season, soil N-rich organic matter may have significantly decreased compared to the legume harvest in the spring (Ahmad et al., 2014), followed by lower MBN concentration in the soil in the fall than Spring season. Globally, the concentration of CO₂ exhaled by legume roots may be comparable to or even greater than CO₂ produced during N-fertilizer production. However, the CO₂ evolved from the legume root system via photosynthesis, hence, the CO₂ concentration that was not recaptured by the plant and eventually escaped from the legume canopy would be C neutral. In contrast, all the CO₂ released during the synthesis of fertilizer N is derived from fossil fuels and represents a net contribution to CO₂ levels in the atmosphere. In interaction, Biozote-N's higher potential for N fixation and a more favorable soil environment at the <2 mm size biochar might have resulted in higher MBN (μg g⁻¹ soil) followed by Rhizogold's performance at the <2 mm and 2–5 mm biochar size than without biochar.

Reduced mineralizable C with the smaller size of biochar alone and in interaction with biofertilizer products could be because of the enhanced microbial population due to the inoculation of biofertilizer products and increased C immobilization by them at the smaller size of biochar because of the improved soil environment. All biochar size fractions had much lower mineralizable N values than the control values. This could be because of biochar's wide C:N ratio rendering reduced soil mobility and less N availability for mineralization, especially in the short-term experiments (Deenik et al., 2010). Using biochar in the soil is one of the most effective and highly debated aspects to combat climate change and improve soil fertility (Zimmerman et al., 2011). It is claimed that biochar changes how nitrogen (N) moves through soils (Clough and Condron, 2010; Clough et al., 2013). These results demonstrated that biochar application to soil helped keep carbon and nitrogen from escaping the soil. The current study revealed that C and N sequestration is directly linked to the biochar surface area increase when the biochar particle size fraction is decreased. Recent research has shown that adding biochar to mineral soils may directly affect N changes (Castaldi and Aragosa, 2002; Zheng et al., 2013). Mineralizable N increased significantly ($p < 0.05$) with both the Biozote-N and Rhizogold, and after legumes rather than maize. Microorganisms recycle the plant nutrients, reducing the need for artificial fertilizers (Pouryoucef et al., 2007). The results showed that legumes

responded to inoculation by increasing the number of microorganisms and possibly by increasing the rate and composition of exudation (Bashan et al., 2004; Kawasaki et al., 2012) and crop performance (O'Callaghan, 2016), all of which could have led to enhancing soil organic matter. However, Biozote-N was better than Rhizogold at reducing the amount of mineralizable nitrogen. This showed that Biozote-N is better able to fix nitrogen than Rhizogold.

5 Conclusion

Biochar improved microbial biomass C and N concentration ($\mu\text{g g}^{-1}$ soil) but did not improve their contents g^{-1} soil OC. The impact of the particle size range of <2 mm was more positive. Because of low MBC ($\mu\text{g g}^{-1}$ OC) at <2 mm particle size range, higher CO_2 evolution at day 6 and 10 incubation is more likely due to abiotic factors. Similarly, the <2 mm size is better for N sequestration due to its larger MBN and lower mineralizable N. Biozote-N treatment substantially increased MBC and MBN, was low in mineralizable N, and may be advantageous for C sequestration due to its delayed CO_2 evolution. All metrics were considerably higher in the Spring after the legume harvest than the maize in the fall, except for mineralizable C. The results showed that for enhanced microbiological characteristics, the grain size of the biochar amendment should be kept below 2 mm. Even after applying Biozote-N and the smallest biochar particle together for a year to a lentil crop, their combined performance was synergistic and beneficial for most of the microbiological parameters of the organic fertility. Further research should focus on nano-char being most effective in a smaller size to evaluate their impact on crop quality, yield, and environment.

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Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

WA: project conceptualization, resources, writing, editing and review. JN: writing, review and editing, data analysis, validation, ZZ: project administration, supervision, funding acquisition, FM: data analysis, software, review and editing. AK: methodology, review and editing. IA: methodology, review and editing. SZ: review and editing, validation. MK: visualization, methodology, SAJ: review and editing and DT: software, writing and editing and resources. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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