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Optimizing cover crop and fertilizer timing for high maize yield and nitrogen cycle control

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ABSTRACT

Residues of cover crop grasses release nitrogen (N) to subsequent crops, which can contribute to sustainable agricultural management and prevent increases in N-loss-related microorganisms. Moreover, applying N fertilizer to cover crops can enhance the N-use efficiency and yields of subsequent cash crops and tighten the N cycle in the soil. However, the long-term effects of N fertilization of cover crops on soil microbiota and the N cycle in tropical grass-crop no-till systems are unknown. The aim of this study was to evaluate the long-term effects of the timing of N fertilization of cover crops or maize on crop yields, total microbial abundances and N-cycle gene abundances at the time of maize harvest. We carried out a field experiment with two cover crops (palisade grass (*Urochloa brizantha*) and ruzigrass (*U. ruziziensis*) fertilized with 120 kg N ha^{-1} (ammonium sulfate) at one of three times: (i) broadcast over the green cover crops at 35 days before maize seeding (35 DBS), (ii) broadcast over the cover crop straw residues at 1 day before maize seeding (1 DBS), and (iii) as side-dressing at the maize V_4 growth stage according to the conventional method (band-applied 0.05 m from the maize row). A control treatment without N application was also carried out for both cover crop species. Except for the control, 40 kg N ha^{-1} as ammonium sulfate was subsurface band-applied in all treatments 0.05–0.10 m from the maize row at maize seeding, corresponding to 160 kg N ha^{-1}. The total bacterial, archaeal and fungal abundances and abundances of microbial genes encoding enzymes of the N cycle in the soil were quantified by real-time PCR at the maize harvest stage. Overall, maize yield increased significantly in all N fertilizer applications (average 13 Mg ha^{-1}) compared with the control (6 Mg ha^{-1}) over three growing seasons, with maize following palisade grass having the highest yield. The abundances of archaea and fungi in soil were highest under palisade grass that received N at 35 DBS, with values of 4.6×10^6 and 1.7×10^7 gene copies/g of dry soil, respectively. Both cover crop straw production and N release to the soil were positively correlated with the total microbe densities. When ruzigrass was the cover crop, low N enhanced *nifH* abundance. Archaeal *amoA* abundance was positively correlated with cover crop biomass and N release regardless of the N treatment and was highest under palisade grass. Bacterial *amoA*, *nirK*, and *nirS* abundances were highest in soil under ruzigrass and were not linked to cover crop biomass mineralization. We conclude that N fertilizer should be applied using the currently recommended method (40 and 120 kg N ha^{-1} at seeding and side-dressed in maize, respectively) following palisade grass to achieve high maize yield while controlling the level of N loss from tropical soil via nitrification and denitrification.

1. Introduction

Cover cropping in no-tillage (NT) systems promote sustainable management and high food production simultaneously. Cover cropping improves soil conservation by reducing erosion and increases soil quality by enhancing the physical, chemical, and biological properties of

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the soil (Derpsch et al., 2014; Smith et al., 2015). Decomposition of the crop residues remaining on the soil surface releases nutrients back to the soil for uptake by the subsequent cash crop during yearlong cultivation in tropical regions (de Freitas and Landers, 2014; Mueller et al., 2003). As a result, cover cropping may reduce the need for fertilizer input, especially nitrogen (N). However, proper management of the fertilization of the cover crop and/or subsequent cash crop requires a comprehensive understanding of the cover crop species, biomass yield, root system architecture, soil properties and microbial communities (Dignam et al., 2019; Heijboer et al., 2016; Lal et al., 2007). In particular, the selection of an appropriate cover crop species depends on the environment and subsequent cash crop. In tropical agricultural systems, forage grasses belonging to the genus *Urochloa* are a suitable options for cultivation before or intercropping with maize (Bossolani et al., 2020; Crusciol et al., 2015; Momesso et al., 2019) and in integrated crop-livestock systems (Crusciol et al., 2020; Teutscherová et al., 2021; Vázquez et al., 2020) due to their robust stoloniferous growth, deep rooting ability and high N cycling (Williams and Baruch, 2000), even under conditions of drought and low soil fertility (Baruch, 1994; Fisher et al., 1995; Rao, 1998).

Although the high biomass and N content of cover crop residues are returned to the soil, additional applications of N fertilizer are still necessary to supply the subsequent maize demand and achieve high yields in tropical cropping systems (Marcillo and Miguez, 2017; Momesso et al., 2019; Pacheco et al., 2017). Applying fertilizer to the cover crop to ensure N release over the growing season of the subsequent cash crop may improve the effectiveness of fertilization in tropical systems (Momesso et al., 2020; Pöttker and Wiethölter, 2004). For maize, the current recommendation for N fertilization is to divide the application into two stages: at maize seeding and as side-dressing when the maize plants have 5 to 7 leaves (Cantarella et al., 1997; Ciampitti and Vyn, 2012). Early N application on live cover crops or cover crop residues might serve as a replacement for the second N application for maize, since cover crops have high N uptake via the root system and gradually release nutrients through straw decomposition over the maize growing season. Moreover, early N application could increase the flexibility of N application timing for farmers, since side-dressing must be applied during a certain development stage of the cash crop and typical side-dress equipment for N application often causes maize leaf damage (Schepers and Raun, 2008).

The release of biomass and N content by cover crops and N fertilization can affect not only maize yield but also the soil microbial community and microbial activities associated with N cycling. Nitrogen inputs in NT systems increase C and N stocks in the soil, which are key to achieving long-term sustainability and productivity (Babujia et al., 2010). N release by crop residues leads to an increase in microbial biomass in tropical agricultural systems under conservation management (Babujia et al., 2010; Kaschuk et al., 2010). The organic compounds released by plants vary among species and differ in their impact on the responses of soil properties and the soil microbial community to plant decomposition (Bani et al., 2018; King and Hofmockel, 2017). The benefits of sustainable agricultural practices, including large amounts of biomass from cover crops, soil fertility maintenance and cover crop/rotation, strongly favor soil microbial functioning (Lienhard et al., 2014; Zechmeister-Boltenstern et al., 2015). However, the long-term effects of crop residues under N fertilization in tropical systems on the bacterial, archaeal, and fungal populations remain unclear.

Excessive N fertilizer application is a common agricultural practice and alters soil microbiota composition and function (Cassman et al., 2016; Pan et al., 2014), in addition to negatively affecting the environment (Pitombo et al., 2016; Soares et al., 2016). Cover crop cultivation is a strategy to minimize environmental pollution from fertilizers and increase N use efficiency by plants (Kaye and Quemada, 2017; Thapa et al., 2018). One potential consequence of long-term maintenance of cover crop residues and N management over the growing season is tightening of the N cycle (Karwat et al., 2017; Teutscherova et al., 2019). Nitrogen uptake by the cover crop and residues covering the soil can reduce N losses to the soil, which in turn can decrease contractions of NH_4^+ and NO_3^- in the soil, as well as processes of nitrification and denitrification (Kuypers et al., 2018). Given the link of N losses via NO_3^- leaching with N_2O emissions to the atmosphere, limiting the impact of microorganisms involved in specific parts of the N cycle is an important goal in sustainable food production (Kuypers et al., 2018; Lourenço et al., 2018a; Soares et al., 2016). However, the effects of N fertilization on *Urochloa* cover crops in maize crop cultivation systems on soil microbial N-cycle functions are not known.

To help meet the challenge of matching sustainable yields of agricultural systems with beneficial impacts on the soil microbiome, the current study examined combinations of N management with cover crops as potential strategies to bridge the gap between enhancing crop yields and benefiting soil microbes. The following questions are addressed: (i) what is the best N management strategy for forage grass-maize systems to improve crop yields, and (ii) what timing of N application has the smallest impact on microbial populations and the N cycle? A field experiment was conducted to evaluate the long-term effects of different timings of N application on cover crops on maize N uptake, maize yield, soil total bacterial, archaeal and fungal abundances, and N-cycle genes at the harvest stage of the maize cash crop. We hypothesized that compared with ruzigrass, palisade grass as cover crop improves maize N uptake and yield when N is applied either to green palisade grass or its straw residue and that (ii) applying N fertilizer to grass cover crops increases total bacterial, archaeal and fungal abundances and (iii) decreases the abundances of genes related to N fixation, nitrification and denitrification within the soil microbial community at the maize harvest stage.

