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Nitrogen dynamics and carbon sequestration in cropping systems including legumes

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I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice"

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Summary

Soil carbon (C) and nitrogen (N) dynamics sustain to a large extent the productivity of cropping land to provide food, feed and fibre to satisfy the increasing requirements of the global population. At the plot level C and N dynamics are tightly bound and decoupling results in potential damages for soil, water and air quality. On this basis, decreased soil C and N stocks together with increased nitrate leaching in waters and gaseous N emissions towards the atmosphere, are of major concern. To reduce these environmental impacts, innovative and sustainable farming systems are promoted, such as conservation agriculture and organic farming, which favour soil organic matter restoration practices. Within these systems inclusion of legumes in crop rotations plays a prime role in providing multiple benefits in line with agorecological principles, with N provision through biological N fixation (BNF) topping them all. At a larger scale, the implementation of diversified crop rotations with legumes could play an important role in mitigating and adapting agriculture to climate change, assuming that holistic assessments in the short and long term prove beneficial. The objectives of this thesis were to 1) quantify the N dynamics of six grain legumes across pedoclimatic conditions 2) assess the contributions of legume cover crops to soil organic carbon (SOC) stocks. For this purpose, studies at the experimental farms of Gladbacherhof (Germany), Oberfeld (Germany) and El Dorado do Sul (Brazil) were conducted.

The six tested grain legumes at the German sites performed differently across locations, underlying genotypic adaptation strategies of nutrient acquisition and nutrient transformations. At Gladbacherhof the mean rate of biological N fixation was 54.5% much lower than that at Oberfeld where some varieties were almost completely reliant on BNF; nevertheless tested legumes accumulated 99.7 kg ha^{-1} at the first site and 52.4 kg ha^{-1} at the second since, due to sound differences in biomass production. Allocation of N to grains was substantial (~5% N concentration) which led to N balances (N inputs from BNF – N exports through grains) between -214.9 to 41.9 kg ha^{-1}, highlighting a fine line between provisions of protein-rich grains

at the expense of N rich residues. Nitrate leaching under legumes during the cropping season was low probably because no external fertilizers were applied and losses resulted from the mineralization of SOM. Sowing of winter legumes did not decrease winter nitrate or nitrous oxide losses, but extending the cultivation period, did lead to significantly higher shares of BNF. Weekly measurements of nitrous oxide emissions at Gladbacherhof shed important lights on timing and quantifying legume field scale emissions. I found that mineralization of crop residues can increase N_2O release when coupled to warmer and more moist conditions, therefore sowing of subsequent crops should be done shortly after legume harvest to maximize N transfer and decrease mineralization losses.

In the Brazilian study, soil samples were taken at seven increments until the one meter depth from maize-based cropping systems with increasing shares of leguminous cover crops. After 39 years of continuous management, SOC concentrations increased linearly ($R^2=0.85$) with amounts of C inputs suggesting that saturation may have not yet occurred. Significant correlations were also found between amounts of C inputs and thermally labile or thermally recalcitrant SOC stocks. These suggested that diversification of cropping systems with legumes to be a viable means to enhance top soil (0-30 cm) stocks, whereas only systems at 0N fertilizer applications also enhanced subsoil (30-100 cm) stocks. A further fractionation, combining physical and chemical steps, was performed to study the quality and the origin of SOC stocks. Plant inputs (POM) and mineral associated SOC stocks were enhanced, but not aggregation of soil particles suggesting organo-mineral associations to be the driving pathway of SOC sequestration at the site. $\delta^{13}C$ data helped us confirm that increases in stocks were due to legumes by exploiting the unique isotopic signatures of the C_3 cover crops, and although shares of contributions were high in the top layers, these were low in the subsoil.

This study challenges the common belief that legumes necessarily improve plot scale C and N dynamics and points out to the complex interactions occurring between sustainable cropping

practices and the compensations which can occur between C and N processes. Some of which leading to increased biological N fixation or soil C stocks, or decreased leaching and gaseous N emissions. Further work should focus on combining the obtained molecular-scale and plot-level results into a conceptual framework of short, and long-term SOM dynamics, with the exploitation of legumes´ biological N and C provision as key contributor for the design of integrated cropping systems and the sustainable management of agricultural soils.

Keywords: carbon sequestration, soil organic matter turnover, C_3C_4 vegetation shifts, ^{13}C isotopes, ^{15}N isotopes, biological N fixation, nitrous oxide, cropping systems, legumes.

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Table of Contents

1. General introduction

Legumes are unique

Plants need large amounts of nitrogen (N) because N is an essential component of all proteins and nucleic acids required for new, functioning cells (Muratore et al., 2021). Therefore, limited N availability in cropping systems results in restricted plant growth, as postulated in Justus von Liebig´s law of minimum. Legumes can ameliorate the N limitation by having evolved a capability to form a symbiosis with rhizobia and bradyrhizobia to fix atmospheric N_2 into plant available forms (Giller and Cadisch, 1995; Pate et al., 1998). Symbiotic N fixation was a major evolutionary advantage because legume growth was not limited to N availability in the soil, which makes them unique (Roy et al., 2020). Legumes belong to the *Fabaceae* family, the third largest plant family, which encompasses over 20,000 species ranging from herbs, to shrubs, from trees to grain crops (Peoples et al., 2009; Roy et al., 2020). While legumes´ ability to fix N ensures high quality residues and rhizodeposits which impact soil fertility (Mayer et al., 2004; Shah et al., 2003), grains rich in proteins are integral components of healthy diets (de Roos et al., 2017; Polak et al., 2015). As conventional agricultural practices may include harmful agrochemical applications which contribute to the release of greenhouse gases (GHG), pollution of water bodies and to a reduction in biodiversity (Montgomery and Biklé, 2021), restoration of sustainable soil management is urgent (Menegat et al., 2022), and legume varieties across the globe, are candidates for playing an important role in the future of sustainable delivery of N in cropping systems (Giller and Cadisch, 1995; Ladha et al., 2022). Globally 21.5 Tg of N are fixed by legume crops per year, compared to 85 Tg of N applied as chemical fertilizer (Peoples et al., 2009); therefore a potential for increasing exploitation of N derived from legumes does exist.

Due to their versatile nature, legumes are components of diversified cropping systems all over the world. For example *Calliandra calothyrsus, Gliricidia sepum* and *Senna siamea* are used as legume trees species in agroforestry cut-and-carry systems from Togo (Lehmann et al.,

1998) to Rwanda (Bucagu et al., 2012); chickpeas (*Cicer arietinum*) can be sown in between rows of olives across the Mediterranean region (Amassaghrou et al., 2023); urdbean (*Vigna mungo*) and lentils (*Lens culinaria*) are rotated to paddy rice fields in Sri Lanka (Hazra et al., 2020); cowpea (*Vigna unguiculata*) is a common intercrop with cereals in semi-arid Kenya (Binacchi et al., 2022; Frimpong et al., 2011); high quality pastures including mixtures of alfalfa (*Medicago sativa*) with grasses are grazed by llamas in Bolivian highlands (Robinson et al., 2013); liquorice (*Glycyrrhiza glabra*) is grown in home-gardens in parts of India as a medicinal crop (Dagar et al., 2015); relay cropping (Tanveer et al., 2017) of groundnuts (*Arachis hypogaea*) with wheat is a sustainable option on sandy soils in southern states of the United States of America (Moss et al., 2012); in western Australia, lupins (*Lupinus albus)* are the main grain legume and are alternated to cereals in crop rotations and grazed by sheep in agro-pastoral systems (Nelson and Hawthorne, 2000). Improved management approaches such as organic farming, conservation agriculture or climate-smart agriculture, all also embrace diversification of cropping systems through the inclusion of legumes as one of the means by which to promote regeneration of soil organic matter (SOM) while providing ecosystem services (Barbieri et al., 2017; Pittelkow et al., 2015; van Wijk et al., 2020) broadly highlighting legumes´ uniqueness in cropland, and beyond.

Cultivation in Europe

Since the discovery that atmospheric N_2 and gaseous H_2 react under high pressure (~200 ATM) and temperature (~426˚C) to synthesize ammonia through the Haber-Bosch process, the ecological and economic dependency of European agriculture on legumes has diminished greatly (Magrini et al., 2016; Zander et al., 2016). The farmland area cropped with legumes declined from 11.3 million ha in 1961, to 5.3 million ha in 2013 (Preissel et al., 2015). This was determined by several factors including: (i) low economic returns due to unattractive producer prices resulting in the preference of farmers for growing oil or cereal crops (Cernay et al., 2015; Squire et al., 2019) (ii) planting decisions being mostly based on direct economic returns, and not accounting for the ecological services provided by legumes (Watson et al., 2002; Zander et al., 2016) (iii) the significant import of (cheap) soya (*Glycine max)* bean products from countries like Brazil reduced needs for internal production (Ali et al., 2022) (iv) increased meat consumption replaced much of the requirements of plant derived protein intake and (v) ready availability of synthetic fertilizers to deliver mineral N to cropland (Jensen et al., 2012; Lassaletta et al., 2014; Ritchie et al., 2022). Nonetheless, recently a revitalization in shares of legumes in European cropping systems is taking place (Magrini et al., 2016; Notz et al., 2023), as a response of targeted policies as well as positive social and environmental assets (BMEL, 2021; Ferreira et al., 2021), which have long since been empirically appreciated. For example the Common Agricultural Policy (EU, 2022) aims at increasing inclusion of legumes by 25% in 2023 compared to 2022. Such target is aimed to be achieved both through the increase in subsidizes for crop rotations and pastures, as well as through the expansion of soya bean production across large portions of eastern Europe with the overall goal of increasing shares of domestic protein production while decreasing N application as fertilizers (Wesseler, 2022). Legumes in crop rotations are also regarded as "break crop" as they can break cycles of soil-borne root diseases such as the root rot of cereals that die within a year in the absence of a suitable host. Currently, agroforestry with legumes trees is restricted to few semi-arid areas of southern Europe, whereas inclusion of legume crops in between rows of olive or wine trees is slowly increasing (Paris et al., 2019). Most of the cultivation of legumes occurs as cover, forage or cash crops (with terms and uses also intertwining sometimes). Legume cover crops mostly include peas (*Pisum sativum*), vetches (*Vicia villosa*) or clovers (*Trifolium repens*) sown either as seed mixtures with grasses or short cereals, or grown as sole crops with the main purpose of (i) keeping the soil covered over winter or in the understory of main crops and/or (ii) being used as green manures meaning that one or multiple biomass harvesting may take place to be returned as soil amendments (Hauggaard-Nielsen and Jensen, 2001; Vincent-Caboud et al., 2017). Careful management of leguminous cover crops enhances provision of low C/N organic matter, improves soil aggregation, while decreasing erosion and weed appearance (Carranca et al., 2009b, 2009a; Poeplau and Don, 2015). Main crops such as barley or wheat, can also be sown into “living” legume mulches in no-till systems (Zikeli and Gruber, 2017). Cash crops usually encompass grain legumes, which are sown to be used as food or, in most of the cases, as animal feed, and are integral components of farming systems, for on-site protein production and sustainability of crop rotations. The top five grain legumes in Europe are faba bean (*Vicia faba*), peas, soya bean, lupine, chickpea and lentil. Their protein content varies between 20 – 45% compared to ~12% in cereals (da Silva et al., 2014). Faba and field pea are the most widely grown grain legumes in Europe which can accumulate on average 130 and 153 kg N ha^{-1} in their aboveground biomass and large quantities in their below-ground biomass, accounting 30–60% of the total N accumulated by legumes (Peoples et al., 2009). On different soil types in Denmark, N uptake in subsequent crops was increased by 23–59% after field pea and narrow-leafed lupine (Jensen et al., 2004). However, the increase in yield of durum wheat following vetch in a semi-arid Mediterranean environment was only 14–15% (Giambalvo et al., 2012). In a review of 29 European studies, Preissel et al., (2015) found that including grain legumes in crop rotations leads to an increase by 0.5-1.6 Mg ha^{-1} of subsequent cereals, which compared to a reduction of 23-31 kg ha^{-1} of N fertilizer application.

Silage production with additional red clover (Trifolium pratense), white clover (Trifolium repens) or lucerne (Midicago sativa) can produce higher profits per unit of land than grass-only swards by reducing fertilizer applications and providing higher quality feed (Ergon et al., 2018; Rochon et al., 2004) while galega (Galega orientalis) and lotus (Lotus corniculatus) are usually second-choice varieties due to lower biomass production (Doyle and Topp, 2004). Forage legumes are grown as components of temporary pastures (leys) or in permanent grassland, and unless grown for seed, they are generally in mixtures with grasses, other legumes and/or forbs. In many cases, more than one species or cultivar of a legume will be grown within the mixture to provide the desired forage characteristics, and protein throughout the season. The total amount of N fixed by forage legumes in Europe in 2017 was 586 Gg, comprising $414*10^6$ kg from permanent pastures and $172*10^6$ kg from temporary pastures (Watson et al., 2017).

Soil organic matter and it´s decomposition pathways

SOM can be defined as all organic soil compounds, including living biomass and dead organic SOM, and is involved in soil fertility, as it influences soil structure through the formation of aggregates that promote water retention, aeration and structural stability of the soil, along with nutrient availability for soil organisms and cultivated plants to proliferate upon (Lal, 2005; Piccolo et al., 2005). Carbon and nitrogen represent a significant proportion of SOM, about 58% for the C, the proportion of N being variable depending on soils e.g. C/N ratio varying between 8.5 and 18.2 across topsoils of central and eastern Europe (Batjes, 2002). In natural ecosystems, soil organic C and N are coupled and cycle together, therefore perturbations in the N cycle have repercussions on the carbon cycle, and vice-versa (Batjes, 2002). Since the start of the industrial era, agricultural ecosystems´ C and N cycles have been strongly disturbed and decoupled (Mason et al., 2022) due to:

-The shortening of crop rotations, i.e. the decrease of crop diversity in the rotation, and the withdrawal of perennial grasslands in favour of annual crops, has decreased SOM stocks (King and Blesh, 2018; McDaniel et al., 2014).

-The considerable applications of N fertilizers, which increased nine-fold between 1961 and 2009, amounting to 190 million tons per year, of which 53% is not taken up by plants and is “lost” to the environment (Lassaletta et al., 2014; Ritchie et al., 2022).

- Land use change has caused a total loss of 133 Pg C from the soil through cultivation and disturbance, with losses increasing sharply over the past 200 years (Sanderman et al., 2017).

SOM content in agroecosystems is therefore the result of two opposed fluxes: on one side the inputs of organic matter through returned crop residues (straw, litterfall, roots..) and application of organic fertilizers (manure, slurry, green manure…), while on the other side the losses of

SOM by mineralization, leaching and erosion. In order to predict the dynamics of SOM, compartmental models identify dynamic “pools”, characterized by a specific potential decomposition rate and factors controlling the effective decomposition rate (Six et al., 2002; von Lützow et al., 2007). The mean residence time (MRT) of each soil organic pool is the ratio of the amount of SOC contained in this pool to its incoming C input, which is equal to its output C flux in steady state conditions. Thus, SOM models include various pools with different MRT, usually at least three pools: a labile pool, which is easily degraded (MRT from1 day to 1 year), an active pool (MRT from 1 year to a few years) and a stable pool, which turns over in decades or centuries (Parton et al., 1987; Zimmermann et al., 2007). Both labile and active pools originate predominantly from plant, animal, bacterial and fungal residues. The stable pool originates from labile and active pools and includes the largest part of soil organic C (Lehmann et al., 2020). In this pool, the physical protection of C by soil aggregates and the physico-chemical protection by adsorption, humification or complexation with mineral particles all contribute to the slow degradation rate (Derrien et al., 2023; Six et al., 2002). For cultivated soils, the mean MRT of SOC (all pools mixed) has been estimated at 61 ± 9 years in a synthesis of 10 experiments (Six and Jastrow, 2002) although rates usually differ (are lower) in the lower soil profiles (Poeplau et al., 2011).

The decomposition of SOM is a complex process that depends on many factors including soil minerals (clay, iron oxides…), climate (temperature, rainfall…) and agricultural practices (residue management, soil tillage, crop rotations…) but can be defined as the combination of three processes (Derrien et al., 2023):

(i) the conversion of organic matter from a chemical structure to another in response to enzymatic attacks and chemical reactions;

(i) the incorporation of organic C and N into heterotrophic decomposers, and

(ii) mineralization.

C mineralization is the conversion of organic C into CO_2 by heterotrophic organisms under aerobic conditions, and into methane (CH_4) under anoxic conditions. N mineralization leads to release of ammonium (NH_4^+) through the ammonification process. Nitrification then converts ammonium into nitrite (NO_2^-) first and then nitrate (NO_3^-), both steps being realized by autotrophic bacteria. The denitrification process converts NO_2^- and NO_3^- into gaseous N compounds that are nitric oxide (NO), N_2O and N_2. It involves heterotrophic microorganisms, whose activity is maximum in anoxic environment, in presence of NO_3^- and C sources. The nitrifier denitrification can also lead to the production of NO, N_2O and N_2, from the nitrite produced during the nitritation (Butterbach-Bahl et al., 2011). The rate and fate of organic residues decomposition depend on the amount and nature of the residues, environmental factors and the nature of the microbial populations (Gomes et al., 2009; Mooshammer et al., 2022). Microbes have a low C:N ratio, lower than that of crop residues. Since their N requirement during the decomposition process is rarely satisfied by the N mineralized from the residue, they take up soil mineral N, leading to N immobilization. The higher is the C:N ratio of organic residue, the greater immobilization may occur (Sawada et al., 2015). On the long term, decomposers die and part of their C and N is incorporated into stable SOM (Cotrufo et al., 2013).

Soil organic carbon fractionation techniques

Soil organic carbon fraction techniques have evolved to gain a better understating of pathways and processes that occur in the soil, by isolating functional C compounds with varying turnover times. Fractionation methods have evolved along with the understanding of SOM processes and choosing which one to apply depends on specific aims and resources availability (von Lützow et al., 2007). Traditional views of SOC stabilization where SOM becomes humified, favoured the use of alkaline extraction to isolate humic acid, fulvic acid and humin (Baveye and Wander, 2019; Piccolo et al., 2005) although these may not reflect substances naturally existing under natural conditions (Denef et al., 2009; Poeplau et al., 2018). Physical fractionation is currently the most common approach, whereby soil particles of varying size are separated through wet sieving. Physical fractionation may be accompanied by the study of aggregates to place focus on the binding of inorganic and organic soil components, for the turnover of SOM. Spatial protection provided by aggregation and physical protection provided by organo-mineral associations in fine size soil particles (i.e. <63 µm in Europe, or <50 µm in the Americas), are acknowledged to be fundamental for the stabilization of SOC (Bayer et al., 2006; Gentile et al., 2013; Six et al., 2002). Aggregate fractionations are mostly used to explore the influences of management practices on the ecosystem functions of soil aggregates, or their respective pore spaces and microbial habitats (Denef et al., 2009; Gentile et al., 2013). Density fractionation is applied to separate SOM that is not firmly associated with soil minerals which leads to the distinction of “free” or “light” fractions. These light fractions are usually obtained by floatation with heavy liquids such as sodium polytungstate ($Na_6H_2W_{12}O_{40}$), and consist of plant residues that are either free in the soil matrix or occluded in aggregates, after a light sonication (Witzgall et al., 2021). Chemical fractionation is generally applied in more advanced fractionations to highlight the different chemical characteristics of SOC, as different molecular compositions may reflect different recalcitrance, which is a key component of C stabilization and sequestration (Lehmann et al., 2020). In such cases, oxidation with hydrogen peroxide (H_2O_2), sodium hypochloride (NaOCl) or sodium persulfate ($Na_2S_2O_8$), are used to mimic a strong enzymatic decay (Poeplau et al., 2018). Extraction with water can be used to isolate dissolved organic carbon (DOC), which is a very mobile C fraction (Zimmermann et al., 2007). Thermal fractionation may overcome some shortcomings due to laboratory set-up while rapidly providing results of biologically distinct SOC compositions. By heating samples against a known amount of time and temperature, the energy released may be detected and related to the energy requirements of enzymatic degradation (Rennert and Herrmann, 2022). Spectral methods such as nuclear magnetic resonance can also be used to estimate the spectral composition of SOC and relate this to compounds of known spectral peaks. Although results from both thermal and nuclear approaches have been related to conceptual SOC pools, these methods do to allow for actual isolation of different fractions per se.

Each method may highlight different qualities of SOC although all tend to be limited in terms of deciphering stabilization mechanisms (von Lützow et al., 2007). Solutions may include to use data for initiating and validating mechanistic models of SOC turnover by relating fractions to SOC pools, or to calculate the mean residence time of certain SOC fractions by tracing changes in ^{13}C isotopic signatures, either through labelling approaches, or through field-level shifts in ^{13}C abundance (Del Galdo et al., 2003; Hobley et al., 2018).

Scope and structure of the thesis

This doctoral thesis emerged with the scope of quantifying the contributions of legumes to soil organic matter. This was achieved through innovative approaches for assessing nutrient cycling. Research hypotheses were specific to each experiment and were therefore formulated in each chapter. The two main purposes of this work were as follows:

-compare N dynamics of six grain legumes;

-detangle the contribution of legumes to SOC stocks.

