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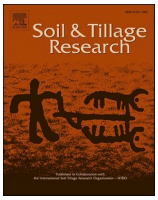

Toward more sustainable tropical agriculture with cover crops: Soil microbiome responses to nitrogen management

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ABSTRACT

Cover crops are a potential pathway for ecological cultivation in agricultural systems. In tropical no-till agricultural systems, the maintenance of residues on the soil surface and the addition of nitrogen (N) benefit the growth and grain yield of cash crops as well as the chemical and physical properties of the soil. However, the effects of these management practices on the soil microbiota are largely unknown. Here, we evaluated the effects of the timing of N application as a pulse disturbance and the growth of different cover crop species before maize in rotation on soil properties, maize productivity, and soil bacterial and fungal community diversity and composition. N fertilizer was applied either on live cover crops (palisade grass or ruzigrass), on cover crop straw just before maize seeding or in the maize V_4 growth stage. Soils previously cultivated with palisade grass established similar microbial communities regardless of N application timing, with increases in total bacteria, total archaea, nutrients, and the C:N ratio. The soil microbial alpha diversity in treatments with palisade grass did not vary with N application timing, whereas the bacterial and fungal diversities in the treatments with ruzigrass decreased when N was applied to live ruzigrass or maize in the V_4 growth stage. We conclude that palisade grass is a more suitable cover crop than ruzigrass, as palisade grass enhanced soil microbial diversity and maize productivity regardless of N application timing. Ruzigrass could be used as an alternative to palisade grass when N is applied during the straw phase. However, considering the entire agricultural system (soil–plant–microbe), ruzigrass is not as efficient as palisade grass in tropical no-till cover crop–maize rotation systems. Palisade grass is a suitable cover crop alternative for enhancing maize productivity, soil chemical properties and nutrient cycling, regardless of the timing of N application. Additionally, this study demonstrates that a holistic approach is valuable for evaluating soil diversity and crop productivity in agricultural systems.

1. Introduction

The cultivation of cover crops under no-till is a common practice in crop rotation systems to improve agroecosystem services, nutrient cycling in soil–plant interactions, and cash crop productivity in the tropics (Ashworth et al., 2020; Crusciol et al., 2021, 2020; Daryanto et al., 2019). Maintaining plant residues on the soil surface is an important agricultural management practice for improving crop yield and soil physicochemical proprieties in tropical no-till systems (Bossolani et al., 2021; Pariz et al., 2017; Tiecher et al., 2017). However, little information is available on the residual effects of cover crops on soil microbial communities and the relationships between microbes or microbial communities in tropical agriculture. The innumerable interactions between microorganisms, plants, and biotic and abiotic factors associated with cover cropping create a very complex environmental system akin to a black box (Cunha et al., 2011; Kim et al., 2020; King and Hofmockel, 2017; Wortman et al., 2013). Microbial diversity is a pillar of ecosystem functioning that directly influences soil processes (Wagg et al., 2019) and food production (Graham et al., 2016; van der Heijden and Wagg, 2013) and therefore should be taken into account when evaluating the soil diversity of these complex agricultural systems.

Decomposition of the straw residues of cover crops in tropical

Abbreviations: N, nitrogen.

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regions gradually releases nutrients to the subsequent crop (Pariz et al., 2011; Reddy et al., 1994; Rosolem et al., 2017). Nitrogen input to cover crops is a common practice employed by farmers to increase crop biomass production and straw residues or provide immediate nutrient availability and cash crop productivity (Chen et al., 2014; Momesso et al., 2019; Tanaka et al., 2019). The N use efficiency of plants and the feasibility of increasing cash crop productivity via N input have been explored in forage grass–maize rotation systems (Momesso et al., 2020; Oliveira et al., 2018; Rocha et al., 2020a). However, N fertilizer application disturbs the soil biota, potentially affecting its composition and interactions and important ecosystem functions such as soil fertility, quality and health, with ensuing effects on crop yields (Bissett et al., 2013; Lourenço et al., 2020). The soil disturbance caused by N fertilization alters microbial communities in the short term (Lourenço et al., 2020, 2018) and soil–plant–microbial ecological interactions in the long term (Cassman et al., 2016; Momesso et al., 2022c).

Soil microbial communities can also be affected by the cover crop species. In tropical agroecosystems, many cover crop species belong to the genus *Urochloa* (Baptistella et al., 2020; Namazzi et al., 2020; Villegas et al., 2020). *U. brizantha* (palisade grass) and *U. ruziziensis* (ruzigrass) can change N dynamics; enhance the efficiency of N use by cash crops; increase water retention capacity, soil aggregation, macrofauna biodiversity, arbuscular mycorrhizal fungi, saprotrophic fungi, and gram-positive bacteria; and improve nutrient uptake due to plant–microbe interactions (Galdos et al., 2020; Rocha et al., 2020; Sarto et al., 2020; Teutscherova et al., 2019, 2021). Compared with ruzigrass, palisade grass produces more biomass, but the management of this biomass is more difficult and ruzigrass can increase NO_3^- losses by nitrification process; however, residues of ruzigrass might negatively affect microbial communities and crop yield in succession (Momesso et al., 2019; Rocha et al., 2020b).

Numerous studies have demonstrated that soil bacterial and fungal community structure and diversity are greatly affected by land management practices, including tillage (Wang et al., 2020; Zhang et al., 2018), crop rotation (Oberholster et al., 2018; Somenahally et al., 2018) and fertilization (Lourenço et al., 2018, 2020; Momesso et al., 2022a). Studies of microbial communities in tropical soil ecosystems under cropping systems with cover crops receiving N inputs are scarce (Lopes et al., 2018), and no study has examined the effects of microbial community interactions and the covariation between the bacterial and fungal communities in these systems. In the present study, we investigated the lasting effect of inorganic N pulse disturbances caused by N application on cover crops on soil bacterial and fungal communities in a cover crop–maize rotation system. Specifically, we sought to answer the following question: How do N application and cover crop grass species affect microbial community structure following maize harvest under no-till? We hypothesized the following:

- H1: Palisade grass positively affects soil microbial community diversity and composition compared to ruzigrass.
- H2: Palisade grass increases soil nutrient content, nutrient cycling and maize productivity at the maize harvest stage compared to ruzigrass.
- H3: The effects of palisade grass hypothesized in (1) and (2) are greater when N is applied on the cover crop than on residue or maize growth.
- H4: N application on live cover crops increases the covariation between the bacterial and fungal communities.

2. Materials and methods

2.1. Field experiment and sample collection

To assess the residual effect of cover crops on the soil microbial communities and below- and aboveground soil properties at the maize harvest stage, we collected samples from a field experiment at the Experimental Farm Station in Botucatu, Brazil (48° 26′ W, 22° 51′ S, 740 m). Samples were collected from soil cultivated with cover crop–maize rotations subject to N inputs at different times. The soil is a clayey, kaolinitic, thermic Typic Haplorthox (USDA soil taxonomy) (Soil Survey Staff, 2014) and was selected due to its use in previous studies reporting the effects of cover crops on maize/upland rice grain yield (Momesso et al., 2020, 2019). The field experiment involved long-term tropical agriculture under no-till for 17 years; in the last 3 years, palisade grass and ruzigrass were cultivated in rotation with maize. The soil physico-chemical properties are shown in Table 1.