2. Material and methods

2.1. Site description, experimental design, crop management and sampling

The field experimental area is in Botucatu, São Paulo, Brazil (48° 26′ W, 22° 51′ S, 740 m above sea level). The climate is classified as Cwa according to the Köppen classification, i.e., dry winters and warm, wet summers. The mean annual temperature is 20.7 °C, and the mean annual precipitation is 1,358 mm. Seasonal precipitation and temperature data during the experiment are shown in Supplementary Fig. S1. The soil is clay (630, 90, and 280 g kg^{-1} of clay, silt, and sand, respectively) and is classified as a kaolinitic, thermic Typic Haplorthox (Soil Survey Staff, 2014). The soil properties prior to the start of the experiment were pH 4.8 ($CaCl_2$); 32 g dm^{-3} SOM; 19 mg dm^{-3}P (resin); 4.9, 34, 18, and 44 $mmol_c$ dm^{-3} exchangeable K, Ca, Mg, and total acidity at pH 7.0 (H + Al), respectively; and base saturation of 56%. The experimental area has been cultivated under NT since 1999.

A cover crop species × N management experiment was carried out during the 2015–2016, 2016–2017, and 2017–2018 growing seasons using a randomized block design with four replicates per treatment. Each year, the cover crops palisade grass (*Urochloa brizantha* cv. Marandu) and ruzigrass (*U. ruziziensis* cv. Comum) were sown at a density of 10 kg seed ha^{-1} (34% viable seed) with no fertilizer addition in early April and chemically desiccated in late October (Fig. 1). For both cover crops, straw residues were obtained by mechanically mowing 0.30 m above the soil level to stimulate growth and N uptake 28 days prior to cover crop desiccation. Chemical desiccation was performed by spraying glyphosate at 1.56 kg ha^{-1} (a.i.) 30 days before maize seeding (Fig. 1). In early December, maize (hybrid P3456, Pioneer) was sown at a depth of 0.03 mm using an NT drill at a density of 65,000 seeds ha^{-1}. The plots were composed of 10 rows of maize with a length of 8 m and a spacing of 0.45 m. Except for the control (no N fertilizer application), all N treatments received 40 kg N ha^{-1} (ammonium sulfate) surface-banded next to the row at maize seeding (Fig. 1). The conventional method of N fertilization was side-dress application of 120 kg N ha^{-1} in single-side surface banding (0.05 m away from the maize row) when the maize

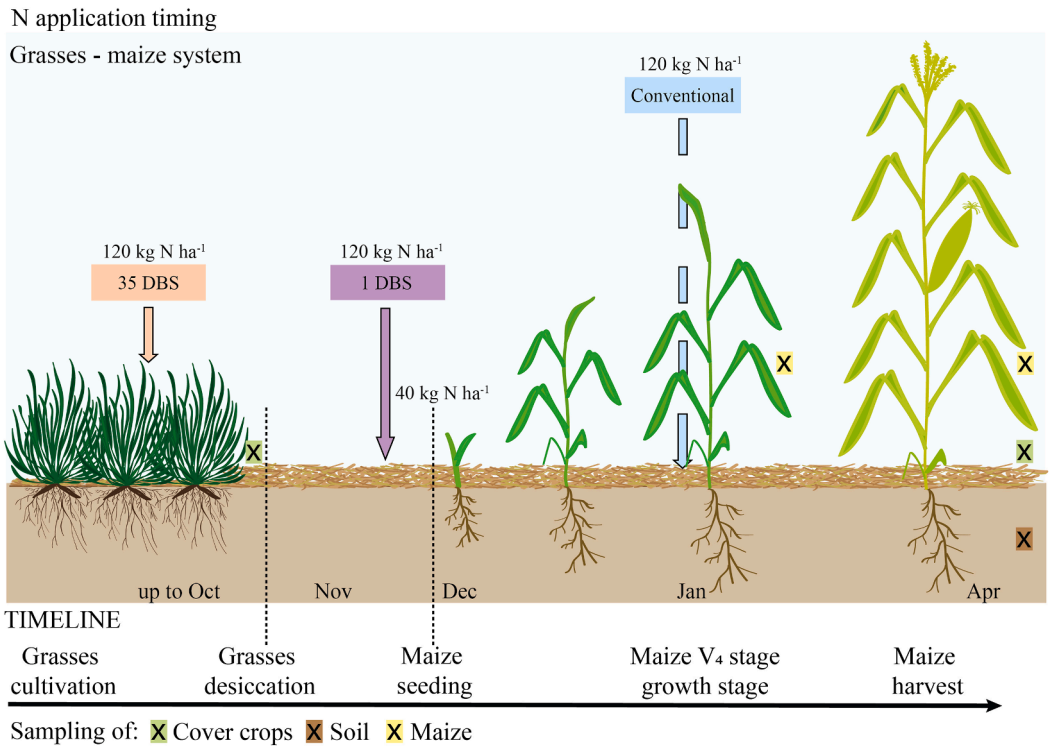

Fig. 1. Schematic illustration of N application on palisade grass (*Urochloa brizantha*) and ruzigrass (*Urochloa ruziziensis*) cover crops, and sampling time. 35 DBS = N applied on green cover crops 35 days before seeding (DBS); 1 DBS = N applied on straw cover crops 1 day before seeding (1 DBS); V_4 = maize with four-leaves.

was at the V_4 growth stage. The basic fertilization in the seeding furrows consisted of 90 kg P_2O_5 ha^{-1} as triple superphosphate and 45 kg K_2O ha^{-1} as potassium chloride.

The treatments consisted of two species of grass cover crops, palisade grass and ruzigrass, and four N treatments: (i) control (no N), (ii) N application on green cover crop 35 days before maize seeding (35 DBS), (iii) N application on cover crop straw residue 1 day before maize seeding (1 DBS), and (iv) conventional side-dress application of N in the V_4 maize growth stage (four leaves), 30 days after maize seeding (Fig. 1). For treatments with N, 120 kg N ha^{-1} was applied either on green cover crops (35 DBS) or on straw cover crops (1 DBS). Because chemical desiccation of the cover crops was performed 30 DBS, the cover crops in treatment (ii) grew for 5 days after fertilization at 35 DBS. Controls (no N) were carried out for both cover crops to compare the grain yields with those of maize without N fertilizer.

When 50% of the maize was in full flowering stage (silking), the leaf opposite the highest ear was collected from 20 randomly selected plants per plot. The leaf samples were ground to pass through a 0.85-mm mesh stainless steel screen and digested with sulfuric acid. The digestion solution was then used for N determination by semi-micro-Kjeldahl distillation. After the final growing period of 125 days, the maize was mechanically harvested from an area of 10.8 m^2 in each plot, and grain yield was determined and expressed per hectare. Additionally, the dry matter of each cover crop was collected on the day of cover crop termination and at the end of the maize growing season in the three years of the experiment. In each plot, two subsamples were collected from an internal area of 0.25 m^2 and pooled. For dry-weight determination, the samples were oven-dried at 65 °C. The carbon (C) and N concentrations in the cover crop dry matter subsamples were determined using an elemental analyzer (LECO-TruSpec® CHNS) and used to calculate the C:N ratio; the accumulated N was extrapolated to Mg ha^{-1} of dry matter.

