Long-term lime and gypsum amendment increase nitrogen fixation and decrease nitrification and denitrification gene abundances in the rhizosphere and soil in a tropical no-till intercropping system

João William Bossolani[a,b], Carlos Alexandre Costa Crusciol[a,*], Luis Fernando Merloti[c], Luiz Gustavo Moretti[a,b], Nídia Raquel Costa[a,b], Siu Mui Tsai[d], Eiko Eurya Kuramae[b,e,*]

[a] College of Agricultural Sciences, São Paulo State University (UNESP), Department of Crop Science, 18610-034 Botucatu, São Paulo, Brazil
[b] Netherlands Institute of Ecology, NIOO-KNAW, Department of Microbial Ecology, 6708 PB Wageningen, the Netherlands
[c] Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo (USP), 13418-900 São Paulo, Brazil
[d] Center for Nuclear Energy in Agriculture (CENA), University of São Paulo (USP), Cell and Molecular Biology Laboratory, 13416-000 Piracicaba, São Paulo, Brazil
[e] Utrecht University, Institute of Environmental Biology, Ecology and Biodiversity, Padualaan 8, 3584 CH, Utrecht, the Netherlands

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ABSTRACT

Liming is widely used to decrease soil acidity, and the application of lime alone or in combination with other amendments, such as gypsum, is a viable agricultural practice to improve soil nutrient status and crop yield. However, the effects of applying lime and gypsum alone or in combination on the microbial population and N cycle in intercropped no-till tropical systems are largely unknown. Here, we determined the lasting effects of applying lime and gypsum individually or in combination on soil chemical properties, N uptake by intercropped plants, maize yield, archaeal and bacterial abundances, and N cycle genes in the maize and ruzigrass rhizospheres in a long-term field experiment in tropical soil with a no-till maize and forage ruzigrass intercropping system. Our results showed that the application of lime or lime + gypsum increased soil fertility and the gene abundances of microorganisms responsible for biological nitrogen fixation and reduced gene abundances of nitrification and denitrification in the soil and rhizosphere of ruzigrass and maize. The accompanying increases in Ca^{2+} and Mg^{2+} availability, reduced Al^{3+} levels, and balance of micronutrient availability, mainly Mn, in the soil strongly influenced the responses of N cycle genes and enhanced plant N-acquisition and maize yield.

1. Introduction

Continuous crop cultivation under tropical conditions typically leads to severe declines in soil quality and, consequently, lower crop productivity (Holland et al., 2018). Soil acidity is one of the primary factors limiting crop development and productive potential (FAO, 2015). Globally, approximately 50% of agricultural areas are affected by problems related to soil acidity (Von Uexküll and Mutert, 1995). Most of these soils are in tropical and subtropical regions and, due to the high degree of weathering, also feature low cation exchange capacity (CEC) and low base saturation (BS) (Allen et al. 2007). In addition, these tropical acidic soils contain high levels of elements that are toxic to plants, such as exchangeable aluminum (Al^{3+}) and manganese (Mn) (Caires et al., 2011; Crusciol et al., 2016, 2019).

There is global interest in more sustainable soil management systems, including the reevaluation of existing management practices (Gibbons et al., 2014; Holland et al., 2018). Liming is an extremely important agricultural practice, particularly in tropical soils, that neutralizes soil acidity, increases nutrient availability, supplies calcium (Ca^{2+}) and magnesium (Mg^{2+}) and relieves the toxicity of some elements, especially in the topsoil layers (Caires et al. 2011). To increase the efficiency of liming in tropical soils, agricultural gypsum can be added (Inagaki et al., 2016; Carmeis Filho et al., 2017; Crusciol et al., 2016). Gypsum has been widely used in the recovery of sodic soils or as a source for Ca and S (Zoca and Penn, 2017), but more recently, it has been widely applied on slightly dispersed and highly acid soils. Unlike liming, gypsum amendment does not correct soil acidity but provides Ca^{2+} and sulfur (S) and reduces Al^{3+} availability throughout the soil profile (Soratto et al., 2010; Caires et al., 2011; Carmeis Filho et al., 2017). The improvement in the soil profile by gypsum benefits crop

root system development (Rosolem et al., 2017) and changes the nutrient dynamics and use due to the increasing nutrient cycling and nutrient uptake in agricultural systems (Holland et al., 2018).

The increase in soil pH induced by liming, which directly impacts soil transformation processes, has been studied extensively (Zhang et al., 2017; Liu et al., 2018; Guo et al., 2019; Pang et al., 2019). The changes induced by liming directly influence the nitrogen (N) supply to plants and N loss to the atmosphere via N_2O emissions and NH_3 volatilization or to groundwater by NO_3^- leaching (Liu et al., 2010; Zhang et al., 2017). According to Jarvis (1984) liming also promotes crop growth due to improved nodulation and increased N fixation through the increased abundance of compatible rhizobia and reduced constraints inhibiting the infection and nodulation of the host plant. It has been shown that liming can either improve N mineralization and subsequent beneficial effects on for cropping systems, including minimizing the inputs of nitrogen fertilizers (Holland et al., 2018). It is known that the soil microbiota influences soil element solubility and availability (Stiehl-Braun et al., 2011; Wachendorf, 2015). In addition, nutrient availability affects microbial metabolic activity (Navarrete et al., 2013). However, the exact mechanisms by which these parameters influence the associated microbial communities remain unclear.

Studies of the long-term effects of lime and gypsum amendment on the composition of the microbial soil community, particularly those related to the abundances of N cycle, are extremely scarce. This information, particularly the long-term effects of lime and gypsum application in agricultural soils, is essential for the development of sustainable management practices that avoid N losses to the environment (Kemmitt et al., 2006; Liu et al., 2018). Moreover, comprehensive investigations of the long-term effects of liming and other soil amendment practices could bring important information on how to reduce the excessive use of fertilizers, due to improved soil quality and increase the sustainability of current nutrient management practices. According to Holland et al., (2018), the impact of liming results in increased activity of soil biota. Consequently, the rate of nutrient cycling processes is increased, and soil function is impacted. The interaction of the changes in soil chemistry promoted by lime and gypsum with the soil microbiota is an important open question, especially in intensive farming systems with a diversity of plants growing at the same time (intercropping). No-till intercropping systems are characterized by alternation between cash crops and forage production during the growing season with or without animal grazing in the off-season (Franzluebbers and Gastal, 2019). These systems can increase soil quality by improving soil chemical, physical and biological properties (Pariz et al., 2017), biodiversity conservation (Kim et al., 2020), and nutrient cycling by plants and soil organic matter (Franzluebbers and Gastal, 2019) and reduce greenhouse gas emissions (Savian et al., 2014).

Nitrogen can be the most limiting nutrient on the crop development in tropical regions. Successive grass-only cultivation can compromise the sustainability of agroecosystems due to soil N depletion through nutrient removal (Garcia et al., 2016). The amount of N required by crops varies according to the environmental conditions and characteristics of the plants used in rotation; however, in crop systems that include only grasses, such as intercropping maize and tropical grass, high amount of N is required (Mateus et al., 2020). These characteristics are extremely important for agriculture in tropical regions around the world. The sustainability of these soils depends on the correct soil acidity management and the input of organic matter content provided by crops in rotation system.

Here, we investigated the long-term effects of lime and gypsum application on soil fertility and their influences on N cycle genes in the soil and rhizosphere of cover and cash crops in a tropical soil. To better understand these impacts in intercropped systems, we analyzed the rhizospheres of each plant and bulk soil separately. We hypothesized that the combined application of lime and gypsum would affect the abundances of N cycle genes in the agricultural system due to changes in soil chemical properties. To test this hypothesis, the following

questions were addressed: (*i*) What are the main changes in properties in soil amended with lime, gypsum and lime + gypsum? (*ii*) What are the impacts of these amendments on total archaea and bacteria and on the relative abundance of N cycle genes in the rhizosphere of each plant species and the soil? (*iii*) What are the main soil chemical properties that influence N cycle genes? (*iv*) How do changes in soil fertility and the N cycle affect mineral nitrogen availability for plant uptake and maize yield?

