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Denitrifying microbial genes quantification attests inference for potential N_2O emissions in sugarcane soils by enzymatic bioanalysis

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This study evaluated the relationships and sensitivity of denitrifying microbial gene abundance, as well as the activities of soil enzymes β-glucosidase (GLU) and arylsulfatase (ARYL), to assess the quality of sugarcane soils managed with vinasse (V), filter cake (FC), and mineral fertilizer (MF). Composite soil samples were collected using a systematic sampling approach that included two soil classes (Ferralsol and Acrisol), two textures (clayey and sandy), three management systems (V, V+FC, and MF), two sampling seasons (rainy and dry), and three replicates, totaling 72 samples. Analysis of soil organic carbon (SOC), and macro- and micronutrients differentiated the Ferralsol and Acrisol samples into distinct groups based on agricultural management (Global R = 0.554) and showed some overlap based on soil texture (Global R = 0.369). The number of *nir*K, *nir*S, and *nos*Z I gene copies per gram of soil, determined by Real-Time Quantitative PCR (qPCR) based on genomic DNA isolated from the 72 soil samples, was higher in the rainy season compared to the dry season ($P<0.05$). None of the genes evaluated revealed a consistent response to different sugarcane soil managements, showing specific response patterns for each soil class and texture. In the Ferralsol, the activities of GLU and ARYL increased in the following order: V < MF < V+FC, regardless of soil texture (sandy or clayey) and sampling season. The average activity of the two enzymes in both V+FC and MF treatments was 1.8 times higher in sandy soil and 3.9 times higher in clayey soil compared to soil managed with vinasse. In the Acrisol, no significant differences among the treatments were observed. Statistical analyses revealed negative correlations ($P<0.05$) between the number of copies of the *nir*K and *nos*Z I genes and GLU and ARYL activities in the soil during both seasonal periods analyzed. The number of copies of these two microbial genes was also negatively correlated with the soil organic matter in the rainy season. Thus, the indications

of sugarcane soil quality based on enzymatic analyses were corroborated by the lower abundance of genes associated with denitrification process. The findings of this study open the possibilities to infer about the potential for N_2O emission from these sugarcane soils based on GLU and ARYL activities.

KEYWORDS

environmental DNA (eDNA), microbial genes, denitrification, soil enzymes, soil bioanalysis technology

1 Introduction

Nitrous oxide (N_2O) is the most important greenhouse gas emitted from agricultural soils, presenting global warming potential 298 times higher than carbon dioxide (CO_2). In soils, N_2O is produced by nitrogen (N) cycling nitrification and denitrification reactions, which are mediated by enzymes encoded by *nir*K, *nir*S, and *nos*Z genes (1). Agriculture and soil management account for 78% of the N_2O anthropogenic emissions (2). The main reasons for the high emissions in the agriculture sector are organic matter oxidation and microbe-mediated processes, which are sensitive to organic amendments (3), nitrogen fertilization (4), and climatic conditions (5).

Since agriculture contributes significantly to N_2O emissions from soil, it is imperative to understand crop productivity while reducing the environmental burden of the agricultural systems, especially for sugarcane (*Saccharum officinarum*) that is the world´s largest crop by production quantity. High yields in sugar and alcohol production have been achieved through the availability of sugarcane varieties that are resistant to pests and adapted to regional soil and climate conditions. This success is complemented by agronomic management including adequate doses of nitrogen (N), phosphorus (P), and potassium (K) fertilizers (6), micronutrients (7), maintenance of sugarcane crop residues on the soil surface (8), and the use of industry waste such as vinasse and filter cake (9, 10). Vinasse, a byproduct of the sugarcane industry composed of water, organic matter, and mineral elements (11), has been used as a liquid fertilizer in sugarcane crops to reduce mineral fertilizer use and address the ecological challenges of their disposal (12). Vinasse provides the K and part of the N needed for sugarcane growth but is low in P. Depending on its chemical composition and soil fertility, vinasse is applied in sugarcane rows at 30-45 m^3 ha^{-1} or sprayed on the plantations at the100-300 m^3 ha^{-1}. Meanwhile, the filter cake—an organic compound rich in P and containing significant amounts of iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) (13)—is derived from the juice filtration in rotary vacuum filter. It is applied at 10-30 t ha^{-1} in the planting furrow and 80-100 t ha^{-1} across the total area during pre-planting, partially or entirely replacing phosphate fertilizer depending on the recommended phosphate dose.

Several studies reported that N_2O emissions from soil are enhanced once N fertilizer is applied in combination with organic residues, and, in some cases, N_2O emissions are higher than those estimated using the IPCC default factors (14–18). These studies showed that increase in emissions of N_2O were especially significant when vinasse and filter cake were used as K and P sources, respectively, in comparison with mineral N fertilizer in sugarcane production fields. In addition to contributing to climate change, N_2O emissions from soil can affect soil quality (19). Although there is consensus among researchers and farmers that maintaining or enhancing soil quality is a key factor for the sustainability of agricultural systems, evaluating soil quality remains challenge. The multitude of chemical, physical, and biological factors that govern biogeochemical processes, along with their temporal and spatial variations and the inherent complexity of soil, are among the factors that hinder the assessment of soil quality and the identification of key indicators of its function. However, expanding the functional aspects of the soil that can be interpreted through the assessment of its quality is necessary given the finding that unsustainable land management practices that degrade soils have led not only to reduction in soils' ability to sequester carbon and support agricultural productivity, but also a greenhouse gas emission (20).

In Brazil, since 2020, two key soil enzymes, β-glucosidase (GLU) and arylsulfatase (ARYL)—associated to the carbon (C) and sulfur (S) cycles, respectively—have been used as indicators of soil health, in large scale on-farm soil quality assessments (21–23) representing an opportunity to engage producers in soil testing beyond the standard chemical test approaches. In addition to their sensitivity (24, 25), low seasonal variability (26), ease of measurement and low cost (27), other advantages that favored the selection of GLU and ARYL for commercial use as bioindicators of soil quality included their good correlation with crop yields and soil organic matter (22, 25, 28), and the fact that they can be measured directly in air-dried soil samples collected at the postharvest stage (22). More recent studies also have shown a linear relationship between GLU and ARYL and the diversity of the soil microbial community (fungus, bacteria, protist, and archaeal groups) assessed by DNA characterization (28). These authors observed that sites with higher crop yields presented higher GLU and ARYL activities and higher soil microbial diversity with an abundance of beneficial soil microorganisms (e.g., Mortierella, Bacillus, Penicillium, Trichoderma, Pseudomonas, Bradyrhizobium and Rhizobium) and lower presence of plant pathogenic organisms

(e.g., Fusarium and Macrophomina). These previous studies focused on GLU and ARYL showed that the determination of activity of both soil enzymes allows an evaluation of the impact of agricultural management practices on soil microbiota. The nature and extent of biological and inorganic interactions between C, S and N biogeochemical cycles have been previously identified, with positive and negative feedbacks recognized (29).

Advances in molecular methods have enhanced our ability to research biodiversity in various environments, including agroecosystems, based on the detection of environmental DNA (eDNA)—genetic material isolated from environmental samples—that can provide information on key taxonomic or functional microbial groups in the environment. Yang et al. (30) and Soares et al. (31) utilized eDNA to evaluate the impact of organic and inorganic fertilizers in agroecosystems on the distribution and abundance of genes associated with the denitrification process (*nir*K, *nir*S, and *nos*Z), demonstrating the sensitivity of these genes to changes in the soil due to agricultural management. Nishisaka et al. (32) showed that these genetic markers of the denitrifiers are sensitive to soil moisture, increasing in abundance from the dry to rainy season in tropical soil. Microbe-mediated N_2O production begins with the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), followed by the reduction of NO_2^- to nitric oxide (NO), which is catalyzed by enzymes encoded by *nir*K and *nir*S genes (33). The conversion of one-third of NO to N_2O is followed by its reduction to N_2 by N_2O reductases encoded by *nos*Z genes, thereby partially mitigating N_2O release to the atmosphere (34, 35).