Soil samples (0–20 cm top layer) were collected using a probe positioned in the maize row in the three growing seasons. Soil samples were oven-dried at 40 °C and ground in a ball mill for N concentration determination using an elemental analyzer (LECO-TruSpec® CHNS). The soil samples were also used to determine pH by preparing a 1:1.5 soil:water suspension, and the moisture content of the soil was determined by gravimetric analysis after drying the soil at 105 °C for 24 h. Potential C mineralization was determined from two 50-g subsamples of soil in 60-mL glass jars that were moistened to a water-filled pore space of 50% and placed in a 1-L canning jar along with 10 mL of 1 M NaOH to trap CO_2 and a vial of water to maintain humidity (Franzluebbers et al., 1999). The soil samples were incubated at 25 ± 1 °C for 24 days. Alkali traps were replaced every 3 days and removed at 24 days. Soil inorganic N (NH_4^+ and NO_3^- contents) was extracted with 2 M KCl solution (Keeney and Nelson, 1982).

2.2. Soil DNA extraction and quantitative real-time PCR

At maize maturity in the 2015–2016 growing season (March 2016), soil was sampled from each plot for microbiological analyses. Five subsamples (0–20 cm top layer) from a single plot were pooled and then divided into two portions; one portion was used to determine soil moisture gravimetrically by drying the soil at 105 °C for 24 h, and the other portion was stored at −80 °C for molecular analyses. Total soil DNA was extracted from 0.25 g of soil stored at −80 °C using the MoBio PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions. DNA quality and quantity were determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, DE, USA) and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). The total extracted soil DNA was visualized on 1% (w/v) agarose gels.

Real-time PCR (qPCR) was performed on three replicates per sample of each plot to quantify the copy numbers (abundances) of total bacteria and archaea (*16S rRNA*), total fungi (*18S rRNA*) and genes encoding the N fixation-associated enzyme nitrogenase (*nifH*), nitrification-associated archaeal and bacterial ammonia monooxygenases (*amoA* bacteria, AOA; *amoA* archaea AOA), and denitrification-associated nitrite reductases (*nirS* and *nirK*) and nitrous oxide reductase (*nosZ*). qPCR was carried out

in 96-well plates (Bio-Rad) using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Details of the primer sets and the PCR conditions for each gene are provided in Supplementary Table S1. Amplicon sizes were confirmed by separation on agarose gels (1%). Standard curves were constructed using serial 10-fold dilutions of a known amount of plasmid DNA (10^9 to 10^1 gene copies) containing the respective gene fragment. Negative controls were performed with water instead of template DNA. Efficiencies for all assays were between 80 and 101%, with R^2 values between 0.91 and 0.99.

2.3. Statistical analyses

Normal distributions of all data for cover crops (dry matter loss, N release and C:N ratio), maize (shoot dry matter, leaf N concentration, and grain yield) and soil (pH, moisture, total-N content, NH_4^+ content, NO_3^- content and mineralizable C) were confirmed using the Shapiro-Wilk test from the UNIVARIATE procedure in the statistical software R (version 3.5.2) ($W \geq 0.90$). Analysis of variance (ANOVA) was performed using the statistical software R (version 3.5.2). Cover crop and N treatments were considered fixed factors. If the null hypothesis was rejected, a comparison of means was performed with the least significant difference (LSD) test ($P \leq 0.05$).

Data on gene abundances in soil were log-transformed, and the normal distribution of residues and variance stability were confirmed. Calculations were performed with cover crop and N application as fixed factors. The data were submitted to analysis of variance (ANOVA), and means were compared by the LSD test at $P \leq 0.05$. For microbial populations, the total archaea:bacteria, total fungi:archaea, and total archaea:fungi ratios were calculated. The ratios of the *nifH*, AOB, *nirK*, *nirS* and *nosZ* genes to 16S rRNA were calculated by dividing the copy number of each N cycle gene by the copy number of total bacteria. For the AOA gene ratio, the copy number of the AOA gene was divided by the copy number of total archaea. The calculated ratios were graphed in a box-plot in the statistical software R (version 3.5.2). Additionally, we calculated the correlation-based networks and Spearman's correlations among the abundances of each N cycle gene; total bacterial, archaeal and fungal abundances; cover crop biomass, N release and C:N ratio; and soil pH, moisture, C mineralizable, and total-N and inorganic N contents using the statistical software R (version 3.5.2). Principal component analysis (PCA) was carried out to determine the differences between treatments and the correlations between gene abundances and plant and soil factors in cover crop and N management. Permutational multivariate analysis of variance (PERMANOVA) was performed to test whether the treatments harbored significant differences. PCA and PERMANOVA were performed using Past4 (version 4.0) (Hammer et al., 2001).

3. Results

3.1. Cover crop and maize performance

At least part of the dry matter and N lost during this period should be transferred or released to the soil system (Fig. 2). At cover crop termination, the cover crop dry matter yield and N content were highest for both cover crops in the 35 DBS (green cover crop) treatment and were higher for palisade grass (10.2 Mg ha^{-1} and 248 kg N ha^{-1}, respectively) than for ruzigrass (7.7 Mg ha^{-1} and 212 kg N ha^{-1}, respectively). At the end of the maize growing season (harvest stage), the dry matter yield (8.2 Mg ha^{-1}) and N release (58 kg N ha^{-1}) of palisade grass were higher in the 35 DBS treatment (green) than the 1 DBS treatment (straw) (results not shown). By contrast, ruzigrass dry matter yield and N release did not differ between the 35 DBS and 1 DBS treatments.

The interaction of cover crop and N management significantly impacted the shoot dry matter, leaf N concentration, and grain yield of maize in the 2015–2016, 2016–2017, and 2017–2018 growing seasons (Fig. 3). Maize shoot dry matter and leaf N concentration were similar in the treatments in which palisade grass received N at 35 DBS or 1 DBS or no N and in which ruzigrass received N at 1 DBS or no N. In all of these treatments, maize shoot dry matter and leaf N concentration were higher than in the treatment in which ruzigrass received N at 35 DBS.

Maize grain yield was greatest following palisade grass that received N fertilizer, regardless of treatment timing, and lowest following unfertilized ruzigrass (control) (Fig. 3). The amount of N applied on green palisade grass (35 DBS) and on straw (1 DBS) was sufficient to achieve maize yields (average 11.9 Mg ha^{-1}) equivalent to those obtained using the conventional N application method (average 12.2 Mg ha^{-1}). However, maize yield was higher following palisade grass compared with ruzigrass in all treatments, in contrast to the patterns of maize shoot dry matter and leaf N concentration. Applying N on green palisade grass (35 DBS) increased maize grain yield compared with applying N on green ruzigrass (35 DBS). For maize following ruzigrass, grain yield was similar between the 1 DBS and conventional treatments.

3.2. Total bacterial, archaeal and fungal abundances and nitrogen cycle gene abundances in soil at maize harvest

The abundances of total bacteria, archaea, and fungi in soil at the time of maize harvest are shown in Fig. 4. The sizes of the bacterial, archaeal, and fungal populations differed for each cover crop and treatment. When the cover crop was palisade grass, the bacterial copy number in soil was similar among the control, 35 DBS (green) and conventional treatments but was significantly higher in the 1 DBS (straw) treatment (9.4×10^7 copies g^{-1} dry soil) (Fig. 4A). The archaeal abundance in soil was similar in the control and conventional treatments (average 2.7×10^6 copies g^{-1} dry soil), higher in the 1 DBS (straw) treatment, and highest in the 35 DBS (green) treatment (Fig. 4B). The total copy number of fungi was lowest in the conventional treatment (7.9×10^6 copies g^{-1} dry soil), higher in the control and 1 DBS (straw) treatments, and highest in the 35 DBS (green) treatment (Fig. 4C).

When the cover crop was ruzigrass, the bacterial gene copy numbers differed among the treatments and increased in the following order: 35 DBS (green), 1 DBS (straw), control and conventional (Fig. 4A). Archaeal abundance was lowest in the 1 DBS (straw) treatment and subsequently increased in the following order: control, 35 DBS (green), and conventional (Fig. 4B). In soil under ruzigrass, the abundance of fungi was lowest in the 1 DBS (straw) treatment, higher in the conventional and 35 DBS (green) treatments, and highest in the control (Fig. 4C).