The experimental design comprised two cover crop–maize systems × three N management treatments [N input on live cover crops (CC), on the straw of the cover crops (SC), and at the maize growth stage (MA)] × four replicates. A randomized complete block design was adopted. The cover crops used in the cover crop–maize systems were palisade grass and ruzigrass. The N management treatments included the application of N fertilizer on (*i*) live cover crops (+CC), (*ii*) on the straw of the cover crops (+ST) just before maize seeding or (*iii*) at the maize V_4 growth stage (+MA) according to the conventional method of N application (Fig. 1). The treatments were chosen based on a prior study of maize management practices (Momesso et al., 2019) that showed that palisade grass–maize rotations increase maize productivity regardless of N application timing, while the effects of ruzigrass–maize rotations are dependent on the time of N application. *Urochloa* spp. were sown in April to November (off-season), and maize was the subsequent crop (summer season) each year under no-till. N was applied at a rate of 120 kg ha^{-1} as ammonium sulfate. N fertilizer was surface broadcast on live cover crops or their residues in October and November 2015, respectively, or band-applied on the soil surface 5 cm from the maize plants in December 2015. Cover crops were seeded at 10 kg seed ha^{-1} with no fertilizer application. In all treatments, grasses were terminated by spraying 1.56 kg glyphosate ha^{-1} (active ingredient), and the straw from the cover crops was left on the soil after mowing. In each plot, maize (*Zea mays* L.) cultivar P3456 was seeded after cover crop cultivation in ten 8-m-long rows with a spacing of 0.45 m between rows. The basic fertilizer applied in the maize seeding furrow consisted of 40 kg N ha^{-1} as ammonium sulfate, 90 kg P_2O_5 ha^{-1} as triple superphosphate and 45 kg K_2O ha^{-1} as potassium chloride in all treatments. The maize was harvested 125 days after emergence.

Soil samples were collected at maize harvest in 2016, i.e., after the entire growing season (Fig. 1). The bulk soil was collected from the top 10-cm layer within the row of maize plants in each of the treatment replicates. Each replicate of bulk soil was composed of 5 random soil cores from a single plot. The soil samples were stored a -80 °C until DNA extraction and at -20 °C until the analysis of soil chemical properties. The NO_3^--N and NH_4^+-N concentrations in the soil were determined by extraction with 2 M KCl solution according to Keeney and Nelson (1982). Another portion of the soil sample was air-dried to characterize pH, P, K^+, Ca^{2+}, Mg^{2+}, S-SO_4^{-2}, C and N (van Raij et al., 2001; Vitti, 1989). Soil pH was measured by preparing a 1:1.5 soil:water suspension. P and exchangeable K^+, Ca^{+2} and Mg^{+2} were extracted using ion-exchange resins; P was determined by colorimetry, and the cations were determined by atomic absorption spectrometry (van Raij et al., 2001). Soil S-SO_4^{2-} was extracted with 0.1 M calcium phosphate in a 1:2:5 soil/solution ratio and determined by the turbidimetric method using $BaSO_4$ (Vitti, 1989). For dry matter determination, the cover crop straw (aboveground) was collected on the day of maize harvest at the end of the growing season. Two subsamples were collected from an internal area of 0.25 m^2 in each plot and pooled. The samples were oven-dried at 65 °C for dry-weight determination. Total organic carbon (TOC) and N concentrations in soil and dry matter were measured using an elemental analyzer (LECO-TruSpec® CHNS) and used to calculate the C:N ratio using 0.2 g of soil.

Table 1
Chemical properties and total-N of soil (0–0.20 m depth), and C:N ratio and straw production of cover crops prior the field experiment installation.

Area	Soil										Cover crop	
	pH	SOM†	P (resin)	H+Al	K^+	Ca^{2+}	Mg^{2+}	CEC‡	BS§	Total-N	C:N ratio	Straw
	($CaCl_2$)	g dm^{-3}	mg dm^{-3}	——— mmol$_c$ dm^{-3} ———					(%)	g kg^{-1}		Mg ha^{-1}
Palisade grass	4.9	32	18.5	44	4.9	34	20	103	59	2.25	27	13.8
Ruzigrass	4.8	29	17.5	39	3.6	37	18	97	57	2.30	30	9.9

† Soil organic matter; ‡ Cation exchange capacity; § Base saturation.

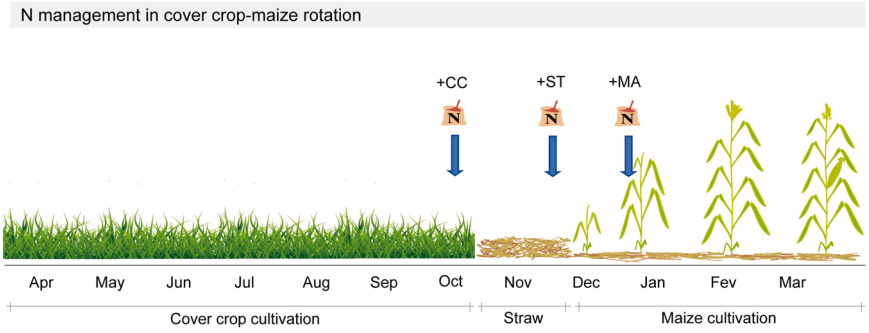

Fig. 1. Scheme of N application on live cover crops (+CC), on cover crop straw (+ST) or at the maize harvest growth stage in a cover crop–maize rotation system.

2.2. Soil DNA extraction, qPCR and 16S rRNA gene and ITS region amplicon sequencing

From the four soil replicates of each treatment, DNA was extracted from soil samples using the MO BIO PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol. DNA from soil was extracted from 0.25 g of soil and stored at–-20 °C until further use. DNA concentrations, quantity and quality were determined using a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific, USA) and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). DNA integrity was assessed by electrophoresis on an agarose gel (1% w/v). Total bacterial and fungal populations were quantified from DNA samples. To assess the abundances of total bacteria (16 S rRNA gene) and fungi (ITS region), copy numbers were determined by quantitative real-time PCR (qPCR) in a 96-well plate (Bio-Rad) using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The details of the primer sets and the PCR conditions for the genes are shown in Supplementary Table S1.

DNA samples from three replicates of soil from each treatment were used to amplify the targets: the primers 515 F (forward primer 5′-GTGC CAGCMGCCGCGGTAA-3′) and 806 R (reverse primer 5′-G GAC-TACHVGGGTWTCTAAT-3′) were used to amplify the variable V4 region with barcodes for 16 S rRNA gene sequencing, and the primers ITS9F (forward primer 5′-AACGCAGCRAAIIGYGA-3′) and ITS2R (reverse primer 5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS2 region with barcodes for fungal ribosomal internal transcribed spacers (ITS amplicon). Amplicon sequencing was performed on an Illumina MiSeq System at Genome Quebec (Quebec, Canada).