Considering i) the ability of healthy soils to mitigate greenhouse gas emissions, particularly by reducing N_2O emissions (36), and ii) the impact of ethanol production by-products used as organic and mineral fertilizers on N_2O emissions, we hypothesized that sugarcane fields with higher GLU and ARYL activities (i.e., healthy soils) would exhibit a lower abundance of denitrifying genes. To test this hypothesis, this study aimed to define the relationships between the abundance of denitrifying microbial genes and the activities of GLU and ARYL in sugarcane soils managed with vinasse (V), vinasse combined with filter cake (V+FC), and mineral fertilizer (MF).

2 Materials and methods

2.1 Study areas and soil sampling

The study areas were located in the northwest region of São Paulo and were identified along with different commercial sugarcane plants. This region is colloquially known as the 'sugarcane valley'. Its soils are generally less fertile compared to other regions of São Paulo, yet they exhibit a variety of soil classes and textures, all under sugarcane cultivation.

Two production units were selected for this research. One is located in Santa Albertina, at (20°2'31.21"S and 50°40'42.91"W), and the other in the municipality of Sebastianópolis do Sul (20° 35'8.05"S and 49°57'0.71"W). In each of these commercial sugarcane production units, six study areas (plots) were selected to correspond with the different scenarios outlined for this research. These were defined based on soil classes and textures and the agricultural management practices employed (Figure 1).

Before planting the sugarcane, 1.0 t ha^{-1} of dolomitic limestone (PRNT = 90%) was applied to the area and mineral fertilization that is common in all plots consisted of 150 kg ha^{-1} P_2O_5 (triple superphosphate) and 80 kg ha^{-1} KCl (potassium chloride). Mineral fertilizer consisted of 600 kg ha^{-1} of 04-28-20 + 2% zinc + 3% manganese + 2% copper. Vinasse was sprayed onto the plantations at a rate of 120 m^3 ha^{-1} as a source of K in addition to organic matter and other nutrients. Raw vinasse wastewater was obtained from ethanol distilleries from both production units selected for this study. Filter cake (25 t ha^{-1}) with 70% moisture was incorporated in the inter-row zone and combined with vinasse spread at 80 m^3 ha^{-1}.

In each of the 12 plots, soil sampling was executed in a completely randomized design. This design included obtaining three samples from the planting row and six samples from inter-rows at three distinct sampling points per plot. Subsequently, the samples from each sampling point—one from the planting row and two from inter-rows—were homogenized by rolling in a plastic bag. This procedure aimed to enhance accuracy in representativeness and facilitate the obtaining of subsamples.

Soil sampling commenced during the dry season (August 2022) and was repeated in the rainy season (January 2023). The soil collection was performed using previously sterilized equipment, employing a 50-mm diameter, 25-cm long PVC tube. This tube was driven 20 cm deep into the soil with a rubber mallet, following standard agricultural soil sampling protocols for microbial analysis, which typically target the root zone with the highest density of roots. For enzymatic analysis, soil samples were collected from the 0-10 cm layer using a 50-mm diameter, 15-cm long PVC tube, following the SoilBio technology protocol (37).

Two soil subsamples were extracted from the composite sample collected from the 0-20 cm layer. One subsample was stored in a plastic bag for chemical analysis, and the other, intended for molecular analysis, was placed in a 50-mL Falcon tube, immediately cooled on ice, and transported to the Agricultural Sciences Laboratory at University Brazil, Fernandópolis Campus. A total of 72 soil subsamples were collected for chemical analysis and an equal number for molecular analysis. This sampling comprised 2 soil classes (Ferralsol and Acrisol) × 2 textures (clayey: 12 to 14.7% clay; and sandy: 35.3 to 56.3% clay) × 3 management systems (V, V+FC, and MF) × 3 plots × 2 seasonal periods (rainy and dry). The subsamples for molecular analysis were preserved at -20°C until processing. Enzymatic soil analysis samples were stored at room temperature until analysis.

2.2 Soil chemical analyses

The soil pH from each of the 72 soil subsamples from the 0-20 cm layer was measured in a 1:2.5 soil-to-water suspension. Exchangeable aluminum (Al), calcium (Ca), and magnesium (Mg) were extracted with 1 M KCl, with Ca, and Mg levels determined by atomic absorption spectrometry and Al by acid-base titration. The P and K were extracted using ion exchange resin. Potential acidity (H + Al) was estimated through an equation based

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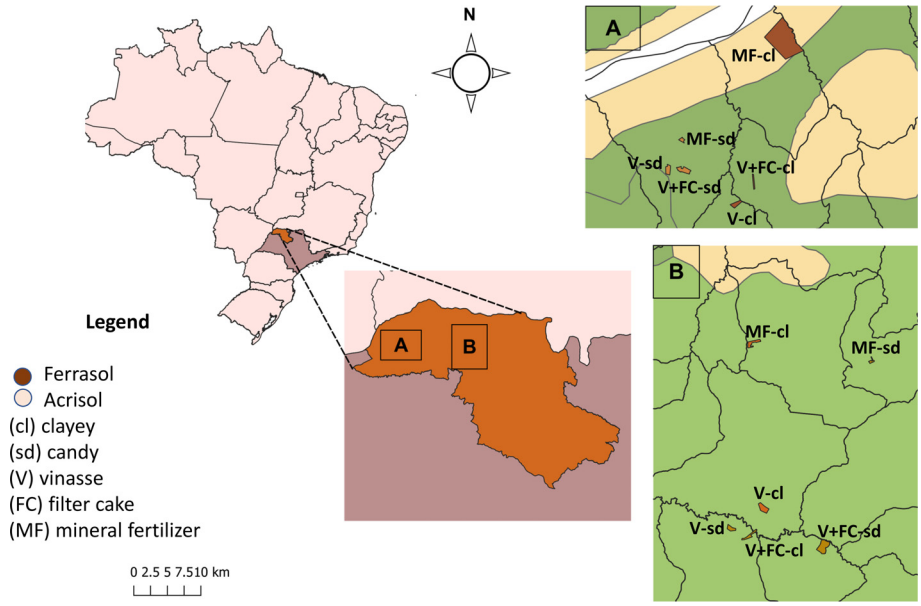

FIGURE 1
Soil sampling plots in commercial sugarcane plantations in the northwest region of São Paulo used for this study.

on the pH determined in an SMP buffer solution (pH SMP). Available micronutrients, including Fe, Mn, Zn, and Cu, were extracted using Mehlich 1 and measured via atomic absorption spectrophotometry. Boron (B) was extracted with hot water and determined by spectrophotometry using azomethine-H at 420 nm. Additional parameters such as exchangeable bases (SB, sum of Ca, Mg, and K), cation-exchange capacity (CEC, sum of Ca, Mg, K, Al, and H), base saturation (BS, percentage of SB to CEC), and Al saturation (percentage of exchangeable Al to CEC) were calculated (38). Soil organic matter (OM) was determined using the Walkley-Black method (39) and calculated according to Jackson (40). Soil organic carbon content (SOC) was calculated by dividing the OM content by a factor of 1.72 (obtained by dividing 100 by 58).

2.3 Extraction of genomic DNA from soil

Genomic DNA from each of the 72 soil subsamples designated for molecular analysis was extracted using the DNeasy® PowerSoil® Pro Kit (Qiagen, Carlsbad, CA, USA) following the manufacturer's instructions. DNA concentration and quality were assessed using a NanoDrop spectrophotometer (NanoDrop® ND-1000 UV/vis-spectrophotometer, Peqlab Biotechnologie GmbH, Erlangen, Germany). This assessment was followed by agarose gel electrophoresis in 1x TBE buffer (10.78 g L^{-1} Tris; 0.58 g L^{-1} EDTA; and 5.50 g L^{-1} boric acid), run at 90 volts for 1 h. The extracted DNA was then stored at -20 °C until needed for further analysis.

