Insights on soil carbon cycling in intercropped maize-forage systems as affected by nitrogen

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ARTICLE INFO

ABSTRACT

Intercropping maize with forage grasses is an economical and environmentally sound practice that is increasingly being adopted to enhance resilience in tropical agriculture. Although intensifying integrated cropping systems can increase the sequestration of carbon (C) from plant residues, it also unleashes priming of old soil C enhancing C cycling, particularly under nitrogen (N) fertilization. However, the extent of these competing processes in intercropped maize–forage systems is poorly understood. This four-year study assessed whether new C inputs from maize (*Zea mays*) intercropped with ruzigrass (*Urochloa ruziziensis*), palisade grass (*Urochloa brizantha*), or Guinea grass (*Megathyrsus maximum*) in the presence or absence of N fertilization affect soil aggregation and C cycling in the soil and within macroaggregates (>0.250 mm) and microaggregates (<0.250 mm) down the soil profile. C cycling was assessed by measuring variations in the abundance of the natural isotope ^{13}C. N fertilization of the intercropped maize–forage systems reduced the proportion of aggregates > 2 mm and the mean weight diameter of aggregates by reducing soil pH. Under N fertilization, the geometric mean diameter of aggregates were 42 % larger under palisade than under Guinea grass. New C inputs from intercropping maize with forage grasses promoted C cycling in bulk soil, particulate organic matter (POM), mineral-associated organic matter (MAOM), and macro- and microaggregates, although these effects were restricted to topsoil. No N fertilization increased ruzigrass C input into MAOM with no clear link with ^{13}C enrichment, suggesting that N fertilization does not impair C stabilization in this pool. Aggregates >2 mm and >0.5 mm were key sinks of C and N up to a soil depth of 40 cm in this intercropped system. Our findings provide insights into the extension of C cycling across SOM pools and aggregates, and the role of N management in intercropping maize forage systems.

1. Introduction

Sustainable food production systems, such as intercropping cash crops with tropical forage grasses, may boost SOC by increasing root and shoot dry biomass inputs (Castro et al., 2015; Yang et al., 2019). Maize intercropped with forage grasses in succession to soybean, which increases plant residue inputs and nutrient use efficiency, is a sound strategy for intensifying food production systems in wet tropical regions (Rosolem et al., 2017). However, whether the new C inputs promote the cycling of the C already stabilized within macro- and microaggregates or in soil organic matter (SOM) fractions is still overlooked in tropical lands. Understanding soil C dynamics in crop management systems is critical for developing strategies to increase C sequestration while strengthening soil resilience against adverse weather events (Droste et al., 2020; Powlson and Galdos, 2023).

Plant physiological traits favor the lighter C isotope ^{12}C over the heavier stable isotope ^{13}C, resulting in natural variations in the ^{13}C/^{12}C isotope ratio—expressed as δ^{13}C—in soils (Bender, 1971; Balesdent et al., 1987). This ratio can be used as a natural tracer to map the contributions of different plant residues (C$_3$ and C$_4$ photosynthesis pathways) to a range of soil C pools (Farquhar et al., 1989; Amelung et al., 2008). Δ^{13}C has also been used as an indicator of SOM decomposition, as

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the microbial breakdown of plant residues leads to ^{13}C enrichment in the microbial byproducts that primarily compose the mineral-associated organic matter (MAOM) fraction (Balesdent et al., 1987; Midwood et al., 2021). Therefore, this technique can be used in experimental areas with a defined crop history, such as conversion from forest to intercropped maize–forage grass systems, to understand C dynamics in tropical soils.

The dense, aggressive root systems of forage grasses reduce nitrate leaching in no-till production systems by increasing the complexity of the soil pore network (Rosolem et al., 2017; Galdos et al., 2020). The minimal perturbation of the soil under no-till enhances aggregate formation, and the dense root systems of forage grasses release exudates that act as cementing agents to stabilize larger aggregates (Oades, 1984; Six et al., 2000). However, the effects of different forage species on soil aggregation in integrated systems and their links with N fertilization have not been comprehensively studied. Furthermore, as aggregates protect C from microbial oxidative enzymes, evaluating the origin of the C inside aggregates may provide important insights into the ability of new C inputs to access and promote C cycling within aggregates (Six et al., 2001; Dungait et al., 2012; Even and Cotrufo, 2024). Galdos et al. (2020) reported that palisade grass had a greater density of fine roots than ruzigrass and maize in a long-term no-till experiment. Finer root systems increase the complexity of the soil pore network, enhancing rhizosphere activity and C turnover in soil hotspots (Kuzyakov and Blagodatskaya, 2015; Pausch and Kuzyakov, 2018). Microbial enzymes can break down recalcitrant organic molecules in the soil into low-molecular-weight molecules, which may be enhanced in integrated systems with root C inputs (Liang et al., 2018; Cotrufo and Lavallee, 2022).

Inputs of nitrogen (N) increase crop grain yields and biomass input in complex intercropped systems (De Almeida et al., 2017; Crusciol et al., 2020). De Almeida et al. (2017) reported that only 5 % of supplied N was taken up by palisade grass intercropped with maize. Rocha et al. (2020) observed a negative N balance in a maize–Guinea grass system despite N supply (210 kg ha^{-1}) due to high N export by grain, which could lead to SOM priming and C loss. N shortages are consistently observed in systems that include ruzigrass, probably because of temporary N immobilization (Rosolem et al., 2012, 2017). Despite extensive research on N dynamics in intercropped systems, the interactions between soil N and C dynamics are not yet thoroughly understood. N plays an important role in the stabilization of C in some SOM fractions, such as MAOM (Jilling et al., 2018, 2020; Possinger et al., 2020; Cotrufo and Lavallee, 2022). MAOM has a low C:N ratio and slower turnover than POM because it is stabilized by electrically charged mineral surfaces (Lavallee et al., 2020). Furthermore, MAOM is formed mainly by low-molecular-weight molecules and microbial byproducts of plant decomposition, for which N availability is critical (Kögel-Knabner et al., 2022).

The present study investigated the extent to which new C inputs from intercropping maize with ruzigrass (*Urochloa ruziziensis*), palisade grass (*Urochloa brizantha* cv. Marandu), or Guinea grass (*Megathyrsus maximus* cv. Tanzania) fertilized or not with N affected soil aggregation and cycling of old C in bulk soil, POM and MAOM fractions, and micro-(<0.250 mm) and macroaggregates (>0.250 mm) down the soil profile in a four-year experiment. We hypothesized that (i) inputs of below- and aboveground biomass, especially under N supply, lead to higher aggregation and C content in macroaggregates. Furthermore, we hypothesized that ii) new C inputs from intercropping maize with forage grasses under N addition promote C cycling in bulk soil, particulate organic matter (POM) and MAOM fractions, and macro- and microaggregates. Although plant biomass inputs are expected to be highest in the uppermost soil layer, we hypothesized that iii) intercropping maize with forage grasses increases C content and cycling along the whole soil profile (0–40 cm).