2. Material and methods

2.1. Field description and sample collection

A long-term lime and gypsum application field experiment was initiated in October 2002 at the Experimental Farm Station in Botucatu, southeast of São Paulo State, Brazil (22° 83′ 3″ S, 48° 42′ 64″ W, elevation 765 m above sea level). The soil was classified as a sandy clay loam kaolinitic and thermic Typic Haplorthox (USDA, 2014). According to Köeppen's classification, the climate is Cwa type, which corresponds to a tropical altitude with a dry winter and a hot, wet summer (Alvares et al., 2013). The long-term (1956–2019) average annual temperatures are 26.1 °C maximum and 15.3 °C minimum, with a 20.7 °C average. The annual rainfall average is approximately 1360 mm (Unicamp, 2020). Climatological data during the experiment period were presented in Supplementary Fig. S1.

Prior to establishing the experiment in 2002, the soil chemical properties and mineral soil fraction were determined (0.00–0.20 m depth), according to van Raij et al. (2001) and Kiehl (1979), respectively. The following results were obtained: soil organic matter (SOM): 21 g kg^{-1}; pH (CaCl$_2$): 4.2; phosphorus (P$_{resin}$): 9.2 mg kg^{-1}; exchangeable potassium (K$^+$), calcium (Ca^{2+}), and magnesium (Mg^{2+}): 1.2, 14, and 5 mmol$_c$ kg^{-1}, respectively; total acidity in pH7 (H + Al): 37 mmol$_c$ kg^{-1}, CEC: 57 mmol$_c$ kg^{-1}; BS: 35%; sand, silt and clay contents: 540, 110, and 350 g kg^{-1}, respectively. In the subsoil (0.20–0.40 m), the clay content was 360 g kg^{-1}. The bulk density at depth 0.00–0.20 m was 1.128 t m^{-3}.

2.2. Experimental design and treatments

The experiment design was a complete randomized block with four replications. The plots had four treatments: (*i*) a control (natural soil conditions; 17 years without soil amendment and intensive crop cultivation with fertilizer inputs); (*ii*) gypsum (10.0 Mg ha^{-1}); (*iii*) lime (13.04 Mg ha^{-1}); (*iv*) lime (13.04 Mg ha^{-1}) + gypsum (10.0 Mg ha^{-1}) application. A graphical scheme of the crop system and respective treatments and samplings are shown in Fig. 1. The dolomitic lime dose (DLD) was calculated to increase the base saturation (BS) in the topsoil (0.00–0.20 m) to 70%, according to the method of Quaggio and van Raij (1997) as shown in Eq. (1):

$$DLD(Mg\,ha^{-1}) = CEC \times (BS_2 - BS_1)/(10 \times ECCE) \quad (1)$$

where BS$_2$ is the estimated base saturation (70%), and BS$_1$ is the base saturation measured in the soil analysis. as shown in Eq. (2). ECCE is the effective calcium carbonate equivalents.

$$BS_1(\%) = (Ca^{2+} + Mg^{2+} + K^+) \times 100/CEC \quad (2)$$

where Ca^{2+}, Mg^{2+}, and K$^+$ are basic exchangeable cations (mmol$_c$ kg^{-1}), and CEC is the total cation exchange capacity, calculated as shown in Eq. (3):

$$CEC(mmol_c kg^{-1}) = Ca^{2+} + Mg^{2+} + K^+ + (H+ Al) \quad (3)$$

The gypsum dose applied in 2002, 2004 and 2010 were calculated by Eq. (4) (Quaggio and van Raij, 1997):

$$GD(Mg\,ha^{-1}) = 6 \times clay\,content\,(g\,kg^{-1})/1000 \quad (4)$$

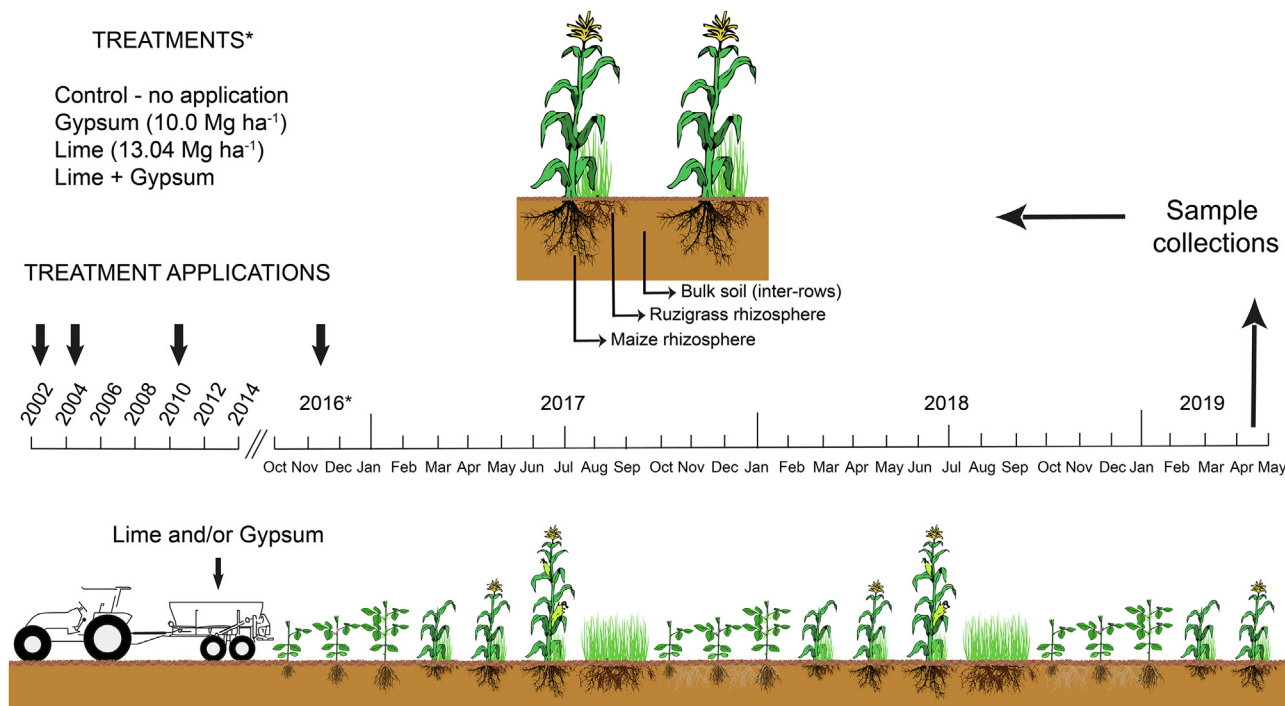where clay content corresponds to the soil layer of 0.20–0.40 m. In

Fig. 1. Schematic graphic representing the timeline of the field experiment and the respective treatments and sample collections.

2016, the gypsum dose was calculated based on the new methodology proposed by Caires and Guimarães (2018), with Eq. (5):

$$GD(Mg\,ha^{-1}) = [(0.6 \times CEC) - Ca^{2+}] \times 0.64 \qquad (5)$$

where CEC and Ca^{2+} were calculated based on 0.20–0.40 m soil layer.

2.3. Lime and gypsum application, tillage and crop management

During the study period, the treatments were applied four times (2002, 2004, 2010 and 2016) (Fig. 1). The criterion for reapplying treatments is based on annual assessments of soil fertility when base saturation of exclusive lime treatment reaches value ≤ 50%. The present study corresponds to third year after the last reapplication (2016), characterized by being a long-term residual effect. Different crops were cultivated during the growing season and the off season from 2002 to 2019. Details of the previous crop sequences and lime and gypsum applications as well as the characteristics of these amendments are shown in Supplementary Table S1. Since the experiment was implemented, it was cultivated under no-tillage system (NTS) and all lime and gypsum applications were performed superficially, except for the first application in 2002, when no-tillage began.