2.4 Real-time quantitative PCR

The copy number of the *nir*K, *nir*S, and *nos*Z I genes was determined from 72 different soil genomic DNA samples using the real-time quantitative PCR (qPCR) technique. Standard curves were constructed using five serial dilutions (ranging from 10^2 to 10^7 copies) of plasmid DNA containing the target genes from strains obtained from the German collection *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ)—*Sinorhizobium meliloti* (DSM 30135) for *nir*K, *Pseudomonas fluorescens* (DSM 50090) for *nir*S, and *Bradyrhizobium japonicum* (DSM 1755) for *nos*Z clade I. The qPCR reactions were performed on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The reaction mixtures were prepared in a total volume of 10 µL, consisting of 5 µL of SybrGreen Master Mix Kit (Applied Biosystem, Foster City, CA, USA), 3.35 µM of each primer for the *nir*K and *nir*S genes (Table 1), 4.70 µM for the *nos*Z I gene (Table 1), 0.5 µL of 0.6% BSA, and 1 µL of soil genomic DNA sample. Amplification conditions were as follows: for the *nir*K gene: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 40 s, with a final melting curve at 95°C for 15 s, 58°C for 1 min, and 95°C for 1 s. For the *nir*S gene: 94°C for 10 min, followed by 50 cycles of 95°C for 15 s, 57°C for 1 min, and 72°C for 1 min, with a melting curve at 95°C for 15 s, 58° C for 1 min, and 95°C for 15 s. For the *nos*Z I gene: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, with a melting curve at 95°C for 15 s, 58°C for 1 min, and 95°C for 15 s. Fluorescence was captured at the end of the extension step in each amplification cycle. Melting curve analysis was performed to ensure the fluorescence signals originated from specific amplicons rather than primer dimers and/or other artifacts. Automated analyses of amplification quality (e.g. background noise subtraction from the plateau phase and threshold cycle [Ct] setting during the linear amplification phase) and quantity were performed using QuantStudio® Design and Analysis Software (Thermo Fisher Scientific, Waltham, MA, USA).

The number of copies of each target gene was calculated per gram of soil using Equation 1.

TABLE 1 Primers used for quantifying the copy number of microbial functional genes associated with the denitrification process.

Gene	Function	Primer sequence (5´ to 3´)	Reference
*nir*K	Reduction of nitrite to nitric oxide or ammonia	*nir*K876 (ATYGGCGGVAYGGCGA) *nir*K 1040 (GCCTCGATCAG(A/G)TT(A/G)TGG)	Henry et al. (41) Braker et al. (42)
*nir*S	Reduction of nitrite to nitric oxide	*nir*S cd3f (GTNAAYGTNAARGARACNGG) *nir*SR3cd (GA(C/G)TTCGG(A/G)TG(C/G)GTCTTGA)	Throbäck et al. (43)
*nos*Z I	Reduction of nitrous oxide to nitrogen gas	*nos*Z2F (CGCRACGGCAASAAGGTSMSSGT) *nos*Z2R (CAKRTGCAKSGCRTGGCAGAA)	Henry et al. (44)

$$CN/g\ soil = QC \times 4 \times 100, \quad (1)$$

where CN/g soil = Copy number of the target gene per gram of soil; QC = Copy number obtained from the qPCR analysis. In this equation, the multiplication by 4 accounts for the fact that 0.25 g of soil was used for genomic DNA extraction. The result is then multiplied by 100, reflecting the final volume in microliters of the DNA suspension at the conclusion of the extraction process.

2.5 Determination of soil enzyme activity

The activities of the enzymes GLU and ARYL were determined as described by Tabatabai (45) with soil samples collected at the 0-10 cm depth. For each soil sample, two analytical replicates and a control (without substrate addition) were used. The method is based on the colorimetric determination of the p-nitrophenol (PNP) released when soil is incubated with the corresponding p-nitrophenyl substrate—p-nitrophenyl-β-D-glucopyranoside (PNG) for β-glucosidase and p-nitrophenyl sulphate (PNS) for arylsulfatase—at the optimal pH for each specific enzymatic reaction. The incubation was performed at 37°C for 1 hour. Due to the short incubation time, toluene was omitted from the assays (25).

Soil samples were processed according to the FERTBIO soil sample concept from Mendes et al. (22). All samples were air-dried at room temperature for two weeks, sieved through a 2-mm mesh, and stored at room temperature until analyzed. To determine the amount of p-nitrophenol released from the samples, a standard curve was prepared with known p-nitrophenol concentrations. The enzyme activities were expressed in mg p-nitrophenol kg^{-1} h^{-1} soil.

2.6 Statistical analysis

Analysis of variance (ANOVA) and Tukey's test were conducted on the datasets obtained from soil chemical analyses, soil enzymatic activities, and quantification of the *nir*K, *nir*S, and *nos*Z I genes, using a significance level of 0.05. These analyses were performed using R software version 4.4.0 (46), a free platform for statistical computing.

Spearman's correlation coefficients were calculated to generate a heatmap correlation graph. This graph illustrates the relationships among the following datasets: *i*. soil organic matter; *ii*. activities of the enzymes β-glucosidase and arylsulfatase; and *iii*. the absolute number of copies of the *nir*K, *nir*S, and *nos*Z I genes in the soil. The analysis was executed in the R v.4.4.0 environment using the "corrplot" package (47). Pearson's correlation was used to confirm the relationship between the copy number of the *nir*K, *nir*S, and *nos*Z I genes and β-glucosidase and arylsulfatase enzyme activity in rainy and dry seasons.

3 Results

3.1 Assessment of soil chemical attributes

Results from soil chemical analyses are shown in Tables 2 and 3 for the rainy and dry seasons, respectively. These results indicated statistically significant differences ($P<0.05$), for some parameters, when comparing sandy and clayey textures, as well as different agricultural management strategies—V, V+FC, and MF—within the same soil class.

For SOC, no significant differences were observed in the Ferralsol, regardless of texture or sampling season. In the clayey Acrisol, a significant reduction in SOC was observed in the V+FC treatment, regardless of the sampling season. Vinasse application led to higher concentrations of macro- and micronutrients in sandy Ferralsol, with increase in P, Ca, and Mg contents in the rainy season (Table 2). Furthermore, the K and Fe contents increased in this soil class and texture with filter cake application. At the same time, mineral fertilization was associated with increases in H + Al and Mn. For the dry season, sandy Ferralsol with vinasse application also resulted in high concentrations of Ca and Mg, whereas mineral fertilization increased Mn levels in this soil.

In the rainy season, the management strategy had a more pronounced impact on the chemical attributes of Ferralsol with a sandy texture, whereas in the dry season these effects were more evident in Acrisol with a sandy texture.

In Acrisol, vinasse application was linked to higher P values, whereas mineral fertilization enhanced the levels of H + Al, Cu, Mn, and Fe. In this soil class, only management with vinasse demonstrated a statistically significant difference in Fe concentration during the rainy season in sandy texture. For the clayey texture, the rainy season highlighted the management with vinasse, and vinasse combined with filter cake, both contributing to significant increases in the levels of P, Ca, Mg, Cu, and Zn.

In general, statistical analyses indicated differences in chemical factors within Ferralsol within the same texture and management strategy when compared to Acrisol under the same conditions during the dry season. Both dry and rainy seasons exhibited variations in soil

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TABLE 2 Chemical properties of soil collected during the rainy season from the 0-20 cm layer in sandy and clayey Ferralsol and Acrisol from the study sites.

