RESPONSE OF SOIL HEALTH INDICATORS TO BIOCHAR AND ITS INTEGRATION WITH BEST AGRICULTURAL PRACTICES

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ABSTRACT

Growing global demand for food production places significant pressure on agricultural soils, particularly those under intensive cultivation. Hence, agricultural producers face the challenge of balancing the need to sustain or improve crop yield while preserving soil resources and minimizing greenhouse gas emissions. Biochar, a carbon-rich material produced from pyrolysis of biomass (e.g., crop residue, animal manure, biosolids), has gained recognition as a "climate-smart" agricultural practice because of its agronomic and environmental benefits. However, there needs to be more understanding of how biochar can be effectively integrated into other management practices, such as no-till, cover cropping, and nitrogen management. The first chapter explored the individual and combined effects of biochar, no-till, cover cropping, and nitrogen management on soil health indicators. Results suggest that integrating these practices, especially biochar, in a traditional corn-soybean system increases soil extracellular enzyme activities (EEAs) and soil organic carbon (SOC). In the second chapter, we explored the impact of biochar application rate and incorporation depth on soil health. Overall, increasing the biochar rate from 5 Mg ha^{-1} to 15 Mg ha^{-1} leads to higher production of EEAs and an increase in SOC, indicating that biochar can aid in nutrient acquisition and C sequestration (i.e., 1-2 years after application). This study will help inform decisions about integrating biochar into existing soil conservation methods.

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CHAPTER ONE: LITERATURE REVIEW

1.1: Conceptual history of soil health

Soil is a complex system serving a fundamental role that encompasses agricultural productivity, human development, ecosystem services, and climate change mitigation (Hou et al., 2020). In the 1990s, research efforts to characterize and establish a concise definition of a complex system became important in both natural and social sciences (Ladyman et al., 2013). Rind (1999) defined a complex system as having multiple components interacting differently. Whitesides and Ismagilov (1999) described it as a system whose evolution is highly sensitive to initial conditions or minor disturbances, with many interacting components or multiple ways to evolve. Scientists and philosophers have provided various definitions of a complex system. However, its overarching feature is having interacting elements that create a robust order without a single factor centrally controlling all its processes (Parrish & Edelstein-Keshet, 1999; Werner, 1999; Weng et al., 1999).

These definitions indeed describe soil, marking the emergence of soil health as a fundamental complex system that provides ecosystem services for food production and sustainability (Lehmann et al., 2020; Silveira & Kohmann, 2019). Henry Wallace first mentioned soil health in his unpublished thesis in 1910 to refer to soil fertility. He used it to describe humus as a central key to maintaining soil health, elaborating that clayey soil, compared to sandy soil, benefits more from adding humus, making the soil more friable and increasing its water-holding capacity. Today, the terminology and concept of soil health are still evolving. The Natural Resources Conservation Service (USDA-NRCS) defines soil health as "the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans." This current definition of soil health evolved from "soil quality" in the 1990s, referring to the soil's

"capacity to function within ecosystem and land-use boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health" (Doran & Zeiss, 2000). Recognizing the complexity of the soil environment is vital to understanding the behavior of soil properties that dictate soil health (Ladyman et al., 2013).

1.2: Distinguishing soil health from soil quality

While soil health and quality are terms often used interchangeably in literature, there is still a distinction between them. Generally, soil quality describes a soil's ability to function mainly for agriculture and sustain human-desired services. In 1994, Doran and Parkin developed a broader definition of soil quality with both ecosystem and soil function perspectives; this included the ability of soils to promote environmental quality and human health. However, several papers have argued that soil quality is only appropriate for soil use, not function (Sojka & Upchurch, 1999; Letey et al., 2003). Only in the early 2000s was soil quality used to describe soil management-related works (Lehmann et al., 2020). Currently, USDA-NRCS defines soil quality as the capacity of soil to provide ecosystem services that sustain plant, animal, and human lives. These services include enhancing air and water quality, sustainable production, and support for human welfare (e.g., health and habitation).

In contrast, soil health extends to a broader scope. It views soils from an ecosystem perspective: to sustain services that are not merely for humans (e.g., provision of food, fuel, and fiber) but also for the benefit of all organisms within its network of ecosystems. It has a broader goal of sustaining planetary health than soil quality, focusing only on ecosystem services centered on human welfare (Lehmann et al., 2020). Soil health also depends on the stability of the whole system, how the soil is maintained, and its resilience (self-regulation) to stress (Tugel, 1995, as cited in Sojka and Upchurch, 1999). Only in the early 2000s did the term soil health

emerge in the scientific literature (Pankhurst et al., 1995; Haberem, 1992). Guo (2021) argued that soil health became a popular term presumably because of the 2007–2008 global food crisis and when the soil's capability to sequester carbon (C) and mitigate climate change was potentially recognized.

Lehmann et al. (2020) presented the importance of considering three aspects of soil health management to support the sustainability of soil ecosystem services: (1) employing a multi-functional management system, (2) recognizing possible disservices or tradeoffs and synergistic effects from managing soil to improve a service, and (3) recognizing that the implemented management should be able to sustain the long-term viability of soil services. Ideal management practices that consider these facets of soil health enable balance among soil functions for crop productivity, environmental quality, and plant and animal welfare – all of which are highly influenced by land management decisions. Doran and Zeiss (2000) stressed that all soil functions must be considered when designing management practices and that focusing on a single function must be avoided (e.g., crop productivity).

1.3: Soil organic matter and its relevance to soil health

Because soil health is central to human and environmental health, it is essential to understand how it changes over time and identify the human-determined factors that influence it. The soil health status of cropping systems can now be evaluated and quantified based on soil health metrics/indicators. To be used as an indicator, a soil property is assessed based on several criteria: relevance to soil health (and its ecosystem functions and services), effectiveness or sensitivity, production readiness, measurement repeatability, and interpretability for management decisions. A soil health indicator is effective if (1) it is sensitive to short-term changes in cropping management, (2) it can represent soil processes that are important for agriculture and

the environment, and (3) it can provide helpful information for soil health assessment (USDA-NRCS, 2019).

An ideal indicator of soil health should be easy and cost-effective to sample and measure for commercial production laboratories (production readiness). Moreover, measurements should exhibit repeatability with acceptable precision and have results easily interpreted for agricultural management decisions. NRCS recommends several indicators to evaluate soil health status as a standard starting point. They categorized indicators based on the most critical soil processes that dictate soil health: organic matter (OM) cycling and carbon (C) sequestration, soil structural stability, general microbial activity, carbon food source, bioavailable nitrogen (N), and microbial diversity (USDA-NRCS, 2019).

Soil organic matter (SOM) is integral to many processes that drive soil health. It supports vital ecosystem services that enable the provision of food and fiber, climate regulation, and biodiversity (Smith et al., 2015). One of the significant functions of SOM is to supply nutrients to crops via decomposition. For example, macronutrients such as sulfur (S) and nitrogen (N), along with the micronutrient boron (B), are stored within SOM and made available to plants once SOM decomposes (Sullivan et al., 2019). The composition of SOM and its decomposition rates impact nutrient availability and contribute to different soil properties (cation exchange capacity, soil structure) and functions (water dynamics, soil biota, and C storage) (Cotrufo & Lavallee, 2022). Additionally, SOM has been a widely used indicator to assess soil health changes based on management practice. It is preferable to combine SOM with other indicators (e.g., enzyme activity, microbial abundance) into models to provide integrated information about soil processes and functioning (Zornoza et al., 2015).

Soil cation exchange capacity (CEC) refers to the ability of soils to store cations (e.g., Ca2+, Mg2+, K+) in their negatively charged sites. CEC is frequently used as an indicator of soil quality and fertility. Predictive models often generate soil texture and SOM as main CEC predictors, while mineralogy is an interacting factor (Seybold et al., 2005). Mineral-associated organic matter (MAOM) fractions (>53 μm) also contribute to higher CEC values than the particulate organic matter (POM) fractions (Oorts et al., 2003). Kaiser et al. (2008) also found that phosphate-soluble OM fractions accounted for 0.8-11.6% of total CEC despite only comprising 0.3-0.9% of total soil mass. These studies demonstrate that the CEC of SOM increases as it breaks down, producing low-molecular-weight SOM compounds with higher proportions of reactive/acidic functional groups such as carboxyl and hydroxyl.

The current understanding of the role of SOM in maintaining soil structure stability is based on studies that highlight the importance of plant roots and fungal hyphae in forming macro-aggregates (>250 micrometers). Roots and hyphae produce net-like structures and mucilage that bind soil particles and micro aggregates, significantly contributing to macro-aggregate formation. While not considered SOM, their structures become POM as they die, releasing soluble compounds as dissolved organic matter (DOM) (Bucka et al., 2021). The decomposition of these plant materials serves as a starting point of aggregate formation by promoting microbial activity, resulting in the deposition of microbial byproducts and proliferation of saprophytic hyphae, which enhances soil aggregation (Bucka et al., 2021; Jastrow & Miller, 1998). In addition, less decomposed and larger fragments (POM) can act as catalysts for macroaggregate formation, while smaller organic compounds (e.g., DOM) act as a "glue" that binds soil particles or precipitates with metal oxides to form mineral-associated organic matter (MAOM) (Wagai et al., 2020).

Another vital function that SOM contributes is the provision of essential nutrients such as N, P, and S. These nutrients are made available to plants and microbes through SOM decomposition via depolymerization and mineralization from organic to inorganic forms, thereby supporting soil health. Typically, SOM contains 20%-80% and 90%-95% of total P and total N, respectively (Cotrufo & Lavallee, 2022; Jones et al., 2011). SOM also acts as a storage and a source of exchangeable cations (Ca, Mg, K), thereby contributing to CEC. Soils with relatively low SOM content and those amended with external N sources acquire most of their total inorganic N from native SOM (Masunga et al., 2016). Although external N fertilization may bypass SOM-N supply, a meta-analysis of 43 15N laboratory and field studies showed that application of either inorganic fertilizers or organic soil amendments increases plant uptake of native soil N (Liu et al., 2017). This increase in N uptake was suggested to be a result of plant-mediated mechanisms (e.g., increased root growth and rhizosphere priming effect) rather than soil microbe-mediated mechanisms (Huo et al., 2017; Clarholm et al., 2015; Liu et al., 2017).

Soil organic matter is essential to factors that contribute to soil health, including tillage, resistance to water and wind erosion, water movement in the soil, nutrient retention, microbial diversity, and microbial abundance (Sullivan et al., 2019.). Given that soils in agricultural and unmanaged (i.e., natural) systems derive their N primarily from SOM, it is imperative to consider SOM when evaluating soil health and, more so, to understand SOM dynamics and controls at a larger scale. Future experiments to better link SOM properties to nutrient provision under various conditions can inform the targeted management of SOM stocks (NRCS, 2019).

The relationship between SOM and soil functions is complex. Hence, SOM is considered the most essential baseline soil health indicator. Soil organic matter can be estimated by measuring soil organic carbon (SOC) using the dry combustion method (Nelson & Sommers,

1996) or wet chemical oxidation (i.e., Walkley-Black wet oxidation) (Walkley & Black, 1934). Significant variations in SOM content from the same field location can occur when soil sampling depth is inconsistent or when different laboratory methods are used (Sullivan & Moore, 2019). Therefore, inconsistent sampling methods can produce inaccurate results. Methods for SOM analysis also vary in cost, accuracy, and reproducibility; hence, selecting an approach that ensures consistent and reliable data quality is essential. Sleutel et al. (2007) demonstrated that the Walkley and Black method has good precision for SOC determination. The traditional conversion factor 1.724 used to convert SOC into organic matter percentages was not valid for the investigated soil, highlighting the need for region-specific factors.

1.4: Biological indicators of soil health

Agricultural practices affect soil physicochemical properties and influence the microbial community. Soil biological properties tend to be more reactive to land-use changes and can be good indicators to understand and improve soil health and ecosystem services (He et al., 2021).

Soil microbial biomass carbon (MBC) and nitrogen (MBN) can be used as indicators of short-term changes in soil microbial functions and diversity and to predict the accumulation of organic C or N in soils (Gan et al., 2013). Anderson and Domsch (2006) claimed that MBN houses the largest proportion of biologically active N in the soil. MBN is crucial in soil N cycling, particularly in converting organic N to plant-available forms. When MBC and MBN increase in soil, it signifies a high level of soil microbial activity. However, this could also deplete SOC since microbes need it as an energy source to perform their functions in soil processes such as decomposition (Muhammad et al., 2021).

A decline in SOC may also indicate that a system can no longer be sustainable. Hence, existing management strategies, such as a ridge furrow system, are being implemented to return

more crop residues to the soil, increase SOC stocks, and improve soil fertility in the long term (Gan et al., 2013). The size of MBN pools in agroecosystems can be highly affected by changes in agronomic management practices (Anderson & Domsch, 2006). For example, a meta-analysis of 203 published studies showed that crop rotation and monocropping stimulate the response ratio of MBN to synthetic N fertilizers, with crop rotation reaching the highest level of MBN response (Xing et al., 2022). Incorporating organic manure in the soil is also considered one of the better ways to increase the build-up of large and active microbial biomass. Organic amendments provide readily available carbon sources for microbial consumption (Santos et al., 2012).

Maintaining an active and large pool of soil microbial biomass is important for improving nutrient availability. The size of microbial biomass and C availability is determined by the ratio of carbon and nitrogen (C/N ratio) in organic substrates (Xing et al., 2022). When the carbon input in the soil is increased (e.g., through cover cropping), microorganisms can access more carbon. They can digest and catabolize this carbon for their energy, hence better C accumulation in microbial biomass. Long-term crop residue retention in organic farming increases microbial biomass C and N compared to organic manure application. The quantity and quality of organic inputs positively affect soil microbial biomass (Melero et al., 2008). Crop rotation in organic agriculture promotes diversity in soil microbes, leads to the strong rhizosphere effect by releasing root exudates, and facilitates the effective carbon and nitrogen mineralization from organic matter to microbial biomass. There was a significant difference in MBC and MBN even in transitional organic plots compared to conventional farming plots, but the effect was only in surface soils (Schjonning et al., 2002; Melero et al., 2006). However, the increase in MBC and MBN was significant in long-term organic farming plots (>5 years), even in deeper soil layers.

Although MBC and MBN increase in organic farming, the carbon conversion efficiency viz. MBC: TOC ratio was higher in conventional agriculture, showing a more efficient conversion of carbon from source to microbial biomass than in organic farming (Melero et al., 2008). An increase in microbial biomass improves soil fertility in an environmentally sustainable way, making organic carbon essential for maintaining soil quality (de Araújo & de Melo, 2010).

Understanding the nature and extent of soil EEAs is critical for characterizing transformation processes related to nutrient acquisition and decomposition in agricultural soils. Several extracellular enzymes are biological indicators of soil health and are associated with SOM, microbial activity, and soil physical processes. They are sensitive to changes in soil management practice and tend to transform quickly, which makes them more suitable than other metrics for detecting short-term changes in soil health status (Dick et al., 1996). Soil enzymes are classified based on their nature and catalyzed reaction: amylases, arylsulfatase, β-glucosidases, cellulases, chitinase, dehydrogenases, phosphatases, proteases, and ureases (Dick et al., 2005). The specificity of each enzyme is related to the nature of the biochemical reaction it catalyzes (Wang et al., 2010).

β-glucosidase (BG) is an enzyme that breaks down cellulose and complex sugars into glucose, an important C energy source for soil bacteria. It contributes to the availability of energy sources for microorganisms and is frequently used as an indicator to assess carbon cycling (Knight & Dick, 2004; Martinez & Tabatabai, 1997). In terms of N-cycling, N-acetyl-glucosaminidase (NAG) and leucine aminopeptidase (LAP) are enzymes that aid in making N available. NAG breaks down chitin and releases N compounds, which can be further metabolized by microorganisms and plants (Liao et al., 2022). Similarly, LAP releases amino acids from polypeptides, especially leucine, which plants and microorganisms can use as an N source (Liao

et al., 2022). Phosphatase, on the other hand, is an enzyme involved in the hydrolysis of organic phosphorus compounds, making P available for plant uptake (Knight & Dick, 2004). Phosphatase is also a good indicator of soil fertility. In cases of P deficiency, a signal is produced to increase the production of phosphatase enzymes from plant roots. The increased levels of phosphatase help enhance the solubility and movement of the phosphate molecule, hence curbing P deficiency (Versaw & Harrison, 2002).

It is also essential to understand how various agricultural practices would affect EEAs. Cover crops, for example, have mixed effects on soil EEAs. A corn-soybean field study established in Missouri from 2016 to 2018 found that cover cropping led to significantly greater N-Acetyl-β-D-glucosaminidase (NAG) activity at 0-10cm depth in 2016. However, higher activity was observed at 10-20cm and 20-30cm depths in 2018 (Rankoth et al., 2019). Intensive tillage, such as plowing or harrowing, can disrupt soil structure and microbial communities. A meta-analysis assessed the effect of tillage on soil microbial properties and found that compared to conventional tillage, no-till and reduced tillage practices promote larger microbial communities and greater enzymatic activity (Zuber & Villamil, 2016). Urease activity is significantly higher in organic farming than in conventional farming systems and can be mainly due to the utilization of organic nitrogen from the applied organic inputs (Melero et al., 2008).

1.5: Agricultural practices for building soil health

Poor soil quality from intensive land cultivation is becoming an imminent problem for agricultural systems worldwide. In addition, increasing GHG emissions (e.g., N2O, CH4) and carbon fluxes from industrial agriculture continue to be an alarming global threat. Implementing agricultural practices that promote soil health can offset these issues that continue to raise concerns about climate change and sustainable crop production.

Soil health is an integral part of agriculture, encompassing physical, chemical, and biological properties vital for crop productivity (Enriqueta Arias et al., 2005). The primary goal of intensive agriculture is to optimize crop yields, which is also a core focus of soil health management (Lehmann et al., 2020). The foundation of soil health relies on the understanding that managing nutrient availability alone through reliance on agrochemicals (e.g., fertilizers) will not be enough to support crop growth (Bünemann et al., 2018). There is a growing recognition that some practices employed in intensive agriculture to improve crop yields are detrimental to soil health. For example, extensive tillage operations destroy soil structure, typically increasing soil erosion, nutrient and moisture losses, and soil organic matter degradation (Phogat et al., 2020). Applying chemical fertilizers is often insufficient to recover soil OM lost from tillage or retain adequate OM levels. Goyal et al. (1999) found that inorganic fertilizers increased soil OM and mineralizable C and N. However, applying animal and green manure with inorganic fertilizers resulted in more significant OM increases.

Managing farming systems for soil health will expand management options from reliance on inorganic fertilizers to using organic amendments, crop residue return, reduced tillage, and crop diversity (Karlen et al., 2019). Choosing appropriate management decisions prioritizing soil health is crucial to maintaining a managed ecosystem's functions (e.g., farms). Soil health management can be possible through applying best agricultural practices (BAPs) that are known to offset the detrimental effects of intensive agriculture. BAPs include the following: (1) application of a sustainable soil amendment (e.g., biochar); (2) proper nutrient management; (3) cover/inter-cropping; and (4) no-till/conservation tillage (Bai et al., 2019a; Haruna & Nkongolo, 2015).

No-till or conservation tillage reduces soil disturbance by eliminating or reducing the rate and intensity of soil cultivation, limiting environmental damage to a minimum (Busari et al., 2015). It also enhances SOC storage, soil biological structure, and aggregate stability (from improved soil texture). It can be more effective when integrated with cover cropping (Nakamoto & Wakahara, 2004; Veloso et al., 2018).

Cover cropping and crop rotation are also beneficial in maintaining cropping systems' soil health through enhanced SOC storage and reduced greenhouse gas (GHG) emissions. For example, cover crops can reduce nitrous oxide (N2O) emissions from excess N fertilizers, contributing to 67% of the global warming potential. The carbon-nitrogen ratio (C: N) in cover crop biomass strongly dictates the reduction efficiency of nitrous oxide (N2O) (Han et al., 2017; Sanz-Cobena et al., 2014). Abdalla et al. (2014) claimed that SOC sequestration could more than offset increased N2O flux with cover crops, resulting in a negative net emission balance. Field studies, data synthesis, and meta-analyses have also documented the C sequestration benefits of cover crops (Olson et al., 2014; Poeplau & Don, 2015). Under no-till, cover crops contributed to the SOC balance of corn-based cropping systems, with a 29%-49% reduction in CO2 emission with cover crops compared to applying compost or manure (Shrestha et al., 2013). In addition, Li et al. (2010) reported that planting soybean after harvesting corn improves yield significantly while enhancing SOC sequestration. A study in 2011 demonstrated that having a corn-soybean crop rotation with cereal cover crops decreases bulk density by 3% (Kichamu-Wachira et al., 2021). Given these benefits from cover crops and crop rotation, it is essential to redesign management strategies to maximize the global warming mitigation benefit from SOC storage under conservation farming (Goglio et al., 2014). Implementing climate-smart management

practices is needed to improve soil nutrient retention and uptake and reduce emissions (Kichamu-Wachira et al., 2021).

1.6: Biochar as a sustainable soil amendment and its integration with other BAPs

Biochar is gaining research attention as an ecologically sound and sustainable soil amendment (Saha & Bauddh, 2020). It is a charcoal-like material produced from plant or animal biomass that was pyrolyzed (or thermally degraded) in an oxygen-depleted environment (Hou et al., 2020). Biochar is highly reactive because of its high porosity and surface area, making it a candidate soil amendment for sustaining soil health, nutrient use efficiency, and reducing GHG emissions (Jiang et al., 2021). This soil amendment has shown potential for enhancing crop yield, SOC sequestration, methane (CH4) uptake, and reducing nitrous oxide N2O emissions (Lehmann et al., 2006; Oladele et al., 2019; Xia et al., 2020). Adding biochar into cropping systems can bring them closer to net-zero emissions. In the next two decades, the predicted amount of CO2 equivalents sequestered by biochar applied to soils might counterbalance the increase in GHG emissions due to soil warming (Bamminger et al., 2018). Furthermore, biochar application contributes to N retention against leaching and thus can improve N use efficiency (El-Naggar et al., 2019).

A meta-analysis comparing three commonly used "climate-smart" agricultural practices (CSAs) found that biochar application led to the most significant improvements in crop yield, along with an increase in soil organic carbon (SOC) and a reduction in greenhouse gas (GHG) emissions. No-till (NT) and cover cropping ranked second and third most effective practices, respectively (Bai et al., 2019a). While these practices have individually demonstrated their benefits for cropping systems, there has yet to be a comprehensive examination of their combined effects on agronomic productivity and soil health. Therefore, it is imperative to

conduct field experiments to evaluate how the integrated use of these best agricultural practices (BAPs) – biochar, cover crop, and no-till – will influence soil health and plant growth.

Similarly, there are logistical questions regarding integrating biochar into soil management strategies, as highlighted by Bass et al. (2016). Biochar is typically incorporated in the soil in cropping systems under conventional tillage or before establishing a no-till system. On the other hand, biochar can only be applied on the surface or incorporated at a very shallow depth in an existing no-till system. The surface application of biochar exposes it to significant losses by wind or run-off, diminishing the benefit from a currently high-cost soil amendment (Cox et al., 2021). Hence, it is also essential to understand the impact of varying biochar application rates and incorporation depths as they also influence soil properties related to soil health.

Current studies on biochar have been promising, but the mechanisms by which it interacts with the environment to improve soil health still need exploration, especially in the long term (Brockamp & Weyers, 2021). To evolve the specific knowledge of biochar decomposition rates, priming effects, and associations with mineral surfaces, field studies are necessary (Wang et al., 2016). While there is a wealth of research on the individual impact of biochar, cover cropping, and N-management, data on their integrated effects (especially for biochar) are limited. Specifically, limited information addresses whether integrating multiple BAPs may improve soil health and crop productivity over traditional tillage systems.

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CHAPTER TWO: COMBINED EFFECTS OF BIOCHAR, COVER CROPPING, AND NITROGEN MANAGEMENT ON SOIL HEALTH INDICATORS

2.1: Abstract

Employing best agricultural practices (BAPs) can offset rising greenhouse gas emissions and declining soil quality. However, there is still a need for field-scale studies on biochar's potential benefits when integrated with BAPs for improving soil health. This study investigated the feasibility of integrating biochar with three BAPs (cover cropping, nitrogen management, and no-till) and their effects on soil health metrics such as extracellular enzyme activity (EEA), soil organic carbon (SOC), microbial biomass, and short-term carbon mineralization. Fluorometric enzyme assays were used to measure soil EEAs. SOC was measured using a combustion analyzer, while microbial biomass was quantified via phospholipid fatty acid (PLFA) analysis. Additionally, a seven-day laboratory incubation was performed to determine short-term carbon respiration. Results showed that the integrated BAPs increased C- and N-acquisition enzymes, whereas SOC in all treatments was significantly higher in 2023 than in 2022. Notably, when incorporated into the BAP system, biochar emerges as a significant contributor to improved EEAs, surpassing the impact of cover cropping and nitrogen management. The study emphasizes that adopting BAPs, particularly when coupled with biochar, can enhance soil health and productivity while mitigating carbon emissions.