More detailed objectives are summarized as follows:

(i) to establish comparable field trials across experimental farms with contrasting soil textures, and study the biomass production of six important grain legumes in central Germany;

(ii) to study biological N fixation and assess shares across crops as well as measuring amounts of fixed nitrogen above, and below ground;

(iii) to monitor mineral N dynamics among winter and summer varieties to estimate leaching losses;

(iv) to provide novel data on nitrous oxide emissions throughout the growth length of grain legumes;
(v) to investigate SOC stocks in long-term subtropical cropping systems;
(vi) to decipher legume contributions to SOC stocks, and
(vii) to summarize findings and propose applicability of results.

The first chapter presents the general framework of the thesis. Chapter 2 focuses on the genotype*environment*management interactions of N cycling in grain legumes. In chapter 3, I assessed the thermal stability of SOC stocks in the top and in the lower soil strata in systems with legume cover crops. Chapter 4 traced shifts in stable C isotopic signatures resulting from shifts in vegetation, to study pathways of SOC stabilization. In chapter 5, I place experimental results in the wider prospectus, to highlight the role of legumes on SOM dynamics.

2. Demystifying the agronomic and environmental N performance of grain legumes across contrasting soil textures of central Germany

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3. Sub-soil organic C stocks in thermally labile and recalcitrant fractions are enhanced with legume cover crops in a subtropical Acrisol depending on fertilizer applications

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Sub-soil organic C stocks in thermally labile and recalcitrant fractions are enhanced with legume cover crops in a subtropical Acrisol depending on fertilizer applications

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Highlights

- Thermally labile and residual oxidizable carbon were evaluated in 39 years old no-till cropping systems
- Organic carbon stocks in labile fractions were correlated with amounts of C inputs from cover crops in the topsoil (0-30 cm) at 0N and 160N application, while subsoil stocks (30-100 cm) only at 0N
- Since the start of the experiment, SOC sequestration rates varied between -0.08 and 0.59 Mg ha^{-1} year^{-1} with 3.9 Mg C ha^{-1} year^{-1} required to maintain initial stocks
- On this previously degraded Acrisol, combination of legumes and fertilizer led to highest SOC stocks.

Keywords C sequestration, thermal stability, subsoil, legume cover crops.

Introduction

Enhancing both the persistence and the bioavailability of soil organic carbon (SOC), is crucial to sequester atmospheric carbon dioxide (CO_2) while restoring the productivity and functionality of degraded soils (Lehmann et al., 2020). SOC content in pristine grasslands reaches a steady state when amounts of C losses through erosion are at balance with amounts of C inputs from decomposing plant materials (Foucher et al., 2023; Pillar et al., 2012). On the other hand, conversion of natural ecosystems to cropland in tropical and subtropical areas, has caused a 20.1-25.8% decline in SOC stocks (Don et al., 2011), due to unbalanced C flows at the plant-soil-atmosphere interface (Caruso et al., 2018; Herzfeld et al., 2021). These losses are exacerbated in southern Brazil where intensively managed monocrop systems (mostly maize, cotton, rice, wheat and/or soya bean) have resulted in disruption of soil structure and low soil organic matter (SOM) concentrations, relying vastly on fertilizer applications to sustain productivity (Babujia et al., 2010; Foucher et al., 2023; Mahal et al., 2019). Conservation practices such as no tillage with crop residue retention (NT), have been employed to restore soil particle aggregation (Bayer et al., 2000; Six et al., 2002), promote mineral-association of OC (Cordeiro et al., 2022; Veloso et al., 2019), soil biological activity (Babujia et al., 2010; Mooshammer et al., 2022) and soil moisture content, by combining high-input cropping systems to decreased soil disturbance. Where pedoclimatic conditions allow it, temporal (e.g. outside the main cereal cropping season) and/or spatial (e.g. intercrops, crop mixtures) integration of leguminous cover crops can provide a continuous source of steadily degradable OM (Barrios et al., 1996; Vieira et al., 2009) which, coupled to more lignified C sources such as these from the main cereal cash crops, may lead to suitable conditions for SOC stabilization (Barrios et al., 1996; Gentile et al., 2013; Wu et al., 2017). It is unknown though, to what extent enhancing shares of leguminous cover crops in maize systems, may compare to fertilizer applications for SOC restoration.

SOC compounds may be grouped into labile, intermediate and non-bioavailable (Poeplau et al., 2018 and references therein) as intricate conditions shape the decomposition and persistence of photosynthetically derived C in the soil (Lehmann et al., 2020; Witzgall et al., 2021). Therefore laboratory techniques have been established to distinguish SOC fractions, and assess the quantity of C within such compartments (Fernández et al., 2011; Petrokofsky et al., 2012). Thermogravimetric analysis of soil C differentiates fractions with varying thermal stabilities by measuring the release of CO_2 at gradually increasing temperature gradients and provide information about the mineralogy, origin, age and quality of soil C (Barreto et al., 2021; Fernández et al., 2011; Manning et al., 2005; Tokarski et al., 2020). Readily biodegradable SOM compounds such as polysaccharides and aliphatic compounds decompose mostly at temperatures below 400 ˚C (Barreto et al., 2021; Fernández et al., 2011) whereas rather stable forms of C due to the binding to clay particles get burned up to 550 ˚C (Kučerík et al., 2018; Tokarski et al., 2020). The recently introduced DIN19539 standard has been used to distinguish three C fractions namely thermally labile OC (OC_{400}) combusted at temperatures between 400 °C, residual oxidizable C (ROC) released up to 600°C, and carbonate minerals which are burned between 650-850°C (inorganic carbon) (Natali et al., 2019; Rennert and Herrmann, 2022). The method offers the advantage of rapidly and precisely measuring SOC by increasing heterogeneity of sample material in crucibles as well as avoiding the need to pre-treat samples for inorganic C separation, compared to classical combustion techniques (Mörchen et al., 2019; Stanley et al., 2023; Stoner et al., 2022). Until now this time/temperature method of CO_2 evaluation has been mostly used to identify stable C forms such as graphite or black carbon (Natali et al., 2021; Rennert and Herrmann, 2020; Zethof et al., 2019) but it is unknown weather plot management options may result in the detection of differences among thermally distinct OC fractions (Rennert and Herrmann, 2022). Therefore we test the hypothesize that high input cereal-legume cropping systems will return SOM compounds of varying sizes and structural stabilities, leading to sequestration of SOC across fractions of varying thermally oxidative nature (Barreto et al., 2021).

Nevertheless, shifts in SOC chemical composition due to field management, can take decades to centuries before being detectable, highlighting the role of long-term experiments in assessing the turn-out of agricultural practices on the structural composition of SOC. While quantification of SOC dynamics in the top soil strata have been vastly assessed to promote NT farming (Leite et al., 2010; Six et al., 2002), deeper layers remain a vastly unexplored domain as well as a hotspot for C sequestration targets. Subsoils are mostly depleted in SOC, and introduction of cover crops may provide OM source for organo-mineral associations beyond the 30 cm depth (Angst et al., 2018; Bolinder et al., 1997; Cordeiro et al., 2022). Particularly in subtropical Oxisols, which report highest shares of clay particles beneath the A horizon, as well as higher Al and Fe particles which can serve as binding sites for decomposing organic matter, increasing shares of deep-rooted crops may be a viable means to meet national CO_2 sequestration agendas (de Oliveira Ferreira et al., 2018, 2013; Diekow et al., 2005; Foucher et al., 2023).

The aim of this study was to quantify SOC stocks in a 39 years old NT experiment and compare them among five maize cropping systems characterized by increasing shares of cover crops, at two N fertilization arrangements. To do so, we measured the thermal stability of SOC and distinguished a bioavailable (OC_{400}) and a non-bioavailable (ROC) fraction and relate these to cropping management. Moreover we assessed SOC stocks in the surface layer and in the subsoil and suggest how the supply of C inputs from maize and/or cover crops impacted the thermal stability of labile and recalcitrant OC stocks. Lastly the study investigated the historical dynamics of SOC stocks at the site, as well as estimating C sequestration rates and SOC stabilization rates.

Materials and Methods

Site description

The ongoing field experiment started in 1983 and it is located at the Agronomic Experimental Station of the Federal University of Rio Grande do Sul, in Eldorado do Sul (30°51´ S and 51°38´W), in Southern Brazil. The regional climate is categorized as subtropical humid, with a mean annual temperature of 19.4°C and a mean annual rainfall of 1440 mm, evenly distributed throughout the year. Soils are classified as Acrisols (FAO, 2002), with a sandy clay loam texture (540 g kg^{-1} sand, 240 g kg^{-1} silt, and 220 g kg^{-1} clay) and the clay fraction is dominated by kaolinite (720 g kg^{-1}) and Fe oxides (109 g kg^{-1} Fe_2O_3). Until 1969, the area was under native grassland (mainly *Paspalum* and *Andropogon*), thereafter it was converted into cropland and the soil was subjected to intensive plowing and disking for 16 years, and presented visible signs of soil degradation (Bayer et al., 2000). The experiment was installed to evaluate the potential of recovering soil fertility, by establishing no-till cropping systems with various levels of N fertilization and lime application. The experimental design was established as a randomized split- plot arrangement with three field replicates. Maize is grown as the main cash crop in summer, to which cover crops are alternated in time (winter) or space (understory) or a combination of the two. We selected 5 cropping systems for this experiment: fallow/maize (*Zea mays* (L.)) (F/M), oat (*Avena strigosa* Schreb)/maize (O/M), oat+vetch (*Vicia sativa* (L.))/maize (OV/M), oat+vetch/maize+cowpea (*Vigna unguiculata* (L.) Walp) (OV/MC) and pigeonpea (*Cajanus cajan* (L.) Millsp) + maize (PM). Main plots measured 8 × 5 m and were divided into two 4 × 5 m subplots at two N fertilization levels (0N or 160N). N was applied to the maize only at a rate of 120 kg N ha^{-1} $year^{-1}$ between 1983 and 1994 and 180 kg N ha^{-1} $year^{-1}$ between 1994 and 2020 which averages at 160N, while 0N treatments did not receive any mineral N applications. Maize was sown with a NT planter in September-October to obtain a plant population ~60´000 with a 90 cm space between rows and harvested in April. 50 kg ha^{-1} of P_2O_5 and 50 kg ha^{-1} of K_2O were applied at maize sowing. Pigeonpea and cowpea were planted between maize rows with a manual seed planter 1–2 weeks after maize, using a seed rate of approximately 25 kg ha^{-1}. After maize harvest, its aboveground biomass was spread on the soil surface using a knife roller and vetch, oat, and vetch + oat were planted with a NT drill with a sowing rate of 80 kg ha^{-1}, 80 kg ha^{-1}, and 60kg ha^{-1} + 30kg ha^{-1} respectively. In the PM treatment, pigeonpea was left standing in the field after maize harvest, and plants stayed until they were killed by frosts in May/June; their residues were left on the soil cover until next maize planting. Glyphosate herbicide was sprayed at a rate of 1.4 kg ha^{-1} to desiccate winter cover crops and weeds, and biomass was left on the soil cover and spread with a knife roller few days after spraying. No agrochemicals were used for pest or disease control and sprinkler irrigation was used during the maize growing phase.

Soil sampling and analysis

Soil samples were obtained at the following seven depths 0-5, 5-10, 10-20, 20-30, 30-50, 50-75 and 75-100 cm with a portable excavator coupled to a spiral auger (Φ=30 cm) in February 2020. Samples were placed in paper bags and air dried for a week to then be ball milled to pass through a 2 mm sieve and stored until further analysis. Determination of C was performed using a soliTOC cube analyser (elementar, Langenselbold, Germany) according to the DIN 19539 standard (Natali et al., 2019; Rennert and Herrmann, 2022) whereby 50 grams of soil were weighed into steel crucibles, fed into the machine and initiated the heating process. The analytical run required approximately 1,600 s and involved a three-step heating of the samples to 400, 600, and 900°C with holding time of 230, 120, and 150 s, respectively (Figure 3.1). CO_2 released from the combustion was continuously analysed by infra-red detectors tracing C concentrations. TIC was detected at very low concentrations (between 0.08-0.15 g kg^{-1} soil) and results were not reported as not being part of the main investigation. Soil organic carbon (SOC) was calculated as the sum of the two fractions

$$SOC = OC_{400} + ROC$$

Moreover, 1.2 m deep and 1.5 m large trenches were opened with an excavator at one end of the 0N plots and metal rings of known volume were hammered at the seven above-mentioned depths to measure bulk density. Nevertheless, to prevent comparisons of different masses across the various cropping systems, we used the equivalent soil mass approach for calculating SOC stocks (Petrokofsky et al., 2012), by relying on bulk density results from the fallow/maize treatment.

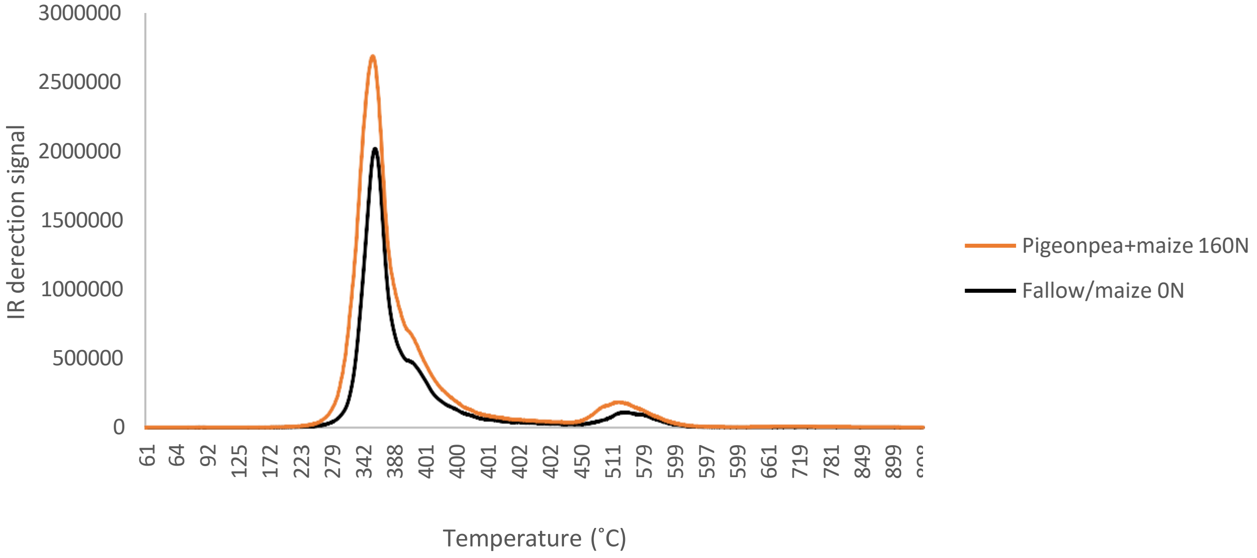


Figure 3.1 Example of changes in Infrared signals at increasing temperatures for contrasting soil samples at the 0-5 cm depth at 0N.

Historical evaluation and SOC sequestration rates

We extracted data from peer-reviewed publications which analysed SOC content until at least the 17.5 cm soil depth across cropping systems which mostly were related to systems without N or lime application (Bayer et al., 2000; Diekow et al., 2005; Vieira et al., 2009). At the time the experiment began in 1983, SOC stock in the 0-17.5 cm was 32.6 Mg ha^{-1} which had been measured through the Walkley-Black analytical method (Bayer et al., 2000). We therefore applied a correction factor of 0.9422 which was obtained by adjusting SOC results between Walkley-Black and dry combustion methods at the same facilities (Vieira et al., 2009). As previous soil sampling campaigns at the site were conducted until the 17.5 cm depth, we transformed data to 20 cm by assuming that SOC concentrations between these two depths were equivalent. SOC sequestration rates were calculated as the difference between changes in SOC stocks between our sampling campaign and the initial SOC stocks, divided by the amount of years since the experiment start, and a C stabilization coefficient was obtained by dividing mean SOC sequestration rates with C inputs (kg of stabilized C ha^{-1} year^{-1} Cinput^{-1}).

Annual carbon inputs by cropping systems

The aboveground biomass of cover crops was calculated as the average of field evaluations at experimental site, by assessing a database from the research group where crop biomass information was recorded since inception of the experiment in 1983. The aboveground biomass of maize was estimated from data of grain yields obtained throughout the years by using the following equation maize biomass = 0.84 Y grain + 2.9, developed by Lovato et al. (2004) at the same experimental site. Belowground biomass was estimated to be 30% of aboveground C as a mean value obtained by several researchers (Bolinder et al., 1997; Cordeiro et al., 2022; Kätterer et al., 2011; Veloso et al., 2019; Vieira et al., 2009). The carbon inputs were then calculated by considering the overall biomass of aboveground plus roots at a mean C concentration of 400 g C kg^{-1} biomass (Bayer et al., 2000; Vieira et al., 2009). As data collected from replicates has been bulked to give one result per treatment until 2008, data are shown without error means.

Statistical analysis

All statistical computations were performed using the software R, version 3.1.1. OC_{400} and ROC concentrations were evaluated across cropping systems, depths and fertilizer applications with linear mixed models by applying the Tukey test and considering results significant when $p<0.05$ (Pinheiro et al., 2009). Statistical analysis of SOC stocks did not include a depth factor in the ANOVA and the relationships between annual C addition and OC stocks was evaluated by comparing means of the coefficient of determination (R^2) of polynomial regressions of Pearson coefficients.

Results

Annual C input across cropping systems

Mean annual C inputs varied between 2.5 - 8.4 Mg ha^{-1} $year^{-1}$ depending on cropping system and fertilizer application, with lowest residues in the fallow/maize 0N and highest for pigeonpea

+ maize 160N (Figure 3.2). The contribution of C inputs by maize was impacted by N fertilization as it represented 34.4-52.5% (median 40.7%) of total annual C inputs in 0N systems, and 40.9-68.5% (median 52.1%) in 160N systems. In 0N systems maize C returns increased with increasing shares of legumes, whereas in 160N systems C inputs by maize were in a close range of between 3.2-3.5 Mg ha. In the fallow/maize systems, C inputs from cover crops are represented by weeds growing during the winter period.

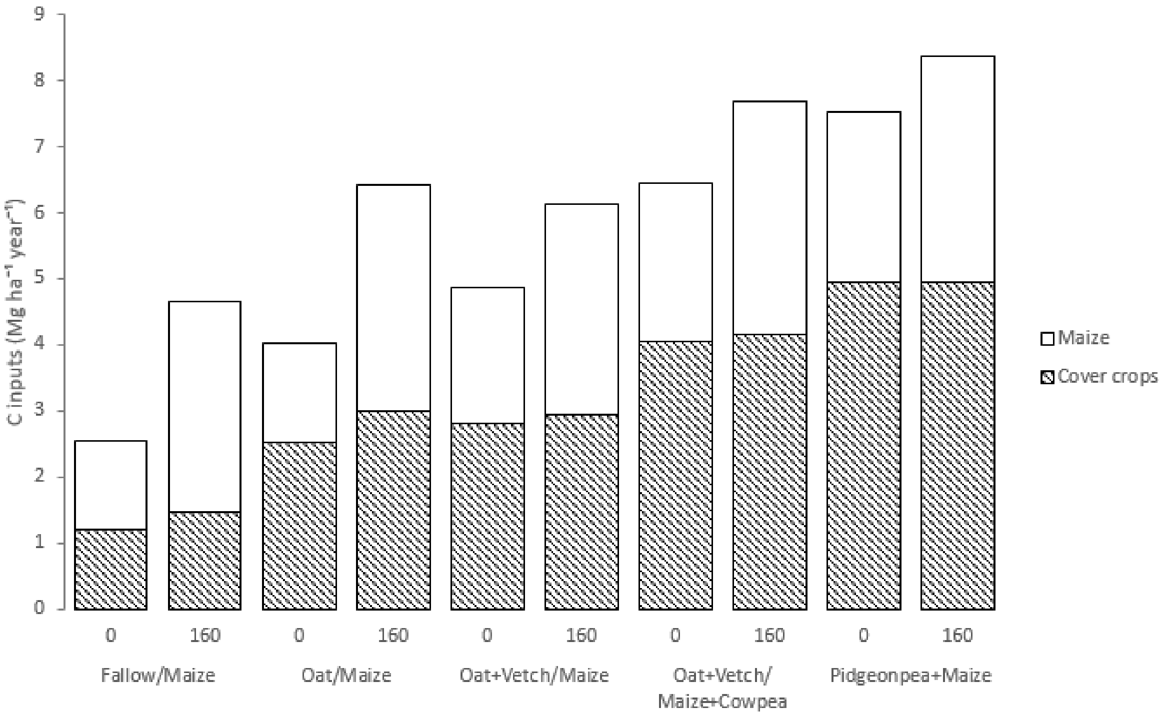


Figure 3.2 Carbon inputs from crop residues of either maize or cover crops origin, from a subtropical Acrisol subjected to no-till cropping systems for 39 years.