2.3. Statistical and bioinformatic analyses

Statistical analyses were conducted in R v4.02 using different packages (R Core Team, 2019). The soil, plant and microbial gene abundance data were non-normally distributed and thus were log-transformed; the normal distribution of the residuals was confirmed using the Shapiro–Wilk test procedure and variance stability ($W \geq 0.90$) (dplyr and devtools packages). Data were submitted to two-way ANOVA to assess the effects of cover crop species, N disturbances, and their interactions. Cover crops and N disturbances were fixed factors. Means were compared with Tukey's test ($p \leq 0.05$) when the F-test was significant (agricolae package).

The bioinformatic pipeline and subsequent analyses of the bacterial and fungal communities were conducted using R v4.02. The forward and reverse PCR primer sequences were removed from the MiSeq reads by using the cutadapt plugin v2.10 (Martin, 2011). The DADA2 v1.8 pipeline was used for demultiplexed paired-end fastq file processing (dada2 package). Forward and reverse reads were trimmed to 240 base pairs and 200 base pairs, respectively, and at the location of the first occurrence of a base call or a sequence containing ≥ 18 estimated errors before merging with a minimum overlap of 12 bases. Chimeric sequences were removed, and the merged reads were dereplicated. Taxonomic assignment of amplicon sequence variants (ASVs) was performed using the "Silva version 138″ database (McLaren, 2020) for the bacterial community and the "UNITE ITS database" for the fungal community (Põlme et al., 2020).

The read counts in the ASV table were filtered based on taxonomy to remove chloroplast reads and unknown taxa at the phylum level. ASV abundance was summarized at the genus level. Microbes present in less than 5 samples were considered low-occurrence microbes and aggregated into a single variable named 'rare'. Then, the data were transformed to the centered log-ratio (CLR) using the Bayesian–multiplicative replacement of count zeros (CZM) method. The Gjam package (Clark et al., 2017) was used to estimate the effects of cover crop species (palisade grass or ruzigrass) and N disturbances (+CC, +ST and +MA) on the soil microbial community together with the soil and plant variables and the abundances of bacteria, archaea, and fungi (qPCR analysis). We extracted regression coefficients for each treatment from the generalized joint attribute model (GJAM) to identify shifts in the microbial community and in the other variables (Leite and Kuramae, 2020). GJAM model diagnosis was evaluated using the Markov chain Monte Carlo (MCMC) method to determine when the estimated coefficients reached a stable value (after 2000 simulations with a burn-in of 500). The regression coefficients were visualized in a redundancy analysis (RDA) plot to explore correlations between soil properties and plant and microbial communities. We used the Hellinger transformation for soil, plant and qPCR data; biological data (qPCR and bacterial and fungal communities) were submitted to redundancy analysis (RDA) to illustrate the similarities between treatments. RDA plots were generated using Canoco 4.5 (Biometrics, Wageningen, the Netherlands). In addition, the filtered ASV table was used to determine

the alpha diversity (Chao index and Shannon index) and beta diversity (Bray–Curtis index distance method, NMDS ordination method and permutational MANOVA (PERMANOVA)) of the bacterial and fungal communities using Microbiome Analyst (Chong et al., 2020). As described by Schlemper et al. (2018), we performed coinertia analysis (COIA) of the Hellinger-transformed datasets (Legendre and Gallagher, 2001) by applying the *coinertia* function of the 'ade4' package (Dray and Dufour, 2007). COIA illustrates the covariation of the bacterial and fungal communities in each treatment and compares the structures of these microbial communities within treatments by means of arrow length. The arrows from COIA analysis were used to generate plots. Arrows in the same direction indicate associations between treatments; longer arrows indicate weaker relationships between the bacterial and fungal communities when comparing treatments (Culhane et al., 2003; Schlemper et al., 2018). We used COIA as a proxy of the codependence between the microbial communities (bacterial and fungal) and evaluate the influence of the different grass species and the regime of N addition in this codependence.

3. Results

3.1. Modulation of soil properties, crop residues and bacterial and fungal communities by cover crop–maize systems

RDA was performed to explore the impact of the two cover crop species and the application of N at different times in a maize rotation agricultural system on microbial community composition (amplicon sequences), soil properties and plant residues at the maize harvest stage. The sum of the first and second axes explained 38.3% of the total variation of environmental and biological factors (Fig. 2). According to RDA followed by Monte Carlo permutation, N in the plant ($p < 0.001$), the C:N ratio ($p < 0.037$) in the cover crops, and total C in the soil ($p < 0.031$) were significantly affected by the treatments.

For both cover crops, total C was higher when N was applied on live palisade grass or ruzigrass (+CC) than in +ST and +MA (Table 2). All N

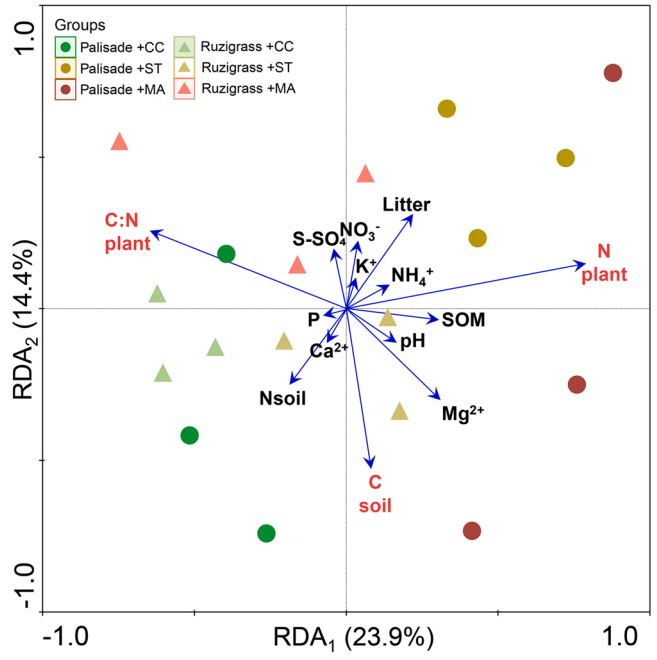

Fig. 2. Redundancy analysis (RDA) based on microbial communities, plant parameters, and soil environment parameters in palisade grass– and ruzigrass–maize systems receiving N on live cover crops (+CC), on cover crop straw (+ST) or in the maize growth stage (+MA). Arrows indicate correlations between factors; correlations in red are significant ($p \leq 0.05$) by the Monte Carlo permutation test (999 permutations).

Table 2
Soil, plant (straw of cover crops), soil genes (bacteria and fungi) and maize grain yield at the end of growing season in cover crop-maize rotation affected by cover crop species and N disturbances. Average data from 3 growing seasons.