2. Material and methods

2.1. Study area

This study used soil samples taken from a four-year experiment (2017–2021) in Botucatu, São Paulo, Brazil (22° 49′ 27.58″ S and 48° 25′ 46.73″ W, altitude of 770 m above sea level and < 3 % slope). The region typically experiences dry winters and hot summers, with historical annual average minimum and maximum temperatures of 15.3 and 26.1 °C, respectively, and an average rainfall of 1450 mm yr^{-1}. Climate data collected from a nearby meteorological station (500 m from the experimental area) during the experiment are shown in Fig. S1. Before the experiment, the area was covered with spontaneous vegetation, mainly perennial grasses (*Urochloa decumbens*). The soil is a Rhodic Hapludox (Soil Survey Staff, 2014) with 614 g kg^{-1} of clay composed of ~70 % kaolinite, ~15 % gibbsite, and small amounts of vermiculite and illite (Grassmann et al., 2020). The soil in the top 0–20 cm was analyzed according to van Raij et al. (2001) and exhibited the following chemical characteristics: pH (CaCl$_2$), 5.9; total C (TC), 19 g kg^{-1}; total N (TN), 1.3 g kg^{-1}; NH$_4^+$–N, 5.4 mg kg^{-1}; NO$_3^-$–N, 6.4 mg kg^{-1}; P, 15 mg kg^{-1}; K, 1.3 mmol$_c$ kg^{-1}; Ca, 35 mmol$_c$ kg^{-1}; Mg, 24 mmol$_c$ kg^{-1}; H+Al, 37 mmol$_c$ kg^{-1}; cation exchange capacity, 97 mmol$_c$ kg^{-1}; and base saturation, 61 %.

2.2. Treatments and experimental design

The experimental design was a complete randomized block with four replicates arranged in a split-plot scheme. Maize was intercropped with ruzigrass (*Urochloa ruziziensis*, cv. Common), palisade grass (*Urochloa brizantha*, cv. Marandu), or Guinea grass (*Megathyrsus maximum*, cv. Tanzania) in the main plots. N rates were applied in the subplots at 0 kg ha^{-1} and 270 kg ha^{-1}. The subplots were 10 m long × 4.5 m wide. The N source was ammonium sulfate.

2.3. Crop management

Before setting up the experiment, spontaneous vegetation was chemically desiccated with glyphosate (2.9 kg ha^{-1}). Forage grasses were sown (7 kg live seed ha^{-1}) in October 2014 under no-till without fertilization and cropped until September 2015. In October 2015, maize was sown over the remaining plant residues using a row spacing of 0.75 m and density of 65,000 plants ha^{-1}, fertilized with 53 kg ha^{-1} phosphorus (P; in the form of triple superphosphate) and 100 kg ha^{-1} potassium (K; in the form of potassium chloride). The N rate (210 kg ha^{-1}) was split: 30 kg ha^{-1} at sowing and 180 kg ha^{-1} sidedressed at the V5 growth stage (fifth leaf with visible collar). The N fertilizer (granular ammonium sulfate) was applied in a single-side surface band approximately 5 cm from the crop row. The maize was hand-harvested in March 2016, and the stover (i.e., leaves, stems, and cobs) was left on the soil surface. Seven months after the maize harvest (October 2016), the forage grasses were seeded again due to a shortage of rainfall and were desiccated 60 days after emergence. Maize was sown in December 2016 and harvested in May 2017 following the same agricultural practices used in the previous growing season. The forage grasses were sown in May and reseeded in August due to the low population and desiccated in November. Dolomite lime (CaCO$_3$•MgCO$_3$) and gypsum (CaSO$_4$ 2H$_2$O) (1.5 and 1.0 Mg ha^{-1}, respectively) were broadcast in October 2017 to raise the soil base saturation to 70 %. In December 2017, maize and forage grasses were jointly sown and grown in an intercropping system. Furthermore, the N rate was increased from 210 kg ha^{-1} to 270 kg ha^{-1} because the N balance had been negative until this point (Rocha et al., 2020). After the maize harvest, the forage grasses were maintained throughout the off-season (April–November). This maize–forage intercropping system was repeated in the following five crop seasons (from 2017/18 to 2021/2022).

2.4. Soil sampling

Soil monoliths were collected in September 2021 by opening a trench (60 cm high × 30 cm wide × 20 cm long) in the center of each plot. Undisturbed soil cores (monoliths 5 cm × 10 cm × 15 cm) were sampled along the exposed soil profile near (approximately 5 cm) the sowing row at soil depths of 0–10 cm, 10–20 cm, and 20–40 cm. Undisturbed soil cores were also taken from a nearby forest area and from a plot alongside the experimental area, which were used as standard samples. The plot alongside the experiment had maize and forage grasses cropped without fertilizer. The monoliths were gently broken into smaller aggregates manually. The samples were dried at room temperature and screened using a set of 8-mm and 4-mm sieves. The aggregates remaining on the 4-mm sieve were retained for aggregate stability analysis. Bulk soil samples were also collected in the experimental site using an auger with internal diameter of 5 cm as a composite of three soil cores diagonally distributed across each subplot at depths of 0–10 cm, 10–20 cm, and 20–40 cm. In the nearby forest and in the plot used alongside the experiment four subsamples were taken randomly 1 m far from the trench in the same soil depths. The samples were dried and screened using a 2-mm sieve before further analysis.

2.5. Aggregate analysis

After air drying the soil at room temperature, 45 g was used for aggregate stability analysis. In addition, a subsample of 20 g was dried for 24 h at 105 °C to determine the initial water content. We used a method adapted to tropical soils that uses a vertical oscillation system based on wet sieving separation (Silva and Mielniczuk, 1998). This method was adapted from pioneering work by Yoder (1936) and modified by Grohmann (1960) and Kemper and Chepil (1965). The aggregates were weighed (45 g) on an aluminum foil surface and moistened with tap water using a sprayer. A set of five sieves—2.0, 1.0, 0.5, 0.25, and 0.105 mm—were immersed inside a water drum assembled with a vertical oscillation system (3.5 cm vertical amplitude and 30 cycles m^{-1}). Ten minutes after rewetting, the aggregates were gently distributed over the 2-mm screen and oscillated vertically for 15 min. The aggregates remaining on each sieve were transferred to plastic cups with lids, dried in a forced-air oven (60 °C), and weighed. The mean weight diameter (MWD) (*Equation 1*), geometric mean diameter (GMD) (*Equation 2*) were assessed as proposed by Zhou et al. (2020).

$$MWD = \sum_{i=1}^{n} (XiWi)$$ (1)

$$GMD = \exp\left\{ \frac{\sum_{i=1}^{n} WilgXi}{\sum_{i=1}^{n} Wi} \right\}$$ (2)

The water-stable aggregate stability (WSAS) was assessed using the equation 3 proposed by Castro Filho et al. (2002).

$$WSAS = \frac{weight\,of\,the\,aggregates - weight\,of\,the < 0.25\,mm\,size\,class\,sample - sand}{weight\,of\,the\,sample - sand} \times 100$$ (3)

The proportion of aggregates recovered on the sieve >2 mm is presented as the percentage (%) of the 45 g soil sample.

2.6. Physical fractionation of soil organic matter

SOM was physically separated following (Cambardella and Elliott, 1993) to separate the soil POM (>53 μm) and MAOM (<53 μm)

fractions. Briefly, 5 g of air-dried 2-mm soil collected in 2021 at a depth of 0–10 cm, 10–20 cm, or 20–40 cm was weighed in a 200-mL plastic cup with a lid and shaken with 20 mL of sodium hexametaphosphate solution (Na$_4$P$_2$O$_7$, 5 g/L) for 15 h. The sample was poured over a 0.053 mm (53 μm) sieve and rinsed with tap water until all clay was eliminated; the fraction remaining on the sieve was deemed POM. The POM fraction was transferred to an aluminum pan and dried in an oven at 50 °C. The fraction that passed through the sieve was deemed MAOM, recovered in plastic cups, and dried at 50 °C. The same analyses were run on soil samples collected from a nearby forest and alongside the experimental area.

2.7. Total carbon and nitrogen measurements

Subsamples of the samples of bulk soil, POM, MAOM, and each aggregate size recovered from 2, 1, 0.5, 0.25, and 0.105 mm sieves were air-dried and ground to a finer texture by shaking in a ball mill for 45 s. TC and TN contents were then measured using a CHN-2000 analyzer (Leco Corp., St. Joseph, MI, USA).