2.2: Introduction

During the Green Revolution, there was a significant shift towards adopting inorganic fertilizers, pesticides, conventional tillage, and crop breeding. One of the key milestones in modern agriculture was the discovery of industrial nitrogen fixation in the 1900s, which led to a six-fold increase in U.S. corn yield within seven decades of nitrogen fertilizer use (Louchheim, 2014; Leigh, 2004). Chemical fertilization has become an essential farm management component, resulting in widespread intensification and population growth (Smil, 2004; Trivedi et al., 2016). Despite improving crop yield and addressing food security, the negative environmental impacts of conventional agriculture, such as declining soil quality, are becoming more evident (Crews et al., 2018). Thus, there is a growing need to focus on understanding the impact of agricultural intensification on food sustainability and soil health.

Managing agriculture for soil health is crucial to maintaining short- and long-term food production and helping mitigate climate change (He et al., 2021). Healthy soils help sustain and increase crop yields and serve as a habitat for microbial populations that provide ecosystem services (e.g., carbon sequestration) (Doran & Zeiss, 2000). For soils to be considered 'healthy,' they should have the capacity to support a diversity of soil organisms that aid in disease, weed, and pest control, form associations with plant roots, produce enzymes for nutrient cycling, improve soil physical properties (e.g., soil structure, aggregate stability) and ultimately increase crop productivity (Food and Agriculture Organization [FAO], 2008).

Adopting management practices for soil health needs to be encouraged to sustain cropping system productivity. In 2012, national adoption rates for soil health practices such as cover cropping and conservation tillage (e.g., mulch, ridge-till) were 2.6% and 27.5%, respectively, whereas 37% of the total acreage for tillage practices were no-till (Census of

Agriculture Historical Archive, 2012). Based on a 2021 survey conducted by NASS and NRCS, approximately 86.4% and 61.3% of the survey respondents use tillage and cover crops, respectively. Although the utilization rates of these practices vary across different regions and crops, the use of no-till or strip-till and cover crops is more common in the southern and eastern regions of the U.S. (National Statistics Service [USDA-NSS] & Economic Research Service [USDA-ERS], 2014). Additionally, 40% to 45% of soybean acres utilized no-till/strip-till from 2006 to 2012, while corn, wheat, and cotton acreage had lower utilization rates. Total cropland planted with cover crops only increased from 2.6% (2012) to 3.9% in 2017 (Soil Health Institute, 2018).

More recently, biochar has been widely investigated for its potential to offset the detrimental effects of intensive agriculture while supporting soil health. Biochar can sequester carbon (C), reduce nutrient leaching, and improve crop yields because of its high surface area and reactive nature (Lehmann et al., 2006; Zhang et al., 2017; Hou et al., 2020). It can also synergistically improve agronomic output and soil organic matter, making it a candidate amendment for soil health improvement (Ding et al., 2017; Hou et al., 2020). Feedstocks, such as plant/animal residues and industrial wastes, are pyrolyzed in an oxygen-limited environment to produce biochar. Using 'waste' biomass to produce biochar is the primary reason biochar is described as a sustainable soil amendment (Lehmann, 2007).

Biochar is also considered a "climate-smart" agricultural practice (CSA) because it offers agronomic and environmental benefits. However, much remains unknown about how biochar might be integrated with other best agricultural practices (BAPs) such as no-till, cover cropping, and nitrogen (N) management. It is essential to investigate the impact of biochar and its combination with BAPs on soil health and agronomic productivity. This will help determine if

integrated BAPs can be suitable for improving soil health in cropping systems. Thus, it is necessary to evaluate its potential benefits or drawbacks by quantifying its effects on soil health.

The overall goal of this study was to examine the effects of biochar integrated with other BAPs (cover crop, N-management, crop rotation) under a no-till system on key indicators of soil health. Specifically, the study investigated how integrated BAPs and their interactions affect soil health, which provides vital ecosystem services. The following soil health indicators were measured over two growing seasons: extracellular enzyme activity (EEA), microbial biomass accumulation, soil organic carbon (SOC), and short-term carbon respiration.

2.3: Materials and Methods

Site description and experimental design

This study was conducted at the Michigan State University Agronomy Farm in East Lansing, Michigan (42°42′52.41″ N, 84°27′42.40″ W) between May 2021 and December 2023. An existing experimental study site, established in the Fall of 2020, was leveraged to evaluate the impact of integrated BAPs on key soil health indicators over two growing seasons. The soil series at the study site is a Riddles (fine-loamy, mixed, active, mesic Typic Hapludalfs)-Hillsdale (coarse-loamy, mixed, active, mesic Typic Hapludalfs) complex. Before establishing the experiment, 20 soil samples were collected from the entire field, homogenized, and tested for soil properties. The initial soil properties showed 1.6% organic matter, with phosphorus (P), potassium (K), magnesium (Mg), and calcium (Ca) concentrations of 67ppm, 187ppm, 172ppm, and 754ppm, respectively. The soil has a pH of 6.0, a cation exchange capacity (CEC) of 6.9 meq/100g of soil, and a sandy clay loam soil texture (60% sand, 27% silt, and 13% clay) (Silva-Pumarada et al., 20203).

The integrated effects of three BAPs (biochar application, cover cropping, and N-

management) on the soil health of a corn-soybean system were examined. The experiment was laid out in a randomized complete block design consisting of nine treatments, including (1) two main treatment controls, with one control having all three BAPs while the other is a traditional system without BAPs and (2) six combinations of biochar, cover cropping, and N management that were each replicated four times. Treatment descriptions are provided in **Table 1.1**.

Table 1.1. Experimental treatment factors involved in the omission trial. BAPs – Best agricultural practices: biochar + cover cropping + nitrogen management. [1]corn-rye-soybean-wheat-double crop soybean; [2]corn-soybean-soybean, [3]corn-soybean + conventional tillage

Cropping system	Treatment	Description
BAPs system	BAP[1]	All BAPs: cover crop, biochar, N management
	BAPC[1]	BAP without cover crop
	BAPB[1]	BAP without biochar
	BAPN[1]	BAP without N management
Traditional	TRD[2]	Traditional system
	TRDC[2]	Traditional system with cover crop
	TRDB[2]	Traditional system with biochar
	TRDN[2]	Traditional system with N management
	TRDT[3]	Tilled system (conventional tillage)

Best agricultural practices

This study evaluated a set of BAPs (biochar, cover crop, N management) that were individually proven to benefit cropping systems (Bai et al., 2019). On May 15, 2021, corn was planted at a seeding rate of 34,000 seeds acre^{-1}, while urea fertilizer was applied at 150 lb N acre^{-1} by hand approximately two weeks after planting. Corn was harvested on October 12, 2021. During the next growing season (2022), soybeans were planted at a rate of 130,000 seeds acre^{-1} and harvested on October 13, 2022. The timeline for production practices is shown in **Fig. 1.1**.

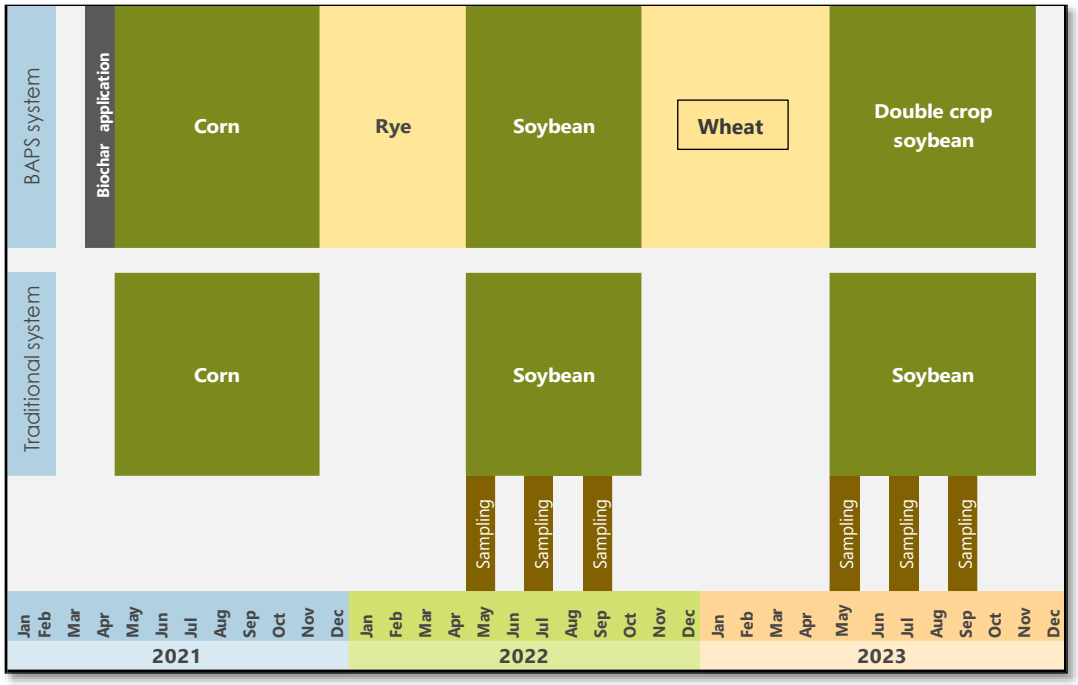

Figure 1.1. Calendar of cropping systems to evaluate the effect of integrated best agricultural practices (BAPs) on soil health metrics.

Biochar. A softwood pine-derived biochar that was pyrolyzed at 425 ºC was applied by hand in the Fall of 2020 at a rate of 10 Mg ha^{-1} while the entire field was tilled at a depth of four inches using a field cultivator. Each experimental block received a one-time biochar application except for the non-biochar control plots. A one-time biochar application can benefit several growing seasons due to its resistance to decomposition and long residence time. As it ages, the physicochemical changes in biochar may increase soil water and nutrient retention (Mia et al., 2017). The experimental plots were maintained under no-till after biochar application except for treatments involving the traditional system (under conventional tillage). The properties of biochar used in this study are described in **Table 1.2**.

Cover crop. Rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) were planted in Fall 2021 and Fall 2022, respectively, to provide fall and winter soil cover in between growing seasons (**Fig. 1**). Wheat was harvested for grain in late June/early July, and soybean (*Glycine max* L.) was planted immediately to promote continuous soil cover; this is referred to as "double crop soybean."

Table 1.2. Properties of biochar used in this study (Control Laboratories, Watsonville, CA).

Property	**Value**	**Rate (lbs acre^{-1})**	**Method**
Organic C	57.10%	5,139	Dry combustion
Total N	0.45%	40.5	Dry combustion
Total P	1853ppm	16.7	Rajkovich et al., 2012
Total K	6089ppm	54.8	U.S. EPA, 2016
pH	9.39	----	U.S. EPA, 2016

Soil sample collection and processing

Soil sampling was carried out three times each growing season: before planting soybean, at the flower development stage (R1), and before harvest (as shown in **Fig. 1.2.1**). Using a soil auger, soil samples were taken at depths of 0-10cm and 10-30cm following a zigzag pattern. Twenty areas per plot were sampled from each soil depth, homogenized to create a composite sample, and transferred to a plastic zip lock bag processing before analysis. Soil samples were sieved through a 2mm sieve to discard rocks, roots, etc, and were analyzed for soil organic carbon and short-term carbon mineralization (STCM). On the other hand, subsamples were taken from the composite samples and immediately frozen at -20˚C to preserve enzyme and microbial activity before performing enzyme assays and phospholipid fatty acid (PLFA) analysis.

Laboratory analysis of soil health metrics

A variety of soil health indicators were examined and listed in **Table 1.3.** The soil samples collected prior to planting and before harvesting were analyzed for STCM, while mid-season samples were mainly analyzed for phospholipid fatty acid (PLFA), soil organic carbon (SOC), and total nitrogen (N).

Table 1.3. Soil response variables assessed in the study to evaluate the effects of integrated BAPs on soil health. Metrics are based on the USDA-defined soil health indicators. [1]measured only in mid-season samples for each growing season, [2]measured at thrice per growing season (pre-planting, mid-season, before harvest).

Soil process and associated health indicator/s*	**Method of analysis**
Soil organic carbon (SOC)[1] Total N, Total C, C:N ratio	Combustion method (Nelson & Sommers, 1996)
Short-term carbon mineralization[2]	Carbon dioxide (CO_2) respired, 7-day incubation
Enzymes associated with C, N, and P cycles[1] β-glucosidase (BG), N- acetyl-glucosamindase (NAG), phosphatase (PHOS), Leucine aminopeptidase (LAP), L-glutamic enzyme (GLU)	Fluorometric Enzyme assay (German et al., 2011)
Microbial biomass and community structure[1] Total PLFA, bacteria, fungi, functional groups of fungi, actinomycetes	Phospholipid Fatty Acid analysis

SOM cycling and C sequestration. Approximately 30g of mid-season samples were sieved and oven-dried at 60℃ until no further mass loss occurred. The samples were then pulverized into fine powder using a ball mill and stored in polyethylene vials. Before total C and N analysis, the presence of carbonates was tested on a separate sample by adding 2-3 drops of 1N hydrochloric acid (HCl) on pulverized soil. No bubbling indicates the absence of carbonates; hence, pre-treatment of soils with acid fumigation was unnecessary. Samples are then weighed in a microbalance and packed into tin capsules for total C and total N analysis using a combustion analyzer.

Enzyme assays. Mid-season samples were analyzed to assess how microbial communities and soil enzymes respond to the integrated BAPs. This study examined the potential activities of five hydrolytic enzymes involved in C, N, and P cycling (**Table 1.4**). Generally, extracellular enzyme activity (EEA) was determined by exposing soil samples to the corresponding BG, NAG, PHOS, LAP, and GLU substrates and recording the fluorescence from substrate hydrolysis using a microplate reader. Enzyme assays were conducted in a black polystyrene 96-well microplate, with three analytical replicates to allow multiple sample analyses. The following

procedure was performed: 1g of frozen soil sample was mixed thoroughly with 125 ml of ultrapure water using a hand-held blender to create a soil slurry. For analytical replicates, 200µL of soil slurry from each sample was pipetted to three consecutive rows of a 96-well black microplate. This was followed by pipetting 50µL of methylumbelliferone (MUB) or methylcoumarin (MC) in columns 1,4,7, 10, and 50 µL of substrate to rows 2,3,5,6,8,9,11, and 12. MUB and MC standard plates and a soil + buffer plate were also prepared. The function of each enzyme evaluated in this study is summarized in **Table 1.4**.

Table 1.4. Soil extracellular enzymes and their associated functions.

Enzyme	Substrate	Catalytic activities
ß-Glucosidase (BG)	4-Methylumbelliferyl β-D-glucopyranoside	Release of glucose from glucoside, cellobiose that is present in plant debris
N-acetyl-β-glucosaminidase (NAG)	4-Methylumbelliferyl N-acetyl-β-D-glucosaminide	Nitrogen degrading enzyme N; hydrolysis of chitin oligomers
Leucine aminopeptidase (LAP)	L-Leucine 7-amido-4-methylcoumarin hydrochloride	Release of amino acids from polypeptides, especially leucine
Phosphatase (PHOS)	4-Methylumbelliferyl phosphate	Phosphate enzyme; releases phosphate ions from the phosphate group
L-glutamic acid enzyme (GLU)	L-glutamic acid γ-(7-amido-4-methylcoumarin)	Catalyzes the hydrolysis of glutamine to produce glutamate and ammonia.

Short-term C mineralization. Composite soil samples were passed through a 2mm sieve to discard roots, rocks, and other debris. Three subsamples were collected from each composite and were incubated in the laboratory for seven days to determine short-term C respiration rates. Precisely, 20g of each subsample was carefully weighed and placed into specimen cups; each sample's water-filled pore space (WFPS) was adjusted to 60%. The cups are placed in labeled incubation jars and flushed with ambient air for 30 minutes in front of an oscillating fan. Jars were capped and incubated in the dark at ambient temperature for seven days; the capping time

was noted for calculation. To measure soil C respiration rates, gas samples from the headspace of each jar were taken at three time points: one day, four days, and seven days after the start of incubation. About 1cc of the gas sample is drawn out from each headspace of the incubation jars and injected into an infrared gas analyzer to measure the amount of CO_2 concentration.

Phospholipid Fatty Acid. The analysis of phospholipid fatty acids (PLFAs) in soil is a commonly used method to assess microbial community composition and diversity. PLFAs are only present in living organisms; hence, they quickly break down when a cell dies. It can be used as a biomarker because each organism has a unique chemical composition of PLFA. Subsamples from fresh mid-season soil samples in 2022 and 2023 were immediately stored at -20℃ to maintain viability before microbial analysis. The PLFAs are extracted from the soil using a solvent mixture, typically a combination of chloroform and methanol. This extraction process helps separate the PLFAs from the soil matrix. Samples were shipped out to a private lab for analysis.

Statistical analysis

By treatment design, this experiment has one factor (farming practice) with nine levels– BAP, BAPB, BAPC, BAPN, TRD, TRDC, TRDB, TRDN, and TRDT (**Table 1.1**). Treatments were laid out in a randomized complete block design (RCBD) and analyzed as a one-way RCBD where the main factor was the type of farming practice (BAPs or traditional practices), and replication was used as a blocking factor. The experiment is comprised of 4 replicates (blocks) where nine treatments were randomly assigned in each block, totaling 36 experimental units. Statistical analysis was performed using Rstudio (2023.06.1 Build 524) to investigate differences in soil health indicators among cropping systems. Farming practices were treated as a fixed effect, while block (replication) was treated as a random effect. EEAs, SOC total N, and PLFAs

were analyzed separately by year, and mean separations were conducted using Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$. Short-term carbon respiration rates were analyzed as a one-way RCBD with time of gas sampling as repeated measures. Means were separated using Fisher's Protected LSD at $\alpha = 0.05$, where cropping system (BAPs, traditional) and time of gas sampling were considered as fixed effects and with replication (block) as a random effect. Assumptions of normality were checked before proceeding with the analysis, and heterogeneous variance was corrected with the appropriate variance-covariance structure.

2.4: Results

Extracellular enzyme activities

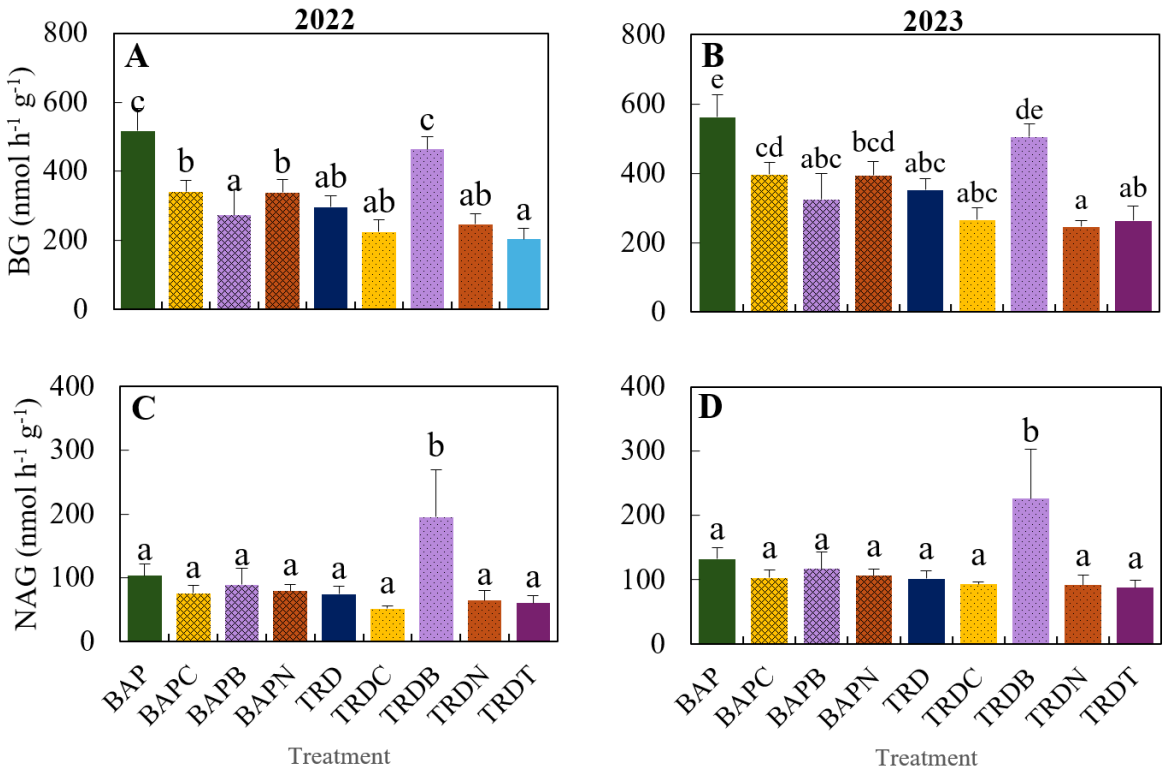

Figure 1.2. Mean soil extracellular enzyme activity of (A-B) β-glucosidase and (C-D) N-acetyl-β-D glucosaminidase under best agricultural practices (BAPs) and traditional practices across two growing seasons of soybean. Error bars represent standard error. For treatments with common letters, no significant differences were observed (Fisher's protected LSD, α=0.05). Y-axis titles are identical between panels A and B, and between C and D. Treatment abbreviations follow Table 1.1.

Activity rates of soil C, N, and P-acquisition enzymes in plots under BAP and traditional practices were measured across two growing seasons (**Fig. 1.2**). These practices had a significant influence on BG ($p=0.003$) and NAG (ANOVA, $p=0.016$) activities. In 2022, BG was 75% ($p = 0.009$) and 154% ($p \leq 0.001$) higher in BAP than TRD and TRDT, respectively. Implementing BAP led to the highest BG activity across all treatments (**Fig. 1.2a-b).** However, the BG activity in plots where biochar was incorporated with traditional practice (TRDB) did not significantly differ from BAP ($p = 0.3782$). On average, BG activities ranged from 203.12 nmol h^{-1} g^{-1} to 516.59 nmol h^{-1} g^{-1} in 2022 and 245.91 nmol h^{-1} g^{-1} to 561.63 nmol h^{-1} g^{-1} in 2023. On the other hand, in 2022, the BAP and traditional practices did not significantly increase NAG activity, and BAP had 41.5% lower NAG than TRDB ($p = 0.0105$) (**Fig. 1.2b-c**). This similar pattern was also observed in 2023, and TRDB had the highest NAG in both years of the study (**Fig. 1.2b, 1.2d**). The average NAG activity in 2022 ranged from 51.57 nmol h^{-1} g^{-1} to 195.48 nmol h^{-1} g^{-1} and 87.9 nmol h^{-1} g^{-1} to 226.22 nmol h^{-1} g^{-1} in 2023.

