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MAIZE-BASED SYSTEMS AS AFFECTED BY FORAGE GRASS AND NITROGEN FERTILIZATION: ELUCIDATING ^{15}N-FERTILIZER RECOVERY, GREENHOUSE GAS EMISSIONS, N-CYCLE FUNCTIONAL GENES IN SOIL AND CROP YIELDS

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ABSTRACT

Due to the interest in N use efficiency (NUE) and sustainable agricultural systems, the adoption of integrated systems, such as the intercropping of maize with forage grasses can be of great relevance, allowing the use of the land throughout the year, besides avoiding losses of N through nitrate (NO_3^-) leaching, nitrous oxide (N_2O) emissions, ammonia volatilization (NH_3), and immobilization. Tropical forage grasses of the genus *Megathyrsus* and *Urochloa* can suppress soil–nitrification by releasing inhibitory substances, reducing N losses and increasing fertilizer N recovery of the cash crop in rotation. In this way, understanding the N transformations in the soil by microorganisms and the fertilizer recovery in the system are very important. Firstly, the first two chapters are about a 3-year (2014-2017) field experiment conducted in southeastern Brazil, were forage grasses Guinea grass (Megathyrsus maximus cv. Tanzânia), palisade grass (Urochloa brizantha cv. Marandu), and ruzigrass (Urochloa ruziziensis cv. Comum) were cultivated in rotation with maize for grain in summer, to analyze the influence of forage grass and N fertilization in each study. In first chapter, maize was fertilized with 140 kg ha^{-1} N as $(^{15}NH_4)_2SO_4$ or not fertilized, and recovery of residual ^{15}N was quantified in the second season. In second chapter, the change was that the N source used was ammonium sulphate not labeled, and were analyzed nitrous oxide (N_2O), methane (CH_4), and NH_3 emissions from the system. In the third and fourth chapter, maize was intercropped with the same grasses previously mentioned. The N rates were 90, 180 and 270 kg ha^{-1} N and treatments without N fertilization. The objective was also to ascertain the effect of grasses and N fertilization from the analyzes carried out. The third chapter characterized the changes in N-cycle genes in the soil and measured the N_2O emissions. The fourth chapter assessed maize grain yield and forage production, bromatological quality, and estimated meat production. In the first season after ^{15}N application, 21%, 65%, and 33% of the N in maize grain, stover, and shoots, respectively, was derived from fertilizer. In the next season, of the total N found in maize grain, stover, and shoots, 2.2%, 1.9%, and 2.0%, respectively, was derived from the residual fertilizer applied in the previous year. There were no differences between forage grass species in the amount of ^{15}N recovered by maize, soil, and total N. In the first season of maize in rotation with forage grasses, Guinea grass, palisade grass, ruzigrass did not affect N_2O and NH_3 emission due to their apparent inability to suppress soil nitrification. However, N fertilization slightly increases cumulative N_2O emission. In maize intercropping with grasses, N fertilization increases the abundance of AOB (*amo*A of bacteria) more than AOA (*amo*A of archaea). N_2O emission was influenced by AOB, water-filled pore space (WFPS) and N fertilization. Nitrogen fertilization positively affects forage growth and nutritional quality, resulting in a higher maize grain yield, higher forage production and quality, and eventually higher estimated meat production. Moreover, Guinea grass resulted in the highest estimated meat production when fertilized with 270 kg ha^{-1} N. However, no evidence of biological inhibition by the grasses were confirmed.

Keywords: *Zea mays* L. *Urochloa*. *Megathyrsus*. N_2O. AOB. AOA. Estimated meat production.

RESUMO

Devido ao interesse na eficiência no uso do N (NUE) e em sistemas agrícolas sustentáveis, a adoção de sistemas integrados, como o consórcio de milho com gramíneas forrageiras, pode ser de grande relevância, permitindo o uso da terra ao longo do ano, além de evitar perdas de N por lixiviação de nitrato (NO_3^-), emissões de óxido nitroso (N_2O), volatilização de amônia (NH_3) e imobilização. Gramíneas forrageiras tropicais do gênero *Megathyrsus* e *Urochloa* podem suprimir a nitrificação do solo ao liberar substâncias inibidoras, reduzindo as perdas de N e aumentando a recuperação de N fertilizante da cultura comercial em sucessão. Desta forma, o entendimento das transformações do N no solo por microrganismos e a recuperação do fertilizante no sistema são muito importantes. Em primeiro lugar, os dois primeiros capítulos são a respeito de um experimento de campo de 3 anos (2014-2017) conduzido no sudeste do Brasil, onde gramíneas forrageiras capim colonião (*Megathyrsus maximus* cv. Tanzânia), capim braquiária (*Urochloa brizantha* cv. Marandu) e capim braquiária *(Urochloa ruziziensis* cv. Comum) foram cultivadas em rotação com milho para grão no verão, para analisar a influência da gramínea forrageira e da fertilização com N em cada estudo. No primeiro capítulo, o milho foi fertilizado com 140 kg ha^{-1} de N na forma de $(^{15}NH_4)_2SO_4$ ou não fertilizado, e a recuperação do ^{15}N residual foi quantificada na segunda safra. No segundo capítulo, a mudança foi que a fonte de N utilizada foi o sulfato de amônio não rotulado, e foram analisadas as emissões de óxido nitroso (N_2O), metano (CH_4) e NH_3 do sistema. No terceiro e quarto capítulos, o milho foi consorciado com as mesmas gramíneas mencionadas anteriormente. As doses de N foram 90, 180 e 270 kg ha^{-1} e os tratamentos sem adubação nitrogenada. O objetivo também foi verificar o efeito das gramíneas e da fertilização com N a partir das análises realizadas. O terceiro capítulo caracterizou as mudanças nos genes do ciclo N no solo e mediu as emissões de N_2O. O quarto capítulo avaliou o rendimento de grãos de milho e a produção de forragem, a qualidade bromatológica e a estimativa da produção de carne. Na primeira safra após a aplicação de ^{15}N, 21%, 65% e 33% do N no grão de milho, palha e brotos, respectivamente, foi derivado de fertilizante. Na safra seguinte, do total de N encontrado nos grãos, caules e ramos de milho, 2,2%, 1,9% e 2,0%, respectivamente, foram derivados do fertilizante residual aplicado no ano anterior. Não houve diferenças entre as espécies de gramíneas forrageiras na quantidade de ^{15}N recuperado pelo milho, solo e N. total. Na primeira temporada de milho em rotação com gramíneas forrageiras, capim-Guiné, capim-paliçada, ruzigrass não afetou a emissão de N_2O e NH_3 devido à sua aparente incapacidade de suprimir a nitrificação do solo. No entanto, a fertilização com N aumenta ligeiramente a emissão cumulativa de N_2O. No consórcio de milho com gramíneas, a fertilização com N aumenta a abundância de AOB (*amo*A de bactérias) mais do que AOA (*amo*A de arquéias). A emissão de N_2O foi influenciada por AOB, espaço poroso cheio de água (WFPS) e fertilização de N. A fertilização nitrogenada afeta positivamente o crescimento das forragens e a qualidade nutricional, resultando em maior rendimento de grãos de milho, maior produção e qualidade das forragens, e eventualmente maior produção de carne estimada. Além disso, o capim colonião resultou na maior produção de carne estimada quando fertilizado com 270 kg ha^{-1} N. No entanto, não foram confirmadas provas de inibição biológica por parte das gramíneas.

Palavras-chave: *Zea mays* L. *Urochloa*. *Megathyrsus*. N_2O. AOB. AOA. Produção estimada de carne.

FIGURE LIST

Figure 1 - Monthly precipitation, and average minimum and maximum air temperatures in the first (2015–2016) and second (2016–2017) growing seasons, and long-term average (period 1955-2015)……32

Figure 2 -Schematic representation of the whole plot and its microplot (deployed exclusively in N–fertilized treatments), in addition to the sampling procedures of plant, litter, and soil……36

Figure 3 - Maize (partitioned into grains and stover) and litter N derived from fertilizer as affected by forage grass in the first (2015–2016) and second (2016-2017 growing season after application of ^{15}N-labeled fertilizer……47

Figure 4 - Soil N derived from fertilizer at four depth layers as affected by forage grass in the first (2015–2016) and second (2016–2017) growing season after application of ^{15}N-labeled fertilizer……..48

Figure 5 -The fate of ^{15}N–labeled fertilizer in plant–litter–soil system as affected by forage grass in the first (2015–2016) and second (2016–2017) growing season after application of 15N-labeled fertilizer……49

Figure 6 - Daily minimum and maximum air temperatures and rainfall recorded during the field experiment period (2014-2017)……..69

Figure 7 - Seasonal variation of water-filled pore space (a) and soil temperature (b) at depth of 5.5 cm in forage grass-maize rotations from 2015 to 2017 as affected by forage grass specie and N fertilization……70

Figure 8 - Seasonal variation of NH_4^+-N (a) and NO_3^--N content in the soil (b) at depth of 0-10 cm in forage grass-maize rotations from 2014 to 2017 as affected by forage grass specie and N fertilization……71

Figure 9 - Seasonal variation of N_2O (a) and CH_4 flux (b), in addition to NH_3 loss rate (c) in forage grass-maize rotations from 2014 to 2017 as affected by forage grass specie and N fertilization……..73

Supplementary Figure 1 - Cumulative emissions (or uptake) of N_2O (a), CH_4 (b), and NH_3 (c) in forage grass-maize rotations as affected by forage grass specie and N fertilization over the experimental period……76

Supplementary Figure 2 - Cumulative crop biomass (forage plus maize) production (a) and yield-scaled emission (or uptake) of N_2O (b), CH_4 (c), and NH_3 (d) in

forage grass-maize rotations as affected by forage grass specie and N fertilization over the experimental period…….79
Figure 10 - Heatmap showing Spearman rank-order correlation coefficients (*r*) between greenhouse gas flux and soil properties (*n* = 237) throughout the experimental period (2015-2017)……..80
Figure 11 - Crop succession occurred in the experimental site from 2014 to 2018….96
Figure 12- Daily minimum, maximum and average air temperatures and rainfall recorded during the growing season period (Dec.2017-May 2018)…….103
Figure 13 -Contents of NO_3^--N (a), NH_4^+-N (b), and total N (c) at the 0-20 cm soil layer as affected by forage grass specie, N fertilization, and time after planting……..….105
Figure 14 - Abundance of 16S rRNA of bacteria (a) and archaea (b and c), *nif*H (d), *amo*A of bacteria (e), *amo*A of archaea (f), *nir*S (g) and *nos*Z (h) at the 0-20 cm soil layer as affected by forage grass species, N fertilization and days after planting.....107
Figure 15 - Cumulative N_2O emission in forage grass-maize intercropping as affected by forage specie and N fertilization……109
Supplementary Figure 3 - Variation of water-filled pore space (a) and soil temperature at depth of 5.5 cm (b) as well as N_2O flux (c) in forage grass-maize intercropping as affected by forage grass specie and N fertilization…….110
Figure 16 - Heatmap of the Spearman rank correlation order among the relative abundance of N-cycle functional genes, soil properties, and N_2O flux. *, **, and *** significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively…….111
Figure 17 - Principal component analysis of the soil microbial genes (a) and chemical properties (b) with N_2O fluxes, water-filled pore space (WFPS) and temperature (TºC) from soil of 72 sampling points across three times……..……...……113
Figure 18 - Monthly minimum, maximum and average air temperatures and rainfall recorded during the growing season period (Dec.2017-Nov.2018)……..135
Figure 19 - Forage dry matter yield (a) and maize grain yield (b) at maize harvest. The error bars represent the SEM (*n* = 8 and 4 for panels a and b, respectively)……..138

Figure 20 - Light interception in Guinea grass (a) and palisade grass (b) from 14 days after maize harvest until the forage grass reach 95% of LI; SPAD index in forage grass cut (c)……...141
Figure 21 - Estimated meat production by the LRNS Cornell model……....…143

TABLE LIST

Table 1 - Net nitrification rate from 10–d incubation of soil samples taken at the 0–20 cm depth layer before maize planting in the first (2015–2016) and second (2016–2017) growing season, in which the latter was also affected by fertilizer N rate……..…....39

Table 2 - Dry matter yield and N accumulation of forage grass species grown before maize in the first (2015–2016) and second (2016–2017) growing season, in wich the later was also affected by fertilizer N rate……..40

Table 3 -Maize (partitioned into grains and stover) and litter dry matter yield and harvest index (HI) as affected by forage grass and fertilizer N rate in the first (2015-2016) and second (2016-2017) growing season……..42

Table 4 – Maize (partitioned into grains and stover) and litter N accumulation and N harvest index (NHI) as affected by forage grass and fertilizer N rate in the first (2015-2016) and second (2016-2017) growing season……..44

Table 5 - Seasonal N_2O and CH_4, and NH_3 emissions (or uptake) in forage grass-maize rotations from 2014 to 2017 as affected by forage grass specie and N fertilization……..74

Table 6 - Seasonal crop biomass and yield-scaled (YS) emission (or uptake) of N_2O, CH_4, and NH_3in forage grass-maize rotations from 2014 to 2017 as affected by forage grass specie and N fertilization ……77

Table 7 - Seasonal N_2O emission factor in forage grass-maize rotations as influenced by forage grasses……..80

Table 8 - Description of primers, standards DNA and amplification conditions used in qPCR analysis……98

Supplementary Table 1 - Soil properties as affected by N fertilization and days after planting (DAP)……104

Supplementary Table 2 - Probability (P) values for microbial genes in the soil as affected by forage grass species, N fertilization and days after planting (DAP)..……108

Supplementary Table 3 - Scores of principal components analysis relative to temperature (T^{o}C), water-filled pore space (WFPS), N_2O flux, and abundance of microbial genes as presented in Fig. 17a……114

Supplementary Table 4 - Scores of principal components analysis of the soil chemical characteristics, water-filled pore space (WFPS), temperature (TºC), and N_2O flux presented in Fig. 17b……115
Table 9 - Bromatological quality of forage grass at maize harvest. The error bars represent the SEM (n = 16, 8 and 4 for forage grasses, N rate and forage grass x N rate, respectively)……..……..…139
Table 10 - Dry matter yield and bromatological quality of forage grass at cutting in Nov. 2018…….……....142

SUMMARY

GENERAL INTRODUCTION……...25

CHAPTER 1: FATE OF ^{15}N FERTILIZER APPLIED TO MAIZE IN ROTATION WITH TROPICAL FORAGE GRASSES……...……...29

1.1 Introduction……30

1.2 Material and methods……31

1.2.1 Study site……31

1.2.2 Study design……33

1.2.3 Crop management……33

1.2.4 Soil nitrification assessed by laboratory incubation……34

1.2.5 ^{15}N microplots……34

1.2.6 Sampling procedure and ^{15}N analyses ……36

1.2.7 Calculations and statistical analysis……37

1.3 Results……38

1.3.1 Net nitrification rates……38

1.3.2 Forage yield and N accumulation……39

1.3.3 Maize yield and N accumulation……40

1.3.4 Distribution and fate of ^{15}N–labeled fertilizer applied to maize……46

1.4 Discussion……49

1.4.1 Dry matter yield and n accumulation……49

1.4.2 Distribution of ^{15}N–Labeled fertilizer……51

1.4.3 Fate of ^{15}N–labeled fertilizer……52

1.5 Conclusion……55

References……....56

CHAPTER 2: EFFECT OF TROPICAL GRASS NITROGEN FERTILIZATION ON NITROUS OXIDE, METHANE, AND AMMONIA EMISSIONS OF MAIZE-BASED-ROTATION SYSTEMS…….61

2.1 Introduction…….62

2.2 Material and methods……64

2.2.1. Study site and experimental setup……64

2.2.2 Gas sampling procedure and auxiliary measurement……65

2.2.3 Ammonia measurements……66

2.2.4. Calculations and statistical analysis……67

2.3 Results……68

2.3.1 Environmental conditions and soil properties……68

2.3.2 Nitrous oxide, methane, and ammonia emissions……71

2.3.3 Crop biomass, yield-scaled emissions, and N_2O emission factor……77

2.3.4 Relationships between greenhouse gas flux and soil properties……80

2.4 Discussion……81

2.4.1 Greenhouse gas flux and rate of ammonia volatilization……81

2.4.2 Cumulative emissions of greenhouse gases and ammonia……82

2.4.3 N_2O emission factor……84

2.5 Conclusion……84

References……85

CHAPTER 3: FUNCTIONAL N-CYCLE GENES IN SOIL AND N2O EMISSIONS IN A MAIZE/TROPICAL FORAGE GRASSES INTERCROPPING SYSTEM……91

3.1 Introduction……..……..……..….92

3.2 Material and methods…….……..……...…94

3.2.1 Site description and experimental design…….……94

3.2.2 Soil sampling……..…….95

3.2.3 Soil chemical analysis…….……...96

3.2.4 Isolation of dna and quantitative real-time PCR…….....96

3.2.5 Gas sampling, temperature and water-filled pore space procedure……..101

3.2.6 Statistical analysis……..……..……102

3.3. Results……..……..……..102

3.3.1 Environmental conditions and soil characteristics……102

3.3.2 N-cycle functional genes……..……..……...106

3.3.3 Nitrous oxide emissions……...108

3.3.4 Correlations between the abundance of N cycle genes, soil properties and N_2O flux…….……111

3.3.5 Principal component analysis……..……112

3.4 Discussion…….……115

3.4.1 Linking N-cycle genes, forage grass and N fertilization……...115

3.4.2 N-cycle gene abundance and soil chemical characteristics……118

3.4.3 Relationships of N_2O emissions with N-cycle genes, N fertilization and soil properties…….……..……...119

3.5….Conclusions……...……..……...120

References……..……..……..……...122

CHAPTER 4: BROMATOLOGICAL QUALITY AND ESTIMATED MEAT PRODUCTION IN MAIZE INTERCROPPING WITH TROPICAL FORAGE GRASSES WITH NITROGEN FERTILIZATION……131

4.1 Introduction……..132

4.2 Materials and methods……...133

4.2.1 Study site……..133

4.2.2 Study design……134

4.2.3 Crop management…… 134

4.2.4 Forage dry matter and maize grain yield……135

4.2.5 Bromatological analysis of forage grass……135

4.2.6 Light interception, SPAD index and height of grasses……..136

4.2.7 Estimated meat production……...136

4.2.8 Statistical analysis……137

4.3 Results……...……...137

4.3.1 Forage dry matter and bromatological quality, and grain yield at maize harvest…..……137

4.3.2 Forage light interception and SPAD index in grass cut……140

4.3.3 Forage dry matter yield and bromatological quality at the final……..140

4.3.4 Estimated meat production……...……...143

4.4 Discussion……143

4.4.1 Forage yield and bromatological quality yield at maize harvest……143

4.4.2 Light interception, height and SPAD index after maize-harvest……..……..145

4.4.3 Dry matter, bromatological quality and estimated meat production……146

4.5 Conclusions.....147

References……..……..……..……..….148

FINAL CONSIDERATIONS……..……...155

REFERENCES……..……..……..…….157

GENERAL INTRODUCTION

Maize (*Zea Mays* L.) is among the most important food sources in the world, playing a key role (Ileri et al., 2018) in ensuring food security. It has been used for animal feed, human nutrition and, more recently, for bioethanol production (Ranum et al., 2014). As a C_4 plant, maize is capable of achieving high dry matter yields accumulating large amounts of nutrients (Uzun et al., 2020). Usually, nitrogen (N) is the nutrient most required by maize (Teixeira et al., 2014; Wang et al., 2017). Nitrogen defficiency can limit crop yields, since this element is a constituent of important molecules such as amino acids, proteins, nucleic acids, nitrogenous bases, and chlorophyll (Moreira and Siqueira, 2002). One of the most studied topics regarding N in agricultural systems refers to strategies to improve N use efficiency (NUE) by crops, which can be achieved through conservation practices, like systems with different species intercropped (Adewopo et al., 2014; Rosolem et al., 2017).

The adoption of integrated systems, such as the intercropping of maize with forage grasses has been of great relevance for tropical agriculture, allowing the use of land throughout the year (Crusciol et al., 2009; Kichel et al., 2009). However, increasing the NUE through conservation systems is paramount, as it is necessary to ensure adequate soil N availability for plants and to reduce losses in the agricultural systems (Rosolem et al., 2017). The volatilization of NH_3 from fertilizer sources is an important issue when urea is applied on the soil surface in no-till systems (Mariano et al., 2012), due to the higher activity of the urease enzyme when compared to conventional tillage (Silva et al., 2017). However, in Brazil, with the N rates currently used and split applications, the risk of NO_3^- reaching the groundwater is relatively low (Villalba et al., 2014). In addition, since N fertilizer is critical to sustain or increase the crop yield, the application of high N rates can lead to subsequent high N_2O emissions in N-fertilized soils compared with those unfertilized (McSwiney and Robertson, 2005; Martins et al., 2015). No-till systems also largely affect the organic matter in the soil (Sá et al., 2015., Souza et al., 2016), and during the anaerobic decomposition of organic matter, CH_4 production can occur (Dutaur and Verchot, 2007). Therefore, crop management strategies to decrease leaching losses and N_2O emissions, as well as to increase NUE are fundamental for achieving adequate sustainability levels in agricultural systems (Rosolem, et al., 2017).

Despite the several benefits from the no-till systems in relation to conventional cropping systems, such as improvements in the chemical, physical and biological properties of the soil, and reduction of CO_2 emissions (Lal et al., 2007), maize-grass intercropped systems can result in competition between species for N, which can compromise crop yields (Borghi et al. 2014). The rotation and intercropping of maize with tropical grasses of the genus *Urochloa* (syn. *Brachiaria*) and *Megathyrsus* (syn. *Panicum*) is very common for integrated crop-livestock systems in tropical Brazil (Salton et al., 2014). In addition, it has been suggested that forage species of the genus *Urochloa* and *Megathyrsus* can affect microbiological processes within the N cycle, as well as N availability and losses (Subbarao et al., 2012). Subbarao et al. (2012) reported that biological nitrification inhibition (BNI) capacity was much higher in *U. humidicola* than in *U. decumbens*, *M. maximus*, *Lolium perenne*, *U. brizantha,* cereal and vegetable crops studied in a sand-vermiculite culture for 60 days. Thus, using ^{15}N to study fertilizer N recovery by cash crops intercropped with forage species is fundamental (Rocha et al., 2019) to assess whether the fate of fertilizer N in the soil-plant system is affected by BNI, when this process is active.

The N transformations in the soil by microorganisms can occur in several ways (Zhang et al., 2006), and the understanding of these processes is essential in the search for efficient and sustainable agricultural systems. In the atmosphere, N is found as a diatomic molecule (N_2), which can be fixed by a specialized microbiota, by a process known as biological N fixation (BNF; Cardoso, 1992). In the soil, this element can be found in organic and mineral forms (Cantarella, 2007). The soil N dynamics is complex, mostly driven by soil microorganisms as follows: i) BNF: the enzyme nitrogenase, which is encoded by the *nif*H gene, breaks the N_2 triple bond to reduce N_2 to ammonia (NH_3; Zhang et al., 2006); ii) nitrification: NH_4^+ is converted to NO_3^- via the action of ammonia monooxygenase (*amo*A). This process comprises two phases: oxidation of NH_4^+ to nitrite (NO_2^-) and oxidation of NO_2^- to NO_3^-; iii) denitrification: copper nitrite reductase (*nir*K), iron nitrite reductase (*nir*S) and nitric oxide reductase (*nor*B; Levy-Booth et al., 2014) oxidize NO_3^- successively to NO_2^-, NO and finally N_2O; iv) the only route for converting N_2O to N_2 (Sun et al., 2019) is nitrous oxide reductase (*nos*Z; Henry et al., 2006).

Considering all these aspects, the following hypotheses can be formulated:

1. Forage species with high BNI capacity would increase maize grain yield and ^{15}N recovery in the soil-plant system by suppressing soil nitrification and therefore decreasing fertilizer N losses;
2. Tropical forage grasses would affect N cycle-associated genes and mitigate N_2O emissions in N-fertilized maize;
3. Nitrogen fertilization would increase maize yield, as well as dry matter yield and bromatological quality of forage grasses.

Based on these hypotheses, the objectives of this study were: i) to estimate N_2O, CH_4, and NH_3 emissions in maize-based rotation systems as affected by tropical forage grasses; ii) assess whether rotation with tropical forage grasses influences maize dry matter yield, N accumulation, ^{15}N recovery, and the fate of the N fertilizer in the plant-litter-soil system over two growing seasons; iii) to characterize changes in total bacterial and archaeal abundances and in microbial populations involved in N-fixation (*nif*H), ammonia oxidation (AOA and AOB), and denitrification (*nir*S and *nos*Z) and to measure N_2O emissions in the maize intercropped with forage grasses; and iv) to assess maize grain yield and forage production, bromatological quality and estimated meat production in maize-forage grass intercropped systems.

The first chapter of this thesis, entitled: “Fate of ^{15}N fertilizer applied to maize in rotation with tropical forage grasses” was published in *Field Crops Research*. Chapter 2, entitled: “Effect of tropical grass and nitrogen fertilization on nitrous oxide, methane, and ammonia emissions of maize-based rotation systems” was published in *Atmospheric Environment*; and the third chapter, entitled: “Functional N-cycle genes in soil and N_2O emissions in a maize/tropical forage grasses intercropping system” was recently submitted to *Science of the Total Environment*. Lastly, chapter 4, entitled “Bromatological quality and estimated meat production in maize intercropping with tropical forage grasses with N fertilization” will be submitted in due course to *Grass and Forage Science*.

CHAPTER 1

FATE OF ^{15}N FERTILIZER APPLIED TO MAIZE IN ROTATION WITH TROPICAL FORAGE GRASSES

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ABSTRACT

Tropical forage grasses of the genus *Megathyrsus* and *Urochloa* can suppress soil–nitrification by releasing inhibitory substances, reducing N losses and increasing fertilizer N recovery of the cash crop in rotation. In contrast, ruzigrass (*Urochloa ruziziensis*) has been reported to decrease the yield and N accumulation of the subsequent crop and hence can affect N use efficiency and the fate of applied N. We investigated the effects of Guinea grass (*M. maximum*), palisade grass (*U. brizantha*), and ruzigrass on succeeding crop yield, N accumulation, and the fate of ^{15}N–labeled fertilizer applied to maize (*Zea mays* L.) in a 2–year field experiment in Brazil. Maize was fertilized with 140 kg ha^{-1} N as $(^{15}NH_4)_2SO_4$ or not fertilized, and recovery of residual ^{15}N was quantified in the second season. Net nitrification rates through an incubation study had no differences among grasses. Nitrogen application increased maize yield and N accumulation in both seasons, whereas maize yield decreased by 9.5% following ruzigrass compared with the other forages. The grasses had no effect on ^{15}N recovery by maize or in the system. On average, the recovery of ^{15}N in maize and soil was 34% and 46% in the first growing season and 2.9% and 20% in the second season, respectively. Our results indicated that tropical perennial grasses had no differential effects on nitrification rates and the fate of ^{15}N–labeled fertilizer in the plant–litter–soil system in the season of application nor in the subsequent crop (residual effect).

Keywords**:** *Zea mays* L.; Brachiaria; ^{15}N; nitrogen uptake efficiency; soil N loss.

1.1 Introduction

The benefits of no–till over conventional tillage systems include improvements in chemical, biological, and physical soil properties, such as increased C sequestration, microbial activity, and water and nutrient availability and reduced CO_2 emissions, soil erosion, and weed incidence (Lal et al., 2007). These benefits are attained by growing crops without disturbing the soil and by maintaining plant residues on the soil surface. In addition, eliminating bare fallow periods in favor of growing leguminous or non–leguminous cover crops is a widely recognized method for increasing soil C stocks and improving nutrient cycling (Tonitto et al, 2006). Forage grasses grown as cover crops in the off–season have been also successfully used in integrated crop–livestock systems (Moraes et al., 2014).

Leguminous cover crops can increase soil N supply through biological N fixation (Baligar and Fageria, 2007), but the rapid decomposition of their residues in tropical regions (Thomas and Asakawa, 1993) is a drawback in terms of soil protection. Therefore, non–leguminous species such as tropical perennial grasses have been introduced to increase the amount and persistence of the litter layer over the soil surface. In tropical Brazil, Guinea grass (*Megathyrsus maximum* cv. Tanzânia; syn. *Panicum maximum* cv. Tanzânia), palisade grass (*Urochloa brizantha* cv. Marandu; syn. *Brachiaria brizantha* cv. Marandu), andruzigrass (*U*. *ruziziensis* cv. Comum; syn. *B. ruziziensis* cv. Comum) are the main forage grass species used as cover crops in the off–season (April–September), while soybean [*Glycine max* (L.) Merrill] and maize (*Zea mays* L.) are the typical cash crops grown in summer (October–March). These tropical grasses have good drought tolerance during fall and winter due to their deep root systems (Fisher et al., 1994).

Several earlier studies reported that forage grasses from the genus *Urochloa*, especially *U. humidicola* (syn. *B. humidicola*), can suppress soil nitrification through the exudation of inhibitory substances (Subbarao et al., 2012; 2015). Inhibition of soil nitrification has been proposed as a practical way to decrease environmental pollution caused by N fertilization (e.g., denitrification and NO_3^- leaching) and to improve N uptake and fertilizer N recovery, primarily for plants preferring NH_4^+ over NO_3^- (Subbarao et al., 2012). Despite the potential benefits of *U. humidicola* for plant N acquisition and reduced N loss to the environment, its cultivation in Brazil is essentially

limited to seasonally flooded soils. However, Guinea grass roots also release moderate amounts of nitrification inhibitors, whereas the suppressive effects of palisade grass seem to be lower (Subbarao et al., 2012). Although the inhibitory effects of ruzigrass on biological nitrification are unknown, adverse effects of its residues on the succeeding crop have been observed, such as lower yields and lower N accumulation (Echer et al., 2012; Souza et al., 2014; Marques et al., 2019). Microbial immobilization of mineral N during decomposition of forage residues and release of allelopathic substances have been suggested to be responsible for this effect (Echer et al., 2012; Souza et al. 2014).

Various field studies have used the ^{15}N method to assess fertilizer N recovery (here termed ^{15}N recovery) by maize in monoculture or intercropped with grass species, and no interference of these forage crops has been confirmed (Coser et al., 2016; Almeida et al., 2017). However, the extent to which previously grown tropical forage grasses influence fertilizer N acquisition by maize and its fate in systems with crop rotation remain unclear. Furthermore, the ability of forage species to alter the recovery of residual fertilizer–derived N in the subsequent maize crop is also poorly understood. We hypothesized that forage species with high biological nitrification inhibition capacity could increase maize grain yield and ^{15}N recovery in the soil–plant system, thus decreasing N fertilizer losses. We aimed to (i) test the nitrification inhibition from Guinea grass, palisade grass, and ruzigrass; and (ii) assess whether rotation with the tropical forage grasses influences maize dry matter yield, N accumulation, ^{15}N recovery, and the fate of ^{15}N–labeled fertilizer in the plant–litter–soil system over two growing seasons.

1.2 Material and Methods

1.2.1 Study site

A rainfed field experiment was conducted in Botucatu, SP, Brazil (22°49’ S, 48°26’ W; 700 m a.s.l.) for two consecutive cropping seasons (October 2015–May 2017) of maize affected by previously grown of tropical grasses. The local soil is a clay Rhodic Hapludox (Soil Survey Staff, 2014), with 190, 196, and 614 g kg^{-1} of sand, silt, and clay, respectively, at a depth of 0–20 cm. The clay fraction has ~70% kaolinite, ~15% gibbsite, and small amounts of vermiculite and illite. The study region typically

experiences dry winters and hot summers, with historical annual average minimum and maximum temperatures of 15.3 and 26.1°C, respectively. The average annual precipitation is 1360 mm. During the first (2015–2016) and second growing seasons (2016–2017), the average annual minimum temperatures were 17.0 and 16.4 °C, and the average annual maximum temperatures were 26.1 and 26.1°C, respectively (Fig. 1). The annual precipitation was 1859 mm and 1683 mm in the first and second seasons, 37% and 24% higher, respectively, than the long–term average (Fig. 1). Maize accumulated 1820 growing–degree days (GDD) and received 74% of the annual precipitation in the first growing season and 1919 GDD and 54% of the annual precipitation in the second season. The weather station used to measure the climate parameters was located 2.6 km from the study site. Prior to the experiment, the basic soil properties at the top 20 cm were: pH 5.9, total C 19 g kg^{-1}, total N 1.3 g kg^{-1}, NH_4^+–N 5.4 mg kg^{-1}, NO_3^-–N 6.4 mg kg^{-1}, P 15 mg dm^{-3}, K 1.3 $mmol_c$ dm^{-3}, Ca 35 $mmol_c$ dm^{-3}, and Mg 24 $mmol_c$ dm^{-3}, H+Al 37 $mmol_c$ dm^{-3}, cation exchange capacity 97 $mmol_c$ dm^{-3}, and base saturation 61%.