In March 2019 (off season), maize (*Zea mays* L.) was sowing intercropped with ruzigrass [*Urochloa ruziziensis* (R. Germ. & C.M. Evrard) Crins (Syn. *Brachiaria ruziziensis* Germ. & Evrard)]. Fertilization was performed at sowing time with rates of 350 kg ha^{-1} of N–P$_2$O$_5$–K$_2$O 08–28–16 and 100 kg N ha^{-1} as ammonium sulfate in topdressing (Cantarella et al., 1997) when maize was at V$_4$ phenological stage (Ritchie et al., 1993). The field plots consisted of 14 rows that were 9 m long and spaced 0.45 m apart, a total plot area of 56.7 m^2. Plots were separated by a space of 8 m to avoid contamination during treatments applications and subsequent cropping seasons. Eight soil subsamples per plot were randomly obtained from rhizosphere of maize and ruzigrass and bulk soil (between rows) to form a composite sample. Samples were collected when maize was at V$_{10}$ phenological stage (Ritchie et al., 1993). The rhizosphere samples were strictly defined as the soil within 2 mm of the root surface (DeAngelis et al., 2009). After gently shaking the roots to remove loosely attached soil clumps, the rhizosphere samples were carefully collected by brushing the remaining soil

from the roots (Schlemper et al., 2017). The bulk soil was collected between maize + ruzigrass rows at the depth 0.00–0.10 m.

2.4. Soil chemical properties analysis

Eight soil subsamples were randomly obtained at depth 0.00–0.10 m from useable areas of each plot to form a composite sample and were processed according to the standard methods for Brazilian tropical soils (Cantarella et al., 1998). Samples were air-dried and homogenized using a 2 mm sieve. The soil organic matter (SOM) was determined by the Walkley–Black method (Walkley and Black 1934). The pH values were measured in 0.01 M CaCl$_2$ (the most stable pH used in soil analysis). The exchangeable cations (K$^+$, Ca^{2+}, and Mg^{2+}) and available P-phosphate were extracted using ion exchange resins and, in the extract, P as orthophosphate was determined colorimetrically, and cations by atomic absorption spectrometry (Shimadzu AA-7000) (van Raij et al. 2001). Soil sulfur-sulfate (S-SO$_4^{2-}$) extraction were performed by calcium phosphate (0.01 mol L^{-1}) in a 1:2.5 soil/solution ratio and later determined by the turbidimetric method using BaSO$_4$ (Vitti, 1989). The cationic micronutrients [iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn)] were extracted by solution (pH 7.3) containing 0.005 mol L^{-1} of diethylenetriaminepentaacetic acid (DTPA), 0.1 mol L^{-1} of triethanolamine (TEA) and 0.01 mol L^{-1} of CaCl$_2$ and determined by atomic absorption spectrometry. Potential acidity (H + Al) was estimated by the Shoemaker-McLean-Pratt buffer solution method (van Raij et al. 2001). The exchangeable Al^{3+} was extracted with 1 mol L^{-1} KCl (pH 7.0) at 1:10 soil/solution ratio, kept under agitation for 15 min and determined by titration with a 0.025 mol L^{-1} NaOH solution. After extraction, the content of ammonium (NH$_4^+$) and nitrate (NO$_3^-$) was determined by colorimetry by sodium salicylate method and vanadium chloride reduction, respectively (Mulvaney, 1996; Miranda et al., 2001). Total inorganic nitrogen (N$_i$) was obtained by summing the measured concentrations of mineral forms (NH$_4^+$ and NO$_3^-$).

2.5. Soil DNA extraction

Total DNA extraction from 250 mg of soil sample was performed for

Table 1
Soil chemical properties.

Soil chemical properties[†]	units	Control	Gypsum	Lime	Lime + Gypsum	p value
pH (CaCl$_2$)	–	3.75 c	4.15 b	5.99 a	6.15 a	0.001
SOM	g kg^{-1}	28.6 b	30.1 b	33.4 a	34.7 a	0.002
NO$_3^-$	mg kg^{-1}	22.9 c	24.5 c	30.0 b	32.5 a	0.001
NH$_4^+$	mg kg^{-1}	20.7 b	18.3 b	29.6 ab	35.1 a	0.041
N$_i$	mg kg^{-1}	43.5 b	42.8 b	59.7 a	67.6 a	0.001
P $_{(resin)}$	mg kg^{-1}	22.9 c	39.9 b	48.0 b	63.7 a	0.001
K$^+$	mmol$_c$ kg^{-1}	3.84 a	3.57 a	3.55 a	3.01 a	0.079
Ca^{2+}	mmol$_c$ kg^{-1}	13.3 c	28.1 b	66.6 ab	81.8 a	0.001
Mg^{2+}	mmol$_c$ kg^{-1}	6.72 c	4.88 c	34.1 a	28.3 b	0.001
S-SO$_4^{2-}$	mg kg^{-1}	12.2 b	22.8 a	14.5 b	17.3 ab	0.002
Al^{3+}	mmol$_c$ kg^{-1}	10.3 a	8.41 a	0.00 b	0.00 b	0.001
H + Al	mmol$_c$ kg^{-1}	65.5 a	61.8 a	21.2 b	20.0 b	0.001
EB	mmol$_c$ kg^{-1}	23.9 c	36.6 b	104 a	113 a	0.001
CEC	mmol$_c$ kg^{-1}	89.4 b	98.4 b	125 a	133 a	0.003
BS	%	26.7 c	37.2 b	83.1 a	84.9 a	0.001
Fe	mg kg^{-1}	33.9 a	31.7 a	29.3 ab	27.1 b	0.040
Mn	mg kg^{-1}	22.0 a	12.7 b	11.4 bc	9.47 c	0.001
Cu	mg kg^{-1}	3.76 a	2.46 b	2.15 b	1.95 b	0.005
Zn	mg kg^{-1}	1.36 a	1.26 a	1.00 b	1.00 b	0.002

Different lower-case letters indicate significant differences between treatments by LSD test at $p \leq 0.05$.

[†] Soil organic matter (SOM), nitrate (NO^{3-}), ammonium (NH$_4^+$), total inorganic nitrogen (N$_i$), phosphorus available (P$_{resin}$), exchangeable potassium (K$^+$), calcium (Ca^{2+}) and magnesium (Mg^{2+}), sulfur-sulfate (S-SO$_4^{2-}$), exchangeable Al^{3+}, potential acidity (H + Al), sum of exchangeable bases (EB), cation exchange capacity (CEC), base saturation (BS) and available iron (Fe), manganese (Mn), cupper (Cu) and zinc (Zn).

each sampled point (maize and ruzigrass rhizospheres and bulk soil) using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), according to manufacturer's protocol. DNA quality and concentration were measured using NanoDrop 1000 spectrophotometry (Thermo Scientific, Wilmington, DE, USA) and 1% sodium boric acid agarose gel electrophoresis (Brody and Kern, 2004).

2.6. Quantitative PCR analyses

The abundance of total archaea and bacteria, nitrogen fixers, nitrifiers and denitrifiers were estimated by quantitative PCR targeting archaeal and bacterial genes (16S rRNA), nitrogenase gene (*nifH*), ammonia monooxygenase gene (*amoA*) of archaea (AOA) and bacteria (AOB), nitrite reductase gene (*nirK*) and nitrous oxide reductase gene (*nosZ*), respectively. Analyses were performed using the StepOnePlus™ Real-Time PCR System with 96-well plates (Applied Biosystems, Foster City, CA, USA). Standard curves were created after serial dilutions of known gene quantity, amplified by PCR previously. Strains that were used to construct the standard curves, primers, and reaction conditions for genes amplifications are described in Supplementary Table S2. Analyses were performed in triplicate, and for each reaction was used 5 µL absolute qPCR SYBR Green/ROX qPCR Master Mix (2 ×) (Abgene, Epsom, UK); 1 µL of each primer (30 µM); 1.5 µL of sterilized water, 1 µL template DNA and 0.5 µL bovine serum albumin (BSA; 10 mg ml^{-1}). Analysis of melting curves were performed from 68 to 95 °C, and all standard curves will have R^2 greater than 0.98 as well efficiency between 98 and 102%. Results were analyzed using the StepOnePlus™ Real-Time software (Applied Biosystems, Foster City, CA, USA). Abundance data from each sample were transformed into the number of copies of DNA g soil^{-1}.

2.7. Determination of plant nitrogen content and crop yield

At the maize flowering, phenological stage R$_1$ (Ritchie et al., 1993), the leaf at the ear base (only the central third parts) were sampled from 30 random maize plants within the useful area of the plot (Cantarella et al. 1997), totaling 30 leaves per plot for N determination (Malavolta et al. 1997). The same was done for ruzigrass plants. The maize grain yield (moisture content of 130 g kg^{-1} of water) was evaluated at harvest maize stage.