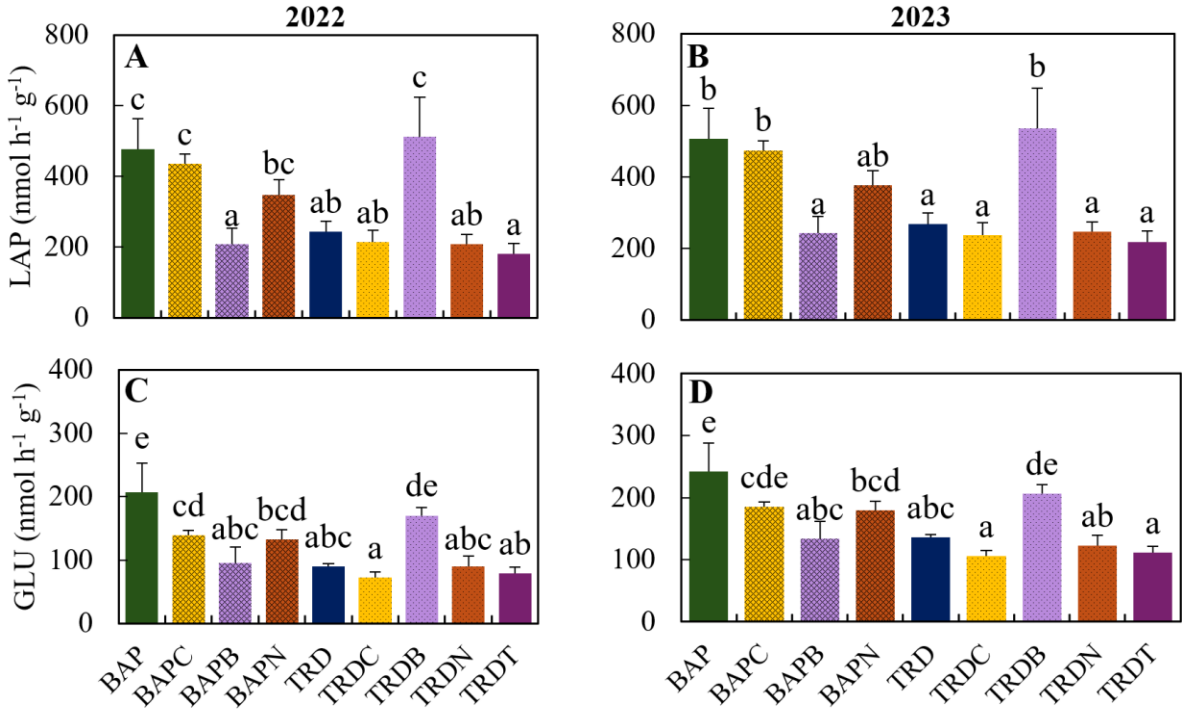

Figure 1.3. Mean soil extracellular enzyme activity of (A-B) Leucine aminopeptidase and (C-D) L-glutamic acid enzyme under best agricultural practices (BAPs) and traditional practices across two growing seasons of soybean and wheat. Error bars represent standard error. For treatments with common letters, no significant differences were observed (Fisher's protected LSD, α=0.05). Y-axis titles are identical between panels A and B, and between C and D. Treatment abbreviations follow Table 1.1.

In terms of LAP activity, on average, measured values ranged from 180.47 nmol h^{-1} g^{-1} to 511.86 nmol h^{-1} g^{-1} in 2022 and 218.73 nmol h^{-1} g^{-1} to 505.89 nmol h^{-1} g^{-1} in 2023. The farming practices had a significant influence on LAP in 2022 ($p = 0.0006$) and 2023 ($p = 0.0007$) (**Fig. 1.3a-b**). The LAP activity of plots under BAP in 2022 was 95.83% ($p = 0.0072$), 123.47% ($p = 0.0029$), 129.26% ($p = 0.0024$), and 164.66% ($p = 0.001$) higher than TRD, TRDC, TRDN, and TRDT, respectively (**Fig. 1.3a**). However, when biochar was absent in the BAP system (BAPB), there was a 47.85% ($p = 0.0084$) reduction in LAP activity relative to BAP. In contrast, biochar application in the traditional system (TRDB) increased LAP activity by 146.68% ($p = 0.0008$). Similarly, the BAP and traditional practices had a significant effect on GLU activities in 2022 (p

= 0.0004) and 2023 ($p \leq 0.0001$) (**Fig. 1.3c-d**). Results also showed that the BAP system had the highest LAP and GLU activity in both years but did not vary significantly from TRDB.

1.4.2 Soil organic carbon, total nitrogen, and C/N ratio

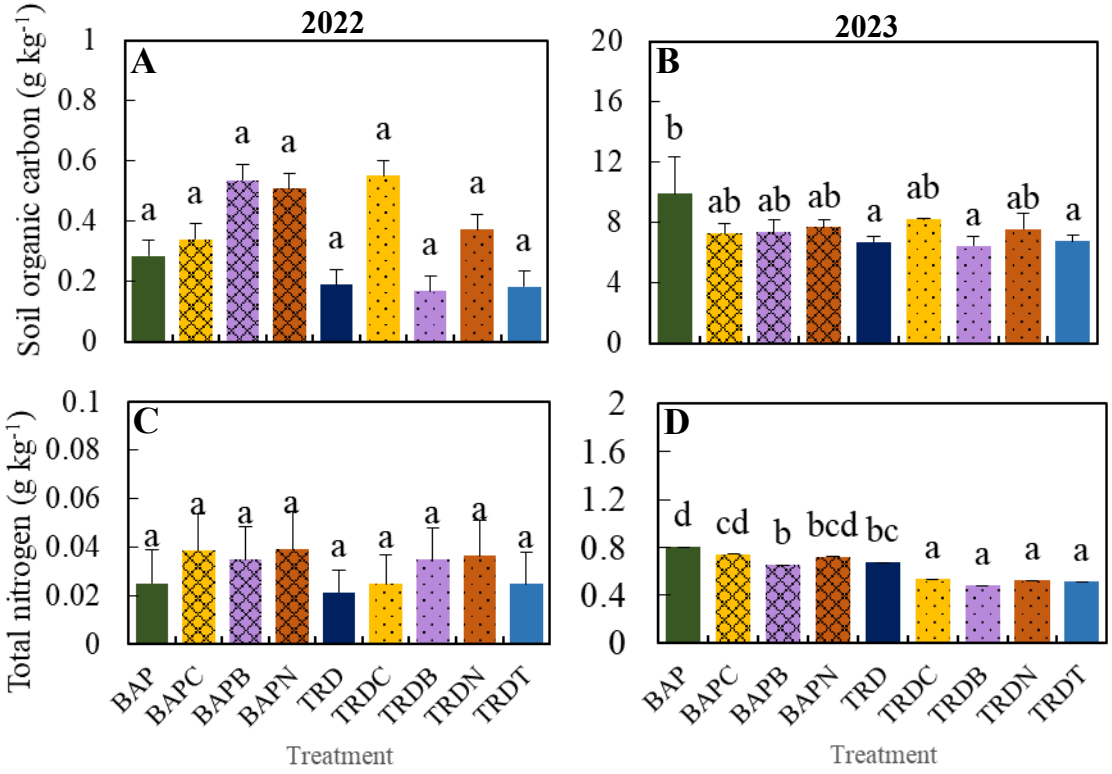

Figure 1.4. Mean (A-B) soil organic carbon and (C-D) total nitrogen concentration as affected by best agricultural practices (BAPs) and traditional practices across two growing seasons of soybean and wheat. Error bars represent standard error. For treatments with common letters, no significant differences were observed (Fisher's protected LSD, α=0.05). Y-axis titles are identical between panels A and B, and between C and D. Treatment abbreviations follow Table 1.1.

Based on the statistical analysis, BAP and traditional practices did not significantly influence soil organic carbon (SOC) in 2022 and 2023 (**Fig. 1.4a, 1.4c**; $p_1 = 0.3883$, $p_2 = 0.4043$). No significant differences in SOC were observed in 2022, while values ranged from 0.022 g kg^{-1} (TRD) to 0.039 g kg^{-1} (BAPN). In 2023, BAP increased SOC by 47.58% ($p = 0.0322$), 52.69% (0.0227), and 47.97% ($p = 0.0347$) compared to TRD, TRDB, and TRDT,

respectively (**Fig. 1.4b**). The SOC in BAP systems where biochar, cover crop, or N-management is absent (TRDB, TRDC, TRDN) did not vary significantly from the BAP system containing all three practices (BAP). In 2023, SOC ranged from 6.5 g kg^{-1} (TRDB) to 9.93 g kg^{-1} (BAP) on average.

No significant differences for total nitrogen were observed in 2022, but the practices significantly affected this soil health indicator in 2023 ($p < 0.0001$) (**Fig. 1.4c-d**). Plots that were maintained under the BAP system (BAP, BAPC, BAPB, BAPN) had higher total N than those under the traditional system (TRDC, TRDB, TRDN, TRDT) (**Fig, 1.4d**). Removing cover crop or N-management in the BAP system did not significantly change SOC and total N in 2023, but removing biochar reduced total N (**Fig. 1.4d**).

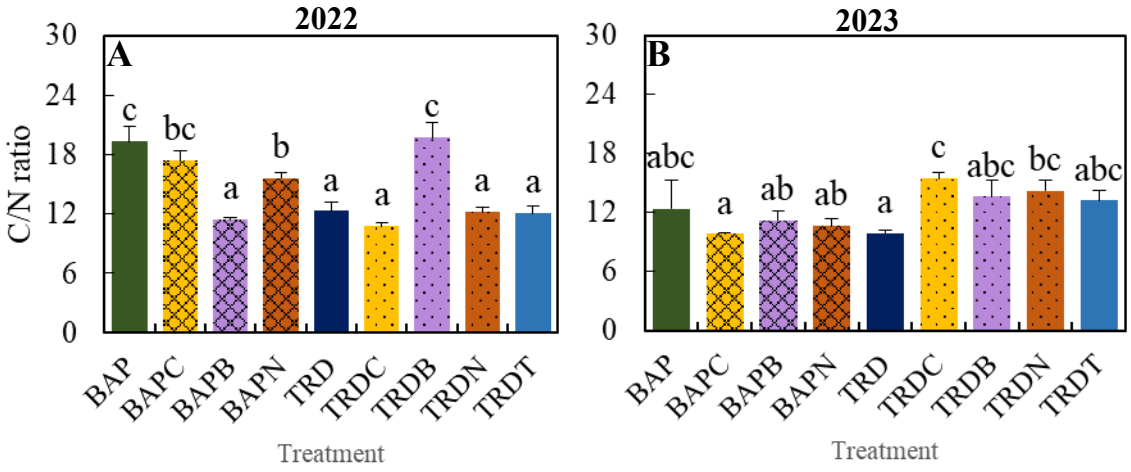

Figure 1.5. Mean soil carbon to nitrogen ratio in the (A) first and (B) second year as affected by best agricultural practices (BAPs) and traditional practices across two growing seasons of soybean and wheat. Error bars represent standard error. For treatments with common letters, no significant differences were observed (Fisher's protected LSD, α=0.05). Y-axis titles are identical between panels A and B. Treatment abbreviations follow Table 1.1.

Soil carbon and nitrogen ratio (C/N ratio) was significantly affected in both years (ANOVA, $p_1 < 0.0001$; $p_2 = 0.0492$). Results in 2022 showed that TRDB had the highest C/N ratio, which did not differ significantly from BAP ($p = 0.7403$) and BAPC ($p = 0.0683$) (**Fig.**

1.5a). Compared to BAP, removing biochar from the BAP system (BAPB) reduced the C/N ratio by 41.07% ($p < 0.0001$), whereas adding it in a traditional system (TRDB) increased the C/N ratio by 73.19% ($p < 0.001$). In 2023, TRDC increased the C/N ratio by 56.26% ($p = 0.0058$) compared to TRD; however, it did not differ significantly from the other traditional treatments (TRDB, TRDN, TRDT) and BAP ($p = 0.1035$) (**Fig. 1.5b**). The contribution of BAP factors to the C/N ratio is in the order of biochar > N management > cover cropping, a similar pattern observed in results for extracellular enzyme activities.

Soil microbial community composition

The total PLFA in soil was not significantly affected by the type of farming practice across the two years of the study ($p_1 = 0.16$, $p_2 = 0.543$). However, treatment differences for PLFA groups measured in this study were observed (**Fig. S1.2**). In 2022, it was observed that N-management in a traditional system (TRDN) significantly reduced total PLFA compared to BAP by 39.96% ($p = 0.021$), whereas eliminating it in the BAP system (BAPN) increased total PLFA by 42.82% ($p = 0.0104$) (**Fig. S1.2a**). In 2023, total PLFA among treatments was not significantly different; however, total PLFA in all treatments was higher in 2023 than in 2022 (**Fig. S1.2b**). A similar pattern was observed in bacterial (Fig. S1.3c-d) and fungal (Fig. S1.3e-f) PLFAs, except for TRDN, which had the highest fungal PLFA. Overall, these results indicate a neutral response of the microbial community to BAPs.

Short-term soil carbon respiration

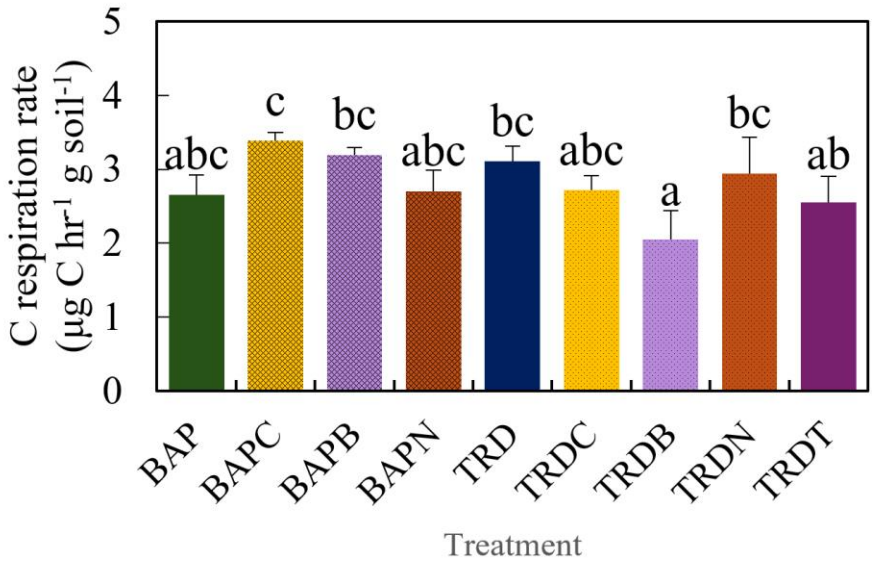

Figure 1.6. After seven days of laboratory incubation, the mean soil carbon respiration rate of late-season samples in 2022 was affected by best agricultural practices (BAPs) and traditional practices across two soybean and wheat growing seasons. Error bars represent standard error. For treatments with common letters, no significant differences were observed (Fisher's protected LSD, α=0.05). Treatment abbreviations follow Table 1.1.

Like PLFA, farming practices did not significantly affect soil C respiration rate after seven days of incubating pre-planting, mid-season, and late-season samples (**Fig. S1.3a-b**). No significant differences among treatments were detected in pre-planting and mid-season soil samples; however, soil C respiration responded differently during the late season (**Fig. 1.6**). The highest rate was observed in the BAP system without cover crop (BAPC). However, it was not significantly different than the other BAP treatments (BAP, BAPC, BAPN) and TRDN. Adding nitrogen or employing conventional tillage increases soil C respiration, while biochar application significantly reduces it relative to TRDN, showing that biochar can reduce the CO_2 release caused by N fertilization.

2.5: Discussion

Extracellular enzyme activities

Here, we found that the farming practices employed in this study significantly influenced extracellular enzyme activities (EEAs), particularly for BG, NAG, LAP, and GLU. The BAP system containing biochar, cover cropping, and N-management had the highest BG activity, which was significantly higher than treatments under a traditional system (TRD) and a traditional system with cover crop (TRDC), N-management (TRDN) and conventional tillage (TRDT). This shows that the combined effect of biochar application, cover cropping, and N-management has a greater benefit on BG than their individual effects. Soils that have been subjected to climate-smart practices such as organic amendment application, crop rotation, and cover cropping have been shown to have elevated enzyme activities that enhance nutrient cycling (Du et al., 2014). Leaving the crop residue on the field and no-till also improved enzyme activities, specifically for BG, PHOS, and dehydrogenase. At the same time, SOC plays a vital role in regulating EEAs (Jat et al., 2021).

Despite the farming practices significantly influencing NAG activity, we did not see significant differences in NAG between BAP and traditional treatments. This could be because of the significant variation among enzymes in the rhizosphere and the bulk soil. Microbial activities are generally higher in the rhizosphere because of C deposition through roots. Therefore, we might see more variations in NAG activity among treatments if we collect samples from the rhizosphere (Jat et al., 2021). On the other hand, we saw the greatest increase in NAG when biochar was incorporated into the traditional system. Integrated BAPs were also found to be more beneficial for LAP and GLU activities than traditional practices, while phosphatase

activity did not respond significantly to the BAP treatments. This is congruent with the findings of Zhang et al. (2019), who recorded non-significant changes in PHOS after biochar application.

Results also showed that biochar had the greatest contribution to EEAs compared to cover cropping and N-management. The lowest EEA was found when biochar was absent in the BAP system (BAPB). On the other hand, adding biochar in a traditional system (TRDB) led to the greatest increase in NAG and LAP activities. In contrast, its BG, NAG, and LAP activity did not vary significantly from activity rates found in BAP treatments. This is congruent with the results shown by Rankoth et al. (2019), where BG activity in cover crop treatment did not differ from treatments without cover crop. However, field-scale studies can demonstrate high spatial and temporal variability in soil EEAs in response to cover crop treatments. It has been reported that biochar application can have contrasting impacts on the activities of soil C mineralizing enzymes and those relevant to N transformation (Du et al., 2014). Biochar's influence on soil EEAs is complex as it can, directly and indirectly, alter enzyme dynamics by modifying soil properties (Liao et al., 2022). The biochar used in this study was produced from pyrolyzing soft pinewood, resulting in a high carbon content. Biochar with high C:N ratios are known to cause microbial N limitation, driving soil microbes to produce more N-acquisition enzymes (Ameloot et al., 2013). This can explain the overall significant response of NAG when biochar was added to the traditional system. At least in this study, combining biochar with traditional practices (e.g., conventional tillage) was seen as a cost-efficient way to promote soil EEAs. Overall, these results showed that integrated BAPs can promote BG activity but not NAG, while adding biochar in a traditional system could be more beneficial for improving the activity of C- and N-acquisition enzymes such as BG and NAG.

Soil microbial community/microbial biomass accumulation

Soil microbial biomass (SMB) is a sensitive indicator of soil health as it responds quickly to changes in management practices. Quantifying the amount of phospholipid fatty acid (PLFA) in the soil has been used as a proxy for SMB. PLFA is only present in living microorganisms and is specific to microbial groups, making it a good biomarker. In this study, the BAP treatments did not significantly impact the PLFAs that were measured. It was also found that adding nitrogen to a traditional system reduces PLFAs. Limited literature explores the combined effects of biochar, cover cropping, and N-management on various soil microbial communities. However, an extensive number of published studies have explored the individual effect of these practices on soil microbial communities. For example, Muhammad et al. (2021) performed a meta-analysis showing that cover crops can enhance biological soil health by increasing microbial community abundance. They observed higher total PLFA in cover crop treatments than fallow, and that cover crops favor fungal growth over bacteria. Chaudhary et al. (2015) reported that applying 50% of the recommended urea fertilizer led to higher total PLFA, gram-negative bacteria, and actinomycetes PLFA concentrations than applying 100%N. Stewart et al. (2018) also claimed that N fertilizer can significantly influence microbial community composition.

Soil organic carbon, total nitrogen, and C/N ratio

This study demonstrated that employing best agricultural practices (BAPs) such as biochar application, cover cropping, and N-management benefited SOC more than conventional practices. Treatments under the BAP system were maintained under no-till; hence, this system has less soil disturbance. Conservation tillage can reduce the rate of soil organic matter decomposition, thereby improving SOC stabilization (Bai et al., 2019). Meanwhile, planting

cover crops provides additional above- and belowground biomass inputs and better soil aggregation, which could promote microbial diversity and reduce carbon loss from erosion. In addition, biochar amendment can affect SOC dynamics by improving soil aggregation and providing physical protection of aggregate-associated SOC against microbial degradation. The stable carbon in biochar can also increase the pool of stable organic substrates in the soil, resulting in slower SOC decomposition.

In a meta-analysis comparing three widely used "climate-smart" agricultural practices (CSAs), biochar application provided the greatest improvements in crop yield while also increasing soil organic carbon (SOC) and decreasing GHG emissions. This is compared with conservation tillage or no-till (NT) and cover cropping, which are ranked second and third most effective (Bai et al., 2019b). However, in this study, adding biochar in a traditional system reduced SOC. As biochar ages in the soil, a part of the biochar is microbially accessible and can be mineralized to CO_2 with rates as high as 15% to 20%. However, this mineralization rate can decrease over time and stay at a daily rate of 0.001% to 0.003% (Han et al., 2020). It is possible that the observed decrease in SOC among treatments is because of the rapid mineralization of biochar's labile C pool and can only last short-term (1-2 years). At the same time, the remaining stable OC will be mineralized very slowly by microorganisms.

2.6: Conclusion

This study evaluated best agricultural practices (BAPs) and their combined effects on important soil health indicators such as EEAs, SOC, microbial biomass, and short-term C respiration. Based on the results, integrating biochar with cover cropping and N-management effectively increased C- and N-acquisition enzymes and soil organic carbon (SOC), especially in a no-till system within 1-2 years. This can be attributed to various factors, including reduced soil

disturbance, additional biomass inputs from cover cropping, enhanced soil aggregation, and biochar's ability to improve SOC dynamics.

Compared with cover cropping and N-management, biochar contributed significantly to improved EEAs. Its application in the conventional corn-soybean system also did not show significant variations in EEAs compared to the BAP system. While an elevated β-glucosidase activity after biochar application may stimulate microbial activity and C respiration, results showed no significant changes in PLFA and soil C respiration. It is worth noting that the rapid mineralization of biochar's labile carbon pool may lead to short-term reductions in SOC but will stabilize in the long term. Adopting BAPs, especially biochar incorporation, has the potential to sustain or improve soil health and productivity. However, the long-term impacts of BAPs on soil carbon dynamics and microbial communities need further investigation. It will also be interesting to explore if increases in soil health indicators will translate to crop yield benefits.

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APPENDIX A: Chapter 1 Supplemental Information

Table S1.1. ANOVA results for extracellular enzyme activities showing degrees of freedom (df), F-values and p-values for all response variables.

Enzyme	Effect	df	F		p	
			2022	2023	2022	2023
β-glucosidase	Farming practice	8	6.675	5.883	0.0001	0.0003
N-acetyl glucosaminidase	Farming practice	8	3.38	3.076	0.0097	0.0156
Phosphatase	Farming practice	8	1.578	1.727	0.1838	0.1431
Leucine aminopeptidase	Farming practice	8	5.423	5.283	0.0006	0.0007
Glutamic acid enzyme	Farming practice	8	5.733	5.679	0.0004	0.0004

Table S1.2. ANOVA results for phospholipid fatty acid (PLFA) degrees of freedom (df), F-values and p-values for all response variables.

PLFA	Effect	df	F		p	
			2022	2023	2022	2023
Total	Farming practice	8	1.661	0.895	0.16	0.5386
Bacteria	Farming practice	8	1.182	0.888	0.3501	0.5432
Fungi	Farming practice	8	0.822	0.962	0.5912	0.4876
Saprophytic fungi	Farming practice	8	0.615	0.965	0.7565	0.4854
Arbuscular mycorrhizal fungi	Farming practice	8	0.931	1	0.6617	0.4613
Actinomycetes	Farming practice	8	0.911	1.266	0.5241	0.3064

Table S1.3. ANOVA results for soil organic carbon, total nitrogen, and carbon to nitrogen ratio showing degrees of freedom (df), F-values and p-values for all response variables.

Response variable	Effect	df	F		p	
			2022	2023	2022	2023
Soil organic carbon	Farming practice	8	1.115	1.088	0.3883	0.4043
Total nitrogen	Farming practice	8	0.684	16.931	0.7012	<.0001
Carbon to nitrogen ratio	Farming practice	8	17.759	2.364	<.0001	0.0492

Table S1.4. Soil extracellular enzyme activity under best agricultural practices (2022). BG – β-glucosidase, NAG – N-acetyl-glucosaminidase, PHOS – phosphatase, LAP – Leucine aminopeptidase, GLU – glutamic acid enzyme. Treatment abbreviations follow Table 1.1.