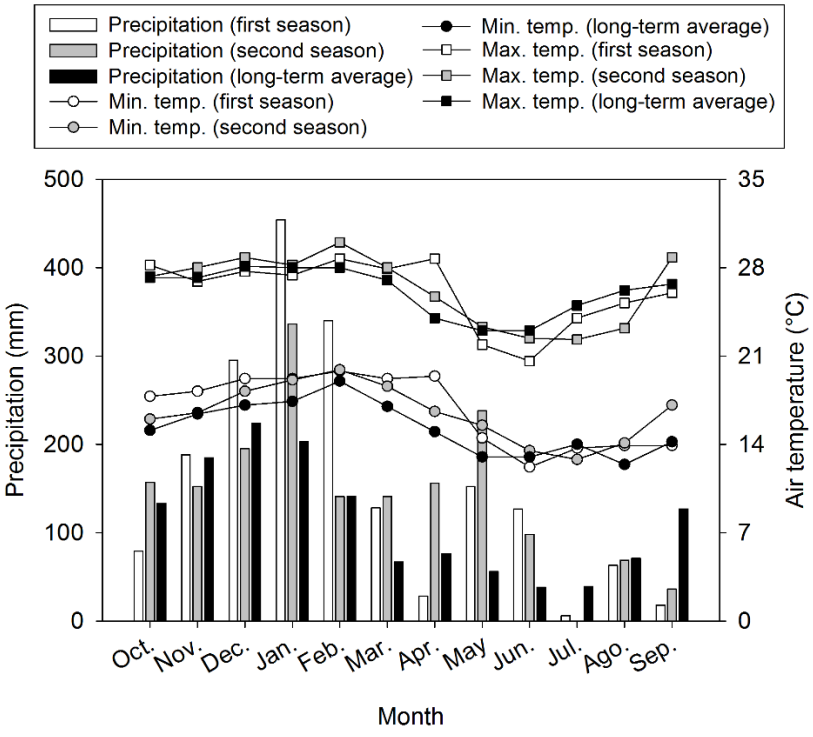

Fig. 1. Monthly precipitation, and average minimum and maximum air temperatures in the first (2015–2016) and second (2016–2017) growing seasons, and long–term average (period 1955–2015).

1.2.2 Study design

The experiment was conducted in split plots arranged in completely randomized blocks, with four replicates. Forage grass species were grown for eleven months (2014–2015) as cover crops, followed by planting of maize over the residues. In 2016, grasses were grown only in the maize off–season, followed by this grain crop. The forage species Guinea grass, palisade grass, and ruzigrass were grown in the main plots, while the subplots were assigned to N fertilization (140 kg ha^{-1} N) or the unfertilized control in maize. The subplots measured 4.5 m ×10 m (Fig. 2). Within each subplot, a microplot was set up to follow the fate of the ^{15}N–labeled fertilizer applied to maize. A static plot design was deployed, with forage species and N fertilization treatments assigned repeatedly to the same plots.

1.2.3 Crop management

Forage grasses were planted in November 2014 using a no–till drill at 7 kg of live seeds ha^{-1} with a row spacing of 0.17 m and no application of fertilizer. The forage grasses were cut twice, in April and June of the following year, at a height of 30 cm. Of the total dry matter yield of Guinea grass, palisade grass, and ruzigrass, 32%, 34%, and 50% was removed through cuts, while N removal was 43%, 45%, and 63% of the total accumulated N, respectively. In September 2015, the forage grasses were terminated using glyphosate (2.9 kg ha^{-1}a.i.) and a mixture of paraquat and diuron (0.6 and 0.3 kg ha^{-1} a.i., respectively). The crop residues were left on the soil surface. Maize (hybrid 2B810PW, Dow AgroSciences, São Paulo, Brazil) was planted in October using the above-mentioned drill at a row spacing of 0.75 m to achieve a final stand of 65,000 plants ha^{-1}. The hybrid used is glyphosate–resistant and insect–tolerant. Each main plot received 53 kg ha^{-1} P as triple superphosphate and 100 kg ha^{-1} K as potassium chloride at planting. The N fertilizer (granular ammonium sulfate) application was split twice, 30 kg ha^{-1} N at planting and 110 kg ha^{-1} N topdressed at growth stage V5 (five leaves with visible leaf collars). The topdressed N fertilizer was hand–applied to the soil surface in single–side banding (3 cm wide), ~5 cm from the crop row. The crop was hand–harvested in April 2016, and the maize stover (leaves, stems, and

cobs) was left in the field. Due to unfavorable climatic conditions after the first maize harvest, forage grasses were planted in October of the second season and desiccated 60 d after plant emergence. The forage grasses were not cut in the second season because growth was much less than in the first season. Maize (cv. hybrid 2B587PW, Dow AgroSciences, São Paulo, Brazil) was planted in December 2016. Apart from the maize cultivar, all agricultural practices (row spacing, plant density, and rate and timing of NPK fertilizers) were the same as in the first growing season. The maize was harvested in May of the following year.

1.2.4 Soil nitrification assessed by laboratory incubation

To assess the influence of the forage grasses in the microbial oxidation of NH_4^+ over NO_3^-, a soil incubation study was performed. Soil samples at the 0–20 cm were taken before maize planting in 2015 and 2016, oven–dried at 40°C to constant weight, and ground (< 2 mm mesh sieve). Two subsamples of 7 g of dry soil were transferred to 50–mL polypropylene centrifuge tubes and rewetted to 65% of water–holding capacity (Mariano et al., 2017). Soil samples were pre–incubated at 25°C for 10 d to decrease the mineral N flush. One subsample received 500 µL of 71 mM $(NH_4)_2SO_4$ (140 µg N g^{-1}) and was reincubated, while the other (untreated soil) was shaken with 2 M KCl (at a soil:solution ratio of 1:5; w/v) on an orbital shaker (200 rev min^{-1}, 1 h). The supernatant was filtered using No. 42 filter paper, and NO_3^-–N content at zero–time was determined by colorimetry (Miranda et al., 2001). Ten days following N addition, soil samples were extracted and analyzed for NO_3^- as above. Net nitrification rate was calculated by subtraction of NO_3^-–N of treated from untreated samples, divided by the incubation period.

1.2.5 ^{15}N microplots

Unconfined microplots measuring 2.25 m × 1.50 m were set up in each N–fertilized (140 kg ha^{-1} N) subplot in the first growing season (Fig. 2). All agricultural factors in the microplots matched those of the subplot. Each microplot contained three rows of maize, with seven plants in each row. ^{15}N–labeled ammonium sulfate [$(^{15}NH_4)_2SO_4$] with an abundance of 4.5 atom % ^{15}N excess was obtained from Sigma–

Aldrich Inc. (St. Louis, MO, USA). Powder ^{15}N–labeled fertilizer was applied at planting (30 kg ha^{-1} N) and as topdressing (110 kg ha^{-1} N) at the V5 stage of the maize. In the second growing season, unlabeled ammonium sulfate was applied on the microplots to assess the recovery of residual ^{15}N–labeled fertilizer from the first season. All other agricultural practices remained the same as in the first season.

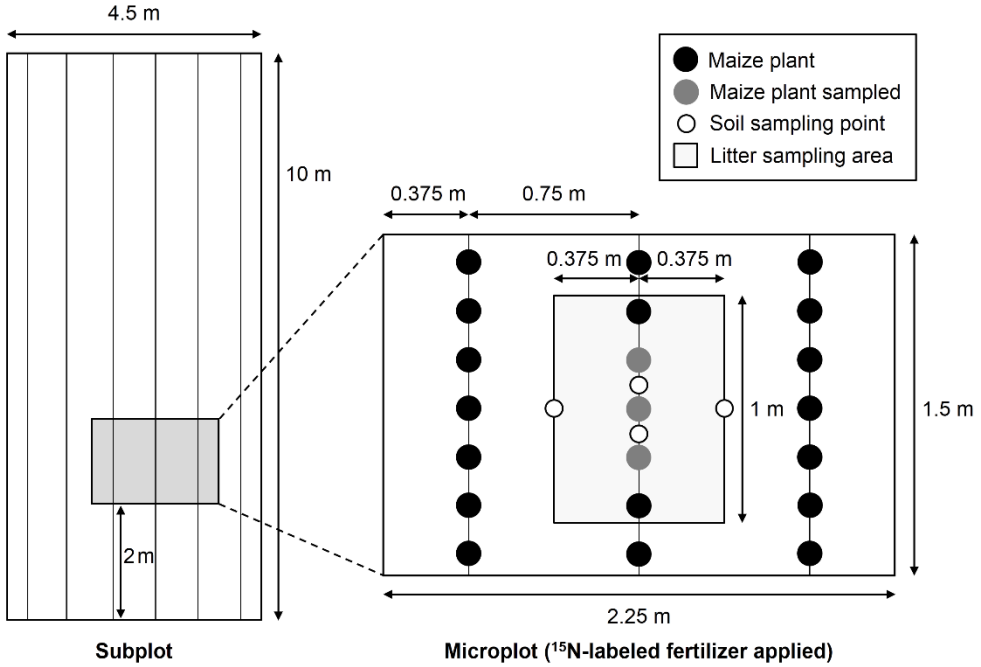

Fig. 2. Schematic representation of the whole plot and its microplot (deployed exclusively in N–fertilized treatments), in addition to the sampling procedures of plant, litter, and soil. Fertilizer N was applied over the litter layer in single–side bandings, ~3 cm from the maize row.

1.2.6 Sampling procedure and ^{15}N analyses

The plant, litter, and soil sampling procedures were the same in both growing seasons. At physiological maturity (R6 growth stage), three maize plants in the middle of each microplot were clipped at the stem base. The plants were partitioned into grains, cob cores, stem, and leaves (including sheaths). Fresh samples were oven–dried at 65°C to constant weight to assess the dry weight. The dry biomass was ground in a Wiley mill and passed through a 0.50–mm sieve for total N concentration and ^{15}N measurements. The cob cores were added to the stem and leaves fraction to form the stover sample. Three plants from each control plot were randomly harvested and subjected to the protocol described above to assess the natural ^{15}N abundance. The litter on the soil surface of each microplot was also sampled. All litter biomass (forage residues from the first growing season and forage plus maize residues from the second season) found in a central area of 0.75 m^2 (0.75 m × 1.00 m; Fig. 2) of each microplot was collected and weighted. A similar protocol was used for the control plots. A subsample of the litter biomass was oven–dried at 65°C for dry weight, ground in a

Wiley mill, and passed through a 0.50–mm sieve for ^{15}N analysis. The remaining fresh litter was returned to the field.

Soil samples were taken using a core sampler at depths of 0–10, 10–20, and 20–40 cm from four points in each microplot: (i) two samples from the central maize row, which received ^{15}N–labeled fertilizer and (ii) two samples from the middle of the two outer maize rows (Fig. 2). Samples from the same depth and sampling position were combined (n = 2), oven–dried at 40°C, ground in a ball mill, and passed through a 0.0059mm sieve (equivalent to 100 mesh) for total N concentration and ^{15}N analyses. To estimate the soil N accumulation, the soil bulk density at each soil depth and position was assessed using the volumetric ring method (Blake and Hartge, 1986) after the maize harvest. The natural ^{15}N abundance in the soil was also measured.

The grain, stover, litter, and soil samples were analyzed for total N concentration and ^{15}N abundance using an automatic N analyzer (PDZ Europa ANCA–GSL, Sercon Ltd., Crewe, UK) interfaced with an isotope ratio mass spectrometer (PDZ Europa 20–20, Sercon Ltd., Crewe, UK).

1.2.7 Calculations and statistical analysis

The grain harvest index (HI) and N harvest index (NHI) of the maize were calculated as follows:

$$\mathrm{HI} = \left(\mathrm{DM_G}/\mathrm{DM_S}\right)\times 100 \quad (1)$$

$$\mathrm{NHI} = \left(\mathrm{NC_G}/\mathrm{NC_S}\right)\times 100 \quad (2)$$

in which *HI* is the grain harvest index; DM_G and DM_S are the grain and shoot dry matter (kg ha^{-1}), respectively; *NHI* is the N harvest index; and NC_G and NC_S are the N accumulation (kg ha^{-1}) in the grains and shoots, respectively.

The amount of N derived from fertilizer (Ndff), ^{15}N recovery in maize, litter, and soil, and unrecovered N were calculated using the following equations:

$$\mathrm{Ndff\ (kg\ ha^{-1})} = \left(\mathrm{a}/\mathrm{b}\right)\times \mathrm{NC} \quad (3)$$

$$^{15}\mathrm{N\ recovery\ (\%)} = \left(\mathrm{Ndff}/\mathrm{FNR}\right)\times 100 \quad (4)$$

$$\mathrm{Unrecovered\ N_{FS}\ (\%)} = 100 - {}^{15}\mathrm{N\ recovery_{TFS}} \quad (5)$$

$$\mathrm{Unrecovered\ N_{SS}\ (\%)} = \left\{\left[\left(\mathrm{Ndff_{TFS}} - \mathrm{Ndff_{GFS}}\right) - \mathrm{Ndff_{TSS}}\right]/\mathrm{FNR}\right\}\times 100 \quad (6)$$

where *Ndff* is the N derived from fertilizer; *a* and *b* are the ^{15}N enrichment (atom % ^{15}N excess) in the product (plant, litter, or soil) and substrate (fertilizer), respectively, both obtained by deducting the natural abundance (~0.368 atom % ^{15}N); *NC* is the N accumulation (kg ha^{-1}) in the product; ^{15}*N recovery* is the percentage of fertilizer N recovery; *FNR* is the fertilizer N rate applied (kg ha^{-1}); *Unrecovered* N_{FS} and *Unrecovered* N_{SS} are the percentage of fertilizer N unaccounted for (i.e., potential losses) in the first and second growing seasons after application of ^{15}N–labeled fertilizer, respectively; $^{15}N\ recovery_{TFS}$ is the total N recovery (%; sum of plant, litter and soil) in the first growing season; $Ndff_{TFS}$ and $Ndff_{GFS}$ are the N derived from fertilizer (kg ha^{-1}) in the plant–litter–soil system and grains, respectively, in the first growing season; and $Ndff_{TSS}$ is the N derived from fertilizer (kg ha^{-1}) in the plant–litter–soil system in the second growing season.

Generalized linear models were performed using the GLM procedure of SAS (version 9.3, SAS Institute, Inc., Cary, NC, USA). Block, forage grass, and N fertilization were considered fixed effects. Net nitrification rate, dry matter yield, and N accumulation of forage in the first and second growing seasons was subjected to one–way (effect of forage grass) and split plot (effect of forage grass and N fertilization) ANOVA, respectively. Splitplot ANOVA was conducted for dry matter yield and N accumulation of maize and litter, in addition to maize harvest indices (HI and NHI). The Ndff, ^{15}N recovery, and unrecovered N results were subjected to one–way ANOVA. Fisher's least significant difference (LSD) test was used to compare least square means through the LS MEANS statement. Statistical significance is reported at the 5% level of significance.

1.3 Results

1.3.1 Net nitrification rates

Net nitrification rate of soils sampled before maize planting did not differ among forage grasses in the first growing season, while nitrification increased by 34% following N fertilizer application relative to the control in the subsequent season (Table 1).

Table 1. Net nitrification rate from 10–d incubation of soil samples taken at the 0–20 cm depth layer before maize planting in the first (2015–2016) and second (2016–2017) growing season, in which the latter was also affected by fertilizer N rate. Values represent means ± SEM (*n* = 8 for the main effect of forage grass; *n* = 12 for the main effect of N rate; and *n* = 4 for the interaction between the forage grass and N rate).

Growing season	**Forage grass**	**N rate** kg ha^{-1}	**Net nitrification rate** mg NO_3^-–N kg^{-1} d^{-1}
First			
	Guinea grass	Control	3.2 ± 0.2
	Palisade grass	Control	3.2 ± 0.3
	Ruzigrass	Control	3.5 ± 0.5
			P = 0.763
Second			
	Guinea grass	–	2.7 ± 0.4
	Palisade grass	–	2.2 ± 0.3
	Ruzigrass	–	2.6 ± 0.5
			P = 0.788
	–	Control	2.2 ± 0.2b
	–	140	2.9 ± 0.4a
			P = 0.050
	Guinea grass	Control	2.5 ± 0.6
	Palisade grass	Control	1.8 ± 0.1
	Ruzigrass	Control	2.2 ± 0.5
	Guinea grass	140	2.9 ± 0.7
	Palisade grass	140	2.7 ± 0.6
	Ruzigrass	140	3.0 ± 1.0
			P = 0.800

Means followed by a common letter within a column are not significantly different by the LSD–test at the 5% level of significance.

1.3.2 Forage yield and N accumulation

In the first growing season, the dry matter yield of palisade grass was 17% higher than that of the other forage grass species on average (Table 2). Nitrogen accumulation followed the results observed for dry matter yield and was highest for Guinea grass and palisade grass. In the second growing season, the dry matter yield and N accumulation of ruzigrass were 42% and 47% higher than those of palisade grass, respectively (Table 2).

Table 2. Dry matter yield and N accumulation of forage grass species grown before maize in the first (2015–2016) and second (2016–2017) growing season, in which the latter was also affected by fertilizer N rate.

Growing season	Forage grass	N rate kg ha^{-1}	Dry matter yield Mg ha^{-1}	N accumulation kg ha^{-1}
First				
	Guinea grass	Control	11.7 ± 0.7b	118 ± 9a
	Palisade grass	Control	13.1 ± 0.5a	111 ± 12a
	Ruzigrass	Control	10.6 ± 0.2b	80 ± 5b
			P= 0.008	*P* = 0.006
Second				
	Guinea grass	–	4.7 ± 0.6ab	48 ± 6ab
	Palisade grass	–	4.2 ± 0.6b	38 ± 6b
	Ruzigrass	–	6.3 ± 0.7a	63 ± 8a
			P = 0.041	*P* = 0.028
	–	Control	5.1 ± 0.5	51 ± 5
	–	140	5.0 ± 0.7	48 ± 7
			P = 0.892	*P* = 0.757
	Guinea grass	Control	4.6 ± 0.9	51 ± 9
	Palisade grass	Control	4.6 ± 0.5	42 ± 1
	Ruzigrass	Control	6.1 ± 1.1	61 ± 11
	Guinea grass	140	4.8 ± 0.9	46 ± 8
	Palisade grass	140	3.7 ± 1.2	34 ± 12
	Ruzigrass	140	6.5 ± 1.1	65 ± 14
			P = 0.861	*P* = 0.898

Means followed by a common letter within a column are not significantly different by the LSD–test at the 5% level of significance. Values represent means ± SEM (*n* = 8 for the main effect of forage grass; *n* = 12 for the main effect of N rate; and *n* = 4 for the interaction between the forage grass and N rate).

1.3.3 Maize yield and N accumulation

The maize grain yield was 11% higher in succession to Guinea grass and palisade grass compared with ruzigrass in the first season, on average (Table 3). Grain and stover yield increased by 206% and 84%, respectively, following fertilizer N addition (140 kg ha^{-1} N)compared with the unfertilized control. An interaction of grass species with the N rate was observed for maize shoot biomass, which was greatest following palisade grass with N application and lowest following ruzigrass without N addition. Litter biomass was not affected by forage or N fertilization. However, HI

increased by 32% following N fertilization over the unfertilized control. In the second season, maize (grain, stover, and shoots) and litter yields and HI increased in response to N fertilization, and there was no effect of grass species (Table 3). Nitrogen accumulation in maize grain, stover, shoots, and NHI increased by 239%, 95%, 179%, and 22%, respectively, following fertilizer N compared with the unfertilized control in the first growing season (Table 4). In the second season, similar to the effects on dry matter yield, N accumulation in maize and litter increased following fertilizer N addition but had no effect on N accumulation in forage grasses (Table 4).

Table 3. Maize (partitioned into grains and stover) and litter dry matter yield and harvest index (HI) as affected by forage grass and fertilizer N rate in the first (2015–2016) and second (2016–2017) growing season. Values represent means ± SEM (*n* = 8 for the main effect of forage grass; *n* = 12 for the main effect of N rate; and *n* = 4 for the interaction between the forage grass and N rate).

Growing season	Forage grass	N rate	Dry matter yield (Mg ha^{-1})				HI
		kg ha^{-1}	Grains	Stover	Shoots	Litter	
First							
	Guinea grass	–	7.1 ± 1.2a	8.0 ± 0.9	15.1 ± 2.0	3.8 ± 0.3	0.46 ± 0.03
	Palisade grass	–	7.1 ± 1.4a	8.4 ± 1.1	15.5 ± 2.5	5.1 ± 0.3	0.43 ± 0.03
	Ruzigrass	–	6.4 ± 1.3b	8.0 ± 1.0	14.4 ± 2.3	4.7 ± 0.4	0.42 ± 0.03
			P = 0.016	*P* = 0.737	*P* = 0.291	*P* = 0.078	*P* = 0.226
	–	Control	3.4 ± 0.2b	5.7 ± 0.4b	9.1 ± 0.4b	4.5 ± 0.3	0.37 ±
	–	140	10.4 ± 0.2a	10.5 ± 0.3a	20.9 ± 0.4a	4.6 ± 0.3	0.50 ±
			P< 0.001	*P*< 0.001	*P*< 0.001	*P* = 0.710	*P*< 0.001
	Guinea grass	Control	4.0 ± 0.2	6.1 ± 1.0	10.0 ± 1.0c	4.1 ± 0.5	0.41 ± 0.03
	Palisade grass	Control	3.3 ± 0.1	5.6 ± 0.6	9.0 ± 0.6cd	5.1 ± 0.6	0.37 ± 0.02
	Ruzigrass	Control	2.9 ± 0.1	5.5 ± 0.3	8.3 ± 0.3d	4.3 ± 0.6	0.35 ± 0.01
	Guinea grass	140	10.3 ± 0.3	9.9 ± 0.4	20.1 ± 0.3b	3.5 ± 0.3	0.51 ± 0.02
	Palisade grass	140	10.9 ± 0.3	11.2 ± 0.6	22.1 ± 0.5a	5.2 ± 0.2	0.49 ± 0.02
	Ruzigrass	140	10.0 ± 0.2	10.5 ± 0.4	20.5 ± 0.6ab	5.2 ± 0.4	0.49 ± 0.01
			P = 0.053	*P* = 0.116	*P* = 0.046	*P* = 0.315	*P* = 0.411
Second							
	Guinea grass	–	6.2 ± 0.8	8.5 ± 1.0	14.8 ± 1.9	6.7 ± 0.8	0.43 ± 0.02
	Palisade grass	–	6.6 ± 1.0	8.5 ± 1.1	15.1 ± 2.0	6.6 ± 0.8	0.43 ± 0.01
	Ruzigrass	–	6.2 ± 1.1	8.6 ± 1.3	14.7 ± 2.4	5.8 ± 0.7	0.41 ± 0.02

			P = 0.593	*P* = 0.996	*P* = 0.963	*P* = 0.420	*P* = 0.996
	–	Control	4.0 ± 0.3b	6.2 ± 0.5b	10.2 ± 0.8b	5.0 ± 0.4b	0.40 ±
	–	140	8.6 ± 0.4a	10.9 ± 0.6a	19.6 ± 1.0a	7.7 ± 0.6a	0.45 ±
			P< 0.001	*P*< 0.001	*P*< 0.001	*P* = 0.005	*P* = 0.003
	Guinea grass	Control	4.2 ± 0.6	6.3 ± 1.0	10.4 ± 1.6	5.0 ± 0.5	0.40 ± 0.01
	Palisade grass	Control	4.4 ± 0.7	6.2 ± 0.8	10.6 ± 1.5	5.5 ± 1.1	0.41 ± 0.01
	Ruzigrass	Control	3.5 ± 0.3	6.0 ± 1.2	9.6 ± 1.4	4.5 ± 0.4	0.41 ± 0.01
	Guinea grass	140	8.3 ± 0.3	10.7 ± 0.8	19.1 ± 1.0	8.4 ± 1.1	0.46 ± 0.02
	Palisade grass	140	8.8 ± 0.8	10.8 ± 1.1	19.6 ± 1.8	7.7 ± 1.0	0.45 ± 0.02
	Ruzigrass	140	8.8 ± 1.0	11.1 ± 1.5	19.9 ± 2.4	7.0 ± 1.0	0.45 ± 0.01
			P = 0.696	*P* = 0.944	*P* = 0.871	*P* = 0.775	*P* = 0.662

Means followed by a common letter within a column are not significantly different by the LSD–test at the 5% level of significance.

Table 4. Maize (partitioned into grains and stover) and litter N accumulation and N harvest index (NHI) as affected by forage grass and fertilizer N rate in the first (2015–2016) and second (2016–2017) growing season. Values represent means ± SEM (*n* = 8 for the main effect of forage grass; *n* = 12 for the main effect of N rate; and *n* = 4 for the interaction between the forage grass and N rate).

Growing season	Forage grass	N rate	N accumulation (kg ha^{-1})				NHI
		kg ha^{-1}	Grains	Stover	Shoots	Litter	
First							
	Guinea grass	–	70 ± 13	31 ± 4	101 ± 16	46 ± 7	0.67 ± 0.02
	Palisade grass	–	68 ± 15	32 ± 4	100 ± 19	67 ± 9	0.64 ± 0.03
	Ruzigrass	–	63 ± 14	32 ± 5	96 ± 19	48 ± 6	0.63 ± 0.03
			P = 0.118	*P* = 0.881	*P* = 0.524	*P* = 0.239	*P* = 0.319
	–	Control	30 ± 2b	22 ± 1b	52 ± 2b	52 ± 8	0.59 ± 0.02b
	–	140	103 ± 2a	42 ± 2a	146 ± 3a	56 ± 5	0.71 ± 0.01a
			P< 0.001	*P*< 0.001	*P*< 0.001	*P* = 0.581	*P*< 0.001
	Guinea grass	Control	37 ± 2	23 ± 4	59 ± 5	48 ± 14	0.62 ± 0.03
	Palisade grass	Control	29 ± 1	22 ± 2	51 ± 2	66 ± 18	0.56 ± 0.03
	Ruzigrass	Control	26 ± 1	20 ± 1	46 ± 1	41 ± 7	0.57 ± 0.03
	Guinea grass	140	103 ± 4	40 ± 2	142 ± 5	45 ± 7	0.72 ± 0.01
	Palisade grass	140	106 ± 5	42 ± 4	148 ± 8	68 ± 9	0.72 ± 0.01
	Ruzigrass	140	101 ± 2	45 ± 3	146 ± 4	55 ± 8	0.69 ± 0.01
			P = 0.251	*P* = 0.302	*P* = 0.279	*P* = 0.607	*P* = 0.418
Second							
	Guinea grass	–	96 ± 18	53 ± 11	149 ± 29	43 ± 6	0.66 ± 0.01
	Palisade grass	–	95 ± 19	39 ± 6	133 ± 25	42 ± 7	0.69 ± 0.02
	Ruzigrass	–	81 ± 16	41 ± 6	123 ± 21	37 ± 5	0.65 ± 0.03
			P = 0.052	*P* = 0.136	*P* = 0.104	*P* = 0.758	*P* = 0.196

	–	Control	47 ± 4b	27 ± 3b	74 ± 6b	31 ± 3b	0.65 ± 0.02b
	–	140	135 ± 6a	62 ± 5a	196 ± 10a	51 ± 4a	0.70 ± 0.01a
			P< 0.001	*P*< 0.001	*P*< 0.001	*P* = 0.001	*P* = 0.009
	Guinea grass	Control	49 ± 8	28 ± 6	78 ± 14	32 ± 5	0.64 ± 0.01
	Palisade grass	Control	50 ± 8	26 ± 4	76 ± 12	33 ± 8	0.66 ± 0.01
	Ruzigrass	Control	41± 4	27 ± 6	68 ± 7	28 ± 3	0.66 ± 0.01
	Guinea grass	140	143 ± 6	78 ± 9	221 ± 15	54 ± 8	0.67 ± 0.02
	Palisade grass	140	139 ± 16	52 ± 8	191 ± 22	52 ± 9	0.73 ± 0.02
	Ruzigrass	140	122 ± 8	56 ± 2	177 ± 10	47 ± 7	0.68 ± 0.01
			P = 0.825	*P* = 0.232	*P* = 0.576	*P* = 0.901	*P* = 0.351

Means followed by a common letter within a column are not significantly different by the LSD–test at the 5% level of significance.

1.3.4 Distribution and fate of ^{15}N–labeled fertilizer applied to maize

In the first season after application of ^{15}N–labeled fertilizer, Ndff in maize and litter did not differ in response to the preceding grass species (Fig. 3). Overall, 21%, 65%, and 33% of the N in maize grain, stover, and shoots, respectively, was derived from fertilizer. Of the total ^{15}N detected in maize shoots (48 kg ha^{-1}, on average), 43% was in grain, and the remainder was in stover. In the litter, only 10% of the N was derived from ^{15}N–labeled fertilizer. Comparable results were observed in the second season, with no effect of forage species on maize and litter Ndff (Fig. 3). Overall, of the total N found in maize grain, stover, and shoots, 2.2%, 1.9%, and 2.0%, respectively, was derived from the residual fertilizer applied in the previous year. In contrast to the first season, most of the ^{15}N accumulated in maize shoots (73%, on average) was found in grains rather than in stover. In the litter, 4.1% of the N was derived from the fertilizer. There was no difference in soil Ndff among the forage grasses (Fig. 3). Of the 140 kg ha^{-1} N applied in the first season, on average 64 kg ha^{-1} was found in the soil profile of 0 to 40 cm, of which 69% was in the topsoil (0–10 cm), 17% was in the 10–20 cm layer, and 14% was in the 20–40 cm layer (Fig. 4). Similarly, most of the residual ^{15}N found in the 0–40 cm soil profile (29 kg ha^{-1}, on average) at harvest in the second season was recovered from the upper layer (0–10 cm; Fig. 4).

There were no differences among the forage grass species in the amount of ^{15}N recovered in maize, soil, and total N in the first season (Fig. 5). However, ^{15}N recovery from litter was 33% higher for palisade grass than for the other forage grasses. The average ^{15}N recovery in maize, litter, and soil was 35%, 4%, and 46%, respectively, whereas 15% (21 kg ha^{-1}) was unaccounted for (unrecovered N). Similarly, in the second season, forage grasses did not affect ^{15}N recovery in maize (grain, stover, and shoots), litter, soil, and total N (Fig. 5). Overall, 2.9%, 1.5%, and 20% of the ^{15}N–labeled fertilizer applied in the first growing season was recovered from maize shoots, litter, and soil, respectively, whereas 43% (60 kg ha^{-1}) was unaccounted for in the plant–litter–soil system.

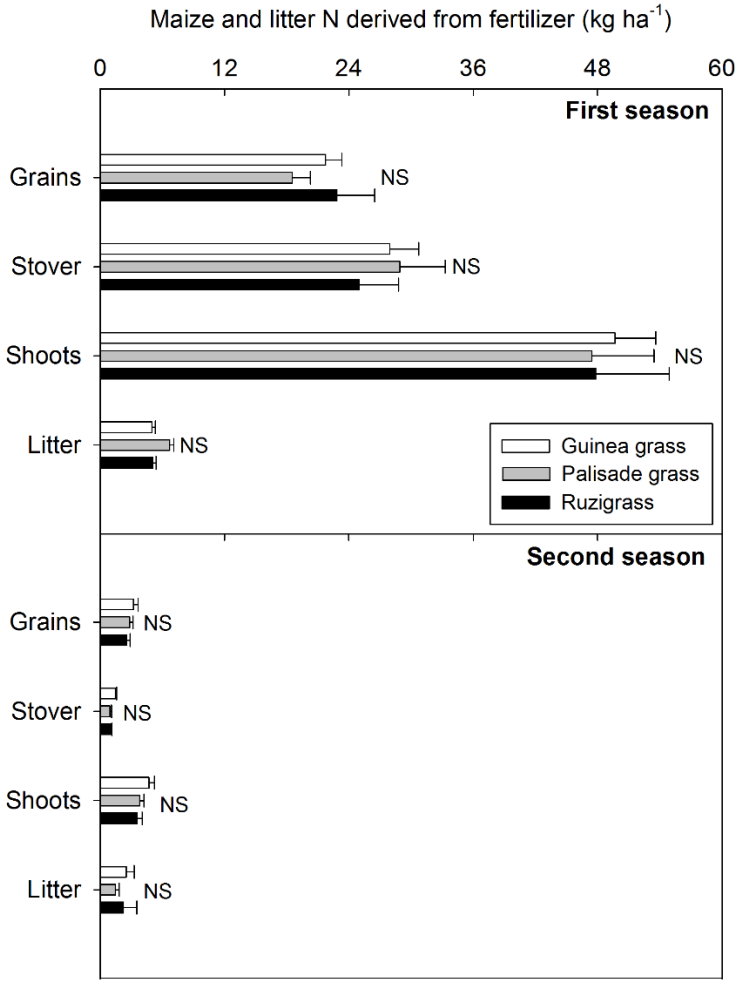

Fig. 3. Maize (partitioned into grains and stover) and litter N derived from fertilizer as affected by forage grass in the first (2015–2016) and second (2016–2017) growing season after application of ^{15}N–labeled fertilizer. The error bars indicate the SEM (*n* = 4). NS: no significantly differences between forage grasses by the LSD–test at the 5% level of significance.