Soil organic carbon concentrations and stocks

Differences in OC_{400} due to management were found up to the 20 cm depth and up to the 30 cm depth for ROC (Table 3.1). Increasing complexity of cropping systems by means of introducing one or more cover crops, tended to yield similar OC_{400} concentrations as systems with less cover crops but receiving N fertilization. Inclusion of summer legumes (cowpea, pigeonpea) resulted in higher OC_{400} concentrations compared to systems with fallow or grasses over winter. ROC reported a similar trend as OC_{400} of increasing concentrations with increasing shares of summer legumes and N fertilization until the 10 cm depth, thereafter, systems left fallow over winter, especially without N fertilization, had as high ROC contents as other systems, and significantly higher than oat/maize 0N. In all cropping systems OC_{400} and ROC decreased with increasing depth ($p<0.001$).

Table 3.1 OC$_{400}$ and ROC concentrations (g kg^{-1}) from a subtropical Acrisol subjected to no-till

Depth	**N fertilization**	**Cropping system**									
		Fallow / Maize	Oat / Maize	Oat+Vetch / Maize	Oat+Vetch / Cowpea+Maize	Pigeonpea + Maize	Fallow / Maize	Oat / Maize	Oat+Vetch / Maize	Oat+Vetch / Cowpea+Maize	Pigeonpea + Maize
cm	kg ha^{-1}	OC$_{400}$ (g C kg^{-1} soil)					ROC (g C kg^{-1} soil)				
0-5	0	13.2 ^{d}	13.8 ^{d}	15.1 ^{cd}	18.5 ^{c}	26.2 ^{ab}	1.5 ^{de}	1.3 ^{e}	1.5 ^{de}	1.8 ^{bcd}	2.0 ^{abc}
	160	14.5 ^{d}	15.2 ^{cd}	15.7 ^{cd}	23.8 ^{b}	32.5 ^{a}	1.6 ^{cde}	1.7 ^{cde}	1.5 ^{de}	2.2 ^{ab}	2.5 ^{a}
5-10	0	8.3 ^{e}	9.0 ^{e}	9.3 ^{e}	12.0 ^{bc}	13.7 ^{ab}	1.1 ^{cd}	1.0 ^{d}	1.1 ^{cd}	1.4 ^{abc}	1.4 ^{abc}
	160	8.8 ^{e}	9.6 ^{cd}	10.0 ^{cd}	15.7 ^{a}	16.5 ^{a}	1.2 ^{bcd}	1.3 ^{bcd}	1.2 ^{cd}	1.7 ^{a}	1.6 ^{ab}
10-20	0	7.0 ^{c}	7.3 ^{bc}	7.4 ^{bc}	7.9 ^{abc}	8.7 ^{abc}	1.2 ^{a}	0.9 ^{b}	1.0 ^{ab}	1.1 ^{ab}	1.1 ^{a}
	160	7.0 ^{c}	7.7 ^{bc}	7.7 ^{bc}	9.1 ^{ab}	9.7 ^{a}	1.1 ^{ab}	1.0 ^{ab}	1.1 ^{ab}	1.2 ^{a}	1.1 ^{ab}
20-30	0	6.9	7.6	8.0	7.8	8.2	1.5 ^{a}	1.1 ^{b}	1.1 ^{ab}	1.1 ^{b}	1.1 ^{ab}
	160	7.4	7.5	7.6	8.2	8.6	1.3 ^{ab}	1.1 ^{ab}	1.2 ^{ab}	1.2 ^{ab}	1.2 ^{ab}
30-50	0	7.1	8.0	7.8	7.8	8.1	1.3	1.1	1.1	1.1	1.1
	160	7.7	7.6	7.8	7.8	7.9	1.2	1.1	1.2	1.3	1.2
50-75	0	6.3	6.2	6.2	6.6	7.0	1.0	1 .0	0.9	1.0	1.0
	160	6.4	6.5	6.1	6.7	6.8	0.9	1.0	1.0	1.0	1 .0
75-100	0	4.9	5.0	4.7	5.0	5.0	0.8	0.7	0.7	0.8	0.7

160	5.2	4.8	4.6	5.2	5.1	0.8	0.7	0.7	0.8	0.9

Different letters indicate significant differences (P<0.05) among cropping systems with or without fertilizer applications at each soil depth

SOC stocks varied between 44.5-73.1 Mg ha^{-1} in the top 30 cm and between 119.5-153.9 Mg ha^{-1} in the 0-100 cm soil depth with the fallow/maize 0N system reporting lowest stocks and pigeonpea+maize 160N the highest, at both depths. Contributions of ROC to SOC stocks in the upper 30 cm summed up to between 4.8-6.8 Mg ha^{-1} with highest ($P<0.05$) shares in fallow/maize 0N systems (16.3%) and lowest in pigeonpea+maize 160N (10.1%). Slightly higher contributions were found in the 0-100 cm depths, where ROC represented 12.6-16.5% of SOC, highest in fallow/maize 0N and lowest pigeonpea+maize 0N with stocks of ROC varying between 14.5 Mg ha^{-1} (oat/maize 0N) and 17.7 Mg ha^{-1} (pigeonpea+maize 160N).

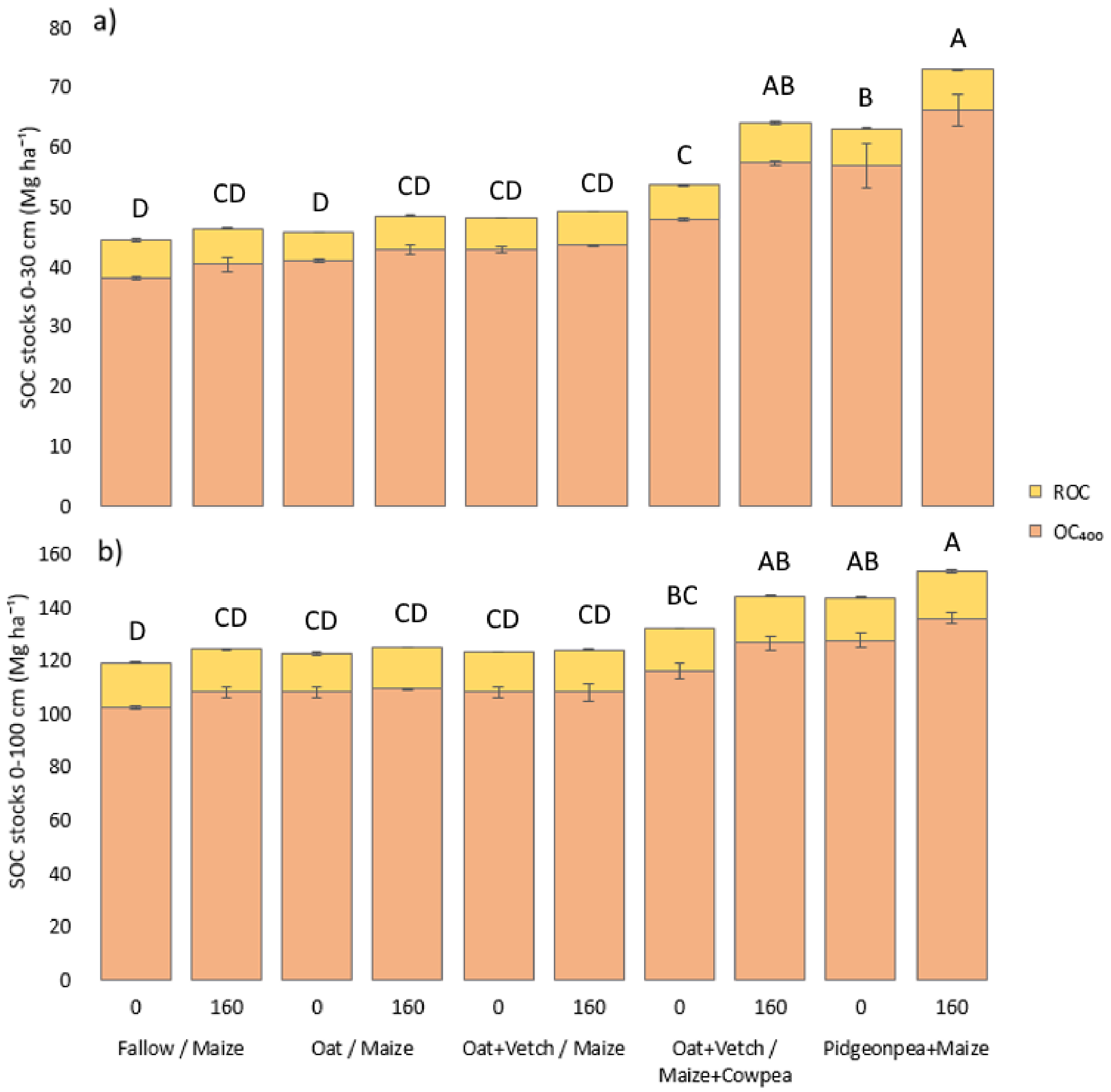


Figure 3.3 SOC stocks as sums of the OC_{400} and ROC stocks in the a) 0-30 cm and b) 0-100 cm depths from a subtropical Acrisol subjected to no-till cropping systems for 39 years. Different letters indicate significant differences in SOC stocks.

The C/N ratio in the 0-30 depth was highest ($p<0.05$) in fallow/maize 160N but similar across remaining cropping systems (Figure 3.4). On the other hand, pigeonpea+maize 160N had highest C/N ratios while oat+vetch/maize 0N the lowest. CN ratios in the top soil varied between 14.9 - 21.4 lower than the ratios in the subsoil which varied between 20.2-26.9.

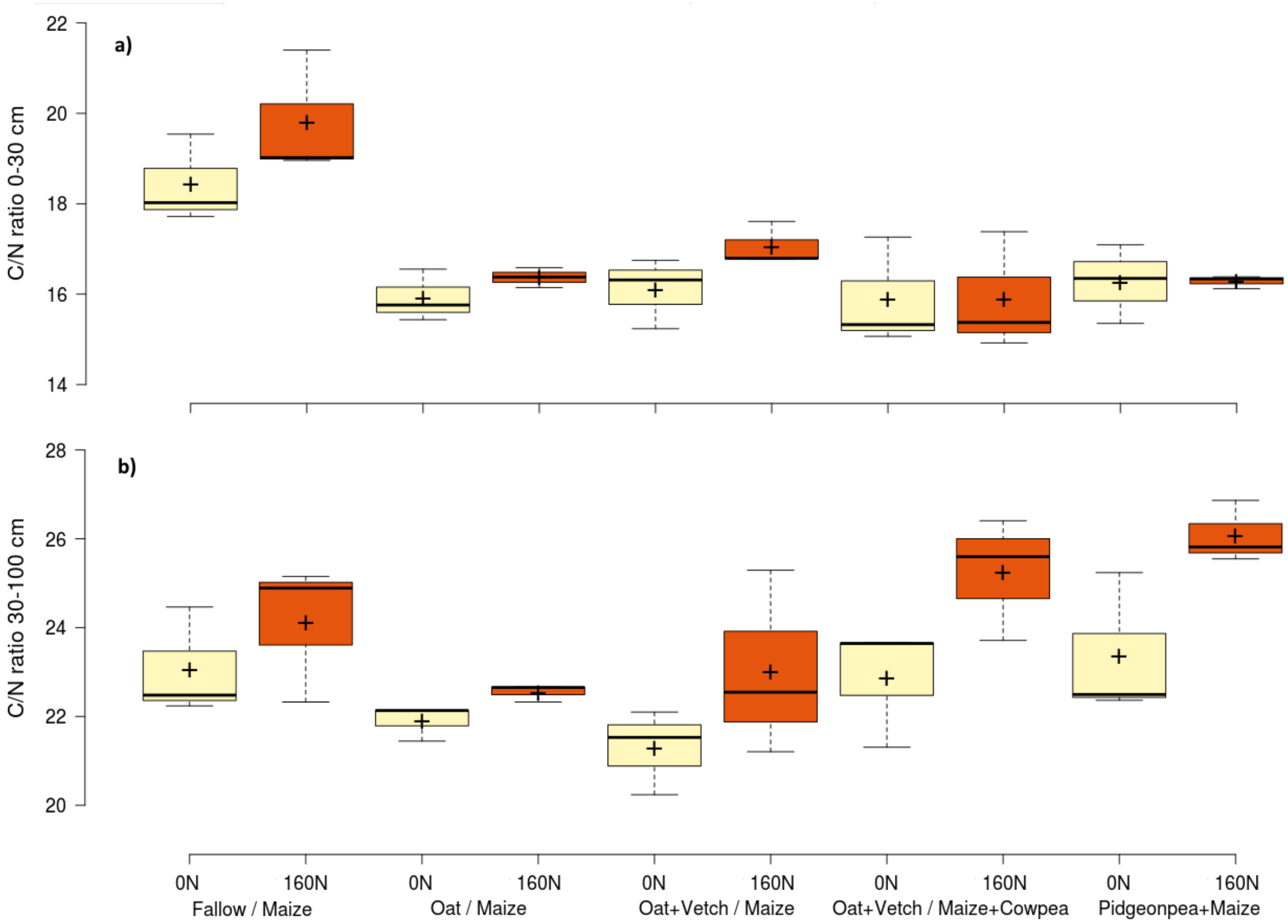
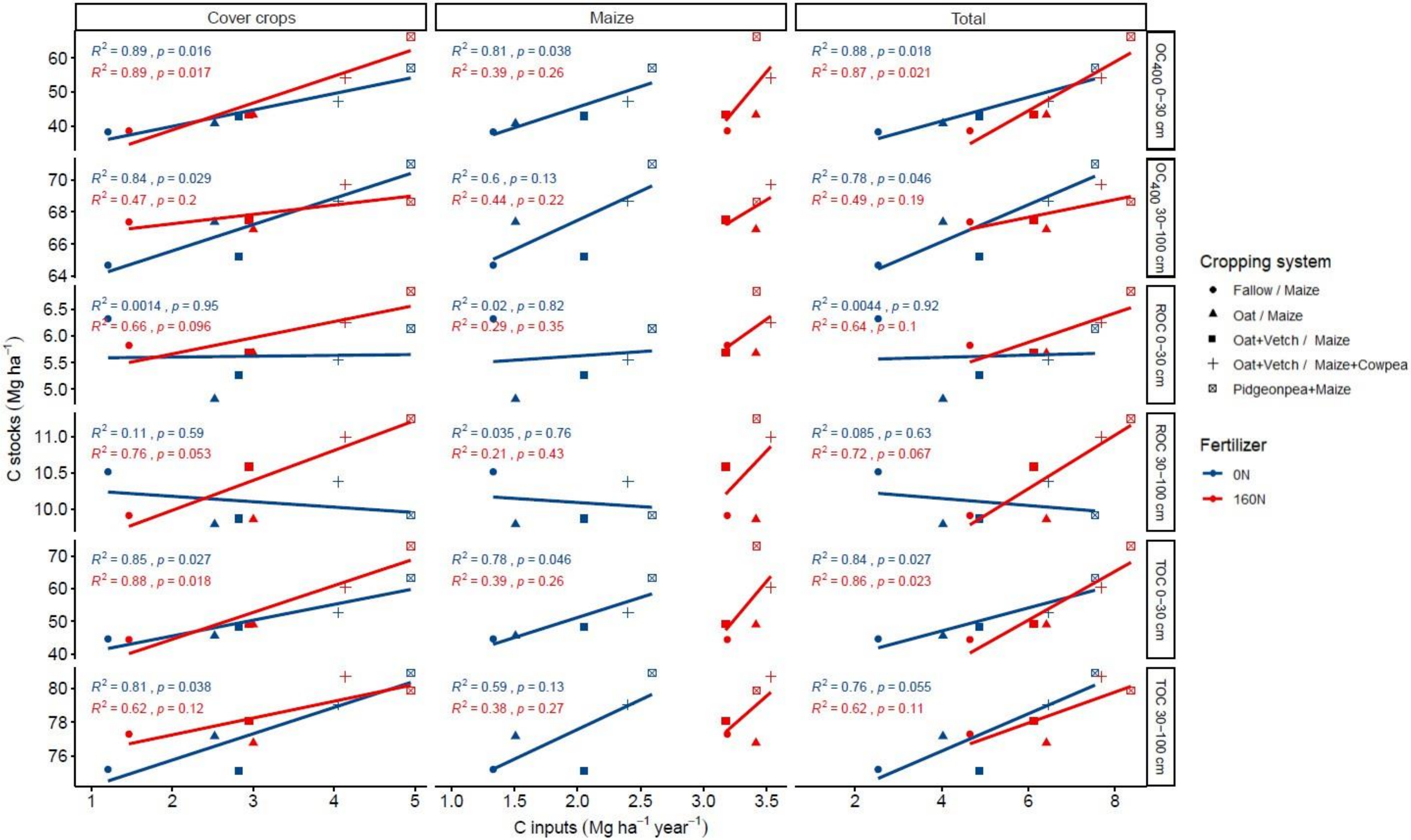


Figure 3.4 C/N ratios in the a) 0-30 cm and b) 30-100 cm depths from a subtropical Acrisol subjected to no-till cropping systems for 39 years.

Total C inputs from crops to the soil, could explain to a large extent (R^2 =0.84-0.86) the increase in SOC stocks in the top 30 cm although the response varied significantly between source of C inputs (cover crop or maize), fertilizer application (0N and 160N) and quality (OC_{400} and ROC) of SOC (Figure 3.5); amounts of C inputs from cover crop and maize were good predictors of OC_{400} stocks at 0N, whereas only C contributions from cover crops correlated with topsoil stocks at 160N. No significant relation was found between source of crop residues and ROC in the 0-30 layer although C inputs from fertilized crop residues did relate ($P<0.01$) to ROC stocks in the subsoil (30-100 cm). On the other hand, amounts of cover crops inputs at 0N were strongly correlated to OC_{400} stocks in the 30-100 cm depth and in overall subsoil SOC stock.



Cover crops
Maize
Total
OC400 0–30 cm
OC400 30–100 cm
ROC 0–30 cm
ROC 30–100 cm
TOC 0–30 cm
TOC 30–100 cm
C stocks (Mg ha⁻¹)
C inputs (Mg ha⁻¹ year⁻¹)
R² = 0.89 , p = 0.016
R² = 0.89 , p = 0.017
R² = 0.81 , p = 0.038
R² = 0.39 , p = 0.26
R² = 0.88 , p = 0.018
R² = 0.87 , p = 0.021
R² = 0.84 , p = 0.029
R² = 0.47 , p = 0.2
R² = 0.6 , p = 0.13
R² = 0.44 , p = 0.22
R² = 0.78 , p = 0.046
R² = 0.49 , p = 0.19
R² = 0.0014 , p = 0.95
R² = 0.66 , p = 0.096
R² = 0.02 , p = 0.82
R² = 0.29 , p = 0.35
R² = 0.0044 , p = 0.92
R² = 0.64 , p = 0.1
R² = 0.11 , p = 0.59
R² = 0.76 , p = 0.053
R² = 0.035 , p = 0.76
R² = 0.21 , p = 0.43
R² = 0.085 , p = 0.63
R² = 0.72 , p = 0.067
R² = 0.85 , p = 0.027
R² = 0.88 , p = 0.018
R² = 0.78 , p = 0.046
R² = 0.39 , p = 0.26
R² = 0.84 , p = 0.027
R² = 0.86 , p = 0.023
R² = 0.81 , p = 0.038
R² = 0.62 , p = 0.12
R² = 0.59 , p = 0.13
R² = 0.38 , p = 0.27
R² = 0.76 , p = 0.055
R² = 0.62 , p = 0.11
60
50
40
70
68
66
64
6.5
6.0
5.5
5.0
11.0
10.5
10.0
70
60
50
40
80
78
76
1
2
3
4
5
1.0
1.5
2.0
2.5
3.0
3.5
2
4
6
8
Cropping system
● Fallow / Maize
▲ Oat / Maize
■ Oat+Vetch / Maize
+ Oat+Vetch / Maize+Cowpea
⊠ Pidgeonpea+Maize
Fertilizer
0N
160N

Figure 3.5 Regression analysis between source of C input (either from cover crop or from maize or the sum of the two) on quality (OC_{400} or ROC) and quantity of SOC stocks in the 0-30 cm and 30-100 cm depths from a subtropical Acrisol subjected to no-till cropping systems for 39 years.