Variable	Ferralsol						Acrisol					
	Sandy			Clayey			Sandy			Clayey		
	V	V+FC	MF	V	V+FC	MF	V	V+FC	MF	V	V+FC	MF
SOC	7.6[¶]aa**a** ± 1.0[§]	8.5aa**a** ± 1.2	6.8ab**a** ± 2.2	8.7aa**b** ± 1.0	8.9aa**b** ± 1.3	14.7aa**a** ± 1.5	5.6aa**a** ± 1.5	4.5bb**a** ± 1.2	5.0aa**a** ±0.3	5.2ba**a** ± 0.6	8.5aa**a** ± 3.8	6.0ba**a** ± 0.9
pH	6.5aa**a** ± 0.2	6.3aa**a** ± 0.2	5.4ba**b** ± 0.2	6.5aa**a** ± 0.1	5.8bb**b** ± 0.3	5.2ba**b** ± 0.2	5.8ba**a** ± 0.8	6.3ab**a** ± 0.2	6.2aa**a** ± 0.1	6.3aa**b** ± 0.2	7.2aa**a** ± 0.2	6.0aa**b** ± 0.3
P	167.3aa**a** ± 48.2	110.7aa**a** ± 15.8	24.3aa**b** ± 11.7	60.0ab**a** ± 15.0	16.0bb**ab** ± 4.2	25.0aa**b** ± 3.3	46.0ba**a** ± 3.5	21.7bb**a** ± 7.5	37.0aa**a** ± 6.0	37.3aa**b** ± 29.0	143.3aa**a** ± 65.0	27.0aa**b** ± 19.0
K	2.6 ab**b** ± 0.2	5.5aa**a** ± 1.5	1.3ab**b** ± 0.8	6.9aa**a** ± 1.1	6.8aa**a** ± 0.5	4.6aa**b** ± 1.6	3.1aa**a** ± 0.2	2.4ba**a** ± 0.7	2.1aa**a** ± 0.1	3.2ba**a** ± 0.1	3.3ba**a** ± 0.4	2.1ba**a** ± 0.8
Ca	195.3aa**a** ± 70.0	42.7aa**b** ± 4.2	17.7bb**c** ± 3.2	45.3ab**a** ± 2.1	36.0ba**a** ± 7.0	52.7aa**a** ± 7.5	28.0ba**ab** ± 11.5	18.3bb**b** ± 5.5	39.3aa**a** ± 5.1	25.7ba**b** ± 5.5	159.3aa**a** ± 91.5	30.7ba**b** ± 8.1
Mg	55.3aa**a** ± 15.6	20.7aa**b** ± 2.5	7.3bb**c** ± 1.5	22.3ab**a** ± 1.2	17.3ba**a** ± 2.5	18.7aa**a** ± 3.3	10.7ba**ab** ± 3.2	10.0bb**b** ± 3.0	17.3aa**a** ± 4.2	12.7ba**b** ± 2.5	48.7aa**a** ± 34.1	12.0ba**b** ± 2.6
H+Al	8.3bb**b** ± 0.6	11.7ab**a** ± 1.2	14.7ab**a** ± 2.5	11.7aa**c** ± 0.6	19.3aa**b** ± 1.5	31.0aa**a** ± 5.4	12.3aa**a** ± 4.0	9.3aa**a** ± 0.6	9.3ba**a** ± 0.6	10.0aa**a** ± 1.0	7.0bb**b** ± 1.7	10.7ba**a** ± 1.2
SB	253.3aa**a** ± 62.2	68.8aa**b** ± 6.2	26.3bb**c** ± 3.9	74.5ab**a** ± 2.1	60.1ba**a** ± 9.9	75.9aa**a** ± 9.5	41.8ba**ab** ± 14.6	30.7bb**b** ± 9.2	58.8aa**a** ± 9.0	41.6ba**b** ± 8.9	211.2aa**a** ± 121.6	44.7ba**b** ± 10.9
CEC	261.6aa**a** ± 62.8	80.5aa**b** ± 5.1	40.9bb**c** ± 3.4	86.2ab**a** ± 2.0	79.4ba**a** ± 8.4	106.9aa**a** ± 6.7	54.0ba**ab** ± 11.0	40.1bb**b** ± 9.7	68.1aa**a** ± 8.7	51.6ba**b** ± 7.9	218.3aa**a** ± 120.1	55.4ba**b** ± 9.9
BS	96.7aa**a** ± 0.6	85.7aa**a** ± 2.3	64.0ba**b** ± 6.1	86.3bb**a** ± 0.6	75.7bb**ab** ± 4.5	70.7ba**b** ± 5.8	75.7ba**a** ± 12.9	76.0bb**a** ± 4.6	86.0aa**a** ± 2.0	80.0aa**b** ± 5.6	96.0aa**a** ± 3.0	80.3aa**b** ± 5.7
B	0.27aa**a** ± 0.0	0.22aa**a** ± 0.1	0.26aa**a** ± 0.0	0.22bb**a** ± 0.6	0.25ba**a** ± 0.1	0.30bb**a** ± 0.1	0.23aa**a** ± 0.1	0.36aa**a** ± 0.0	0.31aa**a** ± 0.0	0.83aa**a** ± 0.1	0.47aa**a** ± 0.1	0.68aa**a** ± 0.1
Cu	1.3ab**a** ± 0.3	1.4ab**a** ± 0.1	0.4bb**b** ± 0.1	4.27aa**c** ± 0.3	11.1aa**b** ± 0.6	17.5aa**a** ± 1.0	0.8ba**ab** ± 0.1	0.53bb**b** ± 0.2	0.87aa**a** ± 0.1	0.73ba**b** ± 0.1	1.17ba**a** ± 0.3	0.77ba**b** ± 0.2
Fe	22.7aa**b** ± 3.2	44.7ba**a** ± 14.0	24.3ba**b** ± 8.0	15.0aa**a** ± 2.6	25.0aa**ab** ± 3.0	37.0ba**b** ± 10.4	30.0ab**b** ± 13.1	12.3aa**a** ± 0.6	12.3aa**a** ± 1.2	16.7aa**a** ± 0.6	21.7ab**a** ± 10.7	15.7aa**a** ± 2.5
Mn	6.77bb**b** ± 2.5	3.5bb**c** ± 0.6	20.6ab**a** ± 3.3	22.5 aa**b** ± 2.1	69.3 aa**a** ± 3.0	81.4aa**a** ± 16.9	17.3aa**a** ± 5.3	23.9aa**a** ± 8.1	19.1aa**a** ± 3.4	15.4ba**ab** ± 1.2	10.0 bb**b** ± 0.8	20.8ba**a** ± 0.5
Zn	1.4aa**a** ± 0.6	1.9aa**a** ± 0.3	1.7aa**a** ± 0.9	2.1 aa**a** ± 0.5	2.0 aa**a** ± 0.6	2.2 aa**a** ± 0.2	1.0aa**a** ± 0.2	0.8bb**a** ± 0.2	1.0aa**a** ± 0.1	1.1ba**b** ± 0.3	2.3 aa**a** ± 1.2	1.0ba**b** ± 0.3
pH_{SMP}	7.5aa**a** ± 0.1	7.2aa**ab** ± 0.1	7.0ba**b** ± 0.2	7.2 aa**a** ± 0.1	6.7 bb**b** ± 0.1	6.3 bb**c** ± 0.2	7.2ba**a** ± 0.3	7.5ab**a** ± 0.1	7.4aa**a** ± 0.0	7.4aa**b** ± 0.1	7.8 aa**a** ± 0.3	7.3aa**b** ± 0.1

SOC is expressed in $g.dm^{-3}$; P is expressed in $mg.dm^{-3}$; Ca, Mg, K, Al, potential acidity (H+Al), sum of base (SB), and base saturation (BS) are expressed in $mmol_c.dm^{-3}$; B, Fe, Mn, Zn, K, Cu and cation exchange capacity in pH 7 (T) are expressed in $mg.dm^{-3}$.

[¶]Mean calculated from three replicates.

[§]Standard deviation calculated from three replicates.