2.8. Statistical analysis

The results were subjected to the outliers' test followed by Anderson-Darling normality (Nelson, 1998), and to evaluate the homogeneity the Levene's test was applied by the Minitab statistical program. Then, the means were subjected to the analysis of individual variance ANOVA by the F test ($p \leq 0.05$), and when there was significant difference, the means were compared by the LSD test ($p \leq 0.05$). Redundancy analysis (RDA) was used to determine the correlation between nitrogeN cycle genes and soil chemical attributes. Forward selection (FS) and Monte Carlo permutation test were applied with 1000 random permutations to verify the significance of soil chemical properties on N cycle gene variation. RDA plots were generated using Canoco 4.5 (Biometrics, Wageningen, the Netherlands). We used the two-way PERMANOVA (Anderson, 2005) to group the treatments for similarity. The heatmap was performed calculating the Spearman correlation coefficients ($p \leq 0.05$) to evaluate the relationship between relative abundance of N cycle functional genes and soil chemical properties.

3. Results

3.1. Soil chemical properties

The greatest change in soil chemical properties was observed in the liming treatment, followed by the lime + gypsum treatment (Table 1). The most notable effect of gypsum application on soil properties was an increase in soil S-SO$_4^{2-}$ levels. Lime increased SOM; reduced soil acidity (pH) and aluminum toxicity; increased exchangeable K$^+$, Ca^{2+} and Mg^{2+} and, consequently, exchangeable bases (EB), BS and CEC; improved the availability of P and S-SO$_4^{2-}$; and increased NO$_3^-$ and total inorganic nitrogen. However, liming with or without gypsum reduced the availability of cationic micronutrients (Fe, Mn, Cu and Zn) in the soil.

3.2. N cycle functional genes (real-time PCR)

Quantitative PCR based on the 16S rRNA gene was used to estimate the total abundance of archaea and bacteria in bulk soil and in the rhizospheres of maize and ruzigrass cultivated in soils under different
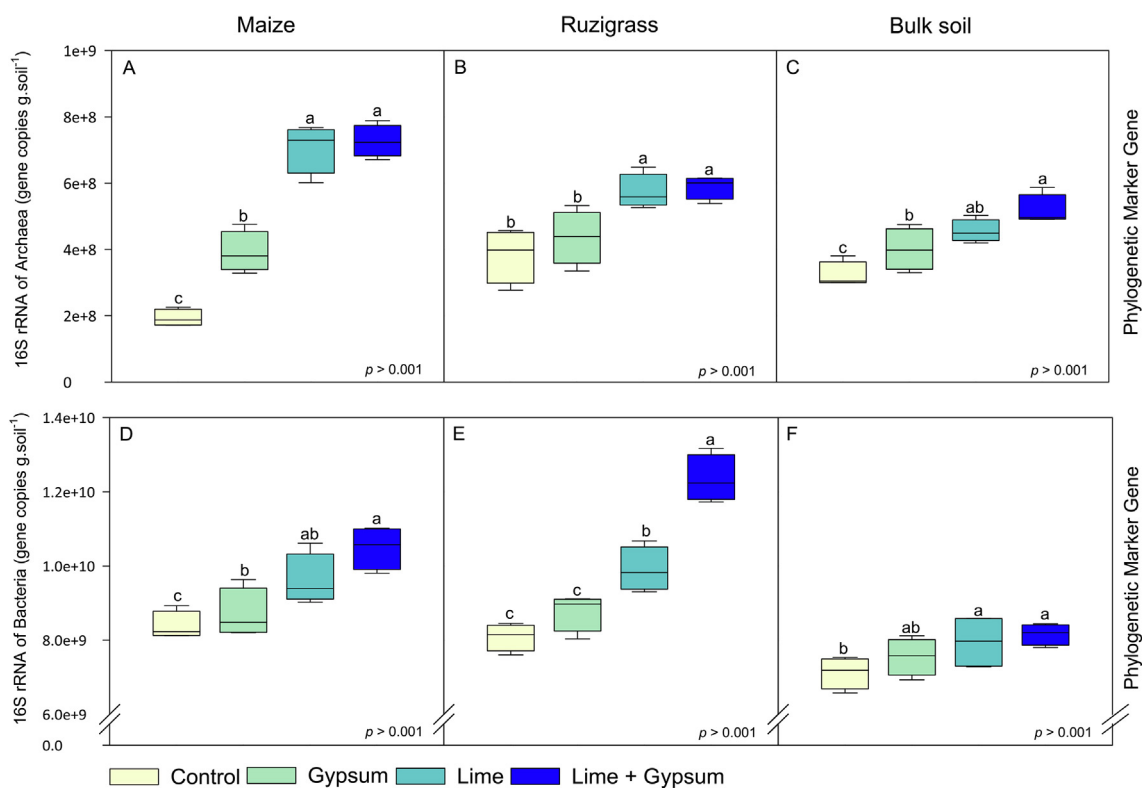
Fig. 2. Archaeal and bacterial *16S rRNA* copy numbers in the rhizospheres of maize (A, D) and ruzigrass (B, E) and in bulk soil (C, F) under different treatments (control, gypsum, lime and lime + gypsum). Different lowercase letters indicate significant differences between treatments by LSD test at $p \leq 0.05$. Error bars express the standard error of the mean ($n = 12$).

treatments (control, gypsum, lime and lime + gypsum) (Fig. 2). The total archaeal abundance was higher in the rhizospheres of maize ($p < 0.001$) and ruzigrass ($p < 0.001$) in the lime and lime + gypsum treatments than in the gypsum and control treatments (Fig. 2A, 2B). Similarly, in the bulk soil samples, the total archaeal abundance was higher ($p < 0.001$) in the lime and lime + gypsum treatments than in the gypsum and control treatments (Fig. 2C). For total bacteria, higher abundances were observed in the rhizospheres (Fig. 2D, 2E) than in the bulk soil (Fig. 2F). The total bacterial abundance in the ruzigrass rhizosphere was highest ($p < 0.001$) in the lime + gypsum treatment followed by the lime and gypsum treatments (Fig. 2E).

The lime + gypsum application resulted in the greatest increase in *nifH* copy numbers compared with the control (Supplementary Fig. S2). The *nifH* copy number increased significantly ($p < 0.001$) in the rhizospheres of both plants (Supplementary Fig. S2A and S2B) under the different treatments and was highest in the lime + gypsum treatment, followed by the lime and gypsum treatments. In bulk soil, the abundance of *nifH* was highest ($p < 0.001$) in the lime and lime + gypsum treatments (Supplementary Fig. S2C)

The abundances of *amoA* archaeal (AOA) and *amoA* bacterial (AOB) genes in the maize rhizosphere increased ($p < 0.001$) in the treatments compared with the control, and the abundances of both genes were highest in the lime + gypsum treatment (Supplementary Fig. S2D and S2G). By contrast, in the ruzigrass rhizosphere, the abundances of AOA ($p < 0.001$) and AOB ($p < 0.001$) decreased in the lime + gypsum treatment. In bulk soil, the abundances of AOA ($p < 0.001$) were the same regardless of treatment (Supplementary Fig. S2F), while the abundances of AOB were lower ($p < 0.001$) in the lime and lime + gypsum treatments than in the control and gypsum treatments (Supplementary Fig. S2I).

The copy numbers of *nirK* and *nosZ*, genes related to the denitrification process, in the maize rhizosphere did not differ ($p = 0.544$; $p = 0.682$) among the treatments and the control (Supplementary Fig.

S2J and S2M). However, in the ruzigrass rhizosphere, the abundances of these two genes were lower ($p < 0.001$) in the lime and lime + gypsum treatments than in the control treatment (Supplementary Fig. S2K and S2N). By contrast, in bulk soil, the *nirK* ($p < 0.001$) and *nosZ* ($p < 0.001$) abundances showed similar decreasing patterns from the control to the gypsum, lime and lime + gypsum treatments (Supplementary Fig. S2L and S2O). In general, the increase in the abundance of phylogenetic marker genes correlated positively with the abundance of genes related to the nitrogeN cycle (Supplementary Fig. S3). On the other hand, *nifH* was negatively correlated with denitrification genes.