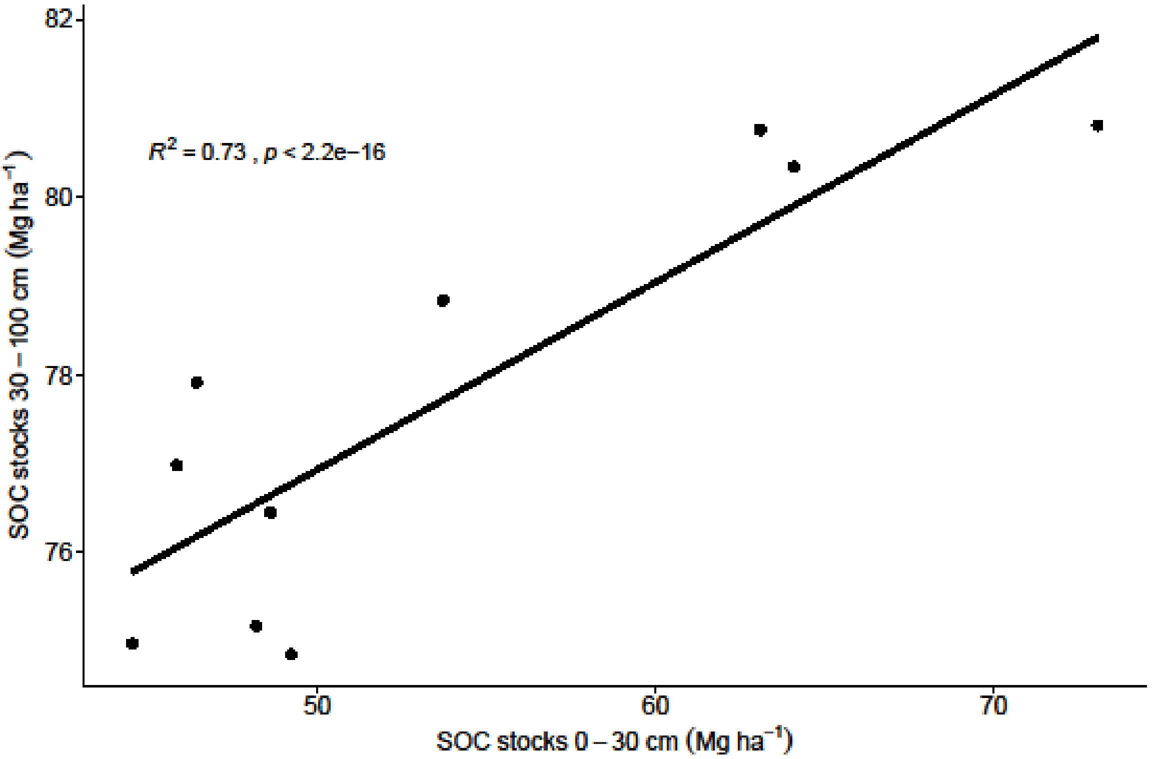


Figure 3.6. Correlation between SOC stocks in the topsoil (0-30 cm) and the subsoil (30-100 cm) from a subtropical Acrisol subjected to no-till cropping systems for 39 years.

SOC temporal evolution and sequestration rates

At the offset of the experiment in 1983, initial SOC stock in the 0-20 was 34.7 Mg ha^{-1} (Figure 3.7). After 11 years steep SOC increases were reported in systems including at least one legume (vetch, cowpea, pigeonpea) while stocks remained stable in oat/maize and decreased by in fallow/maize. In the year 2000, 17 years after the start of the experiment a decrease in stocks compared to the previous sampling was observed in oat/maize and pigeonpea+maize and confirmed two years later for remaining cropping systems. After 39 years from experimental set-up, we found that systems including summer legumes (cowpea, pigeonpea) increased SOC stocks whereas systems without summer legumes had lower SOC stocks than year 0. This resulted in negative sequestration rates and C stabilization coefficients for systems where SOC stocks had decreased compared to the initial SOC status (Table 3.2).

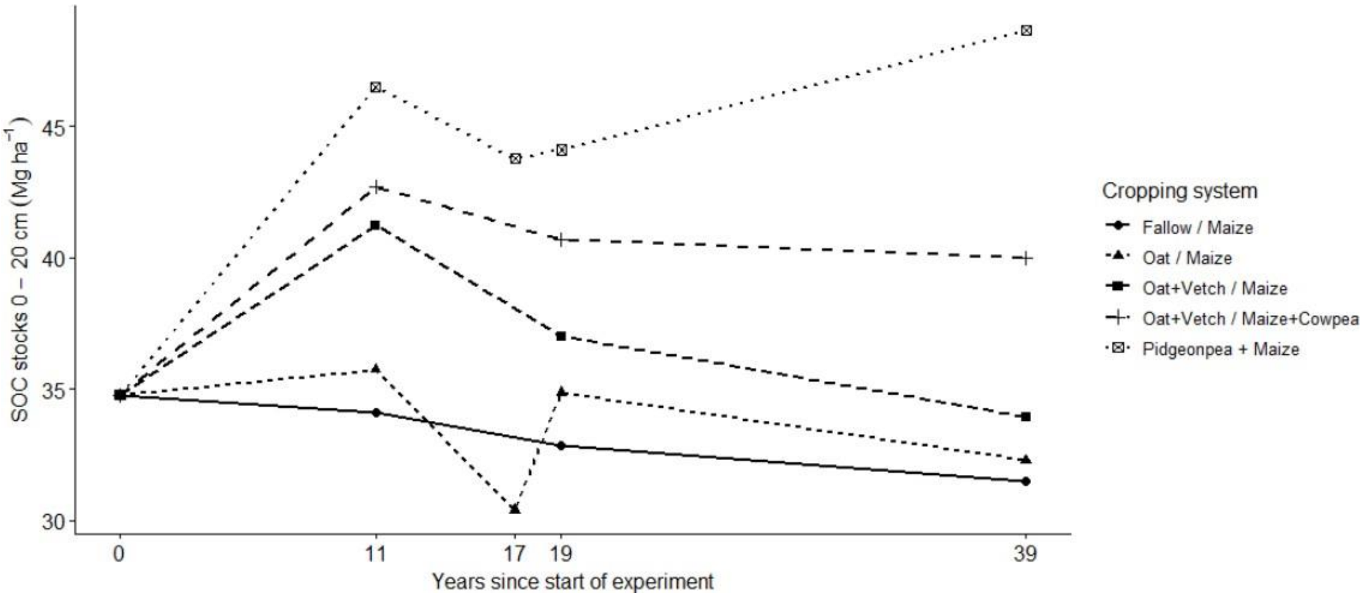


Figure 3.7. Soil organic C (SOC) stocks in the 0-20 cm depth from a subtropical Acrisol subjected to no-till cropping systems for 39 years. Data obtained from Bayer et al., 2000; Diekow et al., 2005; Vieira et al., 2009 and this study.

Table 3.2. SOC sequestration rates and C stabilization coefficients at the 0-20 cm depth from a subtropical Acrisol subjected to 39 years of no-till cropping systems.

Cropping system	**N fertilization**	SOC sequestration rates (Mg ha^{-1} year^{-1})ꞙ	C stabilization coefficient (kg ha^{-1} year^{-1})ψ
Fallow / Maize	0	-0.08	-33.0
	160	-0.05	-10.4
Oat / Maize	0	-0.06	-15.8
	160	0.01	1.8
Oat+Vetch / Maize	0	-0.02	-4.4
	160	0.02	3.7
Oat+Vetch / Maize+Cowpea	0	0.13	20.7
	160	0.38	49.4
Pigeonpea+Maize	0	0.36	47.2
	160	0.59	70.4

ꞙ SOC sequestration rates were calculated as the change in stocks between 1983 and 2020 divided by amount of years the experiment had been running for (39)

ψ The C stabilization coefficient was calculated by dividing SOC sequestration rates (Mg ha^{-1} year^{-1}) by annual C inputs from crop residues, therefore show annual amounts (kg) of C stabilized as SOC for every 1000 kg annually applied as crop residues

Discussion

SOC concentrations and stocks across managerial conditions

Diversification of maize cropping systems through the inclusion of winter and/or summer cover crops, increased SOC concentrations and stocks compared to systems which were left fallow over winter, especially in oat+vetch/cowpea+maize and pigeonpea+maize systems which increased whole profile SOC stocks by 9.8-22.3%. Our results are in line with previous studies from the region which found that spatiotemporal sowing of cover crops enhances the biomass production per unit of area by exploiting functional differences between crop traits, hence resulting in higher C returns from residues (Cordeiro et al., 2022; Leite et al., 2010; Vieira et al., 2009) which positively relate to SOC stocks (Corbeels et al., 2016; de Oliveira Ferreira et al., 2013; Locatelli et al., 2020). Different and overlapping pathways of SOC stabilization may have occurred across cropping systems at the various soil depths. First of all, avoidance of soil particles physical disruption in sub-tropical NT cropping systems, increases the stability of C compounds through the binding to clay particles (Diekow et al., 2005; Veloso et al., 2019), resulting in higher structural complexity which provides resistance from microbial decomposition (Babujia et al., 2010; Poeplau et al., 2018) and exacerbated by spatial inaccessibility of microbes to access C clustered within aggregates (Barreto et al., 2021; Six et al., 2002), overall increasing the mean residence time of OC in the soil with increasing C applications (Barrios et al., 1996; Locatelli et al., 2020; Vieira et al., 2009). This was reflected by a clear correlation between cumulative amounts of C inputs at both fertilization levels and SOC stocks in the topsoil. The humification process requires N availability for SOC retention (Lehmann et al., 2020), which was supplied to the sampled systems as either mineral N or as N derived from legumes´ biological N fixation (BNF-N), or a combination of the two. The increasing stabilization of SOC with increasing N application (legume residues and/or fertilizer) may be due to C immobilization under limited N availability (Blankenau et al., 2000; Sawada et al., 2015) as C sources stimulate microbial activity, but decreases the substrate use efficiency of microbes as additional energy is used to decompose N from recalcitrant SOM (Mahal et al., 2019). Therefore in the absence of N, C stabilized in the soil was low resulting in negative SOC sequestration rates. Increasing crop species diversity, can increase microbial abundance and ability of soil fauna to favour SOM stabilization (Wu et al., 2017) particularly with legumes which may provide continuous sources of N as rhizodeposits and anchors for arbuscular mycorrhizal fungi which can stimulate plant nutrient uptake in cropping systems (Babujia et al., 2010).

Interestingly, C inputs from cover crops significantly related to SOC stocks in the 30-100 cm depth when no fertilizer was applied which may have been due to crops developing deeper rooting patterns as means of nutrient acquisition at lower soil strata, hence providing more C from roots beyond the 30 cm depth as well as higher BNF-N as a response to N depletion by the maize crop (Pampana et al., 2018). Fertilizer application may have resulted in an increase in N availability in the top soil restraining roots´ requirements to obtain nutrients at deeper depths (Shahbaz et al., 2017) as well as decreasing BNF contributions by intercropped summer legumes as BNF is inhibited with increasing N availability (Askegaard and Eriksen, 2007).

Higher top soil OC was linearly associated ($R^2=0.72$, $p<0.001$) with higher subsoil OC, suggesting percolation of dissolved organic carbon from upper layers with higher SOC concentrations to lower strata with a C deficiency (Gross and Harrison, 2019). Vertical percolation of soluble OC can enhance subsoil carbon pools especially in these regions which are prone to high precipitation events (Dou et al., 2008), although roots and root exudates represent the vast portion of newly sequestered SOC in the subsoil (Kätterer et al., 2011). Moreover soil C/N ratios can be a reliable indicator of soil organic matter quality and its expected decomposability (Palm et al., 2001). C/N ratios across treatments in the top soil were around 16.2, except for fallow/maize where the mean ratio across fertilizer levels reached 18.4 which tended to be in line with topsoil data for the region (Sisti et al., 2004). For organic residues, microbial decomposition is generally expected to promote net N immobilization when initial C/N ratios are >30, whereas values <20 commonly result in N mineralization (Mooshammer et al., 2022; Palm et al., 2001), including losses via nitrification (Gomes et al., 2009). For instance, soil organic matter decomposition as measured by CO_2 and N_2O emissions was negatively correlated with soil C/N ratios at the same experimental site (Gomes et al., 2009). However, C/N ratios in soils vary widely due to crop, depth and percent of plant litter in regard to total SOC. C/N ratios were lower in the topsoil probably, due to a more advanced stage of decay than the subsurface (Rumpel and Kögel-Knabner, 2011; Ye et al., 2019) although limited response of cropping system and fertilizer applications were found in the topsoil, whereas ratios did vary in the subsoil layer. Soil texture can also affect soil C/N ratios, as the mechanisms of organic matter retention vary among soil particle sizes. In a Ferralsol field experiment in Uganda, C/N ratios ranged from 15 to 21 in clay fractions, and from 32 to 47 in sand fractions (Musinguzi et al., 2015). As amount of clay increased with depth (Diekow et al., 2005) this may have resulted in additional binding sites for decomposing SOM leading to overall subsoil C/N ratios being higher than the topsoil where more OM was available, but still <30 (between 20.2-26.9), meaning bioavailability of C/N which may be exploited by roots to acquire nutrients at deeper depths (Kätterer et al., 2011). Selecting for crops with a deep rooted architecture is therefore suggested as a favourable means to sequester C in the subsoil, as less exposed to mineralization due to environmental or managerial events in Acrisols of southern Brazil.

Temporal dynamics of SOC and sequestration rates

As soils were degraded by the time the experiment was established, large gains in SOC were reported after 11 years (Bayer et al., 2000) implying a rapid response from soil fauna to the surplus of easily degradable organic matter (Corbeels et al., 2016; Locatelli et al., 2020). Whereas changes in SOC within shorter periods may be due to increases in labile organic matter, stabilization of SOC into more stable fractions can take decades to millennia (Parton et al., 1987). The decrease in SOC stocks between the 11th year and subsequent samplings was unexpected, but may have occurred due to seasonal shifts in SOC composition across the cropping season. SOM decompositions rates are high in the region, which may result in lower SOM levels in early summer, while amounts of OM inputs may be higher at the end of the season due to accumulation of litterfall, residue decomposition and rhizodeposits which may have resulted in the higher than expected stocks in year 11, being ~7% (~2 Mg) higher than in year 17. For example in a paddy rice experiment in Thailand year to year fluctuations in SOC stocks were found, due to the sampling after various crop rotations which depleted or enhanced the labile C pools significantly (Cha-un et al., 2015).

Although winter cropping of oat and vetch is feasible in the area, these could not produce enough organic matter to return to the soil to sustain SOC stocks, which was instead achievable with the introduction of a third cover crop (cowpea) as well as with a high biomass producing legume such as pigeonpea. Bayer et al., (2000) calculated than on average it was required to apply 3.9 Mg C ha^{-1} $year^{-1}$ to maintain initial SOC stocks, which was just slightly higher than what calculated by Vieira et al., (2009) of 4.05 Mg C ha^{-1} $year^{-1}$ (~10 Mg ha^{-1} of crop residues). These rates are higher compared to Mediterranean cropping systems where Kong et al., (2005) found that 3.1 Mg C ha^{-1} $year^{-1}$ were required to sustain SOC stocks, highlighting the fairly favourable climatic conditions for SOM decomposition at our sampling site and the importance of high input cropping systems to guarantee provision of OM for maintaining SOC stocks. In Brazil, no-tillage with leguminous cover crops has led to the C sequestration rates between 1.48-1.61 Mg C ha^{-1} $year^{-1}$ in the Cerrado region (Corbeels et al., 2016) and of 2.61 Mg C ha^{-1} $year^{-1}$ on a sandy soil in the Sao Paolo region (Cordeiro et al., 2022) whereas we report lower sequestration rates (-0.08 and 0.59 Mg SOC ha^{-1} $year^{-1}$) as these largely depend on initial SOC levels, age of experimental site and interactions between biotic and abiotic variables (Angst et al., 2018). In fact, while rapid increases in SOC may be recorded 5-10 years after the rehabilitation of degraded soils, with the application of high quantities of SOC, long-term rates tend to be lower, due to the finite capacity of soils to store C. As linear increases in the PM cropping system were still reported, it may indicate that the upper limit of C saturation has not been met yet in the sampled systems.

Thermal stability of SOC

Enhancing storage of recalcitrant SOC stocks is desirable for sequestrating atmospheric CO_2, as these fractions have highest persistence rates and lowest decomposability (Herzfeld et al., 2021; Poeplau et al., 2018). We found highest shares of ROC in systems with highest C inputs as well as in systems with lowest C inputs which may highlight opposing pathways of C sequestration. Residual inputs in fallow/maize systems without N application, were derived from maize mostly and partially by weeds during winter which led to the input of mostly lignified residues resulting in SOC stocks with the highest percentage of ROC (16.7%) as well as highest topsoil C/N ratios (18.4). Nevertheless recent reviews stress that soils with high bioavailability have higher potential in storing SOC and N, even if this can result in higher gaseous emissions from SOM decomposition, as the biological activity of microbes, earthworms and fungi plays a key role in the stabilization of SOC and overall soil functioning (Herzfeld et al., 2021). This tended to be in line with our results, as OV/MC and P+M systems had lower shares of ROC, but highest stocks of both OC_{400} and ROC. Combination of different quality of residues, especially from N-fixing legumes, is of paramount importance to build up SOM which comprises of both biologically active as well as persistent C fractions (Gentile et al., 2013) as sources of nutrient and energy for decomposition processes.

Conclusions

The retention of organic matter from crop residues in soils plays a crucial role on future scenarios of SOC sequestration potential in cropland. We demonstrate that high input cropping systems under NT enhance SOC concentration in the topsoil. Furthermore, we provide new insights on SOC dynamics in the subsoil, by showing that inclusion of leguminous cover crops can lead to enhancing subsoil SOC stocks which is a core achievement in the light of C sequestration agendas. Most of the C which was sequestered was reported in the pool oxidized up to 400 °C suggesting that SOC sequestration may result through enhancing the rather bioavailable fraction which in turn may lead to mechanisms of C stabilization in more (thermally) recalcitrant forms. Diversification of maize cropping systems with legumes under no-till agriculture, is a viable means for enhancing not only the quantity, but also the quality of SOC on subtropical Acrisols in southern Brazil.

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4. Carbon isotopic signatures in SOM fractions from mixed C_3/C_4 cropping systems suggest mechanisms of soil fertility build-up thanks to legumes

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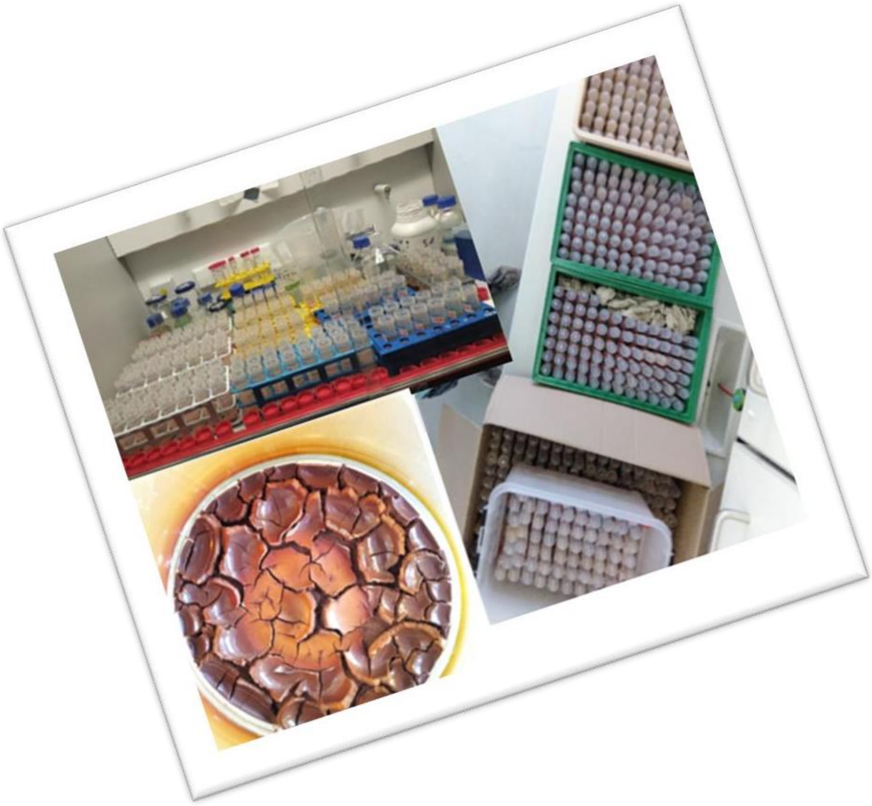

Carbon isotopic signatures in SOM fractions from mixed C_3/C_4 cropping systems suggests mechanisms of soil fertility build-up thanks to legumes

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Abstract

In our previous work we speculated that quantities of C inputs from leguminous cover crops enhanced soil organic carbon (SOC) stocks of subtropical no-till maize cropping systems, whereas in the current experiment, we prove it. Plants with C_3 or C_4 photosynthesis have unique $\delta\ ^{13}C$ signatures which are not altered during incorporation in soil organic matter (SOM) or during decomposition to SOC. $\delta\ ^{13}C$ values have been used to address the contributions of plants with differing photosynthetic pathways to SOC, but the mechanisms associated with isotopic signatures in maize-legume cropping systems and SOC fractions with varying biochemical stability, remains unaddressed. Therefore we combined physical, density and chemical fractionation of soil samples to obtain five fractions namely: silt+clay (SC), recalcitrant SOC (rSOC), stable aggregates + sand (S+A), dissolved organic carbon (DOC) and particulate organic matter (POM) and measured $\delta^{13}C$ signatures within. We hypothesize that legumes, which have a C_3 photosynthetic pathway, will decrease the isotopic signatures differently across different fractions, and that this will allow us to assess mechanisms of SOC formation in mixed C_3/C_4 cropping systems. Soil samples were obtained from 5 cropping systems with increasing shares of C_3 vegetation, at four soil depths, bulked into 0-10 and 20-50 cm depths to calculate stocks, from a 39 years old field experiment on an Acrisol in Southern Brazil. Relative shifts in fraction´s SOC stocks occurred mostly in labile POM, DOC and S+A fractions, although absolute changes occurred mostly in the rSOC and SC fractions; this correlated to sample´s isotopic signatures in the top 0-10 cm, where C_3 derived C accounted to up to 65% of total SOC corresponding to contribution of 23.7 Mg ha^{-1} of SOC. Although no differences were found in fraction´s SOC stocks at 20-50 cm depths, C_3 derived C from cover crops can represent a major pathway for sequestrating atmospheric CO_2.