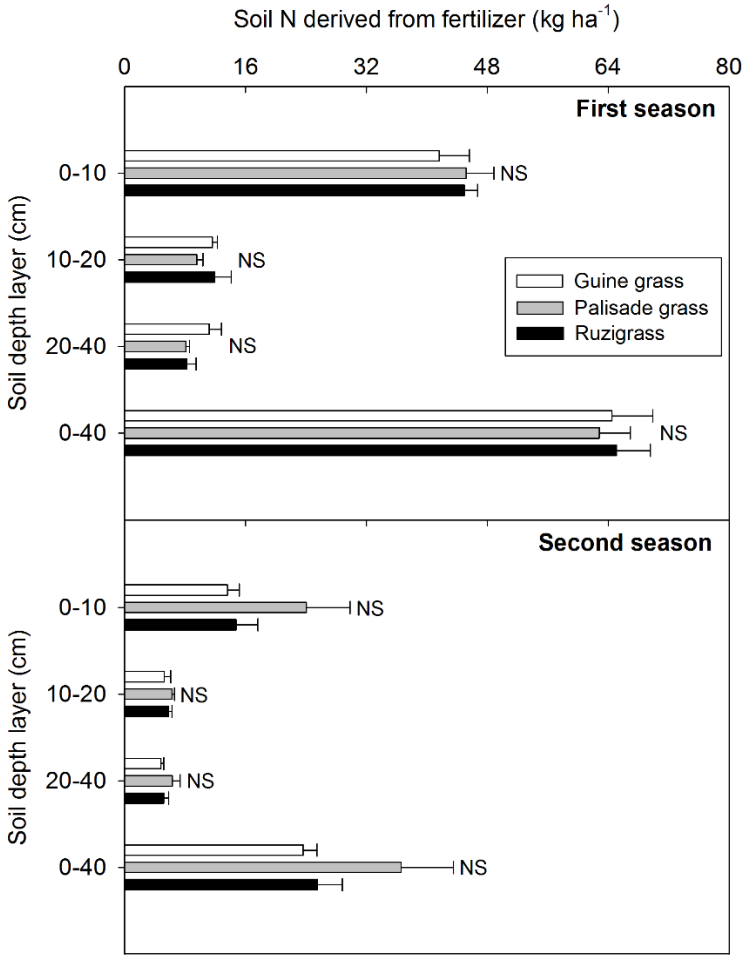

Fig. 4. Soil N derived from fertilizer at four depth layers as affected by forage grass in the first (2015–2016) and second (2016–2017) growing season after application of ^{15}N–labeled fertilizer. The error bars indicate the SEM (*n* = 4). NS: no significantly differences between forage grasses by the LSD–test at the 5% level of significance.

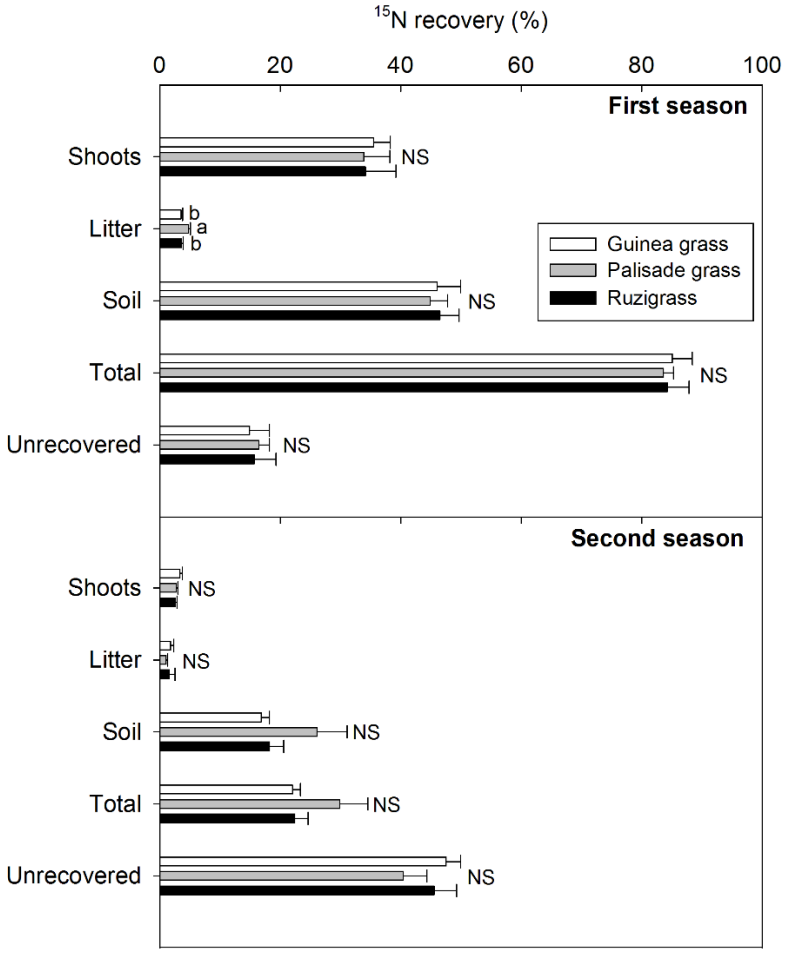

Fig. 5. The fate of ^{15}N–labeled fertilizer in plant–litter–soil system as affected by forage grass in the first (2015–2016) and second (2016–2017) growing season after application of ^{15}N–labeled fertilizer. The error bars indicate the SEM (*n* = 4). Means followed by a common letter are not significantly different, while NS indicates no significantly differences between forage grasses, both by the LSD–test at the 5% level of significance.

1.4 Discussion

1.4.1 Dry matter yield and N accumulation

The dry matter yield of the three grass species in the first and second growing seasons was within the range of 5.3 to 13.1 Mg ha^{-1} reported in other studies (Borghi et al., 2013; Pacheco et al., 2017; Marques et al., 2019), whereas N accumulation was lower than that reported by Marques et al. (2019). The wide variability of forage yields among these studies is directly related to the planting time (in–season and off–

season), duration of the growth cycle, and climatic conditions. Hence, the much lower forage dry matter yield recorded in the second season compared to the previous year (57% lower, on average) is explained by the short period of forage growth and the significant drought period during fall and winter.

The lower maize grain yield following ruzigrass compared with Guinea grass and palisade grass in the first growing season is congruent with the findings of Marques et al. (2019). Two alternative hypotheses have been suggested to explain this observation: (i) a decrease in N availability to maize arising from high microbial N immobilization in the soil due to crop residues of ruzigrass (Echer et al., 2012); and (ii) allelopathic suppression due to secondary metabolites of forage grasses that inhibit maize growth (Weston and Duke, 2003; Souza et al., 2014). However, it is virtually impossible to separate allelopathic interferences from interferences by competition (i.e., utilization or competition for space, light, nutrients, and moisture) under field conditions (Weston and Duke, 2003). In addition to the lower grain yield, the lowest maize shoot biomass following ruzigrass in the unfertilized control is further evidence of the adverse effect of this grass species. The lack of an effect of forage grass species on maize yield and shoot biomass in the second growing season probably reflects the low dry matter yield of the forage crops.

Increased dry matter yield and N accumulation by maize is a well–known and documented effect of N fertilizer application (Setiyono et al., 2010; Ciampitti and Vyn, 2012). In an extensive review, Ciampitti and Vyn (2012) reported that the average (n = 2074) shoot biomass production and N accumulation of modern maize hybrids at physiological maturity were 18 Mg ha^{-1} and 170 kg ha^{-1}, respectively. The lack of an effect of forage species and N fertilization on litter dry matter yield and N accumulation in the first growing season may be attributed to the following mechanisms: (i) the narrow dry matter yield range (10.6–13.1 Mg ha^{-1}) after desiccation; and (ii) the low amount of fertilizer–N retained in the litter layer following maize fertilization, which could lead to faster litter decomposition (Kuzyakov et al., 2000) in the case of significant retention. However, the higher dry matter yield and N accumulation in the litter with N fertilizer application in the subsequent season were due to the inclusion of maize stover from the previous season as a major component of the total litter biomass. In the first season, the dry matter yield and N accumulation of stover at harvest were, on average, 54% and 51% higher in the N–fertilized treatments than in the control, respectively, supporting this hypothesis. The average values of 0.43 for HI and 0.60

for NHI obtained in this study are within the ranges reported for modern maize hybrids (Setiyono et al., 2010; Ciampitti and Vyn, 2012). The lower HI and NHI values in the control treatments compared with N fertilization are associated with suboptimal N rates, which result in reduced grain filling and lower N remobilization from the stover to ears during the critical phases bracketing the silking period (Setiyono et al., 2010).

1.4.2 Distribution of ^{15}N–labeled fertilizer

In the first growing season, the Ndff in maize shoots (48 kg ha^{-1}, on average) was within the range of 34 to 64 kg ha^{-1} found in previous studies (Coelho et al., 1991; Schindler and Knighton, 1999; Gava et al., 2006; Liu et al., 2015). The lower percentage of Ndff in grains (43%, on average) compared with NHI (0.60, considering the N–fertilized treatments) was probably due to partial ^{15}N remobilization from other sources and/or plant N uptake at later stages (e.g., post–silking), which is typical for modern maize hybrids (Ciampitti and Vyn, 2012). In line with previous studies (Gava et al., 2006; Dourado–Neto et al., 2010; Wang et al., 2016), most of the plant N in the present study was derived from soil, primarily through mineralization processes. However, N inputs from litter decomposition, biological N fixation, and wet and dry deposition cannot be neglected as additional N sources for maize (Dentener et al., 2006; Silva et al., 2008; Montañez et al., 2009). The comparable and low values of Ndff in litter (5.6 kg ha^{-1}, on average) among the forage grasses indicate that fertilizer N was weakly retained in this crop residue, even though microbial N immobilization and/or adsorption of NH_4^+ from ammonium sulfate in organic residues may be possible (Mariano et al., 2016). Therefore, the frequent rainfalls after fertilization most likely leached fertilizer N from plant residues into the soil. Accordingly, the substantially higher amount of ^{15}N found in the 0–10 cm depth relative to the subjacent soil layers, irrespective of treatment, suggests considerable immobilization and some adsorption of mineral N forms (presumably NH_4^+) in exchangeable sites of the topsoil, in line with the conclusions of other studies (Coelho et al., 1991; Liu et al., 2015; Karwat et al., 2017). However, the potential for NH_4^+ fixation in this soil (i.e., a highly weathered tropical soil) is low due to the high proportion of kaolinite, a 1:1 clay mineral (Nieder et al., 2011). While sampling deeper soil layers could increase residual N by around 22% in the first season (Reddy and Reddy, 1993; Liu et al., 2015; Wang et al., 2016), deep N leaching below 1.0 m was shown to be low in most of Brazilian regions, usually less

then 5.0% of the N applied (Villalba et al., 2014). The amount of NO_3^-–N leached could not be related to rainfall (Rosolem et al., 2017). Furthermore, when deep rooted grasses are introduced in the system, N leaching is strongly decreased, and it is cycled to the topsoil (Rosolem et al., 2017).

Although a large proportion of the fertilizer N (97 kg ha^{-1}, on average) remained in the system after the first season, only a small amount of residual ^{15}N (4.1 kg ha^{-1}, on average) was taken up by maize. Considering the low percentage of Ndff in the shoots (2.1% of total N, on average), it can be inferred that 97.9% of the total N taken up by maize was derived from other sources (e.g., soil, unlabeled fertilizer, atmospheric deposition, etc) than the residual labeled N. In contrast to the first growing season, the values of the Ndff percentage in grains (73%, on average) and NHI (0.69, considering N–fertilized treatments) were similar, indicating that the residual ^{15}N was taken up by maize mainly at later stages (e.g., during grain filling). Despite the retention of maize stover on the soil surface after the previous harvest, which resulted in Ndff of 27.3 kg ha^{-1}, only 7.7% (considering all treatments) was recovered in the litter biomass in the following year as stover became a component of this organic layer. Although the isotope distribution in the soil in the second season was similar to that in the previous season, with most of the residual ^{15}N found in the upper layer (0–10 cm), a substantial decrease in Ndff (55%, on average) was observed in the 0–40 cm soil layer. Different loss pathways most likely explain these observations, as will be detailed below.

1.4.3 Fate of ^{15}N–labeled fertilizer

To our knowledge, this is the first report on N recovery (using a ^{15}N tracer) by maize grown in rotation with perennial tropical forage grasses. Previous studies have focused on land–use change, such as the shift from cultivation of *U*. *humidicola* or native vegetation to maize, in addition to maize monoculture as a control (Moreta et al., 2014; Karwat et al., 2017). The ^{15}N recovery in maize shoots in the first growing season was within the previously reported range of 12%–57% (Coelho et al., 1991; Gava et al., 2006; Almeida et al., 2017; Karwat et al., 2017) and close to the global estimate of 33% (Raun and Johnson, 1999). Based on the lack of difference in Ndff and ^{15}N recovery in shoots among forage grass species and the lower grain yield of maize succeeding ruzigrass, it may be inferred that ruzigrass residues did not affect the N uptake efficiency (i.e., ^{15}N recovery) of maize but impaired crop yield, as

discussed above. The underlying mechanisms remain unclear but may involve allelopathy (Souza et al., 2014). Furthermore, the higher amount of palisade grass residue before maize planting likely explains the increased ^{15}N recovery from litter. The similarities in ^{15}N distribution and ^{15}N recovery in the soil profile among the treatments show that either the decomposition of forage grass roots during maize growth had no effect on these two factors or that the potential effects were the same for all grasses. The lack of difference in the net nitrification rates between forage grasses clearly supports the^{15}N recovery results. Conversely, the higher soil nitrification following N fertilization in the second season suggests an increased turnover rate of soil organic matter caused by the mineral fertilizer N applied to maize in the first season, increasing NH_4^+oxidation to NO_3^- (Kuzyakov et al., 2000).Thus, the similar soil nitrification rates of the forage grasses, despite the reported higher nitrification suppression capacity of Guinea grass over palisade grass (Subbarao et al., 2012) is possibly explained by two factors. First, the majority of studies evaluating biological nitrification inhibition were performed with *U. Humidicola* pastures established for years (more than 10–years–old), where the nitrification suppression is assumed to be high due to the cumulative release of inhibitory substances essentially from root exudation and root turnover(Subbarao et al., 2007, 2008; Moreta et al., 2014; Subbarao et al., 2015). Secondly, Karwat et al. (2017) postulated that the residual effect of biological nitrification inhibition is short and limited to the subsequent crop, as inhibitory substances can be leached or mineralized by microorganisms. Based on these factors, the residual nitrification suppression effect of the tropical forage grasses in rotation with maize (a plant with very low nitrification inhibition capacity) is questionable and may not reach the critical threshold levels to decrease soil nitrification rates and promote benefits to the agriculture and environment.

The low recovery (<3.4%) of residual ^{15}N–labeled fertilizer in maize shoots after harvest in the second season indicates that the contribution of previous N fertilization to a succeeding crop is limited or even negligible, since most of the residual ^{15}N remains in the soil as organic N due to immobilization and will be released slowly by remineralization (Reddy and Reddy, 1993; Liu et al., 2015; Smith and Chalk, 2018). In a recent meta–analysis performed by Smith and Chalk (2018) with more than 100 studies on the residual value of ^{15}N–labeled fertilizers, the authors reported that a consistent value of 5.4% of the initial applied N was recovered in subsequent crops. This result demonstrates the need for fertilizer application to each crop in order to

maintain or achieve high yields. The observed lack of influence of the forage grasses on ^{15}N recovery in maize, litter, and soil in the second growth season confirms the low potential of these plants to alter fertilizer N dynamics in this rotation system, at least in the short term.

The amount of unrecovered N (16%, or 22 kg ha^{-1}, on average) in the first growing season is consistent with the results of many other studies (Gava et al., 2006; Wang et al., 2016; Almeida et al., 2017) but is lower than the value of 43 kg ha^{-1} reported in a meta–analysis by Gardner and Drinkwater (2009). The following pathways are associated with ^{15}N deficits in the plant–litter–soil system: (i) ammonia volatilization from senescing leaves (Farquhar et al, 1980); (ii) ammonia volatilization following fertilizer addition (Sommer et al., 2004); (iii) leaching of NO_3^- below the crop–rooting zone (Di and Cameron, 2002); (iii) nitrous oxide emission from plants during reduction of NO_3^- (Smart and Bloom, 2001); and (iv) nitrous oxide emission from soil (Bouwman, 1996). However, during the first growing season, NO_3^- leaching and nitrous oxide emission from the soil were negligible, whereas volatilization losses of ammonia from the canopy and soil accounted for ~3.0 kg ha^{-1} (Rocha, 2018). The substantial increase in unrecovered N in the second season compared with the first growing season (60 versus 22 kg ha^{-1}, on average) may have been caused by the leaching of residual ^{15}N below the sampling depth (40 cm.; Liu et al., 2015). We therefore suggest that the downward motion of hydrophilic organic N (Kalbitz et al., 2000) derived from fertilizer can be an important pathway of N loss from upper to lower soil layers in subsequent crops.

1.5 Conclusions

The results of this study provide important information on the effects of tropical forage grasses grown in rotation with maize on the fate of ^{15}N–labeled fertilizer in a no–till system. Soil nitrification assessed by laboratory incubation does not differ among forage species in the first and second growing seasons. While maize yield and N accumulation increase substantially following N fertilization than the unfertilized control, these crop parameters are generally not affected by the forage grass grown in rotation, except for ruzigrass, where maize grain yield in the first season is lower. The mechanism underlying this effect of ruzigrass is not completely understood. There is

no difference among the forage grass species in the distribution and fate of ^{15}N in the plant–litter–soil system and, consequently, in unrecovered–N (i.e., potential losses). The amount of residual labeled N taken up by maize in the second growing season is very low. Guinea grass and palisade grass can be used interchangeably in rotation with summer maize; however, the effect of ruzigrass should be further investigated to prevent potential yield losses.

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CHAPTER 2

EFFECT OF TROPICAL GRASS NITROGEN FERTILIZATION ON NITROUS OXIDE, METHANE, AND AMMONIA EMISSIONS OF MAIZE-BASED ROTATION SYSTEMS

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Abstract

While tropical grasses were shown to inhibit the activity of soil nitrifiers, their role in greenhouse gas (GHG) and ammonia (NH_3) emissions in N fertilized maize-based rotations are poorly understood. A 3-year (2014-2017) field experiment was conducted in southeastern Brazil to assess the influence of forage grass and N fertilization on nitrous oxide (N_2O), methane (CH_4), and NH_3 emissions from maize (*Zea mays* L.)-grass rotations. Guinea grass (*Megathyrsus maximus* cv. Tanzânia), palisade grass (*Urochloa brizantha* cv. Marandu), and ruzigrass (*Urochloa ruziziensis* cv. Comum) were grown in the main plots, while an unfertilized control and 140 kg N ha^{-1} were applied annually to maize in sub-plots. No apparent nitrification suppression by the grasses was detected.N_2O fluxes increased following N fertilizer addition in maize, particularly in the second season, where slightly higher cumulative N_2O emission was observed with N fertilization in comparison with the control. CH_4 fluxes showed high variation in the first forage and maize growing seasons. Residual N fertilizer decreased soil CH_4 uptake of palisade grass and ruzigrass compared with unfertilized palisade grass in the second forage season. Cumulative NH_3 emissions were unaffected by forage species and N fertilization. However, in both maize seasons, yield-scaled NH_3 emission was the lowest following N addition. Throughout the seasons, the differences between the three grasses inN_2O, CH_4, and NH_3 emissions were minimal. We conclude that the tropical perennial grasses rotated with maize were similar regarding GHG and NH_3 emissions, while N fertilization slightly increased N_2O emission and decreased soil CH_4 uptake.

Keywords: *Zea mays* L.; *Brachiaria*; *Panicum*; Nitrogen fertilizer; Nitrogen losses.

2.1 Introduction

From the “Green Revolution” (starting in the 1950s) to 2012, the emission of greenhouse gases (GHGs) from the agriculture sector increased by 112% globally (Gütschow et al. 2017). Soil degassing is a major source of GHG, where the increase of nitrous oxide (N_2O) and methane (CH_4) emissions from soils has been essentially attributed to agricultural practices and land-use changes (IPCC, 2007; Benbi, 2013; Oertel et al., 2016). Biogenic emissions of N_2O are generally driven by nitrification and denitrification (Butterbach-Bahl et al., 2013). In turn, soil CH_4 is produced during the anaerobic decomposition of organic matter, while methanotrophs consume CH_4 (Dutaur and Verchot, 2007). In addition to GHG, emission of ammonia (NH_3) also has adverse effects, leading to the formation atmospheric particulate matter, acidification and eutrophication of terrestrial bodies, degradation of visibility, as well as public health concerns (Erisman et al., 2007; Behera et al., 2013). Additionally, about 1% of the evolved NH_3 is converted into N_2O after its deposition to land (IPCC, 2006). From 1970 to 2012, NH_3 emissions increased by 127% worldwide (Crippa et al., 2018). Agriculture is the largest source of NH_3, and this gaseous pollutant can be emitted to the atmosphere from fertilizers, soil, decomposing litter, and crop foliage (Sommer et al., 2004; Behera et al., 2013).

In Brazil, the production and land area devoted to maize (*Zea mays* L.) is growing and currently occupies ~17 Mha, in which 29% of the total is cultivated in the rainy season (October-March) and the remaining part in the off-season (CONAB, 2019). Maize is key to ensure food security and has been used for animal feed, human nutrition, and more recently, for bioethanol production (Ranum et al., 2014). Although N fertilizer is critical to sustain or achieve high-yielding levels in maize (Ciampitti and Vyn, 2012), higher N_2O emissions have been extensively reported in fertilized soils relative to those without N addition (McSwiney and Robertson, 2005; Martins et al., 2015). Besides environmental risks related to N_2O emissions, the application of ammonium-based fertilizers can also decrease soil CH_4 uptake, allowing the conversion of a sink to a source (Mosier et al., 2004).To reduce reliance on N fertilizer and lowering the emission of N_2O, one of the most recommended cropland management systems involves rotations with legume crops (Lötjönen and Ollikainen, 2017). However, the rapid decomposition of leguminous crop residue in tropical

regions can extend bare fallow and increase soil erosion and consequently carbon dioxide (CO_2) emission (Benbi et al., 2013). Therefore, plants with wider C/N ratio (e.g., tropical perennial grasses) are usually grown in no-till systems as cover crops due to their prolonged persistence onto the soil surface. Moreover, the rotation of maize with tropical grasses of the genus *Urochloa* (syn. *Brachiaria*) and *Megathyrsus* (syn. *Panicum*) is a feasible approach for integrated crop-livestock systems (Salton et al., 2014).

While the cultivation of forage grasses does not increase soil N supply in comparison with leguminous plants (Baligar and Fageria, 2007), forage grasses (primarily *U. humidicola*) have been reported to suppress soil nitrification, thereby decreasing fertilizer-derived N_2O emissions (Subbarao et al., 2009; 2015; Byrnes et al., 2017). Despite the well-known potential of *U. humidicola* to suppress NO_3^- formation (Subbarao et al., 2012), its cultivation occurs mainly in lowlands than in tropical uplands. The so-called “biological nitrification inhibition” (BNI) is a plant-mediated process where exuded and/or released substances inhibit the ammonia monooxygenase and hydroxylamine oxidoreductase ammonia oxidizing enzymatic pathways, in a similar way to synthetic inhibitors (Subbarao et al., 2012). One of the drawbacks of synthetic nitrification inhibitors relies on the increased emission of NH_3 due to NH_4^+ accumulation in the soil (Qiao et al., 2015; Lam et al., 2016). For tropical grasses with high BNI capacity, the underlying effect on NH_3 losses is unknown. In addition, most of the studies on GHG emissions of rotation systems under no-till in tropical Brazil were deployed in soybean-maize rotations (Siqueira Neto et al., 2011; Carvalho et al., 2014; Salton et al., 2014). The effect of tropical grasses with supposed BNI capacity on GHG emissions of N fertilized rotations have not been sufficiently addressed, especially for long-term monitoring periods.

The increase of crop production to meet the forecasted increasing global demand for food will lead to an increase of GHG and NH_3 emissions from agricultural activities. Nevertheless, sustainable agricultural systems must be implemented to protect the environment and lower human health risks from polluting gases. Our objective was to estimate N_2O, CH_4, and NH_3 emissions in maize-based rotation systems affected by tropical forage grasses of the genus *Urochloa* [palisade grass (*U. brizantha* cv. Marandu) and ruzigrass (*U. ruziziensis* cv. Comum)] and *Megathyrsus* [Guinea grass (*M. maximus* cv. Tanzânia)] and N fertilization over three years. We hypothesized that:(i) forage grasses with high BNI capacity suppress soil nitrification, decreasingN_2O

emissions but increasing NH_3 losses from ammonium-based fertilizer, and (ii) N fertilization decreases soil CH_4 uptake, regardless of the forage grass grown in the off-season.

2.2 Material and Methods

2.2.1. Study site and experimental setup

A rainfed field experiment under no-till was established in 2014 in Botucatu (22°49' S, 48°26' W; 750 m a.s.l.), Southeastern region of Brazil. The region typically experiences dry winters and hot summers, with historical annual average minimum and maximum temperatures of 15.3 and 26.1°C, and an average rainfall of 1360 mm yr^{-1}. Before experiment establishment, the vegetation consisted of mixed tropical perennial grasses (i.e., palisade grass and ruzigrass). The local soil is a clay Rhodic Hapludox (Soil Survey Staff, 2014), and the clay fraction has ~70% kaolinite, ~15% gibbsite, and small amounts of vermiculite and illite. Before the experiment set up, selected soil physical and chemical properties in the top 20 cm were: sand 190 g kg^{-1}, silt 196 g kg^{-1}, clay 614 g kg^{-1}, pH 5.9, total C 19 g kg^{-1}, total N 1.3 g kg^{-1}, NH_4^+-N 5.4 mg kg^{-1}, NO_3^--N 6.4 mg kg^{-1}, P 15 mg dm^{-3}, K 1.3 $mmol_c$ dm^{-3}, Ca 35 $mmol_c$ dm^{-3}, and Mg 24 $mmol_c$ dm^{-3}, H+Al 37 $mmol_c$ dm^{-3}, cation exchange capacity 97 $mmol_c$ dm^{-3}, and base saturation 61%. Except for of total C and N, both assessed by dry combustion, physical and chemical soil properties were analyzed according to Gee and Bauder (1986) and van Raij et al. (2001), respectively.

The experiment had a split plot arrangement of three forage grass and two N rates in a randomized complete block design with four replications. The forage species Guinea grass, palisade grass, and ruzigrass were assigned to the main plots, while the subplots comprised the maize N fertilization levels (140 kg ha^{-1} N and control). The subplots were10 m long × 4.5 m wide. Forage grasses were planted in November 2014 using a no-till planter with a row spacing of 0.17 m and no fertilizer application. In September 2015, the grasses were chemically terminated using glyphosate and paraquat plus diuron. The plant residues remained in the field as a cover crop for the no-till system. Maize was planted in October using the above-cited planter at a row spacing of 0.75 m and stand of 65,000 plants ha^{-1}. Single basal application of triple superphosphate and KCl was performed at a rate of 53 kg P ha^{-1} and 100 kg K ha^{-1},

respectively. Nitrogen fertilizer (as granular ammonium sulfate) was split as follows: 30 kg N ha^{-1} at planting and 110 kg N ha^{-1} topdressed at the V5 stage. The N fertilization was performed in single-side surface banding, ~5 cm away from the crop row. No-N was applied in the control. Mature maize (R6 stage) was hand-harvested in March 2016, and the stover (i.e., leaves, stems, and cobs) was left on the soil surface. Due to severe drought conditions following maize harvest, forage planting was delayed to October. Grasses were then desiccated 60 d after plant emergence and residues were retained in the field. Measurements of crop biomass, GHG, and NH_3 emission were not performed between March and November 2016. Maize was planted in December 2016 following all agricultural practices used in the previous growing season. The crop was harvested in May 2017. Forage species were planted in May and August (herbicide was applied before the second replanting) due to the low tiller population. Dolomite ($CaCO_3 \cdot MgCO_3$) and gypsum ($CaSO_4 \cdot 2H_2O$) were surface-applied in October 2017 to ameliorate soil acidity, and forages were desiccated in November. Rainfall and air temperature were monitored at a weather station located near to the study site.

2.2.2 Gas sampling procedure and auxiliary measurements

N_2O and CH_4 fluxes were measured from January 2015 to December 2017 using manual static chambers (Pavelka et al., 2018). A galvanized steel collar (30.0 cm diameter and 9.3 cm height) was inserted ~5 cm into soil, comprising two rows in forage and between-row during maize growth. An opaque (white) and non-vented polypropylene lid (32.8 cm diameter and 7.3 cm height) was fitted through a flange around the upper edge of the collar. Water was added to the flange space to create an airtight seal. A rubber septum (0.5 cm diameter) was placed in the center of the lid. Before sampling, 40 mL of gases were taken with a polypropylene syringe and reinjected inside the chamber to promote air mixing. A gas sample (10 mL) was taken and kept in a syringe at 0, 10, 20, and 40 min after chamber closure, between 09:00 and 10:00 am, as recommended by Alves et al. (2012). Gas sampling was performed at 1, 3, 5, 8, 15, and 30 d following agricultural practices that could alter GHG emissions (i.e., planting, N fertilization, forage desiccation, and application of soil correctives), and monthly in the remaining season. Samples were analyzed for N_2O and CH_4 within 24 h after sampling with a gas chromatograph (GC-2014, Shimadzu Corp., Japan)

equipped with aflame ionization detection (FID) for CH_4 and a ^{63}Ni electron-capture detector (ECD) for N_2O. The FID and ECD operated at 250 and 325°C, respectively. N_2 (99.999%) was used as a carrier gas. Four standards of each GHG were used to plot the calibration curve.

In addition to gas sampling, soil temperature and volumetric water content were measured to a depth of 5.5 cm near the chamber using moisture and temperature sensors (Teros 11, Meter Group Inc., Pullman, WA, USA). The volumetric soil water content was converted into water-filled pore space (WFPS) as follows

$$WFPS = VWC/[1 - (BD/PD)]$$

where *WFPS* is the water-filled pore space (%); *VWC* is the volumetric water content (%); *BD* is the bulk density (g cm^{-3}); and *PD* is the particle density of the soil (g cm^{-3}). The *BD* and *PD* were assessed by the volumetric ring and volumetric flask method, respectively (Blake and Hartge, 1986a, 1986b).

To monitor the mineral N content, soil samples were taken using a core sampler at depth of 0-10 cm. Soil sampling was performed more regularly throughout the first forage season and semiannually from the second maize season. To extract mineral N forms, 5 g of field-moist soil was shaken with 2 M KCl (at a soil:solution ratio of 1:5; w/v) on a reciprocating shaker (200 rev min^{-1}; 1 h) and filtered using No.42 filter paper. The NH_4^+ and NO_3^- (NO_2^- is included) was determined using the salicylate-hypochlorite and VCl_3-Griess method, respectively (Mulvaney, 1996; Miranda et al., 2001).