The residual effects of the timing of N fertilization of different cover crops on the abundances of the N-cycle genes *nifH*, *amoA* AOB, *amoA* AOA, *nirS*, *nirK*, and *nosZ* in soil are depicted in Supplementary Fig. S2. Because the total bacterial, archaeal and fungal abundances varied across treatments and between cover crop species (Fig. 4), we calculated the ratios of abundances of each N gene (*nifH*, AOB, *nirS*, *nirK* and *nosZ*) to total bacteria (*16S rRNA*), AOA to total archaea (*16S rRNA*), total bacteria to total archaea, total bacteria to total fungi (*18S rRNA*), and total archaea to total fungi to determine the relative increases in each gene in each treatment with different cover crops (Figs. 4 and 5). The total archaea:bacteria ratio in soil was highest under ruzigrass in the 35 DBS (green) treatment, while the total fungi:bacteria ratio was similar between the cover crops and highest in the 35 DBS (green) treatment (Fig. 4D). When palisade grass was the cover crop, the abundance of *nifH* in soil was similar among the control, 35 DBS (green) and 1 DBS (straw) treatments and lower than in the conventional treatment (Supplementary Fig. S2A). When ruzigrass was the cover crop, the abundance of *nifH* differed in each treatment and increased in the following order: conventional, control, 1 DBS (straw), and 35 DBS (green).

The soil abundances of the bacterial nitrification gene *amoA* (AOB) under palisade grass were similar in the control and conventional treatments and lower than those in the 35 DBS (green) and 1 DBS (straw) treatments (Fig. 5B). Under ruzigrass, the abundance of AOB was highest in the 35 DBS (green) treatment (Fig. 5B). The soil abundances of the archaeal nitrification gene *amoA* (AOA) under palisade grass were similar regardless of the timing of N application (Fig. 5F). Under

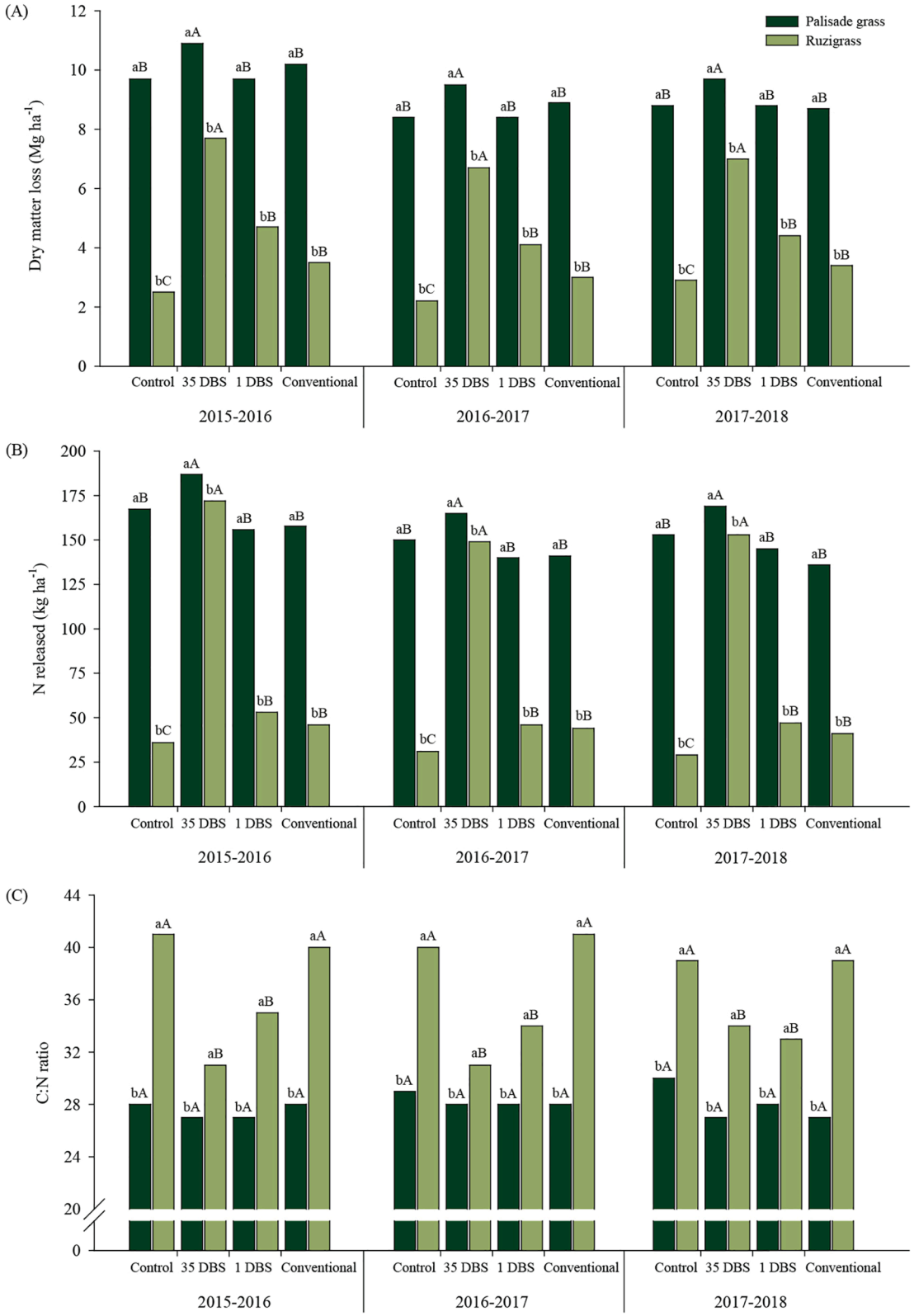

Fig. 2. Effects of cover crop species and timing of N application on dry matter loss (A) and N released (B) from cover crop residues during maize cycle, and C:N ratio of cover crops (C) at maize harvest in the three growing seasons (2015–2016, 2016–2017, and 2017–2018). The dry matter loss and the N release from cover crop are the difference between measurements performed in cover crop samples at termination and at the end of the maize cycle. Nitrogen treatments treatments are as follows: Control: no N application; 35 DBS: 120 kg N ha^{-1} broadcast over cover crop 35 days before maize seeding plus 40 kg N ha^{-1} at seeding; 1 DBS: 120 kg N ha^{-1} broadcast over crop 1 day before maize seeding plus 40 kg N ha^{-1} at seeding; and Conventional method: 40 kg N ha^{-1} at seeding furrow plus 120 kg N ha^{-1} side-dressed at V_4 growth stage of maize. Different lowercase letters denote significant difference between cover crops and different uppercase letters denote significant difference between N treatments (LSD, $P \leq 0.05$).