Values followed by common letters do not differ significantly ($P<0.05$), based on Tukey's test between contrasted samples. Letters in roman, italic, and bold styles contrast soil classes, textures, and soil management strategies, respectively.

chemical factors, with the dry season generally showing lower means compared to the rainy season, except for K, Fe and H+Al.

3.2 Copy number of the *nir*K, *nir*S, and *nos*Z genes

Overall, the copy number of the *nir*K, *nir*S, and *nos*Z I genes in sugarcane soils, as determined by qPCR (expressed as 10^5 gene copies per gram of soil), was higher during the rainy season compared to the dry season (Table 4). The *nir*K gene was the most abundant in the evaluated samples, followed by *nos*Z I and *nir*S. The *nirS* gene fragment had the lowest abundance across all sampling sites, regardless of the season or agricultural soil management strategy.

None of the genes evaluated revealed a consistent response to different sugarcane soil managements, showing a response pattern for each soil class and texture (sandy Ferralsol: MF < V+FC < V; clayey Ferralsol: V < MF < V+FC; Sandy Acrisol: V+FC < V < MF; clayey Acrisol: MF < V < V+FC).

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TABLE 3 Chemical properties of soil collected during the dry season from the 0-20 cm layer in sandy and clayey Ferralsol and Acrisol from the study sites.

Variable	Ferralsol						Acrisol					
	Sandy			Clayey			Sandy			Clayey		
	V	V+FC	MF	V	V+FC	MF	V	V+FC	MF	V	V+FC	MF
SOC	4.8[¶]*ab***a** ± 0.3[§]	6.0*aa***a** ± 0.9	4.5*ab***a** ± 1.5	8.9*aa***a** ± 1.2	10.7*aa***a** ± 0.9	12.6*aa***a** ± 0.7	6.6*aa***a** ± 0.7	6.8*aa***a** ± 1.5	7.0*aa***a** ± 2.0	4.1*ba***a** ± 0.6	7.0*aa***a** ± 3.4	4.3*ba***a** ± 0.9
pH	6.1*aa***a** ± 0.5	5.8*ba***a** ± 0.2	5.5*ba***a** ± 0.1	6.1*aa***a** ± 0.1	5.3*aa***a** ± 0.3	5.9*aa***a** ± 0.1	6.2*aa***a** ± 0.4	6.6*aa***a** ± 0.7	6.4*aa***a** ± 0.1	5.9*aa***a** ± 0.2	5.8*ab***a** ± 1.1	4.9*ab***b** ± 0.1
P	63.7*aa***a** ± 12.7	62.3*aa***a** ± 6.8	32.7*ba***a** ± 24.7	39.0*aa***b** ± 61	88.0*aa***a** ± 2.9	16.3*aa***b** ± 3.1	48.7*aa***a** ± 11.5	53.0*aa***a** ± 29.0	68.7*aa***a** ± 2.2	67.7*aa***a** ± 31.2	58.3*aa***a** ± 27.0	11.7*ab***b** ± 4.5
K	4.0*aa***a** ± 3.9	4.9*aa***a** ± 0.8	3.6*aa***a** ± 1.2	9.0*ab***a** ± 2.6	5.3*aa***a** ± 9.7	4.2*aa***a** ± 0.4	4.5*aa***a** ± 0.7	6.5*aa***a** ± 1.6	3.2*aa***a** ± 0.5	5.1*aa***a** ± 0.7	6.2*aa***a** ± 0.4	4.0*aa***a** ± 0.9
Ca	72.3*aa***a** ± 34.3	30.0*bb***b** ± 10.6	30.3*bb***b** ± 5.9	46.3*aa***a** ± 47	47.7*aa***a** ± 33.7	56.6*aa***a** ± 3.1	48.7*aa***a** ± 11.9	60.3*aa***a** ± 6.8	55.3*aa***a** ± 7.5	42.7*aa***a** ± 10.1	39.0*aa***a** ± 3.6	21.7*bb***b** ± 2.1
Mg	28.3*aa***a** ± 9.0	12.7*ab***b** ± 2.5	9.7*ab***b** ± 2.5	21.7*aa***a** ± 1.5	19.0*aa***a** ± 5.5	16.7*aa***a** ± 1.5	19.7*aa***a** ± 6.7	21.3*aa***a** ± 4.6	23.0*aa***a** ± 4.0	16.7*aa***a** ± 6.7	16.0*aa***a** ± 3.5	5.0*bb***b** ± 1.0
H+Al	10.0*aa***a** ± 1.7	13.3*aa***a** ± 3.2	14.0*ab***a** ± 2.0	13.3*aa***b** ± 0.6	15.0*aa***b** ± 3.1	29.7*aa***a** ± 1.2	10.0*aa***a** ± 1.0	9.3*ab***a** ± 4.2	10.3*ab***a** ± 1.2	10.7*aa***a** ± 1.5	16.7*aa***a** ± 1.0	15.0*ba***a** ± 12.1
SB	104.7*aa***a** ± 42.2	47.5*ab***b** ± 13.1	43.6*bb***b** ± 6.4	77.1*aa***a** ± 4.9	71.9*aa***a** ± 41.8	77.6*aa***a** ± 3.7	72.8*aa***a** ± 18.5	88.2*aa***a** ± 11.1	81.6*aa***a** ± 12.0	64.4*aa***a** ± 15.9	61.2*aa***a** ± 5.2	30.7*bb***b** ± 1.7
CEC	114.7*aa***a** ± 40.8	60.9*aa***b** ± 10.8	114.7*bb***b** ± 8.4	90.4*aa***a** ± 5.4	86.9*aa***a** ± 39.1	107.3*aa***a** ± 4.7	82.8*aa***a** ± 18.9	97.5*aa***a** ± 12.0	91.9*aa***a** ± 13.0	75.1*aa***a** ± 14.5	77.9*aa***a** ± 11.1	44.7*bb***b** ± 2.7
BS	90.0*aa***a** ± 4.6	77.3*ba***b** ± 8.6	75.7*ba***b** ± 0.6	85.0*aa***a** ± 0.0	82.7*aa***ab** ± 5.5	72.3*aa***b** ± 0.6	87.3*aa***a** ± 2.3	88.3*aa***a** ± 4.7	89.0*aa***a** ± 1.0	85.3*aa***a** ± 0.5	77.3*ab***b** ± 15.0	67.3*ab***b** ± 0.6
B	0.25*ab***a** ± 0.1	0.40*aa***a** ± 0.3	0.30*aa***a** ± 0.2	0.56*aa***a** ± 0.1	0.33*aa***a** ± 0.0	0.57*aa***a** ± 0.4	0.21*aa***a** ± 0.1	0.22*aa***a** ± 0.1	0.24*aa***a** ± 0.1	0.28*aa***a** ± 0.1	0.30*aa***a** ± 0.2	0.30*aa***a** ± 0.1
Cu	1.2*ab***a** ± 0.2	0.57*aa***a** ± 0.3	1.1*ab***a** ± 0.3	5.5 *aa***b** ± 0.9	2.9*aa***b** ± 1.4	13.9*aa***a** ± 0.3	0.8*aa***a** ± 0.1	3.6*aa***a** ± 0.1	1.1*aa***a** ± 0.2	0.7 *ba***a** ± 0.1	3.6*aa***a** ± 0.4	0.53*ba***a** ± 0.2
Fe	26.3*aa***a** ± 6.4	33.7*aa***a** ± 17.6	24.3*aa***a** ± 9.2	21.0*aa***a** ± 1.7	35.7*aa***a** ± 11.4	26.0*aa***a** ± 2.0	28.7*aa***a** ± 12.4	15.7*bb***a** ± 14.6	19.3*aa***a** ± 2.9	21.0*aa***a** ± 1.7	52.7*aa***a** ± 22.9	36.0*aa***a** ± 14.9
Mn	9.8*bb***b** ± 3.0	5.2*bb***c** ± 1.3	19.8*ab***a** ± 3.4	21.8*aa***b** ± 2.6	22.7*aa***b** ± 4.6	47.8*aa***a** ± 2.3	14.3*aa***a** ± 1.0	19.8*aa***a** ± 1.0	13.6*aa***a** ± 1.0	11.8*ba***b** ± 0.8	24.8*aa***a** ± 8.3	17.3*ba***ab** ± 3.2
Zn	1.8*aa***a** ± 0.6	1.7*aa***a** ± 0.9	1.4*aa***a** ± 0.1	1.7*aa***a** ± 0.3	2.6*aa***a** ± 0.6	1.1*aa***a** ± 0.1	1.0*aa***a** ± 0.3	1.3*aa***a** ± 1.3	1.5*aa***a** ± 0.2	1.7*aa***a**0.3	4.4*aa***a** ± 5.1	0.9*ab***b** ± 0.5
pH_{SMP}	7.4*aa***a** ± 0.2	7.1*ba***a** ± 0.2	7.1*aa***a** ± 0.1	7.1*aa***a** ± 0.1	7.0*aa***a** ± 0.2	6.3*bb***b** ± 0.0	7.4*aa***a** ± 0.1	7.5*aa***a** ± 0.3	7.4*aa***a** ± 0.1	7.3*aa***a** ± 0.1	7.1*ab***a** ± 0.7	7.0*ab***a** ± 0.1