2.2.3 Ammonia measurements

A static open chamber system was used to measure NH_3 loss from the soil-plant system according to Pacheco et al. (2017), with modifications. A polyurethane foam (12 cm × 12cm × 2 cm) was treated with 22 mL of 0.17 M H_3PO_4 in 4% glycerol solution (v/v) and placed in an opaque polystyrene box (11.5cm × 11.5cm × 3.5 cm) without wrapping with PTFE film. The open chamber was affixed to a steel bar using a burette clamp and held above the canopy (the height of the chamber was changed periodically) to trap any NH_3 evolved from crop foliage and soil. Traps were changed every ~21 d in the first forage season (2014-2015) and every ~12 d on the following dates. The NH_3 trapped in the acidified foam was extracted with 100 mL of deionized water. $NH_4^+$was determined colorimetrically as described above (Mulvaney, 1996).

Although the measured emission of open chambers may be lower than closed chambers, they can be used to compare treatments under identical environmental conditions (Sommer et al., 2004; Mariano et al., 2012).

2.2.4. Calculations and statistical analysis

Greenhouse gas (N_2O and CH_4) fluxes were calculated using the equation below

$$GHGflux = (\Delta C/\Delta t) \times (V/A) \times m \times [P/(T \times R)] \times n \times 24$$

where *GHG flux* is the N_2O (mg N m^{-2} d^{-1}) and CH_4 flux (mg C m^{-2} d^{-1}); $\Delta C/\Delta t$ is the linear slope of GHG concentration (μmol mol^{-1}) change during the sampling period Δt (h)*; V* and *A* are the volume (m^3) and area (m^2) of the chamber, respectively; *m* is the molecular weight (g) of each GHG; *P* is the atmospheric pressure (atm); *T* is the soil temperature (K); *R* is the gas constant; *n* is the ratio between the molecular weight of N with N_2O, and C with CH_4; and *24* is the factor to convert hour into day. The seasonal GHG emission was calculated by trapezoidal rule integration using SigmaPlot (version 14.0, Systat Software Inc., San Jose, CA, USA).

The NH_3 loss rate (NH_3LR) was calculated as

$$NH_3LR = NH_3/\Delta t$$

where NH_3LR is NH_3 loss rate (g N ha^{-1} d^{-1}); NH_3 is the NH_3 emission (g N ha^{-1}) at each sub-period; and *Δt* is the time interval (d) between the replacement of acid traps. The seasonal cumulative emission was estimated by the sum of the NH_3-N loss at different sub-periods.

The yield-scaled emission of N_2O, CH_4, and NH_3 was calculated as follows

$$YS\ emission = E/B$$

where *YS emission* is the yield-scaled emission of N_2O (g N Mg^{-1} DM), CH_4 (g C Mg^{-1} DM), and NH_3(g N Mg^{-1} DM); *E* is the seasonal or cumulative emission of N_2O (kg N ha^{-1}), CH_4 (kg C ha^{-1}), and NH_3 (kg N ha^{-1}); and *B* is the seasonal or cumulative biomass production (Mg ha^{-1}).

The N_2O emission factor was calculated using the following equation

$$EF = [(N_2O_{fert} - N_2O_{con})/NFR] \times 100$$

where *EF* is the fertilizer-induced N_2O emission factor (%); N_2O_{fert} and N_2O_{con} are the seasonal N_2O emissions(kg N ha^{-1}) in N-fertilized and control treatments, respectively; and *NFR* is the N fertilizer rate applied to maize (kg N ha^{-1}).

Statistical analyses were performed using the GLIMMIX procedure in SAS (version 9.4M3,SAS Institute Inc., Cary, NC, USA), at the 5% level of significance. The Kenward-Roger approximation was used to compute denominator degrees of freedom for tests of fixed effects. Block was considered a random effect. Data obtained in the first forage season (2014-2015) were tested using one-way ANOVA. Data from subsequent crops were subjected to split plot ANOVA. Non-normal data were treated using lognormal distribution, and values were back-transformed. The cumulative crop biomass production and gas emissions in the control (2014-2017) and N-fertilized treatments (2015-2017) were tested separately using one-way ANOVA due to the non-similar measuring period. LSMEANS with the simulate adjustment was used to separate least square means. Relationships between GHG flux and soil properties were assessed by Spearman rank-order correlation using the CORR procedure.

2.3 Results

2.3.1 Environmental conditions and soil properties

Rainfall in the first and second forage season totaled 1844 and 745 mm, respectively, and 1381 and 924 mm in the first and second maize crops (Fig. 6).Minimum air temperature over the three years varied from 6.4 to 23.5°C, whereas maximum air temperature ranged from 14.2 to 35.8°C (Fig. 6). The WFPS and primary soil temperature were similar across treatments (Fig. 7). The soil WFPS ranged from 17% to 86% over the experimental period, but the mean value across seasons was comparable, averaging 49% and 55% in the first and second forage season, respectively, and 61% and 47% during the first and second maize season (Fig. 7a). The soil temperature followed the air temperature pattern, with values varying from 15.6 to 34.8°C over the experimental period (Fig. 7b).In the first and second forage season, soil temperatures averaged 21.6 and 24.3°C, respectively, while corresponding values in maize growing seasons were 23.8 and 26.8°C.

In the first forage season, the extractable soil NH_4^+-N content in April and early October 2015 was higher in the ruzigrass in comparison to the other forage grasses, while palisade grass and ruzigrass were higher than Guinea grass in late October (Fig. 8a). In addition, extractable soil NH_4^+-N in fertilized Guinea grass was highest in February 2016 (first maize season), while residual N fertilization increased NH_4^+-N

content compared with the control (main effect) in the second forage season (December 2017).The supposed BNI of tropical forage grasses was not found over the experimental period (Fig. 8b). Although soil NO_3^--N content in the palisade grass was higher than that of Guinea grass in early October 2015, the former forage treatment was the lowest some days later (late October 2015). Lastly, N fertilization (main effect) increased soil NO_3^--N content compared with the control in the first maize season.

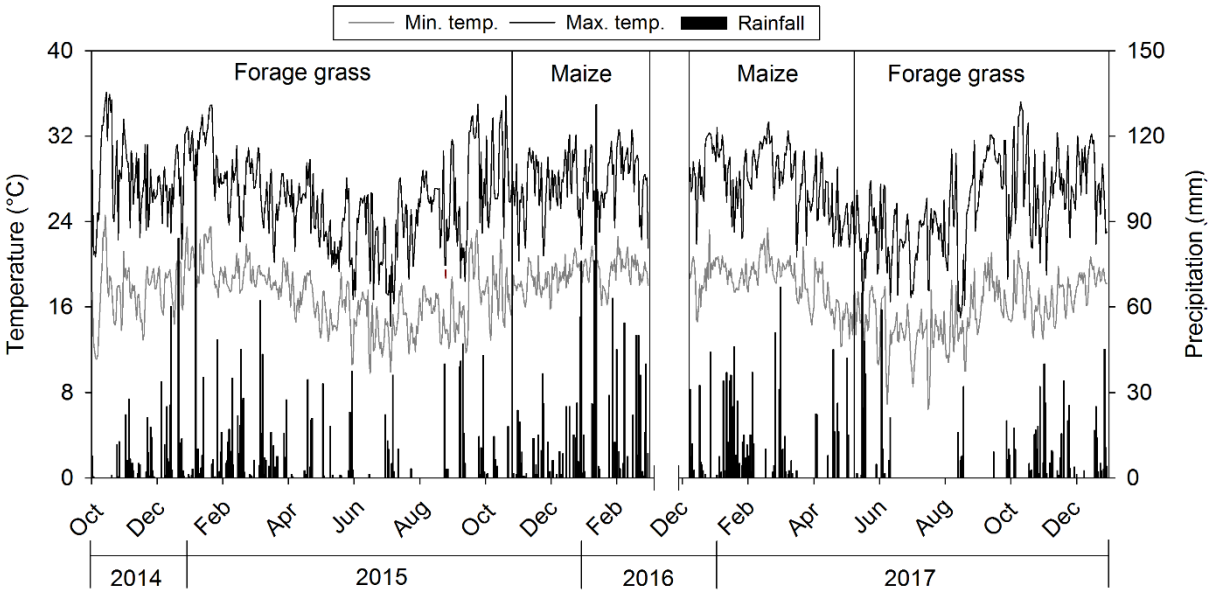


Fig. 6. Daily minimum and maximum air temperatures and rainfall recorded during the field experiment period (2014-2017). Measurements were not conducted from March to November 2016 (off-season).

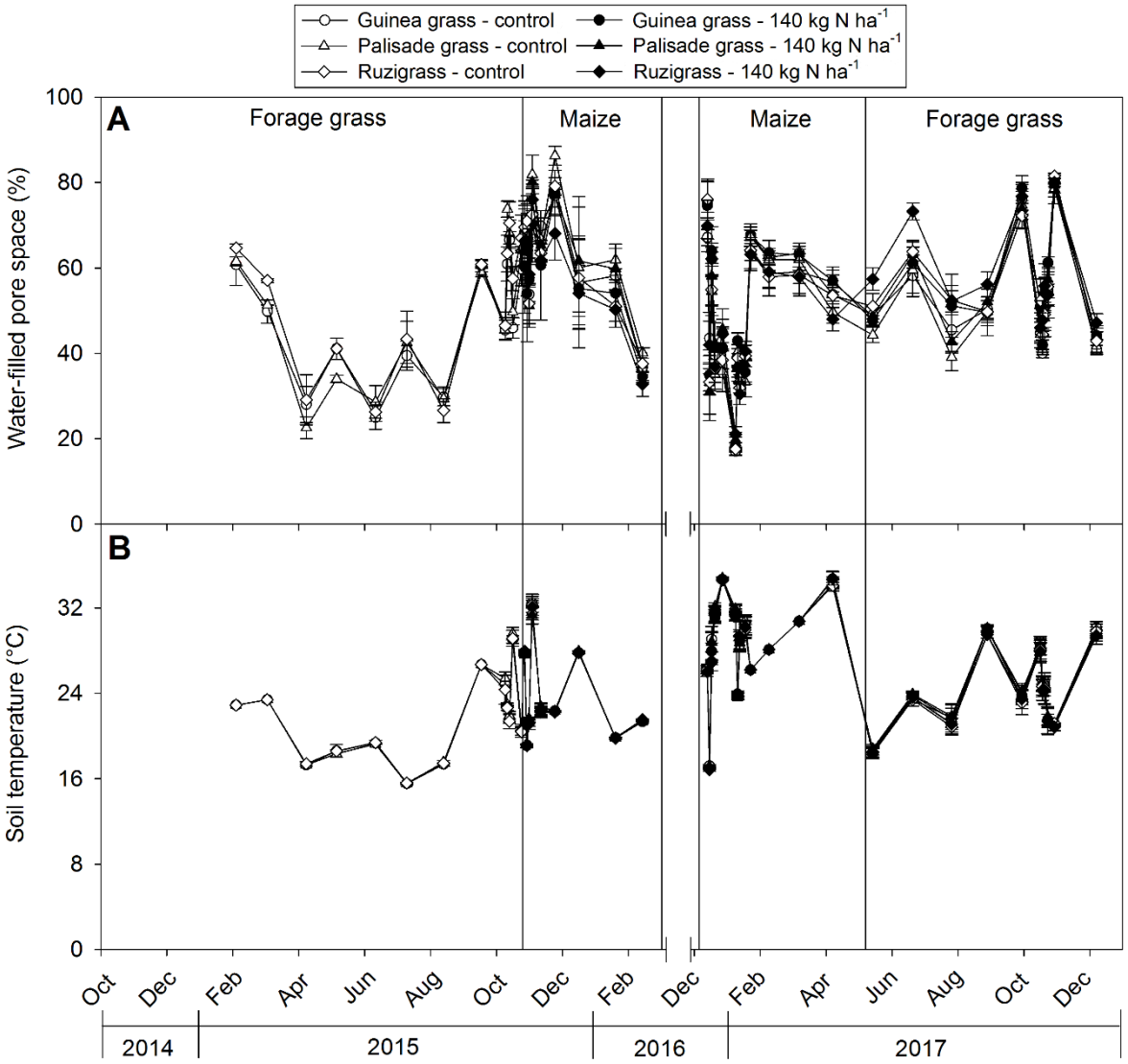


Fig. 7. Seasonal variation of water-filled pore space (a) and soil temperature (b) at depth of 5.5 cm in forage grass-maize rotations from 2015 to 2017 as affected by forage grass specie and N fertilization. Measurements were not conducted from March to November 2016 (off-season).Symbols represent mean values, and the error bars represent the SEM (*n* = 4).

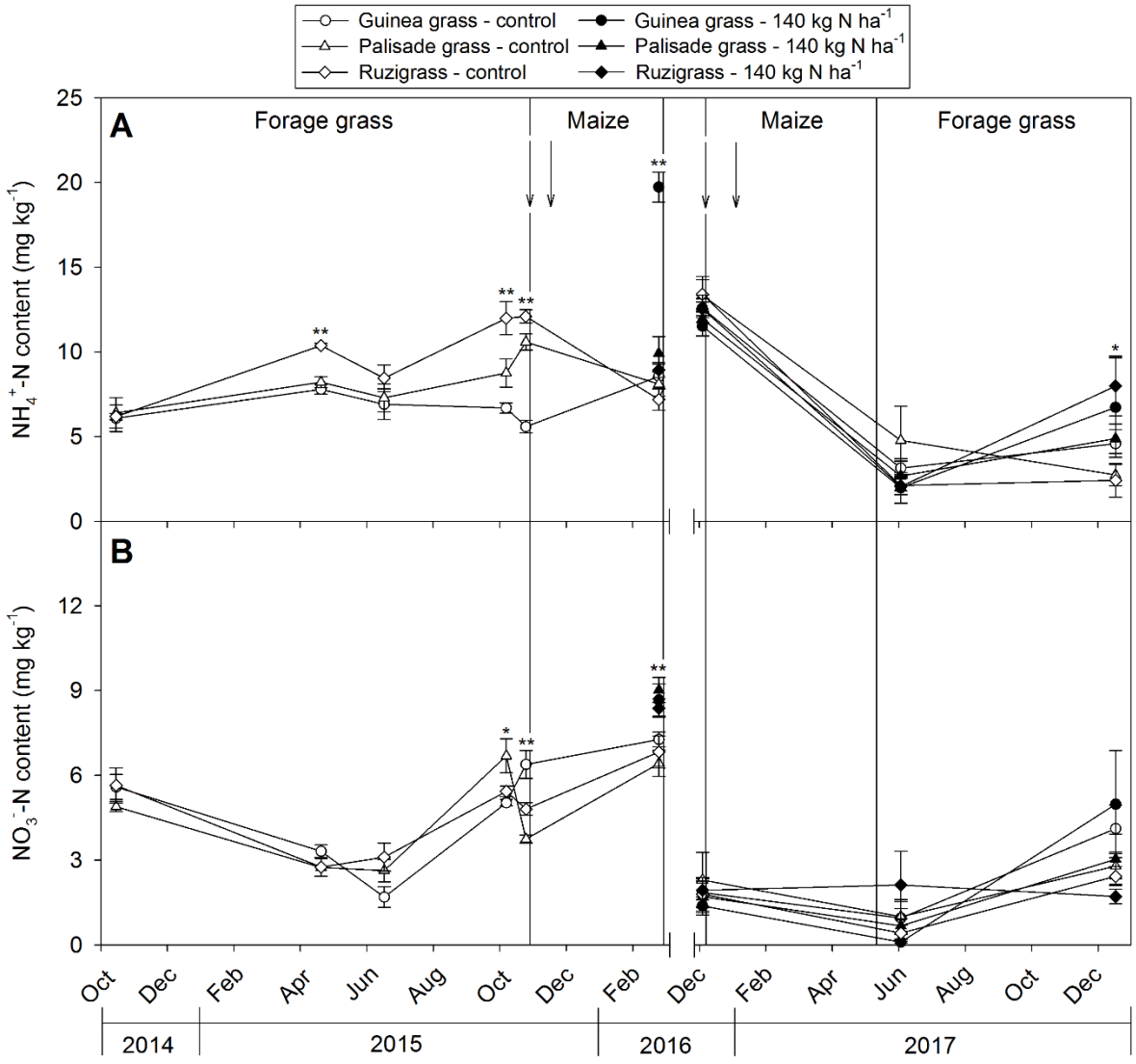


Fig. 8. Seasonal variation of NH_4^+-N (a) and NO_3^--N content in the soil (b) at depth of 0-10 cm in forage grass-maize rotations from 2014 to 2017 as affected by forage grass specie and N fertilization. Arrows indicate N fertilizer application in maize. Measurements were not conducted from March to November 2016 (off-season). Symbols represent mean values, and the error bars represent the SEM (n = 4). * and **: significant at the 5% and 1% level, respectively.

2.3.2 Nitrous oxide, methane, and ammonia emissions

Soil N_2O and CH_4flux and NH_3 loss rate are exhibited in Fig. 9.Over the 3-year period, the N_2O fluxes ranged from -0.1 to 2.0 mg N m^{-2} d^{-1} across treatments, and the highest fluxes occurred following basal and topdressing N fertilization in maize regardless of the growing season and forage previously grown(Fig. 9a). Moreover, the

N_2O fluxes during forage grown were lower compared with maize, averaging 0.14 and 0.08 mg N m^{-2} d^{-1} during the first and second forage season, respectively, and 0.20 and 0.15 mg N m^{-2} d^{-1} in maize seasons. The CH_4 flux throughout the experimental period ranged from -2.4 to 4.9 mg C m^{-2} d^{-1}, with consistent dominance of negative CH_4 flux (i.e., CH_4 uptake) notably initiated in the first maize growing season (Fig. 9b). High positive CH_4 flux was observed from the end of the first forage growing season to topdressing N application in maize, matching with moderately high WFPS values (64%, on average) within this period (October-November 2015). The CH_4 flux averaged 0.00 and -0.20 mg C m^{-2} d^{-1} in the first and second forage season, respectively, and -0.12 and -0.11 mg C m^{-2} d^{-1} during maize seasons. The NH_3 loss rate ranged between 4 and 147 g N ha^{-1} d^{-1} from 2014 and 2017 (3-year period), whereas the daily loss averaged 25 and 30 g N ha^{-1} d^{-1} in the first and second forage season, respectively, and 32 and 38 g N ha^{-1} d^{-1} during maize grown (first and second season, respectively; Fig. 9c).

In the second maize season, N fertilization increased cumulative N_2O emission by 39% relative to the control, while soil CH_4 uptake was 142% lower in the palisade grass and ruzigrass treatments with residual N application compared with the unfertilized palisade grass in the second forage season (Table 5). Conversely, seasonal cumulative NH_3 emissions were not altered by forage grown and N addition. Due to the longer growth period, higher GHG and NH_3 emissions were verified in the first forage season than those of subsequent crops. Likewise, there was no effect of forage grasses on cumulative N_2O, CH_4, and NH_3 emissions over the experimental period (2014-2017 for the control; 2015-2017 for N-fertilized treatments; Suppl. Fig. 1).

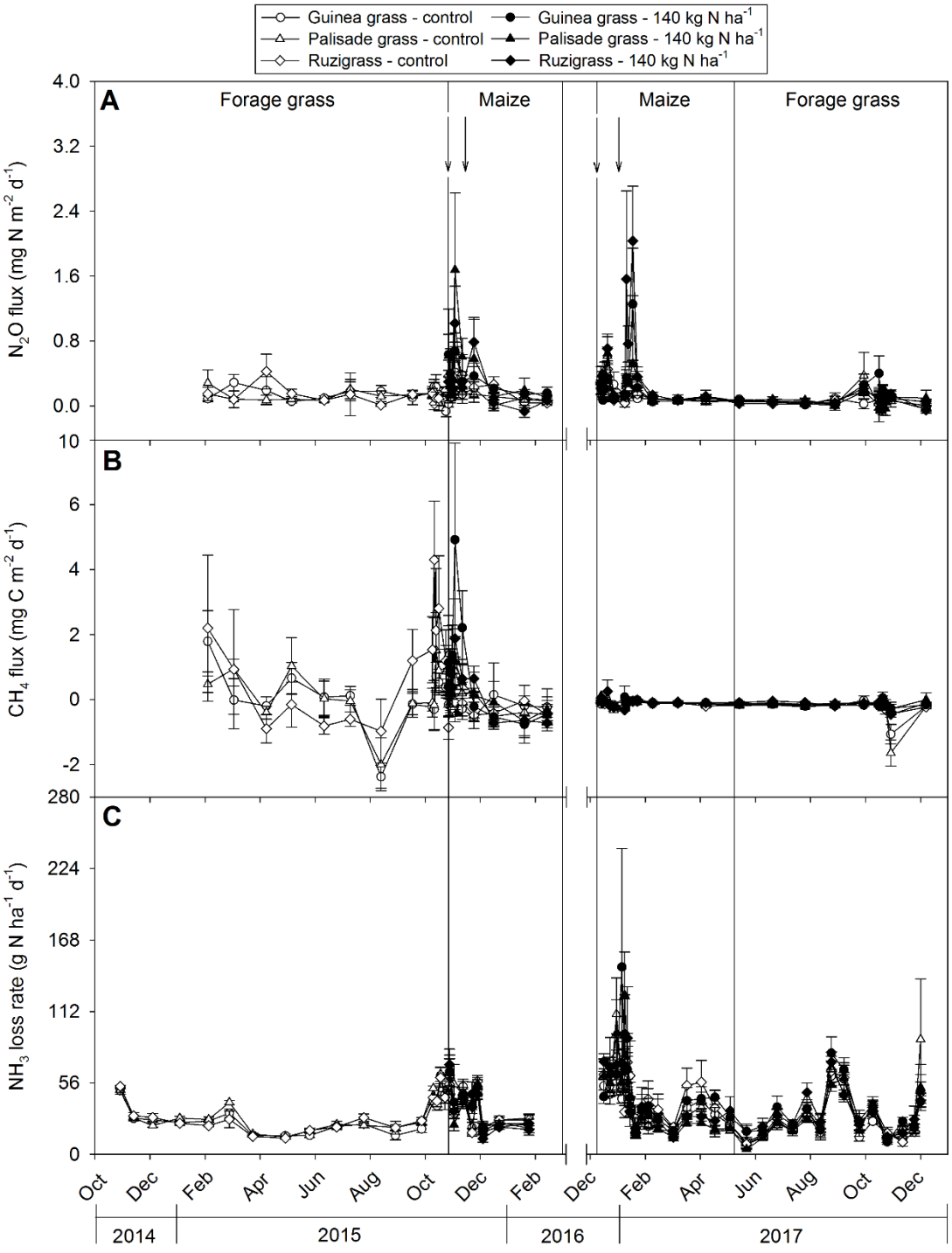


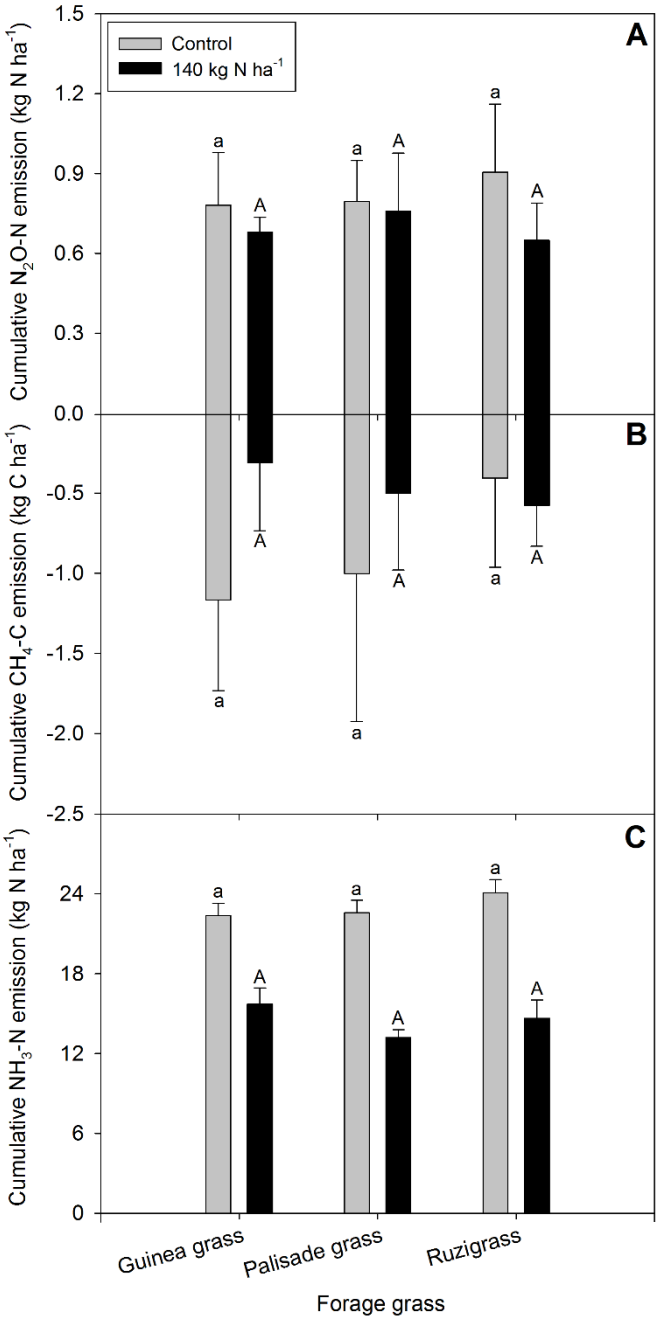
Fig. 9. Seasonal variation of N_2O (a) and CH_4 flux (b), in addition to NH_3 loss rate (c) in forage grass-maize rotations from 2014 to 2017 as affected by forage grass specie and N fertilization. Arrows indicate N fertilizer application in maize. Measurements were not conducted from March to November 2016 (off-season). Symbols represent mean values, and the error bars represent the SEM (*n* = 4).

Table 5. Seasonal N_2O and CH_4, and NH_3 emissions (or uptake) in forage grass-maize rotations from 2014 to 2017 as affected by forage grass specie and N fertilization.

Forage grass	**N rate**	**N_2O**	**CH_4**	**NH_3**
	kg N ha^{-1}	**kg N ha^{-1}**	**kg C ha^{-1}**	**kg N ha^{-1}**
Forage (2014-2015)				
Guinea grass	Control	0.41 ± 0.17	-0.36 ± 0.57	8.5 ± 0.2
Palisade grass	Control	0.32 ± 0.06	-0.04 ± 0.88	9.1 ± 0.1
Ruzigrass	Control	0.38 ± 0.21	0.37 ± 0.44	8.9 ± 0.2
		P = 0.852	*P* = 0.535	*P* = 0.058
Maize (2015-2016)				
Guinea grass	-	0.18 ± 0.04	-0.04 ± 0.29	3.4 ± 0.2
Palisade grass	-	0.18 ± 0.04	-0.13 ± 0.22	3.2 ± 0.2
Ruzigrass	-	0.18 ± 0.04	-0.23 ± 0.15	3.2 ± 0.2
		P = 0.994	*P* = 0.894	*P* = 0.558
-	Control	0.14 ± 0.03	-0.22 ± 0.14	3.3 ± 0.1
-	140	0.22 ± 0.04	-0.06 ± 0.21	3.2 ± 0.1
		P = 0.125	*P* = 0.472	*P* = 0.535
Guinea grass	Control	0.12 ± 0.04	-0.18 ± 0.39	3.5 ± 0.3
Palisade grass	Control	0.13 ± 0.04	-0.17 ± 0.09	3.1 ± 0.2
Ruzigrass	Control	0.22 ± 0.08	-0.29 ± 0.23	3.3 ± 0.3
Guinea grass	140	0.27 ± 0.10	-0.10 ± 0.48	3.3 ± 0.3
Palisade grass	140	0.27 ± 0.09	-0.09 ± 0.46	3.3 ± 0.3
Ruzigrass	140	0.16 ± 0.06	-0.18 ± 0.22	3.0 ± 0.2
		P = 0.189	*P* = 0.918	*P* = 0.685
Maize (2016-2017)				
Guinea grass	-	0.19 ± 0.02	-0.15 ± 0.03	5.6 ± 0.6
Palisade grass	-	0.20 ± 0.02	-0.20 ± 0.01	4.9 ± 0.6
Ruzigrass	-	0.25 ± 0.03	-0.17 ± 0.02	5.8 ± 0.7
		P = 0.213	*P* = 0.385	*P* = 0.421
-	Control	0.18 ± 0.02b	-0.19 ± 0.02	5.4 ± 0.6
-	140	0.25 ± 0.02a	-0.15 ± 0.01	5.4 ± 0.6
		P = 0.007	*P* = 0.128	*P* = 0.948
Guinea grass	Control	0.17 ± 0.03	-0.18 ± 0.05	5.2 ± 0.7
Palisade grass	Control	0.18 ± 0.03	-0.21 ± 0.03	5.0 ± 0.7
Ruzigrass	Control	0.18 ± 0.03	-0.19 ± 0.03	6.2 ± 0.8
Guinea grass	140	0.21 ± 0.03	-0.12 ± 0.03	6.0 ± 0.8
Palisade grass	140	0.22 ± 0.03	-0.19 ± 0.01	4.9 ± 0.6
Ruzigrass	140	0.35 ± 0.05	-0.15 ± 0.03	5.5 ± 0.7
		P = 0.195	*P* = 0.835	*P* = 0.305
Forage (2017)				
Guinea grass	-	0.13 ± 0.03	-0.36 ± 0.04	5.7 ± 0.4

Palisade grass	-	0.15 ± 0.03	-0.40 ± 0.10	5.2 ± 0.3
Ruzigrass	-	0.11 ± 0.02	-0.27 ± 0.03	5.7 ± 0.3
		P = 0.692	*P* = 0.294	*P* = 0.514
-	Control	0.12 ± 0.03	-0.44 ± 0.05b	5.2 ± 0.2
-	140	0.14 ± 0.02	-0.25 ± 0.04a	5.8 ± 0.3
		P = 0.532	*P*< 0.001	*P* = 0.116
Guinea grass	Control	0.08 ± 0.05	-0.44 ± 0.04ab	5.1 ± 0.4
Palisade grass	Control	0.15 ± 0.06	-0.58 ± 0.10b	5.3 ± 0.4
Ruzigrass	Control	0.13 ± 0.03	-0.29 ± 0.04ab	5.3 ± 0.2
Guinea grass	140	0.17 ± 0.04	-0.29 ± 0.03ab	6.2 ± 0.6
Palisade grass	140	0.15 ± 0.04	-0.23 ± 0.12a	5.1 ± 0.4
Ruzigrass	140	0.10 ± 0.04	-0.25 ± 0.05a	6.0 ± 0.4
		P = 0.346	*P* = 0.014	*P* = 0.276

Means followed by a common letter are not significantly different by the LSD-test at the 5% level of significance.



Suppl. Fig. 1. Cumulative emissions (or uptake) of N_2O (a), CH_4 (b), and NH_3 (c) in forage grass-maize rotations as affected by forage grass specie and N fertilization over the experimental period. The error bars represent the SEM (*n* = 4). Means followed by a common lowercase and capital letter do not indicate differences between unfertilized and N-fertilized treatments, respectively, by the LSD-test at the 5% level of significance.