Keywords C_3/C_4 vegetation shift, SOM fractionation, ^{13}C abundance

Introduction

Retention of crop residues on the soil surface directly contributes to greenhouse gas emissions, and their use could be diverted towards the production of bioenergy; nevertheless retention of above and below ground residues in subtropical cropland is crucial for maintaining soil organic carbon (SOC) stocks and guaranteeing soil functions (Derrien et al., 2023; Herzfeld et al., 2021).

While plant residues´ chemical recalcitrance has historically been regarded as the main explanatory factor for describing the persistence of C in the soil (Derrien et al., 2023), experiments on the reduction of tillage intensity have brought forward the importance of SOC aggregation as means of allowing for the physical and spatial protection of labile C compounds through occlusion within micro and macroaggregates, by stopping disruption due to ploughing while slowing down access to microorganisms, leading to less rapid mineralization (Bayer et al., 2001; Guo et al., 2016). Binding of organic carbon compounds to clay and minerals instead, can be considered as structural units of soil aggregates and nanoparticulate fractions of the smaller aggregates themselves, which are important for biochemical stabilization in the form of more stable SOC compounds (Derrien et al., 2023). Recent conceptual frameworks also stress out the role of microbes in assessing, immobilizing and digesting SOM leading to microbial by-products which become the main precursors of stable SOM and are integrated in a complex continuum of chemical bonding and aggregations in the soil matrix across space and time (Cotrufo et al., 2013).

Labelling plant residues with ^{13}C isotopes has helped scientists tracing the fate and the dynamics of SOC turnover across the various pathways of C assimilation in the soil (De Clercq et al., 2015; Hupe et al., 2018a; Kuzyakov et al., 2006; Putz et al., 2011). The preferential fixation of ^{12}C over ^{13}C during photosynthesis, results in the ^{13}C signature of C_3 plants (Calvin cycle) being lower (more negative) than that of C_4 plants (Hatch-Slack pathway) (Calvin, 1989).

Within the C_3 photosynthetic pathway, the degree of $\delta^{13}C$ discrimination during photosynthesis is closely linked to the opening and closing of plants´ stomata. Open stomata, which permit the escape of water (cooling the plant and facilitating the movement of nutrients), ensure free assimilation of CO_2, such that discrimination against $\delta^{13}C$ is at its maximum (Berriel et al., 2020; Farquhar et al., 1982) to cope with high temperatures. "Natural" isotopic labelling can occur when growing C_4 plants on a soil that has previously been under C_3 vegetation, or viceversa (Rao et al., 1994). Soils dominated by either C_3 or C_4 vegetation tend to resemble the isotopic abundance of the plant residue input (Sisti et al., 2004), since microbial decomposers of plant parts have similar ratios of carbon isotopes as their decomposing material (Pausch and Kuzyakov, 2018). Most $\delta^{13}C$ values for C_3 plants are often reported to be between -30 and -24‰ while for C_4 species the isotopic discrimination is less pronounced and often falls between –16 and –10‰ (Balesdent et al., 1987; Farquhar et al., 1982). These can help calculating the C contributions to SOC stocks caused by vegetation changes, by using an isotope end-member mixing model (Balesdentet al.1987), with the assumptions of similar isotopic fractionation during humification of C_3 and C_4 plant residues and a constant temporal ^{13}C abundance of the input C (Cadisch and Giller, 1996).

Although assessment of bulk soils can give a good result on how managerial practices impact SOC, this may mask the evaluation of structural C compartments which may suggest pathways of SOC formation with suitable controls. For example in Germany, diversification of crop rotations with the inclusion of legumes opposed to continuous maize, increased SOC storage by 4.1% per year after 34 years, which was traced to be attributed to the presence of vetch, as $\delta^{13}C$ signatures across the soil profile were largely decreased in such crop rotations, confirming the contribution of C_3 species in the C_4 cropping system. Lab fractionation techniques have been established to distinguish SOC fractions which are more or less bioavailable, with various degrees of structural stability and mid-residence times (Parton et al., 1987). Common lab fractionation techniques encompass physical, chemical and/or density steps, with most complex ones, including all three (Poeplau et al., 2018) and results may be fitted into C pools to model long-term SOC dynamics (Gollany et al., 2013; Liu et al., 2021). Barrios et al., (1996) found no short-term differences in total SOC stocks in mixed legume-maize systems compared to monocropped maize in Kenya, although different compositions on the quality of stocks were found, as systems with legumes enhanced availability of light fractions bound to sand particles, which may have led to enhancing more stable fractions in the long-run.

Combining the study of the quality of SOM, with the isotopic shift due to shifts in C_3/C_4 vegetation, may therefore aid understanding changes due to different crop species as well as stabilization mechanisms of SOC. Although the method has been applied to other settings, never has this combined approach been applied to mixed C_3/C_4 species including legumes and cereals, possibly due to the fact that few experiments with a suitable design exist, especially which have been running for several decades to allow for changes to be detectable.

We therefore hypothesize that diversification of cropping systems through the inclusion of leguminous cover crops will lead to a distinct biochemical SOM composition compared to maize monoculture. Moreover we expect that SOC fractions and their isotopic signatures will shed light on mechanisms of SOC formation due to C_3 crops.

Experimental

Site description

The ongoing field experiment started in 1983 and it is located at the Agronomic Experimental Station of the Federal University of Rio Grande do Sul, in Eldorado do Sul (30°51´ S and 51°38´W), in Southern Brazil as described in Binacchi et al., (*in preparation*). Until 1969, the area was under native C_4 grassland (mainly *Paspalum* and *Andropogon*), thereafter it was converted into cropland with a mostly C_3 vegetation (*Brassica napus)* and the soil was subjected to intensive plowing and disking for 16 years, and presented visible signs of soil degradation. Briefly, the regional climate is categorized as subtropical humid, with a mean annual temperature of 19.4°C and a mean annual rainfall of 1440 mm, evenly distributed throughout the year. Soils are classified as Acrisols (FAO, 2002), with a sandy clay loam texture (540 g kg^{-1} sand, 240 g kg^{-1} silt, and 220 g kg^{-1} clay) and the clay fraction is dominated by kaolinite (720 g kg^{-1}) and Fe oxides (109 g kg^{-1} Fe_2O_3). The experiment comprises 10 cropping systems, where maize is grown as the main cash crop in summer. We selected 5 cropping systems: a control fallow/maize (*Zea mays* (L.)) (F/M), and then increasing shares of C_3 crops: oat (*Avena strigosa* Schreb)/maize (O/M), oat+vetch (*Vicia sativa* (L.))/maize (OV/M), oat+vetch/maize+cowpea (*Vigna unguiculata* (L.) Walp) (OV/MC) and pigeonpea (*Cajanus cajan* (L.) Millsp) + maize (PM) where "/" differentiates between winter and summer cropping and "+" indicates plants were intercropped. Main plots measured 8 × 5 m and were divided into two 4 × 5 m subplots at two N fertilization levels (0 or 160 kg ha^{-1}).

Soil sampling

Soil samples for the fractionation were selected from the 0-5, 5-10, 20-30 and 30-50 cm depths by excavating with a mechanical spiral auger at two locations within each plot and bulking samples into one which were then air-dried for a week and milled to pass through a 5 mm sieve as described in Binacchi et al., (*in preparation*).

SOC fractionation and analysis

SOC fractionation was performed as initially described by Zimmermann et al., (2007) and refined by Poeplau et al., (2013). First, 20 g dry soil was mixed with 150 mL deionised water in a 250-mL beaker glass and treated with ultrasound using a total energy of 22 J mL^{-1} at an output power of 30 W. The resultant dispersion was wet-sieved over a 63-mm sieve to separate the fine silt and clay (SC) fraction from a coarse fraction (>63 mm). An aerosol pump spray was used to flush the sample with a fixed amount (1600 mL) of water. The suspension was centrifuged for 30 min at 1000g. An aliquot of decanted water (~200 mL) was filtered through a 0.45 mm membrane filter and analysed for dissolved organic carbon (DOC) by freeze/drying liquids. The SC fraction and the coarse sieve residues were dried at 60˚C and weighed. The coarse fraction (>63 mm) was further separated into a heavy sand and aggregates (SA) fraction and a light particulate organic matter (POM) fraction by density fractionation with sodium polytungstate (SPT) solution at a density of 1.8 g cm^{-3}. After adding SPT to the coarse soil, the sample was centrifuged for 15 min at 1000g and allowed to stand for several hours until the POM fraction was entirely floating on the SPT. The POM was then carefully transferred to sieve bags with a mesh size of 50 mm. The entire density fractionation step was repeated once more to ensure complete separation of the SA and POM fractions. Also, the SA fraction was transferred to sieve bags, which had the advantage that soil material could be easily washed under the deionised water tap to remove SPT and subsequently quickly dried and weighed without changing the vessel. Finally, a chemically resistant SOC (rSOC) fraction was isolated from the fine SC fraction by oxidation with 6% sodium hypochlorite (NaOCl). The NaOCl solution was first adjusted to pH 8 using hydrochloric acid (HCl) and then 50 mL of the solution was added to a 1 g subsample of the SC fraction. After 18 h of oxidation at room temperature, the sample was centrifuged (15 min, 1000g) and decanted. This procedure was repeated twice more and after each oxidation and decanting step the sample was washed once with 50 mL deionised water. To prevent waterlogging in the fine-textured soil pellet, a vortex shaker was used every time liquid was added to the soil to thoroughly mix liquid and solid, ensuring complete oxidation and cleaning. The fractionation procedure thus yielded five fractions, two fast cycling (POM, DOC), one intermediate (SA), one slow cycling (SC) and one passive (rSOC) (Figure 1).

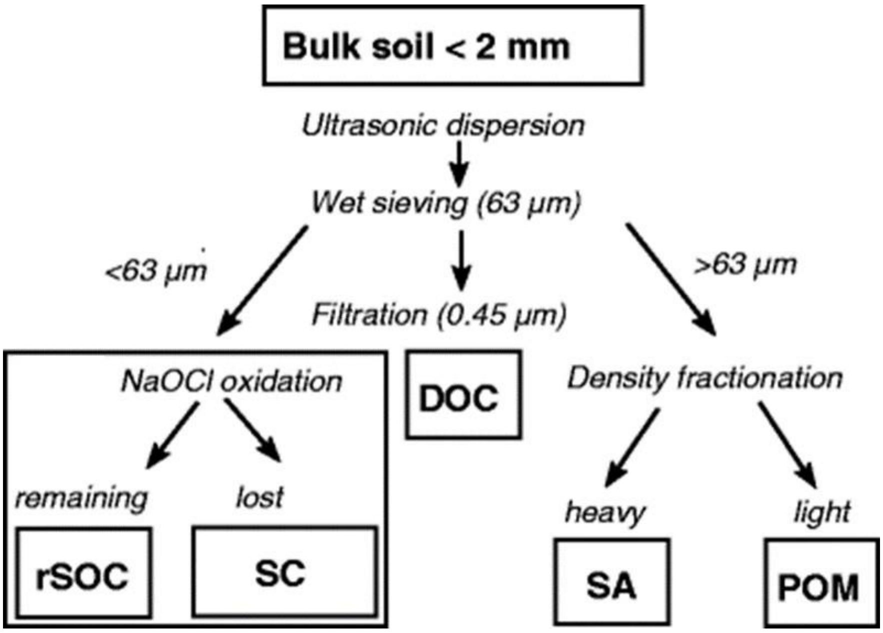

Figure 4.1 Overview of the fractionation technique and resulting fractions, modified after Zimmermann et al., (2007).

Elemental and isotopic analysis

Elemental and isotopic analysis was conducted using elemental analyser (Euro EA, Eurovector, Italy) coupled to an isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Organic C, $\delta^{13}C$ and $\delta^{15}N$ values were determined in all of the above-mentioned fractions as well as in the bulk soil. The samples were weighed into, 8 × 5 mm tin capsules and analysed against a known set of standards (Acetanilide chemical standard for organic C and N plus ANU sucrose for $\delta^{13}C$). Overall precision, including machine and sample preparation error was < 0.001% and met set quality control standards. Isotope results were reported in the conventional delta (δ) notation, with units of per mil or parts per thousand (‰), relative to the international standard of Vienna-Pee Dee Belemnite (V-PDB):

$$\delta^{13}C\ (‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000$$

In this equation, R is the molar ratio of the heavy to light isotope (i.e. ^{13}C /^{12}C) in the sample or standard under analysis. Similarly we obtained $\delta^{15}N$, but instead of V-PDB we calculated $\delta^{15}N$ (ratio of $^{14}N/^{15}N$) relative to atmospheric ^{15}N abundance which is stable at 0.3663%.

Calculation of C_3C and C_4C stocks across fractions

Soil organic carbon stocks were calculated, using the bulk density (g cm^{-3}) of the fallow/maize 0N system, to allow for comparison among different treatments with a fixed mass basis and multiplied by the %C in each of the 5 fractions, at the four depths (cm). We then grouped C stocks in two: the 0-10 cm layer and the 20-50 cm layer by simple addition of C stocks at the corresponding depths.

The proportion of organic C derived from C_3 vegetation in the mixed C_3-C_4 systems (f C_3C), was estimated with a mass balance equation based on a two-compartment mixing model (Cadisch and Giller, 1996) for each of the five fractions (SC, rSC, SA, POM, DOC) as well as for the bulk soil:

$$\text{f } C_3C\ (\%) = \left(\frac{\delta^{13}C_{soil} - \delta^{13}C_{C4\ soil}}{\delta^{13}C_3 - \delta^{13}C_{C4\ soil}}\right) \times 100$$

Where $\delta^{13}C_{soil}$ is the measured $\delta^{13}\mathrm{C}$ value in each fraction of mixed C_3-C_4 systems, $\delta^{13}C_{C4\ soil}$ is the measured $\delta^{13}\mathrm{C}$ value in each fraction of fallow/maize 0N treatment, and $\delta^{13}C_3$ is an average value taken from literature of mean (above and below ground) $\delta^{13}\mathrm{C}$ signatures of the C_3 plants taken into account: -27.0 (Jahren and Kraft, 2008). The proportion of grass and maize derived C_4C was calculated by subtracting C_3C from the totality:

Soil C_4C (%) = 100- f C_3C

Statistical analysis

Data were checked for normality with the Shapiro-Wilk normality test. Four-factorial ANOVAs including “cropping system”, “depth”, “fertilizer”, and “fractions” as explanatory variables were run to assess the absolute and relative changes in SOC stocks – with both total stocks and C_3 derived stocks used as responsive variables, and accepted as significant with a $p<0.05$.

Post-hoc tests with the function pairwise T-test were ran for the chosen explanatory variables independently, when the ANOVAs detected significant interactions. No statistical test were made for C concentrations, fractions´ size or $\delta^{13}C$ signatures, as these were calculated with values already processed in ANOVAs and data is presented accompanied by the standard error of the mean (s.e.m). Mathematical calculations and descriptive statistical analysis were done with Microsoft Excel. Statistical testing was done using the statistical program R version 2.8.1 (R Development Core Team 2008) using linear mixed models (Piepho, 2019).

Results and discussion

Size and C content of fractions

C concentration within fractions decreased in the following order: POM<DOC<SC<rSOC<S+A as it reflected the forms of C found across the fractions and the different compounds which form SOC, as was found by other studies using the same fractionation scheme in cropland settings (Sun et al., 2019; Trigalet et al., 2016). As POM includes plant material and microbial detritus at initial stages of decomposition, the higher C content confirms the plant-derived origin of POM with its associated higher C concentration than SOC (Gosling et al., 2013). The quantity of particulate organic matter in soil is determined by the balance between plant litter inputs and microbial decomposition. POM has a turnover time of less than 10 years (Blagodatskaya et al., 2011; Bol et al., 2009), although data from the region suggests even shorter turnover rates due to warm and moist pedoclimatic conditions which may lead to rapid mineralization under sub-tropical conditions (Six et al., 2002). Although F/M systems had the lowest biomass input (Vieira et al., 2009), the C concentration and share of fraction´s weight (Table 4.1) were fairly high which may have been due to a low availability of N compared to the quantities of applied C, leading to inhibition of decomposition of crop residues as the rate

of decay of SOC is positively correlated with the availability of N (Gomes et al., 2019). Across all systems, POM in the top 5 cm was found to be 8.2-20.1 % of sample´s total mass, moreover shares of POM decreased with depth, and little to no material could be recovered in the 30-50 cm layer. As we sampled during the maize growing period, approximately 2 months after sowing, this could explain the low recovery of fresh organic matter inputs beyond 30 cm as roots from maize or summer crops may have not have had enough time to develop. Lastly, recovery of POM in the subsoil may be “patchy” as uneven distribution in a vast context can result in difficulties in recovering rapidly mineralized organic inputs.

Poeplau et al., (2013) defined the S+A fraction as ”probably the most heterogeneous SOC fraction in terms of composition and stability” as it encompasses POM occluded in aggregates with varying cohesive strength, as well as SOC from clay-sized organo-minerals, as aggregates coating sand grains. In fact the S+A fraction was dispersed through a lighter sonication compared to other fractionation techniques, to allow for recovery not only old SOC, but also include stable aggregates and is considered as an intermediate fraction (Zimmermann et al., 2007). Nevertheless, while others found S+A fraction to be less responsive to plot management than DOC, we found the opposite due to a) no responsiveness of DOC (quantity or C concentration) to changes across treatments b) the long-term nature of our field trial which may have allowed the aggregation of mineral associated OM to coarse sand particles in the top soil. DOC is a mobile, reactive, fast-changing C pool comprising a small fraction of SOC (Zimmermann et al., 2007), and in our case <2.1% of samples´ mass. DOC was defined as particles dissolved in water solution passing through a <0.45 µm sieve (Zimmermann et al., 2007). DOC contains a wide variety of organic molecules, from easily decomposable sugars to the relatively stable phenolic lignin (Al-Graiti et al., 2022). The former, highly available molecules are generally related to the living organic part of the soil such as the microbiome, whereas the latter group reflects the humified and recalcitrant components with a higher residence time (Manninen et al., 2017) which may explain the high C content of this fraction, but not as high as POM. DOC was obtained after centrifugation of samples and its concentration was found to be proportional to the original sample´s C content in other studies (Manninen et al., 2017) although this was not the case in our analysis.

The C content in the SC and rSOC fractions were the ones that most correlated with soil bulk density, probably due to the largest share of these fractions which combined comprised 70.2% of sample mass in the top 5 cm increasing up to 95.1% in the 30-50 cm depth. The difference in C content between these two fractions was due to the oxidization step with NaOCl, which led to distinguishing a recalcitrant fraction (rSOC), resistant to enzymatic breakdown (Zimmermann et al., 2007).