Interestingly, by calculating the relative abundances (ratio of N cycle gene copy numbers to 16S rRNA gene (bacterial or archaeal) copy numbers), we found that the relative abundance of *nifH* in the maize and ruzigrass rhizospheres and in bulk soil increased ($p < 0.001$) successively from the control to the gypsum, lime and lime + gypsum treatments (Fig. 3A-3C). The increases in *nifH* abundance were higher in both plant rhizospheres than in bulk soil. Opposite results were obtained ($p < 0.001$) for the copy numbers of genes related to nitrification (*amoA* AOA and *amoA* AOB; Fig. 3D-3I) and denitrification (*nirK* and *nosZ*; Fig. 3J-3O), which showed similar decreasing patterns from the control to the gypsum, lime, and lime + gypsum treatments. The decreases in the relative abundances of these genes due to liming treatment were observed in both rhizospheres and bulk soil.

3.3. Redundancy analysis (RDA) and correlation between the relative abundance of N cycle functional genes and soil chemical properties

Redundancy analysis (RDA) was performed to evaluate the soil chemical properties and the relative abundance of N cycle genes in the maize (Fig. 4A) and ruzigrass (Fig. 4B) rhizospheres and in bulk soil (Fig. 4C). The sum of the first and second axes explained 90.9% of the total variation of N cycle gene abundance in the maize rhizosphere
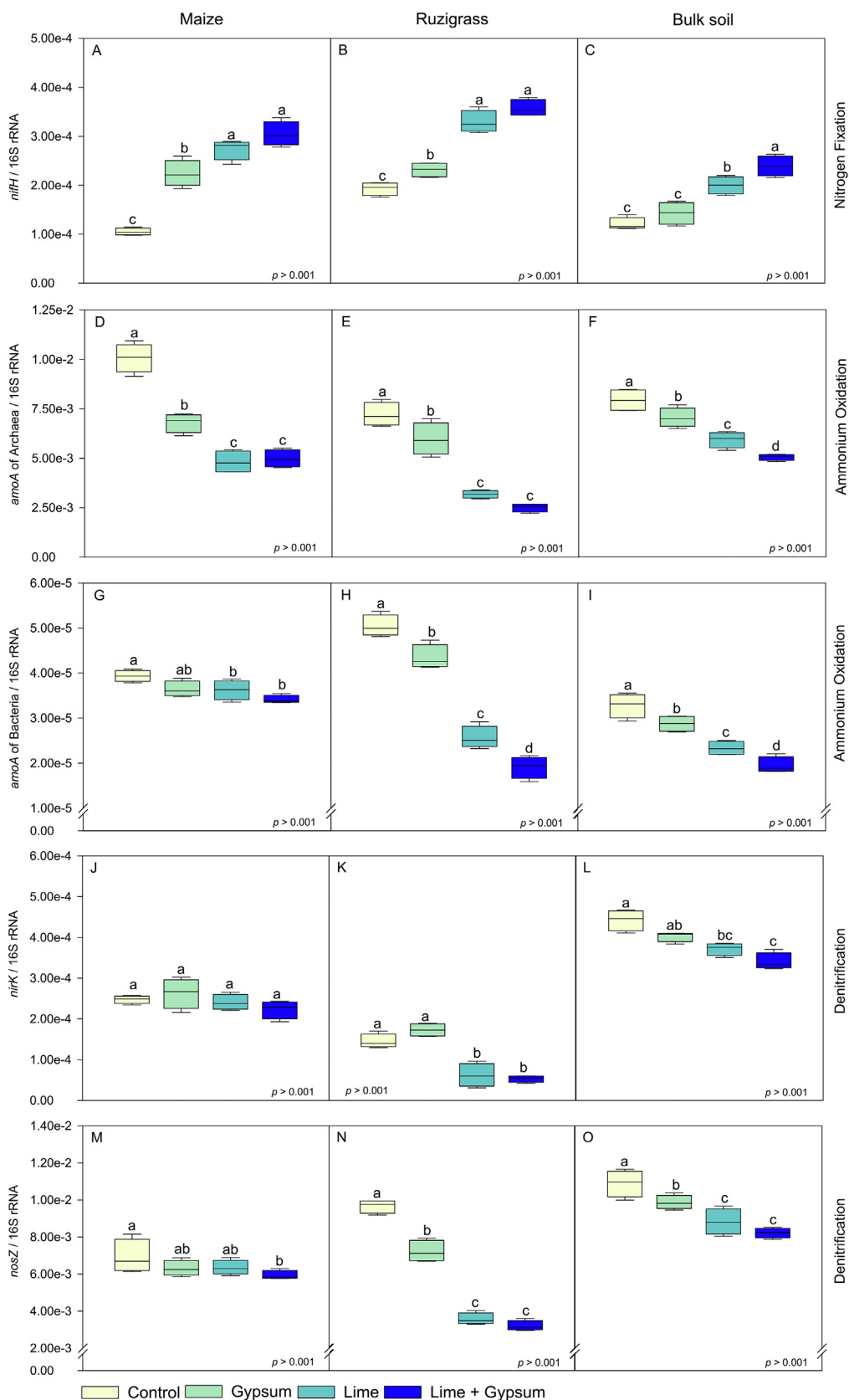
Fig. 3. Ratio of the copy number of N cycle functional genes to the copy number of archaeal or bacterial *16S rRNA* in the rhizospheres of maize (A, D, G, J, M) and ruzigrass (B, E, H, K, N) and in bulk soil (C, F, I, L and O) under different treatments (control, gypsum, lime and lime + gypsum). Different lowercase letters indicate significant differences between treatments by LSD test at $p \leq 0.05$. Error bars express the standard error of the mean ($n = 12$).
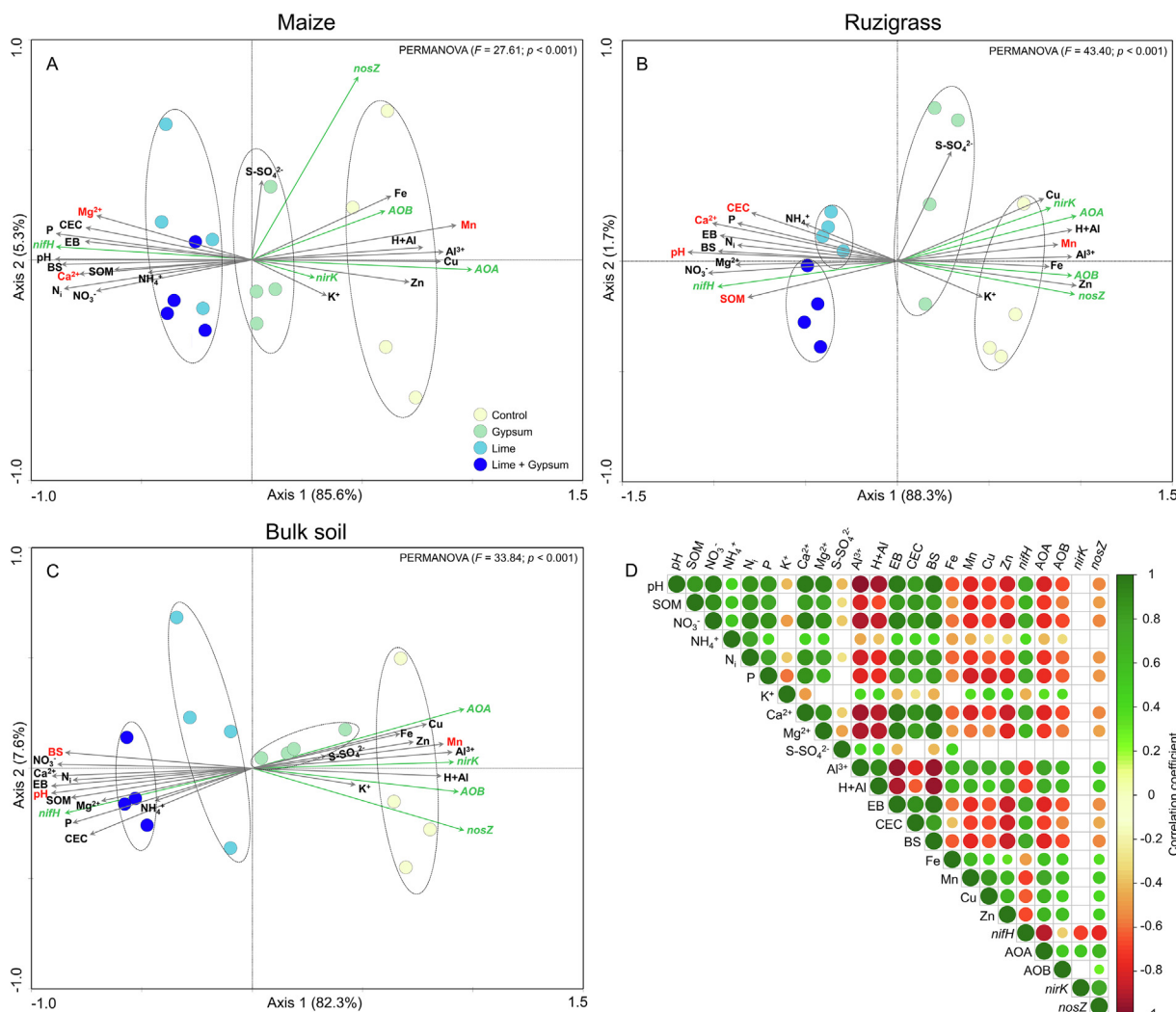
Fig. 4. Redundancy analysis (RDA) of the relative abundance of N cycle functional genes and soil chemical properties (A-C). The arrows indicate correlations between factors. The significance of these correlations was evaluated by a Monte Carlo permutation test and is indicated by red color ($p \leq 0.05$). The dashed lines indicate significant clusters by permutation analysis (PERMANOVA, $p \leq 0.05$). Heatmap of the correlation coefficients (Spearman) among the relative abundance of N cycle functional genes and soil chemical properties (D). Only significant correlations at $p \leq 0.05$ are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4A). Axis 1 explained 85.6% of the separation of N cycle gene abundance into 3 main groups (PERMANOVA $F = 27.61$, $p < 0.001$): group 1, control; group 2, gypsum; and group 3, lime and lime + gypsum (Fig. 4A). The gene abundances in the maize rhizosphere in the control treatment were significantly different ($p < 0.05$) from those in the gypsum, lime and lime + gypsum treatments (Fig. 4A). Group 2, which comprised the gypsum-only treatment, showed intermediate behavior between groups 1 and 3. In group 3, the N cycle gene abundance in the maize rhizosphere was not significantly different between the lime and lime + gypsum treatments. According to RDA analysis followed by Monte Carlo permutation, N cycle gene abundance showed significant correlations with Ca^{2+} ($F = 78.95$; $p < 0.001$), Mg^{2+} ($F = 3.92$; $p = 0.027$) and Mn ($F = 5.38$; $p = 0.033$). Ca^{2+} and Mg^{2+} availability was related to *nifH* abundance, while Mn was related to the abundances of AOA, AOB, *nirK* and *nosZ* (Fig. 4A).