SOC is expressed in $g.dm^{-3}$; P is expressed in $mg.dm^{-3}$; Ca, Mg, K, Al, potential acidity (H+Al), sum of base (SB), and base saturation (BS) are expressed in $mmol_c.dm^{-3}$; B, Fe, Mn, Zn, K, Cu and cation exchange capacity in pH 7 (T) are expressed in $mg.dm^{-3}$.

[¶]Mean calculated from three replicates.

[§]Standard deviation calculated from three replicates.

Values followed by common letters do not differ significantly ($P<0.05$), based on Tukey's test between contrasted samples. Letters in roman, italic, and bold styles contrast soil classes, textures, and soil management strategies, respectively.

3.3 Activity of β-glucosidase and arylsulfatase enzymes

In the Ferralsol, the activities of the enzymes GLU and ARYL increased in the following order: V < MF < V+FC, regardless of soil texture (sandy or clayey) and sampling season (Table 5). The average activity of the two enzymes in the V +FC and MF treatments was 1.8 times higher in sandy soil and 3.9 times higher in clayey soil compared to soil managed with vinasse.

In the Acrisol, no significant differences among the treatments were observed (Table 5).

3.4 Correlation between functional genes, soil enzymatic activity, and soil organic matter

Correlation analyses showed negative correlations ($P<0.05$) between the number of copies of the *nir*K and *nos*ZI genes and

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TABLE 4 Copy number of the *nirK*, *nirS*, and *nosZI* genes as determined by quantitative real-time PCR from sugarcane soil samples collected during the rainy and dry seasons.

Functional gene	Ferralsol						Acrisol					
	Sandy			Clayey			Sandy			Clayey		
	V	V+FC	MF	V	V+FC	MF	V	V+FC	MF	V	V+FC	MF
Rainy season												
*nir*K	38.2[¶]a*b***b** ± 3.0[§]	28.1a*a***a** ± 2.5	27.6a*a***a** ± 2.4	24.5a*a***a** ± 2.2	39.4b*b***b** ± 1.2	37.3b*b***b** ± 3.1	35.7a*a***a** ± 5.5	33.9b*b***a** ± 2.3	43.2b*b***a** ± 3.4	21.3a*b***b** ± 3.4	24.6a*a***b** ± 1.7	18.1a*a***a** ± 1.4
*nir*S	0.4a*b***b** ± 0.03	0.3a*a***a** ± 0.005	0.3a*a***a** ± 0.02	0.3a*a***a** ± 0.01	0.4b*b***b** ± 0.02	0.4b*b***b** ± 0.01	0.4a*a***a** ± 0.02	0.4b*a***a** ± 0.01	0.5b*b***b** ± 0.03	0.3a*a***a** ± 0.01	0.3a*a***a** ± 0.03	0.3a*a***a** ± 0.01
*nos*ZI	4.3a*b***b** ± 1.1	2.8a*a***a** ± 0.1	2.6a*a***a** ± 0.2	2.5a*a***a** ± 0.3	3.2a*b***b** ± 0.2	3.2b*b***b** ± 0.1	3.7a*a***a** ± 0.3	3.0a*a***a** ± 0.8	4.7b*b***a** ± 0.9	3.2a*a***a** ± 1.3	3.5a*a***a** ± 0.2	2.7a*a***a** ± 0.2
Dry season												
*nir*K	28.1a*b***b** ± 2.4	18.4a*a***a** ± 1.9	16.3a*a***a** ± 1.5	13.3a*a***a** ± 1.4	18.7b*b***b** ± 0.8	17.2b*b***b** ± 1.9	22.8a*a***a** ± 2.7	16.4b*b***a** ± 1.1	24.9b*b***a** ± 2.0	11.5a*b***b** ± 2.5	13.3a*a***b** ± 0.3	10.8a*a***a** ± 0.1
*nir*S	0.04a*b***a** ± 0.02	0.04b*a***a** ± 0.03	0.4a*a***a** ± 0.01	0.03a*a***a** ± 0.01	0.03b*b***a** ± 0.02	0.3b*b***a** ± 0.01	0.02a*a***a** ± 0.01	0.03b*a***a** ± 0.01	0.3b*b***a** ± 0.2	0.01a*a***a** ± 0.01	0.02a*a***a** ± 0.02	0.02a*a***a** ± 0.01
*nos*ZI	3.5a*b***b** ± 0.9	1.7a*b***a** ± 0.4	1.8a*a***a** ± 0.3	1.3a*a***a** ± 0.1	2.0a*b***b** ± 0.3	2.3b*b***b** ± 0.2	2.8a*b***a** ± 0.1	2.4a*a***a** ± 0.3	3.6b*b***a** ± 0.2	2.3a*a***a** ± 0.1	2.6a*a***a** ± 0.2	1.7a*a***a** ± 0.3

Values are expressed as 10^5 copies of the gene per gram of soil.
[¶]Mean calculated from three replicates.
[§]Standard deviation calculated from three replicates.
Values followed by common letters do not differ significantly ($P<0.05$), based on Tukey's test between contrasted samples. Letters in roman, italic and bold styles contrast soil classes, textures, and soil management strategies respectively.

the activity of the GLU and ARYL enzymes in both seasonal periods analyzed (Figure 2), more notably in the rainy season. These gene copies also negatively correlated ($P<0.05$) with the soil organic carbon content during the rainy season (Figure 2). Conversely, the activity of these enzymes positively correlated with the soil organic carbon content in both the dry and rainy seasons (Figure 2), more notably in the latter.

Pearson's correlation confirmed the negative relationship between the copy number of the *nir*K, *nir*S, and *nos*ZI genes and GLU and ARYL enzyme activity in dry and rainy seasons (Figure 3).

4 Discussion

The sugarcane soils classified as Ferralsol and Acrisol in this study, with clayey and sandy textures, exhibited differences in soil chemical attributes based on the agricultural management, including the application of V, MF and V+FC. Such differences in agricultural management in sugarcane cultivated soils were consistently corroborated by the activity of GLU and ARYL. Since GLU and ARYL are biological indicators of agricultural soil quality (21, 22), it is expected that healthy soils—characterized by high levels of enzyme activity—have a greater potential for greenhouse gas mitigation. In this study, the indications of healthy sugarcane soils based on enzymatic analyses were reinforced by the lower abundance of genes associated with denitrification, indirectly allowing the activity of GLU and ARYL to provide evidence of the potential for N_2O production in the sugarcane soils analyzed.