2.8. ^{13}C natural isotope abundance measurement

The natural ^{13}C stable isotope abundance in bulk soil, POM, and MAOM was determined. The aggregates were separated into macroaggregates (>0.25 mm) and microaggregates (<0.25 mm) before isotopic measurements. A sample of 10–20 mg was placed in a tin capsule (scale XP6, Mettler Toledo, Switzerland, 0.001 mg resolution). The analyses were performed using a continuous flow isotope ratio mass spectrometry (CF-IRMS) system coupled to an elemental analyzer (Flash 2000 – Delta Advantage, Thermo Scientific, Germany) with a gas interface (ConFlo IV, Thermo Scientific, Germany). The isotopic ratio of C $R(^{13}C/^{12}C)$ was expressed as the relative difference in the isotopic ratio ($\delta^{13}C$) in parts per thousand (‰) from the Vienna Pee Dee Belemnite (V-PDB) standard according to *Equation 4* (Coplen, 2011). The estimated uncertainly of the analysis was ± 0.15 ‰ ($n = 10$), and the results were normalized using the NBS-22 certified reference standard.

$$\delta^{13}C_{(‰)} = \frac{R\left(^{13}C/^{12}C\right)_{sample}}{R\left(^{13}C/^{12}C\right)_{V-PDB}} - 1$$ (4)

2.9. Statistical analysis

The data were analyzed using generalized linear mixed models (GLMMs) with a 5 % level of significance in R (version 4.2.2, R Core Team) in the RStudio environment (version 2022.12.0 Build 353, 2022, Posit Software, Boston, MA, USA). The data were manipulated using the "*tidyverse*" package (Wickham et al., 2019). The normality and distribution of the data were evaluated using the Shapiro–Wilk test and the "*fitdistrplus*" package, respectively (Delignette-Muller and Dutang, 2015). Forage grass species (F), N supply (N), and soil depth (D) were considered fixed effects (split–split plot ANOVA). Soil depths were used as a repeated measure (factor: plot). Furthermore, block and F were assigned as random effects in the model. To compute the degree of freedom for the fixed effects, we applied either the "*lmerTest*" or "*pbkrtest*" package while running the GLMMs with Kenward-Roger approximation (Halekoh and Højsgaard, 2014; Kuznetsova et al., 2017). The model was assessed using the "*redres*" package (Goode et al.,

2022) through visual scrutiny of residuals *vs.* fitted values and normal quantile–quantile plots. Data for which the hypothesis of normality and residual distribution were rejected were transformed by Tukey's Ladder of Powers with the "*rcompanion*" package (Mangiafico, 2022) to increase the data's homoscedasticity. Thereafter, we reran the models but kept the original values for plot visualization. The means were separated with Tukey's post-hoc test using the "*emmeans*" and "*multcomp*" packages, respectively (Hothorn et al., 2008). The GMLM to test TC and TN contents within the aggregates was built using the aggregate classes (A) as a fixed factor instead of soil depths (D). Notwithstanding, we studied the effects of F, N and A for each soil depth.

3. Results

3.1. Soil aggregate properties

The percentage of aggregates > 2 mm and MWD differed significantly between N levels and soil depths (Fig. 1, Table S1). Supplying N to the intercropped maize–forage system reduced the percentage of aggregates > 2 mm by 16 % and MWD by 13 % compared with no N addition (Fig. 1a, b). Furthermore, the percentage of aggregates > 2 mm and MWD were 57 % and 35 % larger in topsoil (0–10 cm) than in subsoil (20–40 cm) (Fig. 1c, d).

The interaction between forage grass species, N supply, and soil depth significantly affected the GMD of the aggregates (Fig. 2, Table S1). When the forage grass was Guinea grass, GMD in topsoil was 46 % larger without N fertilization than with N fertilization (Fig. 2a). Furthermore, when N was applied, GMD was 42 % smaller when maize was intercropped with Guinea grass than when maize was intercropped with palisade grass or ruzigrass (Fig. 2a). GMD was generally larger in topsoil than in subsoil (Fig. 2a, c). When maize was intercropped with Guinea grass, ruzigrass, or palisade grass without N fertilization, GMD was 49 %, 40 %, and 38 % larger in topsoil than in subsoil, respectively (Fig. 1a, c).

In addition, the interaction between soil depth and N significantly affected WSAS (Fig. 2d, Table S1). Under N supply, WSAS was significantly higher in topsoil than at a depth of 10–20 cm, but the differences in WSAS with and without N fertilization were not significant (Fig. 2d).

3.2. Carbon content in bulk soil and SOM fractions and $\delta^{13}C$ values

Neither the TC content of bulk soil nor $\delta^{13}C$ values were affected by N fertilization (Fig. 3, Table S1). Among all systems, bulk soil TC content was 42 % lower in subsoil than in topsoil (Fig. 3a). Compared with TC content in subsoil, TC content in topsoil was 37 %, 35 %, and 29 % higher when maize was intercropped with ruzigrass, palisade grass, or
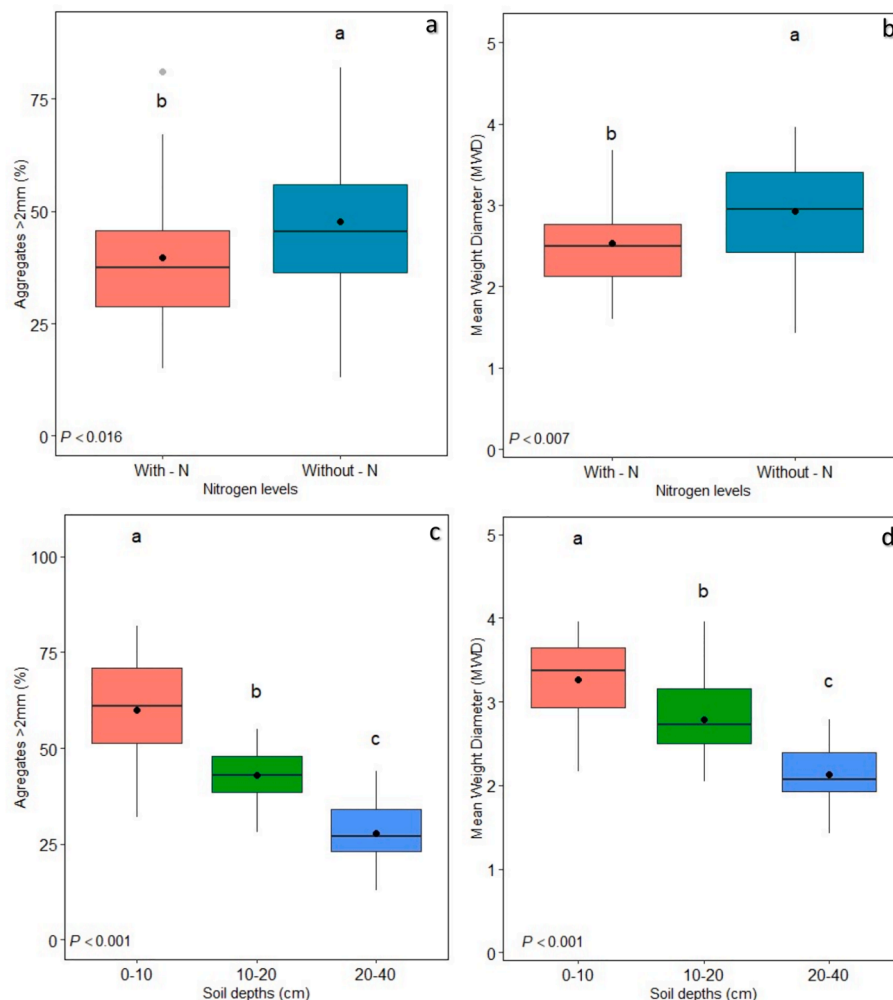