Plot	Treatment ID	Enzyme activity (μmol h^{-1} g^{-1})					
		Block	BG	NAG	PHOS	LAP	GLU
108	BAP	R1	393.71	66.58	46.69	459.70	137.29
202	BAP	R2	414.66	81.37	54.66	466.78	155.13
304	BAP	R3	619.24	138.15	147.66	702.05	194.50
403	BAP	R4	638.77	128.53	82.34	281.98	341.20
103	BAPB	R1	159.81	71.66	30.40	175.05	78.19
206	BAPB	R2	234.38	41.58	30.40	182.48	77.21
308	BAPB	R3	205.57	79.39	38.43	133.12	59.41
408	BAPB	R4	488.13	163.34	101.15	339.36	169.07
105	BAPC	R1	340.93	71.55	69.77	407.74	121.91
203	BAPC	R2	261.19	42.30	31.69	405.12	154.74
301	BAPC	R3	426.07	102.06	63.85	517.28	149.33
409	BAPC	R4	335.70	87.24	46.94	414.03	129.80
109	BAPN	R1	243.30	60.79	37.10	271.89	99.74
201	BAPN	R2	382.14	83.31	67.72	443.07	149.91
305	BAPN	R3	307.96	64.23	93.03	395.51	115.34
402	BAPN	R4	418.84	108.60	73.67	282.76	166.90
107	TRD	R1	228.12	54.40	25.56	166.41	78.94
204	TRD	R2	281.13	57.14	38.27	254.57	90.97
306	TRD	R3	288.09	80.81	48.46	242.18	91.86
401	TRD	R4	386.68	105.94	68.25	312.41	98.58
102	TRDB	R1	564.19	126.61	120.82	784.87	197.43
205	TRDB	R2	454.51	125.68	52.39	559.72	183.69
309	TRDB	R3	448.73	114.30	79.15	462.84	146.15
405	TRDB	R4	388.99	415.33	27.46	240.01	154.09
106	TRDC	R1	175.70	39.72	21.95	145.80	65.39
208	TRDC	R2	285.14	53.05	33.53	198.95	97.00
307	TRDC	R3	286.34	57.70	46.59	199.90	74.03
404	TRDC	R4	150.73	55.81	14.86	310.27	54.89
101	TRDN	R1	229.01	77.35	31.29	218.46	136.54
207	TRDN	R2	218.47	40.72	25.52	166.41	73.21
302	TRDN	R3	201.03	40.33	31.48	165.91	60.75
406	TRDN	R4	335.80	100.87	89.55	282.56	90.69
104	TRDT	R1	213.72	52.29	38.51	213.43	76.83
209	TRDT	R2	130.87	50.71	21.66	107.75	81.64
303	TRDT	R3	188.11	45.83	21.58	159.78	54.84
407	TRDT	R4	279.79	94.17	57.41	240.92	101.99

Table S1.5. Soil extracellular enzyme activity under best agricultural practices (2023). BG – β-glucosidase, NAG – N-acetyl-glucosaminidase, PHOS – phosphatase, LAP – Leucine aminopeptidase, GLU – glutamic acid enzyme. Treatment abbreviations follow Table 1.1.

Plot	Treatment ID	Enzyme activity (μmol h^{-1} g^{-1})					
		Block	BG	NAG	PHOS	LAP	GLU
108	BAP	R1	438.75	97.10	78.18	486.97	172.60
202	BAP	R2	459.69	109.42	87.49	495.38	190.44
304	BAP	R3	664.28	166.20	180.49	730.65	229.82
403	BAP	R4	683.81	156.58	115.17	310.58	376.51
103	BAPB	R1	204.85	99.71	63.23	203.66	113.51
206	BAPB	R2	290.42	69.64	58.52	220.75	112.53
308	BAPB	R3	261.61	107.45	66.55	171.38	94.72
408	BAPB	R4	544.17	191.39	129.27	377.63	215.39
105	BAPC	R1	396.97	99.60	97.89	446.01	168.23
203	BAPC	R2	317.22	70.35	59.80	443.39	201.06
301	BAPC	R3	482.11	129.21	91.97	555.54	195.65
409	BAPC	R4	391.74	114.39	75.06	452.30	176.12
109	BAPN	R1	299.34	87.94	65.22	310.15	146.06
201	BAPN	R2	438.18	110.46	99.21	467.84	196.23
305	BAPN	R3	364.00	91.38	124.52	420.28	161.66
402	BAPN	R4	474.88	135.75	105.16	307.53	213.22
107	TRD	R1	284.16	81.55	57.05	191.18	125.26
204	TRD	R2	337.17	84.29	69.76	279.34	137.29
306	TRD	R3	344.13	107.96	79.95	266.95	138.18
401	TRD	R4	442.72	133.09	99.74	337.18	144.90
102	TRDB	R1	605.85	153.76	152.31	808.76	243.76
205	TRDB	R2	496.17	152.83	83.88	583.61	216.51
309	TRDB	R3	490.39	141.44	110.64	486.73	178.97
405	TRDB	R4	430.65	456.86	59.72	263.90	186.91
106	TRDC	R1	217.36	81.24	54.21	169.69	98.21
208	TRDC	R2	326.81	94.57	65.79	222.84	129.82
307	TRDC	R3	328.00	99.23	78.85	223.79	106.85
404	TRDC	R4	192.39	97.34	47.12	334.16	87.71
101	TRDN	R1	196.84	104.50	47.42	256.73	169.36
207	TRDN	R2	275.11	67.87	63.85	204.68	106.03
302	TRDN	R3	264.57	67.48	58.08	204.18	93.58
406	TRDN	R4	247.13	128.02	64.04	320.83	123.51
104	TRDT	R1	381.91	79.44	122.11	251.69	109.65
209	TRDT	R2	259.82	77.86	71.07	146.01	114.46
303	TRDT	R3	176.97	72.98	54.22	198.04	87.66
407	TRDT	R4	234.21	121.32	54.15	279.19	134.81

Table S1.6. Soil organic carbon, total nitrogen, and carbon-nitrogen ratio under best agricultural practices (2022). BG – β-glucosidase, NAG – N-acetyl-glucosaminidase, PHOS – phosphatase, LAP – Leucine aminopeptidase, GLU – glutamic acid enzyme. Treatment abbreviations follow Table 1.1.

Plot	Treatment ID	Block	Soil organic carbon (g kg^{-1})	Total N (g kg^{-1})	C:N ratio
108	TRDT	R4	0.139	0.012	11.44
202	TRDT	R3	0.152	0.013	11.99
304	TRDT	R2	0.139	0.010	14.10
403	TRDT	R1	0.699	0.065	10.83
103	TRDN	R4	0.137	0.010	13.51
206	TRDN	R3	0.142	0.012	11.63
308	TRDN	R2	0.776	0.064	12.21
408	TRDN	R1	0.691	0.060	11.49
105	TRDC	R4	0.126	0.013	9.98
203	TRDC	R3	0.147	0.013	11.24
301	TRDC	R2	0.139	0.012	11.50
409	TRDC	R1	0.638	0.062	10.36
109	TRDB	R4	0.314	0.013	23.57
201	TRDB	R3	0.208	0.012	17.59
305	TRDB	R2	1.147	0.056	20.34
402	TRDB	R1	1.033	0.059	17.46
107	TRD	R4	0.184	0.016	11.31
204	TRD	R3	0.119	0.010	11.34
306	TRD	R2	0.719	0.049	14.77
401	TRD	R1	0.127	0.011	11.80
102	BAPN	R4	0.200	0.013	15.44
205	BAPN	R3	0.206	0.013	16.09
309	BAPN	R2	0.960	0.058	16.64
405	BAPN	R1	1.050	0.074	14.29
106	BAPC	R4	0.269	0.016	16.37
208	BAPC	R3	0.203	0.010	20.14
307	BAPC	R2	0.989	0.057	17.41
404	BAPC	R1	1.135	0.071	15.96
101	BAPB	R4	0.136	0.012	11.00
207	BAPB	R3	0.127	0.011	11.56
302	BAPB	R2	0.674	0.056	11.98
406	BAPB	R1	0.668	0.060	11.06
104	BAP	R4	0.294	0.013	23.40
209	BAP	R3	0.218	0.011	19.61
303	BAP	R2	1.108	0.067	16.64
407	BAP	R1	0.193	0.011	17.72

Table S1.7. Soil organic carbon, total nitrogen, and carbon-nitrogen ratio under best agricultural practices (2023). Treatment abbreviations follow Table 1.1.

Plot	Treatment number	Treatment ID	Block	Soil organic carbon (g kg^{-1})	Total N	C:N ratio
108	T9	TRDT	R4	6.4	0.55	11.64
202	T9	TRDT	R3	8	0.49	16.33
304	T9	TRDT	R2	6.1	0.5	12.20
403	T9	TRDT	R1	6.6	0.52	12.69
103	T8	TRDN	R4	9.6	0.59	16.27
206	T8	TRDN	R3	6.3	0.49	12.86
308	T8	TRDN	R2	5.4	0.45	12.00
408	T8	TRDN	R1	9.1	0.58	15.69
105	T6	TRDC	R4	8.2	0.58	14.14
203	T6	TRDC	R3	8.2	0.55	14.91
301	T6	TRDC	R2	8.2	0.51	16.08
409	T6	TRDC	R1	8.4	0.5	16.80
109	T7	TRDB	R4	5.9	0.5	11.80
201	T7	TRDB	R3	8.3	0.45	18.44
305	T7	TRDB	R2	6	0.52	11.54
402	T7	TRDB	R1	5.8	0.46	12.61
107	T5	TRD	R4	6	0.65	9.23
204	T5	TRD	R3	6.2	0.64	9.69
306	T5	TRD	R2	7.4	0.7	10.57
401	T5	TRD	R1	7.3	0.72	10.14
102	T4	BAPN	R4	6.7	0.68	9.85
205	T4	BAPN	R3	8.6	0.75	11.47
309	T4	BAPN	R2	8.7	0.72	12.08
405	T4	BAPN	R1	6.8	0.74	9.19
106	T2	BAPC	R4	6.3	0.64	9.84
208	T2	BAPC	R3	6.2	0.65	9.54
307	T2	BAPC	R2	8.7	0.85	10.24
404	T2	BAPC	R1	8	0.83	9.64
101	T3	BAPB	R4	8.1	0.64	12.66
207	T3	BAPB	R3	6	0.62	9.68
302	T3	BAPB	R2	9.4	0.72	13.06
406	T3	BAPB	R1	6.1	0.64	9.53
104	T1	BAP	R4	7.6	0.87	8.74
209	T1	BAP	R3	6.8	0.69	9.86
303	T1	BAP	R2	8.2	0.84	9.76
407	T1	BAP	R1	17.1	0.81	21.11

Table S1.8. Phospholipid fatty acid concentrations under best agricultural practices (2022). Treatment abbreviations follow Table 1.1.

			Phospholipid Fatty Acid (ng g^{-1})		
Plot	Treatment ID	Block	Total PLFA	Bacterial PLFA	Fungal PLFA
108	TRDN	1	3087.38	832.43	147.11
202	TRDB	1	3295.28	888.85	419.15
304	BAPB	1	4038.52	935.92	221.41
403	TRDT	1	3871.05	990.69	550.51
103	BAPC	1	3599.37	874.76	392.77
206	TRDC	1	3145.18	779.98	314.3
308	TRD	1	3947.55	973.27	276.25
408	BAP	1	3421.5	745.02	210.52
105	BAPN	1	3344.93	886.73	128.7
203	BAPN	2	3781.91	751.9	179.28
301	BAP	2	3240.07	750.05	52.55
409	BAPC	2	1053.69	431.23	12.69
109	TRD	2	3121.87	737.64	350.96
201	TRDB	2	3110.88	832.44	227.31
305	BAPB	2	1209.66	493.72	16.45
402	TRDN	2	1157.39	488.79	45.15
107	TRDC	2	1266.11	495.28	8.63
204	TRDT	2	1547.77	616.65	66.53
306	BAPC	3	2686.02	943.37	138.41
401	TRDN	3	1480.74	565.42	57.78
102	TRDT	3	1567.75	613.85	60.85
205	BAP	3	1929.7	722.41	106.58
309	BAPN	3	2309.15	726.26	114.3
405	TRD	3	1744.65	590.13	17.58
106	TRDC	3	1692.63	701.1	19.27
208	BAPB	3	1639.85	685.35	79.68
307	TRDB	3	2312.97	837.26	120.98
404	TRD	4	1497.85	433.59	40.23
101	BAPN	4	1719.78	568.66	160.06
207	BAP	4	2033.54	499.34	121.38
302	TRDC	4	1666.2	394.77	66.63
406	TRDB	4	1074.55	292.32	36.62
104	TRDN	4	653.5	224.28	0
209	TRDT	4	1389.06	412.71	47.83
303	BAPB	4	1285.66	475.13	47.01
407	BAPC	4	922.69	340.87	8.98

Table S1.9. Phospholipid fatty acid concentrations under best agricultural practices (2023). Treatment abbreviations follow Table 1.1.

			Phospholipid Fatty Acid (ng g^{-1})		
Plot	Treatment ID	Block	Total PLFA	Bacterial PLFA	Fungal PLFA
108	TRDN	1	1424.14	209.53	201.98
202	TRDB	1	742.65	190.27	4.03
304	BAPB	1	639.44	108.18	0
403	TRDT	1	424.27	83.74	0
103	BAPC	1	616.72	121.67	0
206	TRDC	1	338.41	64.48	0
308	TRD	1	1011.61	197.35	0
408	BAP	1	463.82	115.22	0
105	BAPN	1	612.9	115.88	0
203	BAPN	2	956.69	197.43	8.29
301	BAP	2	927.56	161.19	7.04
409	BAPC	2	820.28	139.41	0
109	TRD	2	480.47	74.51	0
201	TRDB	2	372.44	58.29	0
305	BAPB	2	711.29	119.4	3.81
402	TRDN	2	379.57	73.28	0
107	TRDC	2	471.45	76.23	0
204	TRDT	2	363.03	60.59	0
306	BAPC	3	232.53	46.27	0
401	TRDN	3	684.24	171.87	3.34
102	TRDT	3	278.57	64.09	0
205	BAP	3	195.19	47.84	0
309	BAPN	3	466.92	134.52	0
405	TRD	3	555.41	142.88	3
106	TRDC	3	350.53	76.34	0
208	BAPB	3	702.45	178.2	7.79
307	TRDB	3	420.45	134.71	3.89
404	TRD	4	425	133.01	5.43
101	BAPN	4	569.32	121.37	13.72
207	BAP	4	458.06	124.13	2.84
302	TRDC	4	630.77	188.58	6.97
406	TRDB	4	248.69	72.36	0
104	TRDN	4	265.05	95.67	0
209	TRDT	4	525.27	122.76	0
303	BAPB	4	400.45	100.45	0
407	BAPC	4	555.8	149.97	0

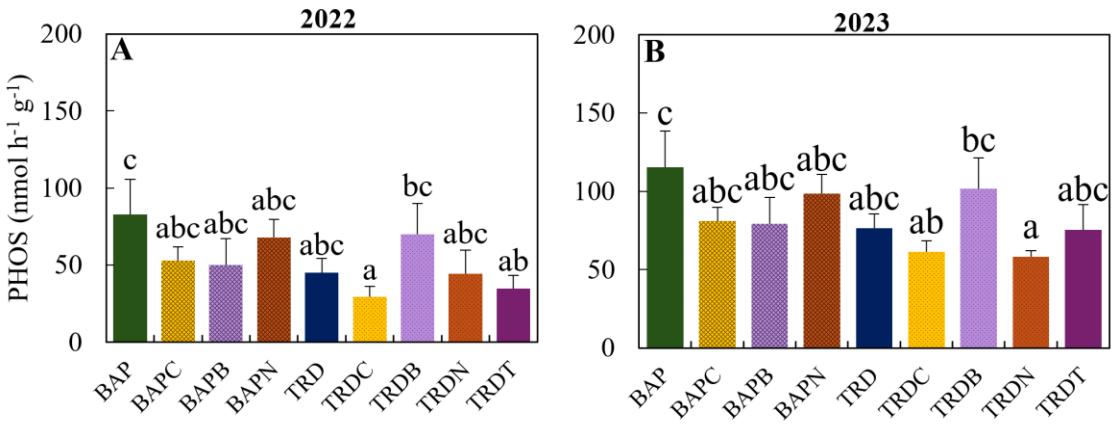

Figure S1.1. Mean soil extracellular enzyme activity of Phosphatase after (A) one year and (B) two years as affected by best agricultural practices (BAPs) and traditional practices across two growing seasons of soybean and wheat. Error bars represent standard error. For treatments with common letters, no significant differences were observed (Fisher's protected LSD, α=0.05). Y-axis titles are identical between panels A and B, and between C and D. Treatment abbreviations follow Table 1.1.

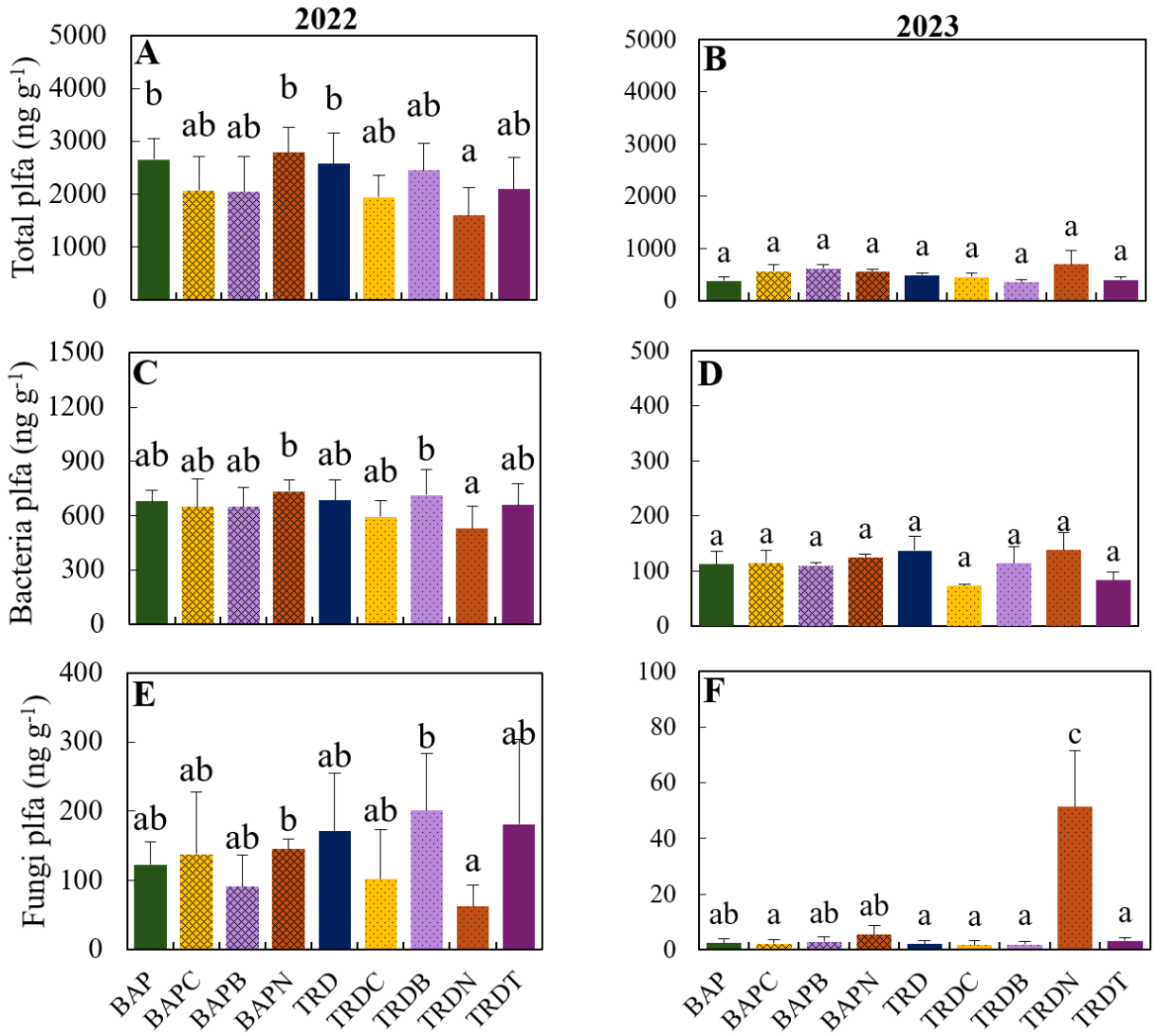

Figure S1.2. Mean (A-B) total PLFA, (C-D) bacterial PLFA, and (E-F) fungal PLFA as affected by best agricultural practices (BAPs) and traditional practices across two growing seasons of soybean and wheat. Error bars represent standard error. For treatments with common letters, no significant differences were observed (Fisher's protected LSD, α=0.05). Y-axis titles are identical between panels A and B, C and D, and E and F. Treatment abbreviations follow Table 1.1.

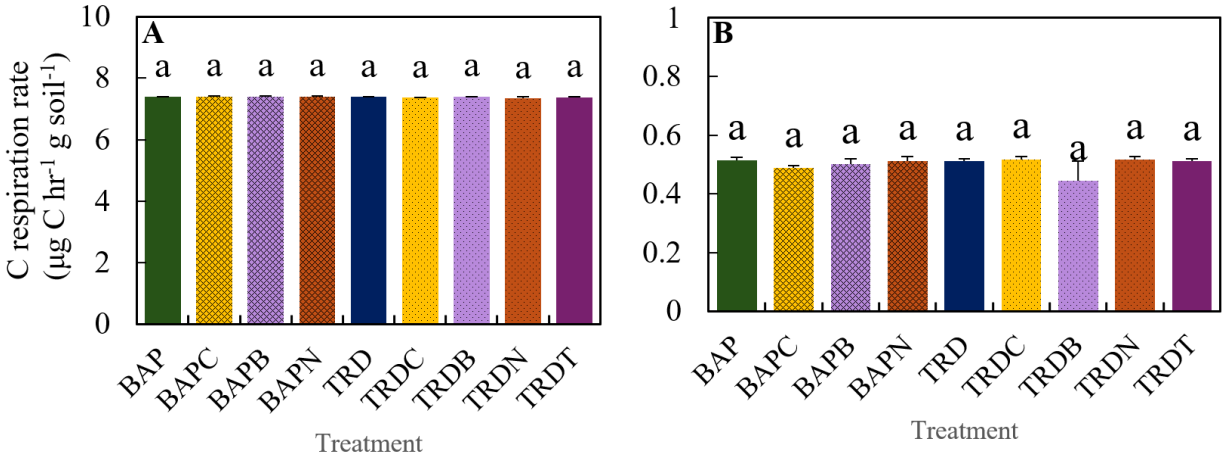

Figure S1.3. Mean soil C respiration rate of (A) pre-planting and (B) mid-season samples after seven days of laboratory incubation as affected by best agricultural practices (BAPs) and traditional practices across two growing seasons of soybean and wheat. Error bars represent standard error. For treatments with common letters, no significant differences were observed (Fisher's protected LSD, α=0.05). Y-axis titles are identical between panels A and B, and between C and D. Treatment abbreviations follow Table 1.1

CHAPTER THREE: BIOCHAR INCORPORATION AND ITS EFFECTS ON SOIL ORGANIC CARBON, ENZYME ACTIVITY, AND MICROBIAL COMMUNITIES

3.1: Abstract

Soil amendments such as biochar have been used in agricultural soils with low fertility to improve their productivity and crop yield. In conventional cropping systems, biochar is typically incorporated in the soil. However, its application, especially in no-till systems, is limited to either narrow incorporation or surface application, exposing biochar to losses via erosion. Over two growing seasons (2022-2023), a field trial was implemented to compare the responses of soil health indicators to two biochar application rates (5Mg ha^{-1} and 15Mg ha^{-1}) incorporated at three depths (surface application, 8cm-10cm, 13cm-18cm). An oakwood-derived biochar was applied before planting corn in May 2021, while soybean (*Glycine max* L.) and wheat (*Triticum aestivum* L.) were seeded in the Spring of 2022 and 2023, respectively. Results showed a neutral response of soil extracellular enzymes to biochar application. On the other hand, applying 5 Mg ha^{-1} of biochar and incorporating it at 8cm-10cm had the highest phospholipid fatty acid (PLFA) concentration for different bacterial and fungal groups. In 2022, incorporating 15 Mg ha^{-1} of biochar at 13cm-18cm increased soil organic carbon (SOC) by 5.12% compared to the control treatment. This increase in SOC is due to the additional carbon credit from biochar, which also stimulated microbial biomass production and favored bacterial growth over fungi. However, this effect only lasted for one growing season. Based on these indicators, oakwood-derived biochar had a short-term benefit to soil health. Establishing long-term field experiments can further explore the duration of biochar effects on yield and soil health of cropping systems.

3.2: Introduction

Climate-smart agriculture (CSA) practices such as conservation tillage increase farmers' capacity to adapt and mitigate the effects of climate change and have been widely researched and recommended (Nyambo et al., 2021). Biochar application is also considered a CSA practice, and it has been widely investigated for its potential to offset the detrimental effects of intensive agriculture and support soil health, synergistically improving agronomic output and soil organic matter (Kumar Mishra et al., 2023; Ding et al., 2017). Biochar is a black carbon material derived from pyrolyzing biomass in an oxygen-limited environment (Lehmann et al., 2006). Using feedstocks such as plant, animal residues, and industrial wastes to produce biochar makes it a sustainable soil amendment (Lehmann, 2007). Over the years, interest in biochar has grown mainly because of its capacity to sequester carbon (C), reduce nutrient leaching, and improve crop yield (Lehmann et al., 2006; Zhang et al., 2017). Biochar is also considered a candidate amendment for soil health improvement due to its high surface area and highly reactive nature (Hou et al., 2020).