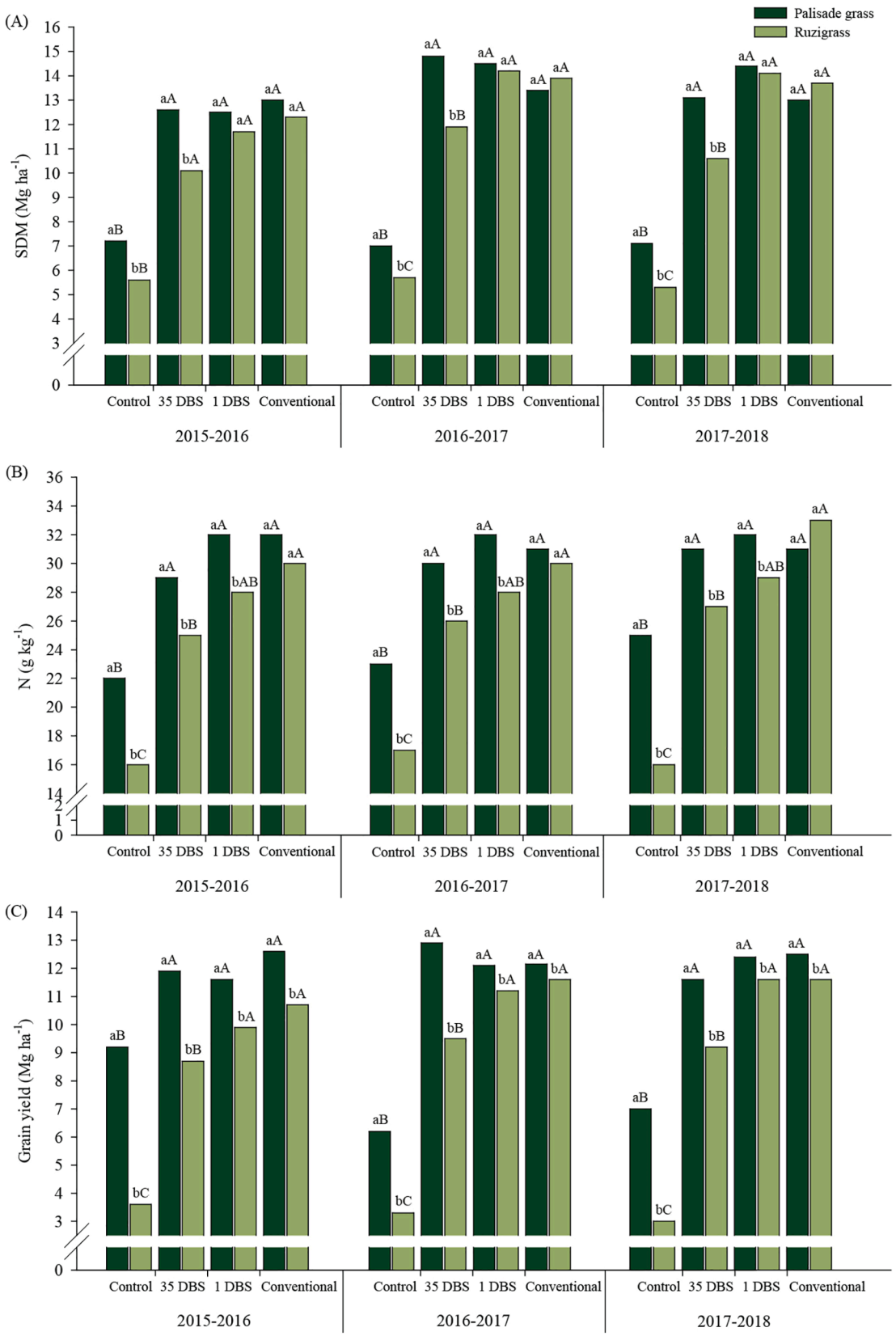

Fig. 3. Effects of cover crop and timing of N application on shoot dry matter (A), N concentration in leaf at 60 days after maize emergence (flowering stage) (B), and grain yield (C) of maize in three growing seasons (2015–2016, 2016–2017, and 2017–2018). Nitrogen treatments are as follows: Control: no N application; 35 DBS: 120 kg N ha^{-1} broadcast over cover crop 35 days before maize seeding plus 40 kg N ha^{-1} at seeding; 1 DBS: 120 kg N ha^{-1} broadcast over crop 1 day before maize seeding plus 40 kg N ha^{-1} at seeding; and Conventional method: 40 kg N ha^{-1} at seeding furrow plus 120 kg N ha^{-1} side-dressed at V_4 growth stage of maize. Different lowercase letters denote significant difference between cover crops and different uppercase letters denote significant difference between N treatments (LSD, $P \leq 0.05$).

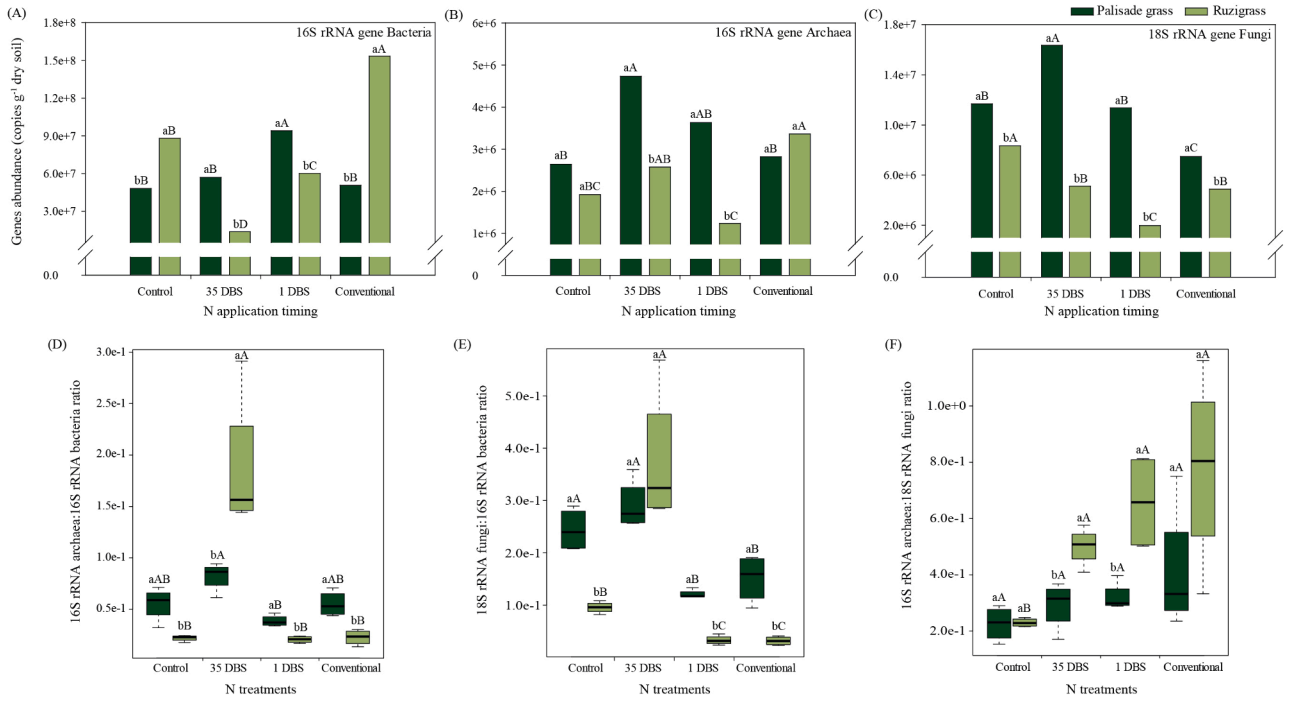

Fig. 4. Impacts of cover crop and N treatments on (A) total bacteria, (B) archaea and (C) fungi in soil at maize harvest stage, and on genes abundances ratio between (D) total archaea (16S rRNA):total bacteria (16S rRNA), (E) total fungi (18S rRNA):total bacteria (16S rRNA), (F) total archaea:total fungi. 35 DBS: N applied on green cover crops 35 days before maize seeding; 1 DBS: N applied on straw cover crops 1 day before maize seeding; Conventional: N applied at side-dressed in V_4 maize growth stage. Different lowercase letters denote significant difference between cover crops and different uppercase letters denote significant difference between N treatments (LSD, $P \leq 0.05$).

ruzigrass, the abundance of AOA was lowest in the control, increased in the 1 DBS (straw) and conventional treatments, and was highest in the 35 DBS (green) treatment (Fig. 5F).

The soil abundance patterns of the denitrification genes *nirK*, *nirS* and *nosZ* differed depending on the treatment and cover crop (Fig. 5C, 5D, 5E). Under palisade grass, the soil abundances of *nirS* were similar in all treatments, whereas under ruzigrass, the abundance of *nirS* was lowest in the 1 DBS (straw) treatment, higher in the control and conventional treatments, and highest in the 35 DBS (green) treatment (Fig. 5C). In general, the soil abundances of *nirK* and *nosZ* were similarly low, except for higher levels under ruzigrass in the 35 DBS (green) treatment (Fig. 5D, 5E).