Fig. 1. Aggregates >2 mm (%) and mean weight diameter (MWD, mm) influenced by nitrogen (a, b) and soil depth sampling (c, d), respectively. The box represents the median (50th percentile), 25th and 75th percentile of the data. The whiskers represent 1.5 times the inter-quartile range. The points >1.5 times the interquartile range were highlighted individually in gray. *P*-values are calculated with linear mixed-effect models. Mean and median values are the round dot and solid line within the box, respectively. Different letters indicate significant differences across N levels and soil depths based on the analysis of linear mixed-effect models followed by Tukey's post-hoc tests.
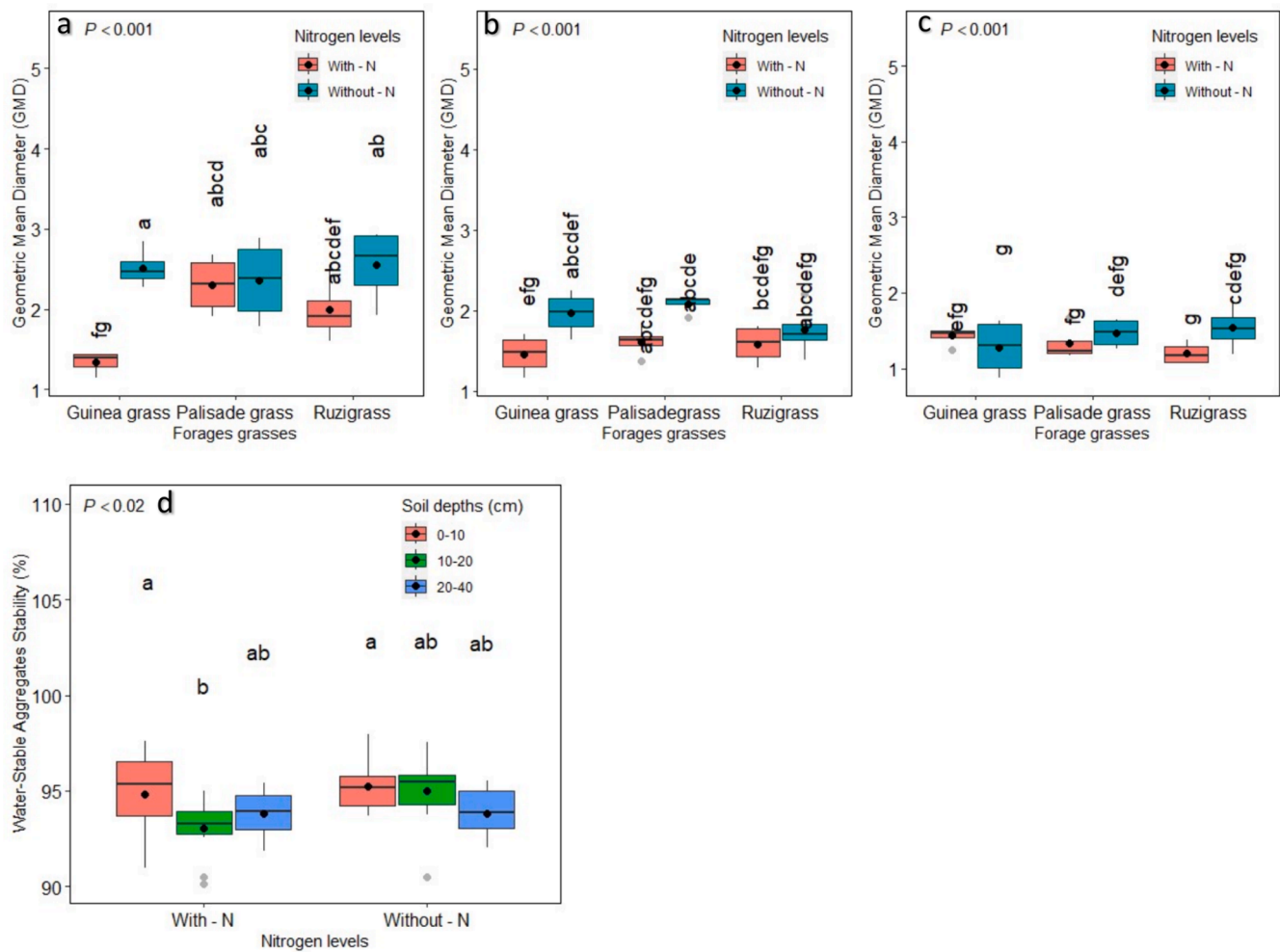
Fig. 2. Effect of forage grasses and nitrogen for the geometric mean diameter (GMD, mm) at the 0–10 cm (a), 10–20 cm (b) and 20–40 cm (c) soil depths and water-stable aggregates stability (WSAS, %) (d) as influenced by the nitrogen levels across the soil profile. The box represents the median (50th percentile), 25th and 75th percentile of the data. The whiskers represent 1.5 times the inter-quartile range. The points > 1.5 times the interquartile range were highlighted individually in gray. *P*-values are calculated with linear mixed-effect models. Mean and median values are the round dot and solid line within the box, respectively. Different letters indicate significant differences among forage grasses and N levels across soil depths based on the analysis of linear mixed-effect models followed by Tukey's post-hoc tests.

Guinea grass, respectively. Topsoil was 8.9 % more enriched in ^{13}C isotopes than subsoil (Fig. 3c).

The POM fraction and POM δ^{13}C value decreased throughout the soil profile, and POM δ^{13}C was correlated with N supply but not forage species (Table S1, Fig. 3). The POM fraction was 37 % and 32 % greater in topsoil than at depths of 10–20 cm and 20–40 cm, respectively (Fig. 3b). At a soil depth of 10–20 cm, POM was 6.3 % richer in ^{13}C after four years without N fertilization than with N fertilization. Furthermore, with N fertilization, POM was richer in ^{13}C in topsoil than at depths of 10–20 cm and 20–40 cm. In the absence of N fertilization, POM δ^{13}C did not differ between the 0–10 cm and 10–20 cm layers and was, on average, 13 % higher in these layers than in subsoil (Fig. 3d).

Among all maize–forage intercropping systems, the MAOM fraction was 50 % greater in topsoil than in subsoil (Fig. 3f–h). The MAOM fraction was greatest at a depth of 10–20 cm when maize was intercropped with ruzigrass without N fertilization (Fig. 3g). At a depth of 10–20 cm, intercropping maize with ruzigrass instead of palisade grass increased the MAOM fraction by 33 % in the absence of N. When maize was intercropped with Guinea grass, the MAOM fraction across the soil profile was 20 % higher without N fertilization than with N fertilization. However, when maize was intercropped with ruzigrass, the MAOM fraction in topsoil was 19 % smaller without N fertilization than with N

fertilization (Fig. 3f, h). The cycling of C into the MAOM pool was only influenced by soil depth, with no effect of N supply or forage grass species (Table S1). Notwithstanding, MAOM was 7 % more enriched in ^{13}C in topsoil than in subsoil (Fig. 3e).

3.3. Carbon within macro- and microaggregates and δ^{13}C values

TC content and δ^{13}C values in macro- and microaggregates differed across soil depths with no effect of N supply or forage grass species (Table S1). Compared with topsoil, TC content within macroaggregates was 10.2 % and 26 % lower at depths of 10–20 cm and 20–40 cm, respectively (Fig. 4a). By contrast, the TC content within micro-aggregates was, on average, 15 % lower in subsoil than at depths of 0–20 cm (Fig. 4b).

