

**University of São Paulo
"Luiz de Queiroz" College of Agriculture**

**Fungal community structure and functional genes abundance in Brazilian
drylands under desertification process**

Danilo Ferreira da Silva

Dissertation presented to obtain the degree of Master in
Science: Area: Soil and Plant Nutrition

**Piracicaba
2023**

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Tell me and I forget. Teach me and I remember. Involve me and I learn.

~ Benjamin Franklin

*We have nothing to lose, nothing to gain, nothing we desired anymore,
except to make our lives into a work of art.*

~ Lana Del Rey

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RESUMO

Estrutura da comunidade de fungos e abundância de genes funcionais em terras secas do Brasil sob processo de desertificação

A desertificação em terras secas causa o esgotamento dos recursos naturais. A degradação altera processos ecológicos que ocorrem naturalmente no solo. Essa degradação se deve em grande parte a alterações nas comunidades microbianas presentes no ecossistema solo-planta-atmosfera. Uma das principais comunidades responsáveis pela manutenção do equilíbrio biológico do solo são as comunidades fúngicas, que participam de inúmeros processos, como ciclagem de nutrientes, decomposição de matéria orgânica, processos de solubilização por ácidos orgânicos, transporte de nutrientes e água. No entanto, estudos que relatam as consequências do processo de desertificação em comunidades de fungos e acessam funções ecológicas são escassos. Neste sentido, esse estudo foi realizado no núcleo de desertificação de Irauçuba, estado do Ceará, Brasil, onde foram implantadas 9 áreas de restauração por exclusão do sobrepastejo de animais em 2002. O objetivo deste estudo foi descrever a modulação das comunidades fúngicas do solo, assim como acessar genes funcionais relacionados aos ciclos de nitrogênio e fósforo em um gradiente de desertificação. Dois capítulos foram elaborados, o primeiro mostrando que o sobrepastoreio altera a estrutura da comunidade fúngica e que a exclusão do pastoreio é eficaz na restauração dessa comunidade. Enquanto o segundo acessa genes funcionais, mostrando que o processo de desertificação reduz a abundância de genes relacionados aos ciclos de N e P enquanto a exclusão foi capaz de mitigar em partes essas perdas. Assim, demos um passo rumo a uma fundamentação teórica que busca uma estratégia eficiente no combate à desertificação das terras secas.

Palavras-chave: Bioma Caatinga, Processos ecológicos, Comunidades microbianas, Ciclagem de N e P, Sustentabilidade ambiental

ABSTRACT

Fungal community structure and functional genes abundance in Brazilian drylands under desertification process

Desertification in drylands causes the depletion of the soil's natural resources. Degradation alters all ecological processes that occur naturally in soil. This degradation is largely due to changes in the microbial communities present in this complex soil-plant-atmosphere ecosystem. One of the main communities responsible for maintaining the biological balance of the soil is the fungal communities, which participate in numerous processes, such as nutrient cycling, decomposition of organic matter, solubilization processes by organic acids, transport of nutrients and water. However, studies that report the consequences of the desertification process on soil fungal communities and access to specific ecological functions are scarce, especially in the Caatinga biome in Brazil, one of the most populous semi-arid region in the world. The study was conducted in the desertification area of Irauçuba, state of Ceará, Brazil, where nine restoration areas were established to prevent overgrazing by animals in 2002. The aim of this study was to describe the modulation of the soil fungal communities as well as the abundance of functional genes related to nitrogen and phosphorus cycles in a desertification gradient. Two chapters were considered, the first showing that overgrazing alters fungal community structure and that grazing exclusion is effective in restoring this community. Whilst the second shed light on the functional genes, showing that the desertification process reduces the abundance of genes related to N and P cycles while grazing exclusion could recover. Therefore, we took an important step towards a theoretical foundation that seeks an efficient strategy in combating the desertification of drylands.

Keywords: Caatinga biome, Ecological processes, Microbial communities, N and P cycling, Environmental sustainability

1. INTRODUCTION

The semiarid Caatinga biome is unique to Brazil, covering ~10% of the country's territory and supporting a population of ~26 million (Silva et al., 2017). Importantly, it presents a high biodiversity of plants and animals (Albuquerque et al., 2012), including microorganisms (Pereira et al., 2020, Silva et al., 2022), which are essential to Caatinga biome maintenance. Although important, this biodiversity is threatened by deforestation, unsuitable soil management, and overgrazing of native vegetation (Souza et al., 2021; Almeida et al., 2017; Oliveira Filho et al., 2019). Between 1985 and 2020, an estimated more than 6 million hectares of native Caatinga vegetation were lost, mainly to livestock activities (MapBiomas, 2022), and it has brought adverse effects on plant diversity in this biome (Schulz et al., 2019). In addition to its impacts aboveground, this widespread vegetation loss is causing degradation due to soil erosion, which promotes losses of soil organic matter (Fraga and Salcedo, 2004; Oliveira-Filho et al., 2019) and bacterial diversity (Pereira et al., 2021).

To reduce and restore this high degradation in Brazilian semiarid regions, the Federal Government established the National Policy to Combat Desertification and Mitigate the Effects of Drought (Brazil, 2015). A grazing-exclusion strategy has been used in the Brazilian semiarid to reduce and restore degraded areas (Araujo et al., 2022) by promoting natural soil fertility and microbial diversity. The experiment was initiated in 2000 (Oliveira et al., 2021)). However, whether the desertification process and this restoration strategy (grazing exclusion) respond to soil functioning and fungal community structure is unclear.