Table 4.1 SOC concentration (g kg⁻¹ ± sem) across fractions, cropping systems and depths

Depth	**Cropping system**	**0N**					**160N**				
		SC	**rSOC**	**S+A**	**DOC**	**POM**	**SC**	**rSOC**	**S+A**	**DOC**	**POM**
0-5	F/M	25.6 ± 1.2	10.5 ± 0.7	3.7 ± 0.7	40.6 ± 3.5	259.1 ± 8.5	23.3 ± 1.8	11.2 ± 0.6	2.9 ± 0.1	44.6 ± 5.6	262.5 ± 14.2
	O/M	25.7 ± 1.3	11 ± 0.2	3.3 ± 0.2	42.3 ± 7.8	170.8 ± 18.3	28.8 ± 1.3	12.3 ± 0.5	3.4 ± 0.2	52.2 ± 1.5	148.1 ± 65.6
	O+V/M	27.5 ± 0.6	11.6 ± 0.3	3.1 ± 0.8	55.1 ± 11.7	274.1 ± 15.4	29.4 ± 0.5	12.5 ± 0.4	2.2 ± 0.7	50.5 ± 10.2	277.2 ± 10.3
	O+V/M+C	32.2 ± 0.3	13.2 ± 0.2	5 ± 0.6	57 ± 6	207.6 ± 9.2	38.5 ± 1.2	16.2 ± 0.2	5.9 ± 0.5	59.4 ± 18	272.2 ± 11.5
	P+M	37.6 ± 1.2	14.8 ± 0.4	7.4 ± 2.6	56.2 ± 12.4	230 ± 3.4	48.9 ± 3.3	19.7 ± 1	5.1 ± 0.2	56.1 ± 6.5	316.9 ± 8.7
5-10	F/M	17.6 ± 0.4	8.1 ± 0.2	0.8 ± 0	33.9 ± 4.7	228.2 ± 13	18.8 ± 0.3	8.6 ± 0.1	1 ± 0.1	41.4 ± 6.2	189.5 ± 46.4
	O/M	18.1 ± 0.1	8.2 ± 0.1	0.6 ± 0.1	31.3 ± 0.4	111.8 ± 11.7	19.6 ± 0.7	9 ± 0.2	0.9 ± 0.1	30.7 ± 1.2	294.8 ± 2.5
	O+V/M	20.5 ± 0.7	8.9 ± 0.1	0.7 ± 0	33.3 ± 2.7	260.9 ± 19.2	20.5 ± 0.2	9.1 ± 0.2	1 ± 0.2	35.3 ± 6.1	330.5 ± 2.3
	O+V/M+C	23 ± 1.9	10.3 ± 1	1 ± 0.1	34.6 ± 3.4	280.4 ± 8.9	28.3 ± 1	12.7 ± 0.5	1.8 ± 0.5	48.4 ± 6.5	312.5 ± 10.2
	P+M	26.3 ± 1	11.3 ± 0.3	2.5 ± 0.3	44 ± 4.6	253.9 ± 17.8	31.5 ± 1.3	13.5 ± 0.4	1.8 ± 0.6	59.5 ± 9.8	292.6 ± 23.4
20-30	F/M	14.4 ± 0.3	7.2 ± 0.1	0.3 ± 0	27 ± 1.6	193.6 ± 1.6	14.6 ± 0.1	7.7 ± 0.2	0.6 ± 0.1	36.1 ± 3.4	309.7 ± 0
	O/M	14.7 ± 0.1	7.1 ± 0.1	0.4 ± 0	30.3 ± 3.6	196 ± 11.1	14.7 ± 0.3	7.5 ± 0.1	0.9 ± 0.2	34.8 ± 1.5	191.6 ± 0
	O+V/M	14.9 ± 0.4	7.2 ± 0.2	0.8 ± 0.3	29.2 ± 0.9	215.5 ± 0.2	15.5 ± 0.2	7.5 ± 0.1	2.5 ± 1.5	62.9 ± 7	0
	O+V/M+C	15.5 ± 0.1	8.1 ± 0.5	0.5 ± 0	35.9 ± 1.1	297.2 ± 11.3	16.8 ± 0.1	8.1 ± 0.1	3 ± 0.9	49.2 ± 6.8	283.7 ± 0
	P+M	15.5 ± 0.2	7.1 ± 0.1	0.5 ± 0.1	32.3 ± 0.5	288.4 ± 6.5	16.7 ± 0.4	7.7 ± 0.1	0.7 ± 0.1	35.7 ± 1.9	228.9 ± 41.1
30-50	F/M	13.6 ± 0.3	7.2 ± 0.2	0.7 ± 0.1	38.7 ± 6.4	0	14 ± 0.5	7.7 ± 0.1	0.8 ± 0.2	29.9 ± 3.8	328.4 ± 17.2
	O/M	13.7 ± 0.5	7.2 ± 0.1	0.6 ± 0.1	30.2 ± 2.6	0	13.1 ± 0.4	7.3 ± 0.1	0.5 ± 0	31.9 ± 2.7	0
	O+V/M	13.7 ± 0.1	7.4 ± 0.1	0.5 ± 0	30.7 ± 1.4	224 ± 38.9	13.3 ± 0.2	7.7 ± 0.1	0.5 ± 0.1	33.5 ± 2.2	323.1 ± 0
	O+V/M+C	14.6 ± 0.7	8 ± 0.2	0.5 ± 0	33.2 ± 3.9	196.6 ± 3	15.4 ± 0.5	8.1 ± 0.2	0.5 ± 0	41.2 ± 2.1	300.5 ± 0
	P+M	14.5 ± 0.1	7.7 ± 0.2	0.7 ± 0	29.8 ± 2.7	0	14.3 ± 0.5	7.5 ± 0.2	0.3 ± 0	30.8 ± 1.5	174.1 ± 49.4

F/M= fallow/maize, O/M= oat/maize, O+V/M= oat+vetch/maize, O+V/M+C= oat+vetch, maize+cowpea, P+M= pigeonpea+maize;

Table 4.2 Fraction mass as a percentage (% ± sem) of whole soil sample.

Depth	**Cropping system**	**0N**					**160N**				
		SC	**rSOC**	**SA**	**DOC**	**POM**	**SC**	**rSOC**	**SA**	**DOC**	**POM**
0-5	F/M	26.3 ± 0.7	45.6 ± 1.5	17.8 ± 2.4	2.1 ± 0.5	15.2 ± 1.2	29 ± 2.7	37.8 ± 2.3	11.5 ± 0.9	1.5 ± 0.2	20.1 ± 5
	O/M	29.8 ± 1.2	46.2 ± 1.3	14.2 ± 0.6	1.5 ± 0.3	8.3 ± 0.5	29.5 ± 0.3	47.9 ± 0.3	13 ± 0.4	1.2 ± 0.5	8.4 ± 0.7
	O+V/M	28.2 ± 1.5	45.7 ± 2.7	13.5 ± 3.6	2 ± 0.5	10.6 ± 1.3	30.5 ± 0.2	49.6 ± 1.4	8 ± 2.1	1.7 ± 0.3	10.3 ± 1
	O+V/M+C	24 ± 0.5	41.7 ± 1.5	18 ± 1.6	1.6 ± 0.2	14.7 ± 1.5	25.2 ± 1.8	42.3 ± 2.4	17.3 ± 2.1	0.9 ± 0.4	14.3 ± 3.9
	P+M	20.6 ± 2.6	37.7 ± 4.8	21.5 ± 7.6	1.3 ± 0.3	18.9 ± 0.9	26 ± 1.5	47.5 ± 1	9.1 ± 0.8	0.9 ± 0.1	16.5 ± 3.1
5-10	F/M	37.2 ± 1.4	51.8 ± 1.3	5.3 ± 0.2	1.9 ± 0.2	5.8 ± 0.6	33.9 ± 2.4	46.8 ± 3.4	5.8 ± 0.7	2 ± 0.3	11.5 ± 5.4
	O/M	37.7 ± 0.3	52.8 ± 0.3	4 ± 0.5	1.8 ± 0	3.7 ± 0.7	37.7 ± 1	52.1 ± 0.2	4.8 ± 0.6	1.5 ± 0.1	3.8 ± 0.6
	O+V/M	35.1 ± 0.7	53.9 ± 0.9	4 ± 0.2	1.6 ± 0.1	5.4 ± 0.7	35.9 ± 0.8	54.3 ± 0.8	5.8 ± 1.1	1.7 ± 0.3	2.3 ± 0.3
	O+V/M+C	35.8 ± 1.1	53.5 ± 0.5	5.1 ± 0.6	1.6 ± 0.3	3.9 ± 0.4	34.1 ± 0.9	50 ± 0.7	8.3 ± 2.4	1.8 ± 0.3	5.8 ± 1.2
	P+M	32.6 ± 0.8	50.9 ± 1.1	10.1 ± 0.6	1.6 ± 0.1	4.8 ± 0.9	33.6 ± 0.5	54.4 ± 2	5.8 ± 2.2	1.2 ± 0.5	5.1 ± 0.4
20-30	F/M	44.1 ± 0.9	51.7 ± 0.6	1.7 ± 0.1	1.6 ± 0.1	0.8 ± 0.4	45.1 ± 1.9	48.3 ± 1.6	3.6 ± 0.7	2.2 ± 0.2	0.8 ± 0.7
	O/M	42.1 ± 0.8	53.5 ± 0.4	2.1 ± 0.1	1.9 ± 0.2	0.4 ± 0.4	43.4 ± 1.1	48.6 ± 0.7	5.5 ± 1.2	2.2 ± 0.1	0.4 ± 0.3
	O+V/M	41.1 ± 0.1	52.1 ± 1.3	4.2 ± 1.4	1.7 ± 0.1	0.9 ± 0.4	38.2 ± 2.4	47.5 ± 3.5	11 ± 5.8	3.3 ± 0.1	0
	O+V/M+C	45.2 ± 3.3	48.3 ± 3.2	3 ± 0.2	2.5 ± 0.2	1 ± 0.4	37.2 ± 2.1	45.9 ± 1.8	14.1 ± 3.8	2.5 ± 0.2	0.2 ± 0.2
	P+M	39.4 ± 0.8	54.3 ± 0.7	2.8 ± 0.3	1.9 ± 0	1.7 ± 0.2	40.6 ± 1	53.2 ± 0.9	3.7 ± 0.2	1.9 ± 0.1	0.7 ± 0.3
30-50	F/M	46 ± 0.7	48.2 ± 0.3	3.5 ± 0.4	2.3 ± 0.4	0	47.2 ± 1.4	46.6 ± 0.9	4.2 ± 1.2	1.8 ± 0.2	0.2 ± 0.1
	O/M	46.7 ± 1.1	48.4 ± 1.1	3 ± 0.4	1.9 ± 0.2	0	49.7 ± 0.9	45.6 ± 0.9	2.7 ± 0	2 ± 0.2	0
	O+V/M	47.4 ± 1.2	47.8 ± 1.2	2.6 ± 0.2	1.9 ± 0.1	0.4 ± 0.2	50.2 ± 0.6	44.7 ± 0.3	2.9 ± 0.5	2.1 ± 0.1	0.1 ± 0.1
	O+V/M+C	47.7 ± 1.3	47 ± 1.4	2.8 ± 0.2	2 ± 0.3	0.5 ± 0.2	46.6 ± 1.7	48.2 ± 1.7	2.5 ± 0.3	2.4 ± 0.1	0.3 ± 0.3
	P+M	45.9 ± 1.3	49 ± 1.3	3.3 ± 0.1	1.7 ± 0.1	0	46.2 ± 0.4	49.5 ± 0.3	1.7 ± 0	1.8 ± 0.1	0.7 ± 0.3

F/M= fallow/maize, O/M= oat/maize, O+V/M= oat+vetch/maize, O+V/M+C= oat+vetch, maize+cowpea, P+M= pigeonpea+maize

$\delta^{13}C$ values in fractions

Soil $\delta^{13}C$ content increased with depth across all treatments and the increment was statistically significant for all fractions and cropping systems and consistent with other findings (Rao et al., 1994; Sisti et al., 2004). The enrichment of $\delta^{13}C$ with depth can be due to several factors including greater contributions of $\delta^{13}C$ -enriched microbial necromass to SOC, more stabilized forms of SOC in the subsoil and older age of SOC which can be reflected by the increasing $\delta^{13}C$ values of atmospheric CO_2 in history. Although POM $\delta^{13}C$ was hypothesized to reflect soils $\delta^{13}C$, this tended to be halfway true. We hypothesized that systems with C_3 components to show a shift towards -27 ‰ while expected the F/M treatment to show a $\delta^{13}C$ ~ -14‰ as these values are often reported in literature in pure systems. The site had been for hundreds of years under natural pampas landscape comprising mostly a C_4 vegetation (Bayer et al., 2001) to then shift to a C_3 cultivation as fields were used for canola breeding, which may have caused a decrease in top soil $\delta^{13}C$ signatures. Although shifts were not as marked as assumed and as commonly found under intact natural settings dominated by plant with one C photosynthetic pathway only, these were enough to estimate differential C_3C and C_4C contributions as leguminous´ above and below ground incorporated in SOM lowered the ^{13}C signature of all fractions and of bulk soil in the upper layers. These data indicate that together with the humification process, stable C from legume residues may have been responsible for the low ^{13}C values in the superficial soil layers. The isotopic composition of SOM in upper soil layers reflected the relative abundance of legume (C_3) and due to contributing plant residues. In the lower layers shifts in signatures across treatments were limited.

Table 4.3 $\delta^{13}C$ signatures (‰ ± sem) across fractions, cropping systems and depths.

Depth	**Cropping system**	**0N**						**160N**					
		SC	rSOC	SA	POM	DOC	Bulk	SC	rSOC	SA	POM	DOC	Bulk
0-5	F/M	-18.5 ± 0.0	-19.1 ± 0.1	-19.2 ± 0.2	-17.8 ± 0.6	-22.6 ± 1.2	-19.1 ± 0.1	-18.2 ± 0.4	-19.4 ± 0.3	-18.7 ± 0.5	-17.1 ± 0.4	-22.4 ± 0.7	-18.6 ± 0.3
	O/M	-19.6 ± 0.2	-19.9 ± 0.2	-19.9 ± 0.2	-20.9 ± 0.3	-23.3 ± 0.6	-19.6 ± 0.2	-19.5 ± 0.3	-20 ± 0.2	-19.7 ± 0.3	-19.8 ± 0.8	-24.5 ± 0.8	-19.4 ± 0.3
	O+V/M	-19.9 ± 0.1	-20.2 ± 0.1	-19.6 ± 0.7	-21.1 ± 0.5	-23.7 ± 1.2	-20.2 ± 0.1	-19.8 ± 0.2	-20.1 ± 0.2	-20.0 ± 0.3	-21.3 ± 0.7	-23 ± 0.4	-19.8 ± 0.1
	O+V/M+C	-20.3 ± 0.2	-20.4 ± 0.2	-20.6 ± 0.3	-20.5 ± 1.2	-23.4 ± 0.6	-20.0 ± 0.4	-20.9 ± 0.1	-21.8 ± 0.2	-21.1 ± 0.1	-20.8 ± 0.4	-25.3 ± 0.6	-20.7 ± 0.1
	P+M	-22.4 ± 0.2	-22.2 ± 0.1	-22.3 ± 0.9	-21.7 ± 0.7	-24.3 ± 1.1	-22.4 ± 0.3	-23.6 ± 0.6	-23.7 ± 0.3	-23.3 ± 0.4	-23.5 ± 0.7	-24.2 ± 0.5	-23.2 ± 0.6
5-10	F/M	-17.4 ± 0.5	-18.0 ± 0.6	-18.1 ± 0.1	-18.1 ± 0.1	-20.9 ± 0.5	-17.3 ± 0.1	-17.5 ± 0.0	-18.3 ± 0	-19.0 ± 0.1	-18.3 ± 0.4	-22.2 ± 0.6	-17.9 ± 0.1
	O/M	-17.9 ± 0.1	-18.7 ± 0.0	-18.8 ± 0.2	-17.9 ± 0.6	-21.7 ± 0.6	-17.8 ± 0.1	-18.1 ± 0.2	-18.8 ± 0.2	-19.0 ± 0.2	-18.1 ± 0.6	-21.7 ± 0.6	-18.0 ± 0.2
	O+V/M	-18.3 ± 0.3	-19.0 ± 0.1	-19.4 ± 0.3	-18.6 ± 0.7	-21.8 ± 0.6	-18 ± 0.1	-18.2 ± 0.3	-18.9 ± 0.3	-19.5 ± 0.2	-19.5 ± 0.6	-21.6 ± 1.1	-18.2 ± 0.3
	O+V/M+C	-18.9 ± 0.2	-19.3 ± 0.1	-19.5 ± 0.2	-18.0 ± 0.7	-22.4 ± 0.4	-19.1 ± 0.1	-19.9 ± 0.1	-20.3 ± 0.1	-20.0 ± 0.5	-19.0 ± 1	-22.9 ± 0.4	-19.6 ± 0.1
	P+M	-21.3 ± 0.0	-21.3 ± 0.0	-21.9 ± 0.1	-19.8 ± 0.5	-24.1 ± 0.6	-21.4 ± 0.1	-21.8 ± 0.1	-21.7 ± 0.2	-21.8 ± 0.6	-22.3 ± 0.5	-24.3 ± 0.9	-21.4 ± 0.2
20-30	F/M	-15.6 ± 0.1	-16.3 ± 0.1	-19.1 ± 0.2	-19.3 ± 0.0	-19.5 ± 0.2	-15.7 ± 0.2	-15.8 ± 0.1	-16.4 ± 0.2	-18.4 ± 0.3	-17.1 ± 0.0	-21 ± 0.4	-15.5 ± 0.1
	O/M	-15.7 ± 0.1	-16.6 ± 0.1	-19.2 ± 0.3	-16.7 ± 0.2	-20.5 ± 0.8	-15.6 ± 0.1	-15.9 ± 0.1	-16.7 ± 0.1	-17.9 ± 0.4	-16.9 ± 0.0	-20.5 ± 0.3	-15.7 ± 0.1
	O+V/M	-15.7 ± 0.1	-16.5 ± 0.1	-19.3 ± 1.2	-19.1 ± 0.7	-19.7 ± 0.3	-15.7 ± 0.1	-16 ± 0.2	-16.7 ± 0.2	-19.2 ± 1.2	na	-23.9 ± 0.5	-15.8 ± 0.2
	O+V/M+C	-16.1 ± 0.2	-16.5 ± 0.2	-18.5 ± 0.3	-18 ± 0.7	-20.1 ± 0.1	-16 ± 0.1	-16.6 ± 0.2	-17.4 ± 0.3	-19.8 ± 0.5	-17.5 ± 0.0	-22.3 ± 0.9	-16.4 ± 0.2
	P+M	-16.4 ± 0.1	-16.9 ± 0.0	-19.6 ± 0.1	-17.9 ± 0.4	-20.1 ± 0.5	-16.4 ± 0.1	-17.1 ± 0.1	-17.6 ± 0.2	-19.9 ± 0.3	-19.8 ± 0.5	-21.1 ± 0.2	-17.0 ± 0.2
30-50	F/M	-15.7 ± 0.0	-16.5 ± 0.1	-17.5 ± 0.2	na	-23.6 ± 1	-15.4 ± 0.0	-15.5 ± 0.1	-16.3 ± 0.1	-17.3 ± 0.2	-18.7 ± 1.5	-21.6 ± 1.0	-15.4 ± 0.1
	O/M	-15.5 ± 0.1	-16.3 ± 0.1	-18.4 ± 0.4	na	-21.8 ± 0.7	-15.4 ± 0.0	-15.6 ± 0.1	-16.3 ± 0.1	-18.9 ± 0.3	na	-22.2 ± 0.8	-15.6 ± 0.1
	O+V/M	-15.5 ± 0.1	-16.4 ± 0.2	-19.1 ± 0.4	-18.5 ± 2.5	-22.4 ± 0.6	-15.5 ± 0.2	-15.7 ± 0.1	-16.3 ± 0.2	-18.4 ± 0.4	-20.3 ± 0.0	-23 ± 0.8	-15.4 ± 0.1
	O+V/M+C	-15.5 ± 0.1	-16.2 ± 0.2	-19.6 ± 0.4	-17.1 ± 0.3	-21.7 ± 1.2	-15.4 ± 0.1	-15.7 ± 0.1	-16.3 ± 0	-18.7 ± 0.4	-19.2 ± 0.0	-22.7 ± 0.5	-15.5 ± 0.1
	P+M	-15.7 ± 0.2	-16.2 ± 0.1	-18.4 ± 0.2	na	-21.7 ± 0.9	-15.8 ± 0.1	-16.1 ± 0.1	-17.0 ± 0.3	-20.0 ± 0.3	-19.1 ± 0.5	-22.2 ± 0.4	-16.0 ± 0.1

F/M= fallow/maize, O/M= oat/maize, O+V/M= oat+vetch/maize, O+V/M+C= oat+vetch, maize+cowpea, P+M= pigeonpea+maize; 0N= no nitrogen, 160N= 160 kg N ha^{-1} $year^{-1}$

SOC stocks

Significant differences were reported in the cumulative C stocks at the 0-10 cm depth whereas no differences were found in the 20-50 cm depth (Figure 4.2). C stocks in the top 0-10 cm varied between 16.8-37.0 Mg ha^{-1} while in the 20-50 depth the range was more narrow, oscillating between 38.1 and 40.3 Mg ha^{-1}. Interestingly the PM system stored 34.1 Mg ha^{-1} C in the top 10 cm which was similar to the mean OC stocks in the 20-50 depth (38.9 Mg ha^{-1}), of three times higher volume. SOC stocks in PM were more than double these of F/M at the 0-10 depth. Pigeonpea intercropping with cereals was found to enhance system´s grain yields and dry matter returns in several occasions under tropical and subtropical conditions (Jayaraman et al., 2022; Liu et al., 2021; Sepat et al., 2014). Substantial amount of green foliage at maturity, serves as a valuable organic input, while providing abundant litterfall with an N content of over 4% throughout the vegetative growth (Mandal et al., 2013). Pigeonpea was sown every two years which may have allowed for the establishment of a complex rooting architecture which enhanced amounts of rhizobial associations, leading to enhanced biological N fixation (Primieri et al., 2022), which managed to sustain yields of intercropped maize and to significantly enhance SOC stocks. Enhancing top soil SOC through the establishment of mixed perennial and annual crops may be a means to turn cropping systems into more sustainable by increasing the amount of time plants have for accumulating biomass with reduced soil operations and erosion (King and Blesh, 2018).