In the ruzigrass rhizosphere, soil factors explained 90% of the N cycle gene abundance variation (Fig. 4B). Axis 1 explained 88.3% of the separation among the control and gypsum, lime, and lime + gypsum. In contrast to the maize rhizosphere (Fig. 4A), the ruzigrass rhizosphere samples were separated into four distinct groups by PERMANOVA analysis ($p < 0.001$), representing the four different treatments. The

abundance of N cycle genes in the ruzigrass rhizosphere in the control treatment was significantly different ($p < 0.05$) from those in the gypsum, lime and lime + gypsum treatments (Fig. 4B). Furthermore, Monte Carlo permutation analysis showed significant correlations of ruzigrass rhizosphere N cycle gene abundance with pH ($F = 237$; $p < 0.001$), SOM ($F = 6.42$; $p = 0.008$), Ca^{2+} ($F = 6.79$; $p = 0.004$), CEC ($F = 3.21$; $p = 0.049$) and Mn availability ($F = 3.67$; $p = 0.044$). pH, SOM, exchangeable Ca^{2+} and CEC were correlated with *nifH* abundance, whereas Mn availability was mostly related to AOA, AOB, *nirK* and *nosZ* abundance (Fig. 4B).

PERMANOVA analysis segregated the N cycle gene abundance in bulk soil into four groups ($p < 0.001$) (Fig. 3C), similar to the ruzigrass rhizosphere (Fig. 4B). Soil chemistry was responsible for 89.9% of the variation in N cycle gene abundance (Fig. 4C). Monte Carlo permutation analysis showed significant correlations of N cycle gene abundance with pH ($F = 57.2$; $p < 0.001$), K^+ ($F = 5.83$; $p = 0.032$) and BS ($F = 4.72$; $p = 0.043$). pH and BS were related to *nifH* gene abundance, and Mn availability was related to AOA, AOB, *nirK* and *nosZ* abundance.

Correlation analysis of N cycle gene abundance with soil chemical factors showed that the improvement in soil chemical properties due to lime and/or gypsum amendment increased the abundances of genes related to the N cycle (Fig. 4D). Increases in SOM, inorganic N, P, Ca^{2+},
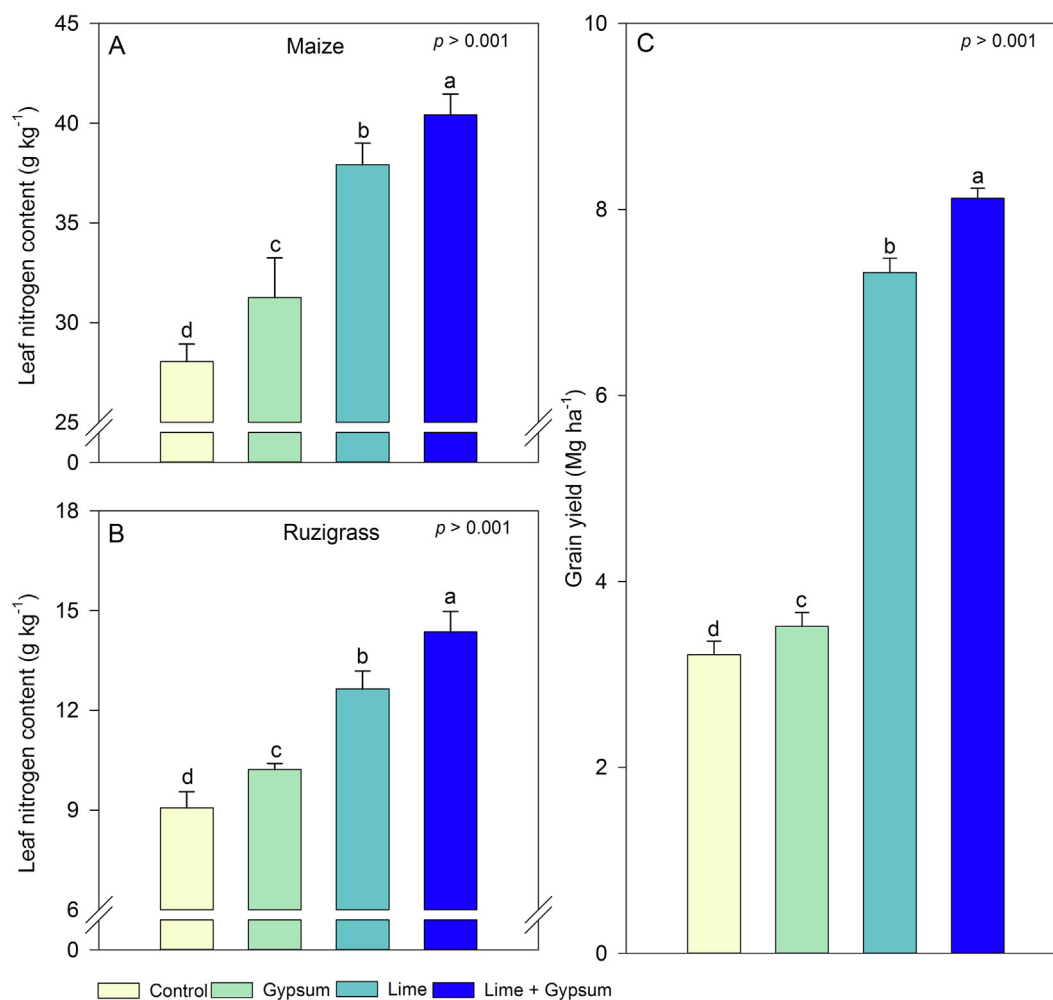
Fig. 5. Maize and ruzigrass leaf nitrogen content (A and B) and maize grain yield (C) under different treatments (control, gypsum, lime and lime + gypsum). Different lowercase letters indicate significant differences between treatments by LSD test at $p \leq 0.05$. Error bars express the standard error of the mean ($n = 4$).