Therefore, this dissertation consists of two chapters structured to accommodate two papers. This first chapter examines how desertification modulated the soil fungal community. In this study, it was possible to notice that the soil desertification in the Caatinga biome seriously affects the diversity and composition of soil fungal communities. On the other hand, grazing exclusion has been shown to present potential in recovering the diversity and composition of soil fungal communities, especially for mycorrhizal, by altering soil chemistry and biological parameters and the relationships among dominant fungal taxa. In the second chapter, we show how desertification alters the abundance of functional genes in the nitrogen and phosphorus cycles. Our findings suggest that degraded soils have lower copies of the quantified genes, indicating a microbiological limitation in N and P. This study also demonstrated that long-term grazing exclusion might effectively restore the ecological functions of N and P cycles in the Caatinga biome. This finding is particularly relevant for managing and conserving this unique and fragile ecosystem.

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2. FUNGAL COMMUNITY STRUCTURE IN A DESERTIFICATION GRADIENT IN THE CAATINGA BIOME

Abstract

Desertification is the most serious worldwide ecological problem in arid and semiarid climate environments. The decrease in the soil's productive capacity caused by desertification is due to the loss of ecological processes carried out by microbial communities, which are essential for the maintenance of life in this environment. Fungi are one of the main microbial communities in the soil, as they act on the decomposition of organic matter and the availability and transport of nutrients and water for plants. However, it is still unclear how desertification in semiarid regions affects the structure of the fungal community, especially how grazing exclusion reduces the negative effects of overgrazing on the microbial community in the Caatinga biome. In our study, we assessed the fungal community structure in areas of natural vegetation (native), 2. grazing exclusion (restored), and degradation by overgrazing (degraded), at 0-10 cm soil depth in two seasons (dry and rainy). The fungal communities were assessed by sequencing the internal transcribed spacer (ITS) for the total fungal community and the small-subunit ribosomal region for the mycorrhizal fungal community (MF). This study is one of the first to report that the MF families Acaulosporaceae and Glomeraceae should be included as crucial groups to mitigate climate change in dryland soils and also that the orders Pleosporales and Capnodiales are the most abundant in soil total fungal communities in the Caatinga biome. Here, we report that desertification severely modifies soil fungal communities, and grazing exclusion potentially improves soil properties and recovers communities under climate change globally.

Keywords: Mycorrhizal symbiosis, Microbial communities, Soil degradation, Semiarid.

Introduction

About 40% of the Earth's surface comprises drylands, including arid and semiarid habitats (Nickayin et al., 2022). However, in many of these ecosystems, the combined impact of natural and human activities accelerated soil degradation and desertification (Araujo et al., 2022). Globally, 1.9 billion hectares and 1.5 billion people are affected by land degradation brought on by drought and desertification (Albuquerque et al., 2020). Due to its geological and climatic environmental circumstances, the 1.2 million km² Brazilian semiarid shows a vast area with soils highly vulnerable to desertification. Additionally, improper land use practices carried out by humans have hastened the development of desertification (CGEE, 2016).

Overgrazing of native vegetation is one of the many factors contributing to desertification, and it has had a detrimental impact on the soil health (Feltran-Barbieri and F'eres, 2021; Vieira et al., 2021). Overgrazing can reduce plant cover and, consequently, causes soil erosion, depletes nutrients, and reduces soil organic matter (SOM) (Oliveira Filho et al., 2019). As an impact, the soil microorganisms are affected, and their activities are

impaired (Pereira et al., 2021). Recent research has shown that overgrazing considerably worsens soil desertification in the semi-arid region of Brazil by lowering the diversity, richness, and functions of the soil bacterial community (Oliveira et al., 2021; Pereira et al., 2021).

The loss of soil ecological functions performed by microbial communities, essential for the survival of life in the soil in places with some aridity, is mainly responsible for reduce the soil's productive capacity. Through soil quality indicators, which evaluate a wide range of properties of interest, evaluating biological activity is becoming routine (Mendes et al., 2020). In this sense, some enzymes' activity has been used to access functions in the soil in a simple and economically viable way. Furthermore, when correlated to the biogeochemical cycles of their respective target nutrients, the enzymatic activity is an excellent tool for assessing soil health.

Most soil enzymes come from microbial biomass. On average, 80% of soil microbial biomass comprises fungi (Joergensen; Emmerling, 2006). The soil fungal community represents one of the most diverse assemblages of organisms on Earth. They are vital to ecological and biogeochemical processes and live in various ecological niches (Dix and Webster, 2012), such as decomposing organic matter, carbon storage, and transporting nutrients and water to plants. Taxonomically, they are mainly grouped within the nine phyla – Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Basidiomycota and Ascomycota (Naranjo-Ortiz and Gabaldón, 2019).

Arbuscular mycorrhizal fungi (AMF), Glomeromycota phylum, play several vital roles in soil services (Genre et al., 2020) and plant growth (Silva et al., 2023), including improved plant nutrition (Averill et al., 2019; Silva et al., 2023) and water absorption (Zhang et al., 2018), protection against pathogens (Wehner et al., 2011) and amelioration of heavy metal contamination (Garcia et al., 2020). Especially in degraded lands, AMF has been recognized to be a sensitive indicator of both degradation and restoration, affecting soil health (Vasar et al., 2021). In addition, AMF groups can play an essential role in mitigating the ongoing climatic changes worldwide (Duarte and Maherali, 2022). In contrast, some restoration practices, such as grazing exclusion, may effectively mitigate the negative impacts of overgrazing (Pereira et al., 2021).