2.3.3 Crop biomass, yield-scaled emissions, and N_2O emission factor

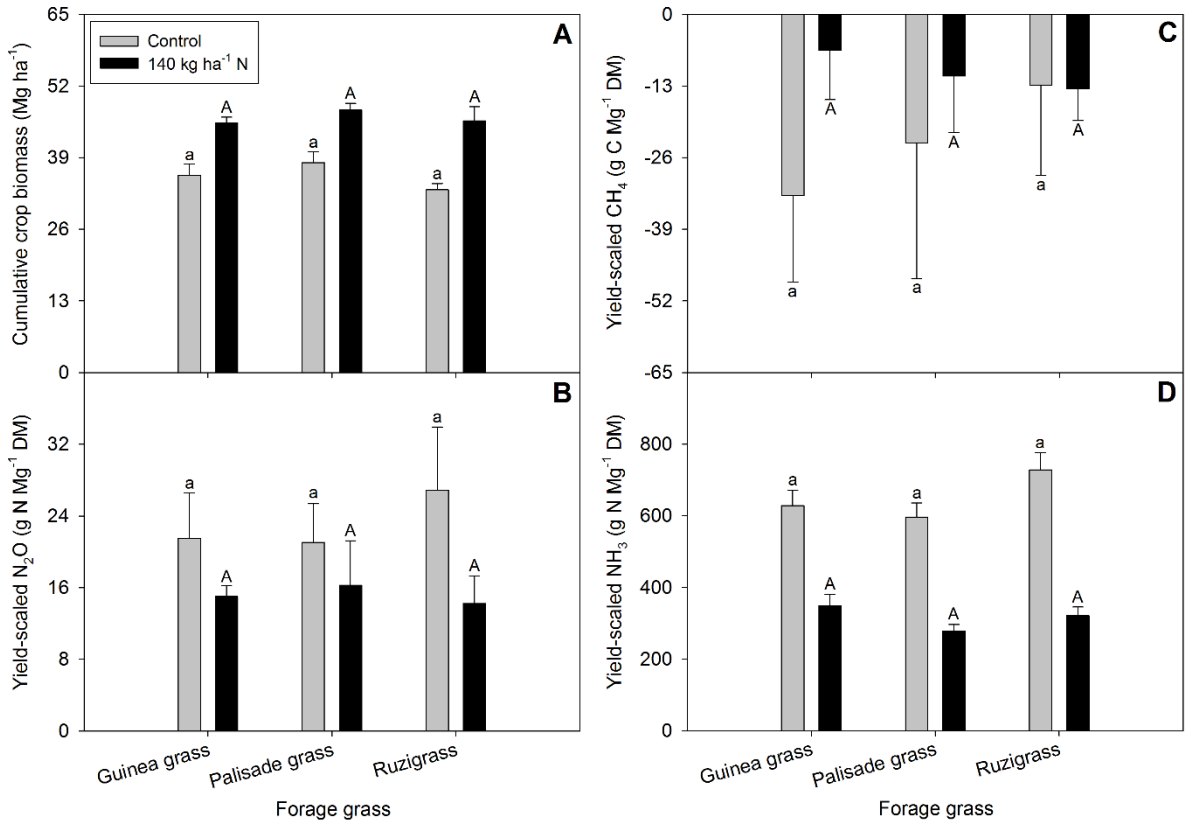
The biomass of palisade grass was 24% higher than that of ruzigrass in the first season (Table 6). Maize (first and second season) and forage biomass (second season) increased by 132%, 92%, and 26% following N application in comparison with the control, respectively. While the yield-scaled N_2O emission was not affected by grasses and N fertilizer, the yield-scaled CH_4uptake in the second season of maize and forage decreased by 62% and 55%, respectively, following N fertilization compared with the control (Table 6). Ruzigrass increased yield-scaled NH_3 emission by 20% compared with palisade grass in the first forage season (Table 6). The yield-scaled NH_3 emission was 141% and 97% higher in the control than in the N-fertilized soil in the first and second maize season, respectively. Forage grasses did not alter cumulative crop biomass production (considering both forage and maize dry matter yield) as well as yield-scaled emission of GHG and NH_3 over the 3-year period (Suppl. Fig. 2). In addition, the N_2O emission factor was low, ranging from -0.03 to 0.17%, with no differences among forage species regardless of the season (Table 7).

Table 6. Seasonal crop biomass and yield-scaled (YS) emission (or uptake) of N_2O, CH_4, and NH_3in forage grass-maize rotations from 2014 to 2017 as affected by forage grass specie and N fertilization.

Forage grass	**N rate kg N**	**Crop Mg ha^{-1}**	**YS N_2O g N Mg^{-1}**	**YS CH_4 g C Mg^{-1}**	**YS NH_3 g N Mg^{-1}**
Forage (2014-2015)					
Guinea grass	Control	11.7 ± 0.7ab	35 ± 15	-36 ± 48	738 ± 57ab
Palisade grass	Control	13.1 ± 0.5a	25 ± 6	-6 ± 63	700 ± 26b
Ruzigrass	Control	10.6 ± 0.2b	37 ± 21	37 ± 43	843 ± 25a
		P = 0.008	*P* = 0.675	*P* = 0.382	*P* = 0.021
Maize (2015-2016)					
Guinea grass	-	14.1 ± 0.6	12 ± 3	-12 ± 22	242 ± 16
Palisade grass	-	14.0 ± 0.6	13 ± 3	-11 ± 11	228 ± 15
Ruzigrass	-	13.0 ± 0.5	14 ± 3	-22 ± 14	242 ± 16
		P = 0.253	*P* = 0.936	*P* = 0.918	*P* = 0.771
-	Control	9.0 ± 0.3b	16 ± 3	-28 ± 15	368 ± 20a
-	140	20.9 ± 0.8a	11 ± 2	-3 ± 10	153 ± 8b
		P< 0.001	*P* = 0.145	*P* = 0.134	*P*< 0.001
Guinea grass	Control	9.9 ± 0.5	12 ± 4	-29 ± 40	357 ± 34
Palisade grass	Control	8.9 ± 0.5	14 ± 5	-18 ± 9	164 ± 33

Ruzigrass	Control	8.3 ± 0.5	26 ± 9	-35 ± 27	353 ± 38
Guinea grass	140	20.1 ± 1.1	14 ± 5	-5 ± 24	148 ± 15
Palisade grass	140	22.1 ± 1.2	12 ± 4	-5 ± 21	398 ± 14
Ruzigrass	140	20.5 ± 1.1	8 ± 3	-9 ± 11	148 ± 14
		P = 0.116	*P* = 0.156	*P* = 0.843	*P* = 0.529
Maize (2016-2017)					
Guinea grass	-	14.8 ± 1.9	15 ± 2	-13 ± 4	403 ± 54
Palisade grass	-	15.1 ± 2.0	15 ± 2	-15 ± 3	350 ± 47
Ruzigrass	-	14.7 ± 2.4	19 ± 3	-15 ± 3	434 ± 59
		P = 0.965	*P* = 0.241	*P* = 0.873	*P* = 0.543
-	Control	10.2 ± 0.8b	18 ± 1	-21 ± 3b	551 ± 60a
-	140	19.6 ± 1.0a	14 ± 2	-8 ± 1a	280 ± 31b
		P< 0.001	*P* = 0.129	*P*< 0.001	*P* = 0.002
Guinea grass	Control	10.4 ± 1.6	18 ± 2	-20 ± 7	520 ± 99
Palisade grass	Control	10.6 ± 1.5	18 ± 3	-21 ± 4	494 ± 94
Ruzigrass	Control	9.5 ± 1.4	19 ± 3	-21 ± 4	674 ± 13
Guinea grass	140	19.1 ± 1.0	12 ± 2	-7 ± 2	318 ± 60
Palisade grass	140	19.6 ± 1.8	12 ± 2	-10 ± 1	253 ± 48
Ruzigrass	140	19.9 ± 2.4	20 ± 5	-8 ± 2	284 ± 54
		P = 0.849	*P* = 0.513	*P* = 0.939	*P* = 0.621
Forage (2017)					
Guinea grass	-	4.9 ± 0.6	25 ± 6	-92 ± 23	1267 ± 142
Palisade grass	-	5.6 ± 0.6	27 ± 7	-80 ± 21	970 ± 86
Ruzigrass	-	5.0 ± 0.6	25 ± 6	-55 ± 6	1170 ± 78
		P = 0.430	*P* = 0.972	*P* = 0.100	*P* = 0.205
-	Control	4.6 ± 0.4b	27 ± 6	-104 ± 16b	1239 ± 102
-	140	5.8 ± 0.3a	25 ± 4	-47 ± 8a	1033 ± 69
		P = 0.038	*P* = 0.795	*P*< 0.001	*P* = 0.082
Guinea grass	Control	3.7 ± 0.5	21 ± 12	-134 ± 35	1487 ± 205
Palisade grass	Control	5.4 ± 0.9	29 ± 12	-115 ± 24	1024 ± 94
Ruzigrass	Control	4.7 ± 0.6	30 ± 9	-64 ± 7	1205 ± 162
Guinea grass	140	6.1 ± 0.6	29 ± 7	-49 ± 9	1048 ± 142
Palisade grass	140	5.9 ± 0.7	25 ± 7	-46 ± 25	916 ± 0.154
Ruzigrass	140	5.3 ± 0.3	20 ± 8	-47 ± 10	1134 ± 31
		P = 0.251	*P* = 0.663	*P* = 0.120	*P* = 0.343

Means followed by a common letter are not significantly different by the LSD-test at the 5% level of significance.



Suppl. Fig. 2. Cumulative crop biomass (forage plus maize) production (a) and yield-scaled emission (or uptake) of N_2O (b), CH_4 (c), and NH_3 (d) in forage grass-maize rotations as affected by forage grass specie and N fertilization over the experimental period. The error bars represent the SEM (*n* = 4). Means followed by a common lowercase and capital letter do not indicate differences between unfertilized and N-fertilized treatments, respectively, by the LSD-test at the 5% level of significance.

Table 7. Seasonal N_2O emission factor in forage grass-maize rotations as influenced by forage grasses.

Forage grass	N rate kg N ha^{-1}	Emission factor %
Maize (2015-2016)		
Guinea grass	140	0.11 ± 0.04
Palisade grass	140	0.17 ± 0.11
Ruzigrass	140	-0.03 ± 0.07
		P = 0.239
Maize (2016-2017)		
Guinea grass	140	0.04 ± 0.04
Palisade grass	140	0.03 ± 0.04
Ruzigrass	140	0.13 ± 0.05
		P = 0.188

Means followed by a common letter are not significantly different by the LSD-test at the 5% level of significance.

2.3.4 Relationships between greenhouse gas flux and soil properties

A weak positive correlation was observed between N_2Oflux withCH_4flux(Fig. 5). The soil WFPS was positively correlated with the two GHG fluxes (*r* varying from 0.14 to 0.24), while soil temperature did not correlate with any variable.

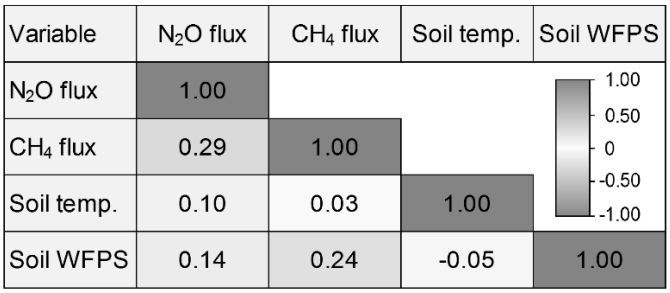


Fig. 10. Heatmap showing Spearman rank-order correlation coefficients (*r*) between greenhouse gas flux and soil properties (*n* = 237) throughout the experimental period (2015-2017). $r \geq 0.14$ and $r \geq 0.24$ are significant at the 5% and 1% level, respectively. Soil temp., soil temperature; Soil WFPS, soil water-filled pore space.

2.4 Discussion

2.4.1 Greenhouse gas flux and rate of ammonia volatilization

The prominent flux of N_2O following basal and topdressing N fertilization in maize is congruent with previous studies reporting the role of N fertilizer as a key factor driving N_2O emissions (Stehfest and Bouwman, 2006; Shcherbak et al., 2014).This hypothesis is supported by consistent low N_2O flux during forage growth, which was not fertilized. The application of synthetic N fertilizers readily increases the availability of NH_4^+ and NO_3^-, which are substrates for N_2O production (Butterbach-Bahl et al., 2013). Soil water content is the single most important factor in regulating N_2O emissions (Butterbach-Bahl et al., 2013; Oertel et al., 2016). Although variable across soils, Davidson et al. (2000) proposed that N_2O emissions are highest when the WFPS is in the range of 60-70%. With WFPS between 40% and 65%, the relative contribution of nitrification and denitrification to N_2O production is~70% and ~30%, respectively, whereas denitrification is the dominant process in wet soils with >85% WFPS (Stevens et al., 1997; Senbayram et al., 2009; Liu et al., 2016). The highest N_2O flux in the first maize season was verified at~76% WFPS, suggesting that nitrification and denitrification contributed to N_2O production. Conversely, we can infer that nitrification was the dominant process of N-fertilized treatments (primarily Guinea grass and ruzigrass) in the second maize season owing to the much lower soil WFPS (38%, on average) detected in the highest N_2O flux.

Mainly in the first forage and maize season (from October 2014 to March 2016), CH_4 flux followed the seasonal rainfall pattern (r = 0.37; P< 0.001). Increased CH_4 emission was observed as soil WFPS increased in the wet season (November-March), followed by a decrease(or even the transition to CH_4 uptake flux) with lower soil water content in the dry season, in line with previous reports (Verchot et al., 2000; Kiese et al., 2003).The intense methanogenesis from the end of the first forage season to topdressing N in maize (primarily in the unfertilized ruzigrass and fertilized Guinea grass treatments) is explained by the concomitant high soil WFPS, since $CH_4$is produced under hypoxic conditions, primarily in wetlands and rice paddies, but also in water-saturated aggregates of upland soils (Moiser et al., 2004; Dutaur and Verchot, 2007). In contrast, the consistent CH_4 uptake detected from the second maize season indicates that methanotrophs prevailed over methanogens. The oxidation of CH_4 in the

soil is mediated by the methane monooxygenase (MMO) that requires O_2 as a terminal electron acceptor (Topp and Pattey, 1997; Mosier et al. 2004).

Crop foliage and soil disruption by planting were the main factors for NH_3 emissions across the experimental period. Loss of NH_3 from senescing leaves occurs when the ambient concentration of this gas is lower than the plant NH_3 compensation point (Farqhuar et al., 1980; Sommer et al., 2004). The NH_3 is emitted by crops through natural aging or following herbicide application. Indeed, herbicides can alter the plant N metabolism, that is, senescence, stomatal conductance, NH_4^+ concentration in leaf tissues, as well as activity of the glutamine synthetase enzyme (Manderscheid et al. 2005; Pacheco et al., 2017). Similarly, Damin et al. (2008) found that glyphosate application in signal grass (*U. decumbens*) decreased ^{15}N recovery in the soil-plant system than that of the control and attributed this result to higher NH_3 emission following desiccation. Moreover, herbicide application for further replanting of the forage grasses in August 2017 also increased NH_3 loss rate. We also suggest that soil disturbance from seed and fertilizer incorporation during mechanized planting of maize, primarily in the second growing season, likely increased availability of C substrates for microbes, stimulating N mineralization and NH_3 emissions (Silgram and Sheperd, 1999).The role of N fertilizer seems to be negligible since all treatments, including those unfertilized, experienced increase NH_3 loss rates following maize planting.

2.4.2 Cumulative emissions of greenhouse gases and ammonia

The similar seasonal and cumulative N_2O emissions between the forage grass treatments suggest that the BNI influence through the exudation and/or release of inhibitory compounds was consistently similar across species, or more likely negligible to suppress the oxidation of NH_4^+ to NO_3^- and lowering N_2O emission. We recently showed that soil nitrification rates were similar between the three-forage grass (Rocha et al., 2019), which supports the above hypothesis. Moreover, the NH_4^+-N and NO_3^--N content in the soil can be used as a proxy to assess the BNI (Nuñez et al., 2018). However, we unable to identify an apparent inhibition of NO_3^- formation across sampling dates. Although Guinea grass has the highest BNI capacity among the three forage species (Subbarao et al., 2012), the similar N_2O emission between can be explained by the following factors: (i) most of the studies assessing BNI from tropical

forage grasses were carried out in pastures established for years, continuously, where the cumulative release and/or exudation of inhibitory substances are presumed to be high; and (ii) the BNI capacity of the three grasses may be much lower than that of *U. humidicola* (Subbarao et al.,2012)] and therefore insufficient to decrease soil nitrification rates. The small effect of the N fertilizer in stimulating N_2O emission compared with the control needs some caution. The placement of chambers only in between-row during maize season may have led to underestimating of N_2O emissions in comparison with their placement in the crop row and between two rows. However, the supposed higher N_2O flux of chambers placed in the crop row over between-row was not confirmed for banded-applied fertilizer in sugarcane (Allen et al., 2010), which does not invalidate our results.

Global budgets advocate that upland soils are a major sink for atmospheric CH_4 (Mosier and Delgado, 1997; Dutaur and Verchot, 2007). However, we found that the tropical clayey soil was a weak sink for CH_4 through methanotrophy. Soil CH_4uptake is dependent upon WFPS and controlled by gas diffusivity and biological activity, in which high water content restricts diffusion and oxidation of CH_4, while water deficit limits biological activity (Mosier et al., 2004; Dutaur and Verchot, 2007).Moreover, the postulated ability of the MMO to co-oxidize NH_3 explains the lower CH_4 uptake of N-fertilized treatments than that of unfertilized palisade grass in the second forage season since CH_4 and NH_3 are competitive substrates for this enzyme (Mosier et al., 2004).

The hypothesis that forage grass with differential BNI capacity would increase NH_3 volatilization was not confirmed. Additionally, the often negligible NH_3 loss from ammonium sulfate surface-applied to acidic soils (Fontoura and Bayer, 2010) also supports the similar NH_3loss between N-fertilized and control treatments. Although crop NH_3emissionsare expected to increase with plant N accumulation [N-fertilized treatments had the highest plant N accumulation (Rocha et al., 2019)], no relationship between N fertilization and foliage NH_3losshasbeenreported (Schjoerring and Mattsson, 2001).

The high responsiveness of maize to N fertilization is consistent with previous knowledge (Setiyono et al., 2010). Thus, the report of N_2O, CH_4, and NH_3emissions per unit yield (i.e., yield-scaled emission), in addition to the conventional metric per unit area, allow us to integrate environmental protection with increasing demand for food production towards to agricultural intensification instead of expanding to new farmland

(Pittelkow et al., 2013). Here, N fertilizer substantially increased maize biomass and decreased yield-scaled NH_3 emissions, translating into effective N use efficiency and lower environmental risks. More importantly, the take-home message is that without N fertilization additional land area would be needed to produce the same amount of biomass from fertilized fields. This result is consistent with the findings of Venterea et al. (2011) for yield-scaled N_2O emissions from maize under no-till. In contrast, the lower yield-scaled CH_4 uptake with N fertilization in the second maize season is a trade-off.

2.4.3 N_2O emission factor

The N_2O emission factor was remarkably lower than the default IPCC emission factor for N fertilizer of 1% (IPCC, 2007). Based on the possible underestimate quantification of N_2O emissions due to the chamber placement in between-rows of maize, caution is advised in interpreting these results. However, studies under Brazilian conditions also reported lower N_2O emission factors from synthetic N fertilizer than that of IPCC (Jantalia et al., 2008; Martins et al., 2015). Lastly, the similar N_2O emission factor among forage grasses supports the negligible effect of tropical pastures in mitigating N_2O production.

2.5 Conclusion

This study provides previously unavailable information on GHG and NH_3 emissions of maize-based rotations using different tropical grasses. Based on our results, Guinea grass, palisade grass, ruzigrass do not affect $N_2O$and NH_3 emission sowing to their apparent inability to suppress soil nitrification. However, N fertilization slightly increases cumulative N_2O emission in the second maize season and decreases soil CH_4 uptake in the fertilized palisade grass and ruzigrass relative to unfertilized palisade grass in the second forage season. As future research, the development and evaluation under field conditions of improved forage grass species (e.g., *Urochloa* hybrids) with remarkable high BNI is a suggested approach aiming low-nitrifying agricultural systems in tropical uplands. Moreover, the BNI potential of aged pastures (e.g., established for over 10 years) should also be assessed.

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CHAPTER 3

FUNCTIONAL N-CYCLE GENES IN SOIL AND N_2O EMISSIONS IN A MAIZE/TROPICAL FORAGE GRASSES INTERCROPPING SYSTEM

Submitted to Science of the Total Environment

Abstract

Studies suggest a relatively minor contribution of AOA (ammonia-oxidizing archaea) to nitrification in nitrogen (N)-rich soils and greater importance of AOB (ammonia-oxidizing bacteria) in soils under aerobic or semi-aerobic conditions. There is also evidence that forage grasses such as *Megathyrsus* and *Urochloa* affect nitrification to directly or indirectly impact soil N dynamics and N_2O emissions. However, the influence of soil chemical properties on the dynamics of functional genes in the N cycle and losses of N in maize intercropped with forage grasses under N fertilization is poorly understood. In this study, soil samples and N_2O emissions were analyzed from a field experiment in which maize was cropped for two years in rotation and one year intercropped with guinea grass (*Megathyrsus maximus* cv. Tanzânia), palisade grass (*Urochloa brizantha* cv. Marandu), and ruzigrass (*Urochloa ruziziensis* cv. Comum) with or without fertilization with NH_4^+-based fertilizer. Soil N-cycle microorganisms were influenced by grasses and N fertilization and timing, but no evidence of biological nitrification inhibition (BNI) was found. Palisade grass was associated with a higher abundance of *nif*H (average of 7.0×10^5 gene copies g^{-1} soil) in the absence of N compared with the other grasses (average of 4.3×10^5 gene copies g^{-1} soil). N fertilization increased the abundance of AOB more than AOA. Over time, AOB abundance decreased from 1.8×10^5 [12 days after planting (DAP)] to 1.02×10^5 (100 DAP) gene copies g^{-1} soil (43%), and AOA abundance increased from 3.8×10^7 (12 DAP) to 6.1×10^7 (100 DAP) gene copies g^{-1} soil (60%). However, the higher AOA abundance was probably the result of reduced NH_3^-availability in the soil. Furthermore, N_2O flux was influenced by AOB, water-filled pore space (WFPS) and N fertilization. This study reveals through nitrification, a strong dominance of AOB under ammonium supply, potentially stimulating N_2O emissions in intercropping systems.

Keywords: *Zea mays* L*; Urochloa; Megathyrsus;* N fertilization*;* N-cycle genes

3.1 Introduction

The soil microbiota impacts nitrogen (N) dynamics in several ways, including N_2 biological fixation, nitrification and denitrification. In biological N fixation (BNF), the enzyme nitrogenase, which is encoded by the *nif*H gene in free-living and symbiotic bacteria and archaea (diazotrophics), breaks the N_2 triple bond to reduce N_2 to ammonia (NH_3) (Zhang et al., 2006). In chemoautotrophic or heterotrophic organisms, nitrification converts ammonium (NH_4^+) to nitrate (NO_3^-) via the action of ammonia monooxygenase (*amoA*). This process consists of two phases: oxidation of NH_4^+ to nitrite (NO_2^-) and oxidation of NO_2^- to NO_3^-. In denitrification, copper nitrite reductase (*nir*K), iron nitrite reductase (*nir*S), nitric oxide reductase (*nor*B) and nitrate reductase (*nap*A and *nar*G) (Levy-Booth et al., 2014) reduce NO_3^- successively to NO_2^-, NO and finally N_2O. The only route for converting N_2O to N_2 (Sun et al., 2019) is nitrous oxide reductase (*nos*Z; Henry et al., 2006).Understanding the above processes is critical because atmospheric concentrations of N_2O now exceed pre-industrial levels by 20%, according to a 2013 assessment by the Intergovernmental Panel on Climate Change (IPCC) (Wu and Mu, 2019).

N_2O is typically produced by nitrifying microorganisms under aerobic or semi-aerobic conditions but by denitrifying microorganisms under anaerobic conditions (Signor and Cerri, 2013). Most studies have suggested that NH_4^+ oxidation in the soil is driven mainly by AOB, although others have suggested dominance of AOA (Tourna et al., 2008; Jia and Conrad, 2009). Beeckman et al. (2018) postulated that N-rich soils may decrease the contribution of AOA to nitrification. However, Enwall et al. (2005) showed that the addition of high concentrations of NH_4^+ increased N_2O emissions and altered the denitrifying community but not the ammonia-oxidizing community.

AOB community structure changes in response to soil temperature, fertilization and pH (He et al., 2007). In addition, long-term fertilization regimes influence the size of microbial guilds responsible for ammonia oxidation, nitrate reduction, and denitrification (Hallin et al., 2009). It is widely accepted that climate change interacts with agricultural management and plant-soil-microorganism relationships to affect the N cycle (Bowles et al., 2018). This interaction could limit the benefits of common practices to reduce N loss and become an increasing barrier to mitigating future agricultural losses. Precipitation and soil moisture are among the strongest determinants of losses of terrestrial N (Bowles et al., 2018). Large N inputs to fertile

soils as well as factors such as long periods with low N uptake rates by plants can further lead to low N use efficiency and consequently high N losses (Bowles et al., 2018).

Plants and cropping systems also directly or indirectly influence soil N dynamics. Forages of the genera *Urochloa* and *Megathyrsus* affect microbiological processes within the N cycle, as well as N availability and losses (Subbarao et al., 2012). It has been speculated that BNF is an important route of N entry in rotational production systems including maize (*Zea mays* L.) and forage grasses and that ruzigrass may increase the abundance of N-fixing microorganisms in the early growth stage (Rocha et al., 2020). However, the data supporting this speculation are from samples taken very early during system implantation. Furthermore, exudates of *Urochloa* species can inhibit the activity of nitrifying bacteria (Subbarao et al., 2003, 2009), which could result in N deficiency and decreased yield in maize grown after ruzigrass compared with palisade grass (Momesso et al., 2019).

Therefore, soils cultivated with some *Urochloa* species are expected to have a higher content of NH_4^+ than NO_3^-.This effect could accumulate through the years but has not yet been studied. Subbarao et al. (2012) reported that biological nitrification inhibition (BNI) capacity was much higher in *U. humidicola* than in *U. decumbens*, *M. maximus, Lolium perenne, U. brizantha* and other pasture, cereal and legume crops studied in sand-vermiculite culture for 60 days. However, Rocha et al. (2019) found no difference in net nitrification in the soil in the presence of three species of forage grasses (*U. brizantha*, *U. ruziziensis* and *M. maximus*) compared with the control, but nitrification increased by 34% relative to the control following N fertilizer application in the subsequent season.

Because exudation of BNI through grass roots, soil characteristics, and N fertilization can impact the microbial population and N_2O emissions, we hypothesized that forage grasses affect N cycle-associated microbiota and can decrease soil nitrification in addition to mitigating N_2O emissions in N-fertilized maize. The objectives of the present medium-term experiment were therefore i) to characterize changes in total bacterial and archaeal abundances and in microbial populations involved in N-fixation (*nif*H), ammonia oxidation (AOA and AOB), and denitrification (*nir*S, and *nos*Z); ii) to measure N_2O emissions in the system; and iii) to relate the chemical parameters of the soil and environmental factors with functional genes and N_2O emissions.

3.2 Material and Methods

3.2.1 Site description and experimental design

A field experiment was conducted under no-till management in Botucatu, SP, Brazil (22°49'S, 48°26'W),in a clay Rhodic Hapludox (Soil Survey Staff, 2014) with ~70% kaolinite, ~15% gibbsite, and small amounts of vermiculite and illite in the clay fraction. Before the experiment, the area had a mixed stand of tropical perennial grasses including but not limited to palisade grass and ruzigrass. Before the onset of the experiment, soil samples were randomly taken to a depth of 20 cm, and analyzed for the following physical (Gee and Bauder, 1986) and chemical properties (Raij et al.,2001): 190, 196, and 614 g kg^{-1} of sand, silt, and clay, respectively; pH($CaCl_2$) 5.9; P 15 mg dm^{-3}; K 1.3 $mmol_c dm^{-3}$; Ca 35 $mmol_c$ dm^{-3}; Mg 24 $mmol_c$ dm^{-3}; H+Al 37 $mmol_c$ dm^{-3};and cation exchange capacity (CEC) 97 $mmol_c$ dm^{-3}. Total N and C were determined by dry combustion using a CHNS-2000 elemental analyzer (Leco Corp., St. Joseph, MI, USA) and averaged 19 and 1.3 g kg^{-1} of total C and N, respectively. Analysis of mineral N forms (Mulvaney, 1996; Miranda et al., 2001) gave results of 5.4 mg kg^{-1} of NH_4^+-N and 6.4 mg kg^{-1} of NO_3-N.

The experiment was initiated in November 2014 by planting Guinea grass (*M. maximus*), palisade grass (*U. brizantha*), and ruzigrass (*U. ruziziensis* cv. Comum) in rows 0.17 m apart using a no-till planter and no fertilizer application. After 10 months, the grasses were chemically desiccated using 4.0 kg ha^{-1} of glyphosate (720 g kg^{-1}a.i.), and one week later 3 L ha^{-1} of paraquat (200 g L^{-1} a.i.) and diuron (100 g L^{-1} a.i.) was applied. Glyphosate is used in most conservation systems under no-till in Brazil, and a recent assessment showed that the occurrence of natural chelators with relatively high chelating potential makes an additional impact of glyphosate on soil microorganisms unlikely (Mertens et al., 2018). Plant residues remained in the field, and maize was planted over the grass residues in October 2015 at a row spacing of 0.75 m and stand density of 65,000 plants ha^{-1}. The plot dimensions were 4.5×10 m. Phosphorus (P) was applied at 53 kg ha^{-1} as triple superphosphate, and potassium (K) was applied at 100 kg ha^{-1}as KCl. N was applied as granular ammonium sulfate as follows: 30 kg ha^{-1}at planting and 110 kg ha^{-1} side-dressed ~5 cm from the maize rows at the V4-V5 stage.

Maize was harvested at the R6 stage in March 2016. Cobs, stems and leaves were left in the field. After maize harvest, the forage grasses were planted again. Due to a very dry winter, the forages did not grow, and they were replanted in October and desiccated after 60 days. Maize was planted in December 2016 and harvested in May 2017. The grasses were planted immediately after the second maize harvest and desiccated in August using 4.5 kg $ha^{-1}$of glyphosate (720 g kg^{-1}a.i.) due to poor growth, replanted in early September and desiccated in November 2017. Dolomite ($CaCO_3.MgCO_3$) and gypsum ($CaSO_4.2H_2O$) were surface-applied (1.5 Mg and 1.0 Mg, respectively) in October 2017 to ameliorate soil acidity and add S to the system. Maize [hybrid 2B587PW (Dow AgroSciences, São Paulo, Brazil)] was planted on December7, 2017, in rows 0.45 m apart and intercropped with the same forage grasses used previously. N was applied at 30 kg ha^{-1}as ammonium sulfate for the N-fertilized treatments, in addition to 50 kg $ha^{-1}$of K as KCl and120 kg ha^{-1} of P as triple superphosphate, on December 8, 2017. The forage seeds were mixed together with the P and K fertilizers at a rate of 12 kg $ha^{-1}$of pure live seeds. At the V4-V5 growth stage of maize, 34 days after planting (DAP), the remaining part of N (150 kg ha^{-1}) and K (60 kg K ha^{-1}) were side-dressed using the same fertilizer sources applied at seeding. The fertilizers were hand-applied over the soil surface~5 cm from the crop row. Maize was harvested in April2018, 134 days after planting.

3.2.2 Soil sampling

In the 2017/2018 season (Fig. 11), soil samples were randomly taken from three locations in each subplot to a depth of 20 cm using a core sampler and pooled as a single sample at (i) 12 DAP (maize at V2 stage) in December2017; (ii) 100 DAP (maize at R3/R4 stage) in March2018; and (iii) 134 DAP (maize at physiological maturity - R6 stage) in April 2018 (Fig. 11). The samples were split into two parts: (i) 50 g of fresh soil was stored at -20°C for molecular analysis, and (ii) 30 g was oven-dried at 40ºC for chemical analysis.

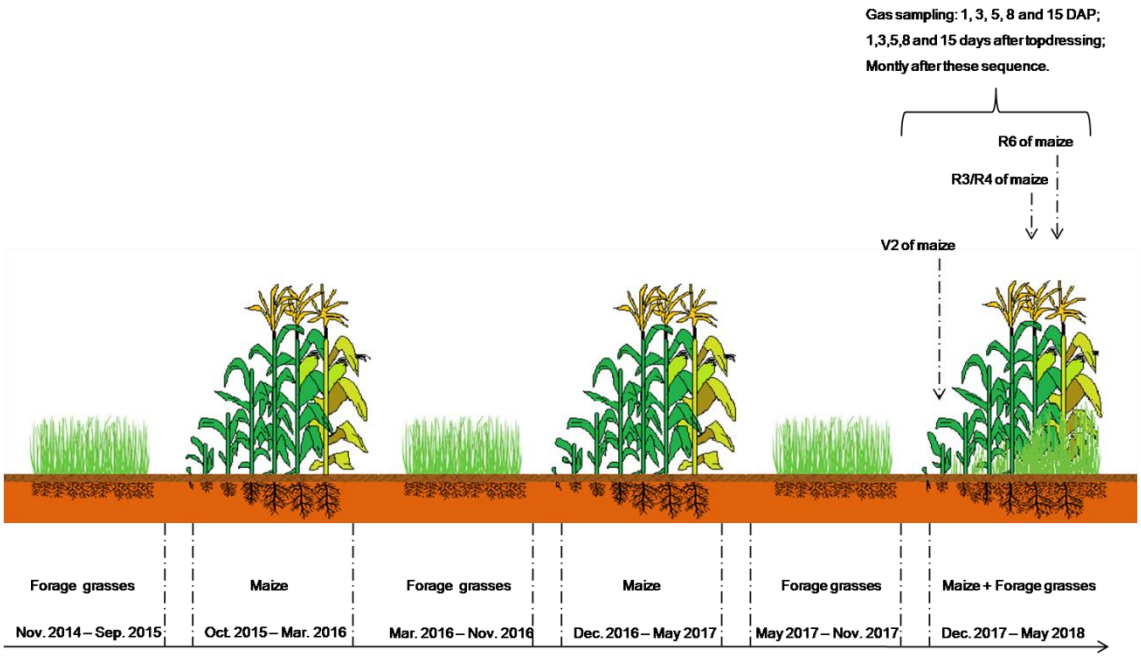


Fig. 11. Crop succession occurred in the experimental site from 2014 to 2018. Arrows represent soil sampling during the last growing season (Dec. 2017- May 2018).