3.3. Correlations between microbial gene abundances and soil and plant properties

To assess the main microbially driven processes of nitrification and denitrification in each treatment under each cover crop, the AOA:AOB and (AOA + AOB):(*nirS* + *nirK*) ratios were calculated (Table 1). Among AOA, AOB, *nirS* and *nirK*, the abundance of AOA was highest in all treatments (Table 1). The AOA:AOB ratio in soil under palisade grass was highest in the control and conventional treatments; under ruzigrass, this ratio was similar among all treatments. Under palisade grass, the soil (AOA + AOB):(*nirS* + *nirK*) ratio, which indicates preferential nitrification, was lowest in the 35 DBS (green) treatment and was similarly high in all other treatments. Under ruzigrass, the soil (AOA + AOB):(*nirS* + *nirK*) ratio was lower in the 35 DBS (green) and conventional treatments than in the 1 DBS (straw) treatment (Table 1).

Correlations were calculated to evaluate the relative influences of the abundances of N-cycle genes, total bacteria, total archaea, and total fungi in all treatments on biomass and N release (amount of biomass/N at maize harvest minus the amount of biomass/N initial at 30 DBS), cover crop C:N ratio, and soil pH, moisture, mineralizable C, total-N, NH_4^+, and NO_3^- content (Supplementary Fig. S3). Based on the results of Spearman's correlation analysis, a general linear model was used. The correlations between pairs of factors are shown in Supplementary Fig. S3. Total bacterial abundance was significantly positively correlated with the abundances of N-cycle genes: *nifH* ($R^2 = 0.4291$, $P = 0.0015$), AOB ($R^2 = 0.2831$, $P = 0.0326$), *nirS* ($R^2 = 0.3223$, $P = 0.0209$), *nirK* ($R^2 = 0.7912$, $P < 0.0001$) and *nosZ* ($R^2 = 0.1713$, $P = 0.0443$). By contrast, total bacterial abundance was negatively correlated with N release by cover crops ($R^2 = 0.1842$, $P = 0.03634$). Total archaeal abundance was significantly positively correlated with the gene abundances of AOA ($R^2 = 0.2822$, $P = 0.0082$) and *nirS* ($R^2 = 0.4275$, $P = 0.0005$), total fungal abundance ($R^2 = 0.4718$, $P = 0.0054$), biomass released by cover crops ($R^2 = 0.1705$, $P = 0.0448$) and total soil N ($R^2 = 0.2208$, $P = 0.0204$). Total fungal abundance was significantly positively correlated with the gene abundances of AOB ($R^2 = 0.3485$, $P = 0.0023$), AOA ($R^2 = 0.5921$, $P < 0.0001$) and *nirS* ($R^2 = 0.3353$, $P = 0.0030$) and total soil N ($R^2 = 0.1923$, $P = 0.0320$) and significantly negatively correlated with *nosZ* ($R^2 = 0.1770$, $P = 0.0405$). The only two significant negative correlations between gene abundances were between *nifH* and AOA ($R^2 = 0.2822$, $P = 0.0326$) and between AOA and *nosZ* ($R^2 = 0.3609$, $P = 0.0142$). Among plant variables, the C:N ratio of straw was positively correlated with *nifH* abundance ($R^2 = 0.2280$, $P = 0.0182$) and negatively correlated with AOA abundance ($R^2 = 0.3862$, $P = 0.0011$), biomass by cover crops ($R^2 = 0.2230$, $P = 0.0197$) and N release by biomass ($R^2 = 0.2074$, $P = 0.0252$).

PCA was performed based on the relative abundances of genes and plant and soil factors in the different treatments (Fig. 6). The first two axes of the plots explained more than 68% of the data variation. In the first set of correlations, positive correlations of the abundances of *nifH* and total bacteria with the C:N ratio of cover crops were observed in soil under ruzigrass in the control and conventional treatments. By contrast, the abundances of *nifH* and total bacteria and the C:N ratio of cover crops were negatively correlated with AOA abundance, biomass and N release. In addition, AOA abundance, biomass, and N release were positively correlated with each other under palisade grass in the control

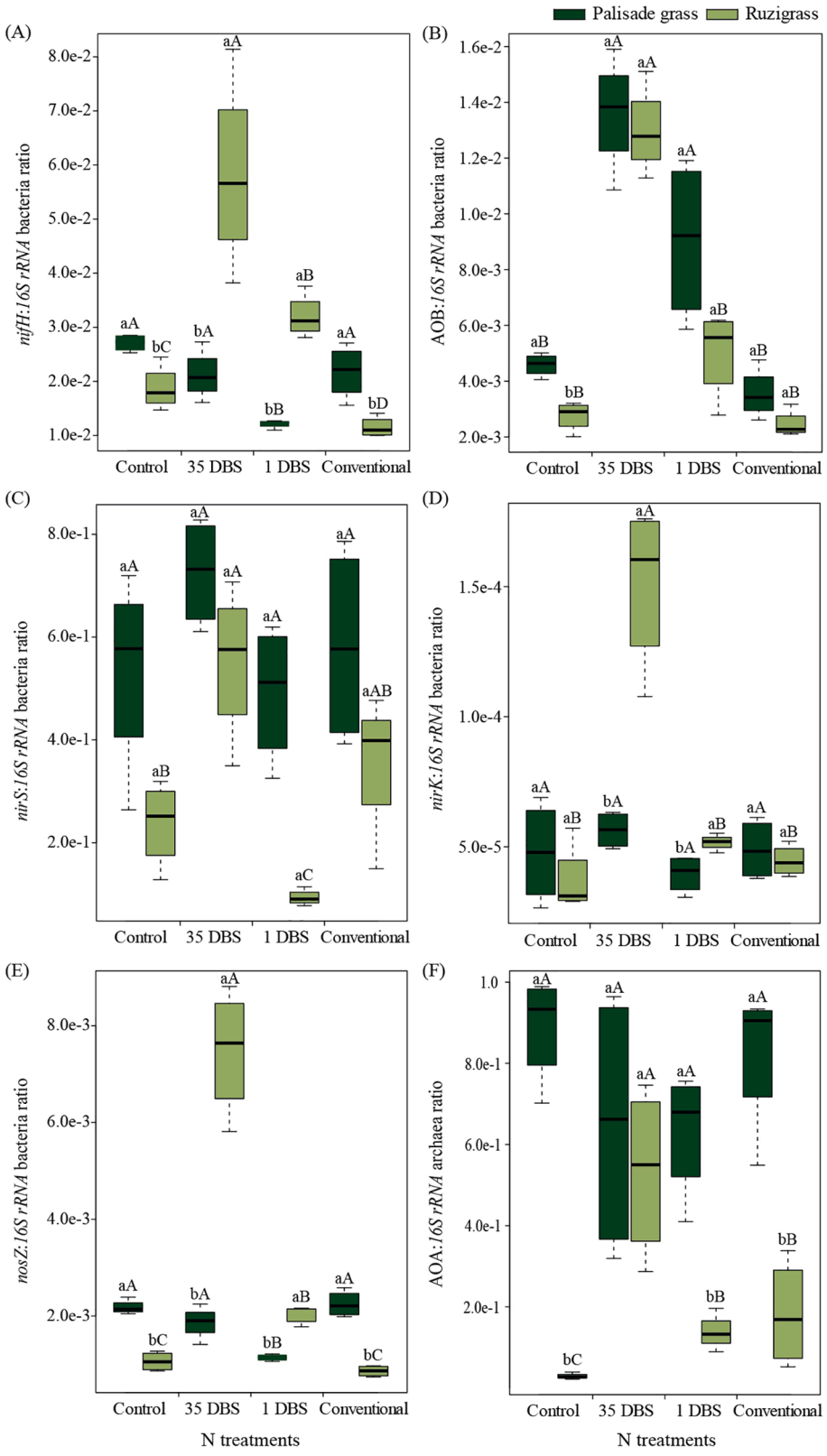

Fig. 5. Impact of cover crop and N treatments on genes abundances ratio between (A) *nifH*, (B) *amoA* bacteria (AOB), (C) *nirS*, (D) *nirK*, (E) *nosZ* and total bacteria (16S rRNA), and (F) *amoA* archaea (AOA) and total archaea (16S rRNA) in soil at maize harvest stage. 35 DBS: N applied on green cover crops 35 days before maize seeding; 1 DBS: N applied on straw cover crops 1 day before maize seeding; Conventional: N applied at side-dressed in V_4 maize growth stage. Different lowercase letters denote significant differences between forage grasses and different uppercase letters denote significant differences among N application systems ($P \leq 0.05$).

and conventional treatments. NO_3^- content and the abundances of *nirS*, AOB, total archaea and total fungi were positively correlated with each other under palisade grass in the 35 DBS (green) and 1 DBS (straw) treatments. Furthermore, under ruzigrass, all treatments were distinct from each other, whereas under palisade grass, the control treatment was significantly different from the remaining treatments according to PERMANOVA ($F = 11.25$ and $p < 0.0001$) (Fig. 6).