The results of previous studies in the Brazilian semi-arid have shown that grazing exclusion can improve soil properties in the long-term, increasing the levels of soil organic matter and nutrients (Oliveira Filho et al., 2019), as well as recovering bacterial diversity and

richness (Pereira et al., 2021). Although the grazing exclusion can effectively restore soil properties worldwide (Liu et al., 2020; Xun et al., 2018; Zhang et al., 2021). Recent findings from classical taxonomy of AMF spores in the semi-arid region of Brazil provided evidence that this community had been severely impacted by desertification and had negative diversity parameters compared to native areas. However, it also demonstrated that grazing exclusion could restore the community's diversity parameters (Silva et al., 2021).

Knowledge of the dynamics and composition of fungal communities, correlated to other bioindicators of soil quality in regions with a semi-arid climate, still needs to be widespread, especially in the Brazilian Northeast (Caatinga biome). This study hypothesized that overgrazing alters fungal community structure and that grazing exclusion is effective in restoring this community. Therefore, we analyzed the mycorrhizal and total fungal communities based on the small-subunit ribosomal region and ITS region sequencing of soils under desertification (overgrazing) and restoration (twenty years of exclusion of pastures) in the Brazilian semi-arid region.

Materials and Methods

Location, climate, and soil classification

The study was conducted in the municipality of Irauçuba, Ceará, Brazil (3°44' 46 "S e 39°47'00" W) (**Figure 1**). The average per year in the last 22 years was 454 mm, mainly between January and May (**Figure 2**). The climate in this region is hot semiarid (BSh) (Köppen's classification system), with mean temperature ranging from 26°C to 28°C (Alvares et al., 2013). The soil was classified as Planosols accordingly WRB/FAO (Oliveira Filho et al., 2019).

Study locations

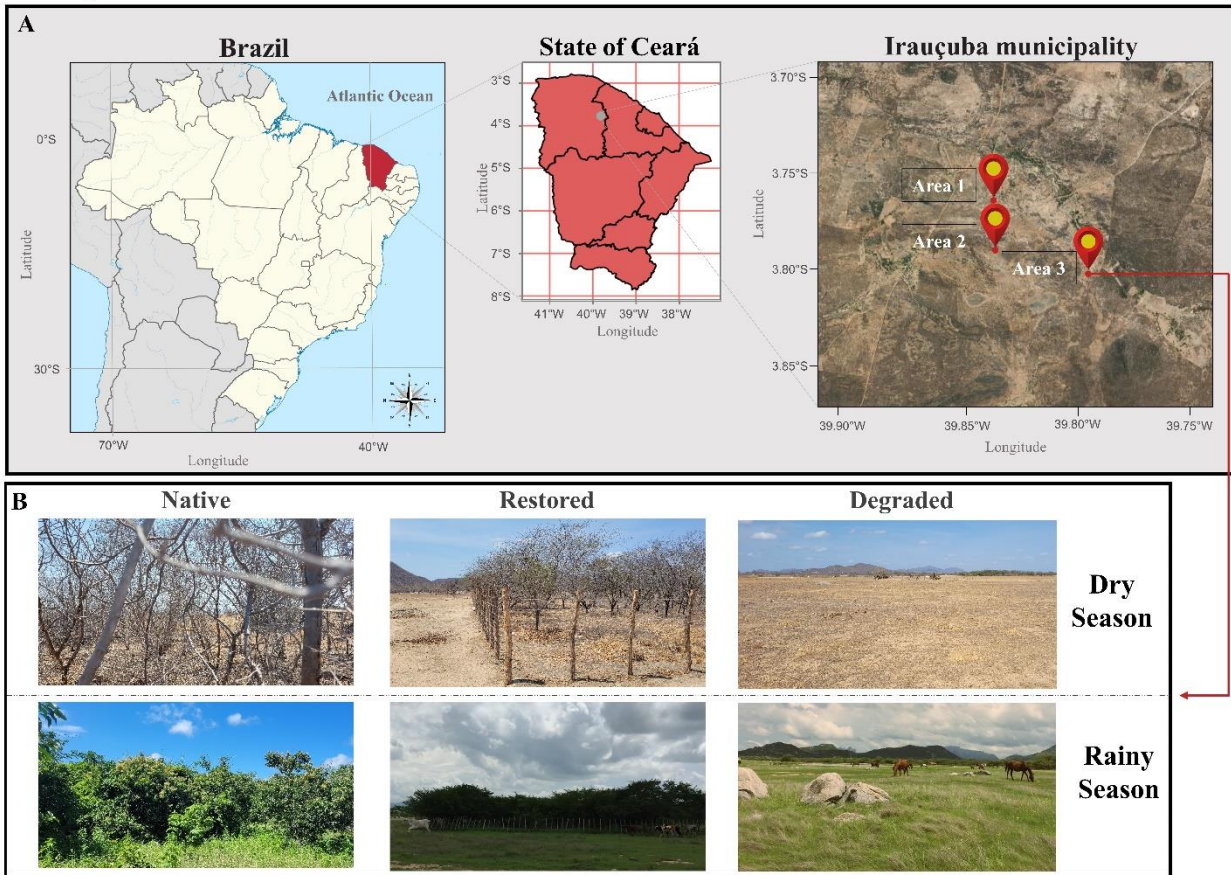


Figure 1. A - Areas 1, 2, and 3 of soil sampling in Irauçuba Municipality, Ceará State, Brazil. B - Caatinga native vegetation, degraded areas due to overgrazing, and restored areas due to grazing exclusion during the dry and rainy seasons of Area 3.