Mg^{2+} and consequently EB, CEC and BS were negatively correlated with Al^{3+}, H + Al, Fe, Mn, Cu and Zn availability (Fig. 4D).

3.4. N content in maize and ruzigrass leaves and maize grain yield

The maize (Fig. 5A) and ruzigrass (Fig. 5B) leaf nitrogen content and maize grain yield (Fig. 5C) increased under the individual amendments compared with the control, but joint application of lime and gypsum provided the largest increases.

4. Discussion

4.1. Changes in soil chemical properties

In general, our results showed lasting effects for at least three years after exclusive liming application in 2002, 2004, 2010 and 2016, with improvements in all soil chemical attributes, including reduced pH, exchangeable Al^{3+}, H + Al and micronutrients (Fe, Mn, Cu and Zn) and greatly increased SOM, inorganic N, P, Ca^{2+} and Mg^{2+} availability and consequently CEC and BS compared with the control treatment (Table 1). Thus, liming triggers soil buffering processes that change the dynamics of exchangeable cations and the dissolution of other elements. Moreover, as pH increases, the availability of metal ions such as Al^{3+} and micronutrients decreases, reducing possible toxic effects on plant development and microbial growth (Caires et al., 2011; Carmeis Filho et al., 2017). Therefore, the liming process has complex impacts

on soil, which are reflected in a series of dependent and simultaneous effects and subsequent changes to soil processes (Holland et al., 2018).

Exclusive gypsum application resulted in a small increase in soil properties compared with the control treatment (Table 1), in agreement with Caires et al. (2011) and Zoca and Penn (2017). Although gypsum does not directly affect soil pH, it increases P, Ca^{2+}, $S-SO_4^{2-}$ and CEC availability and reduces Mn and Cu availability. These changes in turn increase pH due to ligand exchange reactions of $S-SO_4^{2-}$ with terminal hydroxides associated with Al and Fe oxides, which displace OH^- and promote partial neutralization of soil acidity. The improvements in crop yield due to gypsum application are primarily due to the increased Ca^{2+} and S solubility in soil and, consequently, availability in plants and/or reduced Al^{3+} availability in soil, mainly in deep layers (Soratto et al., 2010). Due to the thermodynamics of ion exchange and the properties of Ca^{2+}, gypsum can also potentially increase leaching losses of Mg^{2+} and K^+ to deep layers (Syed-Omar and Sumner, 1991; Zoca and Penn, 2017).

When applied separately, both lime and gypsum amendment improved soil fertility in our study, but the effects of lime were greater than those of gypsum, indicating the impossibility of replacing lime with gypsum in agricultural systems. However, the effects of joint application of lime and gypsum were greater than those of either amendment individually, particularly for N, P, Ca^{2+} and $S-SO_4^{2-}$ availability. The availability of other soil factors remained similar to those obtained by lime application only, particularly Mg^{2+} availability. Several studies have demonstrated that the effects of gypsum on soil

chemical properties are smaller than those of lime (Zoca and Penn, 2017, Bossolani et al., 2018, Crusciol et al., 2019), mainly because gypsum is not an acid-neutralizing or acid-forming substance (Zoca and Penn, 2017). Thus, liming has the greatest effects on soil, but our work demonstrates that gypsum is an important complement to lime application that potentiates its effects, especially under tropical conditions with intensive cropping.

4.2. N cycle genes in an intercropped system with soil amendment

The addition of lime and/or gypsum to soil in the no-till intercropping system changed the soil chemical properties and consequently had positive effects on total archaea, total bacteria and N cycle genes in the bulk soil and rhizospheres of both plant species evaluated. Gypsum increased total archaea and total bacteria, but liming produced the largest increases in these groups in plant rhizospheres and soil.

The responses of N cycle genes in the rhizosphere to amendment differed between maize and ruzigrass, even though both plant species were cultivated under the same soil conditions with intercropping. Plant species can alter rhizosphere characteristics (for example, pH and root exudates) (Chen et al., 2019), and these alterations support the formation of a microhabitat (Schmid et al., 2018) that can be occupied by different amounts and species of microorganisms specialized in various niches (Suleiman et al., 2019). The treatments with lime and/or gypsum changed soil nutrient availability, and these changes interacted strongly with the changes in the crop system induced by the plants themselves, further contributing to shaping the microbial community in these agroecosystems (Navarrete et al., 2013).

The abundances of N cycle genes increased proportionally to population growth of archaea and bacteria (Supplementary Fig. S2 and S3), but the responses differed between the plant rhizospheres and bulk soil. Thus, the relative abundances of N cycle genes in relation to the total population of archaea and bacteria were examined to determine the real contribution of these genes in the microbial community according to the treatments employed (Fig. 3). The relative abundance of *nifH* increased in all treatments compared with the control; however, exclusive liming and lime + gypsum amendment enhanced the abundance of this gene in the soil and plant rhizospheres (Supplementary Fig. S2A-S2C). Likewise, the relative abundance of *nifH* in relation to the total population of bacteria increased under the application of lime exclusively or lime + gypsum, indicating a high contribution of these amendments to the nitrogen-fixing community in the soil. There was a strong correlation between soil factors and the relative abundance of *nifH* (Fig. 4D), indicating that the improvement in soil attributes (e.g., increases in pH and availability of Ca^{2+} and Mg^{2+} and reduced Al^{3+}) increased nitrogen fixers in the microbial community. RDA enabled the identification of the most important soil factors for increasing the relative abundance of the *nifH* gene in each compartment evaluated in the crop system (maize and ruzigrass rhizospheres and bulk soil; Fig. 4A-4C). Different species of plants vary in their recruitment of microorganisms, mainly due to differences in root exudates (Kuzyakov and Razavi, 2019). *nifH* abundance is completely dependent on soil fertility and how each amendment changes soil factors. In this study, nitrogen fixers responded significantly to pH and base saturation, but the responses in the rhizospheres of intercropped maize and ruzigrass differed. Exchangeable Ca^{2+} and Mg^{2+} were the most important soil factors shaping the relative abundance of *nifH* in the maize rhizosphere, whereas in the ruzigrass rhizosphere, SOM, pH, exchangeable Ca^{2+} and CEC provided the greatest changes in *nifH* abundance.

The low pH range in the control and gypsum treatments (Table 1) showed high availability of Al^{3+}, which could be highly toxic to N fixers (Mokolobate and Haynes, 2002; Jaiswal et al., 2018). Mg^{2+} is an essential cofactor for nitrogenase function and assists in electron transfer (Mortenson et al., 1973). Soil factors, mainly Ca^{2+} and Mg^{2+}, have been frequently reported to increase nitrogen fixer populations in different agroecosystems (Wakelin et al. 2010; Mirza et al. 2014; Wang

et al., 2018) and might explain the increase in *nifH* in our study. During the summer, soybean is cultivated in the study system; the increase in the amount of nitrogen fixers in association with soybean may favor higher yields, since long-term nitrogen fertilization of this leguminous crop has not been carried out in the area. The high abundance of *nifH* may be associated with this summer crop, however, although soybean is inoculated with Bradyrhizobium sp. every crop season, the amount of inoculant used is the same in all treatments. Hence, the difference in the abundance of *nifH* obtained is attributed exclusively to the effect of the treatments.The abundances of ammonia-oxidizing communities of both archaea (AOA) and bacteria (AOB) were modified by the application of different amendments, but distinct patterns were observed between the plant rhizospheres and bulk soil (Supplementary Fig. S2D-S2I). In the maize rhizosphere, AOA and AOB increased, whereas in the ruzigrass rhizosphere and bulk soil they decreased. Our data indicate a strong capacity of forage grasses to reduce soil nitrification compared with maize. The improvement of soil components may contribute to reducing soil nitrification, as evidenced by the reduction in the abundance of AOB in bulk soil. Interestingly, we observed decreases in the relative abundances of AOA and AOB in the soil regardless of plant species (maize or ruzigrass), and thus improving soil chemistry through lime and gypsum amendments can reduce the nitrification process in intercropping systems under no-till.