The C within macroaggregates was 7.5 % richer in ^{13}C in topsoil than in subsoil (Fig. 4c). Similar δ^{13}C values were observed in micro-aggregates (Fig. 4d). When averaged across both macro- and micro-aggregates, C was 5.5 % richer in ^{13}C in topsoil than in subsoil and only 2.2 % richer in topsoil than at a depth of 10–20 cm (Fig. 4c, d).
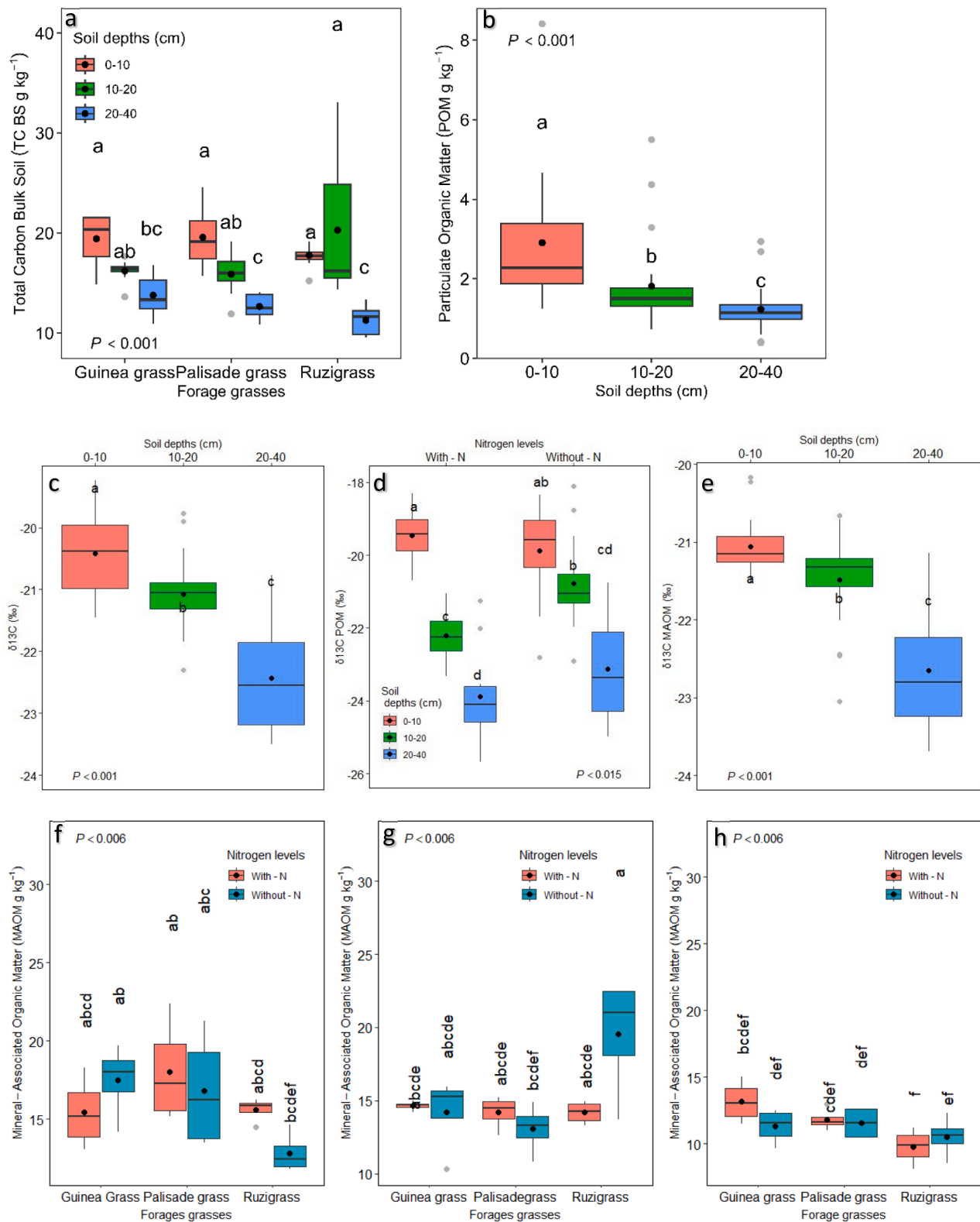
Fig. 3. Total carbon content measured in the bulk soil (a), particulate organic matter (POM) fraction (b), δ^{13}C values measured at the bulk soil (c), POM (d), and mineral-associated organic matter (MAOM) (e), and MAOM determined at the 0–10 cm (f), 10–20 cm (g) and 20–40 cm (h) as affected by forage grasses, nitrogen levels and soil depths in a four-year intercropping experiment. The box represents the median (50th percentile), 25th and 75th percentile of the data. The whiskers represent 1.5 times the inter-quartile range. The points >1.5 times the interquartile range were highlighted individually in gray. *P*-values are calculated with linear mixed-effect models. Mean and median values are the round dot and solid line within the box, respectively. Different letters indicate significant differences among forage grasses, N levels and soil depths based on the analysis of linear mixed-effect models followed by Tukey's post-hoc tests.
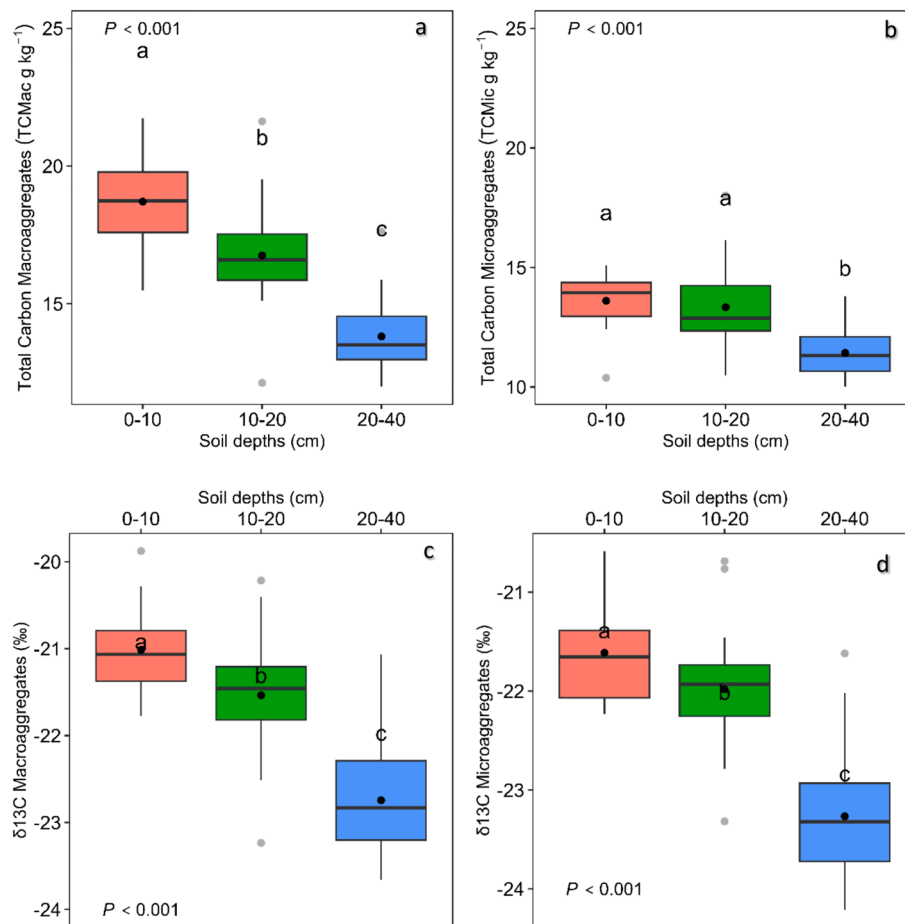
Fig. 4. Total carbon (a, b) and δ^{13}C values (c, d) within macro and microaggregates measured in soil samples gleaned at the 0–10 cm, 10–20 cm and 20–40 cm depths in an intercropped maize-forage system fertilized with nitrogen. The box represents the median (50th percentile), 25th and 75th percentile of the data. The whiskers represent 1.5 times the inter-quartile range. The points > 1.5 times the interquartile range were highlighted individually in gray. *P*-values are calculated with linear mixed-effect models. Mean and median values are the round dot and solid line within the box, respectively. Different letters indicate significant differences among soil depths based on the analysis of linear mixed-effect models followed by Tukey's post-hoc tests.