4.1 Sugarcane soil management practices, seasonality and chemical attributes

This study took into account different sugarcane soil management with organic and mineral fertilizers, physical and chemical characteristics of the soil, and a seasonal sampling design that allowed the evaluation of key variables to find patterns and relationships among chemical, enzymatic and molecular determinations to address the agro-environmental quality of these soils.

The changes in soil chemical properties observed in our sugarcane soils, considering different textures and agricultural management practices, align with findings in tropical soils that reported increased macro- and micronutrient concentrations following vinasse application. This effect may be due to the ability of vinasse, as an organic fertilizer, to modify soil acidity and enhance the dissolution of minerals (48). Additionally, vinasse is known to be rich in organic matter, including organic acids and cations such as K, Mg, and Ca (49), which could account for the observed nutrient increases under this management practice. When applied together, vinasse and filter cake tend to enhance nutrient concentrations and improve the quality of soil chemical properties (50).

Seasonal changes in soil chemical properties varied according to the parameters evaluated and may be associated with several factors, including environmental conditions (such as temperature and rainfall fluctuations that influence soil moisture, leading to either evaporation or leaching), plant growth cycles, and agricultural practices, such as fertilizer application and soil amendments.

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TABLE 5 β-glucosidase and arylsulfatase enzyme activity in sugarcane soils treated with organic and mineral fertilizers during the rainy and dry seasons.

Enzyme activity	Ferralsol						Acrisol					
	Sandy			Clayey			Sandy			Clayey		
	V	V+FC	MF	V	V+FC	MF	V	V+FC	MF	V	V+FC	MF
Rainy season												
β-glucosidase	28.3[¶]aa**a** ± 2.3[$]	50.7ba**b** ± 7.8	46.3ba**b** ± 3.0	37.3a*b***a** ± 7.5	87.7b*b***b** ± 5.1	62.7b*b***b** ± 11.1	25.0aa**a** ± 11.2	25.0aa**a** ± 2.1	18.0aa**a** ± 4.6	32.0aa**a** ± 5.3	32.3a*b***a** ± 1.5	41.3a*b***b** ± 3.5
Arylsulfatase	21.7aa**a** ± 8.0	48.7ba**b** ± 6.6	37.3ba**a** ± 12.7	24.3aa**a** ± 5.0	169.7b*b***b** ± 19.5	166.0b*b***b** ± 33.8	18.0aa**a** ± 7.0	24.7aa**a** ± 3.5	14.0aa**a** ± 4.6	17.0aa**a** ± 2.6	46.3a*b***b** ± 4.2	36.0a*b***b** ± 15.0
Average GLU+ARYL	25	50	42	31	129	114	21	25	16	25	39	39
Dry season												
β-glucosidase	23.7aa**a** ± 1.9	45.5ba**b** ± 5.9	41.1ba**b** ± 2.3	32.6a*b***a** ± 4.4	81.9b*b***b** ± 3.1	57.8b*b***b** ± 8.5	22.3aa**a** ± 9.7	19.4aa**a** ± 1.4	13.0aa**a** ± 2.2	28.4aa**a** ± 3.3	27.7a*b***a** ± 1.1	38.2ba**a** ± 2.9
Arylsulfatase	18.6aa**a** ± 6.2	41.2ba**b** ± 4.6	33.1ba**a** ± 9.8	19.9aa**a** ± 3.7	147.5b*b***b** ± 11.9	138.6b*b***b** ± 27.4	13.4aa**a** ± 5.2	20.3aa**a** ± 2.5	11.4aa**a** ± 2.9	12.4aa**a** ± 1.9	41.4a*b***b** ± 3.8	33.5b*b***b** ± 9.3
Average GLU+ARYL	21	43	37	26	115	98	18	20	12	20	34	36

Values are expressed in milligrams of p-nitrophenol per kilogram of soil per hour.
[¶]Mean calculated from three replicates.
[$]Standard deviation calculated from three replicates.
Values followed by common letters do not differ significantly ($P<0.05$), based on Tukey's test between contrasted samples. Letters in roman, italic and bold styles contrast soil classes, textures, and soil management strategies respectively.

Leinweber et al. (51) pointed out that the mineralization of coarser-sized plant fragments from silt and sand along with enhanced crosslinking within the humic macromolecules appears the best explanation for the clear seasonal variations of SOC between different seasons.

4.2 Denitrification genes abundance

It is essential to understand the sensitivity of the microbial community, which regulates the nitrogen cycle in agroecosystems to changes in environmental conditions and agricultural soil

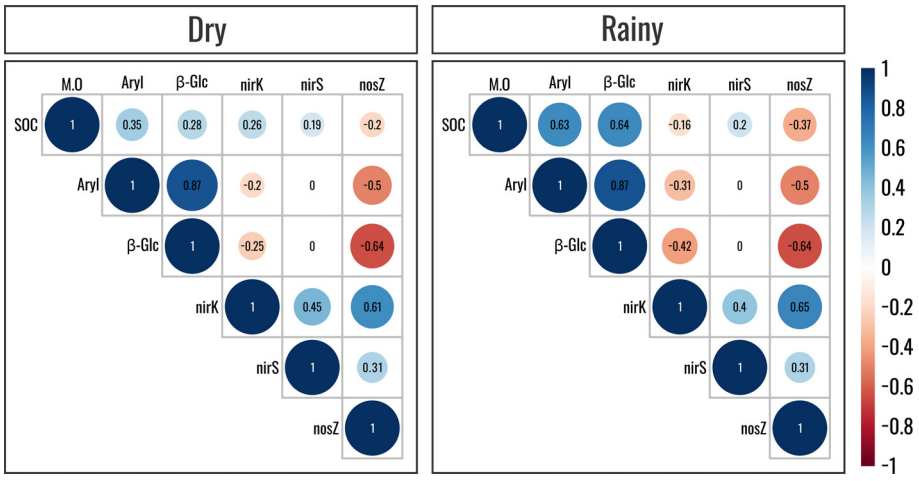

FIGURE 2
Heatmap based on the correlation matrix with soil organic matter content, β-glucosidase and arylsulfatase enzyme activity, and copy number of the *nirK*, *nirS*, and *nosZ*I genes according to significant values ($P<0.05$) of Spearman's correlation coefficient. Map generated within the R environment using the "Hminsk and CorrPlot" package. Cold colors represent positive correlations and warm colors denote negative correlations.