On the other hand, the benefits of residue management strategies such as biochar application are not universally successful, with examples where biochar did not affect soil productivity and crop yield (Jeffery et al., 2011). Published studies that demonstrated the benefits of biochar (e.g., reduction of nitrous oxide emissions) either had biochar applied with compost or incorporated into the soil, enabling close contact between the biochar and rhizosphere (Oo et al., 2018; Paulin & O'Malley, 2008). However, in no-till systems or those under conservation tillage, biochar application is only limited to field surface application or narrow incorporation, often relying on the movement of soluble nutrients with rainfall or irrigation, bioturbation, and vertical transport of solutes over time (Major et al., 2010). Surface-applied biochar can only infiltrate the

soil up to 3cm annually (Wang et al., 2013). This contrasts significant biochar losses after field application, estimated at 7%-55% of surface-applied biochar lost through erosion. Moreover, biochar typically has a low density and is highly porous, making it physically vulnerable to lateral movement after rainfall or irrigation events (Rumpel et al., 2015; Wang et al., 2013). These factors diminish the benefit of a currently high-cost soil amendment (Major et al., 2010; Rumpel et al., 2015).

Based on these challenges, incorporating biochar into the soil has become a standard practice to reduce the risk of lateral movement, but it creates challenges for no-till systems (Major et al., 2010). Moreover, information about the fate and influence of surface-applied and incorporated biochar is limited, as well as their effects on soil health indicators (Bass et al., 2016; Cox et al., 2021). This study evaluated the influence of biochar application rate and incorporation depth on changes in soil health indicators. Specifically, this study intended to (1) quantify and compare activity rates of extracellular enzymes across treatments, (2) elucidate the response of microbial community under biochar-amended and non-biochar treatment plots by quantifying microbial biomass and soil organic carbon, and (3) determine the biochar rate and depth of incorporation at which the most significant effect on soil health was recorded. It is hypothesized that biochar will increase soil health in all application treatments. However, its effects would be more significant when incorporated into the soil than when surface applied and will increase with incorporation depth and application rate.

3.3: Materials and Methods

Experimental study site and design

This study was conducted from May 2022 to December 2023 to determine the influence of biochar incorporation on soil health indicators. It also leveraged an existing field trial

established in 2021 at the Kellog Biological Station (KBS) in Hickory Corners, Michigan, that compares the effects of biochar application rate and incorporation depth on yield and agronomic parameters. Treatments were laid out in a randomized complete block design (RCBD) with four replications. This study uses a two-factor RCBD with four replications representing one block. The two factors included: (1) biochar application rate with two levels – 5 Mg ha^{-1} and 15 Mg ha^{-1}; and (2) incorporation depth with three levels – surface application, shallow incorporation (8cm-10cm), and deep incorporation (13cm-15cm). A total of 8 treatment combinations, including a control treatment (no biochar, no cover crop), were employed in this study, with each treatment plot having a dimension of 15' x 75' (**Table 2.1**).

Table 2.1. Treatment combinations of biochar application rate and incorporation depth.

Treatment ID	Biochar rate	Incorporation depth
Control	-	No biochar, no cover crop
CC	-	Cover crop
S1	5 Mg ha^{-1}	Surface application
ST1		Shallow incorporation (8cm-10cm)
DT1		Deep incorporation (13cm-18cm)
S2	15 Mg ha^{-1}	Surface application
ST2		Shallow incorporation
DT2		Deep incorporation

Farm management practices

Biochar produced from oakwood chips was incorporated in the soil on May 12, 2021, at soil depths of approximately 8cm-10cm and 13cm-18cm using a field cultivator (John Deere 960 10') and chisel plow (John Deere 714), respectively. The characteristics of biochar used in this study are summarized in **Table 2.2**, while the management practices implemented in the study are summarized in **Fig. 2.1**. On May 13, 2021, corn hybrid variety P0414AM was planted in six rows at a seeding rate of 74,131 seeds ha^{-1} using a row planter (7300, John Deere, MO, USA). Primary tillage practice was performed in the Fall of 2020, while secondary tillage was performed in the Spring of 2021 before biochar application. Soybean was planted on May 12,

2022, and harvested on October 5, 2022 (Almaco plot combine 1978, John Deere 9410 combine). After harvesting soybean, wheat was planted in no-till plots at 3cm depth (John Deere 1590 15' no-till drill) at a rate of 3,461,204 seeds $ha^{-1,}$ and they received 102.95 L ha^{-1} of liquid fertilizer (10-34-0). Following fertilizer application, 2.91 kg ha^{-1} of ammonium sulfate and 987.91 mL ha^{-1} of herbicide Huskie were sprayed in wheat, barley, and rye plots to control weeds on November 9, 2022.

Table 2.2. Characteristics of oakwood-derived biochar used in this study.

Biomass source	Oakwood
Moisture	<15%
% Carbon	>85%
Density	0.15 g cc^{-1} - 0.3 g cc^{-1}
pH	9-10
Mesh size	Pellets (0.5" x 0.19")
Ash content	<10%

On March 20, 2023, red clover was frost-seeded in cover crop plots (T4, T7, T8) at a rate of 878.08 mL ha^{-1} (Gandy Air Seeder) and received 135.66 L ha^{-1} of UAN (28-0-0) on March 28, 2023. On the other hand, wheat and rye plots received 186.95 L ha^{-1} of UAN ATS (26.7-0-0-3.6) and 2.34 L ha^{-1} of Horosol 10 (Demco 3pt 100 gallon, 45' boom sprayer). Wheat was harvested on July 11, 2023 (Kincaid 8XP Plot Combine), and on August 1, 2023, red clover was planted at 16.15 kg ha^{-1} since the frost-seeded clover did not survive. Plots were also mowed on August 18 and October 4, 2023, as a mechanical weed control measure.

Soil sampling collection

Soil samples were collected across two growing seasons from May 2022 to October 2023. For each growing season, soil samples were taken at three time points: pre-planting, mid-season, and before harvest (**Fig. 2.1**). Specifically, 2022 soil samples were taken before planting soybean (May 12, 2022), during pod formation (R5; July 27, 2022) and before harvest

(September 30, 2022). In October 2023, wheat was planted in the field, and samples were taken at similar time points. A total of 20 soil cores were taken from each plot and in two depths (0-10cm and 10-30cm), following a zigzag pattern. Collected samples were mixed in a bucket to make a composite sample. They were transported back to the lab in a cooler box for further processing (e.g., air-drying, oven-drying, sieving) prior to analysis. Subsampling was done for soil samples taken from July 2022 and July 2023, and they were analyzed for phospholipid fatty acid (PLFA), extracellular enzyme activity (EEA), soil organic carbon (SOC), and short-term soil carbon respiration. Samples taken in May 2022/May 2023 and October 2022/ October 2023 were also analyzed for these soil response variables and were incubated to determine soil carbon respiration.

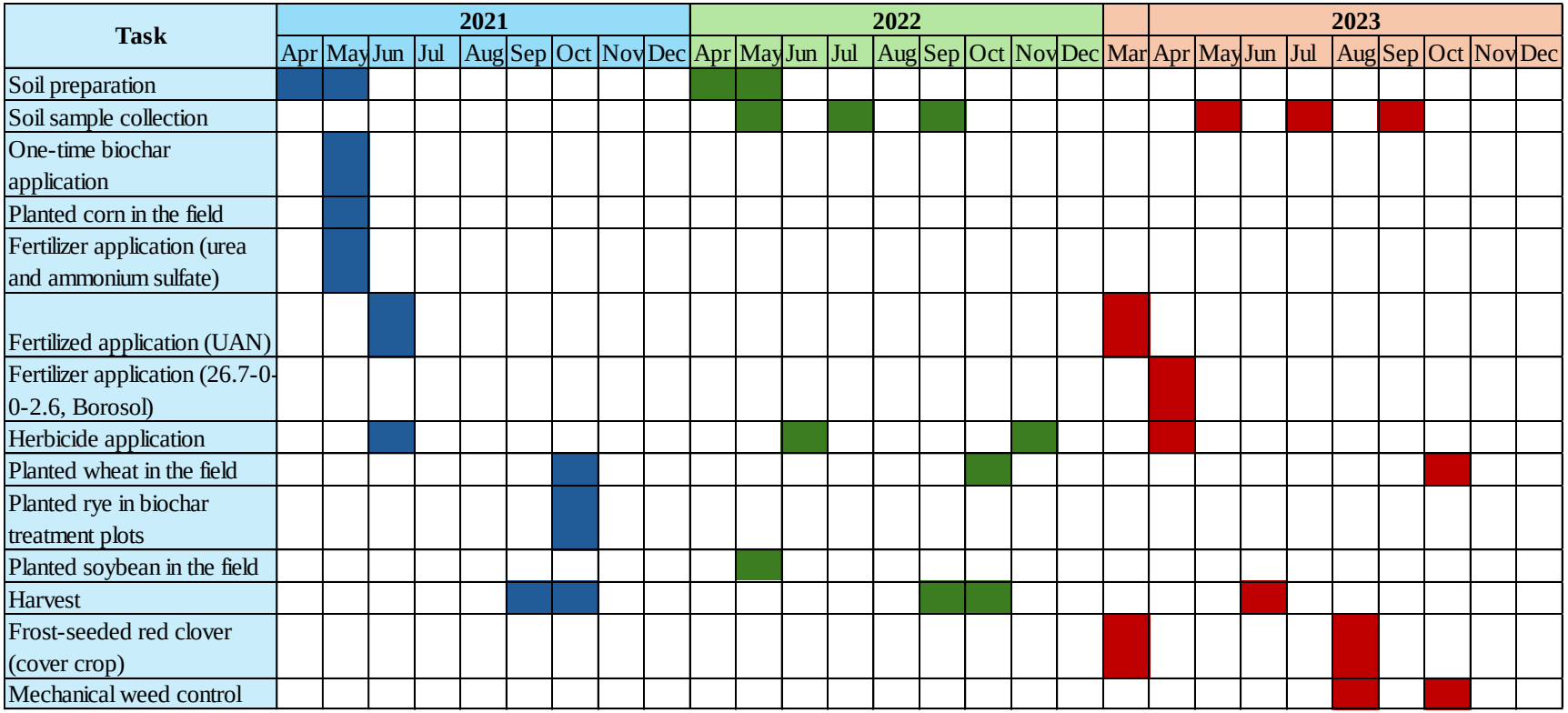

Figure 2.1. Management practices implemented in the field from 2021 to 2023.

Assessment of soil health indicators

Extracellular enzyme activity. In this study, the potential activities of five hydrolytic enzymes involved in C, N, and P cycling were assayed for biochar and non-biochar amended plots. *B-glucosidase* (BG) is a commonly measured indicator for C dynamics and is responsible for hydrolyzing cellulose and polymeric saccharides to glucose. *N-acetyl-glucosaminidase* (NAG) and *phosphatase* (PHOS) often indicate N and P acquisition, respectively (Ferraz-Almeida et al., 2015; Liao et al., 2022). Briefly, 1g of soil sample was homogenized with 125 ml of ultrapure water using a hand-held blender to prepare the soil slurry. To create analytical replicates, 200µL of soil slurry from each sample was pipetted into three consecutive rows of a 96-well black microplate. Specifically, soil slurry and methylumbelliferone (MUB) or methylcoumarin (MC) standards are pipetted in columns 1,4,7 and 10, while 50µL of MUB or MC and 50µL of the substrate are pipetted in columns 2,3,5,6,7,8,9,11 and 12. Standards were also made for MUB and MC-labelled substrates (Saiya-Cork et al., 2002.). The respective substrate used for each enzyme is listed in **Table 1.3.**

Soil microbial community composition. Phospholipid fatty acid (PLFA) is only present in living organisms; hence, they quickly break down when a cell dies. PLFA can be used as a biomarker because each organism has its unique chemical composition of PLFA. For PLFA analysis, soil samples from October 2022 and October 2023 were placed in Nalgene bottles and immediately stored at -20℃ after collection to maintain viability prior to microbial analysis. Soil samples were sent to a private laboratory to determine the quantity and composition of microbial communities.

Soil organic carbon and total nitrogen. Soil samples from October 2022 and October 2023 were oven-dried at 60℃ until no further mass loss occurred. Samples were pulverized into

fine powder using a ball mill and placed into polyethylene vials for storage at ambient temperature. Before analysis, samples were tested for carbonates by adding 2-3 drops of 1N hydrochloric acid (HCl). Fine bubbles were absent, indicating the absence of carbonates; hence, pre-treatment of soils with acid fumigation was unnecessary. Samples are then weighed in a microbalance and packed into tin capsules for CN analysis using a combustion analyzer (Robertson & VanderWulp, 2019).

Short-term soil respiration. Composite soil samples were mixed, sieved using a 2-mm sieve, and sub-sampled to determine short-term soil respiration rate. Three sub-samples were taken from each composite and were incubated for seven days to determine short-term C mineralization rates. Samples were oven-dried at 105 °C for 24 hours to determine gravimetric moisture content (GMC), bulk density (BD), and the amount of water to add to achieve 60% water-field pore space (WFPS).

Triplicate 20g subsamples were weighed into specimen cups and adjusted to 60% water-filled pore space (WFPS) before starting the incubation. The amount of water added to each specimen cup was determined by multiplying the weight of incubated soil by the values determined using Eq.1. To calculate the total volume of water needed to achieve 60% WFPS, the target WFPS is multiplied by the quotient of total pore space (PS) and BD (Eq. 1). Once the total volume of water needed is calculated, the amount of water to add for every gram of soil to achieve 60% WFPS is determined using Eq. 2. Total pore space was calculated using Eq. 3, while the value used for bulk density is based on the KBS LTER data from 2021.

$$\textit{Total Water Volume Needed (mL/gram soil)} = \textit{(Target WFPS x (PS/BD))/10000)} \quad \textbf{Eq. 1}$$

$$\textit{Water to add (ml/gram soil)} = \textit{Total Water Volume Needed} - \textit{(GMC/100)} \quad \textbf{Eq. 2}$$

$$\textit{PS (\%)} = \textit{(1-(BD/2.65))*100} \quad \textbf{Eq. 3}$$

After adding water, specimen cups were placed in quart-size wide-mouth jars and flushed with ambient air; this was done by placing the jars in front of an oscillating fan for 30 minutes. Jars were capped and incubated in the dark for 24 hours, recording the capping time to calculate the C respiration rate. The CO_2 concentration in the headspace of each jar was measured at three intervals (after one day, four days, and seven days of incubation) by taking 1cc of gas samples and injecting them into an infrared gas analyzer (IRGA). Each jar was flushed with ambient air 24 hours prior to every measurement.

Statistical analysis

This study used the following soil health indicators as response variables: enzyme activity, microbial biomass, soil organic carbon, total nitrogen, carbon-to-nitrogen ratio, and soil C respiration rate. The normality of the residuals was assessed by visual inspection of the normal probability plots and histogram of residuals. Based on the normal probability plots, there is no funnel-shaped pattern distribution to the residuals as the predicted responses/means increase. This indicates that the residual variances are proportional to the means and that the residuals follow a normal distribution. No skewness in the distribution was also observed in the histogram plots. Hence, there is no need for a logarithmic transformation of soil health responses across the eight biochar treatment groups. The effect of biochar on soil health metrics were evaluated using analysis of variance (ANOVA). Linear mixed models were specified with the biochar application rate and incorporation depth as fixed effects and block (replication) as the random effect in the lme4 package in R. Significance of the biochar treatment effect was determined using the emmeans package and through conducting a Type III test with Kenward-Roger adjusted degrees of freedom. When treatment effect was significant (α=0.05), pairwise comparisons were conducted among treatments using the cld function in multcomp package in R.

3.4: Results

Soil extracellular enzyme activity

In 2022, biochar application rate and incorporation depth did not significantly affect activity rates of β-1,4-glucosidase (BG), N-acetyl-glucosaminidase (NAG), phosphatase (PHOS), leucine aminopeptidase (LAP) and L-glutamic acid enzyme (GLU) (**Table S2.1**). However, significant differences among treatments were observed (**Fig. 2.2, 2.3, S2.4**). Mean BG activity in 2022 ranged from 0.2294 μmol h^{-1} g^{-1} (ST2) to 0.3045 μmol h^{-1} g^{-1} (DT2) in 2022 (**Fig. 2.2a**). Plots amended with 15 Mg ha^{-1} of biochar incorporated at 13cm-18 cm (DT2) had the highest BG activity rate among treatments. Specifically, DT2 increased BG by 25.43% ($p = 0.033$), 23.7% ($p=0.0481$), and 32.7% ($p = 0.0114$) relative to the control treatment (C), plots with 5 Mg ha^{-1} of biochar incorporated at 8cm-10cm (ST1), and 15 Mg ha^{-1} of biochar incorporated at 8cm-10cm (ST2), respectively. On average, NAG activities ranged from 0.067 μmol h^{-1} g^{-1} (ST2) to 0.10 μmol h^{-1} g^{-1} (DT2) in 2022 (**Fig. 2.2c**). DT2 increased NAG by 31.11% ($p=0.034$) and 33.32% ($p=0.0264$) compared to when 5 Mg ha^{-1} of biochar was surface-applied (S1) and incorporated at 13cm-18cm (ST1). Moreover, DT2 had 51.34% ($p=0.0039$) higher NAG activity than the cover crop treatment.

In 2023, biochar rate and incorporation depth also did not significantly influence BG and NAG activities. However, we observed differences among treatments and higher values than in 2022 (**Fig. 2.2b, 2.2d**). On average, BG activity in 2023 ranged from 1.498 μmol h^{-1} g^{-1} (S1) to 3.25 μmol h^{-1} g^{-1} (DT1). Within plots under 5 Mg ha^{-1} of biochar, incorporation at 8cm-10cm (ST1) and 13cm-15cm (DT1) increased BG by 89.03% ($p=0.0385$) and 116.86% ($p=0.0203$), respectively than surface-applied biochar (S1). In contrast, incorporating 15 Mg ha^{-1} of biochar at 15cm (DT2) increased BG by 97.98% than incorporation at 8cm (ST2) (**Fig. 2.2b**). BG

activity also declined by 37.95% (p=0.0408) when 15 Mg ha^{-1} biochar was incorporated at 8cm-10cm (ST2) compared to when 5Mg ha^{-1} was incorporated at the same depth (ST1).

NAG activity in 2023 ranged between 0.7709 µmol h^{-1} g^{-1} (S2) to 1.338 µmol h^{-1} g^{-1} (C). Relative to the control treatment, surface application at 5 Mg ha^{-1} (S1) and 15 Mg ha^{-1} (S2) reduced NAG activity by 14.09% (p=0.0264) and 24.88% (p=0.0356), respectively (**Fig. 2.2d)**. In plots under 5 Mg ha^{-1} of biochar, there was also a 4.5% (p=0.0467) decline in activity when biochar was incorporated at 8cm-10cm (ST1) and a 2.7% (p = 0.0378) reduction at 13cm-18cm (DT1) incorporation.

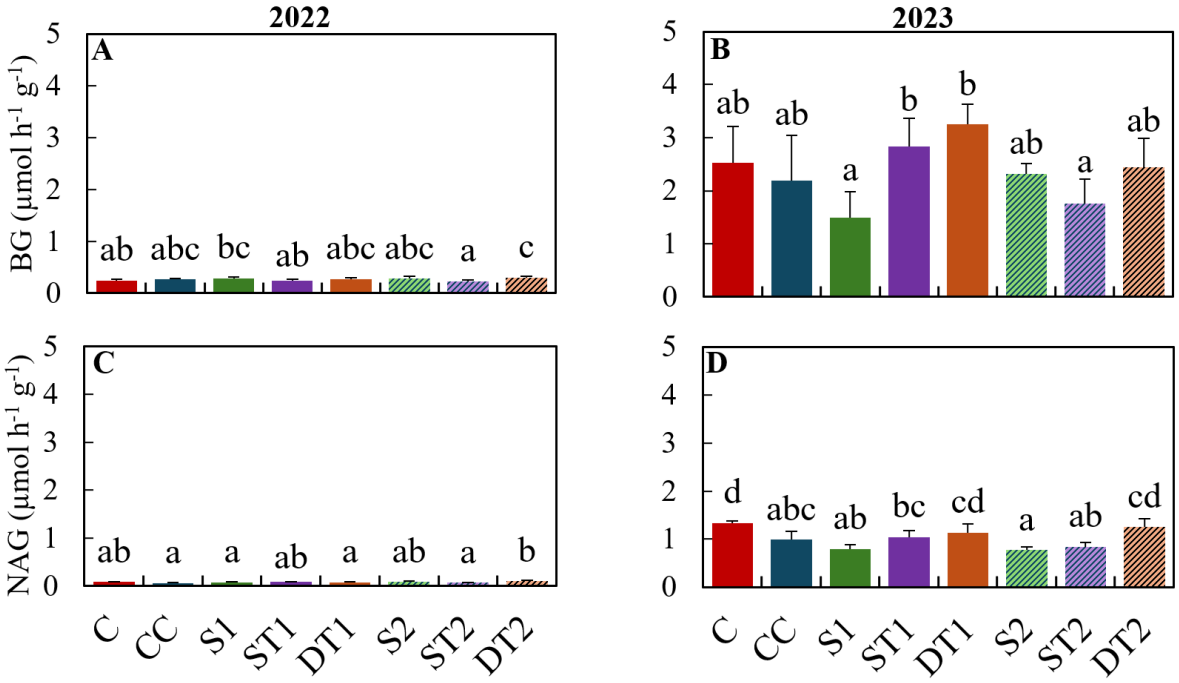

Figure 2.2. Mean activity (±SE) of soil extracellular enzymes β- 1,4-glucosidase (BG) and N-acetyl-glucosaminidase (NAG) as influenced by biochar rates (5 Mg ha^{-1}; 15 Mg ha^{-1}) incorporated via surface application, shallow incorporation (8cm-10cm) and deep incorporation (13cm-18cm). Different lowercase letters show statistically significant differences ($p<0.05$). Y-axis titles are identical between panels A and B, and between C and D. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg ha$^{-1;}$ ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.

In 2022, GLU activity ranged from 0.0441 µmol h^{-1} g^{-1} (ST2) to 0.0662 µmol h^{-1} g^{-1} (S1), with S1 having 31.04% higher GLU than the control treatment (p=0.0359) (**Fig. 2.3a**). Applying 5 Mg ha^{-1} of biochar and incorporating it at 13cm-18cm (DT1) significantly increased GLU by 175% (p=0.0004) relative to the control treatment (**Fig. 2.3a**). For LAP activity, 2022 values ranged from 0.0513 µmol h^{-1} g^{-1} (ST2) to 0.066 µmol h^{-1} g^{-1} (S1) (**Fig. 2.3c**) on average. However, treatments did not vary significantly from each other. LAP activities in biochar-treated soils are also significantly lower than the control treatment, indicating that biochar application reduces LAP except with surface application of 15 Mg ha^{-1} biochar (S2). GLU and LAP activities were also greatly increased in 2023 (**Fig.2.3b, 2.3d**), two years after biochar was applied, which is similar with the trend observed for BG, NAG, and PHOS.