3.4. Carbon and nitrogen contents of each aggregate class

In topsoil, the TC content increased with aggregate size; the TC content of 2 mm aggregates was 41.3 % higher than that of 0.105 mm aggregates. Across all maize–forage intercropping systems, the TC content of 2 mm aggregates was 8 % higher with N fertilization than without N fertilization (Fig. 5a). When N was supplied, the TC content of the aggregates was 10.6 % higher when maize was intercropped with Guinea grass than when maize was intercropped with palisade grass or ruzigrass (Fig. 5b). Along the soil profile, the TC content of 2 mm aggregates was 30 % higher than that of 0.105 mm aggregates (Fig. 5c). Similar to the pattern observed in topsoil, the TC content of aggregates in subsoil was 10.3 % higher when maize was intercropped with Guinea grass than when maize was intercropped with ruzigrass (Fig. 5d).

At all soil depths, TN content did not differ among aggregate sizes (Fig. 5e, f, g). In topsoil, the average TN content of 2, 1, and 0.5 mm aggregates was 12 % and 24 % higher than that of 0.25 mm and 0.105 mm aggregates, respectively (Fig. 5e). Similar patterns of TN content were observed at depths of 10–20 cm and 20–40 cm. At a depth of 10–20 cm, the TN content of 0.25 mm and 0.105 mm aggregates was 16 % and 28 % lower than that of aggregates larger than 0.5 mm, respectively. When averaged throughout the soil profile, the TN content of aggregates larger than 0.5 mm was 13 % and 20 % higher than that of 0.25 mm and 0.105 mm aggregates, respectively (Fig. 5f, g). At a depth of 20–40 cm, TN content was 6.5 % greater under N supply than without N supply.

4. Discussion

4.1. Effects of maize–forage intercropping and N supply on soil aggregate properties across the soil profile

Our first hypothesis was not fully confirmed because the combination of increased above- and belowground plant biomass with N supply did not increase aggregation (Fig. 1a) but instead led to higher C sequestration into aggregates > 2 mm (Fig. 5a). A decrease in MWD and aggregates > 2 mm under N supply was reported in a six-year N fertilization trial in a Korean pine forest (Zhijie et al., 2019). Zhijie et al. (2019) attributed this negative response to a decrease in glomalin-related protein production when N supply was combined with water scarcity, which was not the case in our study. The decreases in aggregates > 2 mm and MWD observed in the present study under N fertilization warrant further investigation.

There is evidence linking N supply to greater soil aggregation (Chang et al., 2019; Lu et al., 2021). In a *meta*-analysis of 72 studies of a range of ecosystems in China, Lu et al. (2021) found that supplying N increased MWD and macroaggregates by 10 % and 6 %, respectively. In a short two-year forest experiment, Chang et al. (2019) observed that N addition increased MWD and C content in soil aggregates.

Our results led us to speculate that the decrease in soil pH resulting from annual N addition could explain the decreases in MWD, GMD, and aggregates >2 mm (Fig. 1a, b; 2a). N can increase soil C sequestration by suppressing lignin-modifying enzyme activity (Chen et al., 2018), but
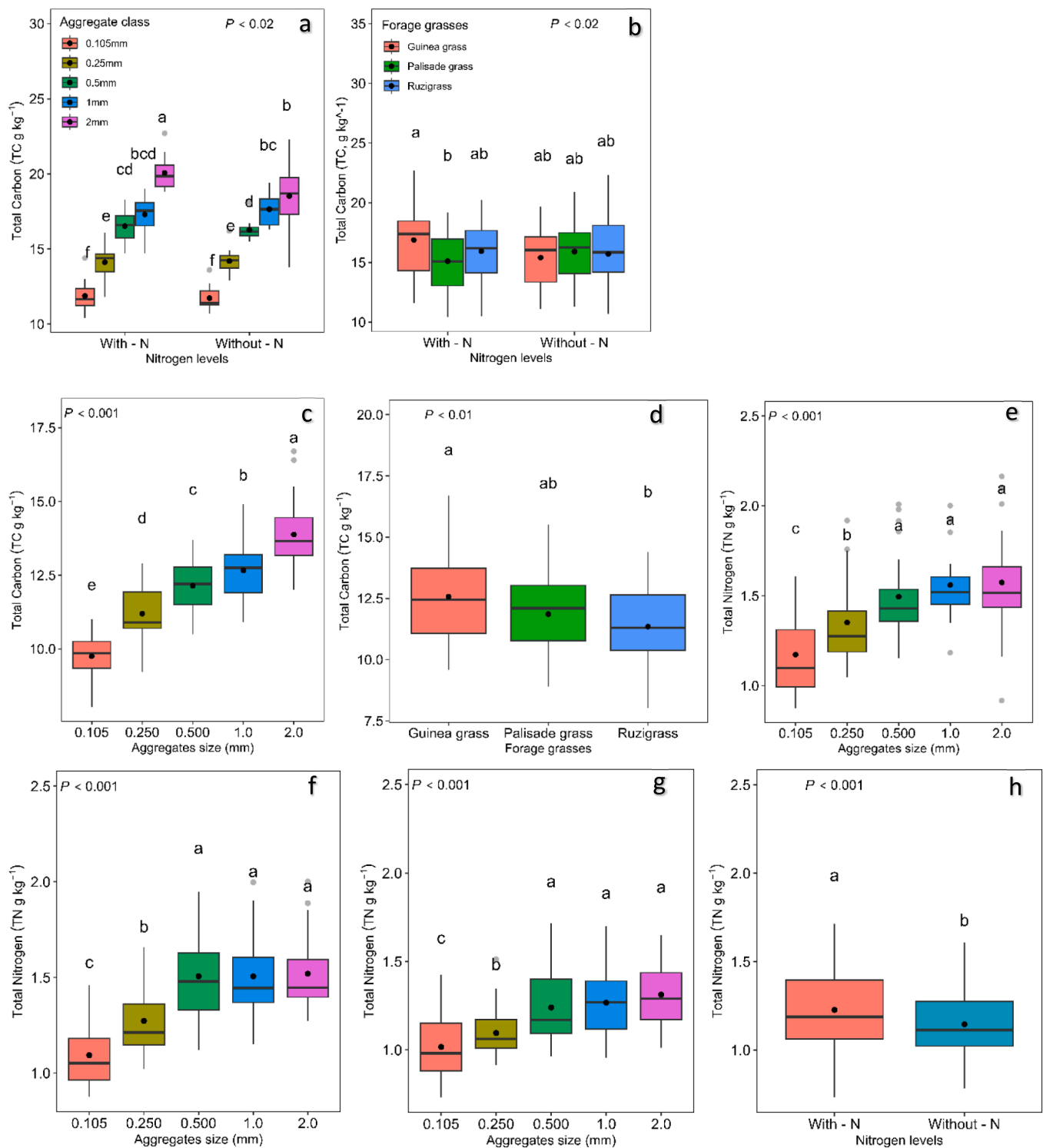
Fig. 5. Total carbon at 0–10 cm (a, b) and 20–40 cm (c, d) and nitrogen content at 0–10 cm, 10–20 cm and 20–40 cm measured within each class of aggregate (1, 2, 0.5, 0.25 and 0.105 mm) sampled at the 0–10 cm (e), 10–20 cm (f) and 20–40 cm (g, h) soil depth. The box represents the median (50th percentile), 25th and 75th percentile of the data. The whiskers represent 1.5 times the inter-quartile range. The points > 1.5 times the interquartile range were highlighted individually in gray. *P*-values are calculated with linear mixed-effect models. Mean and median values are the round dot and solid line within the box, respectively. Different letter indicate significant differences among N levels and aggregates size based on the analysis of linear mixed-effect models followed by Tukey's post-hoc tests.