In our previous work we found a strong correlation between increasing SOC stocks and increasing amounts of inputs, as was found elsewhere (Corbeels et al., 2016; Sisti et al., 2004) although limited long-term data on the quality of stocks exists. Differences among treatments in fractions´ stocks in the top layer, were found for SC, rSOC, POM and SA but not for DOC as results were similar across all treatments within depths. rSOC stocks comprised the largest fraction, followed by SC, SA , POM, DOC in the top layer while POM and DOC were inverted in size order in the lower depth as DOC remained stable while little to no POM was recovered beyond 20 cm. The different soil mineralogy was reflected in the sharp decline in S+A in the subsoil where an increase in SC occurred. S+A C stocks were similar across treatments with the exception of PM 0N and OV/MC 160N being higher than OV/M 160N. Across all treatments, the OV/M 160N system had the highest amounts of C inputs from maize and a higher share of thermally recalcitrant OC (Binacchi et al., *in preparation)* which, coupled to the higher share of fine particle associated OC in the current study, may suggest pathways for mineral-association at the expense of aggregation in systems with proportionally high C inputs and mineral N applications. Overall, low differences in stocks changes were found in the S+A fraction, probably due to the low C content in these samples (<0.1 g kg^{-1} beyond the 5 cm depth) coupled to the high Fe and Al contents of the soils (Bayer et al., 2001) favouring mineral-associations of OC. Sand particles may not provide enough spatial and physical protection against rapid microbial decomposition in subtropical climates. Therefore most differences between treatments occurred in the finer particles which may be due to SOC sequestration on Brazilian oxisols being related to mineral association of clay particles rather than aggregation (Veloso et al., 2019). What distinguishes the heterogeneous class of mineral-associated organic matter from labile forms is the fact that microbes cannot directly break down or assimilate carbon and nitrogen bound to silt and clay; first, they must disrupt the chemical bonds that link organic molecules with the mineral surface (Averill and Waring, 2018). However, the growth efficiency and turnover rate of the microbial biomass regulates the gross flux of microbial necromass, the dominant C inputs of SC and rSOC. Therefore, two key microbial physiological parameters may affect SC dynamics: carbon use efficiency (the fraction of C uptake converted to biomass) and biomass turnover rate (Schmidt et al., 2017). These parameters both influence quantities of C and N in the microbial biomass and necromass pools (Schmidt et al., 2017). While microbial physiology affects inputs, the physicochemical characteristics of the soil minerals affect stabilization, that is, how much of this microbe-derived organic matter can be stabilized via organo-mineral bonds. This Microbial Efficiency and Matrix

Stabilization hypothesis (Cotrufo et al., 2013) has been validated by field studies demonstrating that higher microbial substrate use efficiency enhances soil C accumulation in the organo-mineral fraction. Unexpectedly differences within the rSOC across treatments were the most evident, which conceptually would take hundred years to be detected, which led to the highest sequestration of C in the most stable fractions.

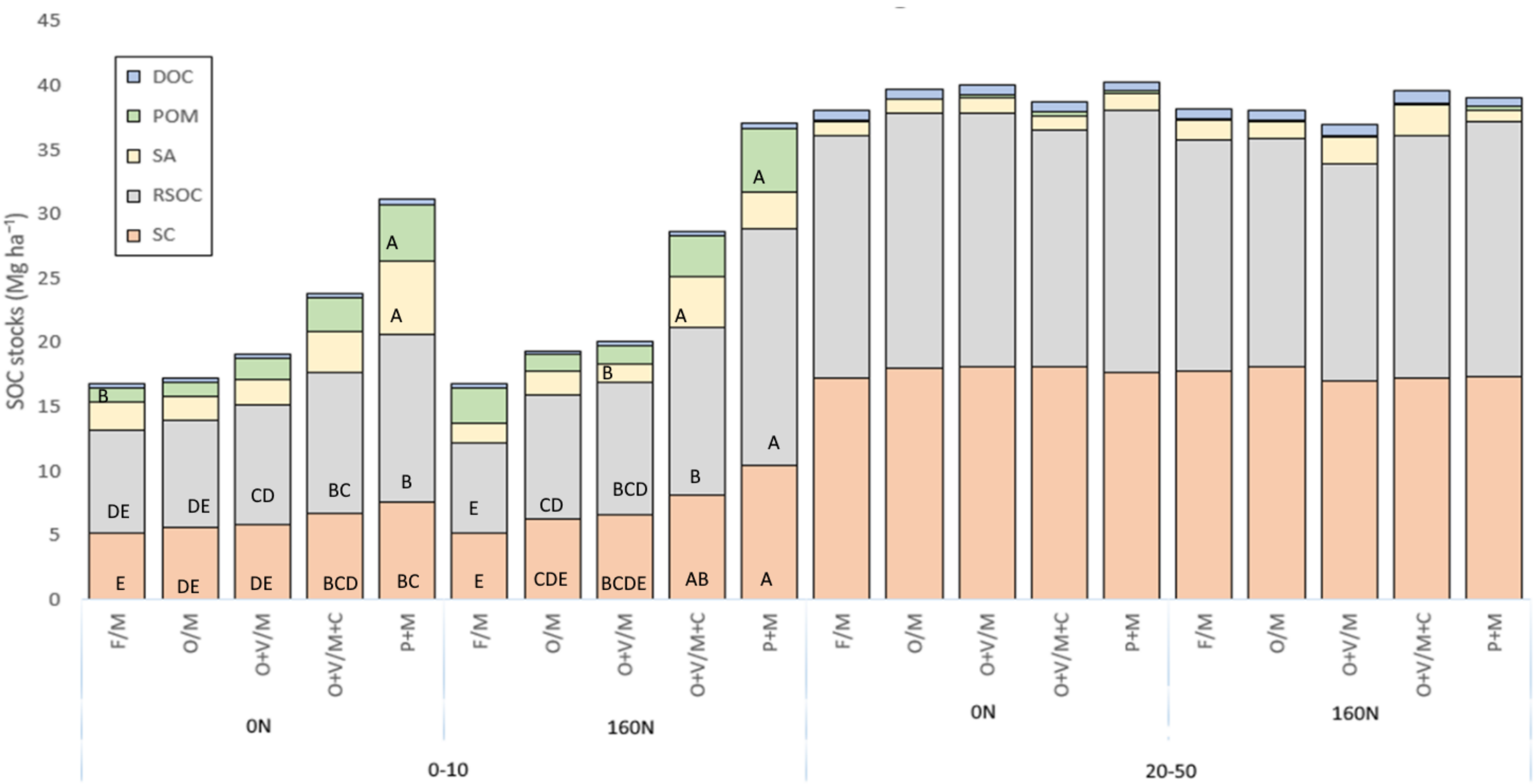

Figure 4.2 SOC stocks across SOM fractions. F/M= fallow/maize. O/M= oat/maize. O+V/M= oat+vetch/maize. O+V/M+C= oat+vetch. maize+cowpea. P+M= pigeonpea+maize. Different letters indicate significant differences in fractions´ stocks across cropping systems at the two fertilizer levels, at the 0-10 depth.

C_3 derived carbon

Integration of legumes in maize systems can lead to substantial SOC gains is some cases. Increases in bulk SOC stocks due to C_3 vegetation was found to be between 14-64.9% in the top layer and between 3.2 and 12.0% in the lower strata, which amounted to SOC gains of 2.6-23.7 Mg ha^{-1} in the top layer and 1.6-4.8 Mg ha^{-1} in the lower strata. In general, PM was the best performing cropping system, application of 160N yielded higher shares of SOC, and topsoil stocks were higher than in the subsoil. Within fractions, the highest absolute changes due to C_3C were found in labile (POM and DOC) fractions and in the SA fraction, whereas SC and rSOC were the ones that changed the least (~ +25%). This seems to be in agreement with the overall conceptual elaboration of the fractions whereby greatest changes, compared to controls, are found in less-stable pools, more prone to management and pedoclimatic conditions and moreover also fits in conceptual models of SOC dynamics, which postulates that for C sequestration to occur C should be converted from labile C to less reactive intermediate or passive. In fact, although absolute changes were highest in labile fractions, relative changes in SOC stocks (Mg ha^{-1}) were found in SC, and rSOC especially. In the top layer the greatest gains in C_3 SOC were found in the rSOC fraction, which were similar to the ones in the SC but higher ($p<0.05$) than in the S+A, POM and DOC. In the lower layer instead, again rSOC stocks had the highest C_3C quantity gains, but these were followed by SA and then SC, POM and DOC ($p<0.05$). Due to its resistance to decomposition the recalcitrant C pool is likely more important for C storage and sequestration than the labile C pool (Zhang and Zhou, 2018). The labile fractions of SOC exhibit relatively fast turnover rates and therefore may not contribute significantly to C sequestration in soils. In an incubation study in China (Zhang and Zhou, 2018) found that most of the mineralized carbon derived from the recalcitrant pool as labile carbon fractions were rapidly digested by soil microbes during early phases of residue decomposition and this was increased at increasing temperatures. This was in disagreement with a review of 95 papers investigating soil C isotopic shifts due to tillage management which found that the largest C gains as calculated from increases in $\delta^{13}C$ values

were observed in the coarse sand fraction and the smallest changes occurred in the clay fraction which may relate to the impact of ploughing on C aggregation (Smith and Chalk, 2021) suggesting that adoption of no-till systems may be a viable means for enhancing structural SOC fractions.

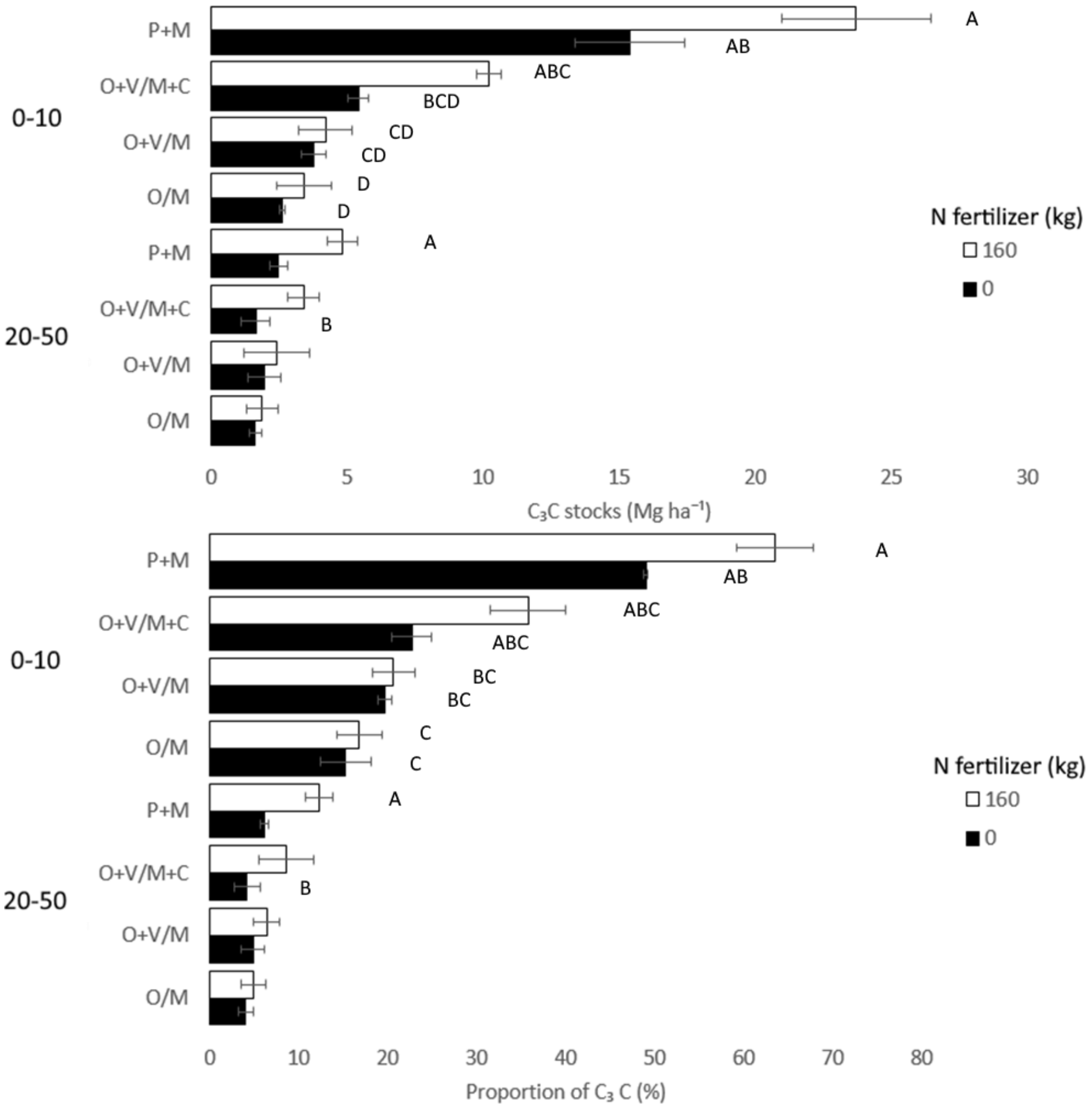

Figure 4.3. Contributions of C_3 derived C to SOC stocks. Top: quantities of C_3C stocks (Mg ha^{-1}) bottom: proportions of C_3C stocks (%). Different letters indicate treatment effects (cropping systems*fertilizer) at the different depths.

Table 4.4 Changes (%) in SOC stocks across fractions due to C_3C vegetation. Different letters indicate treatment (cropping system*fertilizer) effects on SOM fractions, per each depth.

Depth	N fertilizer	Cropping system	SC	rSOC	SA	POM	DOC
			%				
0-10	0	O/M	14.7 ^{c}	18.2 ^{b}	18.9 ^{b}	32.4 ^{ab}	63.6 ^{a}
		O+V/M	18.5 ^{bc}	22.3 ^{ab}	15.3 ^{b}	35.1 ^{b}	69.3 ^{a}
		O+V/M+C	23.4 ^{abc}	24.8 ^{ab}	27.3 ^{ab}	26.9 ^{ab}	65.7 ^{a}
		P+M	51.7 ^{ab}	48.8 ^{ab}	50.6 ^{ab}	43.0 ^{b}	77.6 ^{a}
	160	O/M	16.5 ^{c}	22.2 ^{b}	19.2 ^{b}	21.0 ^{b}	-17.2 ^{a}
		O+V/M	19.8 ^{bc}	24.7 ^{ab}	23.2 ^{ab}	38.8 ^{b}	61.5 ^{a}
		O+V/M+C	33.8 ^{abc}	45.8 ^{ab}	37.0 ^{ab}	33.2 ^{b}	-11.0 ^{a}
		P+M	69.6 ^{a}	69.9 ^{a}	65.5 ^{a}	69.2 ^{b}	76.8 ^{a}
20-50	0	O/M	1.0 ^{c}	9.9	35.2	-41.9	47.7
		O+V/M	1.0 ^{c}	9.1	35.9	-26.3	39.2
		O+V/M+C	4.8 ^{bc}	9.0	28.3	-33.3	43.5
		P+M	8.1 ^{ab}	13.0	38.4	22.4	43.1
	160	O/M	1.3 ^{bc}	8.7	20.8	-99.2	46.2
		O+V/M	1.6 ^{bc}	8.5	33.4	-155.0	79.5
		O+V/M+C	7.6 ^{abc}	15.9	39.0	-97.0	64.0
		P+M	13.2 ^{a}	17.6	39.9	-24.6	52.2

Table 4.5 Contributions of C_3C vegetation to SOC stocks (Mg ha^{-1}) across SOM fractions. Different letters indicate distribution of SOC stocks per each cropping system, at the two N fertilizer levels

Depth	**N fertilizer**	**Cropping system**	**SC**	**rSOC**	**S+A**	**POM**	**DOC**
					Mg ha^{-1}		
0-10	0	O/M	0 .6^{a}	1 .4^{a}	0 .3^{a}	0 .3^{ab}	0 .0^{b}
		O+V/M	0 .9^{ab}	1 .9^{a}	0 .4^{bc}	0 .5^{abc}	0 .1^{c}
		O+V/M+C	1 .4^{ab}	2 .5^{a}	0 .8^{abc}	0 .6^{bc}	0 .1^{c}
		P+M	3 .7^{a}	6 .1^{a}	3 .5^{a}	1 .9^{ab}	0 .2^{b}
	160	O/M	0 .8^{a}	2 .0^{a}	0 .4^{ab}	0 .2^{ab}	0 .0^{b}
		O+V/M	1 .0^{a}	2 .2^{a}	0 .4^{ab}	0 .6^{ab}	0 .1^{b}
		O+V/M+C	2 .6^{a}	5 .2^{a}	1 .4^{a}	0 .9^{ab}	0 .1^{ab}
		P+M	6 .6^{a}	11 .4^{a}	1 .8^{ab}	3 .6^{a}	0 .2^{b}

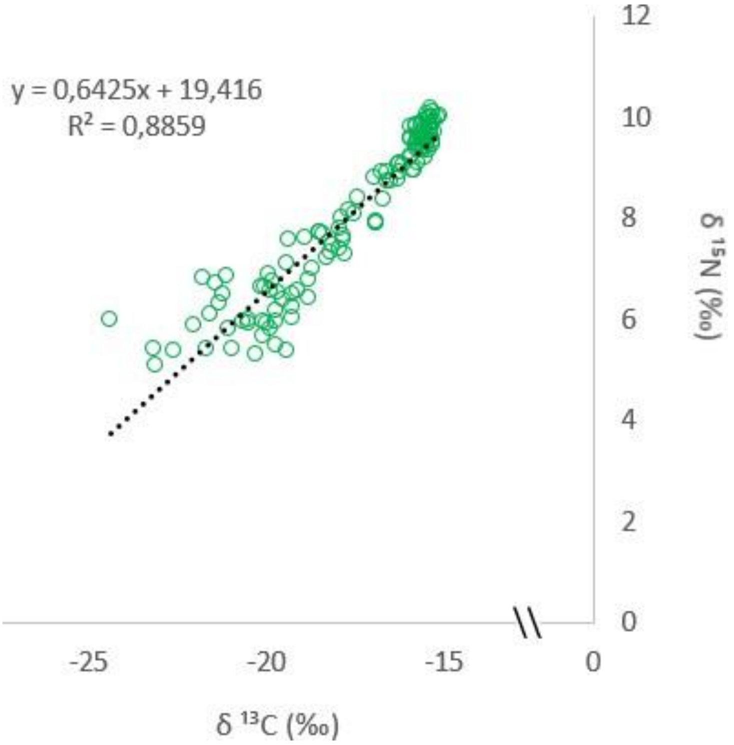

Figure 4.4 Linear correlation between δ^{15}N and δ^{13}C signatures across all bulk samples (*n=120*).

As δ ^{13}C decreased due to shifts in C_3 vegetation so did δ^{15}N which may be a unique feature of legume cropping systems. As volatilization of N from SOM tends to favour the release of the lighter ^{14}N isotope, soils are slightly enriched in ^{15}N compared to the atmosphere. On the other hand, legumes fix atmospheric N_2 and then transfer this ^{14}N-N into the soil through the decomposition of residues causing the differential signatures in samples. We propose that in settings were shift in C_3 and C_4 vegetation exists and these differentiate between leguminous and non-leguminous species, ^{15}N signatures may explored to assess contributions to SOM.

Conclusions

The proportion of labile and stable carbon fractions specified the quality of SOC, and determined the magnitude of SOC sequestration. SOC stocks were significantly influenced by cropping system and fertilizer application and reached the highest value for pigeonpea+maize 160N. The amount of C_3 derived C was highest in the topsoil where contributions from legumes were as high as 65%. The combination of SOM fractionation and isotopic tracing aided detangling the contributions of legumes to soil fertility and mechanisms of SOC formation as organo-mineral associations.