Table 1
Ratio between abundances of AOA and AOB, and nitrifiers (AOA + AOB) and denitrifiers (*nirS* + *nirK*) in the soil at maize harvest, as affected by cover crop and N treatments.

Cover crop (C)	N treatments AOA/AOB			
	Control†	35 DBS‡	1 DBS§	Conventional¥
Palisade grass	227aB††	46aC	84aC	375aA
Ruzigrass	10bA	42aA	30aA	72bA
	(AOA + AOB):(*nirS* + *nirK*)			
	Control	35 DBS	1 DBS	Conventional
Palisade grass	2.5aA	0.9aA	1.5aA	2.0aA
Ruzigrass	0.1bA	0.9aA	1.5aA	0.5bA

† Control: no N application.
‡ 35 DBS: 120 kg N ha^{-1} broadcast over cover crop 35 days before maize seeding plus 40 kg N ha^{-1} at seeding.
§ 1 DBS: 120 kg N ha^{-1} broadcast over crop 1 day before maize seeding plus 40 kg N ha^{-1} at seeding.
¥ Conventional method: 40 kg N ha^{-1} at seeding furrow plus 120 kg N ha^{-1} side-dressed at V_4 growth stage of maize.
†† Different lowercase letters denote significant difference between cover crops and different uppercase letters denote significant difference between N treatments ($P \leq 0.05$) according to LSD test.

4. Discussion

4.1. Cover crops and N-fertilizer management in maize production

The cultivation of cover crops from April to October before maize seeding resulted in high yields of grass biomass, especially when N was applied on green cover crops (35 DBS). N application was expected to increase biomass by stimulating vegetative growth, and the amount of grass cover crop biomass obtained in the current study (13.8 Mg ha^{-1} average) exceeds the minimum mulch requirement (4 Mg ha^{-1}) for NT systems in tropical regions (Lopes et al., 1987). The N input from cover crops was 1.76 times higher under palisade grass than under ruzigrass (123 kg N ha^{-1}). The growth performance of N-fertilized palisade grass and its higher dry matter yield compared with ruzigrass resulted in the release of a high amount of N from straw residues. By contrast, the various N-fertilization treatments did not affect the release of N from straw residues of ruzigrass at maize harvest due to the higher C:N ratio of ruzigrass (values from 31 to 41) compared with palisade grass (values from 27 to 28). The advantages of palisade grass over ruzigrass include its easy adaptation capacity, broad seasonal distribution, and high responsiveness to N application (Fisher et al., 1995). The greater dry matter and N release of palisade grass compared with ruzigrass in the current study are consistent with previous observations (Momesso et al., 2020; Pacheco et al., 2017; Tanaka et al., 2019). However, this study is the first to show an effect of the remaining cover crop straw on the yield of the subsequent crop. The high growth, N release, and soil surface coverage of palisade grass fertilized with N at the green (35 DBS) or straw (1 DBS) stage enhanced subsequent maize production in this tropical NT system.

Compared with the conventional timing of N application, applying N fertilizer to green cover crops benefits the system by increasing plant N uptake, as the N fertilizer absorbed by the cover crops is released gradually by straw decomposition during maize growth and thus supplies the N demand of maize (Rosolem et al., 2017). However, the supply of N depends on the cover crop species. Applying N fertilizer to green ruzigrass (35 DBS) increased ruzigrass dry matter and N release (Fig. 2), but these increases did not translate into higher maize yields compared with N application later in the cycle (1 DBS or conventional). It is likely that most of the N released upon cover crop desiccation enters N cycling in the soil organic fractions rather than directly supplying the subsequent maize crop (Canisares et al., 2021). In the absence of N fertilization, ruzigrass had a negative effect on maize growth compared with palisade grass, corroborating previous studies using the same grass species (Momesso et al., 2020, 2019; Tanaka et al., 2019).

In summary, palisade grass appears to be a better cover crop for cultivation in rotation with maize in NT systems. Palisade grass had large dry matter production and N release during the subsequent maize growing cycle, even in the treatments without N fertilization (Fig. 2A), indicating a superior capacity to exploit residual soil N as compared to ruzigrass. Nonetheless, despite the high amount of N recycled in the control treatments (210 kg ha^{-1}), maize still responded to N fertilization (Fig. 3).

4.2. Effect of cover crops and fertilizer timing on microbial groups and N-cycle gene abundances

In the tropics, effects of straw and fertilization on soil bacteria, archaea and fungi have been reported in agricultural systems for sugarcane cultivation for bioethanol production (Lourenço et al., 2018a;

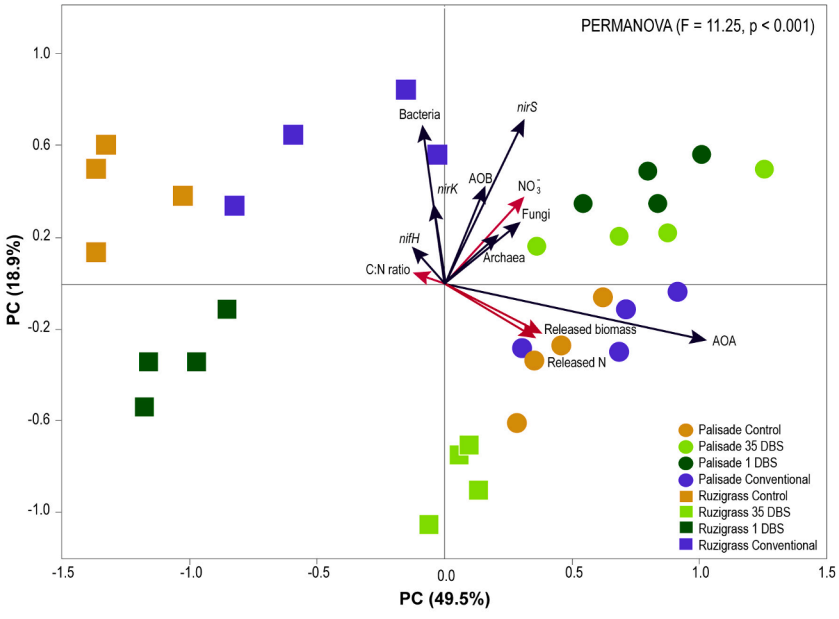

Fig. 6. Principal component analysis summarizing the variance of microbial functions and soil and plant factors according to cover crops (palisade grass and ruzigrass) and N treatments (Control: no-N application; 35 DBS: N applied on green cover crops 35 days before maize seeding; 1 DBS: N applied on straw cover crops 1 day before maize seeding; Conventional: N applied at side-dressed in V_4 maize growth stage). Black arrows are microbial and N cycle abundances and red arrows are plant and soil variables. Arrows are projected variables showing variables with the highest squared correlation coefficients. Different colors indicate the different sampled treatments as indicated.