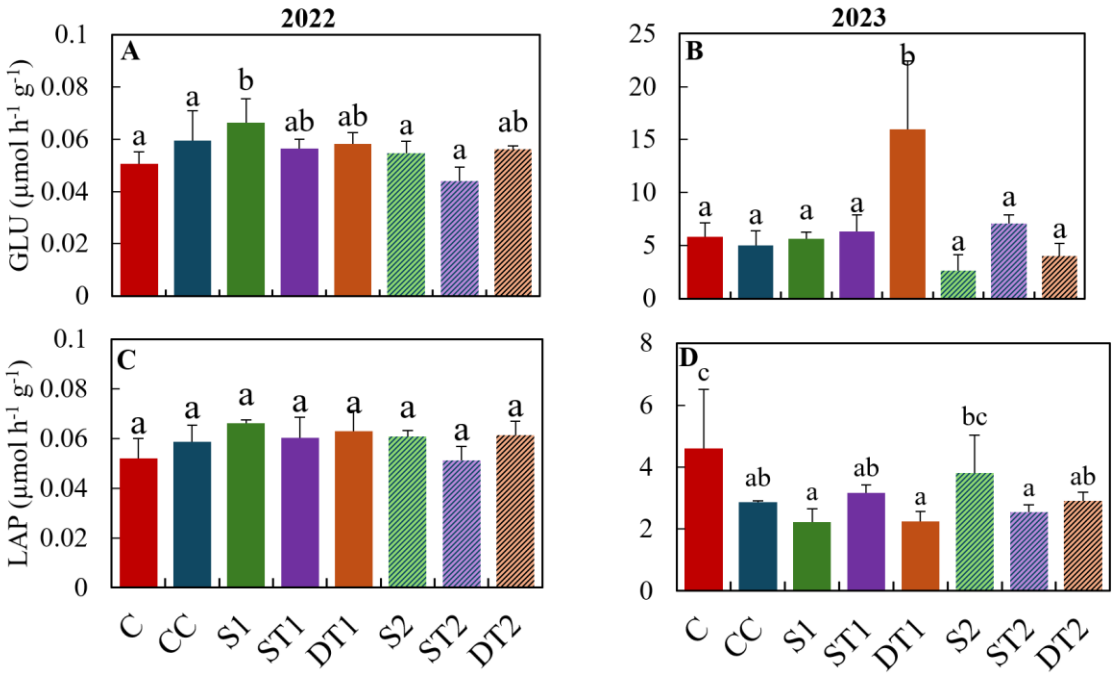

Figure 2.3. Mean activity (±SE) of leucine aminopeptidase (LAP) and L-glutamic acid enzyme (GLU) as influenced by biochar rates (5 Mg ha-1; 15 Mg ha-1) incorporated via surface application, shallow incorporation (8cm) and deep incorporation (15cm). Different lowercase letters show statistically significant differences (p<0.05). Y-axis titles are identical between panels A and B, and between C and D. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg ha^{-1}; ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.

Microbial community composition and diversity

In 2022, only the biochar application rate significantly affected total (bacterial + fungal) PLFA (p=0.0217). On average, total PLFA values in 2022 ranged from 2035.48 ng g^1 (ST1) to 3546.87 ng g^1 (DT1) (**Fig. 2.4a**). In all treatment plots, the highest total PLFA was recorded when biochar was applied at 5 Mg ha^{-1} and incorporated at 13cm-18cm of soil depth (DT2). Compared to the control treatment, DT2 increased total PLFA by 31.52% (p=0.0494). DT2 also had higher total PLFA than S1, ST1 and treatments with 15 Mg ha^{-1} of biochar (S2, ST2, DT2). Total PLFA in treatments declined in 2023 (**Fig. 2.4b**). Compared to the control treatment, 42.25% of total PLFA declined when 15 Mg ha^{-1} biochar was incorporated at 8cm-10cm (ST2) (p=0.0231). In addition, biochar application rate (p = 0.6921) and incorporation depth (p=0.838) did not significantly influence total PLFA in 2023.

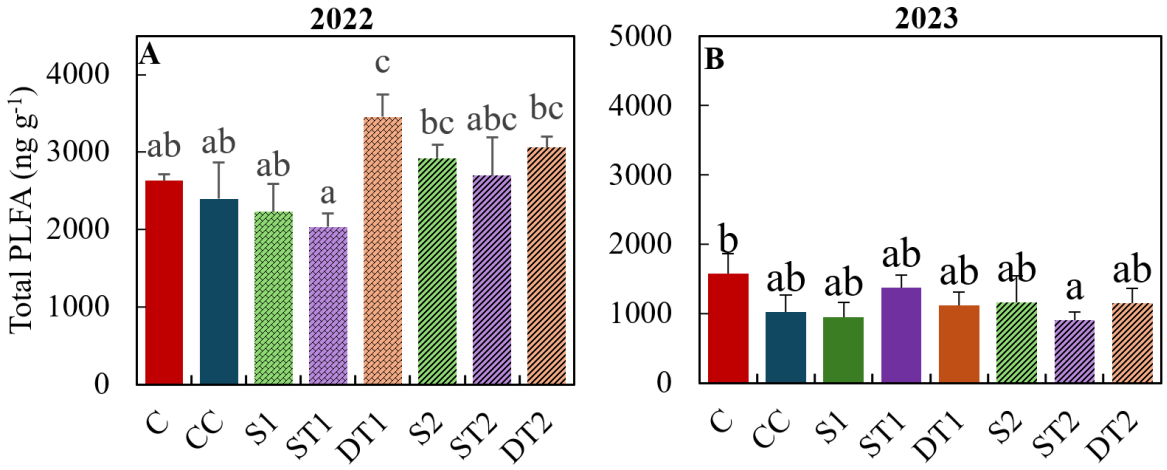

Figure 2.4. Total microbial biomass under different biochar rates (5 Mg $ha^{-1,}$ 15 Mg ha^{-1}) and incorporation depth (surface application, 8cm, 15cm). Bars show mean values and standard errors for each treatment. Different lower-case letters among treatments denote significant differences at $p < 0.05$. Y-axis titles are identical between panels A and B. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg $ha^{-1;}$ ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.

When examining PLFAs within the major microbial group (i.e., bacteria alone or fungi alone), biochar application rate significantly affected bacterial ($p = 0.0081$) and fungal PLFAs ($p=0.0051$) in 2022 (**Fig. 2.5a, 2.5c**). On average, bacterial PLFA ranged from 999.16 ng g^{1} (ST1) to 1738.03 ng g^{1} (DT1) (**Fig. 2.5a**) and 104.3 ng g^{1} (ST1) to 284.9 ng g^{1} (DT1) for fungal PLFA (**Fig. 2.5c**). Increasing the application rate did not significantly improve these parameters, except ST2 having 110% higher fungal PLFA than ST1. In addition, incorporation at higher rates did not improve bacterial and fungal PLFAs when treatments under 15 Mg ha^{-1} were compared (e.g., S2 vs. ST2 vs. DT2). Biochar application rate and incorporation depth also significantly affected bacterial and fungal PLFAs in 2023; however, all treatments had lower PLFAs in 2023 than in 2022 (**Fig. 2.3b, 2.3d**).

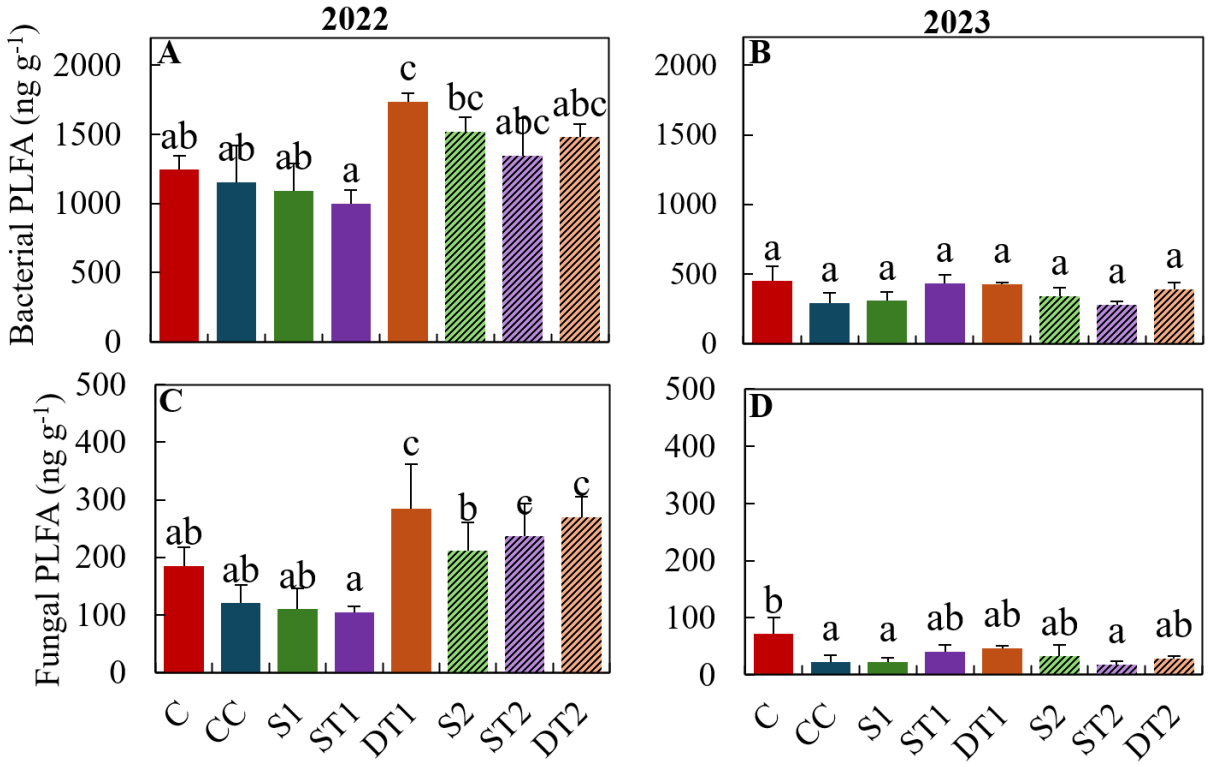

Figure 2.5. Microbial biomass of (a-b) bacteria and (c-d) fungi under different biochar rates (5 Mg ha$^{-1,}$ 15 Mg ha^{-1}) and incorporation depth (surface application, 8cm, 15cm) across two growing seasons. Error bars indicate standard error. Different lower-case letters among treatments denote significant differences at $p < 0.05$. Y-axis titles are identical between panels A and B, and between C and D. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg ha$^{-1;}$ ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.

Biochar rate had a significant influence on PLFAs for saprophytic fungi (p=0.0041), arbuscular mycorrhizal fungi (p=0.0195), and actinomycetes (p=0.0175) in 2022 (**Fig. 2.6a, 2.6c, 2.6e**). These are functional groups within soil fungi, and they responded positively to biochar application rate. Specifically, incorporating 5 Mg ha^{-1} of biochar at 13cm-18cm (DT1) had the highest saprophytic fungal PLFA and AMF PLFA among treatments (**Fig. 2.6a, 2.6c),** whereas increasing the application rate did not lead to significant improvement in PLFAs of SF, AMF, and actinomycetes. This pattern was also observed for fungal and bacterial PLFAs (**Fig. 2.5a, 2.5c**). Results showed that using 5 Mg ha^{-1} of biochar and incorporation at 13cm-18cm (DT1)

can benefit the growth of these fungal functional groups. However, during the second year, there was a significant decline in saprophytic fungi, AMF, and actinomycetes (**Fig. 2.6b, 2.6d, 2.6e**), and no significant differences were observed across treatments.

Soil organic carbon, total nitrogen, and C:N ratio

In 2022, the average amount of soil organic carbon in treatments ranged from 13.62 g kg^{-1} (CC) to 23.29 g kg^{-1} (DT2) (**Fig. 2.7a**). Biochar application rate significantly influenced SOC ($p = 0.0005$). Increasing the rate led to higher SOC, while biochar incorporation did not further improve this soil health indicator. For example, ST2 had 43.94% higher SOC than ST1, whereas the SOC in DT2 was 52.77% higher than DT1. The following year (2023), SOC in treatments significantly declined, especially those with 15 Mg ha^{-1} of biochar (S2, ST2, DT2) (**Fig. 2.7b**). On the other hand, total nitrogen was not significantly affected by biochar rate ($p = 0.166$) and incorporation depth ($p=0.2792$) (**Fig. S2.3**). Total N did not vary significantly among treatments in 2022 and 2023, except for CC having a 10.14% higher total N than S2 in 2022 (**Fig. 2.7c, 2.7d**).

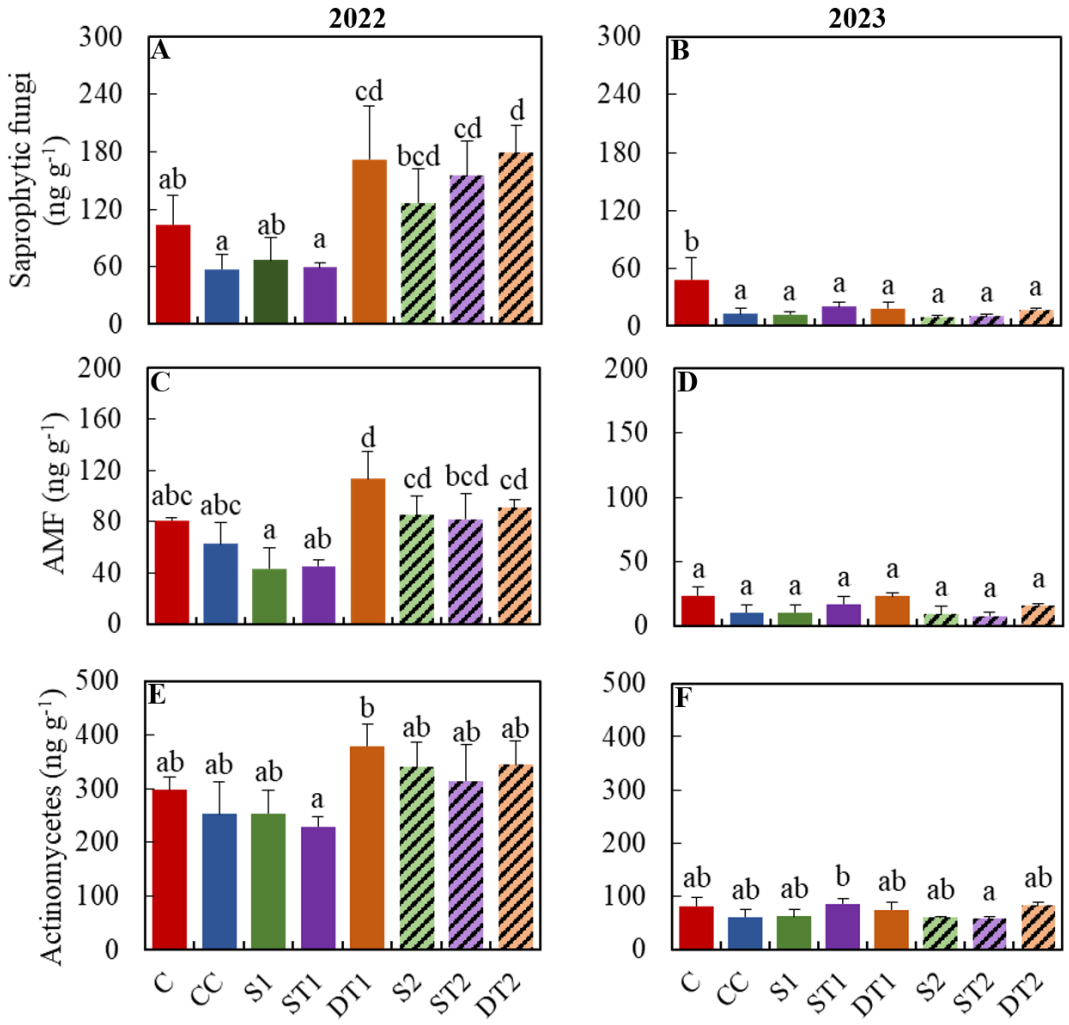

Figure 2.6. Microbial biomass of (a-b) saprophytic fungi, (c-d) arbuscular mycorrhizal fungi, and (e-f) actinomycetes biomass under different biochar rates (5 Mg ha$^{-1,}$ 15 Mg ha^{-1}) and incorporation depth (surface application, 8cm, 15cm) across two growing seasons. Error bars indicate standard error. Different lower-case letters among treatments denote significant differences at $p < 0.05$. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg ha$^{-1;}$ ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.

Biochar rate significantly affected soil carbon to nitrogen ratio ($p<0.001$) (**Fig. 2.7e, 2.7f**), with values ranging from 9.72 (CC) to 18.06 (DT2) in 2022 (**Fig. 2.7e**). Like SOC, increasing the biochar application rate from 5 Mg ha^{-1} to 15 Mg ha^{-1} also increased the C/N ratio. Specifically, S2, ST2, and DT2 had 10.5%, 50.04%, and 61.02% higher C/N ratio than S1, ST2, and DT2, respectively.

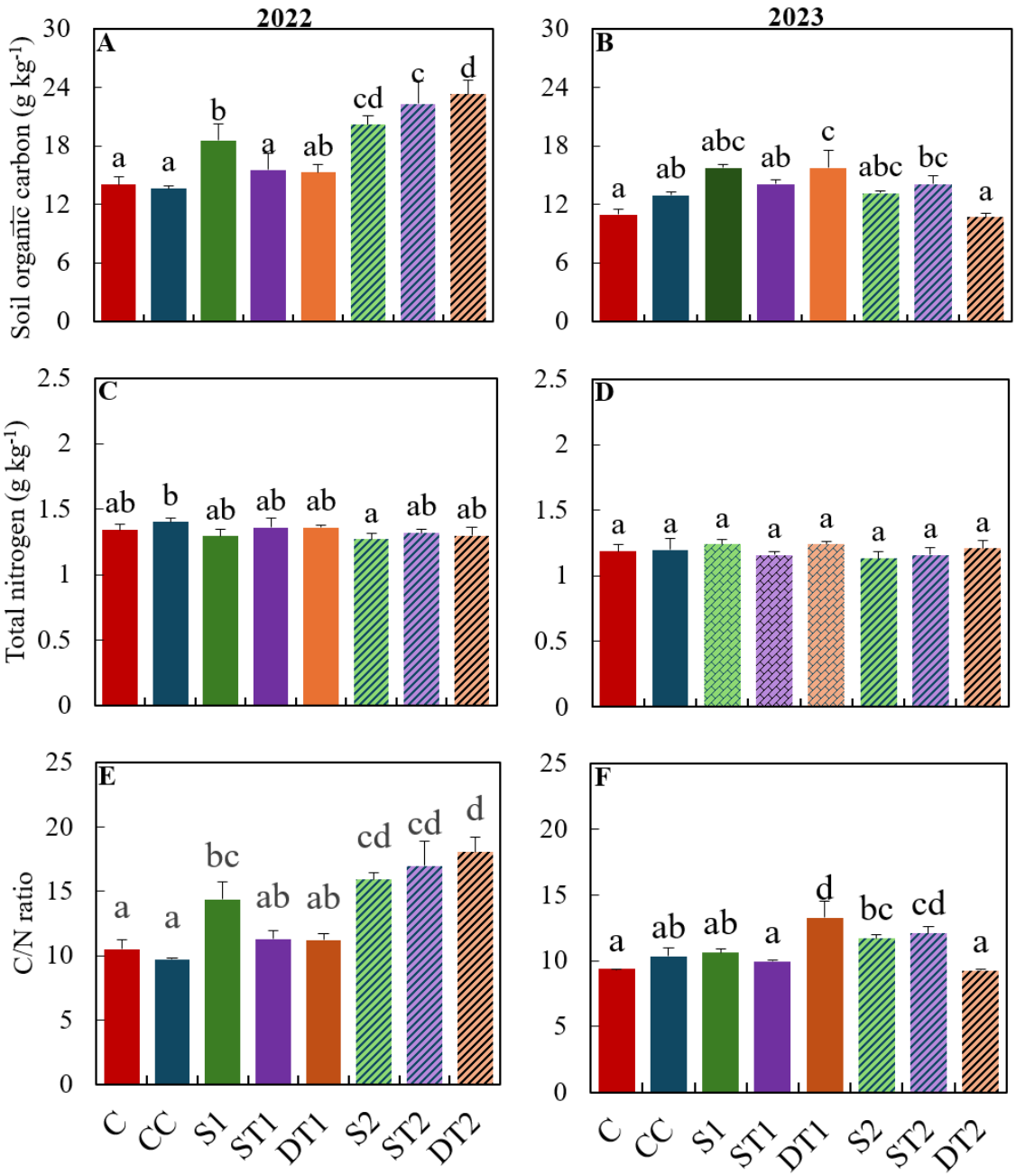

Figure 2.7. Soil organic carbon (a-b), total nitrogen (c-d), and carbon to nitrogen ratio under different biochar rates (5 Mg ha$^{-1,}$ 15 Mg ha^{-1}) and incorporation depth (surface application, 8cm-10cm, 13cm-18cm) across two growing seasons. Error bars indicate standard error. Different lower-case letters among treatments denote significant differences at $p < 0.05$. Y-axis titles are identical between panels A and B, C and D, and between E and F. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg ha$^{-1;}$ ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.

Short-term soil carbon respiration

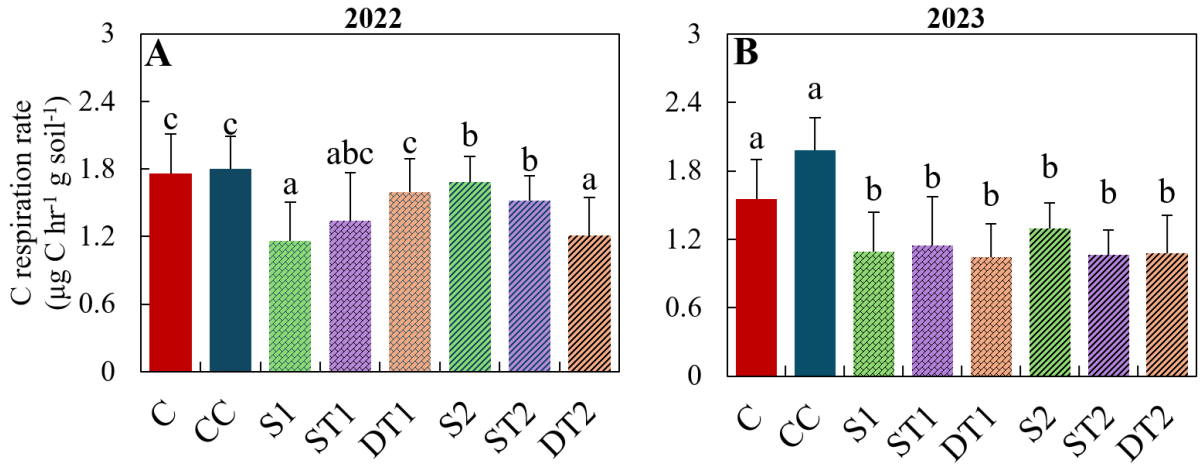

Figure 2.8. Soil carbon respiration rate of mid-season samples after seven days of laboratory incubation as influenced by biochar rates (5 Mg $ha^{-1,}$ 15 Mg ha^{-1}) and incorporation depth (surface application, 8cm-10cm, 13cm-18cm). Soil samples used in the incubation were taken in (a) October 2022 and (b) October 2023. Error bars indicate standard error. Different lower-case letters among treatments denote significant differences at $p < 0.05$. Y-axis titles are identical between panels A and B. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg $ha^{-1;}$ ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.

For each growing season, soil samples were taken at three time points (pre-planting, mid-season, late-season) and were incubated for seven days to determine soil C respiration rates. Overall, biochar application rate and incorporation depth did not affect the mean soil respiration rate after seven days of incubation. For pre-planting samples, the mean soil C respiration rate after seven days did not vary significantly across treatments ($p>0.05$) (**Fig. S2.2a**). On average, soil C respiration rate values ranged from 1.45 µg C hr^{-1} g $soil^{-1}$ (C) to 2.027 µg C hr^{-1} g $soil^{-1}$.

For mid-season samples, the mean soil C respiration rate varied across treatments after seven days of incubation (**Fig. 2.8**). Soil C respiration rates in 2022 ranged from 1.07 µg C hr^{-1} g $soil^{-1}$(S1) to 1.75 µg C hr^{-1} g $soil^{-1}$ (C) (**Fig. 2.8a**). Surface-applied biochar at 5 Mg ha^{-1} (S1) had significantly lower soil C respiration rates than plots under the control (C), cover crop (CC), S2, and ST2 treatments. On the other hand, the mean soil C respiration rate in DT1 is significantly

higher than in S1 and DT2. Surface application at 5 Mg ha^{-1} (S1) and incorporating 15 Mg ha^{-1} of biochar at 15cm-18cm (DT2) may reduce soil C respiration. However, this pattern is inconsistent with generated results in 2022 and 2023 (**Fig. S2.2**). During the late season, mean soil C respiration rate did not vary among treatments except for S1, which had a significantly higher CO_2 flux than ST2 (**Fig. S2.2c)**. Soil respiration rates in 2022 did not vary significantly from 2023 rates.

3.5: Discussion

Effects of biochar on soil extracellular enzymes involved in C-, N- and P-cycling

Results showed no significant effects of biochar application on BG, NAG, and LAP activity rates, which are soil extracellular enzymes participating in C and N acquisition. A negative or neutral response of these enzymes to biochar can be attributed to biochar's high specific surface area and porosity, which can slow down degradation by making substrates unavailable (Lammirato et al., 2011). This response may arise from various mechanisms: (1) biochar inducing the liberation of soluble organic compounds, which can bind and hinder the production of extracellular enzyme activities; and (2) enzymes being adsorbed into the biochar's surface, leading to their deactivation and separation in space from potential substrates (Jones et al., 2011). It is also possible that the oakwood biochar did not stimulate soil enzyme activities mainly because of its high C:N ratio, rendering the biochar C resistant to microbial degradation (Ameloot et al., 2014).