Precipitation in Irauçuba, Brazil

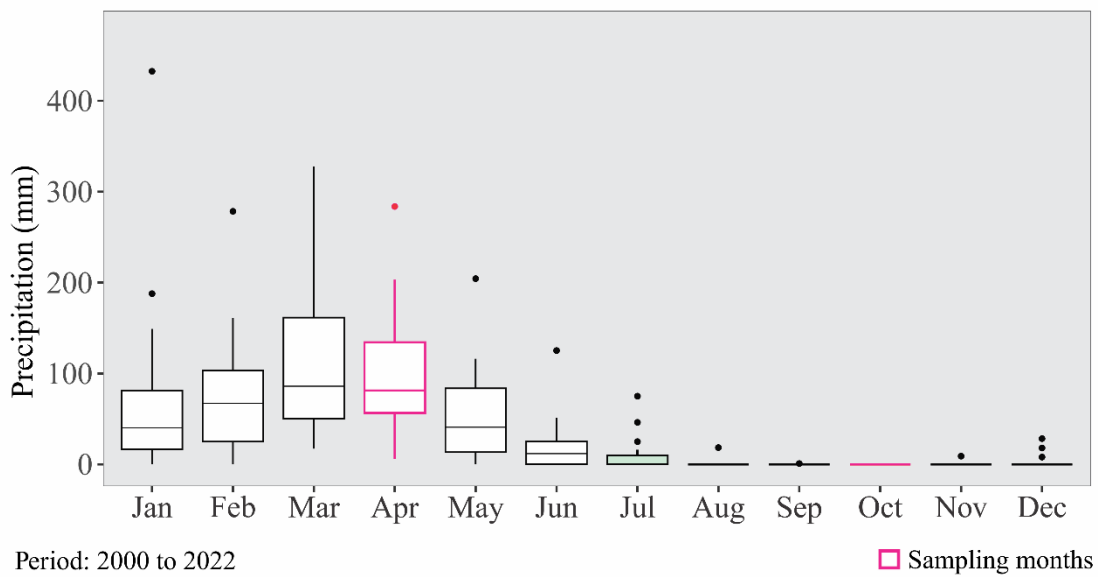


Figure 2. Monthly averages of precipitation for the last 22 years in Irauçuba, Brazil. Sampling periods are highlighted in pink color (i.e., April and October).

The use of natural pasture is a frequent practice by farmers in this region. However, it has been improperly used in an overgrazing system, contributing to the intensification of soil desertification in large areas of the Caatinga biome for the last 50 years. In 2000, grazing-exclusion systems were implemented in 50 m x 50 m (2,500 m²) areas, separated by fences to avoid animal grazing (**Figure 3 - D**). To avoid edge effects, we sampled in an area of 40 m x 40 m.

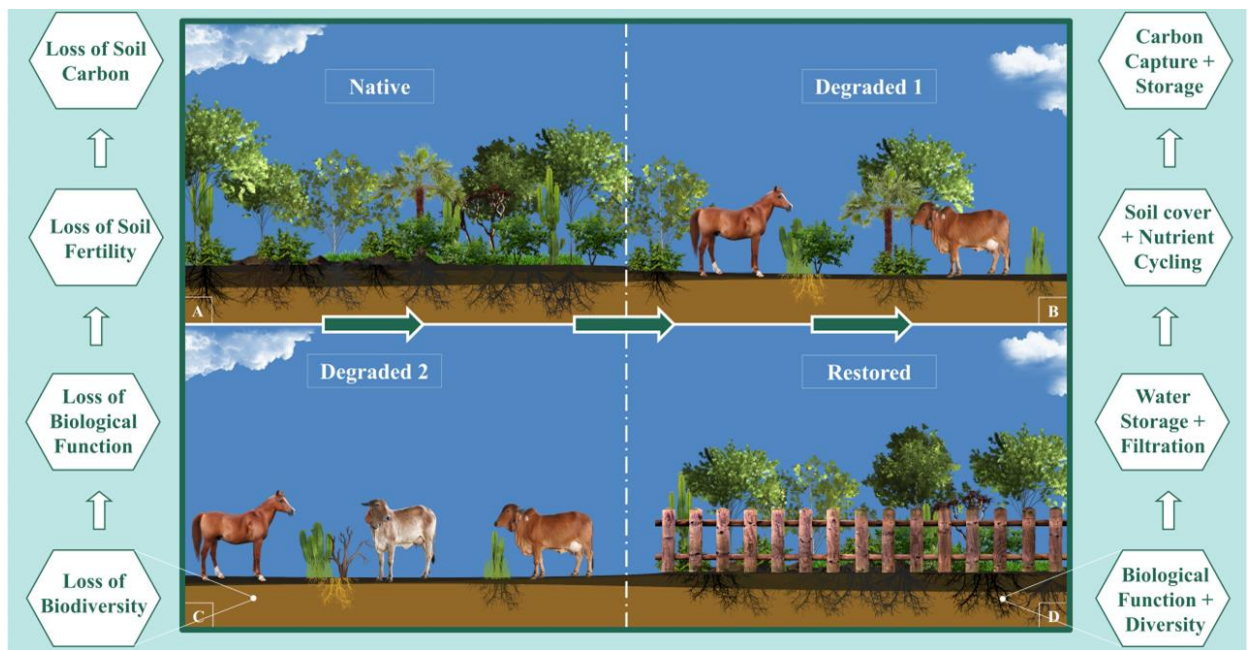


Figure 3. Representation of native Caatinga vegetation (A - Native), soil degradation through overgrazing (B and C - Degraded) and 20 years of grazing exclusion (D – Restored).