Soil factors that were positively correlated with the increases in the relative abundances of nitrifier and denitrifier genes were identified by RDA (Fig. 4A-4C) and further supported by correlation analysis (Fig. 4D). In general, high levels of micronutrients (mainly Mn), Al^{3+} and H + Al were associated with greater abundances of AOA, AOB, *nirK* and *nosZ*. Exclusive liming and liming + gypsum reduced the abundances of *nirK* and *nosZ* in the ruzigrass rhizosphere and bulk soil but not in the maize rhizosphere (Supplementary Fig. S2J-S2O). The relative abundances of these genes exhibited the same patterns (Fig. 3J-3O), and in general, the improvement of soil attributes correlated negatively with nitrifier and denitrifier gene abundance (Fig. 4D). Thus, our results demonstrate that soil chemistry is an important factor linked to the abundance of genes related to nitrification and denitrification and that micronutrients are important factors in these processes.

Generally, acidic environments favor the availability of micronutrients, which can be toxic to plants and microorganisms; thus, soil acidity correction is an important management strategy to balance the availability of these micronutrients and reduce the abundances of nitrifiers and denitrifiers (Liu et al., 2010). Almost every enzymatic pathway in the N cycle involves a metallic cofactor containing Fe, Cu or sometimes Zn (Godfrey and Glass, 2011). In addition, in tropical soils with low SOM, oxidation of NH_4^+ can occur in the presence of metal oxides like manganese oxides (Swathi et al., 2017), which explains the strong influence of Mn in our results (Fig. 4A-4C). This element plays a major role in biogeochemical nitrogen cycling (Hulth et al., 1999). MnO_x acts as a terminal electron acceptor (Lin and Taillefert, 2014) and mediates the reduction of NH_4^+. Bru et al. (2011) and Levy-Booth et al. (2014) reported that in addition to pH, Mn levels were positively correlated with the abundances of *nirK*, *nirS* and *nosZ* genes. Ligi et al. (2014) demonstrated that *nirK* and *nosZ* gene abundances are most similarly influenced by the same environmental parameters.

Changes in bacterial community composition have been shown to influence the nitrogen cycling potential of soils (Wakelin et al., 2009). These observations suggest that liming, regardless of gypsum addition in the present research, is an important strategy for efficiently reducing overall N_2O emissions through nitrification and denitrification in crop systems that utilize nitrogen fertilizers. Our results corroborate with those of Holland et al. (2018) and Kunhikrishnan et al. (2016) showing that impacts on N transformation due to liming decreased N_2O emissions and oxidation rates of CH_4 and increased CO_2 flux. Quantification of liming effect showed that there was four times decreased N_2O emissions from a limed soil (pH 7.0) compared to a soil with pH 4.5 (Baggs et al., 2010). Another key gaseous process is volatilization, that

has been shown to increase by liming, which raises NH_3 emissions (Sommer and Ersbøll, 1996). In terms of the direct net liming impact on gases flux there is evidence that liming is not a sound mitigation strategy (Gibbons et al., 2014) and thus, overall greenhouse gas emissions are increased after liming. According to Holland et al. (2018), the impacts of liming on greenhouse gas emissions are complex and there are markedly different potential changes in emissions between different gases.

Despite the vast literature indicating that pH is the greatest determinant of microbial assembly (Bartram et al. 2014; Lammel et al., 2018; Holland et al., 2018), this study clearly shows that the increase in soil pH due to liming directly impacts many other soil factors that in turn directly affect microbial enzymes related to the N cycle, i.e., EB, CEC, BS, Al^{3+} and micronutrient availability. However, most studies of the N cycle in agricultural soils and the soil microbial community do not consider soil chemical factors and attach importance only to pH and SOM (Zhang et al., 2017; Liu et al., 2018; Holland et al., 2018). Thus, the development of sustainable crop systems in tropical regions is necessary to improve overall agricultural functionality (Moraes et al., 2019), as most tropical agricultural soils are acidic with low fertility.

4.3. N content in maize and ruzigrass leaves and maize grain yield

The high N concentration in maize and ruzigrass leaves (Fig. 5A and 5B) may be a consequence of the higher N availability in the soil amended with lime + gypsum (Table 1). Soil ameliorants such as lime can increase biological processes that govern the activity of soil microorganisms responsible for SOM mineralization (Kemmitt et al., 2006; Stiehl-Braun et al., 2011; Wachendorf, 2015). The responses to liming vary according to the crop system and whether long-term or recent effects are examined. The positive effects of liming on soil fertility induce changes in soil properties and can promote cascade effects on soil N transformation processes (Holland et al., 2018). Several studies have associated liming with increased nitrification and denitrification in subtropical environments, but studies in tropical soils like the current study are rare. The research shows that repeated liming applications will increase soil N mineralization (Holland et al., 2018), but the overall impact of liming will depend on immobilization that occur based on the C:N ratio of plant residues returned to soils and quality of the organic matter (Bailey, 1995; Kliemman et al., 2006; Wachendorf, 2015). Nitrogen is considered the nutrient that better returns to the soil-plant system through plant residues and can be reused by the crops in subsequent agricultural cycles (Hungria et al., 2015).

Other factors must also be considered. (a) Exclusive liming or, in particular, gypsum addition increases the depth and volume of root system exploration (Costa et al., 2018), which reduces losses of N by leaching. (b) Plants growing in chemically fertile environments are more vigorous and can compete with microorganisms for soil resources, mainly N, which reduces the availability of N for nitrification and subsequent denitrification processes. (c) Most studies reporting increased nitrification in amended soils do not consider losses due to denitrification or the possibility of reduced nitrification in intercropping systems with grain crops and tropical grasses. Soils cultivated with forage grasses reduce nitrifying activity. Consequently, the residual N in the soil, whether from top dressing fertilization in maize or from nutrient cycling, remains in the form of NH_4^+ and is no longer linked to soil CEC, thereby reducing losses by leaching and denitrification (Subbarao et al., 2015, Coskun et al., 2017).

Finally, our findings demonstrated that liming and lime + gypsum amendment affect the microbiota and soil chemical properties, thereby influencing maize development and grain yield (Fig. 5C) through resource availability. In addition, the increase in nitrogen absorption by plants increases the absorption and metabolism of other nutrients (Marschner, 2012), which is reflected in crop yield. Understanding how different cultivated plants respond to these changes is of paramount importance for developing more productive and sustainable

agricultural systems. Therefore, the combination of lime and gypsum can be considered an important practice to improve the yield capacity of acidic tropical soils managed under no-till systems (Inagaki et al., 2016; Carmeis Filho et al., 2017).

5. Conclusions

Our results reveal how the lasting effects of long-term exclusive and joint application of lime and gypsum change soil chemical properties in a tropical intercropping no-till system and how these changes influence total prokaryotes (archaea and bacteria) and genes related to the N cycle. These soil amendments increased soil fertility (mainly Ca^{2+} and Mg^{2+} status), reduced Al^{3+} availability and balanced the levels of soil micronutrients. The influences of these soil amendments on N cycle genes were similar in the rhizospheres of ruzigrass and maize and bulk soil, indicating that soil chemical properties provide strong selection of microorganisms and thus may be as relevant as the type of plant in intercropping systems. In addition, the improvement in soil quality led to greater N uptake by plants and higher maize yield. This study demonstrates that soil amendments are an important alternative for increasing the efficiency of nitrogen use in no-till agricultural systems and provides a basis for future research characterizing the active community and expression of functional genes of microorganisms related to the N cycle.

Declaration of Competing Interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2020.114476.

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