3.2.3 Soil chemical analysis

Soil chemical analyses were performed according to van Raij et al. (2001), except for total C and N, which were determined by dry combustion as stated previously. The chemical and microorganism analysis were performed using the same samples. Soil pH was determined in 0.01 ml $L^{-1}CaCl_2$, while potential acidity was measured with SMP (Shoemaker-McLean-Pratt) buffer solution. Aluminum was extracted with 1.0 M KCl and determined by titration with HCl. Plant-available P, K, Ca, and Mg were extracted with ion-exchange resin. Calcium and Mg were determined by atomic absorption spectrometry, K was measured by flame photometry, and P was analyzed by colorimetry. Mineral N forms (NH_4^+ and NO_3^-) were extracted with 2 M KCl (at a soil:solution ratio of 1:5,w/v) and determined by colorimetry (Mulvaney, 1996; Miranda et al., 2001). The cation exchange capacity (CEC) was calculated as the sum of exchangeable cations (K, Ca, and Mg, represented by EC) and potential acidity (H+Al). Base saturation (BS) was calculated as the ratio of EC to CEC, multiplied by 100.

3.2.4 Isolation of DNA and quantitative real-time PCR

DNA was extracted from soil with the DNeasy PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany). To confirm DNA quality, a 5-□L aliquot was subjected to electrophoresis on a 1% (w/v) agarose gel stained with GelRed™ (Uniscience) in SB buffer (Brody and Kern, 2004). As a molecular standard, 2 µL of Low Mass DNA Ladder (Invitrogen) was used. The gel was subjected to an 85 V electric field for ~30 min. Subsequently, the DNA was quantified in a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at 260 nm (Sambrook et al., 1989).

The abundances of bacteria (16S rRNA), archaea (16S rRNA) and functional genes of the N cycle were determined by real-time quantitative PCR (qPCR) and expressed as numbers of gene copies per g of dry soil. The following genes were assessed: bacteria and archaea (16S rRNA - taxonomic gene); nitrogenase (*nif*H) for biological N fixation; bacterial and archaeal ammonia monooxygenase (*amo*A) for nitrification (NH_4^+ to NH_2OH); and nitrite reductase (*nir*S; NO_2^- to NO) and nitrous oxide reductase (*nos*Z; N_2O to N_2) for denitrification. The analyses were performed using the StepOnePlus™ Real-Time PCR System with 96-well plates (Applied Biosystems, Foster City, CA, USA).Standard curves were created based on serial dilutions of a known quantity of a gene previously amplified by PCR. For better efficiency of the standard curve, all genes used in the curves were purified with the Illustra™ GFX™ PCR DNA Kit and Gel Band Purification (GE Healthcare, UK) following the manufacturer's protocols. The DNA standards, primers, and amplification conditions are shown in Table 8. All samples had a final reaction volume of 10 µL comprising 5 µL of PowerUp™SYBR™ Green Master Mix (Thermo Fisher Scientific), 0.67 to 2.0 µL of each primer (Table 8), 0.5 µL of bovine serum albumin (6 $mgmL^{-1}$), 1 µL of 10-fold diluted DNA, and ultra-pure water to complete the volume. The specificity of the primer set was checked by observing melting of a single peak, which confirmed the purity of the amplified product and was observed as a single band in an agarose gel 1%. For all assays, the amplification efficiency ranged between 86% and 102%, and R^2 values were centered around 0.98. All microbial genes were expressed per mass of dry soil.

Table 8. Description of primers, standards DNA and amplification conditions used in qPCR analysis.

Target gene	*DSMZ or **BR code	Primers	Sequences (5' – 3')	Frag. length (pb)	Primer amount (ul)	Primer content (pmol)	Reference	Amplif. conditions
16S rRNA Of Bacteria	DSMZ 50090 *Pseudomonas fluorescens*	Eub 338f	ACTCCTACGGGAGGCAGCAG	180	1	5	Bakke et al. (2011)	95°C-10 min, 40 cycles of 95°C-30s, 53°C-40s, 72°C-40s; 95ºC-15s, 53ºC-1min, 95ºC-15s
		Eub 518r	ATTACCGCGGCTGCTGG					
16SrRNA of Archaea	DSMZ 23604 *Methanolinea mesophila*	ARC519f	CAGCCGCCGCGGTAA	397	1	5	Coolen et al. (2004), Stahl and Amann (1991)	95ºC-10 min, 45 cycles of 95ºC-30s, 58ºC-30s, 72ºC-50s; 95ºC-15s, 58ºC-1min, 95ºC-15s
		ARC915r	GTGCTCCCCCGCCAATTCCT					
*nif*H	DSMZ 17167 *Paraburkholderia phymatum*	F	AAA GGY GGW ATC GGY AAR TCC ACC AC	457	1	5	Wallensteinand Vilgalys (2005)	95°C-10 min, 40 cycles of 95°C-1 min, 53°C-27s, 72°C-1 min; 95ºC-15s, 53ºC-1min, 95ºC-15s
		R	TTG TTS GCS GCR TAC ATS GCC ATC AT					
		amoB 1F	GGG GTT TCT ACT GGT GGT	491	1	0.2		

*amo*A of Bacteria	DSMZ 28437 *Nitrosomonas europaea*	*amoB* 2R	CCC CTC KGS AAA GCC TTC TTC				Rotthauwe et al. (1997)	95°C-10 min, 40 cycles of 95°C-45s, 60°C-45s, 72°C-45s; 95ºC-15s, 60ºC-1min, 95ºC-15s
*amo*A *of* Archaea	Environmental DNA	*amoA* 1F	STA ATG GTC TGG CTT AGA CG	635	0.56	0.7	Francis et al. (2005)	95°C-5 min, 40 cycles of 95°C-40s, 56°C-30s, 72°C-1 min; 95ºC-15s, 56ºC-1min, 95ºC-15s
		amoA 2R	GCG GCC ATC CAT CTG TAT GT					
*nir*S	DSMZ 1690 *Nitrospirillum brasilense* Sp7	4 QF	GTSAACGYSAAGGARACSGG	410	0.67	0.3	Kandeler et al. (2006)	95°C-5 min; 10 cycles of 95°C-5s, 63°C-40s, 72°C-40s; 40 cycles of 95°C-15s, 58°C-40s, 72°C- 40s; 95ºC-15s, 58ºC-1min, 95ºC-15s
		6 QR	GASTTCGGRTGSGTCTTSAYGAA					
*nos*Z		2F	CGC RAC GGC AAS AAG GTS MSS GT	267	2	5	Henry et al. (2006)	95°C-10 min; 4 cycles of 95°C-

	BR 11003 *Nitrospirillum brasilensi*	2R	CAK RTG CAK SGC RTG GCA GAA	20s, 63°C-30s, 72°C-30s; 40 cycles of 95°C-20s, 60ºC-20s, 72°C–30s; 95ºC-15s, 60ºC-1min, 95ºC-15s

***** DSMZ code: reference code of the cell catalog of the Leibniz Institute DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*), used in the construction of the standard curves for qPCR analysis; BR code: reference code of the catalog of the Johanna Döbereiner Biological Resources Center (CRB-JD), Embrapa Agrobiology, Seropédica, RJ, used in the construction of the standard curves for qPCR.

3.2.5 Gas sampling, temperature and water-filled pore space procedure

Greenhouse gas emission (GHG) was quantified by placing 30 cm in diameter, 9.3 cm in high cylindrical galvanized steel chambers (Pavelka et al., 2018) over the maize row. An opaque (white), non-ventilated polypropylene lid (32.8 cm in diameter and 7.3 cm in height) was used and adjusted through an external "U"- shaped gutter around the chamber, to which water was added at the time of each measurement for sealing. A rubber septum (0.5 cm in diameter) was placed in the center of the lid. Measurements were performed from December $_3$2017 to May 2018 following Grassmann et al. (2020). The N_2O flux used in principal component analysis (PCA) and Spearman's correlation was measured at 15, 89 and 122 DAP. Air samples were taken at 0, 10, 20 and 40 min after closing the chambers. Samples were sent to the laboratory, stored at 4ºC and analyzed for N_2O within 24h using a gas chromatograph (GC-2014, Shimadzu Corp., Japan) equipped with a ^{63}Ni electron-capture detector (ECD). The ECD operated at 325°C. N_2 (99.999%) was used as the carrier gas. Standard curves were built using four standard gas solutions containing concentrations of 305, 693, 1092 and 1885 ppb of N_2O. Gas collections were carried out at 1, 3, 5, 8, 15, 30 and 60 DAP and at side dressing of maize, starting at 08:30 am (Alves et al., 2012). In the remaining season, air samples were taken monthly.

N_2O flux was estimated according to Grassmann et al. (2020):

$$GHGflux = (\Delta C/\Delta t) \times (V/A) \times m \times [P/(T \times R)] \times n \times 24$$

where *GHG flux* is the N_2O flux (mg m^{-2} d^{-1} of N) and CH_4 flux (mg m^{-2} d^{-1} of C); $\Delta C/\Delta t$ is the linear slope of GHG concentration (µmol mol^{-1}) change during the sampling period Δt (h)*; V* and *A* are the volume (m^3) and area (m^{-2}) of the chamber, respectively; *m* is the molecular weight (g) of each GHG; *P* is the atmospheric pressure (atm); *T* is the soil temperature (K); *R* is the gas constant; *n* is the ratio of the molecular weights of N and N_2O; and *24* is the factor for converting hours into days.

Seasonal GHG emissions was calculated by trapezoidal rule integration using SigmaPlot (version 12.5, Systat Software Inc., San Jose, CA, USA). In addition to N_2O emissions, soil temperature and volumetric water content were measured at a depth of 5.5 cm near the chamber using moisture and temperature sensors (Teros 11, Meter Group Inc., Pullman, WA, USA). The volumetric soil water content was converted into the water-filled pore space (WFPS) according to Grassmann et al. (2020).

3.2.6 Statistical analysis

Statistical analyses were performed in SAS (version 9.4M3, SAS Institute Inc., Cary, NC, USA). Data regarding gene abundance and soil properties were analyzed using split-split plot ANOVA with the GLIMMIX procedure. Block was considered a random effect, while sampling time was treated as a repeated measure. Restricted maximum likelihood (ReML) was used to estimate the covariance parameters, while compound symmetry (CS) was adopted as the covariance structure based on the corrected Akaike information criterion (AICc). Emissions of N_2O were subjected to split-plot ANOVA. Skewed data were treated using a lognormal distribution, and values were back-transformed. Means were separated using the LSMEANS ($P \leq 0.05$) statement with the SIMULATE adjustment. Relationships between N-cycle gene abundances, soil chemical properties, WFPS, soil temperature and N_2O flux were assessed by Spearman rank-order correlation using the CORR procedure. Principal component analysis (PCA) using the Bray-Curtis distance to test relationships between variables across sampling dates and NPMANOVA to check if the factors were statistically different were performed in R (version 3.6.3, The R Foundation for Statistical Computing, Boston, MA, USA) and RStudio (version 1.2.5019, RStudio, Vienna, Austria) using the "*lme4*" and "*vegan*" packages (Bates et al., 2015; Oksanen et al., 2019).

3.3. Results

3.3.1 Environmental conditions and soil characteristics

Precipitation during the maize season totaled 713 mm (Fig. 12). The minimum air temperature ranged from 6.9 to 22.1°C, while the maximum air temperature ranged from 17.3 to 32.9°C.

There were no differences in soil N between forage grasses. Soil NO_3^- decreased by 43% (DAP: $P<0.001$) from 12 to 100 DAP, with relative stabilization afterwards (Fig. 13a). For NH_4^+, there was an interaction of N rate and DAP ($P<0.001$; Fig. 13b). Initially, N fertilization increased soil NH_4^+-N by 114% compared with the unfertilized treatment at 12 DAP (Fig. 13b). However, this difference disappeared as the season progressed and was not significant at 134 DAP. Total N in the soil decreased from 12 to 134 DAP ($P<0.05$; Fig. 13c). No differences were found for the other soil parameters regardless of the factor (Suppl. Table 1). However, soil pH and

Mg were higher in the absence of N application (control), while H+Al was higher in the fertilized treatment. Soil pH, Mg and BS were higher at 12 DAP, and C total, C/N ratio, P, Ca, H+Al, CEC and BS were higher at 100 and 134 DAP.

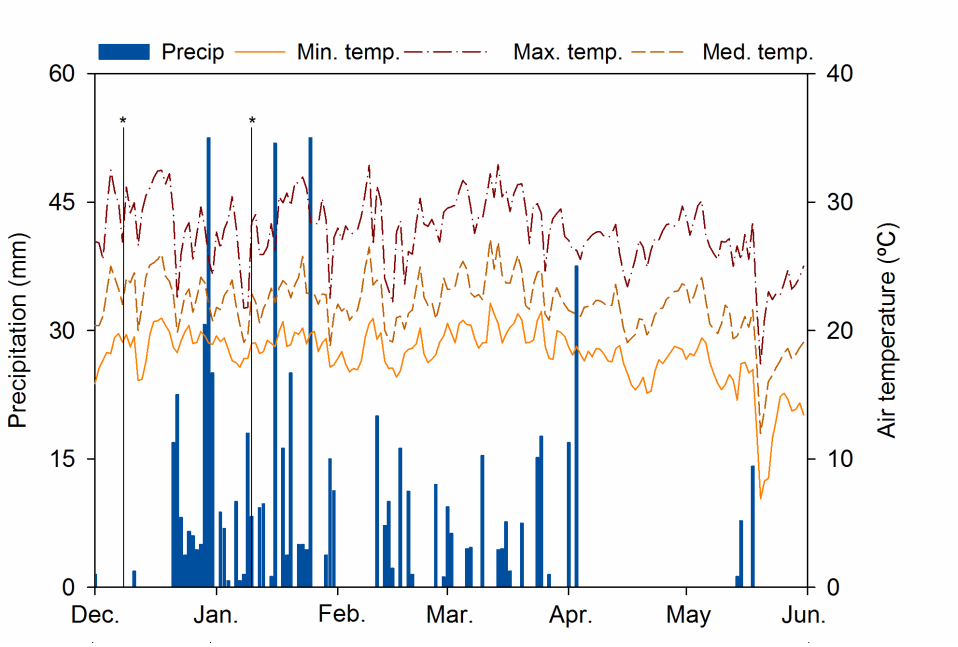


Fig. 12. Daily minimum, maximum and average air temperatures and rainfall recorded during the growing season period (Dec. 2017- May 2018). Asterisk represent N fertilization events.

Suppl. Table 1. Soil properties as affected by N fertilization and days after planting (DAP). Values for forage grass were ommited since they were not significant. The error bars represent the SEM (*n* = 36, 24, 12 for N rate, DAP and DAP × N, respectively.

N rate	DAP	pH	Total C	C/N	P	K	Ca	Mg	H+Al	Al	EC	CEC	BS
(kg ha^{-}			g kg^{-1}		mg kg^{-1}				mmol$_c$ kg^{-}				%
0	–	5.2±0.1a	17±1	9.1±0.1	30±3	2.5±0.1a	40±1	25±0.6a	37±2b	0.8±0.2b	68±1a	105±1	64±1a
180	–	5.1±0.1b	18±1	9.0±0.1	27±3	1.6±0.1b	39±1	22±0.6b	43±2a	1.6±0.3a	63±1b	107±1	59±1b
		P<0.001	*P*=0.353	*P*=0.406	*P*=0.288	*P*<0.001	*P*=0.088	*P*=0.001	*P*<0.001	*P*<0.001	*P*=0.002	*P*<0.296	*P*<0.001
–	12	5.3±0.1a	17±1b	8.7±0.1c	14±2b	2.0±0.1	38±0b	24±0.7a	36±2b	1.0±0.2b	64±1b	101±1c	63±1a
–	100	5.1±0.1b	18±1ab	9.1±0.1b	38±4a	2.1±0.2	43±1a	24±0.8a	41±2a	1.2±0.3ab	69±2a	111±1a	62±2ab
–	134	5.1±0.1b	18±1a	9.4±0.1a	45±5a	2.1±0.2	39±1b	22±0.7b	42±2a	1.4±0.3a	64±2b	106±1b	60±1b
		P<0.001	*P*=0.038	*P*<0.001	*P*<0.001	*P*<0.804	*P*<0.001	*P*<0.001	*P*<0.001	*P*=0.013	*P*<0.001	*P*<0.001	*P*=0.003
0	12	5.4±0.1	17±1	8.7±0.1	13±2	1.9±0.2bc	38±1	25±1.0	35±2	0.8±0.2c	65±2bc	100±1	64±2
0	100	5.2±0.1	17±1	9.2±0.1	41±6	2.8±0.2a	43±1	25±1.0	38±2	0.7±0.2c	71±2a	110±1	65±2
0	134	5.2±0.1	18±1	9.4±0.1	53±7	2.7±0.2a	41±1	24±0.9	38±2	0.9±0.2c	68±2ab	106±1	63±2
180	12	5.2±0.1	17±1	8.7±0.1	15±2	2.0±0.1b	38±1	23±0.8	38±2	1.0±0.2bc	63±2bc	102±1	62±2
180	100	5.0±0.1	18±1	9.0±0.1	35±5	1.3±0.1d	43±2	23±1.1	45±3	1.9±0.5ab	67±3ab	112±2	59±2
180	134	5.0±0.1	18±1	9.4±0.1	38±5	1.4±0.1cd	38±1	20±1.0	46±3	2.0±0.5a	60±2c	106±1	56±2
		P=0.571	*P*=0.590	*P*=0.832	*P*=0.173	*P*<0.001	*P*=0.112	*P*=0.375	*P*=0.108	*P*=0.030	*P*=0.039	*P*=0.602	*P*=0.056

Different lowercase letters indicate differences at *P*≤0.05. DAP: Days after planting; EC: Sum of exchangeable cations; CEC: Cation exchange capacity; BS: Base saturation.

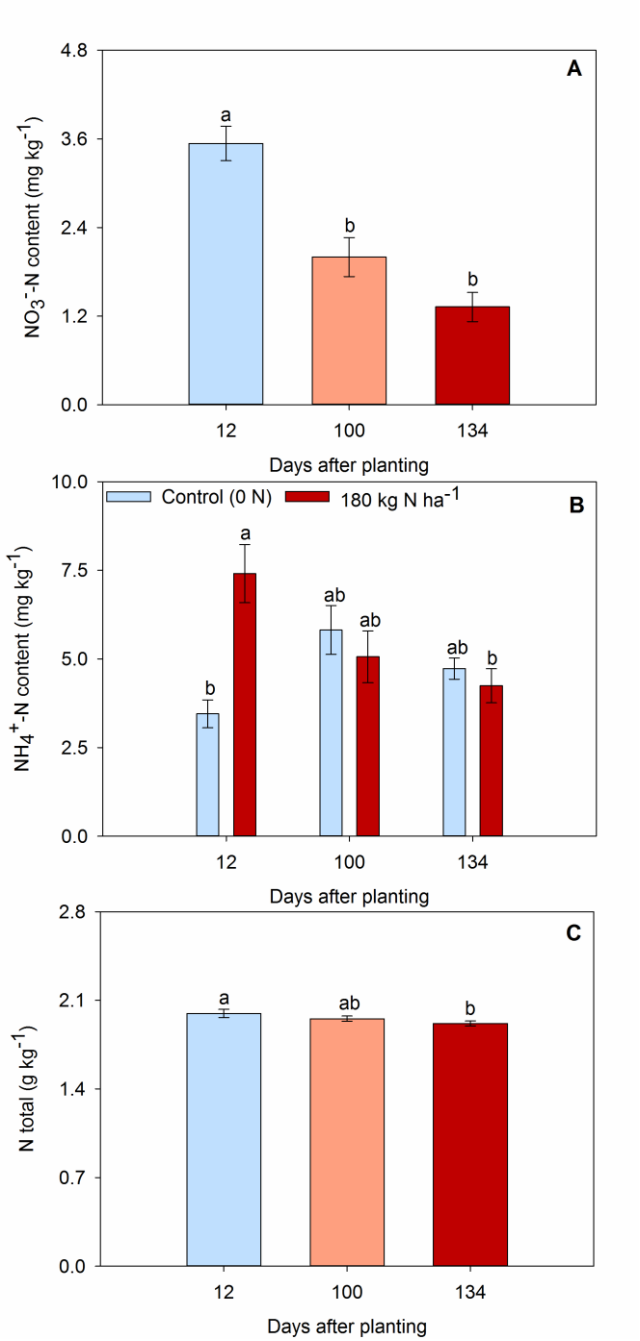


Fig. 13. Contents of NO_3^--N (a), NH_4^+-N (b), and total N (c) at the 0-20 cm soil layer as affected by forage grass specie, N fertilization, and time after planting. The error bars represent the SEM (*n* = 24, 12, and 24 for panels a, b, and c, respectively). This figure shows only the significant results. Different lowercase letters indicate differences at $P \leq 0.05$.

3.3.2 N-cycle functional genes

Bacteria and archaea were analyzed only to demonstrate their presence in the soil (Fig. 14abc). The *nif*H copy number (Fig. 14d) was highest under unfertilized palisade grass (7.0×10^5gene copies g^{-1} soil). The abundance of AOA was higher than AOB (Fig. 14ef). For AOB, averages of 1.8×10^5 and 1.0×10^5 gene copies g^{-1} soil were detected at 12 and 100 DAP, respectively. These results indicate a slight increase in AOB close to the time of N fertilizer application. However, for AOA, the opposite pattern was observed, with average values of 3.8×10^7 and 6.1×10^7 gene copies g^{-1}soil at 12 and 100 DAP respectively. Regarding denitrification genes, the *nir*S copy number was 34% higher in the unfertilized control than in N-fertilized soil (Fig. 14g). The copy number of *nos*Z was 22% higher at 100 DAP than at 12 DAP and 45% higher at 100 DAP than at 134 DAP (Fig. 14h).

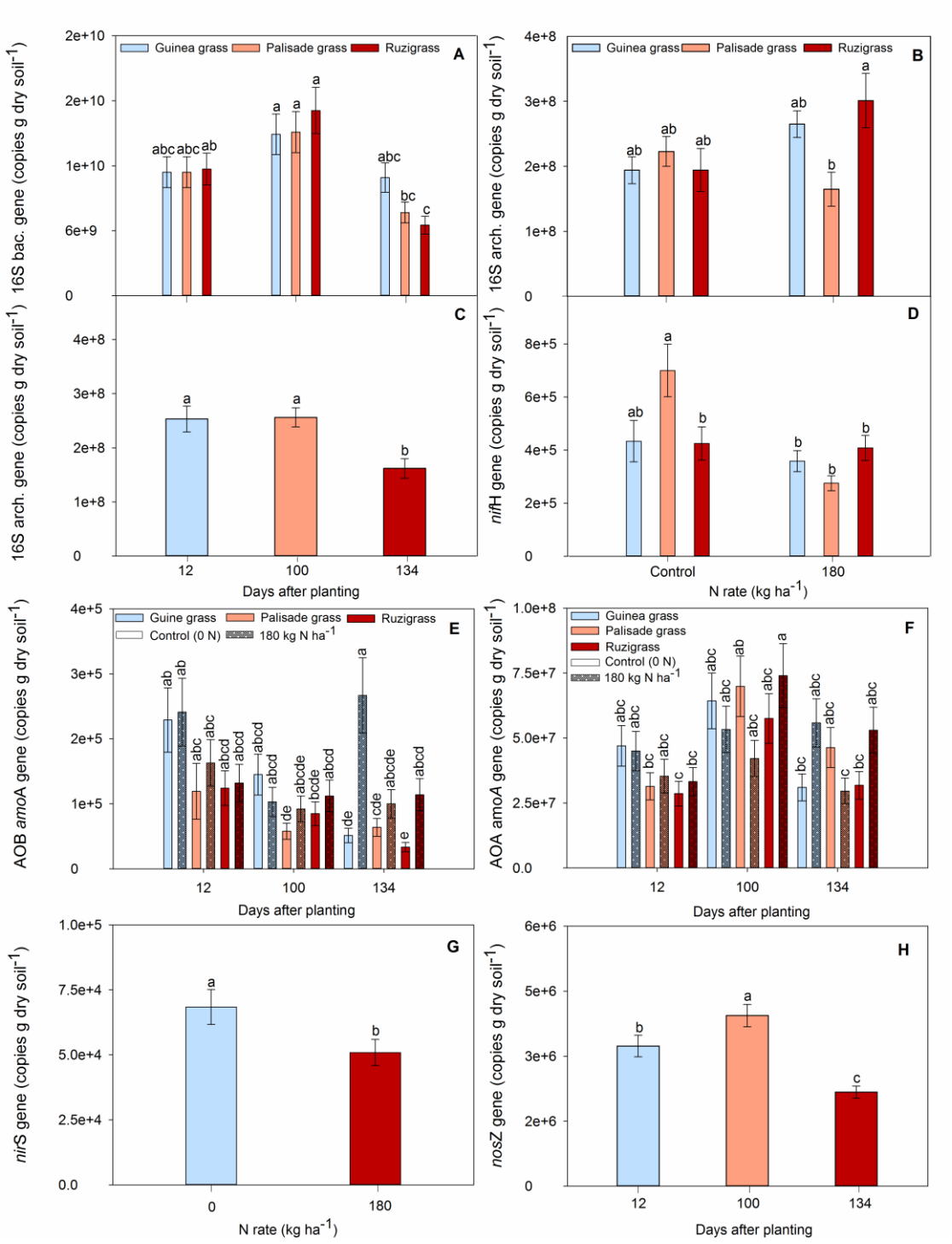


Fig. 14. Abundance of 16S rRNA of bacteria (a) and archaea (b and c), *nif*H (d), *amo*A of bacteria (e), *amo*A of archaea (f), *nir*S (g) and *nos*Z (h) at the 0-20 cm soil layer as affected by forage grass species, N fertilization and days after planting. This figure shows only the significant results. The error bars represent the SEM (n = 8, 12, 24, 12, 4, 4, 36, and 24 for panels a, b, c, d, e, f, g, and h, respectively). Different lowercase letters indicate differences at $P \leq 0.05$. Probability (P) values of each variable can be seen in Suppl. Table. 2.

Suppl. Table 2. Probability (*P)* values for microbial genes in the soil as affected by forage grass species, N fertilization and days after planting (DAP).

N-cycle genes	Forage grass	N rate	DAP	Forage grass x N rate	Forage grass x DAP	N rate x DAP	Forage grass x N rate x DAP
Archaea	0.277	0.060	0.006	0.013	0.207	0.440	0.554
Bacteria	0.624	0.952	<0.001	0.677	0.031	0.463	0.202
*amo*A	0.592	0.457	<0.001	0.008	0.086	0.061	0.024
*amo*B	0.041	<0.001	<0.001	0.195	0.278	<0.001	0.047
*nir*S	0.328	0.036	0.342	0.098	0.852	0.178	0.550
*nos*Z	0.577	0.806	<0.001	0.844	0.490	0.573	0.353
*nif*H	0.352	0.002	0.777	0.004	0.989	0.562	0.742

3.3.3 Nitrous oxide emissions

Cumulative N_2O emissions were 430% higher in the N-fertilized treatment than in the unfertilized control (N rate: $P<0.001$) (Fig. 15). Nevertheless, seasonal cumulative N_2O emissions were not affected by the forage grasses intercropped with maize. WFPS ranged from 31% to 84% over the experimental period (Suppl. Fig. 3a). The soil temperature followed the air temperature pattern, with values ranging from 21.0 to 35.7°C over the experimental period (Suppl. Fig. 1b). The N_2O fluxes ranged from -0.1 to 4.5 mg m^{-2} d^{-1} of N across treatments (Suppl. Fig. 3c), and the highest fluxes occurred following the basal and side dressed N applications, regardless of the forage grass species.

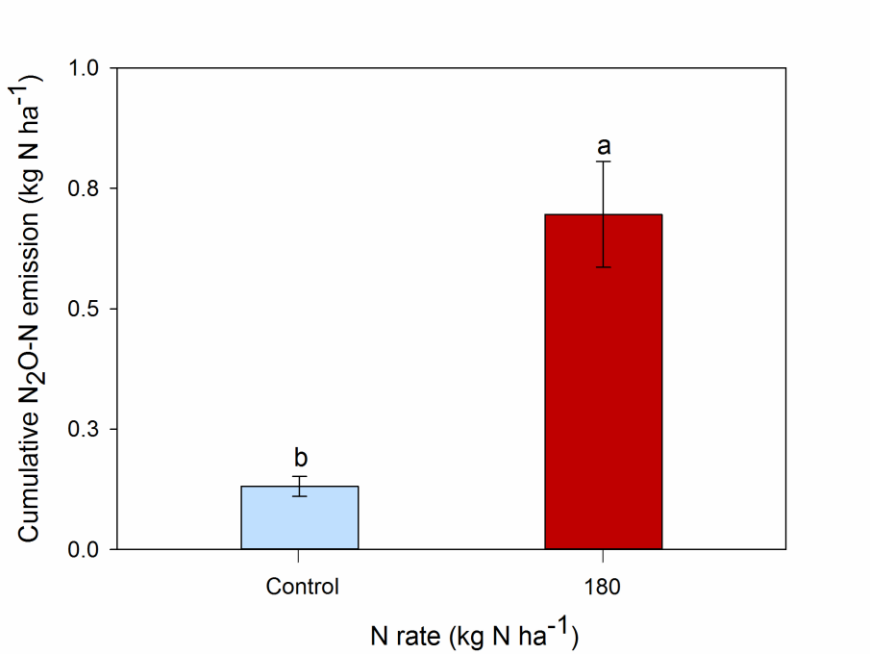
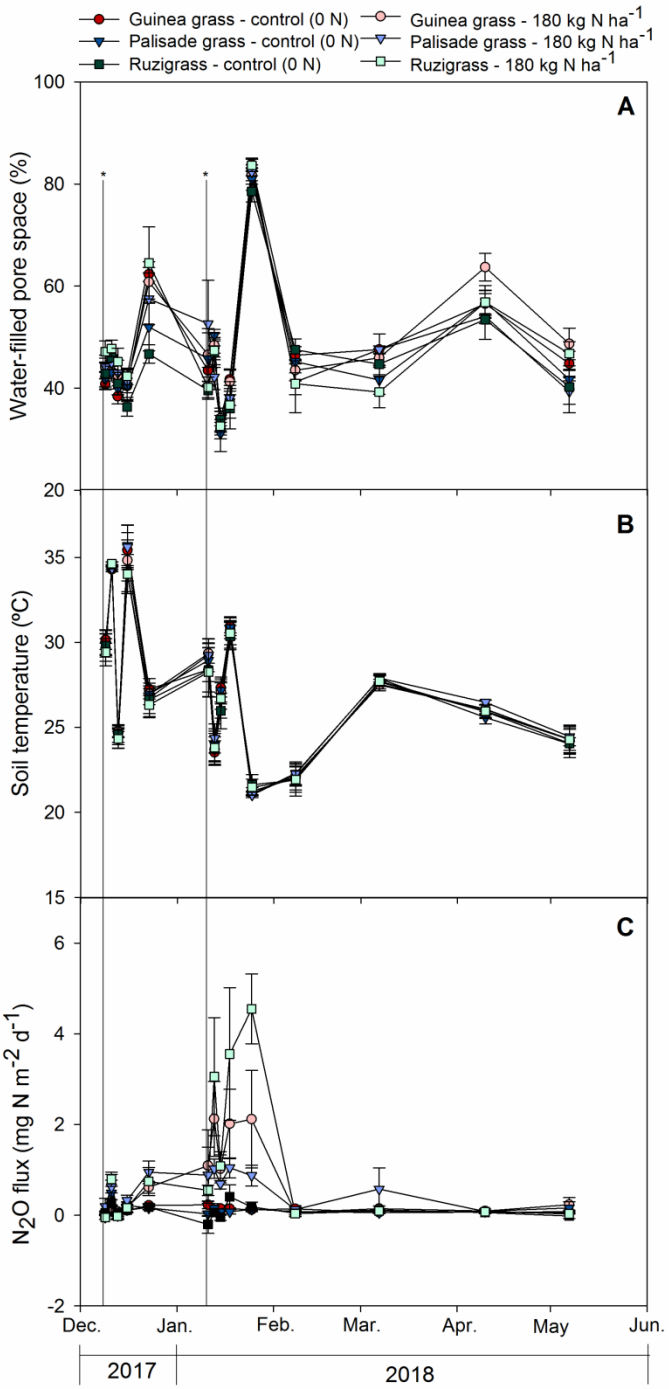


Fig. 15. Cumulative N_2O emission in forage grass-maize intercropping as affected by forage grass specie and N fertilization. The error bars represent the SEM (n = 12). Different lowercase letters indicate differences at $P \leq 0.05$.