	Palisade grass			Ruzigrass		
Variables	+CC†	+ST	+MA	+CC	+ST	+MA
Soil						
pH ($CaCl_2$)	5.09 aA§	4.31 aA	4.61 aA	4.16 aA	4.23 aA	4.62 aA
$P_{(resin)}$ (mg dm^{-3})	27 aA	17 bA	22 aA	24 aA	20 aA	14 bB
K^+ ($mmol_c$ dm^{-3})	0.7 aA	1.8 aA	2.0 aA	1.6 aA	1.7 aA	1.3 aA
Ca^{2+} ($mmol_c$ dm^{-3})	30 aA	12 aA	19 aA	22 aA	16 aA	29 aA
Mg^{2+} ($mmol_c$ dm^{-3})	14 aA	11 aA	11 aA	10 aA	6 aB	12 aA
S-SO_4^{2-} (mg kg^{-1})	1.5 aA	1.8 aA	1.8 aA	1.8 aA	2.0 aA	1.5 aA
TOC (g kg^{-1})	25.7 aA	24.0 bA	23.2 bA	24.9 aA	24.1 abA	23.2 bA
Total-N (g kg^{-1})	2.08 aA	1.92 aA	1.81 aA	1.95 aA	2.01 aA	1.79 aA
NH_4^+ (mg kg^{-1})	11.9 aA	12.5 aA	11.8 aA	7.9 aB	11.5 aA	11.1 aA
NO_3^- (mg kg^{-1})	0.81 aA	1.34 A	0.92 aA	0.09 bB	0.51 aB	0.36 abA
Plant straw						
Litter (kg ha^{-1})	3.84 aA	3.79 abA	3.74 bA	3.67 abB	3.61 bB	3.72 aA
N content	14.6 bA	16.3 aA	14.3 bA	12.3 aB	11.6 bB	11.3 bB
C:N ratio	23 aB	29 aB	29 aB	29 aA	35 aA	41 aA
Soil genes						
16 S rRNA bacteria (log)	7.7 aA	7.9 aA	7.6 bB	7.1 bB	7.7 bB	8.1 aA
18 S rRNA fungi (log)	7.1 aA	7.0 aA	6.8 bA	6.6 aB	6.2 bB	6.7 aB
Maize						
Grain yield (Mg ha^{-1})	13.2 aA	13.2 aA	13.6 aA	9.2 bB	11.1 aA	11.9 aB

† +CC: N applied on live cover crops, +ST: N applied on cover crop straw, +MA: N applied at maize growth stage V4; §Lowercase letters mean significant differences between N application (+CC, +ST, and +MA), and uppercase letters mean significant differences between cover crops (Palisade grass and Ruzigrass) in row by Tukey's test ($p \leq 0.05$).

applications on palisade grass positively influenced soil P, NH_4^+, and NO_3^- contents, cover crop litter, total abundances (qPCR) of bacteria and fungi, and maize productivity. Ruzigrass promoted decreases in N content in +ST and +MA, in bacterial and fungal populations in +ST, and maize productivity in +CC and +MA. The C:N ratio was higher when the cover crop was ruzigrass compared with palisade grass, regardless of N application.

We evaluated which microbial taxa were responsible for the differences in the effects of N application timing between palisade grass and ruzigrass based on regression coefficients (Figs. 3 and 4). In total, 33 amplicon sequence variants (ASVs) in the bacterial community and 54 ASVs in the fungal community were highly responsive to the treatments. Most of the bacterial ASVs were significantly positively influenced by +ST on palisade grass (*Lapillicoccus*, *Singulisphaera*, *Ktedonobacteria*, *Clostridia*, *Ktedonobacterales*, *Solirubrobacterales*, *Acidothermus*, *Xanthobacteraceae*, and *Chloroflexi*) and ruzigrass (*Verrucomicrobia*, *Betaproteobacteria*, *Chloroflexi*, *Chthoniobacterales*, *Nitrospirales*, *Acidobacteria*, *Holophagae*, *Anaerolineaceae*, and *Roseiflexus*) (Fig. 3). However, none of these ASVs were affected by palisade +CC. Interestingly, palisade grass +MA promoted a negative response of 12 bacterial ASVs, including *Nitrospirales*. In the fungal community, N management practices benefited 8, 7, and 12 ASVs in palisade grass +CC, +ST, and +MA,

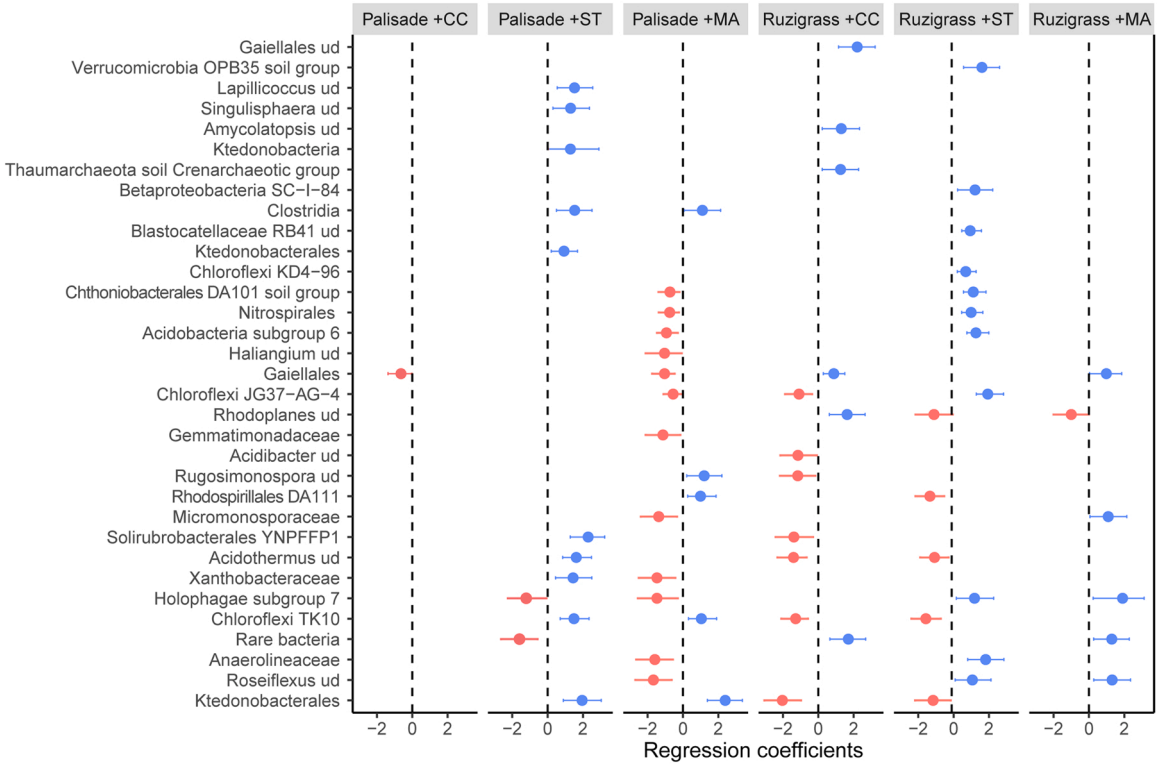

Fig. 3. Regression coefficients showing differential abundances in the soil bacterial community among palisade grass– and ruzigrass–maize systems receiving N on live cover crops (+CC), on cover crop straw (+ST) or in the maize growth stage (+MA). The black dashed lines correspond to a regression coefficient of 0. Red symbols indicate values less than zero and a decrease in the bacterial species. Blue symbols indicate values greater than zero and an increase in the bacterial species. The whiskers indicate 95% confidence intervals. Ud means unidentified.

respectively, and 12, 11, and 9 ASVs in ruzigrass +CC, +ST, +MA (Fig. 4). Palisade grass +CC and +MA positively influenced *Trichoderma* and *Ascomycota*, whereas negative effects were observed for *Mortierella* in ruzigrass +MA, *Fusarium* undefined (ud) in palisade grass +CC, *F. solani* in palisade grass +ST and +MA, and *Chaetomium* in palisade grass +ST and +MA and ruzigrass +CC.