whether these effects extend to soil aggregation is unknown. Indeed, biological suppression after N deposition has been linked to decreased pH (Averill and Waring, 2018). Furthermore, high soil N content is thought to impair the enzymatic breakdown of organic molecules with high N content, potentially increasing C sequestration (Chang et al.,

2019). Xiao et al. (2018) reported that N addition suppressed the activity of extracellular oxidases (β1,4-glucosidase, β-D-cellobiohydrolase, and β-1,4-N-acetyl-glucosaminnidase) by 6.8 % while stimulating the activity of C-acquisition enzymes by 9.1 %. After four years of N fertilization in the current study, soil pH in topsoil (0–10 cm) was lower than

that under no N fertilization (Fig. S2). This decrease in pH under N supply may have been a key modulator of soil aggregation, but the underlying mechanism remains to be investigated. In addition, the suppressing effect of N supply on soil microbial activity and subsequent increase in C sequestration reported in previous investigations (Averill and Waring, 2018; Chen et al., 2018; Xiao et al., 2018) may explain the 8 % increase in TC content of aggregates >2 mm in topsoil (Fig. 5a) under N fertilization compared with no N addition in the intercropping systems.

Aggregates >2 mm, MWD, and WSAS did not differ between intercropping systems. Under N supply, GMD was 42 % larger when maize was intercropped with palisade grass than when maize was intercropped with Guinea grass; likely due palisade grass greater density of fine (<0.5 mm) roots reported by Galdos et al. (2020). Despite the importance of fine roots for macroaggregate formation and stabilization (Oades, 1984; Six et al., 2004), we observed no difference in GWD between the systems with palisade grass and ruzigrass, whose root system is dominated by larger roots (Galdos et al., 2020). Therefore, we hypothesize that some factor other than root morphology influenced the GMD of the aggregates in our study. The lack of information on the root traits of Guinea grass hinders a thorough understanding of our findings and is a knowledge gap to be filled. When maize was intercropped with Guinea grass, GMD in topsoil was 46 % smaller under N supply than without N (Fig. 2a), suggesting a link with lower soil pH (Fig. S2). However, it is not clear why similar effects of N fertilization on GWD were not observed in the systems with ruzigrass and palisade grass. Under N fertilization, soil pH was relatively constant along the soil profile (Fig. S2), suggesting that Guinea grass is more sensitive to soil acidity than *Urochloa* species. The higher soil pH of topsoil in the absence of N fertilization may have led to better root growth, as reported by FURLAN et al. (2020), and therefore larger aggregates (Fig. 2a).

4.2. Carbon in bulk soil, SOM fractions, and macro- and microaggregates and $\delta^{13}C$ values

Enrichment of ^{13}C was observed in bulk soil, POM, MAOM, and macro- and microaggregates (Fig. 3c–e; Fig. 4 c, d), indicating that C inputs from intercropping maize with forage grasses ended up in soil C pools (POM and MAOM) and compartments (bulk soil and macro- and microaggregates), promoting C cycling mainly in topsoil. A study of maize and sorghum intercropped with forage grasses found that 75 %–80 % of root biomass was located at depths of up to 20 cm (Sarto et al., 2021). In no-till systems such as those in the present study, the presence of plant residues on the soil surface may have contributed to the observed cycling of C. These findings partially support our second hypothesis because N supply affected $\delta^{13}C$ values only in POM compared with four years without N supply. This is interesting because previous reports from the same experiment by Grassmann et al. (2020) showed that N supply (140 kg N ha^{-1}) increased maize–forage shoot biomass by 9 Mg ha^{-1} compared with no N fertilization when averaged over two crop seasons. Despite the reported effect of N on plant C inputs, our results suggest that the amount of C was not the main driver of C cycling in our maize–forage grass intercropping systems. Regardless of N fertilization or forage species, the POM fraction of C was 37 % greater in topsoil than deeper in the soil profile (Fig. 3b). However, at a soil depth of 10–20 cm, POM was 6.3 % richer in ^{13}C without N than with N, indicating a greater contribution of C inputs from the intercropped system to this C pool when N was not added. Because C inputs are higher under N supply (Grassmann et al., 2020; Gazola et al., 2023), we expected a greater contribution of C from the intercropped systems to POM under N fertilization, which was not observed. Forage residues—mainly roots—have high C:N ratios (Rosolem et al., 2012, 2019) and result in higher plant inputs from residues, which are linked with increased C inputs into the POM pool in tropical soil (Castro et al., 2015), likely due to the physical transfer of acid-unhydrolyzable material (Cotrufo et al., 2015). Furthermore, weed grasses were also present in the experimental

area. The isotope signatures of these weeds are similar to those of the forage grasses in our treatments (AMELUNG et al., 2008), which may have affected the POM $\delta^{13}C$ values.

Our findings suggest that new C inputs enrich the MAOM pool in ^{13}C, indicating that C cycling occurs in this pool, mainly in topsoil. Although C stabilization in organomineral associations (MAOM) confers higher persistence in soils (Lavallee et al., 2020; Possinger et al., 2020), root exudates and the input of low-molecular-weight compounds may unleash C destabilization through priming effects, resulting in C losses and nutrient cycling (Jilling et al., 2018; Liang et al., 2018; Possinger et al., 2020). The point of entry of plant residues (Sokol et al., 2019)—topsoil—apparently influenced our findings. Liang et al. (2018) observed that under inputs of plant residues with low C:N ratios, C replenishment is 32 % greater than priming of old soil C. By contrast, the high C:N ratios of the forage grasses in our study (Rosolem et al., 2012) enhanced priming and C losses of old C already stabilized in the soil (Liang et al., 2018). As ruzigrass is associated with N immobilization and inadequate N for crop growth (Rosolem et al., 2012, 2017), we hypothesized that intercropping maize with ruzigrass would reduce C inputs into MAOM due to the key role of N in C stabilization (Possinger et al., 2020). Instead, under N restriction, intercropping maize with ruzigrass increased C in the MAOM pool by 33 % compared with intercropping with palisade grass and had no significant effect on C in the MAOM pool compared with intercropping with Guinea grass. These results indicate that N was not mined from the MAOM pool due to the absence of N or immobilization of N by ruzigrass.

There was no apparent link between C input and ^{13}C enrichment in the MAOM pool. The $\delta^{13}C$ values in MAOM at the onset of the trial (Table S3) indicate that C cycling was limited to a soil depth of 20 cm. Midwood et al. (2021) reported ^{13}C enrichment in MAOM in topsoil in a woody perennial crop system, likely due to microbial selection of ^{13}C during the oxidation of plant residues. As the C:N ratio of MAOM is low (Lavallee et al., 2020), the absence of N fertilization may have promoted mineralization as highlighted by Jilling et al. (2018), resulting in a lower proportion of C in MAOM. However, this did not occur in our four-year intercropped system. Indeed, N fertilization had no consistent effect on C stabilization in MAOM. This is interesting because Possinger et al. (2020) reported N-rich patches in organomineral interfaces of MAOM fractions and close links of N with C stabilization in MAOM. Further investigation of the chemical nature and elemental composition of the MAOM fraction in tropical soils may provide a thorough understanding of the role of N in C dynamics in MAOM and the impact of intensified intercropped maize–forage food production systems.