Feedstock type and pyrolysis conditions (e.g., temperature, duration) primarily determine biochar's intrinsic biochemical lability or stability. For example, Wu et al. (2013) found that wheat straw biochar did not affect BG activity, while adding wheat straw significantly increased BG. Since this study used oakwood biochar, it has high lignin and cellulose content, making it

highly stable in the soil and resistant to microbial decomposition (Domingues et al., 2017). Soil microorganisms will then prefer to utilize readily available carbon from compounds that easily degrade or those with lower lignin and cellulose concentrations – a mechanism called preferential substrate utilization (Wang et al., 2016). On the other hand, a meta-analysis showed that wood-based biochar had the greatest stimulating effect on enzyme activity, followed by crop-based and manure-based biochar (Chen et al., 2022).

Biochar can also have contrasting effects on enzyme activities and can be influenced by application rate, soil type, pyrolysis duration, and pyrolysis temperature. For instance, biochar applications of 20 t ha^{-1} and 40 t ha^{-1} in a rice paddy resulted in a 23% and 26% reduction in BG, respectively, compared to the control treatment (Pukalchik et al., 2018). Bailey et al. (2011) studied the effects of biochar produced from fast pyrolysis of switchgrass on four enzymes, including BG and NAG. They found that biochar can have variable effects on soil enzyme activities depending on soil type and the enzyme. Using biochar produced at high pyrolysis temperatures can increase soil pH (due to carbonates and high alkalinity), improving BG and NAG activity (Chen et al., 2022). Song et al. (2018) observed that combining biochar with N, P, and K fertilizers promotes BG and NAG activity, but NAG would decline with increasing biochar pyrolysis temperature, i.e., >300C. The volatile compounds in biochar produced at low pyrolysis temperatures (350–500°C) also stimulated BG and dehydrogenase activities (Ameloot et al., 2013; Bailey et al., 2011). In a study by Sun et al. (2022), LAP activity either increased or remained unchanged when only biochar was applied to the soil. LAP decreased when nitrogen fertilizer was applied with biochar. Moreover, biochar application also increased C and N acquisition enzymes by 9.3% and 15.1% on average, but higher pyrolysis temperatures can have lower stimulating effects on enzyme activity (Chen et al., 2022).

This study also demonstrated a neutral response of phosphatase (PHOS) to biochar application. Soil phosphatases (acid or alkaline) mineralize organic phosphorus by hydrolyzing phosphoric acid esters, making P available to plants and microorganisms (Schimel & Weintraub, 2003). Based on a meta-analysis conducted by Chen et al. (2022), the influence of biochar in P-acquisition enzyme activities was not significant. On the other hand, Khadem and Raiesi (2019) detected an increase (3.1 to 9.7-fold) in PHOS after 90 days of incubating a calcareous soil with biochar made from corn stalks produced at 400◦C and 600◦C. They also found that PHOS activity is higher in plots applied with biochar produced at a lower pyrolysis temperature (400◦C > 600◦C) and those with coarser soil texture (sandy loam>clay). In addition, biochar can significantly enhance the hotspots of phosphatase activity, which are distributed along living plant roots and are highest on the root tips (Wang et al., 2023). Hence, considering the sampling location in the field is also essential when conducting enzyme assays, specifically on PHOS.

As biochar ages in the soil, enzyme activities are expected to decrease. They are highly associated with changes in soil moisture content and oxygenation rather than the amount of soil C and N (Futa et al., 2020). However, this study showed that EEAs increased two years after applying biochar (2023). Based on a meta-analysis, biochar can improve enzyme activities for at most three growing seasons (Chen et al., 2022). There are three mechanisms where biochar can influence EEAs: adsorption, supply of substrates, and improvement of soil physicochemical properties and functions (Zhang et al., 2019; Zhu et al., 2017). Moreover, among other edaphic factors, soil pH, SOC, total N, and clay content were the most critical drivers of extracellular enzyme activities (Chen et al., 2022). Adding biochar in fine-textured soils can act as a binding agent to facilitate greater adsorption of clay minerals and organic matter. This increase in adsorption creates an environment suitable for soil biota, such as improving aggregate formation

and stability, which protects OM and helps with soil water and nutrient retention (Lehmann et al., 2011). Soils with high OM can supply more C and N sources for microbial consumption and growth, thus supporting the accumulation of microbial biomass and the production of extracellular enzymes. Conducting long-term field studies can further reveal the impact of biochar on EEAs and its duration.

Effects of biochar rate and incorporation on soil microbial community composition

Biochar can alter the structure of soil bacterial communities. In this study, incorporating 5 Mg ha^{-1} at 8cm-10cm (DT1) significantly increased total PLFA compared to the surface application (S1) and the control treatment. On the other hand, increasing the application rate to 15 Mg ha^{-1} and incorporation did not further increase the total PLFA. This is also the same trend observed for bacterial and fungal PLFAs. Based on a 3.5-year spring maize field experiment, adding 50 tons ha^{-1} of biochar increased fungal diversity in the upper 20cm soil depth (Luo et al. (2017). Bacterial abundance also increased with biochar addition under 2%, 4%, and 8% biochar doses (based on the total mass of the top 20cm of soil) (Yao et al., 2017). Other studies also showed that biochar increases total PLFAs in sandy loam soil (Chen et al.,2017) or in contrast to conventional tillage (Amoakwah et al., 2022). Our study used a biochar produced from oakwood chips. Its high porosity and large specific area may have stimulated the growth and reproduction of soil bacteria, thereby changing the microbial community composition. Jones et al. (2011) also emphasized the adsorption characteristics of biochar being accountable for the modifications in this soil health indicator.

Microbial groups (e.g., bacteria and fungi) can respond to biochar depending on the application rate and soil residence time (Han et al., 2020). Several mechanisms have been proposed to explain the improved bacterial and fungal abundance after biochar application: (1)

the OC in biochar acting as readily available source of energy and nutrients for effective soil microbial growth and development; (2) biochar providing habitat for soil microorganisms and protecting them from predators; (3) biochar acting as a slow-release fertilizer which benefits microbial growth; and (4) biochar improving the availability of anions and cations due to its large porosity and sorption capacity (He et al., 2021; Palansooriya et al., 2019; Zhu et al., 2017). However, soils with high native SOC can cause priming effects through co-metabolisms of labile C fractions of biochar to stimulate microbial and enzyme activity (Chen et al., 2022). Increasing soil total N can also compensate for high C:N ratios after biochar application to increase soil microbial biomass and enzyme production (Zhang et al., 2018). Hence, enough soil organic C and available N should be provided first to meet microbial stoichiometric requirements and improve crop productivity, especially in low fertile soils under biochar amendment.

Microbial diversity is important in rejuvenating and maintaining soil health to support ecosystem functions and crop productivity. Applying soil amendments and other inputs in an agroecosystem can induce changes in microbial community composition (Liu et al., 2017). This study demonstrated that biochar application rate significantly affected major microbial groups. The PLFA data showed higher $PLFA_{Bacteria}$ than $PLFA_{Fungi}$ in 2022 and 2023. One possible explanation is that the highly alkaline nature of biochar favored the survival of fast-growing bacteria, resulting in a negative fungal response when labile C substrates in biochar favor bacteria (Ippolito et al., 2014). Rousk et al. (2009) also revealed that with a lower pH (pH 4.0), there was a fivefold increase in fungal growth and a fivefold decrease in bacterial growth. Therefore, higher fungal growth than bacterial growth may be observed in acidic soils than in neutral to alkaline soils.

Functional groups of fungi, such as saprophytic fungi (SF) and arbuscular mycorrhizal fungi (AMF), also significantly impact soil health and biochar degradation. Saprophytic fungi, for example, decompose non-living, organic matter and produce enzymes to degrade cellulose, hemicellulose, and pectin (Brundrett, 2002). AMF is known to help plant roots acquire nutrients such as P, S, N, and micronutrients and contribute significantly to soil aggregation (Vanderwolf et al., 2013), while their abundance is usually associated with reduced nutrient availability (Lehmann et al., 2011; Luo et al., 2017b). In this study, $PLFA_{SF}$ and $PLFA_{AMF}$ were highest in soils with 5 Mg ha^{-1} of biochar incorporated at 5cm (DT1). Like total PLFA, incorporating biochar in the soil at a higher rate (15 Mg ha^{-1}) did not further increase $PLFA_{SF}$. $PLFA_{SF}$ was also more abundant than $PLFA_{AMF}$. Saprophytic fungi are the primary degraders of biochar in soils; they excrete oxidative enzymes that can degrade lignin (Gomez et al., 2014a). Hence, they are expected to be abundant in biochar-treated soils. Warnock et al. (2010) observed that AMF abundance decreased or remained unchanged in biochar-treated soils and that applying large quantities of lodgepole pine biochar (2% and 4%, w/w) resulted in 58% and 73% declines in AMF abundance in roots, respectively. On the contrary, other studies have shown that AMF can benefit from biochar while SF is reduced (Luo et al., 2017).

Fungi and actinomycetes are predicted to be the primary consumers of biochar-C as they obtain their energy through decomposing organic materials. They have an advantage over other microbiota due to their metabolic ability to degrade complex biomass, lignocelluloses, and other polysaccharides (Kabuyah et al., 2012). Actinomycetes are versatile microorganisms that can produce enzymes such as cellulases, chitinases, and proteases, essential in sustainable soil health. However, in this study, biochar application did not significantly improve actinomycetes relative to the control treatment. Overall, microbial biomass declined in all treatments two years after

applying biochar. This decrease can result from some toxic compounds in biochar that can inhibit microbial activity, such as benzene, ketones, furans, and PAHs. Hence, identifying the presence of these compounds in biochar has important implications for microbial dynamics in the soil.

While studies explore the effect of biochar application rate on microbial abundance and diversity, they have varying findings on which microbial groups are primarily influenced by biochar and the conditions that drive these effects. Biochar feedstock type may play a crucial role, resulting in varied reactions among fungal and bacterial groups regarding their preferred energy sources (Wang et al., 2016b). However, limited published research specifically looks at the impact of biochar incorporation on microbial communities (Gomez et al., 2014; Lehmann et al., 2011).

Biochar effects on soil organic carbon

This study showed that soil organic carbon increased with biochar application rate, especially when incorporated into the soil. A meta-analysis also demonstrated that SOC increased by 23% to 59% with biochar application rate. Moreover, soils with coarser texture and SOC greater than 20g kg^{-1} responded more strongly to biochar addition. The added carbon from biochar enhances SOC levels, leading to higher biomass accumulation and greater retention of plant residues in the soil Chen et al. (2022). The increased labile organic C from biochar can also rapidly stimulate short-term microbial biomass production (Maestrini et al., 2014) and offer additional C credit due to biochar's high C/N ratio (Lehmann et al., 2006). However, this may cause N or P limitation because of high microbial demands for nutrients (Fang et al., 2018). The nutrient limitation may only spring from using biochar produced at low pyrolysis temperature

because of its high labile C fractions, which can be alleviated by providing available N (Ippolito et al., 2020).

However, according to this study, biochar's benefit to SOC has only lasted within one growing season. As biochar ages in the soil, a part of the biochar is microbially accessible and can be mineralized to CO_2 with rates as high as 15% to 20% of the biochar-C. However, this mineralization rate can decrease over time and stay at a daily rate of 0.001% to 0.003% (Han et al., 2020). Microorganisms can also completely exhaust the labile C in biochar within a year after application (Wang et al., 2016). It is possible that the observed decrease in SOC among treatments is because of the rapid mineralization of biochar's labile C pool and can only last short-term (1-2 years). At the same time, the remaining stable OC will be mineralized very slowly by microorganisms. Aside from biochar's residence time, feedstock type and pyrolysis conditions also play a vital role in the percentage of mineralized OC in biochar. Manure and crop-based biochar mineralize faster than wood-based biochar mainly because they have higher concentrations of aromatic carbon. In contrast, biochar produced at high pyrolysis temperatures has a relatively lower mineralized OC content than that produced at low temperatures (Chen et al., 2017). Soils with a clay content of >20% also tend to reduce the amount of mineralized biochar. The OC fractions in biochar are highly soluble and can be transferred to the soil solution by dissolution (Han et al., 2020). Dissolved organic carbon (DOC) is considered the main form of labile C in biochar. Hence, it can be used as an indicator of the lability of biochar OC (Major et al., 2010). The possibility of biochar impeding the natural turnover of soil organic matter over an extended period warrants additional exploration, as it could imply a dual approach to enhancing carbon sequestration in the soil.

Effect of biochar on short-term soil carbon respiration

This study showed that increasing the biochar rate and incorporation depth did not increase short-term soil carbon respiration across two growing seasons. In a meta-analysis, Liu et al. (2016) revealed that biochar amendment does not significantly affect soil carbon respiration across the entire set of studies. Kuzyakov et al. (2009) and Singh and Cowie (2010) also reported a lack of significant response of soil carbon respiration to biochar. Additionally, the application of biochar produced from biosolids, poultry litter, and papermill waste did not result in a net increase in soil carbon respiration following nitrogen amendment after a 48-day incubation (Van Zwieten et al., 2010). Lehmann and Rondon (2006) proposed that biochar applied at higher rates may suppress soil C mineralization due to the high C/N ratio, leading to low microbial N availability. The decrease in soil carbon respiration at higher biochar application rates may also be associated with specific biochar characteristics and their impacts on soil properties. Biochar created at high temperatures is more resistant to decomposition and, thereby, would be a better candidate for soil C sequestration (Novak et al., 2010; Harvey et al., 2012, as cited in Liu et al., 2016).

However, other laboratory and field incubation studies have often found a short-term increase in soil carbon respiration after adding biochar to the soil. Kimetu and Lehmann (2010) compared the carbon loss in C-rich and C-poor soils after biochar application, and they found that high soil carbon respiration rates are associated with a relatively high SOC. In coarse-textured soils, biochar amendment significantly increased soil carbon respiration, whereas it had the opposite effect in fine-textured soils. Combining biochar with synthetic nitrogen fertilizer significantly increased soil carbon respiration (Liu et al., 2016a). An increase in CO_2 can also originate from applying fresh biochar, which provides labile C to soil microbes. Nevertheless,

short-term soil C respiration in biochar-amended soils should not degrade their potential for long-term C sequestration (Jones et al., 2011).

3.6: Conclusion

This study assessed the impact of biochar application rate and incorporation depth on soil health indicators. Soil EEAs (e.g., BG, NAG, PHOS, LAP, GLU) showed no significant response to oakwood-derived biochar. On the other hand, applying 5 Mg ha^{-1} of biochar at 13cm-18cm depth led to elevated PLFA concentrations for functional groups of fungi such as saprophytic fungi and arbuscular mycorrhizal fungi, indicating that biochar can alter the soil microbial community structure. Results also suggest that higher application rates and incorporation depth amplify the benefit of biochar on SOC. When using 5 Mg ha^{-1} of biochar, surface application or deep incorporation (13cm-18cm) is recommended to prevent a significant decline in SOC. However, this effect persisted within a year after applying biochar in 2021. Generally, the biochar used in this study had a short-term benefit to SOC and microbial community structure, while soil EEAs and short-term C respiration were not significantly affected. As we strive to determine the potential benefits of biochar application to soil health, the findings in this study help provide a foundational understanding of the short-term responses. Future studies should delve deeper into the dynamics involved in long-term biochar persistence and identify biochar's contribution to soil health. It is also recommended to establish studies that will determine if applying biochar annually is needed to replenish its benefits to SOC and other soil health indicators.

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APPENDIX B: Chapter 2 Supplemental Information

Table S2.1. ANOVA results for extracellular enzyme activities showing degrees of freedom (df), F-values and p-values for all response variables.

Enzyme	Effect	df	F		p	
			2022	2023	2022	2023
β-glucosidase (BG)	*Biochar rate*	1	0.11	0.32	0.7467	0.5755
	Incorporation depth	2	1.98	0.79	0.1723	0.4643
	Rate x inc depth	2	0.14	2.5	0.8704	0.1042
N-acetyl glucosaminidase (NAG)	*Biochar rate*	1	0.01	0.08	0.9325	0.7815
	Incorporation depth	2	0.68	1.99	0.5234	0.1589
	Rate x inc depth	2	1.33	1.09	0.2938	0.3522
Phosphatase (PHOS)	*Biochar rate*	1	2.73	1.93	0.1193	0.1781
	Incorporation depth	2	0.58	1.17	0.5729	0.3281
	Rate x inc depth	2	0.13	0.7	0.8817	0.5048
Leucine aminopeptidase (LAP)	*Biochar rate*	1	0.42	0.72	0.5259	0.406
	Incorporation depth	2	0.8	1.02	0.4693	0.3774
	Rate x inc depth	2	0.6	0.29	0.5617	0.7541
Glutamic acid enzyme (GLU)	*Biochar rate*	1	2.73	1.93	0.1193	0.1781
	Incorporation depth	2	0.58	1.17	0.5729	0.3281
	Rate x inc depth	2	0.13	0.7	0.8817	0.5048

Table S2.2. ANOVA results for phospholipid fatty acid (PLFA) showing degrees of freedom (df), F-values and p-values for all response variables.

PLFA	Effect	df	F		p	
			2022	2023	2022	2023
Total	*Biochar rate*	1	6.56	0.16	0.0217	0.6921
	Incorporation depth	2	0.62	0.18	0.5513	0.838
	Rate x inc depth	2	0.06	2.72	0.9457	0.087
Bacteria	*Biochar rate*	1	9.32	0.12	0.0081	0.7336
	Incorporation depth	2	0.55	0.36	0.5898	0.7044
	Rate x inc depth	2	0.03	3.13	0.9738	0.0628
Fungi	*Biochar rate*	1	10.7	0	0.0051	0.9994
	Incorporation depth	2	0.49	0.02	0.622	0.9758
	Rate x inc depth	2	0.52	1.94	0.6071	0.167
Saprophytic fungi (SF)	*Biochar rate*	1	11.47	0.04	0.0041	0.8492
	Incorporation depth	2	0.35	0.07	0.7097	0.9313
	Rate x inc depth	2	0.67	2.56	0.5284	0.0991
Arbuscular mycorrhizal fungi (AMF)	*Biochar rate*	1	6.85	0.05	0.0195	0.8217
	Incorporation depth	2	1.33	0.24	0.2934	0.7889
	Rate x inc depth	2	0.25	1.21	0.7796	0.3158
Actinomycetes	*Biochar rate*	1	7.12	0.01	0.0175	0.9121
	Incorporation depth	2	0.08	0.33	0.9255	0.7213
	Rate x inc depth	2	0.15	3.58	0.8641	0.0443

Table S2.3. ANOVA results for soil organic carbon, total nitrogen, and carbon to nitrogen ratio showing degrees of freedom (df), F-values and p-values for all response variables.

Response variable	Effect	df	F		p	
			2022	2023	2022	2023
Soil organic carbon	*Biochar rate*	1	19.39	13.27	0.0005	0.0014
	Incorporation depth	2	0.05	0.3	0.9537	0.7433
	Rate x inc depth	2	2.48	0.65	0.1178	0.5331
Total nitrogen	*Biochar rate*	1	2.12	1.23	0.166	0.2792
	Incorporation depth	2	1.53	0.22	0.2484	0.8003
	Rate x inc depth	2	0.16	0.95	0.8553	0.4032
Carbon to nitrogen ratio	*Biochar rate*	1	32.95	18	<.0001	0.0003
	Incorporation depth	2	0.51	1.09	0.6118	0.3538
	Rate x inc depth	2	3.95	1.01	0.0418	0.3783

Table S2.4. Soil extracellular enzyme activity rates under biochar and non-biochar treated plots (2022). BG – β-glucosidase, NAG – N-acetyl-glucosaminidase, PHOS – phosphatase, LAP – Leucine aminopeptidase, GLU – glutamic acid enzyme. Treatment abbreviations follow Table 2.1.

Plot	Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Enzyme activity (nmol h^{-1} g^{-1})				
					BG	NAG	PHOS	LAP	GLU
102	CC	Cover crop only	NA	R1	0.262	0.049	0.026	0.074	0.060
103	ST2	15	8cm-10cm	R1	0.226	0.077	0.024	0.052	0.052
104	S1	5	surface application	R1	0.195	0.058	0.051	0.066	0.049
105	DT1	5	13cm-18cm	R1	0.237	0.061	0.039	0.051	0.050
106	S2	15	surface application	R1	0.195	0.057	0.030	0.064	0.059
107	C	No biochar, no cover crop	NA	R1	0.165	0.064	0.031	0.032	0.046
108	ST1	5	8cm-10cm	R1	0.232	0.064	0.047	0.041	0.049
109	DT2	15	13cm-18cm	R1	0.347	0.081	0.085	0.062	0.057
201	CC	Cover crop only	NA	R2	0.292	0.063	0.062	0.061	0.051
202	ST1	5	8cm-10cm	R2	0.304	0.087	0.056	0.078	0.058
205	S1	5	surface application	R2	0.336	0.059	0.065	0.068	0.062
206	DT1	5	13cm-18cm	R2	0.305	0.101	0.054	0.051	0.053
207	C	No biochar, no cover crop	NA	R2	0.314	0.113	0.066	0.046	0.064
208	S2	15	surface application	R2	0.357	0.105	0.078	0.064	0.063
209	DT2	15	13cm-18cm	R2	0.246	0.077	0.063	0.046	0.052
210	ST2	15	8cm-10cm	R2	0.315	0.084	0.080	0.065	0.054
301	S1	5	surface application	R3	0.291	0.104	0.075	0.068	0.062
302	DT2	15	13cm-18cm	R3	0.332	0.141	0.083	0.069	0.057
304	S2	15	surface application	R3	0.349	0.114	0.061	0.061	0.056
305	ST2	15	8cm-10cm	R3	0.199	0.063	0.039	0.049	0.038
306	CC	Cover crop only	NA	R3	0.224	0.070	0.049	0.041	0.037
308	ST1	5	8cm-10cm	R3	0.211	0.093	0.074	0.069	0.065
309	C	No biochar, no cover crop	NA	R3	0.237	0.069	0.051	0.067	0.045
310	DT1	5	13cm-18cm	R3	0.330	0.079	0.048	0.086	0.062
401	DT1	5	13cm-18cm	R4	0.222	0.059	0.050	0.064	0.068

Table S2.4. (cont'd)

Plot	Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Enzyme activity (nmol h^{-1} g^{-1})				
					BG	NAG	PHOS	LAP	GLU
402	S2	15	surface application	R4	0.218	0.063	0.035	0.054	0.041
403	C	No biochar, no cover crop	NA	R4	0.255	0.089	0.077	0.063	0.047
404	DT2	15	13cm-18cm	R4	0.292	0.101	0.071	0.068	0.058
406	CC	Cover crop only	NA	R4	0.291	0.081	0.075	0.059	0.090
407	S1	5	surface application	R4	0.322	0.083	0.054	0.062	0.092
408	ST2	15	8cm-10cm	R4	0.176	0.049	0.029	0.039	0.032
409	ST1	5	8cm-10cm	R4	0.243	0.092	0.044	0.053	0.053

Table S2.5. Extracellular enzyme activity rates under biochar and non-biochar treatments (2023). BG – β-glucosidase, NAG – N-acetyl-glucosaminidase, PHOS – phosphatase, LAP – Leucine aminopeptidase, GLU – glutamic acid enzyme. Treatment abbreviations follow Table 2.1.