Suppl. Fig. 3. Variation of water-filled pore space (a) and soil temperature at depth of 5.5 cm (b) as well as N_2O flux (c) in forage grass-maize intercropping as affected by forage grass specie and N fertilization. Symbols represent mean values, and the error bars represent the SEM (*n* = 4). Asterisk represent N fertilization events.

3.3.4 Correlations between the abundance of N cycle genes, soil properties and N_2O flux

The copy numbers of *nir*S, *nif*H and *nos*Z genes were not correlated with any soil chemical property, temperature, WFPS, or N_2O flux but were correlated with the copy numbers of N-cycle genes involved in nitrification (Fig. 16). The copy number of archaea was positively correlated with total N and negatively correlated with C/N. In addition, the copy number of bacteria was positively correlated with total N and temperature but negatively correlated with WFPS. AOA was positively correlated with C, Ca, Mg, EC, CEC, SB and temperature and negatively correlated with Al. Lastly, AOB was correlated positively with NO_3^- and WFPS and negatively with plant-available P.

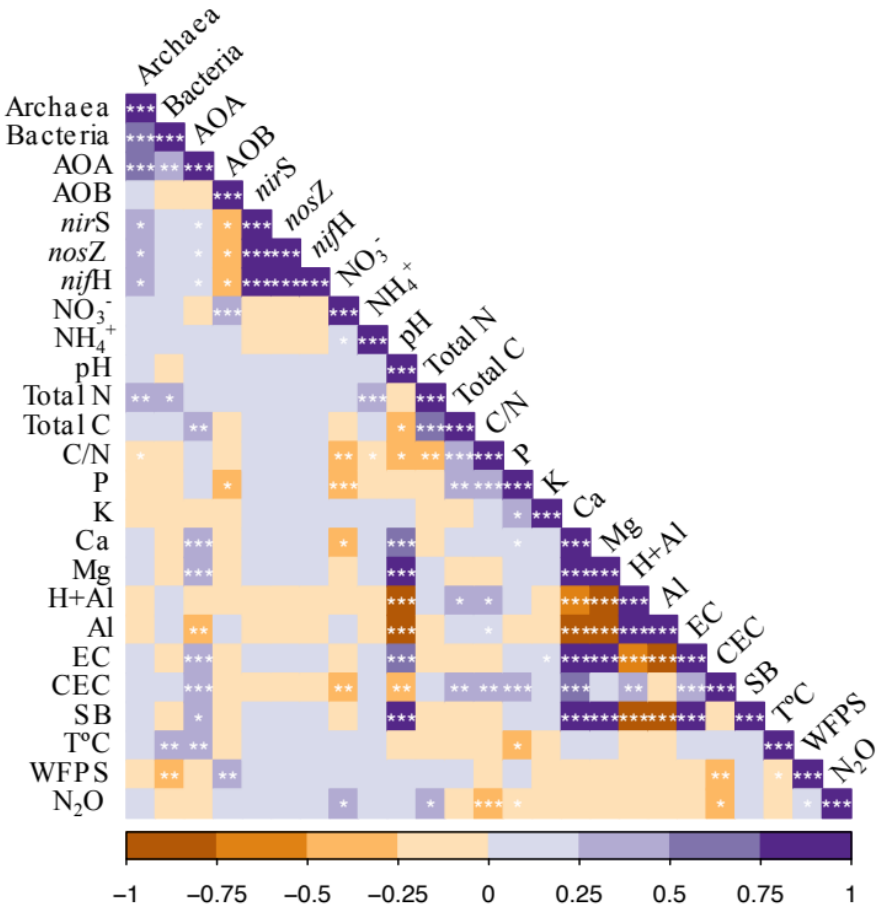


Fig. 16. Heatmap of the Spearman rank correlation order among the relative abundance of N-cycle functional genes, soil properties, and N_2O flux. *, **, and ***: significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. Archaea: 16S rRNA of

archaea; Bacteria: 16S rRNA of Archaea; AOA: *amo*A of archaea; *amo*A of bacteria; EC: Sum of exchangeable cations; CEC: Cation exchange capacity; BS: Base saturation; TºC: Temperature; WFPS: water-filled pore space.

3.3.5 Principal component analysis

The biplot of PCA of the abundance of microbial genes against WFPS, temperature, and N_2O flux revealed a clear separation among sampling timings (12, 100, and 134 DAP) as confirmed by NPMANOVA (P<0.001, Fig. 17a). Additionally, ordination using "*envfit*" significance tests (P< 0.01; Suppl. Table 3) of the axes revealed higher N_2O flux, WFPS and AOB abundance at 12 DAP compared with 100 and 134 DAP. By contrast, at 100 DAP, we observed a high correlation among the abundances of bacteria, archaea, AOA and *nos*Z and soil temperature. However, the ordination of chemical properties sampled at 12, 100, and 134 DAP (Fig. 17B) showed no response, as confirmed by NPMANOVA (R^2=0.18; P=0.06). The biplot of PCA ordination by using the "*envfit*" function of the chemical properties against WFPS, temperature, and N_2O flux (Fig. 17b; Suppl. Table 4) showed higher contents of NH_4^+, NO_3^-, total N, and soil pH associated with N_2O flux at 12 DAP.

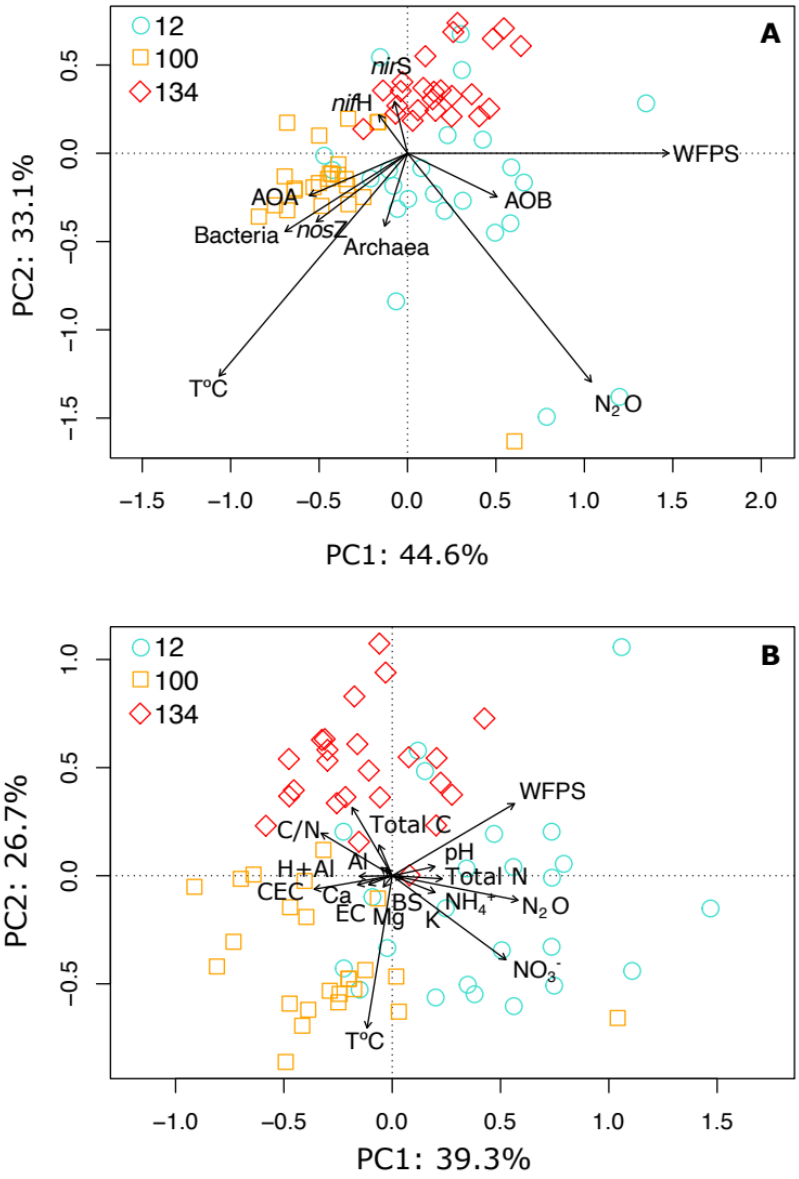


Fig. 17. Principal component analysis of the soil microbial genes (a) and chemical properties (b) with N_2O fluxes, water-filled pore space (WFPS) and temperature (T°C) from soil of 72 sampling points across three times. Archaea: 16S rRNA of archaea; Bacteria: 16S rRNA of Archaea; AOA: *amo*A of archaea; AOB; *amo*A of bacteria; EC: Sum of exchangeable cations; CEC: Cation exchange capacity; BS: Base saturation.

Suppl. Table 3. Scores of principal components analysis relative to temperature (T°C), water-filled pore space (WFPS), N_2O flux, and abundance of microbial genes as presented in Fig. 17a.

Parameter	**PC1**	**PC2**	R^2	***P* value**
Temperature	-0.64	0.76	0.83	0.001
WFPS	1.00	-0.01	0.66	0.001
N_2O flux	0.63	0.78	0.84	0.001
Archaea	-0.30	0.95	0.06	0.139
Bacteria	-0.89	0.46	0.21	0.002
AOA	-0.95	0.31	0.12	0.007
AOB	0.99	0.16	0.12	0.01
*nir*S	0.91	0.40	0.01	0.828
*nos*Z	0.91	0.40	0.01	0.828
*nif*H	0.91	0.40	0.01	0.828

Archaea: 16 S rRNA of archaea; Bacteria: 16S rRNA of bacteria; AOA: *amo*A of archaea; AOB: *amo*A of bacteria.

Suppl. Table 4. Scores of principal components analysis of the soil chemical characteristics, water-filled pore space (WFPS), temperature (TºC), and N_2O flux presented in Fig. 17b.

Evaluated parameters	PC1	PC2	R^2	*P* value
NO_3^-	-0.81	0.59	0.49	0.001
NH_4^+	-0.99	-0.05	0.67	0.001
pH	-0.05	0.99	0.80	0.001
Total N	-0.92	-0.38	0.68	0.001
Total C	-0.58	-0.81	0.40	0.001
C/N	0.86	-0.50	0.26	0.001
P	0.51	-0.86	0.10	0.028
K	0.19	0.98	0.06	0.117
Ca	0.02	0.99	0.10	0.032
Mg	-0.15	0.99	0.37	0.001
H+Al	0.08	-0.99	0.67	0.001
Al	0.19	-0.98	0.37	0.001
EC	-0.07	0.99	0.23	0.001
CEC	0.09	-0.99	0.19	0.002
SB	-0.06	0.99	0.53	0.001
Temperature	-0.99	0.08	0.01	0.846
WFPS	-0.79	0.61	0.08	0.072
N_2O	-0.87	0.49	0.14	0.005

EC: Sum of exchangeable cations; CEC: Cation exchange capacity; BS: Base saturation; WFPS: Water-filled pore space.

3.4 Discussion

3.4.1 Linking N-cycle genes, forage grass and N fertilization

Different genotypes and species of *Urochloa* acquire N in part from BNF (Reis et al., 2001; Roesch et al., 2007). Rocha et al. (2020) suggested that the abundant N-fixing organisms in the rhizosphere of forage grasses promote entry of N into systems with high nutritional responses, as BNF provides NH_4^+ to the plant. In our study, the abundance of *nif*H was highest for palisade grass in the unfertilized control (Fig. 14d), probably due to inhibition of BNF by N fertilization (He et al., 2020). Marques et al. (2017) suggested that well-nourished plants produce less attractive exudates for

microorganisms, and as a result, the non-symbiotic association may not be energetically favorable. However, our results differed from those of Rocha et al. (2020): the abundance of *nif*H was higher for ruzigrass than palisade grass and guinea grass six months after the planting of these grasses without N addition, suggesting that other, unaccounted-for factors influenced the abundance of *nif*H in addition to N fertilization. Differences in *nif*H abundance among forage species like those observed in our study have been reported previously (Boddey and Dobereiner, 1995; Reis et al., 2001; Gupta et al., 2019), and significant effects of plant species and cultivars on the abundance of diazotrophs and the number of *nif*H transcripts have been documented in different root systems (Bouffaud et al., 2016; Gupta et al., 2019). Reis et al. (2001) reported that *U. brizantha* and *M. maximus* obtained up to 41% of accumulated N through biological fixation and suggested that this could be achieved by allocating large amounts of C to root exudates. However, root exudates and secondary plant metabolites have a selective or inhibitory effect on bacteria (Marques et al., 2017). For example, *Sphingomonadales* appears to prefer root exudates from monocot plants over those of other plants (Rosenblueth et al., 2018). Exudate composition can also be modified by nutrient availability in the soil. Fertilization with N and P may alter the types of exudates released in the rhizosphere, with a negative effect on colonization (Marques et al., 2017). Thus, we speculate that N fertilization inhibited BNF for palisade grass but not other forage species. The impact of N fertilization on BNF may have been less pronounced in the other forages because the associated communities did not necessarily fix N; quantifying a target gene does not indicate whether it is functional in the cell.

The abundances of AOA and AOB were affected by the interaction of the grass with N fertilization as well as time (Fig. 14ef). AOB but not AOA increased in response to N fertilization for all three grasses and decreased over time. Studies have suggested that N fertilization increases the abundances of AOA and AOB in agricultural soils, but there is no consensus on the relative responses of AOB and AOA to N fertilization (Hirsh et al., 2015; Beeckman et al., 2018; Oyang et al., 2018). Oyang et al. (2018) observed that the effect of N fertilization on AOB was 9-fold greater than that on AOA. Suggested explanations for the greater AOB response to N fertilization compared with AOA include i) the larger cell size of AOB compared with AOA (Lehtovirta-Morley et al., 2016; Prosser and Nicol, 2012); ii) the distinct ammonia oxidation pathways of AOB and AOA, which affects physiological responses to NH_4^+ availability (Kozlowski et al.,

2016; Ouyang et al., 2017); and iii) the 10- to 20-fold greater maximum activity (V_{max}) and 15- to 40-fold greater half saturation constant (K_m) of AOB compared with AOA in agricultural soil treated with NH_4^+-based fertilizers (Ouyang et al., 2017).

In pasture soils exposed to high rates of animal urine, AOB populations can be enhanced (Di et al., 2009). A study in which a nitrification inhibitor was applied to pasture revealed inhibition of the population growth of AOB; by contrast, the abundance of AOA remained unchanged regardless of N-urine and inhibitor treatments (Di et al., 2009). The apparent responsiveness of AOB to N emphasizes the importance of this group of microorganisms as a target group for N management to reduce losses and improve N use efficiency (Di et al., 2009). AOB but not AOA responded positively to N fertilization at 12 DAP. Since both groups (AOB and AOA) use NH_4^+ as a substrate, these divergent responses may indicate that AOB and AOA do not compete directly for the substrate. Recent studies suggest that the distinct evolution of ammonia oxidation pathways in these groups may have led to differences in physiological responses to ammonia availability (Kozlowski et al., 2016; Ouyang et al., 2017) reflecting niche differentiation, as microorganisms tend to inhabit unique vertical environment niches (Hansel et al., 2008). Over time, the abundance of AOA increased and soil NH_4^+-N availability decreased in the N-fertilized treatment (Fig. 13b), suggesting NH_4^+ sensitivity of AOA.

With respect to genes involved in denitrification, the abundance of *nir*S decreased with N application, whereas the abundance of *nos*Z was highest at 100 DAP (Fig. 14gh). Furthermore, *nir*S and *nos*Z were positively correlated with AOA but negatively correlated with AOB (Fig. 16). The correlation of the nitrifying and denitrifying genes may reflect similar effects of N fertilization: at 100 DAP, *nos*Z and AOA increased in abundance (Fig. 14fh), whereas over time, the availability of N decreased in the soil (Fig 13c). This may explain the positive correlations of AOA with *nir*S and *nos*Z. By contrast, AOB seemed to respond better when N was present in the soil, which may explain the negative correlations of this group with *nir*S and *nos*Z. Oyang et al. (2018) also found correlations between genes involved in nitrification and denitrification and suggested similar effects of N fertilization on these genes. However, the relationship between the abundances of nitrifying and denitrifying genes remains unclear (Jin et al., 2014; Kastl et al., 2015). In unfertilized soils, denitrifiers may depend on nitrifiers, as the latter produce the nitrate required by the former through the microbial oxidation of NH_4^+. Close spatial and temporal interactions with nitrifiers may

therefore be beneficial for denitrifiers to avoid competition with plants for NO_3^- (Regan et al., 2016). The variation of *nos*Z abundance over time (Fig. 14h) seemed to be independent of soil chemical properties, since no correlations were observed. In addition, the abundance of soil denitrifiers depends on the physiological stage of the plant (Maul et al., 2019; Rocha et al., 2020). This mechanism could explain the variation of *nos*Z abundance over time, which was unaffected by forage species. Resolving these uncertainties requires further analyses of nitrification and denitrification genes.

3.4.2 N-cycle gene abundance and soil chemical characteristics

The ability of *Urochloa* grasses to suppress nitrification has been suggested (Subbarao et al., 2012) but remains poorly understood (Ishikawa et al., 2003). The levels of NO_3^--N, and NH_4^+-N found in the soil in our study do not support the occurrence of nitrification inhibition by the forage species, and these levels were also similar among the species (Fig. 13abc). The difference in NH_4^+-N in the soil between the N-fertilized treatment and the unfertilized control at 12 DAP is probably related to the proximity of this time point to the application of ammonium sulfate. In a study at the same experimental site, Rocha et al. (2019) showed that soil nitrification rates were similar among these three forage grasses. In addition, the positive correlation between NO_3^--N and AOB (Fig. 16) suggests that AOB made a larger contribution to nitrification activity, even with the dominance of AOA. This unexpected pattern was also identified by Banning et al. (2015), who reported dominance of AOA but a lesser contribution of this group to soil nitrification. The lack of correlation between AOA and NO_3^--N in the soil supports this result.

The abundance of AOA was higher than that of AOB in our study, probably due to the low pH. A study across an agricultural soil pH gradient (4.9–7.5) found a negative relationship of the abundance of archaeal *amo*A genes and transcripts with soil pH (Jia and Conrad, 2009). Low pH decreases the availability of NH_3, thus favoring the AOA community, which prefers lower NH_3 concentrations than AOB (Sun et al., 2019). However, the mechanism underlying the dominance of AOB over AOA in agricultural soils is not yet understood, and the contributions of AOB and AOA to *in situ* nitrification rates have not been firmly established (O'Sullivan et al., 2013). The higher abundance of AOA and weak positive correlations with total C, Ca, Mg, EC, CEC and SB (Fig. 16) may be related to the capability of these microorganisms to develop an autotrophic,

mixotrophic or heterotrophic lifestyle (Hatzenpichler et al., 2012; Stahl and De La Torre, 2012; Shen et al., 2015).

3.4.3 Relationships of N_2O emissions with N-cycle genes, N fertilization and soil properties

PCA (Fig. 17a) showed a strong influence of WFPS and AOB on N_2O fluxes at 12 DAP, but denitrification genes were not correlated with these parameters. A lack of correlation between the abundance of denitrification genes (e.g., *nirK*, *nirS*, and *nos*Z) and N_2O fluxes has been reported previously (Nadeau et al., 2019) and suggests that soil environmental conditions are the main driver of N_2O fluxes (Nadeau et al., 2019). Denitrification requires specific environmental conditions (Tiejed, 1988), although there are many processes other than nitrification that have the potential to produce N_2O (Butterbach-Bahl et al., 2013). Although both AOA and AOB encode nitrite reductase, most AOB also encode nitric oxide reductase, allowing them to sustain respiratory metabolism under oxygen limitation (O_2) using NO_2^- and NO as alternative electron acceptors via so-called nitrification denitrification (Arp and Stein, 2003; Hink et al., 2017). Our results are in line with other studies supporting AOB as a principal group controlling N_2O emissions in tropical agricultural soils (Pitombo et al., 2015; Lourenço et al., 2018). For instance, in oxide soils with relatively low moisture content (up to 60% WFPS), N_2O is produced mainly by nitrifiers (Bollmann and Conrad, 1998; Bateman and Baggs, 2005; Baggs et al., 2010). These organisms oxidize NH_3^+ to NO_2 via hydroxylamine (NH_2OH) under aerobic conditions (Vajrala et al., 2013). AOB generate N_2O both directly through incomplete oxidation of NH_2OH to NO and later to N_2O and by nitrification denitrification, mainly under reduced O_2 conditions (Shaw et al., 2006).

Among the most important factors in N_2O flux are soil moisture (Butterbach-Bahl et al., 2013) and drainage capacity (Davidson et al., 2001). Events that increase soil moisture, such as rain and irrigation, can stimulate nitrification and denitrification, thus increasing N_2O emissions (Liu et al., 2016). Previous studies have shown optimal N_2O production through ammonia oxidation at 60% WPFS (Hink et al., 2016), and N_2O emission by denitrification predominates in moist soils with >85% WFPS (Liu et al., 2016, Bowles et al., 2018). Therefore, we can infer that nitrification by AOB and WFPS may have been the determinants of N_2O emissions at 12 DAP; during this period, WFPS was below 60%, with an average of 57% (Suppl. Fig. 1). High

evapotranspiration in tropical agricultural soil, which contributes to rapid soil drying, is another potential factor supporting the importance of AOB population growth. The drainage capacity of most Brazilian tropical soils mitigates N_2O emissions (Bustamante et al., 2009).

Although soil water content is one of the most important factors determining N_2O flux, we observed an increase in N_2O flux following basal and side dressing N fertilization in maize, primarily in succession to ruzigrass (Suppl. Fig. 3). The high N_2O fluxes after N fertilizer side dressing are consistent with other studies showing that fertilizer N is important for N_2O fluxes (Stehfest and Bouwman, 2006; Shcherbak et al., 2014, Grassmann et al., 2020). This hypothesis is supported by the low N_2O flux in the remaining season, indicating a lower influence of N fertilization (February-May 2018). NH_4^+ and NO_3^-, which are substrates for N_2O production (Bowles et al., 2018), were also higher in the soil at 12 DAP, around the time of N application (Fig. 3abc). Over the experimental period, cumulative N_2O-N emissions were 0.13 and 0.70 kg ha^{-1}in the unfertilized control and N-fertilized treatments, respectively (Fig. 15), which are relatively low compared with the averages of 0.67 and 0.98 kg N_2O-N found by Jantalia et al. (2008) in a no-till crop rotation system in Brazilian soil. This result clearly supports a key role of N fertilization in N_2O emissions. As the cumulative N_2O emissions were not affected by forage species, BNI must be similar for these forage grasses and much lower, if present, than that found for *U. humidicola* (Subbarao et al., 2012). Differences between species and cultivars might appear after a period of cultivation long enough for the accumulated exudates from the grasses to have a greater influence on the N cycle, as emphasized by Grassmann et al. (2020).

3.5. Conclusions

This study provides previously unavailable information on the correlations of N-cycle functional genes with soil parameters and N_2O fluxes in intercropping systems of maize with tropical forage grasses, which are thought to have the ability to suppress soil nitrification. Cumulative N_2O emissions were higher in the N-fertilized treatments than those without N addition. Moreover, a close relationship was observed among soil characteristics (NH_4^+-N, NO_3^--N, and WFPS), gene abundance (AOB) and N_2O emissions. No relationship was found between denitrifying genes and N_2O emissions. Furthermore, the linkage between N_2O and AOB revealed by PCA may imply that AOB increase N_2O emissions in intercropping systems. Our results support the hypothesis

that soil N-cycle microorganisms are influenced by forage grasses and N fertilization and timing, although there was no evidence of BNI for the three forage species. Future studies should assess the main factors affecting the abundance of N-cycle functional genes and N losses by N_2O in intercropping systems with tropical grasses and maize. Research on the archaeal and bacterial populations involved in the N-cycle in such agroecosystems may provide further insights. A better understanding of these interactions may provide a pathway for achieving synergism between plant demand and N fertilization to mitigate greenhouse gas emissions.

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CHAPTER 4

BROMATOLOGICAL QUALITY AND ESTIMATED MEAT PRODUCTION IN MAIZE INTERCROPPING WITH TROPICAL FORAGE GRASSES WITH NITROGEN FERTILIZATION

Will be submitted to Grass and Forage Science

Abstract

The adoption of integrated production systems such as intercropping and crop rotation in conservationist systems has been of great relevance for agriculture, allowing the use of land throughout the year in addition to the recovery of degraded pastures. The success of integrated crop-livestock systems (ICLSs) under no-till (NT) depends on numerous factors, and the choice of the grass to be introduced is of fundamental importance. In this study, the bromatological quality, dry matter yield, and estimated meat production were analyzed in a field experiment in which maize was intercropped with Guinea grass (*Megathyrsus maximus* cv. Tanzania) and palisade grass (*Urochloa brizantha* cv Marandu) under three N rates (90, 180 and 270 kg N ha^{-1}) and control without N fertilization. Nitrogen fertilization resulted in the highest grass dry matter yield, 144% higher than the N-unfertilized treatments, on average. The maize highest grain yield was observed with 270 kg ha^{-1} of N, 48% greater than all other treatments. Guinea grass fertilized with 270 kg ha^{-1} of N resulted in a calculated meat production 27% higher than palisade grass at the same amount of N added. However, at the final cut, Guinea grass showed the highest values for neutral detergent fiber (NDF), acid detergent fiber (ADF) and cellulose, even when fertilized with 270 kg ha^{-1} of N. Palisade grass seems to impose a lower competition with maize; however, Guinea grass results in greater meat production.

Keywords: *Megathyrsus maximus; Urochloa brizantha*, *Zea mays* L.; dry matter yield; canopy light interception.

4.1 Introduction

The recovery of degraded areas and the maximization of the fertilizer use efficiency are essential factors to increase sustainability in several agricultural systems (Thierfelder et al., 2012; Mateus et al., 2019). The adoption of integrated systems, such as the intercropping of maize with forage grasses that can be pastured after maize harvest has been of great relevance for tropical agriculture, allowing the use of land throughout the year. The system allows for a cheap remediation of degraded pastures, or produce plant residues essential for the success of systems under no-till (Scopel et al., 2013; Almeida et al., 2017b).

After maize harvest, the grass forage is allowed to grow, and can be pastured, or else, it can be left in the area and then desiccated. The straw left on the soil surface offers soil cover and protection against erosion, avoids extreme temperatures, conserves soil water and improves nutrient cycling, soil physical characteristics (Calonego and Rosolem, 2008) avoiding nutrient losses (Rosolem and Calonego, 2013, Franzluebbers and Stuedemann, 2014, Rosolem et al., 2017).

However, in maize-grass intercropped systems, competition for N between species may be present, which can compromise maize and/or forage production (Borghi et al. 2014). Among the nutrients considered essential to the development of forage species, N stands out for promoting the greatest increases in forage production, and the need for this nutrient is greater after the initial development of the grass (Borghi et al., 2014). Among all nutrients, N deficiency in crops is one of the most recurrent (Soratto et al., 2011. However, it is not known whether the amount of N fertilizer applied to maize will be enough to meet the requirements of the system. The N requirement of the crops can vary according to the environmental conditions and the rotation system used, greater when there is only grasses in the system (Borghi et al., 2014; Mateus et al., 2016). Although the intercropping of maize with forage grasses can increase the soil N supply, the usage of N fertilizer is still necessary to sustain high yield levels (Rosolem et al., 2011).

Usually, grasses and forage legumes are the main source of nutrients for ruminants. Thus, raising depends fundamentally on forage production, mainly grasses and legumes from pastures (Franzluebbers and Stuedemann, 2014). The right choice of adapted grass species and a better understanding of fertilizer management will allow

for higher forage yields and eventually in higher meat production (Barbosa et al, 2007). Palisade grass (*Urochloa brizantha*) and Guinea grass (*Megathyrsus maximus*) are among the grass species most used because of their high dry matter yields and palatability (Machado et al., 1998; Rego, 2001). Several edaphoclimatic factors can interfere in the bromatological composition, thus, nor only the yield, but also the quality of the forage can be affected under shade (Leonel et al., 2009). Grass forage production is directly associated with direct light interception by the leaves, the most limiting factor for its growth. The adoption of 95% of light interception (LI) to determine the moment of introducing cattle in the area increases the frequency of grazing, accumulation of dry matter and improves nutritional quality (Bueno, 2003).

Most of the areas cultivated with maize, in Brazil, have been planted using intensive cropping systems in the off-season, often following the early cycle soybean [*Glycine max* (L.) Merr.] harvest (Oliveira et al., 2019).. Therefore, an interdisciplinary study about maize intercropped with forage grasses is essential to generate information for a better understanding of the system, both on the response of the forage species, the grain crops and N fertilization. We hypothesized that N fertilization, besides increasing maize yield, improves forage dry matter quality and yield, eventually increasing meat production. Therefore, the objectives of the present short-term experiment using intercropping systems were to: i) evaluate maize grain yield when intercropped with grasses; ii) assess forage dry matter yield and bromatological quality; iii) monitor the canopy LI of grasses after maize harvest as affected by nitrogen fertilization and grass species.

4.2 Materials and methods

4.2.1 Study site

A field experiment where maize was intercropped with tropical forage grasses was carried out in Botucatu, State of São Paulo, Brazil (22º49’27.68” S, 48º25’46.75”W, 700 m a.s.1) on a clay Rhodic Hapludox (Soil Survey Staff, 2014), with ~70% kaolinite, ~15% gibbsite, and small amounts of 2:1 clay minerals. The climate is Cwa - mesothermic - with dry winter climate according to Alvares et a. (2013). The study was established in December 2017. More details on this experiment can be found in Rocha et al. (2019). Three soil samples were randomly sampled from each plot to the depth of 20 cm to determine physical (Gee and Bauder, 1986) and chemical

properties (van Raij et al., 2001). The soil showed190 g kg^{-1} of sand, 196 g kg^{-1} of silt, 614 g kg^{-1} of clay, pH ($CaCl_2$) 5.1, P 35.9 mg dm^{-3}, K 2.0 $mmol_c$ dm^{-3}, Ca 40.3 $mmol_c$ dm^{-3}, and Mg 23.4 $mmol_c$ dm^{-3}, H+Al 40.5 $mmol_c$ dm^{-3}, cation exchange capacity 106.2 $mmol_c$ dm^{-3} and base saturation 62%. Total N and C were determined by dry combustion using a CHNS-2000 elemental analyzer (Leco Corp., St. Joseph, USA), and the soil showed 17.7 g kg^{-1} of total C and 2.0 g kg^{-1} of total N. Besides that, mineral N forms were analyzed (Mulvaney, 1996; Miranda et al., 2001), and it was found 5.1 mg kg^{-1} of NH_4^+-N and 2.3 mg kg^{-1} of NO_3-N.

4.2.2 Study design

The experimental design was a split plot in randomized complete blocks with four replications. The two forage grasses were planted in the plots, and N was applied at 90, 180 and 270 kg N ha^{-1} as ammonium sulfate (a N-unfertilized control was also used) in subplots measuring 10 m x 4.5 m.

4.2.3 Crop management

Firstly, dolomite ($CaCO_3 \cdot MgCO_3$) and gypsum ($CaSO_4 \cdot 2H_2O$) were surface-applied in October 2017 to ameliorate soil acidity and to add S to the system. Forage grasses and maize were planted simultaneously in Dec. 2017 using a no-till planter with a row spacing of 0.45 m. Forage seeds were mixed with base fertilizer (120 kg ha^{-1} of P as triple superphosphate and 50 kg K ha^{-1} as KCl) and planted at a depth of 8 cm. It was used 12 kg of pure live seeds ha^{-1} of each grass, while maize (cv. hybrid 2B587PW, Dow AgroSciences, São Paulo, Brazil) was planted to a final stand of ~42,000 plants ha^{-1}. The N fertilizer (granular ammonium sulfate) application was split twice, 30 kg ha^{-1} of N at planting and the remaining was side dressed in the N-fertilized treatments at growth stage V4-V5 (five leaves with visible leaf collars, Ritchie and Hanway, 1986). At the same time, 60 kg ha^{-1} of K was also side dressed as KCl. The N fertilizer was hand-applied to the soil surface in single-side banding (3 cm wide), ~5 cm from the crop row. Three central rows (8.1 m^2) of maize were mechanical harvested in April 2018, and the stover remained in the field. The grasses were left to grow up to November 2018, when they were chemically terminated using glyphosate (2.9 kg ha^{-1}a.i.), and the plant residues were left over the soil surface.