We collected soil samples in the dry season (Oct/2021) and rainy season (Apr/22) in three different scenarios, as follows: native Caatinga vegetation (Native), 20 years of grazing exclusion (Restored), and areas in desertification process (Degraded). These three scenarios were repeated in three large areas, separated by ~ 2 km. At each sampling point, we collected nine sub-samples, which were homogenized to create a composite sample. Thus, we analyzed 54 samples (three areas, three scenarios, three composite replicates, and two seasons). The experimental design was detailed extensively by Pereira et al. (2021) and Silva et al. (2022).

Soil chemical and physical characterization

The extraction methods for the nitrogen forms (total N, NH₄⁺, NO₃⁻) were made by the steam distillation method proposed by Kjeldahl method (Nelson and Sommers, 1982; Vezzani et al., 2001; Freitas et al., 2013). Phosphorus (P) was extracted through the ion exchange resin

and pH in 0.01 mol L⁻¹ CaCl₂ solution, following van Raij et al. (2001). Total organic carbon (TOC) was extracted using carbon oxidation in organic form with potassium dichromate (K₂Cr₂O₇) and determined by colorimetry. The NH₄⁺ and NO₃⁻ fractions were determined following the aerobic incubation method proposed by Hart et al. (1994). Soil (Aluminum [Al₃⁺], calcium [Ca₂⁺], and magnesium [Mg₂⁺]) was extracted using 1 mol L⁻¹ KCl solution, while sodium (Na⁺) was extracted by Mehlich-1 solution (EMBRAPA, 2009). Electrical conductivity (EC) was read in an electrical conductivity meter. Cation exchange capacity (CEC) was determined following the methods described by Raij et al. (2001) for tropical soils. A pipette method was used to measure the clay fraction (Gee and Bauder, 1986), sieving was used to measure the sand fraction, and the silt fraction was determined by subtracting the total mass from the sand and clay fractions. A gravimetric method with oven drying was used to determine soil moisture, in which wet soil samples were weighed and then dried in an oven at 105°C until they reached a constant mass. The samples were weighed again in the following step, and the mass difference represented the water mass. Soil characterization can be found in Table 1.

Microbial activity (soil enzymes, glomalin, microbial biomass carbon, respiration and *q*CO₂)

Enzyme activities, i.e., β-glucosidase (BG), urease (U), and acid phosphatase (AP), were determined according to consolidated methods. Briefly, the β-glucosidase (EC 3.2.1.21) activity was measured using *p*-nitrophenyl β-glucopyranoside as substrate under incubation (1 h, 37° C) in a modified buffer adjusted to pH 6.0. The *p*-nitrophenol form was determined spectrophotometrically at 410 nm (Eivazi and Tabatabai, 1988).

The activity of acid phosphatase (EC 3.1.3.2) was measured using disodium *p*-nitrophenyl phosphate as substrate under incubation (1 h, 37° C) in a modified universal buffer adjusted to pH 6.5. The amount of *p*-nitrophenol formed was measured spectrophotometrically at 420 nm (Tabatabai and Bremner, 1969). The urease (EC 3.5.1.5) activity was determined using the method of Kandeler and Gerber (1988) with urea as substrate under incubation (1 h, 37° C). The amount of ammonium (NH₄⁺-N) produced was determined using the Kjeldahl method (Pereira et al., 2018).

Glomalin was extracted following the Wu et al. (2014) and Wright and Upadhyaya (1998) procedures. Briefly, soil samples (1 g) were incubated with 8 mL of 20 mM sodium citrate (C₆H₅Na₃O₇·2 H₂O) buffer solution (pH 7.0) at 120 °C for 30 min and later centrifuged at 2800 RCF for 15 min. The supernatant was used for glomalin determination, which was

analyzed in a spectrophotometer (595 nm) according to Bradford (1976), and contents were estimated by using bovine serum albumin as a standard curve (He et al., 2020).

Microbial biomass carbon (MBC) was determined by the chloroform fumigation-extraction method, where 10 g of soil from each sample was fumigated with chloroform (99.8%) and digested with potassium dichromate for 30 min at 100 °C. Extracts were measured by titration using iron sulfate and ammoniacal ferrous sulfate (Brookes et al., 1985; Vance et al. 1987). Soil respiration was estimated by quantification of CO₂-C released during 31 days of incubation at 28°C. Briefly, soil samples (100 g) were incubated in hermetically sealed flasks containing a (0.5 M) bottle trap with 0.5 M NaOH. CO₂-C emitted was estimated by titration using 0.5 M KCl (Cardoso et al., 2013). In both assays, soil moisture was maintained at 60% of the water-holding capacity (WHC). The qCO₂ was calculated from basal respiration (CO₂-C·h⁻¹) per unit microbial biomass carbon (MBC).

DNA extraction and Illumina sequencing

Genomic DNA was extracted from each sample using 0.25 g soil with the DNeasy PowerSoil Pro Kit (QIAGEN, DE, US), following the manufacturer's protocols.

The ITS amplicon libraries of the total fungal community were prepared following the ITS sequencing library protocol (Illumina®, San Diego, CA, USA). PCR was performed with DNA extracted from soil (~ 50 ng) with ITS1 (CTTGGTCATTTAGAGGAAGTAA) and reverse primer ITS2 (GCTGCGTTCTTCATCGATGC) (5.0 µL) (Smith and Peay, 2014). The KAPA 2x HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) was used for the PCR reaction, and the AMPure XP kit was used for the purification reactions. Negative and positive controls will be introduced in all amplification procedures. From the amplified products, they were sequenced by the MiSeq sequencing platform (Illumina®) with the V3 kit (600 cycles) and paired approach (2 × 250 bp) following Illumina® sequencing protocols.