3.2. Soil microbial community structure in the cover crop–maize systems

The impacts of the two cover crop species (palisade– and ruzigrass–maize systems) and applying N at different times on soil bacterial and fungal diversities were evaluated (Figs. 5 and 6). The bacterial Chao and Shannon indices of the systems with palisade grass were similar regardless of the time of N application (Fig. 5 A and 5B). By contrast, for the systems with ruzigrass, these indices were higher for +ST than for +CC. For both cover crops, the indices were similar between +CC and +MA (Fig. 5 A and 5B). For fungi, the Chao1 index was lowest for palisade grass +MA and highest for ruzigrass +ST (Fig. 6 A). The fungal Shannon indices of the treatments were similar with the exception of ruzigrass +ST (Fig. 6B).

To examine the lasting effect of N applied at different time points in the two cover crop–maize systems on the soil bacterial and fungal communities at the maize harvest stage, we determined the beta diversity of species composition based on Bray–Curtis distances and nonmetric multidimensional scaling (NMDS) (Fig. 5 C, bacteria/archaea; Fig. 6 C, fungi). In the bacteria/archaea NMDS plot, all palisade grass treatments (+CC, +ST and +MA) were similar along the second principal coordinate axis. However, an effect of fertilizer application timing was observed in the ruzigrass treatments, as the bacterial community structures in ruzigrass +CC and +MA were distinct from that in ruzigrass +ST (Fig. 5 A). Additionally, the bacterial community structure in palisade +CC was similar to those in all other treatments except ruzigrass +MA. The bacterial beta diversity in ruzigrass +MA was similar only to that in ruzigrass +CC. For fungi, the lasting effects of N applied at different stages of palisade grass development or in the maize V_4 stage resulted in similar fungal communities in the soil at the maize harvest stage (Fig. 6 C), whereas the fungal community in ruzigrass +ST differed from those in ruzigrass +CC and +MA.

3.3. Covariation of soil bacterial and fungal communities in cover crop–maize systems under the lasting effect of N application at different times

We assessed the covariation of the bacterial and fungal community structures via COIA (Fig. 7). The different treatments in the palisade grass– and ruzigrass–maize rotations explained 85% of the covariation of the soil microbial communities (Fig. 7). The covariation of the bacterial and fungal communities differed significantly depending on the cover crop or N application time ($P = 0.001$). The shorter arrows for palisade grass +CC and ruzigrass +CC indicated stronger relationships between the soil bacterial and fungal communities compared with palisade grass +ST and ruzigrass +ST. The projections of the arrows for ruzigrass +ST and palisade grass +ST were opposite in direction, indicating that N application changed the community interactions within the treatments; however, the arrow lengths were similar, indicating similar interdependences of the bacterial and fungal communities. Similarly, the arrows for treatments with the same timing of N application but different cover crops were the same length but in different directions.

4. Discussion

In this study, N applications on two species of cover crops (palisade and ruzigrass) had lasting effects on the soil bacterial and fungal

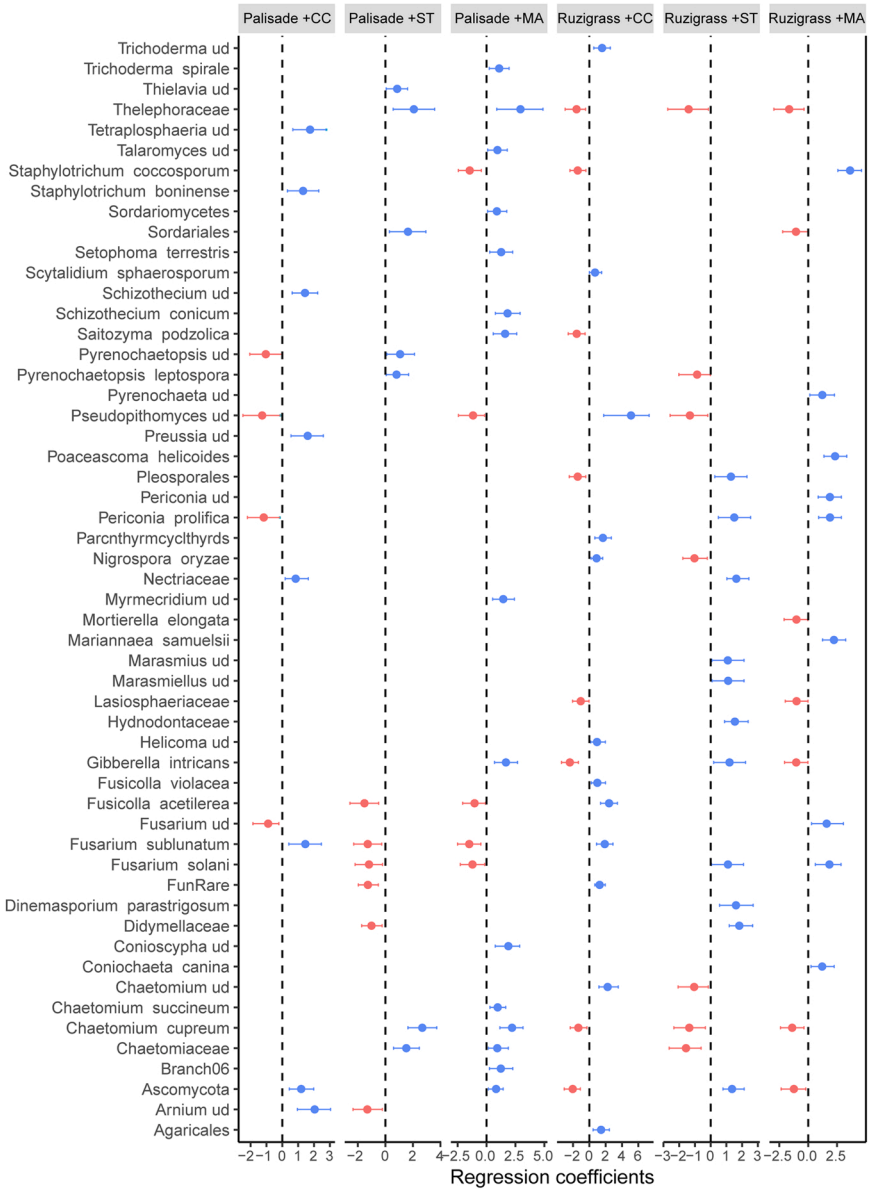

Fig. 4. Regression coefficients showing differential abundances in the soil fungal community among palisade grass– and ruzigrass–maize systems receiving N on live cover crops (+CC), on cover crop straw (+ST) or in the maize growth stage (+MA). The black dashed lines correspond to a regression coefficient of 0. Red symbols indicate values less than zero and a decrease in the abundance of the fungal species. Blue symbols indicate values greater than zero and an increase in the abundance of the fungal species. The whiskers indicate 95% confidence intervals. Ud means unidentified.

communities after the growing season in a cover crop–maize rotation system under no-till. Studies have shown that the use of palisade grass as a cover crop benefits the agricultural system by improving soil aggregation and fertility and nutrient cycling (Boddey et al., 2004; Ferrari Neto et al., 2021; Nascente et al., 2015). Compared with ruzigrass, palisade grass is more resistant to drought due to its deep root system and produces greater amounts of straw (Rosolem et al., 2017; Takamori et al., 2017). The roots of palisade grass absorb large quantities of nutrients from deep soil layers and release these nutrients via straw decomposition to enhance the yield of the subsequent crop (maize) (Table 2) (Momesso et al., 2022b, 2019; Oliveira et al., 2018). In this study, we determined whether the advantages of these two species of cover crop cultivated in rotation with maize also extend to the diversity and composition of the soil bacterial and fungal communities.