Our results showed that C inputs from maize–forage intercropping systems may gain access to the interior of macro- and microaggregates in topsoil (Fig. 4a, d). The enrichment of both macro- and microaggregates in ^{13}C in topsoil may have resulted from the deposition of surface plant residues. Dungait et al. (2012) hypothesized that SOM turnover in soil is a matter of accessibility—substrate *vs* microbial enzymes—not substrate recalcitrance. Macro- and microaggregates protect C because their small pore size hinders oxygen diffusion and the accessibility of C patches to microbial enzymes, reducing metabolic oxidation and C losses (Dungait et al., 2012; Cotrufo and Lavallee, 2022; Even and Cotrufo, 2024). Although our results support the input of new C into aggregates, the mechanism is unknown. A potential hypothesis is that root- and shoot-derived organic compounds influence aggregate build-up and stabilization, resulting in higher C inputs from maize and forage grasses within macro- and microaggregates. It is also possible that the root growth of intercropped maize–forage grasses promoted aggregate turnover (Six et al., 2004), exposing destabilized C to oxidation by microbial enzymes and enhancing the cycling of old C within macro- and microaggregates simultaneously with new C inputs. It has been proposed that the higher density and superficial area of the fine roots of palisade grass facilitate the access of root exudates into aggregate microsites, unlocking C cycling (Dungait et al., 2012; Galdos et al., 2020), but our results do not support such a link. The enrichment of macro- and microaggregates in

^{13}C was accompanied by higher C content in both classes of aggregates. Cooper et al. (2021) reported that long-term zero tillage (31 years) in tropical soil enhanced C protection compared with conventional tillage by increasing total C content in macroaggregates, microaggregates, and microaggregates within macroaggregates. The clayey nature of the soil in the present study, the constant presence of living root inputs, and minimal perturbation may have reduced the turnover of macroaggregate structures and promoted the formation of microaggregates within macroaggregates as conceptualized by Six et al. (2000), increasing C content in these structures. There is also evidence that POM formation within microaggregates protected inside macroaggregates leads to higher C content in aggregates (King et al., 2020).

In the maize–forage grass intercropping systems, TC content in bulk soil was higher in topsoil than in subsoil (Fig. 3c). Consistent with this finding, TC content was 37 %, 35 %, and 29 % higher in topsoil than deeper in the soil profile in the systems with ruzigrass, palisade grass, and Guinea grass, respectively. Contrary to our hypothesis, our results do not support C gains and C cycling across the whole soil profile. The low soil pH across the soil profile (Fig. S2), especially under N supply, may have restricted root growth to the uppermost soil layer (Furlan et al., 2020). In addition, the forage grasses were not grazed in this study, which may have resulted in a shallower root system. Sarto et al. (2021) reported that 75 %–80 % of *Urochloa* and *Megathyrsus* root biomass was located at depths of $\leq$ 20 cm when the grasses were grazed in the off-season after the maize harvest, consistent with the impairment of root growth by soil acidity. Intercropping forage grasses with cash crops is an alternative to the intensification of tropical food production systems that provides significant above- and belowground C inputs (Grassmann et al., 2020; Gazola et al., 2023). Vertical water flow through the soil pore network may carry small soluble organic molecules from the depolymerization of plant residues down the soil profile, where they are stabilized at mineral surfaces and increase C content in the subsoil (Gmach et al., 2020; Cotrufo and Lavallee, 2022). However, our results do not support C movement across the soil profile, as no enrichment of ^{13}C in the assessed C pools and compartments across soil depths was observed (Table S3).

4.3. Carbon and nitrogen contents as a function of aggregate size

Compared with microaggregates, TC content was 41.3 % and 30 % higher in 2 mm aggregates in topsoil and subsoil, respectively. Although the TC content of 2 mm aggregates was lower in subsoil than in topsoil, likely due to lower C inputs, more complex structures were observed at greater depths, representing an important sink of C across the soil profile. Carmeis Filho et al. (2018) reported increased C accumulation in aggregates sizes of >0.25 mm to 2.00 mm up to a depth of 60 cm when lime was surface applied in a tropical no-till system. N fertilization increased the TC content of 2 mm aggregates when maize was intercropped with Guinea grass, which may be attributable to higher plant residue inputs as reported by Grassmann et al. (2020). However, Grassmann et al. (2020) reported similar aboveground inputs from ruzigrass and palisade grass under N addition. It is important to understand why more C was protected in 2 mm aggregates in the Guinea grass system than in the ruzigrass and palisade grass systems. Throughout the soil profile, the TC content of aggregates was 10.3 % higher when maize was intercropped with Guinea grass than when maize was intercropped with ruzigrass.

Compared with 0.105 mm aggregates, the average TN content of aggregates >0.5 mm was 24 % and 20 % higher in topsoil and subsoil, respectively. Exploiting the dense root systems of forage grasses to avoid nitrate leaching and its environmental impacts is a key management strategy in tropical soils. Forage root systems immobilize nitrate within shoot and root debris (Rosolem et al., 2017). Likewise, the TN content of larger aggregates (>0.5 mm) was higher than that of microaggregates (<0.250 mm), indicating the importance of complex aggregates as an N sink. Although soil N dynamics have been shown to differ depending on

the *Urochloa* species (Rosolem et al., 2012, 2017; Galdos et al., 2020), no effect of forage grass species on the distribution of N among aggregate sizes (>2, 1, 0.5, 0.25 and 0.105 mm) was observed in the present study.

5. Conclusions

Our findings support the hypothesis that new C inputs from maize–forage grass intercropping unleash the cycling of old soil C in bulk soil, POM, MAOM, and in the interior of macro- and microaggregate cores, especially in topsoil. The effects of new C inputs thus did not differ significantly between forage grass species or the presence or absence of N fertilization.

N fertilization of the maize–forage intercropping systems reduced the proportion of aggregates > 2 mm and MWD by reducing soil pH. N fertilization of the maize-Guinea grass intercropping system resulted in 10.3 % more C protected within aggregates than ruzigrass and palisade grass, however the GMD of aggregates was 46 % and 42 % smaller with N than that without N supply and when maize was intercropped with palisade grass instead of Guinea grass in the topsoil, respectively. These findings indicate higher sensitivity of Guinea grass to N-driven low soil pH compared to the others forage species, resulting in smaller aggregates; on the other hand Guinea grass ought be an important strategy to increase protected C at the interior of soil aggregates despite still unknow mechanisms.

As hypothesized, N fertilization increased both C cycling and TC content in topsoil but not along the whole profile, likely due to N fertilization-driven decreases in pH across the soil profile.

Further research should focus on the mechanisms leading to higher C inputs in aggregates with Guinea grass as well as drivers controlling the cycling of C at the interior of aggregates unveiled in our study. Furthermore, more detailed information of the chemical nature of SOM fractions across varying forage species and N-driven soil pH may provide insights on the role of N fertilization on C stabilization in tropical soils.

CRediT authorship contribution statement

Laudelino Vieira da Mota Neto: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **José Victor Silva Barros:** Project administration, Methodology, Investigation, Funding acquisition, Data curation. **Vladimir Eliodoro Costa:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Data curation. **Marcelo Valadares Galdos:** . **Amanda Rithieli Pereira dos Santos:** Validation, Software. **Ciro Antonio Rosolem:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

The data and code that support the findings of this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.10576134. Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2024.116998.

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