To optimize the sequencing of the mycorrhizal fungal communities, a nested reaction was performed to increase the sequencing effectiveness (Van Geel et al., 2014). The first PCR reaction was performed with primers NS31 (TTGGAGGGCAAGTCTGGTGCC) (Simon et al., 1992) and reverse AML2 (GAACCCAAACACTTTGGTTTCC) (Lee et al., 2008), a region specific for this microbial classification within the small-subunit ribosomal region. For the second reaction, the primers used were AMV4.5NF (CGAAATTCAACTACGAGCTT) and AMDGR (ATGATTAATAGGGATAGTTGGG) (Sato et al., 2005). In the second set of primers, pre-adapters were inserted for subsequent ligation of the barcodes. The obtained

material was submitted to standard sequencing on the MiSeq platform (Illumina®) with the V3 kit.

Data processing and Statistical analyses

The variance homogeneity and normality were examined for our quantitative results using the Breusch–Pagan and Shapiro–Wilks tests. We then used nested ANOVA and compared groups of means with Tukey's test ($p < 0.05$).

We used RStudio software for bioinformatic analyses. The reads were filtered by quality, and chimeric sequences were removed. Here, we used the high-resolution DADA2 pipeline to infer sequence variants without any fixed threshold, thereby resolving variants that differ by as little as one nucleotide (Callahan et al., 2016). Each ASV (99% identity) was taxonomically classified based on SILVA's ribosomal database-138 (Quast et al., 2013). Shannon's diversity index was calculated as an alpha diversity metric. A Principal coordinates analysis (PCoA) using Bray-Curtis was applied to study the distance of treatments and seasons communities after 1,000 permutations. A differential abundance analysis (DAA) was performed, and taxonomic groups from native and restored sequencing data were compared to degraded data ($p < 0.05$) as the rainy season was compared to the dry season. Log₂ changes were used in the DAA for Order level and Family level taxonomic ranks in the land variable treatment and seasons, respectively, i.e., $\log_2(\text{native}) - \log_2(\text{degraded})$. Using the *rwantshue* of the *devtools* packages, we made a plot composition graph based on relative abundance data. Furthermore, we used a redundancy analysis (RDA) to assess correlations between fungal communities' structure and soil attributes.

Results

Soil pH was generally acid (ranging 4 to 5), with degraded areas more acidic ($p < 0.05$) than restored and native areas in dry and rainy seasons. Soil organic carbon (SOC), Ca^{2+} , and EC were higher in both native and restored, independently of seasons (dry and rainy). During the dry season, the restored area showed higher P contents compared with native and degraded. However, native showed the highest P content than other treatments during the rainy season. The highest N (N) content was found in native, while restored treatment had intermediate levels, and degraded treatment had the lowest. There was a seasonal difference in restored and degraded treatments, with higher N content in the dry season. NO_3^- was found in higher levels in the native treatment, intermediate in restored, and lower in degraded during the dry season. There was no difference in NO_3^- contents between

treatments during the rainy season, and there was no seasonal difference for any of the treatments. NH_4^+ had lower levels in the degraded treatment compared to the other treatments and showed seasonal differences in the native and degraded treatments, with higher contents in the dry season.

The Na^+ content was lower in native (dry) and showed no significant difference between other treatments or seasons. In all treatments, moisture was higher during the rainy season but degraded presented the lowest values. PST increased more in degraded, independent of seasons. The Al^{3+} and Mg^{2+} contents showed no effect of treatments (soil management) or time (seasons) (**Table 1**). MBC was higher in native and restored, mainly in the rainy season. Glomalin was higher in native compared to degraded; the restored areas showed intermediate results in the dry season, and there was no difference in the rainy season. The metabolic quotient ($q\text{CO}_2$) was higher in the dry season in all treatments, in this season, degraded was higher when compared to native, and restored showed intermediate results. While in the rainy season, native and restored were higher than degraded.

Table 1. Soil characterization in Brazilian semiarid soils under Native Caatinga vegetation, Degraded system by overgrazing and Restored area by grazing-exclusion management during dry and rainy seasons.

	Native		Degraded		Restored	
	Dry	Rainy	Dry	Rainy	Dry	Rainy
pH	5.64 Aa*	5.48 Aa	4.62 Ba	4.75 Ba	4.84 Aa	4.67 Ba
P (mg dm^{-3})	11.44 Bb	26.72 Aa	10.44 Ba	10.90 Ba	25.61 Aa	11.77 Bb
Ca^{2+} ($\text{mmol}_c \text{dm}^{-3}$)	74.67 Aa	63.81 Aa	22.16 Ba	21.41 Ba	59.22 Aa	41.68 Aa
SOC (g dm^{-3})	35.99 Aa	35.98 Aa	15.40 Ba	12.79 Ca	44.71 Aa	28.33 Bb
N (mg dm^{-3})	3.5 Aa	3.12 Aa	1.58 Ca	1.26 Cb	2.48 Ba	2.09 Bb
NO_3^- ($\text{mmol}_c \text{dm}^{-3}$)	0.38 Aa	0.28 Aa	0.25 Ba	0.26 Aa	0.30 ABa	0.29 Aa
NH_4^+ ($\text{mmol}_c \text{dm}^{-3}$)	0.31 Aa	0.26 Ab	0.29 Aa	0.24 Bb	0.29 Aa	0.30 Aa
Na^+ ($\text{mmol}_c \text{dm}^{-3}$)	1.02 Ba	2.49 Aa	2.92 Aa	3.38 Aa	2.28 Aa	1.57 Aa
Al^{3+} ($\text{mmol}_c \text{dm}^{-3}$)	0.61 Aa	2.94 Aa	2.26 Aa	2.30 Aa	0.76 Aa	0.99 Aa
EC ($\text{mmol}_c \text{dm}^{-3}$)	106.99 Aa	127.04 Aa	47.08 Bb	73.14 Bb	90.53 Aa	116.43 Aa
Moisture (%)	1.55 Ab	7.28 Aa	0.75 Ab	4.28 Ba	1.16 Ab	6.14 Aa
PST (%)	2.11 Ba	11.78 Ba	3.89 Ab	20.78 Aa	1.44 Ba	1.44 Ba
MBC (mg C g^{-1})	6.79 Aa	7.55 Aa	4.47 Aa	5.06 Ba	7.35 Aa	8.23 Aa
Glomalin (mg g^{-1})	117.67 Aa	99.27 Aa	71.57 Ba	73.17 Aa	90.28 ABa	91.77 Aa
$q\text{CO}_2$	29.01 Ba	17.32 Ab	51.52 Aa	9.66 Bb	39.87 ABa	16.05 Ab