The lasting effects of N applied at different stages of cover crop development or the maize V_4 stage varied. In soils previously cultivated with palisade grass, the microbial community was similar regardless of the timing of N application, consistent with the positive impact of palisade grass on subsequent maize productivity (Table 2). In soils previously cultivated with ruzigrass, the changes in the bacterial and fungal populations depended on when N was added to the system, which also affected maize productivity in this crop rotation system. Cover crop straw production directly benefited the levels of soil nutrients, mainly C, in the agricultural system (Fig. 2). The C:N ratio of cover crop straw determined decomposition processes by microorganisms. The lower C:N ratio of palisade grass (on average 27) compared with ruzigrass (Tables 1 and 2) ensured adequate amount of C and N for plants and microbes and reduced competition among microorganisms. Applying N fertilizer to live ruzigrass or maize decreased the C:N ratio of ruzigrass straw, resulting in residual effects on the soil microbial communities. N and C levels in agroecosystems impact the availability of substrates and nutrients for microbial growth and functioning, as N and C are required for the synthesis of amino acids, proteins, and nucleic acids and as a source of energy for microbes, respectively (Holland and Weitz, 2003). Additionally, the cover crop decomposition rate, N availability (from soil and fertilizer), maize growth rate and maize yield directly impact soil microbial community structure in agroecosystems and vary depending on the cover crop species and N input (Costa et al., 2016; Mbuthia et al., 2015; Nevins et al., 2018). The high temperatures and moisture found in tropical regions accelerate straw decomposition compared with temperate regions. Straw deposited on the soil surface protects against moisture loss depending on the cultivated species and

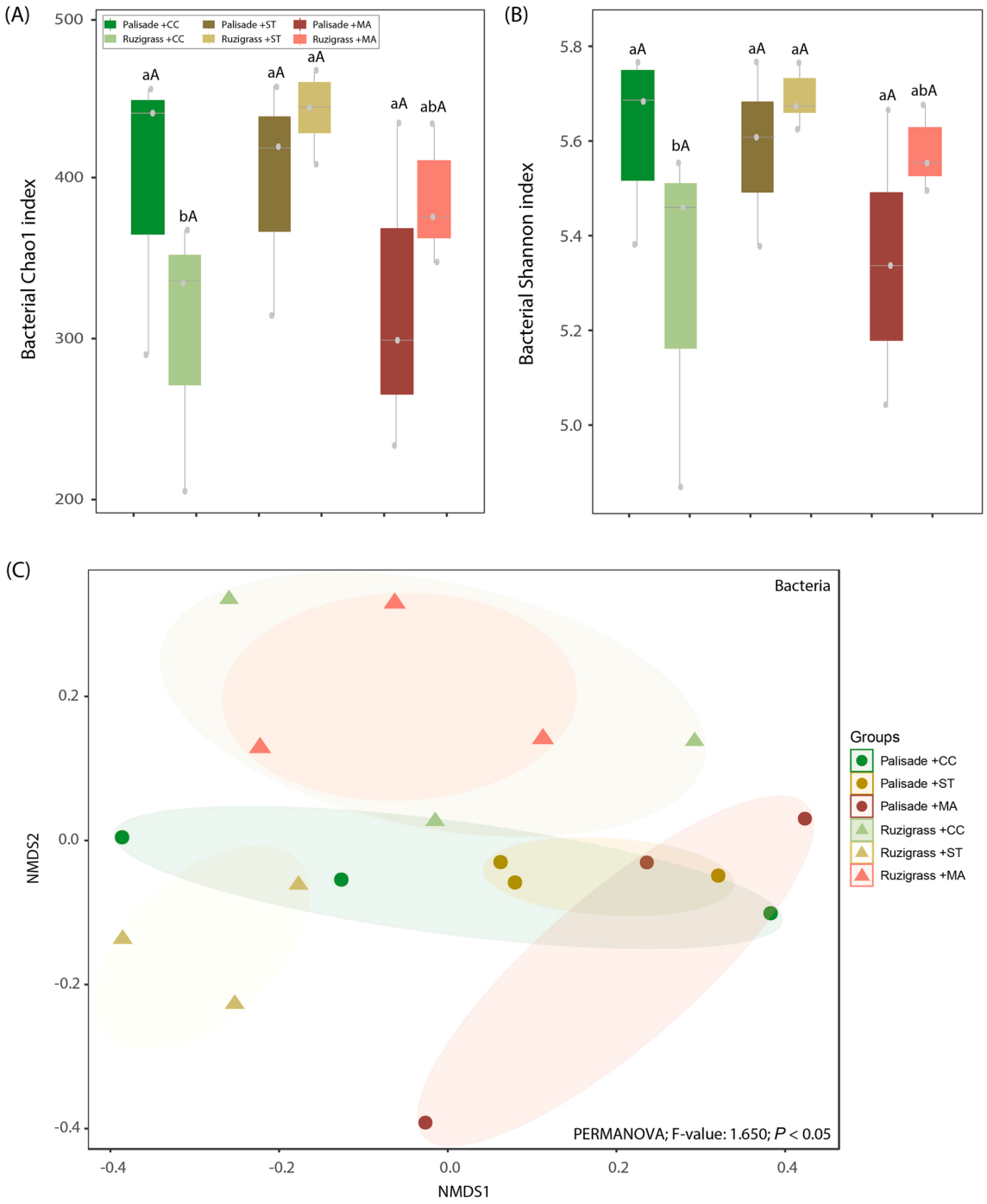

Fig. 5. Alpha diversity (A,B) and beta diversity (C) of the bacterial community in soil cultivated with the palisade grass– or ruzigrass–maize system and receiving N on the live cover crop (+CC), on the cover crop straw (+ST) or in the maize growth stage (+MA). Different lowercase letters indicate significant differences between N application times (+CC, +ST, and +MA), and different uppercase letters indicate significant differences between cover crops (palisade grass and ruzigrass) by two-way ANOVA with the LSD test at $p < 0.05$.

provides nutrients to the subsequent crop (Crusciol et al., 2015; Oliveira et al., 2018; Rosolem et al., 2017). Maize growth and grain production absorb nutrients from the soil solution and compete with microbial communities (Bossolani et al., 2021; Momesso et al., 2022b, 2021), as observed in the reduction of bacterial and fungal abundances in ruzigrass (receiving N on live cover crop and on cover crop straw) (Table 2). Thus, the appropriate choice of cover crop is crucial for favoring soil microbial communities while enhancing crop productivity.