Besides that, a weather station 2.6 km away from the study was used to monitor the climate variables (Fig. 18).

4.2.4 Forage dry matter and maize grain yield

At maize physiological maturity (R6 growth stage) and when grasses were cut in mid-October/November, forage grasses were randomly sampled within each experimental plot, using two frames with 0.75 × 0.45 m (0.67 m^2) and 1 frame with 0.5 × 1 m (0.50 m^2), respectively, and their fresh samples in each period were oven-dried at 65°C to constant weight to assess the dry weight to quantify the dry matter yield. The dry biomass was ground in a Wiley mill and passed through a 1-mm sieve. For grain yield at maize harvesting, three central rows were considered, discarding 0.5 m from each one, making a useful area of 8.1 m^2.

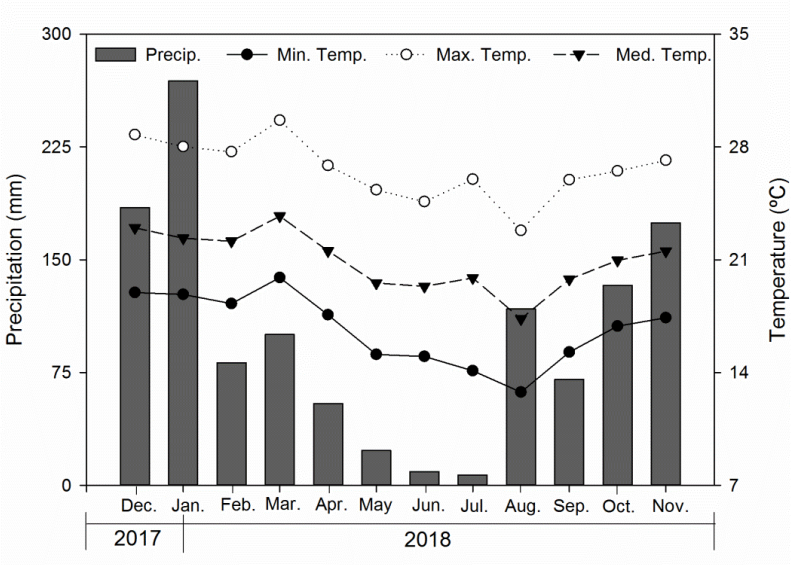

Fig. 18. Monthly minimum, maximum and average air temperatures and rainfall recorded during the growing season period (Dec. 2017- Nov. 2018).

4.2.5 Bromatological analysis of forage grass

Bromatological analysis of the two forage grasses was done in samples collected in two periods: maize harvest and grasses cut in mid-October/November.

After forage cutting, the fresh material was oven-dried at 65°C to constant weight. The dry biomass was ground in a Wiley mill to pass through a 1-mm sieve. Subsequently, bromatological analysis of the forage grasses was performed. For each sample, a sub-sample was oven-dried at 65°C to constant weight to pre-dry the material. A sub sample was then returned to the oven at 105°C for dry matter (DM) determination. The following equation was used to calculate DM:

$$\mathrm{DM}(\%) = [(\mathrm{C} - \mathrm{A}) \times 100]/(\mathrm{B} - \mathrm{A})$$

where *C* is the dry sample weight; *B* is fresh sample weight; and *A* is the weight of the container.

The mineral matter (MM) and crude protein (CP) contents were determined by Micro-Kjeldahl distillation (AOAC, 1995), while CP was calculated using the follow equation:

$$\mathrm{C}(\%) = \%\mathrm{N} \times 6.25$$ (AOC, 1995)

where *%N* is determined by Micro-Kjeldahl distillation (AOAC, 1995).

Fiber in acid detergent (ADF), fiber in neutral detergent (NDF), hemicellulose, cellulose, and lignin (LIG) were analyzed according to van Soest et al. (1991).

4.2.6 Light interception, SPAD index and height of grasses

After maize harvest, a micro area of 1.0 x 0.5 m (0.5 m^2) was delimited in each plot, where the light interception (LI) of the remaining forage grasses was measured weekly at 11:00 am-1:00 pm, using a canopy analyzer (LI-COR model LAI 2000, Lincoln, 1992), up to 95% LI. When each treatment reached 95% LI, the SPAD index was determined using a chlorophyll meter (Soil and Plant Analysis Development).

4.2.7 Estimated meat production

Although grazing by animals was not performed, meat production was estimated using the Large Ruminant Nutrition System model (LRNS) (Crusciol et al., 2019). The LRNS model is based on the Cornell Net Carbohydrate and Protein System (CNCPS) version 5, as described by Fox et al. (2004). Some parameters were used to predict animal performance: Nelore breed, bull sex, 450 kg body weight, 52% carcass yield, 22% body fat grading system and continuous grazing. The following bromatological quality was used to estimate the meat production within the LRNS model: Grass dry

matter yield, mineral material (MM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), hemicellulose (HEM), cellulose (CEL) and lignin (LIG).

4.2.8 Statistical analysis

Statistical analyses were performed in SAS (version 9.4M3, SAS Institute Inc., Cary, NC, USA). Data of crop yield, bromatological quality, and meat production were tested using split plot ANOVA using the GLIMMIX procedure. Block was considered a random effect, while cover crop and N rate were treated as fixed effects. Restricted maximum likelihood (ReML) was used to estimate the covariance parameters, while compound symmetry (CS) was adopted as the covariance structure based on the corrected Akaike information criterion (AICc). Skewed data were treated using lognormal distribution, and values were back-transformed. Means were separated using the LSMEANS ($P \leq 0.05$) statement with the simulate adjustment.

4.3 Results

4.3.1 Forage dry matter and bromatological quality, and grain yield at maize harvest

The forage dry matter yield in treatments fertilized with 90 and 270 kg N ha^{-1} was, on average, 144% higher than in N-unfertilized treatments (Fig. 19a). Grain yield responded positively to N applied, but treatments fertilized with 180 and 270 kg N ha^{-1} for maize intercropped with Guinea grass did not differ from each other (Fig. 19b).

Interaction of grass species and N fertilization were not significant for bromatological quality (Table 9). The MM of Guinea grass was 18% higher than that of palisade grass, with no effect of the grasses on CP, NDF, ADF and HEM. The CP, NDF, ADF and HEM responded to N. For CP, the treatments fertilized with 270 kg N ha^{-1} were 33% higher than the average of all other treatments. For NDF and ADF, the treatments fertilized with N were 5.8 and 16%, respectively, higher than the unfertilized treatments. However, HEM was higher in the absence of N.

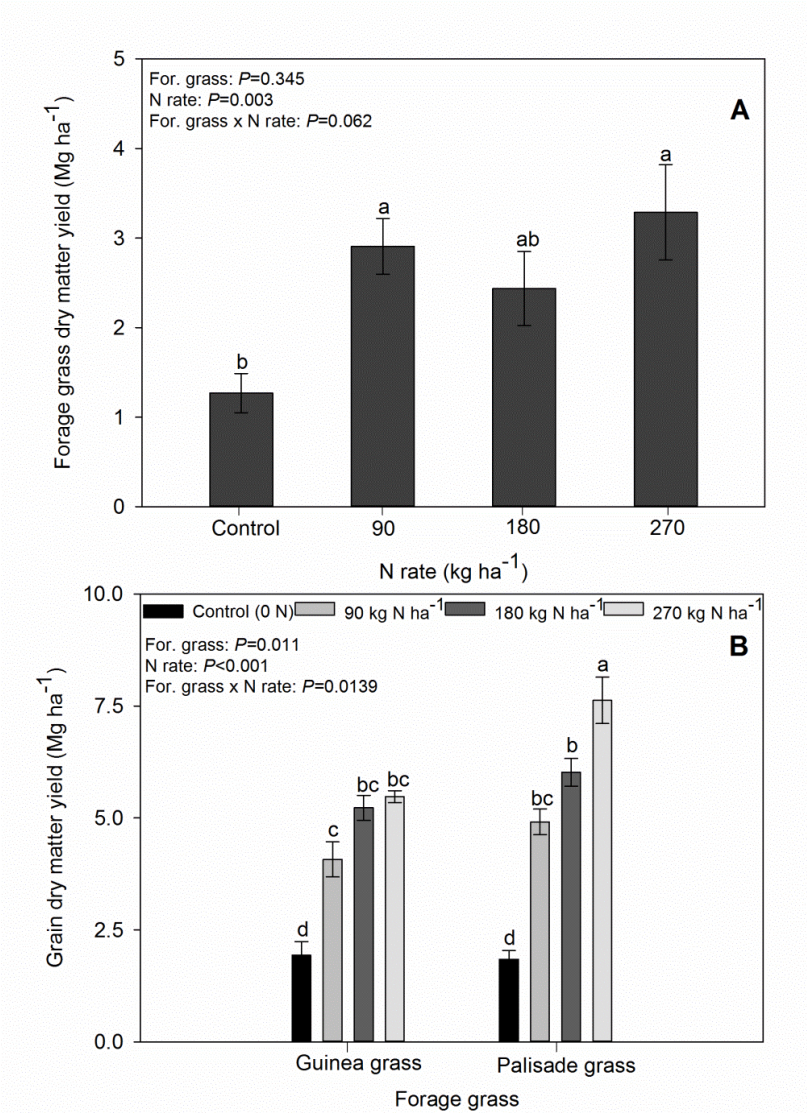

Fig. 19. Forage dry matter yield (a) and maize grain yield (b) at maize harvest. The error bars represent the SEM (*n* = 8 and 4 for panels a and b, respectively). Different lowercase letters indicate differences at the 5% level.

Table 9. Bromatological quality of forage grass at maize harvest. The error bars represent the SEM (n = 16, 8 and 4 for forage grasses, N rate and forage grass × N rate, respectively).

Forage grass	**N rate**	**MM**	**CP**	**NDF**	**ADF**	**HEM**
	kg ha^{-1}	**%**				
Guinea grass	-	9.3±0.2a	5.4±0.2	76±1	45±1	31±1
Palisade grass	-	7.9±0.3b	6.2±0.3	74±1	43±1	31±1
		P=0.018	*P*=0.104	*P*=0.083	*P*=0.097	*P*=0.411
-	0	8.8±0.3	5.2±0.2b	72±1b	39±1b	33±1a
-	90	9.0±0.4	5.7±0.3b	77±1a	46±1a	31±1b
-	180	8.4±0.5	5.4±0.3b	76±1a	45±1a	31±1ab
-	270	8.1±0.4	7.1±0.3a	76±1a	46±1a	30±1b
		P=0.056	*P*<0.001	*P*=0.001	*P*<0.001	*P*=0.004
Guinea grass	0	8.9±0.3	5.1±0.3	72±2	40±1	33±1
Guinea grass	90	9.7±0.4	5.3±0.4	77±1	47±1	30±1
Guinea grass	180	9.4±0.4	5.2±0.4	77±1	46±2	31±1
Guinea grass	270	9.1±0.5	6.2±0.4	77±1	48±2	29±1
Palisade grass	0	8.6±0.6	5.2±0.4	72±1	39±1	33±1
Palisade grass	90	8.3±0.4	6.1±0.4	76±1	45±1	31±1
Palisade grass	180	7.4±0.5	5.6±0.4	75±1	44±1	31±1
Palisade grass	270	7.1±0.1	8.3±0.6	75±1	44±1	30±1
		P=0.080	*P*=0.139	*P*=0.743	*P*=0.791	*P*=0.970

Different lowercase letters indicate significant differences at the 5% level. MM, CP, NDF, ADF and HEM: mineral material, crude protein, neutral detergent fiber, acid detergent fiber and hemicelluloses, respectively.

4.3.2 Forage light interception and SPAD index in grass cut

After maize harvest, the grasses in the 0.5 m^2 micro plots showed a linear increase in LI, with the regression coefficient (R^2) ranging from 0.89 to 0.96 (Fig. 20ab). However, there were no influence of N rate and forage species on the linear regression of LI throughout the season. The SPAD index was increased by 46 % following N fertilization, although no differences were noticed between N rates (Fig. 20c).

4.3.3 Forage dry matter yield and bromatological quality at the final cut

No interactions between grass species and N fertilization were observed for bromatological quality (Table 10). Overall, Guinea grass showed higher NDF, ADF and cellulose relative to palisade grass. The highest forage dry matter yield was obtained with 270 kg N ha^{-1}, with an average of 3.4 Mg ha^{-1}, and CP was 38% higher in fertilized treatments compared with the absence of N fertilization (Table 10).

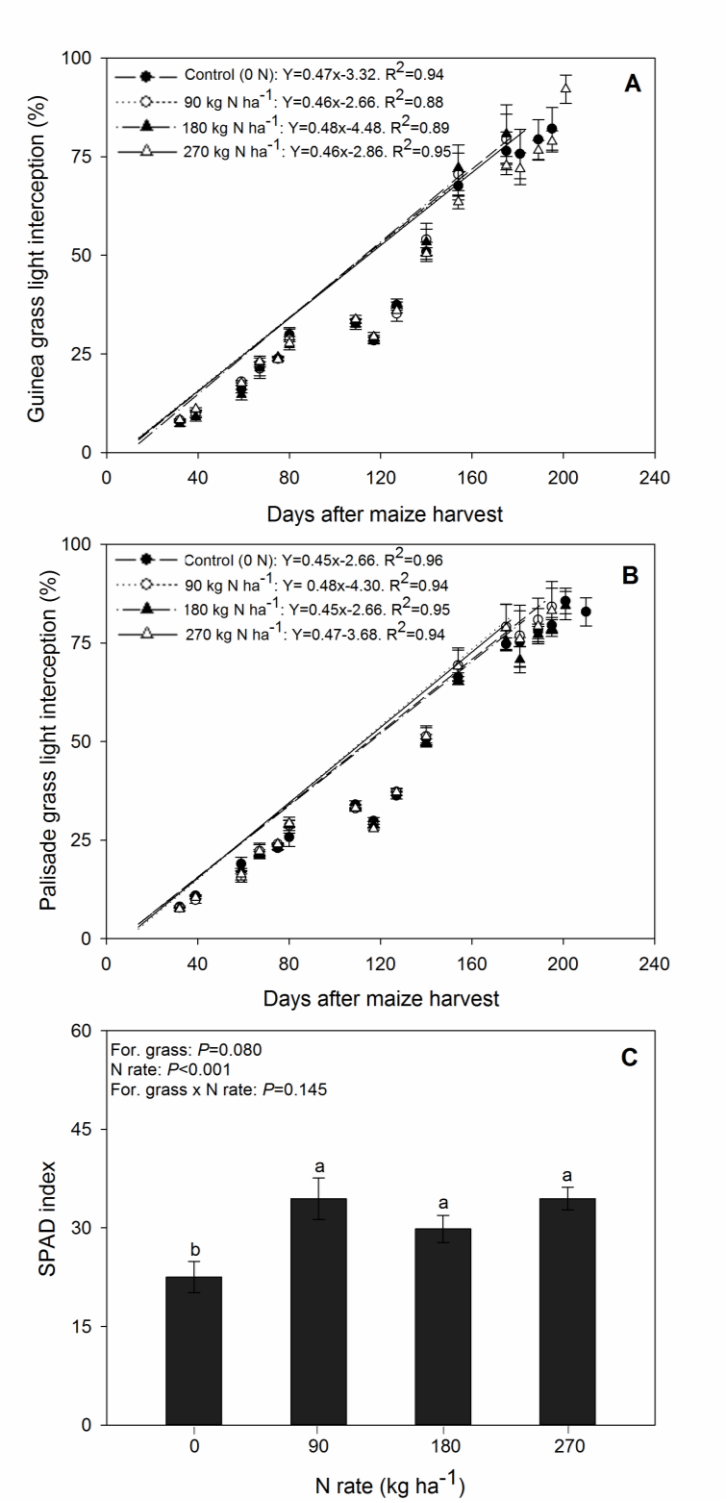

Fig. 20. Light interception in Guinea grass (a) and palisade grass (b) from 14 days after maize harvest until the forage grass reach 95% of LI; SPAD index in forage grass cut (c). The error bars represent the SEM (n = 4, 4 and 8 for panels a, b and c, respectively). Different lowercase letters indicate significant differences at the 5% level.

Table 10. Dry matter yield and bromatological quality of forage grass at cutting in Nov. 2018. The error bars represent the SEM (n = 16, 8 and 4 for forage grass, N rate and forage grass × N rate, respectively).

Forage grass	**N rate**	**Drymatter**	**MM**	**CP**	**NDF**	**ADF**	**HEM**	**CEL**	**LIG**
	kg ha^{-1}	**Mg ha^{-1}**	**%**						
Guinea grass	-	1.9±0.2	10.3±0.2	10.0±0.7	66±1a	34±1a	31±1	30±1a	2.2±0.2
Palisade grass	-	1.9±0.2	10.4±0.4	9.6±0.6	62±1b	31±1b	31±1	27±1b	1.8±0.2
		P=0.948	*P*=0.910	*P*=0.809	*P*=0.030	*P*=0.004	*P*=0.631	*P*=0.009	*P*=0.234
-	0	1.0±0.1c	10.7±0.4	7.6±0.5b	64±2	32±1	32±1	27±1	2.02±0.2
-	90	2.3±0.4ab	9.9±0.6	11.4±0.9a	64±2	32±1	31±1	28±1	2.22±0.2
-	180	1.6±0.3bc	10.9±0.5	10.3±1.1a	64±1	33±1	31±1	29±1	1.93±0.2
-	270	3.4±0.5a	9.8±0.4	9.9±0.5a	64±1	33±1	31±1	29±1	1.98±0.2
		P<0.001	*P*=0.151	*P*=0.002	*P*=0.990	*P*=0.529	*P*=0.383	*P*=0.098	*P*=0.728
Guinea grass	0	0.9±0.2	10.6±0.5	7.8±0.7	66±2	33±1	33±1	28±1	2.4±0.3
Guinea grass	90	2.0±0.4	10.4±1.1	10.8±1.6	65±2	34±1	31±1	29±1	2.3±0.3
Guinea grass	180	1.6±0.4	10.1±0.5	11.3±2.0	64±1	34±1	30±1	30±1	2.1±0.3
Guinea grass	270	4.7±1.1	10.1±0.5	9.9±0.5	67±1	36±1	31±1	32±1	2.2±0.3
Palisade grass	0	1.2±0.3	10.8±0.8	7.4±0.8	60±1	30±1	30±1	26±1	1.7±0.2
Palisade grass	90	2.7±0.6	9.4±0.3	12.0±1.0	62±3	31±2	31±1	27±1	2.1±0.3
Palisade grass	180	1.7±0.4	11.6±0.8	9.2±1.0	63±1	31±1	31±1	27±1	1.8±0.3
Palisade grass	270	2.6±0.6	9.6±0.7	9.9±1.0	61±2	30±1	30±1	27±1	1.8±0.3
		P=0.180	*P*=0.148	*P*=0.320	*P*=0.344	*P*=0.489	*P*=0.070	*P*=0.200	*P*=0.826

Different lowercase letters indicate significant differences at the 5% level. MM, CP, NDF, ADF, HEM., CEL. and LIG: mineral material, crude protein, neutral detergent fiber, acid detergent fiber, hemicellulose, cellulose and lignin, respectively.

4.3.4 Estimated meat production

The estimated meat production showed interaction of the grass species and N rates. In general, the increased application of N resulted in greater estimated meat production. However, Guinea grass fertilized with 270 kg N ha^{-1} was 47 and 290% higher than the average of other N-fertilized and N-unfertilized treatments, respectively (Fig. 20). Guinea grass and palisade grass differed only between treatments fertilized with 270 kg N ha^{-1}.

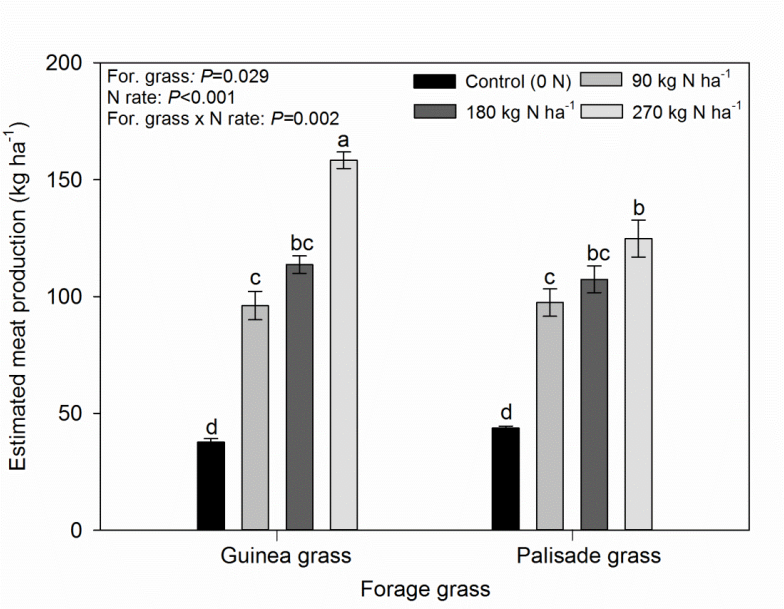

Fig. 21. Estimated meat production by the LRNS Cornell model. The error bars represent the SEM (*n* = 4). Different lowercase letters indicate significant differences at the 5% level.

4.4 Discussion

4.4.1 Forage and grain yield and bromatological quality yield at maize harvest

The importance of N fertilization for forage grasses was remarkable in our experiment, since dry matter yields were increased up to 5-fold with N fertilization (Fig. 19a). It is expected that intercropping maize with grasses increases the N needs for the system (Mateus et al, 2011). Thus, N fertilization has been considered a fundamental practice for increasing maize grain and forage yield (Pariz et al, 2011). Despite maize response N fertilization, there was an apparent competition between species when it was intercropped with Guinea grass (Fig 19b), which was also

observed by Borghi et al. (2013). Probably, this is due to the vigorous development of Guinea grass, which ends up inhibiting maize growth in intercropping systems (Barducci et al., 2009). In addition, Guinea grass has a high nutritional demand, responding to high N inputs (Galindo et al., 2017), which may have competed with maize for plant nutrients. Competition for water must be discarded, because no drought was observed during the maize growing season (Fig 18). There is also a plausible evidence that alelopathic suppression due to secondary metabolites of forage grasses could have been a factor inhibiting maize growth (Souza et al., 2014). Nevertheless, deleterious effects of Guinea grass on maize was not observed by several authors (Borghi and Crusciol (2007); Barducci et al., (2009). In our study, grasses were planted deeper than maize to retard its emergence and minimize competition, what seems not to be enough for Guinea grass. Freitas et al. (2005) suggested the use of post-emergent herbicides in sub-doses in order to suppress the initial grass development during the vegetative growth of maize, when it is more susceptible to competition (Pantano, 2003). Furthermore, although maize yield has been higher when intercropped with palisade grass, is not possible assert that was no effect of palisade grass on maize response to N, because there is no absolute control without forage grass.

The CP was higher with 270 kg $ha^{-1}$of N, at regardless of the grass species. This was expected, since N is a key factor in the biochemical processes, constituent of proteins, chlorophyll, enzymes, coenzymes, and nucleic acids (Fornasieri Filho, 2007). Fiber is considered the least digestible bromatological fraction, represented by the plant cell wall, and is fundamental for the process of rumination and health of the gastrointestinal tract (Weiss, 1993). Bulky foods, i.e., foods containing more than 18% crude fiber have low energy density, so their consumption is limited (Macedo Júnior, 2007).The higher the ADF content, the lower the digestibility, while the NDF has a negative correlation with the consumption of grasses, considering 40% of ADF and 60% of NDF, as limiting digestibility and consumption, respectively (van Soest, 1994). NDF and ADF were higher following N addition (Table 9), and even in the absence of N fertilization, the levels of NDF and ADF were higher in our experiment than those indicated by van Soest (1994) as limiting consumption. Therefore, such results indicate that this forage would have result in lower consumption and lower digestibility. Similar data were found by Martuscello et al. (2005), who observed that an increased grass dry matter yield is interlinked with N fertilization, thus accelerating the senescence of

the grass, becoming more fibrous with a reduction in digestibility. The amount of biomass produced is associated with the chemical composition of the plant material - for example, contents of hemicellulose, cellulose and lignin (Carvalho et al., 2009). Knowing that N stimulates the growth of grasses, the higher hemicellulose found in treatments without N shows that fibrous constituents increase with the thickening and lignification of the cellular walls as the plant matures (Wagner and Wolf, 1999).

4.4.2 Light interception, height and SPAD index after maize harvest

Light interception has been considered as the best criterion for determining ideal pasture events during regrowth (Barbosa et al., 2007; Carnevalli et al., 2006; Zanini et al., 2012). An adequate grass yield is obtained when the pasture intercepts 95% of the photosynthetic active radiation (PAR; Brunett et al., 2016). Above 95% of LI, the grass growth changes, resulting in an increase in the proportion of the stem and accumulation of dead material (Silva et al., 2009). Because maize requires high amounts of N , between 180 and 200 kg N ha^{-1} (Almeida et al., 2017a), differences between the growth of different species of forage grasses after the maize grain harvest can be insignificant, although Guinea grass has a vigorous development (Barducci et al., 2009). This ends up influencing, even if indirectly, the LI by grasses, resulting in no differences between species when cut at the same time. The LI equations fit to the forage grasses in this study were very similar for both species (Fig. 20ab). Without N application, the species seems have been similar. To obtain a forage response to N after maize harvest, it would be advisable to re-apply N in the soil (Pariz et al., 2011). As N is part of the chlorophyll molecule (Fornasieri Filho, 2007), the increase in N fertilization directly reflected in the SPAD index reading. According to Bullock and Anderson (1998), the higher chlorophyll is synthetized with the higher availability of N in plant. We observed that SPAD index ranged from 22.5 to 34.5 for N fertilized treatments and unfertilized treatments, respectively. Lavres Junior et al. (2010) evaluated the SPAD index in Guinea grass in two growing periods, and in the second growth period the average chlorophyll content ranged from 20.6 to 39.0 SPAD units, corresponding to the lowest and the highest N rate, respectively. The range of this characteristic can be explained by the variations in the growth rates and the beginning of leaf senescence. Generally, in the second period of growth of grasses, the leaf

senescence flux is more accentuated than in the first growth (Hay and Walker, 1989). Costa et al. (2008) assessed the effect of palisade grass submitted to increasing N rates (100, 200 and 300 kg of N ha^{-1}) and a N-unfertilized treatment, reporting an increase in (SPAD units with the higher the N rate. These results were similar to those found by Costa et al. (2012).

4.4.3 Dry matter, bromatological quality and estimated meat production

The forage dry matter yield assessed in October and November responded to N fertilization (Table 10). These results differ from Almeida et al. (2017a), which observed that the residual of N applied to maize did not favor palisade grass growth following maize harvest. Our results can be explained by the fact that the field experiment was established in an area which was under no-till since 2014. Thus, the mineralization of organic matter may have made nutrients available to the forage species (Anghinoni, 2007) ensuring the appropriate supply of N to the plants. According to Carvalho et al. (1997), under reduced luminosity, forage plants change their structure and nutrient concentration. From May to September the luminosity and temperature are lower in tropical Brazil (Fig. 18). Leimare and Chartier (1992) explains that there is an ideal percentage of N for a certain level of dry matter yield. If full sun there is greater production of dry matter, that will dilute with more N taken up and translocated to shoots than in shaded plants, in which the production of dry matter will be lower. This occurs because the plant is not metabolizing all N taken up and converting it into dry matter. Plants adapted to shade tend to prioritize reserves for leaf area growth and to increase chlorophyll concentration (Soares et al., 2009). According to Kephart and Buxton (1993), shading can decrease the availability of photo assimilates used for secondary cell wall development, contributing to the reduction of fiber content and increasing digestibility (Soares et al., 2009). This may explain the higher CP content in grass cutting (Table 10) compared with the CP content of grass prior to maize harvesting (Table 9). Gerdes et al. (2000) also found that palisade grass and Guinea grass showed higher PB contents in fall, followed by winter, spring, and summer. In addition, CP levels were adequate even in the absence of N fertilization (above 7%). Crude protein levels below 7% in tropical grasses may result in reduced digestion of these grasses due to inadequate N levels for rumen microorganisms (van Soest, 1994). At the final cutting, CP was also affected by N fertilization, a like those

patterns found at maize harvest discussed earlier and explained by Fornasieri Filho (2007), since N is part of proteins, chlorophyll, enzymes, coenzymes and nucleic acids.

As N fertilization generally increases the forage dry matter yield (Corrêa et al., 2007; Mazza et al., 2009), enhancing the growth, tillering, and leaf production, as well as the expansion of shoots and roots (Galindo et al., 2018), this may explain why cellulose, NDF, and ADF were higher in Guinea grass compared with palisade grass, since the former is very demanding in N (Galindo et al., 2017). Fernandes et al. (2015) considers forage dry matter yields above 1.6 Mg ha^{-1} as satisfactory to ensure lawn stability and animal production, based on the average Brazilian commercial stocking rate, consumption and herd, although it depends on the stocking rate and time of year. Our results of forage dry matter were on average higher than 1.6 Mg ha^{-1} under fertilization, which also influenced the estimated meat production, which was higher when higher N rate.. Indeed, Guinea grass fertilized with 270 kg N ha^{-1} showed the highest estimated meat production (Fig.21) and, as described before, this may also have occurred due to its high demand for nutrients and growth (Galindo et al., 2017).

4.5 Conclusions

This study proves the potential of ICLS's for meat production in the off-season (May to September), when grain production is also targeted. Nitrogen application in paramount in this system, since N fertilization positively affects forage growth and nutritional quality, resulting in a higher maize grain yield, higher forage production and quality, and eventually higher estimated meat production. Guinea grass resulted in the highest estimate of meat production when fertilized with 270 kg N ha^{-1}, despite the high values for NDF, ADF and cellulose. Future studies with economic balance and residual N in the soil after years of cultivation can help us to prove the real benefits of ICLS's for the sustainability of these integrated agricultural systems.

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FINAL CONSIDERATIONS

This study brings the importance of understanding some mechanisms that govern the integrated production systems such as intercropping and crop rotation in conservationist systems, through the choice of N fertilizer rates and forage grass species. These systems prove to be sustainable, since they allow the use of land throughout the year in addition to the recovery of degraded pastures. In addition, the discussions of the 21st century that address climate change across the planet (Oertel et al., 2016) show us the essentiality of studying systems that can result in more sustainable environments, aiming the reducing hunger in the world, by increasing production without increasing the area cultivated. In this way, the NUE, as well as the reduction of losses of this nutrient in the soil and in the atmosphere, become fundamental in the current agricultural systems, since N is one of the most limiting nutrients for plants (Teixeira et al., 2014; Wang et al., 2017). According to the results obtained, it was possible to understand the interactions between agricultural management and plant-soil-microorganism relationships can affect the N cycle (Bowles et al., 2013) and plant yield. Despite reports suggest that BNI by *Urochloa* spp. and *Megathyrsus* spp. decrease N loss in the system, it was not evident in N-rich environments. Regarding to maize rotation with forage grasses, there was no difference among the forage grass species in the distribution and fate of ^{15}N in the plant–litter–soil system and, consequently, in unrecovered–N (i.e., potential losses). The amount of residual labeled N taken up by maize in the second growing season was very low. Guinea grass, palisade grass and ruzigrass did not affect N_2O and NH_3 emission due to their apparent inability to suppress soil nitrification. However, N fertilization slightly increased cumulative N_2O emission in the second maize season and decreased soil CH_4 uptake in the fertilized palisade grass and ruzigrass relative to unfertilized palisade grass in the second forage season.

Regarding the results of maize intercropped with forage grasses, cumulative N_2O emissions were higher in the N-fertilized treatments than those without N addition. Moreover, a close relationship was observed among soil characteristics (NH_4^+-N, NO_3^--N, and WFPS), gene abundance (AOB) and N_2O emissions. No relationship was found between denitrifying genes and N_2O emissions. This suggests that although N modifies the soil microbiome, in addition to the abundance of AOB being highly linked to the increase in N_2O emissions in the intercropped system, the increase in N-N_2O

emissions to the system is small in relation to productivity gains of the system, suggesting a decreasing the specific emission, that is, the emission per unit of product.

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