Pitombo et al., 2016; Soares et al., 2016). However, the responses of these major microbial groups have not been investigated in cover crop systems designed for sustainably high cash crop yields in tropical soils. Our results suggest that applying N on green palisade grass and straw increases total bacterial, archaeal and fungal densities because the cover crop growth promoted by N fertilization provides N, C, and nutrients in the straw residue for archaeal and fungal processes in the soil (Bani et al., 2018; Chavarría et al., 2016; McDaniel et al., 2014). The ratios of cover crop biomass to the abundances of N-cycle genes, total bacteria, total archaea and total fungi showed that the higher dry matter released by palisade grass was associated with greater microbial abundance and decomposition processes. Under ruzigrass, which had a C:N ratio of 36, conventional fertilization of maize with N increased total bacteria, whereas fungal abundances in soil decreased when N was applied on either green ruzigrass or straw residue. Shifts in microbial populations depend on resource competition, the C:N ratio, litter composition and sources of N for decomposition processes (Bani et al., 2018; Li et al., 2019; Zhou et al., 2017). Thus, the combinations of cover crop species and N management evaluated in this study formed distinct agro-ecosystems: the increase in ruzigrass biomass under conventional N fertilization induced a decrease in the bacterial population, whereas the increase in palisade grass biomass under N fertilization of green grass induced increases in archaeal and fungal densities.

The archaea:bacteria, fungi:bacteria and fungi:archaea ratios were also strongly correlated with straw production by both cover crops receiving N. The roles of bacterial, archaeal and fungal groups in biogeochemical processes vary depending on the source of nutrients and environmental disturbances (Kim et al., 2020; Osburn et al., 2019). Many fungi are able to decompose high-molecular-weight organic matter, which reduces the amount of soil N available for microbial reactions as well as soil C storage (Tatsumi et al., 2020). Residues of cover crops with a low C:N ratio decompose more readily, increasing nutrient and soil organic matter content in the soil surface layer (Mariano et al., 2016; Rosolem et al., 2017). High straw production by cover crops and the associated bacterial response may positively influence the archaeal and fungal populations (Kielak et al., 2016), or the bacteria may compete with fungi for resources such as sugar, C and N (de Boer and van der Wal, 2008; Johnston et al., 2019). Plant-microbe associations are often difficult to predict in complex agroecosystems, but the long-term effects of the cropping system on the soil microbial communities in the current study were clearly driven by the low C:N ratio of the cover crops and the biomass released from straw to soil (Bani et al., 2018; Fu et al., 2020; Yuan et al., 2020), which increased total archaea and fungi and decreased bacteria.

Quantifying microbial genes involved in N transformation provided information on N cycling by microbial populations in response to N management and the cultivation of different grasses as cover crops. All genes analyzed in soil at the time of maize harvest were affected by the cover crop species × N treatment interaction. Microorganisms involved in N transformation might contribute to N gain via nitrogen fixation and N losses through nitrification and denitrification when N is applied on green cover crops or on their straw residues.

With respect to N fixers, soil *nifH* abundance increased dramatically under N fertilization of green ruzigrass. Because ruzigrass straw accumulated 45% less N than palisade grass straw, the low N in the ruzigrass-maize system may have stimulated biological N fixation (BNF) in the soil at the time of maize harvest (Boddey and Victoria, 1986; Rodrigues Coelho et al., 2008). Soils subjected to low rates of fertilizer application or with N-poor environmental conditions typically have high levels of microbial N_2 fixation (Bao et al., 2014; Ikeda et al., 2014; Yokoyama et al., 2017). N-fixing bacteria can account for 10 to 40% of N uptake by forage grasses (Boddey and Victoria, 1986; Hungria et al., 2016). In addition, the *nifH* abundance may reflect an increase in the number of N fixers present on maize plants, which provide significant N for uptake by maize (Montañez et al., 2009; Wen et al., 2019). Thus, part of the N uptake by maize may have been provided by N fixation.

The abundance of AOA, which is involved in nitrification, responded to cover crop species but not the timing of N application. Palisade grass enhanced AOA abundance, which was linked to the high straw amount and N release and the low C:N ratio of the crop residues. Unlike AOA abundance, the response of AOB abundance differed depending on the timing of N application. Recent studies have shown that archaeal ammonia oxidizers are less responsive than bacterial ammonia oxidizers to soil N supplied by fertilizer in tropical soils (Carey et al., 2016; Lourenço et al., 2018a; Soares et al., 2016). The different responses of AOA and AOB in the present study may be attributable to niche differentiation between these groups, which are known to possess contrasting traits with respect to cellular structure, metabolism and physiology (Prosser, 2020; Prosser and Nicol, 2012; Trivedi et al., 2020). Both AOA and AOB respond to environmental factors such as soil pH, C:N ratio and substrate concentration, with AOA typically linked to oligotrophic conditions and a low C:N ratio in acid soils. Consistent with previous observations, we found high AOA abundance and a low C:N ratio under palisade grass, whereas AOB densities were stimulated by copiotrophic conditions, higher soil pH and an intermediate C:N ratio (Lu et al., 2015; Soares et al., 2016; Trivedi et al., 2020).

Analysis of the AOA:AOB ratio indicated a predominance of AOA genes under both cover crops and an increase in AOA under palisade grass straw. Both groups of ammonia oxidizers compete for the same energy source (NH_3) in the soil, but are able to coexist in the same environment due to their different niches (Liu et al., 2020). In this study, the increase in AOA might be a consequence of the acidic soil conditions ($pH < 5$) and higher N available under palisade grass, since ammonia oxidizers are strongly dependent on pH and acid soils favor AOA over AOB (Nicol et al., 2008). The timing of N fertilization and the cover crop species may also have influenced the populations of nitrifiers and as well as other groups within the microbial community, with such effects of management having been previously reported in temperate soils (Jia et al., 2020; Liu et al., 2020). However, contrasting results have been reported with respect to the relative dominances of AOB versus AOA across a range of soils (Castellano-Hinojosa et al., 2020; Prosser and Nicol, 2012; Taylor et al., 2012).

Nitrification and denitrification occur simultaneously in the soil, but the ratio of these processes can vary greatly across tropical agricultural systems (Lourenço et al., 2018b). In soil under palisade grass, nitrification was the dominant process, whereas denitrification increased in soil under ruzigrass. N release from straw negatively affected denitrifiers, especially the abundances of *nirK* and *nosZ*, in soil under ruzigrass in the conventional fertilizer treatment. Thus, residues of cover crops were environmental drivers of these processes. In general, nitrification and denitrification rates tend to depend on environmental factors such as soil mineral N, pH, oxygen concentration and the availability of N and C substrates, and these factors are correlated with soil conditions and management (Hofstra and Bouwman, 2005). Our findings suggest that soils with high cover crop coverage had relatively higher amounts of nitrification (by AOA and AOB), whereas denitrification (by *nirK* and *nirS*) was dominant when N was applied on green cover crops or their straw.

We found a lasting effect of cover crop and N management on microbial populations and N-cycle genes in soil depending on the cover crop species receiving N fertilization. The responses of the microbial populations to the lasting effect of N fertilization and cover crops may be linked to the large input of soil organic matter and soil total C due to the C- and N-rich environment provided by the cover crops (McDaniel et al., 2014; Ouyang et al., 2018). The application of N fertilizer on green palisade grass and straw induced increases in nitrification genes in soil (Fig. 5B and Fig. 6). The simultaneous increase in genes involved in nitrification might lead to N losses since such high levels of N may stimulate N-transforming microbial reactions (Liang and MacKenzie, 2011; Robertson and Vitousek, 2009).

In summary, the timing of N application in cover crop agricultural systems is an important factor for maize grain yield and forage

production. We found that applying N fertilizer on palisade cover crops 35 days before maize seeding, on palisade straw residue 1 day before maize seeding or on maize with conventional timing provided equally high grain yields. Residues from N application on green cover crops and on straw significantly increased N-cycling microbial populations of AOA, AOB, *nirK* and *nirS*, as well as total bacterial and total fungil densities under palisade grass. Thus, the conventional method of N fertilizer application on palisade grass used in grass-maize tropical production appears to be the best option to achieve high grain yield, high N fertilizer uptake by maize, and low potential for N losses via nitrification and denitrification. Future studies addressing the effects of N fertilization rates in agricultural management systems that use palisade grass are needed to determine the amount of N fertilizer that is adequate but not excessive.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2021.115423.

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