In general, the analysis of the bacterial and fungal communities combined (Fig. 2) and the effects of the treatments on soil, plant and maize productivity (Table 2) showed that palisade grass mainly increased total bacteria, total C content in the soil, soil nutrients and the soil C:N ratio. Plant–soil interactions alter soil chemical properties via root system growth, which can modulate N, C and nutrient availability and activate or suppress the growth of specific microorganisms (Pascale et al., 2020). These changes lead to differences in bacterial and fungal communities since abiotic and biotic factors usually drive taxa differences and determine environmental attributes, which have selection effects on soil bacteria and fungi (Carroll et al., 2011). Palisade grass benefits soil, as our findings showed negative relationships between

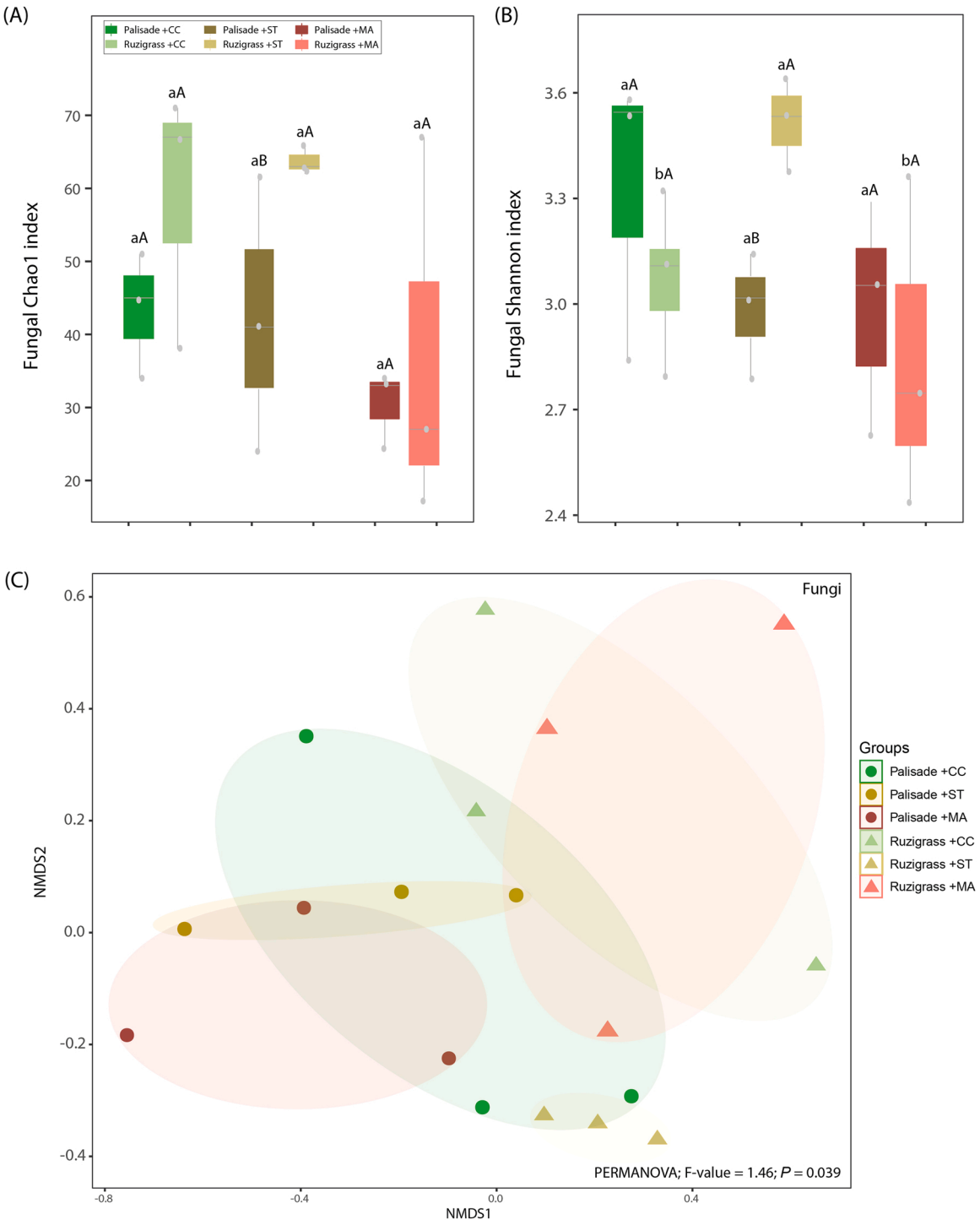

Fig. 6. Alpha diversity (A,B) and beta diversity (C) of the fungal community in soil cultivated with the palisade grass– or ruzigrass–maize system and receiving N on the live cover crop (+CC), on the cover crop straw (+ST) or in the maize growth stage (+MA). Different lowercase letters indicate significant differences between N application timing (+CC, +ST, and +MA), and different uppercase letters indicate significant differences between cover crops (palisade grass and ruzigrass) by two-way ANOVA with the LSD test at $p < 0.05$.

palisade grass and bacteria from the *Nitrospirales* family, which includes chemolithoautotrophic aerobic nitrite-oxidizing bacteria (*Nitrospira*), chemolithoautotrophic aerobic and acidophilic ferrous iron oxidizers (*Leptospirillum*), and anaerobic, thermophilic, chemo-organoheterotrophic or hydrogenotrophic sulfate reducers (*Thermodesulfovibrio*) (Daims, 2014). Additionally, applying N to the live cover crop (+CC) or during maize growth (+MA) increased the abundances of *Trichoderma* and *Ascomycota* in systems with palisade grass. *Trichoderma* is a soil-dwelling fungus that is present in both temperate and tropical regions. It colonizes decaying wood, mushrooms and wood ears and parasitizes other fungi that threaten plant species, such as *Fusarium* spp. *Ascomycota* are excellent decomposers of organic matter and directly improve soil fertility.

Cover crop cultivation reduced the abundances of other bacteria and fungi depending on N application timing. Palisade grass cultivation prior to maize decreased the potential pathogen *Fusarium* (*Fusarium* ud in

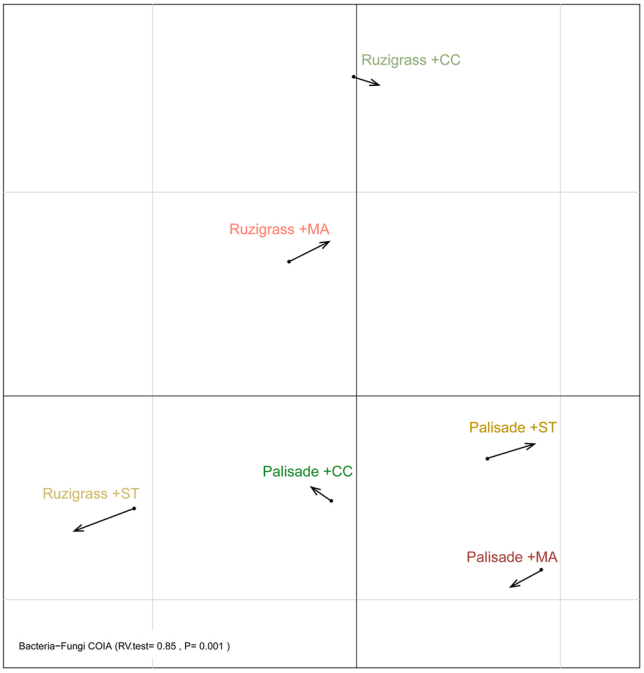

Fig. 7. Coinertia (COIA) analysis of the principal coordinate analysis (PCoA) of the bacterial and fungal microbial communities. The arrows represent the covariation of the bacterial and fungal communities within the treatments in soil cultivated with palisade grass– or ruzigrass–maize systems receiving N on the live cover crop (+CC), on the cover crop straw (+ST) or in the maize growth stage (+MA).