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5. General discussion

Assessment of legume-SOM dynamics through C and N isotopes

The main highlight of legumes´ role on soil fertility is to provide N, and that this N is bound to OC, which makes it less prone to as rapid mineralization as N provided as standard fertilizer and more likely to impact SOM, so a more sustainable approach. Therefore one of the first objectives of this work was to quantify the so often cited “biological N fixation from legumes” in chapter 2, i.e. how do we know that legumes fix N, apart from pink to dark red nodules? Direct and indirect techniques to assess quantities of N_2 fixation by grain legumes in agricultural systems are available. Direct methods relay on the quantification of plant N or its stable isotopes, while indirect methods investigate other substances that can indicate N_2 fixation such as nitrogenase activity or number and quality of nodules. Each method has its own advantages and disadvantages and choosing which technique to use depends on researcher´s investigation question, legume specie, sampling conditions, degree of precision required and available technologies (Unkovich et al., 2008). One reliable and precise method includes the study of the ratio between ^{14}N and ^{15}N in a two way equation with suitable controls. The two stable isotopes of nitrogen have an atomic mass of 14 or 15, and are present in the atmosphere at a mostly standard abundance of 99.6337% and 0.3663% respectively, which can help in estimating shares of BNF through the δ ^{15}N natural abundance method. Slight deviation from these values occurs across a range of chemical and biological pathways due to preferences for, or discrimination against, the heavier ^{15}N atom. Since such differences are in the order of 10^{-3}, results are usually expressed as parts per mill (‰) of the delta deviation (δ) from its atmospheric natural abundance, which is δ=0‰ by definition (Högberg, 1997). In the soil for example, denitrification and volatilization of ammonia result in preferential losses of gaseous ^{14}N, enriching the residual nitrate in the soil in ^{15}N. Soils are therefore usually slightly enriched in ^{15}N compared to the atmosphere and such differences may be exploited to calculate a set of soil-plant metabolic pathways and interactions such as BNF (Chalk et al., 2016). Key requirement for the success of ^{15}N -based methodologies is that the ^{15}N of the soil be homogeneous so that variations in soil mineral N across the test site are smaller than the amount of biologically fixed nitrogen, and that the ^{15}N concentration in plant-available soil N differs significantly from atmospheric N_2 (Baptista et al., 2014). Plants which acquire N from either the soil or fertilizer, are likely to resemble the isotopic composition of its source, since do not report a preferential pathway for either ^{14}N or ^{15}N uptake. N-fixing legumes on the other hand, are expected to report ^{15}N values which lie between atmospheric and soil mineral N sources, depending on efficiency of fixation (100%Ndfa= $\delta\ ^{15}N$ of atmosphere, 0%Ndfa=^{15}N of soil Nmin). Such differences are exploited in BNF studies analysed with sensitive Isotope Ratio Mass Spectrometers (IRMS) (Craine et al., 2015; Dawson and Brooks, 2001). Comparison of the isotopic abundance of soil mineral N or reference plants relying solely on soil N, to that of N fixing plant species, is therefore used to estimate %Ndfa (Unkovich et al., 2008). An ideal non-N_2-fixing reference plant is a non-nodulating genotype of the same specie used for evaluation since these will have similar root architecture and shoot morphology. Soil ^{15}N values can be impacted by application of fertilizers or manures which modify soil isotopic signatures. Peoples et al., (2015) also highlighted that when a non-legume is cropped with a N fixing legume, nutrient interactions in the form of rhizodeposition or litterfall, cause a direct ^{15}N transfer, which may dilute the isotopic signature of the reference plant. ^{15}N natural abundance method is applied in glasshouse or field experiments as long as both N_2 fixing and non N_2 fixing plants are present at the same location, share the same N pools and are sampled at the same time (Unkovich et al., 2008).

Although our $\delta\ ^{15}N$ data from Oberfeld in 2020 indicated that crops (except soya) were almost entirely relaying on BNF, as isotopic signatures approached towards 0 or were even negative, BNF rates were between 80.8-93.9. Literature suggests that this was due to the differential distribution of ^{15}N among legume plant components, such as roots having higher values than above ground biomass e.g. for all our dataset roots had a $\delta^{15}N$ abundance of 2.2., compared to 1.4 of stem and 1.2 of leaves. As whole root system recovery is challenging, especially under field conditions, an adjustment is made in the calculation to account for the different partitioning of ^{15}N (and biomass) among plant parts, by including a B value. Ideally each study should calculate its own B value, as above and below ground biomass partitioning is specific to each crop specie with the specific inoculant, although values collated from other studies are available and may be used as a reference (Unkovich et al., 2008). Nevertheless as we also measured root´s isotopic N composition we tried including this in the equation and thereby avoid using the B value. This lead to insignificant changes in whole plant ^{15}N weighted means, because the biomass of the root was lower than what is reported under control environments (Hupe et al., 2018b) which led to the "weight" of root ^{15}N being low. The only exception was for soya, where root ^{15}N averaged 3.9 (5.5 at Gladbacherhof and 2.3 at Oberfeld) and root biomass was higher than other crops, leading to the weight of root´s ^{15}N being less insignificant and whole plant weighted mean, ~10% higher if accounting for below-ground compartments and avoiding using B values from literature. Similar short-coming were found by others (Nebiyu et al., 2014; Okito et al., 2004) highlighting that when BNF rates are below <60%, variations in B values are of little impact, whereas divergences are more apparent above that threshold. The question remains as whether we could have circumnavigated the B value and include in the calculation weighted means including isotopic data obtained from roots. As legume roots may represent up to 30% of plant´s biomass (Bolinder et al., 1997) and 16% of plants´ N (Hupe et al., 2018b), the partial root recovery of our study would have limited replicability of results. So although ^{15}N plant weighted mean (shoot and root) indicated ~100% BNF rates at Oberfeld in 2020 actual rates were calculated to be lower, due to the unexplored biomass of roots in the deeper soil layers.

Further considerations may also be provided regarding the two-way equation used for calculating shares of C_3 and C_4 contributions to SOC in El Dorado do Sul. As descriped in chapter 4, the differential equation relies on the relation between different plant species to perform photosynthesis underlying mechanisms to heat adaptation, and opening and closing of stomata. A pure lab-lab (*Lablab purpureus*) cropping system was envisaged to be sampled as source of pure C_3 signature, but we found out that this system had collapsed, as legume monocropping for multiple years is not a viable option as pests and diseases limit the adoption as sole crop, whereas on the other hand the fallow/maize system provided the control for the C_4 control, although some weeds may have appeared during the fallow season and possibility dilute SOC signatures. Therefore we had three options of approaching the calculation of mixed crop cultivation contributions to SOC stocks, either to highlight contributions from the cover crops, or from maize or from both. As the focus of the paper and the overall thesis was to study contributions from legumes to SOM, we selected the value of -27 for $\delta^{13}C_3$ in the below equation

$$f\ C_3C\ (\%) = \left(\frac{\delta^{13}C_{soil} - \delta^{13}C_{C4\ soil}}{\delta^{13}C_3 - \delta^{13}C_{C4\ soil}}\right) \times 100$$

whereas the other values were empirically measured. Large differences in literature are found on the isotopic signatures from C_3 crops as these reflect plant species and water use efficiency which varies from one season to the next. For example the mean $\delta^{13}C$ in 2020 across legume crops at Gladbacherhof and Oberfeld was -27.1 ppm and -29.2 ppm in 2021, or -28.4 ppm at Gladbacherhof and -28.8 ppm at Oberfeld, highlighting how efficiency of C assimilation depends on the legume genotype*rhizobial genotype*management*environment. Small variations in the $\delta^{13}C_3$ could have led to large variations in calculating C_3 contributions to SOC, especially as signatures between treatments and controls (fallow/maize with or without N fertilizer), were not as marked as could have been expected. For example Veloso et al., (2018) analysed bulk soil $\delta^{13}C$ from a nearby – comparable field experiment (50 meters away, which had been established a few years after the one I had sampled but focused on comparing till and no-till systems) and found that the oat/maize system to have bulk $\delta^{13}C$ signature of -15.0 ppm in the top soil (0-5 cm) while treatments with legumes were around ~-19, with similar trends all the way down to the 100 cm depth, which was not the case in our study as (i) below 30 cm no differences in isotopic signatures were found (ii) differences in the upper layers were not distinctive of either or photosynthetic pathway, as can be expected in cropland where historical cultivation shifts has occurred (Fuentes et al., 2012; Rao et al., 1994), but laid somewhere in between the -14 ppm expected for C_4 maize or the – 27 ppm expected for C_3 species but were enough to calculate a shift in SOC quality due to differential soil isotopic signatures. This suggests than overall tracing the fate of C and N isotopically labelled plant materials is a powerful tool to study biochemical nutrient transformations in legumes (Craine et al., 2015; Dawson and Brooks, 2001) but assumptions on controls and “adjustments” (e.g. roots) may strongly impact results. The assessment of the natural abundance of ^{15}N and ^{13}C provided a powerful means to assess the impact of legumes on SOM cycling.

Does thermal analysis of SOC overlap with this from physical fractionation?

In chapter 3 and in chapter 4 I employed two different methods to distinguish the quality of SOC. First we applied thermal differentiation which assumes that labile C compounds like carbohydrates require lower activation energies for their oxidations compared to these of recalcitrant compounds such as C found in aromatic rings, therefore at three increasing temperatures, three qualities of carbon were realised: OC_{400}, ROC and TIC, with amounts of TIC being insignificant and not considered part of the study. On the other hand in chapter 4 we combined physical and chemical lab fractionation to obtain five different fractions and analysed samples with a mass spectrometer. Bulk soil from the two machines had a 96% correlation confirming results of sample´s C content, although the SoliTOC reported ~9% higher values than the EA-IRMS, probably due to slightly different calibrations. Initially I assumed that some fractions would have showed high correlations e.g. POM and TOC: as organic matter inputs (POM) increase, so should OC concentrations in the soil; nevertheless by sampling at one point in time, especially at a time where little residues were present on the soil surface, as maize had been recently cropped, this resulted in no correlations between fractions (Table 5.1) although the correlation with cumulative amounts of residues across the years was evident (chapter 3). Next I predicted that rSOC and ROC would correlate, which actually did (R^2=0.74) and this was the highest correlation between ROC from the Solitoc and C concentrations from the lab fractionation, which made me question about the biological, chemical and age characteristics of these two fractions and if overlaps could exist.

Table 5.1 Pearson correlation coefficients between OC results from the lab fractionation (Zimmermann et al., 2007) (with EA-IRMS analysis) and the thermal C results (with Solitoc analyser) (Natali et al., 2019).

	rSOC	*SC*	*SA*	*POM*	*DOC*	*bulk*
ROC	0.74	0.68	0.59	0.21	0.39	0.71
OC_{400}	0.91	0.92	0.71	0.18	0.48	0.95
TOC	0.91	0.92	0.71	0.18	0.48	0.96

ROC=residual oxidizable C; OC_{400}= C burnt up to 400 degrees C; TOC= total organic carbon; rSOC= recalcitrant SOC; SC= silt+clay; SA= sand+aggregates; POM= particulate organic matter; DOC= dissolved organic carbon; bulk= bulk sample.

Although correlations were high, absolute terms differed almost seven fold i.e. overall mean C concentration of ROC=0.14% while rSOC=0.95% with also marked differences in contributions to bulk soil C content, as rSOC made up most of the SOC, while ROC represented <10% of SOC concentrations, with actual sizes unknown as thermal analysis does not yield a tangible size. So shares of C compared to total sample C, and C concentration, differed largely, while the variations in C detection among samples across machines, was related. The Zimmerman et al., (2007), fractionation scheme is a modern, expensive and time consuming technique, involving multiple steps to differentiate five carbon fractions which were designed to fit into conceptual pools of SOC turnover in the RothC and Century models (Poeplau et al., 2018). Thermal analysis of C stability instead is a modern, cheap and fast technique, which has been linked to the structural composition of SOC and questions arise whether thermal analysis could replace physical fractionation in the identification of more or less bioavailable C pools (Kučerík et al., 2018; Tokarski et al., 2020). Chemical fractionation of SC into rSOC, reflects the view that stability and turnover of each fraction is determined by its chemical composition. The decomposition of bioavailable macromolecules in soils is microbially mediated, with animals accounting for 10 to 15%, and abiotic processes for less than 5% of organic matter decomposition (Lorenz et al., 2009). Recalcitrance is therefore defined as an inherent chemical property of a molecule rendering it resistant to decomposition and usually associated with complex aromatic structures such as lignin or SOC that has been 'humified' (Kleber et al., 2011). Differences in molecular-level characteristics among biomacromolecules, including elemental composition, presence of functional groups, and molecular confirmation, affect their biotic (i.e., enzymatic) and abiotic degradation. Oxidization with NaOCl was performed three times for 18h each, to mimic strong, enzymatic attack and yield a fraction with the longest turn-over rate which would be little affected by soil management activities. Nevertheless most of the gains in SOC stocks and in C_3 derived stocks were found in this fraction. With the Solitoc, C concentrations also were significantly affected by management, at even deeper depths that more thermally labile fractions (OC_{400}) which was unexpected as to date, this fraction has been mostly used to identify geogenic organic carbon (Natali et al., 2021, 2019; Zethof et al., 2019). Intrinsic properties of compounds contribute to their long-term stability and resistance to biodegradation namely polyaromatic ring structures (e.g., in lignin, tannin, steranes, hopanes), cross-linked structures (e.g., in cutin, suberin, cutans, suberans), and polymethylenic molecules (e.g., in lipids, waxes) (Lorenz et al., 2009). Furthermore, the inert SOC fraction is assumed to be not biologically decomposable. Biochemically recalcitrant SOC fractions occur in particular in the subsoil, probably because derived from relatively higher microbial-derived origin, compared to plant-derived inputs which was reflected by the lower ^{13}C of our samples. Although concentrations and relative abundance of these two fractions (ROC and rSOC) differed, neither resulted in being a “passive” or an “inherit” compartment with hundreds of years turnover times, as both were impacted by treatments. As no agreement lies in the definition of recalcitrant C fractions (Kleber et al., 2011; Lehmann et al., 2020; Lorenz et al., 2009), and both methods failed at isolating a completely recalcitrant fraction, I propose that a linkage in detecting lignified compounds associated with clay minerals among the two methods occurred, but that oxidation as 600˚C had more impact in detecting tightly bound C complexes with a high energy release requirement compared to chemical oxidation, and that research upon linking thermal SOC analysis and nature of C compounds may lead to a faster and more standardized means of analysing quality of SOC.

When does residual BNF-N enhance SOC cycling?

A number of meta-analysis have been dealing with the topic of carbon sequestration as a consequence of legume inclusion in cropping systems which were summarized in Table 5.2 (Abdalla et al., 2020b; Alvarez et al., 2017; Francaviglia et al., 2019; Jian et al., 2020; King and

Blesh, 2018; McDaniel et al., 2014; Poeplau and Don, 2015). In a meta-analysis of 122 experiments studying crop rotation impacts on C and N pools, McDaniel et al. (2014) found rotations including a cover crop (green manures) increased total C and N levels by 8.5% and 12.8%, respectively, whereas crop rotations without cover crops increased levels by 3.6% and 5.3%, respectively. Impacts of rotation upon microbial biomass C and N, a common indicator of labile SOM dynamics, were more pronounced, with increases of 20.7% and 26.1%, respectively. As 97% of the cover crops included in the meta-analysis were leguminous, the authors suggest that legumes may have an outsized effect on crop rotation influence on SOM levels. Labile C from legume residues and root exudates is likely to lead to efficient microbial decomposition and SOM formation; through the combination of low C/N residue, N provisioning, and complementarity with grass crops in polycultures, legumes play an important role in SOM development and coupled C/N cycling in agroecosystems. On the other hand McClelland et al., (2021) found no significant differences among qualities (C/N ratios) or single classes (legumes, grasses, mixtures) of cover crops on SOC stocks, athough the overall effect was highly significant, leading to a 12% increase on SOC stocks (1.11 Mg C/ha). Interestingly the authours found that the best variable predictors were planting and termination dates, with continuous cover cropping or winter cover cropping leading to up to 20% higher C stocks. King and Blesh, (2018) came to a similar conclusion, suggesting that across 169 cropping systems, these that perennially maintained the soil covered with living mulches, increase SOC concentration by 12.5%, but that including grain legumes decreased total C input by 16% and SOC by 5.3% although results varied greatly depending on levels of N fertilizer applications of controls. Poeplau and Don (2015), estimated a global of 0.12 Pg C/year, or 8% of the direct annial greenhouse gases from agriculture, by including winter (legume or non legume) cover crops which are then used as green manures in spring. Cover cropping green manures also were found to increase SOC by 15.5%, particularly on clay soils with legume crops, with a potential SOC sequestration rate of 0.56 Mg/ha/year or 0.16 Pg C/year (Jian et al., 2020).

Francaviglia et al. (2019) also conducted a meta-analysis on SOC changes as a response to introduction of legumes in European crop rotations. The study found that crop diversification with extended rotations (at least 3 years up to 5 years) and the introduction of legume crops significantly increased SOC content by 18% compared to control treatments. Conversely, SOC content changes decreased by 6% in long rotations without legumes, and by 3–5% in short rotations. Overall management of crop residues, length of cropping season and type of legume, tended to explain the various results from the above mentioned studies. Returning legumes´ residues to the soil, through crops which were sown with the sole purpose of being used as green manures, increased C (and N) returns, benefiting nutrient budgets at the plot level (Abdalla et al., 2019; Alvarez et al., 2017; Jian et al., 2020; Poeplau and Don, 2015). On the other hand, reductions in SOC stocks with crop rotations with grain legumes were found (King and Blesh, 2018; McDaniel et al., 2014) as controls with N fertilized cereals could return more residues than grain legumes, where N exports in the form of seed were found.

Table 5.2. Summary of latest meta-analysis which have assessed the relation between legumes and SOC in cropping systems. CC=cover crop

Control	**Treatment**	**SOC change**	**Unit**	**Depth (cm)**	**Reach**	**Reference**
No CC	Winter legume	1.61	Mg CO_2 eq ha^{-1} $year^{-1}$	0-30	global	Abdalla et al., 2018
no CC	Legume	11.1	%stocks	0-30	global	Jian et al., 2020
fallow	Legume green manure	0.3	Mg ha^{-1} $year^{-1}$	0-30	global	Poeplau and Don, 2015
soja rotation	Soja monocrop	-12.9	%	0-30	global	McDaniel et al., 2014
fallow	Legume	0.1	%	0-30	temperate climates	McClelland et al., 2021
maize +CC	(maize + legume) +CC	-1	%	0-30	global	King and Blesh, 2018
wheat + CC	(wheat + legume) +CC	-2.6	%	0-30	global	King and Blesh, 2018
no CC	Legume CC	7	%	0-20	pampas	Alvarez et al., 2017
fallow	Legume CC	1.54¥	Mg/ha/yr	0-30	Spain	Gonzalez-Sanchez et al., 2012
fallow	Legume CC	0.35ꞙ	Mg/ha/yr	0-30	Spain	Gonzalez-Sanchez et al., 2012
continuous cereal	Legume in crop rotation	18	%	na	Europe	Francaviglia et al., 2019
no CC	Legume CC	9	%	na	global	Bai et al., 2019

¥studies shorter than 10 years

ꞙstudies longer than 10years

Exploiting legume´s BNF is a pathway to climate smart SOM management

Legumes employed in diversified crop rotations and cover cropping systems influence labile SOM by increasing N availability and through readily decomposable plant material inputs leading to more efficient microbial conversion of labile C inputs to SOM (Blesh, 2019). Increased N inputs from legume BNF also increase system´s C inputs, as N availability is a major limiting factor governing systems productivity. High quality, low C/N ratio inputs are preferentially decomposed by bacteria and fungi as they contain high amounts of stored energy and contain a relatively high ratio of metabolic to structural carbon compounds (Mooshammer et al., 2022). That rapid decomposition can result in SOM development would have seemed paradoxical under classical conceptions of SOM. Classical theory explained SOM stabilization through the accumulation of recalcitrant, complex C compounds resistant to decomposition (Piccolo et al., 2005). Inputs of labile C material containing high N content results in higher amounts of microbially processed organic matter and long-term C and N storage (Cotrufo et al., 2013). Structural elements such as lignin and hemicellulose require significantly more energy to decompose, reducing microbial efficiency of decomposition and increasing CO_2 loss per unit of C converted to microbial tissue. Legumes have been shown to increase labile SOM fractions in both perennial and annual systems (King and Blesh, 2018). Positive N effects in crop rotations after cropping of grain legumes are a combination of examined factors including 'N-sparing' and 'quality of residues' and factors such as N rhizodeposition, which impact root-soil interactions and consequent turnover of its residues (Cernay et al., 2017; Chen et al., 2009). This impacts C turnover directly and indirectly and could lead to reduced greenhouse gas emissions by savings of mineral fertilisers produced with fossil resources, increase yields and higher grain quality of subsequent crops due to N-transfer from legumes to the following crops. Spatial-temporal integration of N fixing crops with non-legume crops such as vegetables or cereals also leads to increased land equivalent ratios (LER) by 20% which was found as mean value from 462 field experiments (Jie et al., 2022).

Way forward

Agorecological approaches to crop production can support the transition to sustainable provision of food, fiber and fuel, with concomitant improvements in soil health and on-farm biodiversity. Nevertheless implementing shares of legumes as cash or cover crops remains challenging, largely due to the logics of commodity oriented markets and contingent socio-technical approaches to satisfy neo-liberal production demands (Wezel et al., 2015). Encouraging the reflection, and taking action upon our impacts on environmental degradation and on the exploitation of finite resources, conceals the solution to the advancement towards agricultural production systems which place men and the relationship between men and nature at its centre. This will lead to croplands which “resemble” complex natural system, to a re-carbonization of soils and a fairer retributions to farmers,. Legume cultivation is a small but significant step towards agroecological croplands which support the autonomy of farmers in the face of increased agro-industry control and a deviation towards production systems with lower environmental costs. The thesis was structured to take a step wise approach to detangle some of the major pathways by which legumes may prove sustainable in optimizing soil organic matter development and reduction of nutrient losses in agroecosystems. Results will be of use from policy makers to practitioners, who aim at supporting the resilience of soils in the context of global warming and ecosystem functioning.

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