Plot	Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Enzyme activity (nmol h^{-1} g^{-1})				
					BG	NAG	PHOS	LAP	GLU
102	CC	Cover crop only	NA	1	0.241	1.359	0.500	2.844	8.787
103	ST2	15	8cm-10cm	1	0.066	1.157	0.376	5.188	9.625
103	ST2	15	8cm-10cm	1	0.133	1.144	0.284	2.573	6.481
104	S1	5	surface application	1	0.061	0.543	0.221	8.073	5.820
105	DT1	5	13cm-18cm	1	3.764	1.360	0.350	3.156	12.002
106	S2	15	surface application	1	1.979	0.792	0.193	1.544	0.049
107	C	No biochar, no cover crop	NA	1	1.691	1.408	0.395	2.435	6.024
108	ST1	5	8cm-10cm	1	2.998	1.501	0.412	3.441	2.775
108	ST1	5	8cm-10cm	1	4.486	1.334	0.283	7.638	26.338
109	DT2	15	13cm-18cm	1	3.527	1.609	0.452	2.526	5.037
201	CC	Cover crop only	NA	2	4.434	1.204	0.355	2.801	3.338
202	ST1	5	8cm-10cm	2	4.181	1.004	0.230	2.893	4.201
202	ST1	5	8cm-10cm	2	0.240	0.523	0.270	2.514	13.147
205	S1	5	surface application	2	2.114	0.798	0.242	2.936	4.939
206	DT1	5	13cm-18cm	2	2.692	0.782	0.205	1.728	15.086
207	C	No biochar, no cover crop	NA	2	1.749	0.400	0.256	1.720	8.443
208	S2	15	surface application	2	2.861	0.626	0.434	3.612	113.633
209	DT2	15	13cm-18cm	2	1.779	0.334	0.256	2.362	11.080
210	ST2	15	8cm-10cm	2	2.226	1.071	1.020	3.415	8.763
210	ST2	15	8cm-10cm	2	1.963	0.646	0.193	2.061	7.015
301	S1	5	surface application	3	1.947	0.903	0.199	1.418	7.346
302	DT2	15	13cm-18cm	3	2.014	1.099	0.260	3.226	1.576
304	S2	15	surface application	3	2.308	0.699	0.188	2.884	5.187
305	ST2	15	8cm-10cm	3	3.294	0.946	0.244	1.845	5.129

Table S2.5. (cont'd)

Plot	Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Enzyme activity (nmol h^{-1} g^{-1})				
					BG	NAG	PHOS	LAP	GLU
305	ST2	15	8cm-10cm	3	0.612	0.569	0.157	1.934	3.525
306	CC	Cover crop only	NA	3	2.104	0.614	0.166	3.487	2.677
308	ST1	5	8cm-10cm	3	2.066	1.342	0.223	2.897	4.014
308	ST1	5	8cm-10cm	3	4.401	1.316	0.221	2.975	11.129
309	C	No biochar, no cover crop	NA	3	4.563	1.334	0.426	4.145	2.037
310	DT1	5	13cm-18cm	3	4.038	1.534	0.345	1.952	3.101
401	DT1	5	13cm-18cm	4	2.505	0.854	1.660	2.174	33.735
402	S2	15	surface application	4	2.144	0.966	0.322	7.248	2.682
403	C	No biochar, no cover crop	NA	4	2.122	1.273	0.283	10.108	6.727
404	DT2	15	13cm-18cm	4	6.598	1.052	0.237	3.518	5.372
406	CC	Cover crop only	NA	4	1.962	0.773	0.227	2.954	5.378
407	S1	5	surface application	4	1.871	0.951	0.334	2.307	4.479
408	ST2	15	8cm-10cm	4	3.162	0.466	0.435	3.224	10.309
408	ST2	15	8cm-10cm	4	2.604	0.721	0.294	2.748	5.579
409	ST1	5	8cm-10cm	4	2.689	0.684	0.246	4.475	4.739
409	ST1	5	8cm-10cm	4	1.598	0.646	0.552	3.073	4.351

Table S2.6. Phospholipid fatty acid concentration under biochar and non-biochar treated plots (2022). AMF – arbuscular mycorrhizal fungi, SF – saprophytic fungi. Treatment abbreviations follow Table 2.1.

Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Phospholipid Fatty Acid (ng g^{-1})					
				Total PLFA	Bacterial PLFA	Fungal PLFA	Actinomycetes PLFA	AMF PLFA	SF PLFA
CC	Cover crop only	NA	1	1429.31	530.90	64.70	103.85	26.77	37.93
ST2	15	8cm-10cm	1	1296.88	593.61	76.22	115.12	27.60	48.62
S1	5	surface application	1	1521.22	710.89	38.01	156.56	0.00	38.01
DT1	5	13cm-18cm	1	1319.74	602.15	27.44	121.82	0.00	27.44
S2	15	surface application	1	2122.27	990.02	39.02	204.25	0.00	39.02
C	No biochar, no cover crop	NA	1	2470.23	1047.11	245.61	254.25	83.77	161.84
ST1	5	8cm-10cm	1	1837.27	777.23	91.46	179.44	38.85	52.61
DT2	15	13cm-18cm	1	2777.69	1291.53	200.45	258.29	79.26	121.19
CC	Cover crop only	NA	2	1781.32	925.41	78.04	226.67	45.91	32.13
ST1	5	8cm-10cm	2	2464.94	1204.68	123.63	256.97	54.96	68.67
S1	5	surface application	2	2461.27	1114.25	109.49	263.12	63.41	46.08
DT1	5	13cm-18cm	2	3061.26	1639.19	192.02	321.27	85.67	106.35
C	No biochar, no cover crop	NA	2	2750.68	1367.16	134.37	332.62	75.83	58.54
S2	15	surface application	2	2750.36	1386.58	168.05	281.12	62.71	105.34
DT2	15	13cm-18cm	2	3185.65	1570.53	313.17	373.12	99.17	214.00
ST2	15	8cm-10cm	2	3212.18	1612.09	283.39	382.42	96.46	186.93
S1	5	surface application	3	3133.15	1625.78	211.14	367.03	71.50	139.64
DT2	15	13cm-18cm	3	4268.67	2153.57	363.54	490.09	129.04	234.50

Table S2.6. (cont'd)

Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Phospholipid Fatty Acid (ng g^{-1})					
				Total PLFA	Bacterial PLFA	Fungal PLFA	Actinomycetes PLFA	AMF PLFA	SF PLFA
S2	15	surface application	3	2736.82	1448.56	158.31	305.23	80.57	77.74
ST2	15	8cm-10cm	3	3558.72	1840.79	335.75	419.90	122.36	213.38
CC	Cover crop only	NA	3	3405.77	1725.72	201.07	388.67	104.52	96.55
ST1	5	8cm-10cm	3	2152.09	1131.40	118.94	251.67	53.40	65.54
C	No biochar, no cover crop	NA	3	3430.90	1731.93	354.41	426.98	129.70	224.70
DT1	5	13cm-18cm	4	4010.70	1850.54	437.40	456.32	155.03	282.38
DT1	5	13cm-18cm	4	3298.64	1724.36	225.29	358.07	98.79	126.50
S2	15	surface application	4	3273.38	1725.73	308.94	432.39	112.27	196.68
C	No biochar, no cover crop	NA	4	2664.46	1317.07	174.01	305.54	83.05	90.96
DT2	15	13cm-18cm	4	3212.05	1578.07	296.39	400.94	95.00	201.38
CC	Cover crop only	NA	4	2973.85	1440.31	136.87	289.62	73.65	63.22
S1	5	surface application	4	1801.54	914.87	80.93	228.21	38.88	42.05
ST2	15	8cm-10cm	4	2715.75	1341.00	251.29	337.51	80.28	171.01
ST1	5	8cm-10cm	4	1687.62	883.32	83.18	229.80	33.79	49.39

Table S2.7. Phospholipid fatty acid concentration under biochar and non-biochar treated plots (2023). AMF – arbuscular mycorrhizal fungi, SF – saprophytic fungi. Treatment abbreviations follow Table 2.1.

Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Phospholipid Fatty Acid (ng g^{-1})					
				Total PLFA	Bacterial PLFA	Fungal PLFA	Actinomycetes PLFA	AMF PLFA	SF PLFA
CC	Cover crop only	NA	1	356.01	105.78	0	25.05	0	0
ST2	15	8cm-10cm	1	463.44	129.54	0	26.57	0	0
ST2	15	8cm-10cm	1	816.31	244.52	6.01	51.8	0	6.01
S1	5	surface application	1	390.4	130.17	2.74	27.75	0	2.74
DT1	5	13cm-18cm	1	582.44	179.79	0	39.76	0	0
S2	15	surface application	1	538.25	245.15	4.66	53.8	0	4.66
C	No biochar, no cover crop	NA	1	1722.47	543.62	136.76	88.7	30.41	106.35
ST1	5	8cm-10cm	1	1766.76	640.74	100.3	109.11	44.64	55.66
ST1	5	8cm-10cm	1	807.68	252.25	30.47	51.62	10.4	20.07
DT2	15	13cm-18cm	1	819.22	344.36	27.25	76.84	14.39	12.86
CC	Cover crop only	NA	2	1152.25	376.86	35.9	80.72	19.42	16.48
ST1	5	8cm-10cm	2	951.01	281.98	20.15	62.05	6.26	13.89
ST1	5	8cm-10cm	2	1083.87	523.17	61.24	105.91	22.04	39.2
S1	5	surface application	2	1370.59	434.09	39.58	88.08	23.92	15.66
DT1	5	13cm-18cm	2	1132.13	443.96	53.46	105.7	21.94	31.53
C	No biochar, no cover crop	NA	2	1052.44	304.56	34.79	68.81	16.79	18.01

Table S2.7. (cont'd)

Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Phospholipid Fatty Acid (ng g^{-1})					
				Total PLFA	Bacterial PLFA	Fungal PLFA	Actinomycetes PLFA	AMF PLFA	SF PLFA
S2	15	surface application	2	629.37	263.53	8.34	62.22	0	8.34
DT2	15	13cm-18cm	2	1295.98	412.02	36.06	94.49	19.22	16.84
ST2	15	8cm-10cm	2	724.77	269.98	27.76	59.86	15.06	12.7
ST2	15	8cm-10cm	2	735.45	279.64	20.09	63.56	7.92	12.17
S1	5	surface application	3	1083.66	315.6	11.34	59.76	0	11.34
DT2	15	13cm-18cm	3	797.33	288.97	34.82	62.43	13.24	21.58
S2	15	surface application	3	1228.88	331.67	25.03	61.62	11.44	13.59
ST2	15	8cm-10cm	3	1171.06	389.55	30.16	67.31	14.02	16.14
ST2	15	8cm-10cm	3	1096.72	234.14	5.04	46.87	0	5.04
CC	Cover crop only	NA	3	1047.66	253.44	6.21	49.32	1.78	4.43
ST1	5	8cm-10cm	3	1424.7	360.86	34.81	75.7	19.06	15.75
ST1	5	8cm-10cm	3	879.13	224.44	2.95	46.52	0	2.95
C	No biochar, no cover crop	NA	3	1216.62	237.1	15.01	44.47	7.11	7.9
DT1	5	13cm-18cm	3	1512.15	399.63	41.97	72.02	19.53	22.45
DT1	5	13cm-18cm	4	1248.75	432.38	44.94	81.56	27.77	17.17
S2	15	surface application	4	2244.2	515.55	91.13	105.05	25.5	65.63
C	No biochar, no cover crop	NA	4	2319.29	708.65	99.77	124.08	39.15	60.62
DT2	15	13cm-18cm	4	1692.33	513.97	14.56	93.17	0	14.56
CC	Cover crop only	NA	4	1551.67	434.13	47.95	86.57	20.27	27.68

Table S2.7. (cont'd)

Treatment ID	Biochar rate (Mg ha^{-1})	Incorporation depth	Block	Phospholipid Fatty Acid (ng g^{-1})					
				Total PLFA	Bacterial PLFA	Fungal PLFA	Actinomycetes PLFA	AMF PLFA	SF PLFA
S1	5	surface application	4	974.12	345.8	33.13	71.17	16.87	16.27
ST2	15	8cm-10cm	4	1523.07	477.11	47.09	105.93	24.67	22.42
ST2	15	8cm-10cm	4	754.87	258.06	6.28	55.08	0	6.28
ST1	5	8cm-10cm	4	2049.03	643.87	60.4	127.91	31.7	28.69
ST1	5	8cm-10cm	4	2051.68	538.21	17.29	104.65	0	17.29

Table S2.8. Soil organic carbon, total nitrogen, and carbon-nitrogen ratio of biochar and non-biochar treated plots (2022). Total N – total nitrogen, C:N – carbon-nitrogen ratio. Treatment abbreviations follow Table 2.1.

Plot	Treatment ID	Biochar rate	Incorporation depth	Block	Soil organic carbon (g kg^{-1})	Total N (g kg^{-1})	C:N Ratio
102	CC	Cover crop only	NA	1	19.197	1.15	16.69
103	ST2	15	8cm-10cm	1	21.906	1.107	19.78
104	S1	5	surface application	1	14.987	1.284	11.67
105	DT1	5	13cm-18cm	1	18.453	1.136	16.24
106	S2	15	surface application	1	27.219	1.254	21.70
107	C	No biochar, no cover crop	NA	1	15.972	1.251	12.76
108	ST1	5	8cm-10cm	1	14.218	1.322	10.75
109	DT2	15	13cm-18cm	1	13.281	1.346	9.867
201	CC	Cover crop only	NA	2	17.779	1.304	13.63
202	ST1	5	8cm-10cm	2	14.672	1.408	10.42
205	S1	5	surface application	2	20.073	1.548	12.96
206	DT1	5	13cm-18cm	2	19.222	1.321	14.55
207	C	No biochar, no cover crop	NA	2	12.179	1.283	9.49
208	S2	15	surface application	2	24.462	1.367	17.89
209	DT2	15	13cm-18cm	2	14.014	1.418	9.88
210	ST2	15	8cm-10cm	2	26.61	1.39	19.14
301	S1	5	surface application	3	11.987	1.217	9.84
302	DT2	15	13cm-18cm	3	13.185	1.373	9.60
304	S2	15	surface application	3	21.089	1.34	15.73

Table S2.8. (cont'd)

Plot	Treatment ID	Biochar rate	Incorporation depth	Block	Soil organic carbon (g kg^{-1})	Total N (g kg^{-1})	C:N Ratio
305	ST2	15	8cm-10cm	3	24.447	1.318	18.54
306	CC	Cover crop only	NA	3	22.808	1.395	16.34
308	ST1	5	8cm-10cm	3	17.582	1.374	12.79
309	C	No biochar, no cover crop	NA	3	14.415	1.452	9.92
310	DT1	5	13cm-18cm	3	20.359	1.288	15.80
401	DT1	5	13cm-18cm	4	22.679	1.334	17.00
402	S2	15	surface application	4	16.521	1.316	12.55
403	C	No biochar, no cover crop	NA	4	13.626	1.383	9.85
404	DT2	15	13cm-18cm	4	14.008	1.468	9.54
406	CC	Cover crop only	NA	4	14.392	1.321	10.89
407	S1	5	surface application	4	14.982	1.392	10.76
408	ST2	15	8cm-10cm	4	20.209	1.371	14.74
409	ST1	5	8cm-10cm	4	14.516	1.332	10.89

Table S2.9. Soil organic carbon, total nitrogen, and carbon-nitrogen ratio of biochar and non-biochar treated plots (2023). Total N – total nitrogen, C:N – carbon-nitrogen ratio. Treatment abbreviations follow Table 2.1.

Plot	Treatment ID	Biochar rate	Incorporation depth	Block	Soil organic carbon (g kg^{-1})	Total N (g kg^{-1})	C:N Ratio
102	CC	Cover crop only	NA	1	0.82	0.086	9.53
103	ST2	15	8cm-10cm	1	1.13	0.101	11.19
103	ST2	15	8cm-10cm	1	1.44	0.098	14.69
104	S1	5	surface application	1	0.9	0.09	10.00
105	DT1	5	13cm-18cm	1	1.26	0.101	12.48
106	S2	15	surface application	1	1.28	0.103	12.43
107	C	No biochar, no cover crop	NA	1	1.17	0.125	9.36
108	ST1	5	8cm-10cm	1	1.09	0.116	9.40
108	ST1	5	8cm-10cm	1	1.23	0.119	10.34
109	DT2	15	13cm-18cm	1	0.99	0.108	9.17
201	CC	Cover crop only	NA	2	1.25	0.117	10.68
202	ST1	5	8cm-10cm	2	1.09	0.109	10.00
202	ST1	5	8cm-10cm	2	1.03	0.105	9.81
205	S1	5	surface application	2	1.24	0.115	10.78
206	DT1	5	13cm-18cm	2	1.33	0.122	10.90
207	C	No biochar, no cover crop	NA	2	1	0.113	8.85
208	S2	15	surface application	2	1.35	0.118	11.44
209	DT2	15	13cm-18cm	2	1.28	0.137	9.34
210	ST2	15	8cm-10cm	2	1.54	0.125	12.32
210	ST2	15	8cm-10cm	2	1.53	0.128	11.95

Table S2.9. (cont'd)

Plot	Treatment ID	Biochar rate	Incorporation depth	Block	Soil organic carbon (g kg^{-1})	Total N (g kg^{-1})	C:N Ratio
301	S1	5	surface application	3	1.34	0.117	11.45
302	DT2	15	13cm-18cm	3	1.1	0.12	9.17
304	S2	15	surface application	3	1.72	0.144	11.94
305	ST2	15	8cm-10cm	3	1.82	0.131	13.89
305	ST2	15	8cm-10cm	3	1.05	0.099	10.61
306	CC	Cover crop only	NA	3	1.49	0.125	11.92
308	ST1	5	8cm-10cm	3	1.1	0.113	9.73
308	ST1	5	8cm-10cm	3	1.27	0.126	10.08
309	C	No biochar, no cover crop	NA	3	1.01	0.108	9.35
310	DT1	5	13cm-18cm	3	1.68	0.129	13.02
401	DT1	5	13cm-18cm	4	2.03	0.121	16.78
402	S2	15	surface application	4	1.31	0.119	11.01
403	C	No biochar, no cover crop	NA	4	1.21	0.129	9.38
404	DT2	15	13cm-18cm	4	1.11	0.118	9.41
406	CC	Cover crop only	NA	4	1.03	0.111	9.28
407	S1	5	surface application	4	1.3	0.127	10.24
408	ST2	15	8cm-10cm	4	1.23	0.115	10.70
408	ST2	15	8cm-10cm	4	1.5	0.131	11.45
409	ST1	5	8cm-10cm	4	1.44	0.147	9.80
409	ST1	5	8cm-10cm	4	1.14	0.115	9.91

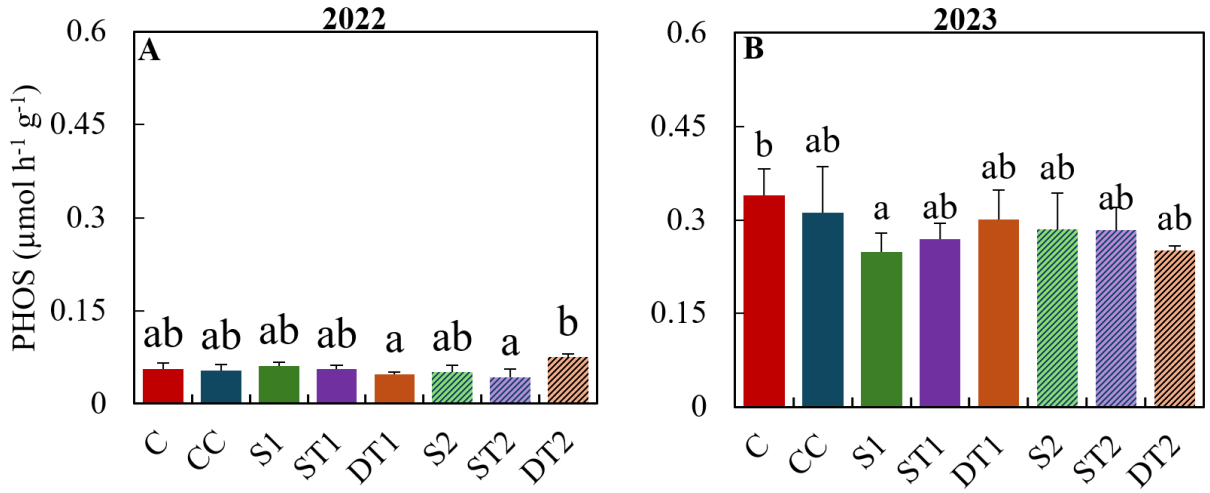

Figure S2.1. Mean activity (±SE) of phosphatase (PHOS) as influenced by biochar rates (5 Mg ha-1; 15 Mg ha-1) incorporated via surface application, shallow incorporation (8cm) and deep incorporation (15cm). Different lowercase letters show statistically significant differences ($p<0.05$). Y-axis titles are identical between panels A and B. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg $ha^{-1;}$ ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.

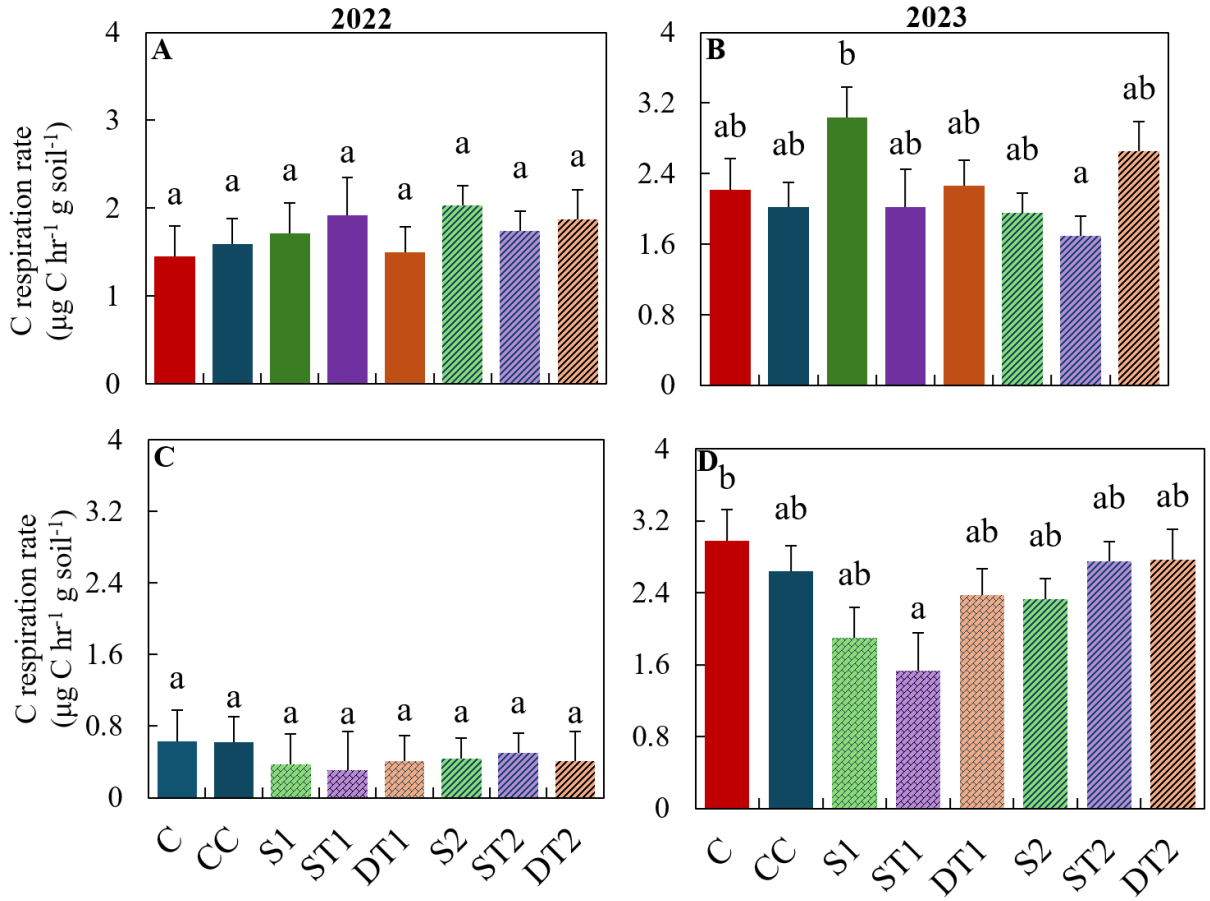

Figure S2.2. Soil carbon respiration of (a-b) pre-planting and (c-d) late-season samples in 2022 and 2023 after 7 days of laboratory incubation under different biochar rates (5 Mg ha^{-1}, 15 Mg ha^{-1}) and incorporation depth (surface application, 8cm, 15cm. Error bars indicate standard error. Different lower-case letters among treatments denote significant differences at $p < 0.05$. Y-axis titles are identical between panels A and B, and between C and D. Treatment description: C – control (no biochar, no cover crop); CC – cover crop; S1 – surface-applied biochar at 5 Mg ha^{-1}; ST1 – shallow incorporation at 5 Mg ha^{-1}; DT1 – deep incorporation at 5 Mg ha^{-1}; S2 – surface-applied biochar at 15 Mg ha^{-1}; ST2 – shallow incorporation at 15 Mg ha^{-1}; DT2 – deep incorporation at 15 Mg ha^{-1}.