+CC; *F. solani* in +ST and +MA). In addition, *Chaetomium* was negatively impacted by ruzigrass +CC but positively impacted by palisade grass +ST and +MA; this fungus colonizes several substrates and has potential as a biological control agent. In addition, ruzigrass as a cover crop decreased nutrients and total bacteria, and the timing of N input influenced maize–microbe competition (Table 2). Previous ruzigrass cultivation in rotation with maize likely resulted in strong competition between the maize cash crop and microorganisms for nutrients in the soil. Ruzigrass straw provides less nutrients to the soil than palisade grass, and thus most of the resources were taken up by the maize cash crop, as evidenced by the increases in maize productivity when N was applied to ruzigrass straw or maize in the V_4 growth stage compared with application to live ruzigrass.

Under field conditions, the cover crop species were grown in rotation with maize in the same location and under the same conditions for three years. We observed differences in the richness and diversity of the microbial communities. Alpha diversity was similar among the treatments that included palisade grass, whereas in the treatments that included ruzigrass, bacterial and fungal diversities were lower when N was applied to straw or maize in the V_4 stage (Figs. 5 and 6). The cultivation of cover crops alters the microbial diversity of soil communities, resulting in an extended impact of the cover crop on soil microbiota composition after crop rotation. Similar clusters of community diversity were observed in the palisade grass–maize rotation treatments. This similarity was driven by the high biomass production of palisade grass, which favors soil quality, nutrient cycling, and crop yields under no-till (Table 2 and Fig. 2) (Kim et al., 2020; Pariz et al., 2017; Tiecher et al., 2017). The lasting effect of N application on live ruzigrass decreased soil bacterial diversity, suggesting selective pressure imposed by N; the opposite effect was observed for fungal diversity. N applied on live ruzigrass decreased bacterial communities and the productivity of the subsequent crop, i.e., maize, compared with palisade grass (Momesso et al., 2020, 2019). Among the most affected bacteria were members of the phyla *Chloroflexi*, which play roles in N fixation and nitrite oxidation, and *Actinobacteria*, which are related to organic matter decomposition (Fig. 5) (Jiménez-Bueno et al., 2016; Trivedi et al., 2016). Thus, losses in processes related to N cycling and soil organic matter decomposition may be responsible for the negative impacts of ruzigrass cultivation.

Consistent with the patterns of alpha diversity and maize productivity, bacterial and fungal beta diversity were similar among the palisade grass treatments. Applying N to ruzigrass straw resulted in completely different bacterial and fungal communities compared with all other treatments. However, when N was applied at the ruzigrass straw stage, the microbial community diversity was similar to those in the systems with palisade grass. N fertilizer application only altered the microbial communities when ruzigrass was the cover crop. This was most evident when ammonium sulfate was applied to ruzigrass straw (Fig. 1), i.e., when N and S were supplied simultaneously when there were no living plants (ruzigrass or maize) competing with the soil microbiota. Thus, at this time point, these two nutrients may increase the soil microbial diversity. Applying N fertilizer on ruzigrass straw just before maize seeding or on maize in succession to ruzigrass could be management alternatives for enhancing maize productivity. Our study clearly highlights the interaction between the agronomic and microbiological aspects of the food production system. However, applying N to maize in succession to ruzigrass is not the best alternative to enhance soil microbial community diversity. Thus, considering the entire agricultural system, applying N to ruzigrass straw is the best option when ruzigrass is used as an alternative to palisade grass, but palisade grass is still more efficient than ruzigrass as a cover crop.

Evaluating the covariation between bacterial and fungal communities is crucial for understanding how nutrient addition influence the codependence between these two soil communities. Previous studies showed that in a N-rich soil bacterial and fungal communities covaried strongly (Schlemper et al., 2017). Similarly, N addition in soil of sugarcane plantlets increased the covariance between root endophytes and the rhizosphere microbiome (Leite et al., 2021). This phenomenon is also observed when a different nutrient is used. For example, when soil P-availability increases, the covariance between bacterial and fungal communities of Eucalyptus plantlets also increased from 54% to 61% (Bulgarelli et al., 2022). In the current study, we provided evidence that the covariance between bacteria and fungi gets stronger when N is applied on the live cover crop, which happened regardless of the grass species. Consequently, our findings confirmed that the management of N addition in the bacterial and fungal codependence.

Our fourth hypothesis, that the bacterial and fungal communities would be strongly linked to each other when N was applied on the live cover crop, was supported. There was an indirect effect of nutrient addition due to increased cover crop residues on community-level composition links. The C:N ratio is an important driver of soil microbial community composition and microbial interactions. The C:N ratio of crop residues is the result of N uptake by live cover crops and may narrow the relationships between bacteria and fungi involved in soil processes. Bacteria and fungi are primarily responsible for straw decomposition, and thus the bacterial and fungal communities are indirectly driven by plant biomass/litter (Cassman et al., 2002; Millard and Singh, 2010). Additionally, the covariation of the bacterial and fungal communities differed between palisade grass and ruzigrass with the same N application timing, which demonstrated that the covariation depends on the interaction of N application timing with the cultivated cover crop. However, additional studies are needed of the microbial processes that are the main drivers of these links between microbial communities and whether they are positively or negatively related to plant residue decomposition (C:N ratio of cover crops) or N and C cycles (Fig. 2 and Table 2).

5. Conclusions

Studies of the entire system of soil–plant–microbial interactions are

necessary to develop smarter agricultural management practices. Based on the results, palisade grass is recommended as a suitable alternative cover crop prior to maize cultivation, as it increases soil microbial diversity at maize harvest regardless of N application timing while enhancing maize productivity, soil chemical properties and nutrient cycling. When ruzigrass is used as the cover crop, the lasting effect of N application on ruzigrass leaves or at the maize V4 stage decreases microbial diversity, whereas applying N to ruzigrass straw results in high soil microbial diversity similar to that obtained under palisade grass cultivation. Therefore, the success of the entire system is the result of interactions among soil microorganisms, soil chemical properties, and plant litter from cover crops. Examining factors such as microbial diversity, soil chemical or maize productivity separately and in isolation can lead to a misunderstanding of the entire system response and, consequently, incorrect recommendations for management practices.

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.

Data availability

The raw sequences of 16S rRNA gene and ITS were submitted to the European Nucleotide Archive (ENA) under study accession number PRJEB41425.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.still.2022.105507.

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