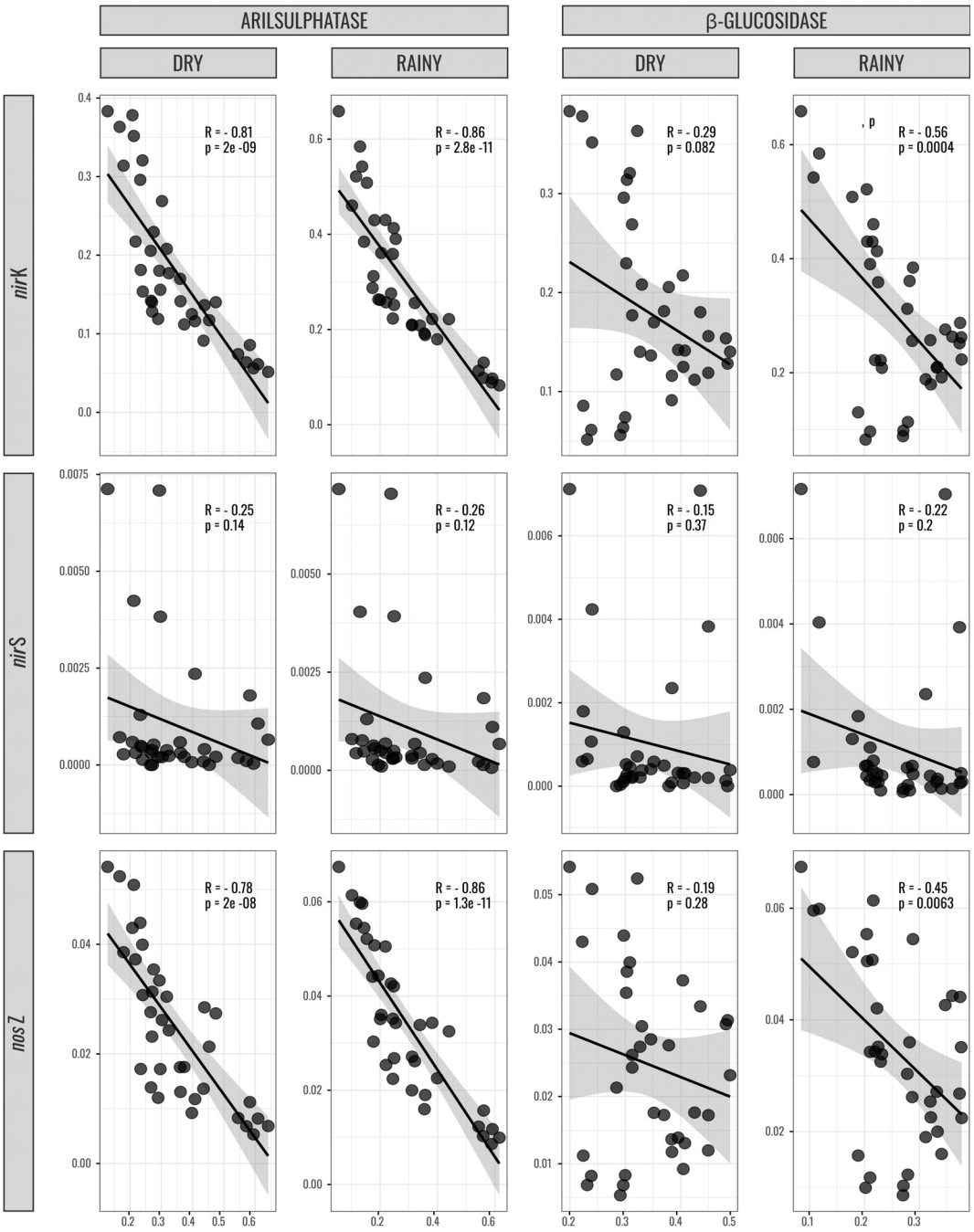

FIGURE 3
Correlation between the copy number of the *nir*K, *nir*S, and *nos*Z I genes and β-glucosidase and arylsulfatase enzyme activity in dry and rainy seasons.

management, especially those related to microbe-mediated emissions of greenhouse gas. Our results for the denitrifying bacterial community revealed by *nir*K, *nir*S, and *nos*Z I genes abundance showed a clear influence of season on the abundance of these genes, with their copy number in sugarcane soils increasing in the rainy season. Nishisaka et al. (32) also showed a positive correlation between rainy season, soil moisture and denitrification gene abundance. High moisture levels in soil decrease oxygen content and, consequently, favor the emergence of anaerobic environments, stimulate denitrification (52).

The relationship between these denitrification gene fertilization-induced changes in functional organic matter fractions and the respective potential denitrification as well as gene abundances have only been rarely addressed. Denitrification in soil mainly occurs in anoxic microhabitats ('hot spots') where enough NO_3^- and C are available (53). Previous studies have shown that addition of well-defined low-molecular weight compounds, such as found in vinasse and filter cake (54, 55), affects denitrification rates, product ratios and denitrifier populations (56–58). The presence of organic compounds in soil affects the

denitrification rate, mainly by increasing the availability of organic carbon, one of the most important factors influencing denitrification (59).

4.3 Soil enzymes activities and their correlations with denitrifying genes and soil chemical properties

Soil microorganisms, extracellular enzymes, and soil organic matter interact in a dynamic and complex manner, with these interactions being influenced by factors such as soil type and agricultural management. This complexity drives many studies about the effects of agricultural practices on soil quality and microbial properties (60). Soil enzymes offer a practical and informative means of evaluating soil quality, providing valuable insights into soil health and management impacts through their sensitivity (61–63), association with soil functions, and ease of measurement (63, 64).

Soil enzyme activity is directly linked to nutrient cycling, the decomposition of organic residues, mineralization (65). Therefore, increased enzymatic activity can indicate trends in soil organic matter accumulation and efficient cycling of C, N, P, and S (66, 67). Fertigation with vinasse in Acrisol is known to enhance mineralization due to its high nutrient and organic matter content (68). Filter cake, on the other hand, can increase the levels of both macro- and micronutrients in the soil (69). When applied together, vinasse and filter cake tend to boost soil nutrient concentrations (50). In turn, low activity of GLU and ARYL was observed in our sugarcane soils amended with vinasse. According to Braga et al. (70), the vinasse application to the soil interferes with competition among microorganisms inhabiting the soil, as the antimicrobial compounds present in the vinasse, such as sulfuric acid and biocides (71), may select some more resistant microbial groups in the soil over others, influencing microbial community composition and activity. This effect may have been responsible for lower soil enzymatic activity in our sugarcane soils amended with vinasse.

Several studies have shown that GLU and ARYL are soil enzymes of particular interest (22, 61, 72). β-Glucosidases are carboxyhidrolases involved in the conversion of cellobiose to glucose, a major source of energy for soil microbial communities (73). Arylsulfatases are enzymes of the esterase class involved in the hydrolysis of ester sulfates to sulfate, a major sulfur source for plant uptake (74). β-glucosidase activity has often been reported to be very sensitive to management practices (75, 76).

In this study, GLU and ARYL activities were positively correlated with soil organic carbon. Decomposition of soil organic matter depends on extracellular enzymes, produced by microorganisms. Microbes exude enzymes to acquire C or limiting nutrients (77), and to target the most abundant substrates (78). Extracellular enzyme activities are therefore often related to the soil organic matter and its C and N content (79, 80). Regarding the relationships of GLU and ARYL with N cycling, our study revealed negative correlation between the activity of these soil enzymes with denitrifying genes abundance in our sugarcane soils. This finding is unprecedented and it opens the possibilities to denitrifying microbial genes quantification to indicate potential N_2O emissions in these soils by soil enzymes determination. The findings of this study reinforce the sensitivity of enzymatic bioanalysis based on GLU and ARYL for soil quality and expand the potential of its interpretation for environmental quality regarding N_2O emission potential by sugarcane soils managed with organic and inorganic fertilizers. Future studies with simultaneous measurements of N_2O emissions, GLU and ARYL activities and quantification of denitrifying genes under field conditions are necessary to support these findings.

5 Conclusions

The results obtained from sandy and clayey Ferrosol and Acrisol cultivated with sugarcane provide evidence that the activity of GLU and ARYL are relevant parameters in studies for assessing the impact of agricultural management practices on soil quality, including the application of vinasse, vinasse combined with filter cake, and mineral fertilizer. The ARYL activity indicated an increase in soil quality in the following order in our study areas: vinasse < mineral fertilizer < vinasse combined with filter cake. Denitrifying genes abundance was negatively correlated with the activity of GLU and ARYL in both sampling seasons evaluated (dry and rainy) in the sugarcane soils. Since these two soil enzymes are biological indicators of soil health, this finding adds more evidence to the greater potential for mitigation of greenhouse gases by healthy soils. More studies are necessary to validate these findings using simultaneous *in situ* measurements of N_2O emissions, GLU and ARYL activities and quantification of denitrifying genes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

LO: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. DK-A: Writing – original draft. GM: Formal analysis, Visualization, Writing – original draft. IM: Formal analysis, Methodology, Visualization, Writing – original draft, Validation. RR: Conceptualization, Methodology, Writing – original draft. LV: Conceptualization, Writing – original draft. GH: Conceptualization, Writing – original draft. AN: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Validation.

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Conflict of interest

Author IM was employed by the company Brazilian Agricultural Research Corporation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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