*Means followed by the same letter do not differ by Tukey (5%). The capital letters in the line indicate how soil management differs within each season; the lowercase letters indicate how soil management differs within seasons.

C- and N-acquiring enzyme levels were similar in both native and restored soils during both seasons. However, C- and P-acquiring enzyme levels in degraded soil were higher during the rainy season. The content of the N-acquiring enzyme did not vary between seasons. During the dry season, both native and restored soils had similar and higher levels of P-

acquiring enzyme compared to degraded soil. Additionally, the restored soil had higher levels of P-acquiring enzyme during the rainy season than the degraded soil (**Figure 3**).

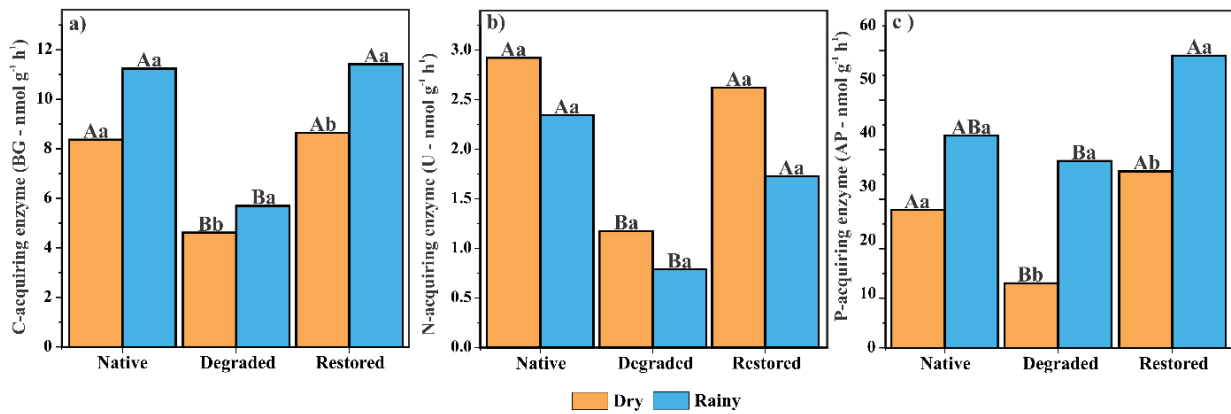


Figure 3. Soil enzymes activity of a) β -glucosidase, b) urease, and c) acid phosphatase under Native Caatinga vegetation, Degraded system by overgrazing and Restored area by grazing-exclusion management during dry and rainy seasons. Means followed by the same letter do not differ by Tukey (5%). Capital letters compared soil management within each season, and lowercase letters compared seasons within each soil management.

For the total fungal community, native areas showed higher diversity values among treatments in the rainy season, degraded showed intermediate results, and restored the lowest values. Degraded presented the highest values for alpha diversity by the Shannon index in the dry season, while native presented intermediate results and restored the lowest values. In the rainy season, native and restored showed greater diversity (**Figure 4 – a**). There were no diversity differences between the treatments in the dry season for the mycorrhizal fungal community. However, in the rainy season, degraded and restored showed higher values of diversity compared to those of native. Degraded and restored showed higher diversity in the rainy season compared to the rainy season (**Figure 4 – b**).

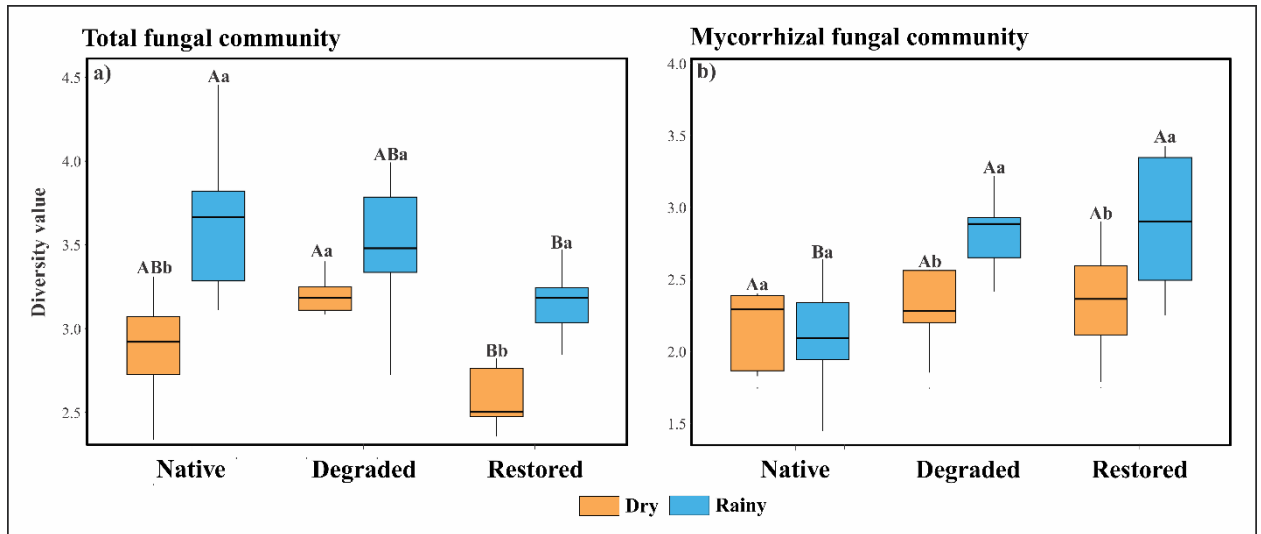


Figure 4 – Alpha diversity, based on Shannon’s index of total fungal community (a) and mycorrhizal fungal community (b) in soil under native Caatinga vegetation, degraded by grazed systems and restored by grazing exclusion. Capital letters compared soil management within each season, and lowercase letters compared seasons within each soil management.

We conducted PCoA to explore the distance in the fungal community structure between three treatments: Native, degraded, and restored and the two seasons (dry and rainy). Fungal communities in native areas differed from those in degraded areas. However, the total fungal community in the restored areas was similar to that in the native regions. Compared to the communities obtained, two groups emerged in degraded and restored areas, one for the dry season and the other for the rainy season. This same design is not seen in natives. (**Figure 5 – a**). On the other hand, the AMF community in restored areas was intermediate between native and degraded areas. Additionally, the PCoA showed that the communities in the rainy and dry seasons were similar in native areas (**Figure 5 – b**)

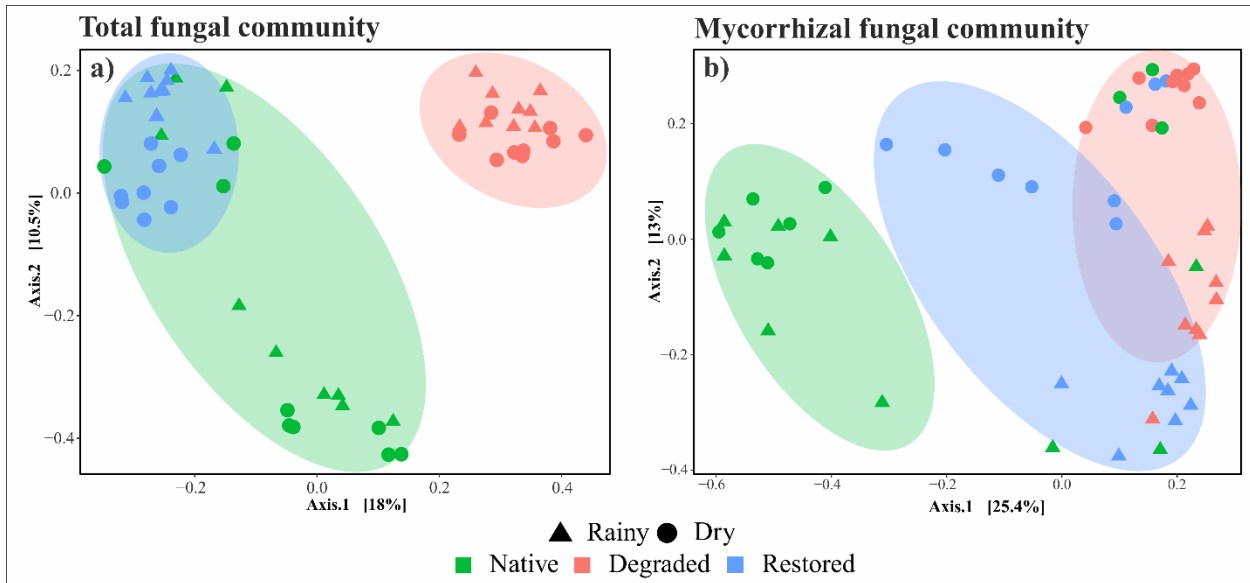


Figure 5 - Principle Coordinate Analysis (PCoA) based on bray-Curtis distances of total fungal community (a) and Mycorrhizal fungal communities (b) under Native Caatinga vegetation (green), Degraded system by overgrazing (pink) and Restored area by grazing-exclusion management (blue) during dry (circle) and rainy (triangle) seasons.

We performed a Redundancy analysis (RDA) to correlate changes in fungal community composition to changes in the environment. Native area was separated from the degraded area especially for the total fungal community, showing the restored area in the transition. Indicates that the soil environment attributes and species found in the restored area were closer to those of the native area. MBC, enzymatic activity, glomalin, OM and moisture, positive soil attributes are generally positively correlated with native and restored treatments. The orders Eurotiales and Hypocleares are strongly correlated with native in both seasons. At the same time, Venturiales and Capnodiales correlate with restoration regardless of season. In degraded, Mycosphaerellales and Botryosphaeriales are strongly correlated in the dry period, while Mycosphaerellales and Chaetothyriales are strongly correlated with degraded in the rainy season. Acaulosporaceae, Claroideoglomeraceae and Gigasporaceae, families of mycorrhizal fungal community, were strongly correlated to degraded in the dry season, while in the rainy season, it was only Glomeraceae. Native is correlated with Diversisporaceae for both seasons, and Archeosporaceae correlate with restored in the dry season.

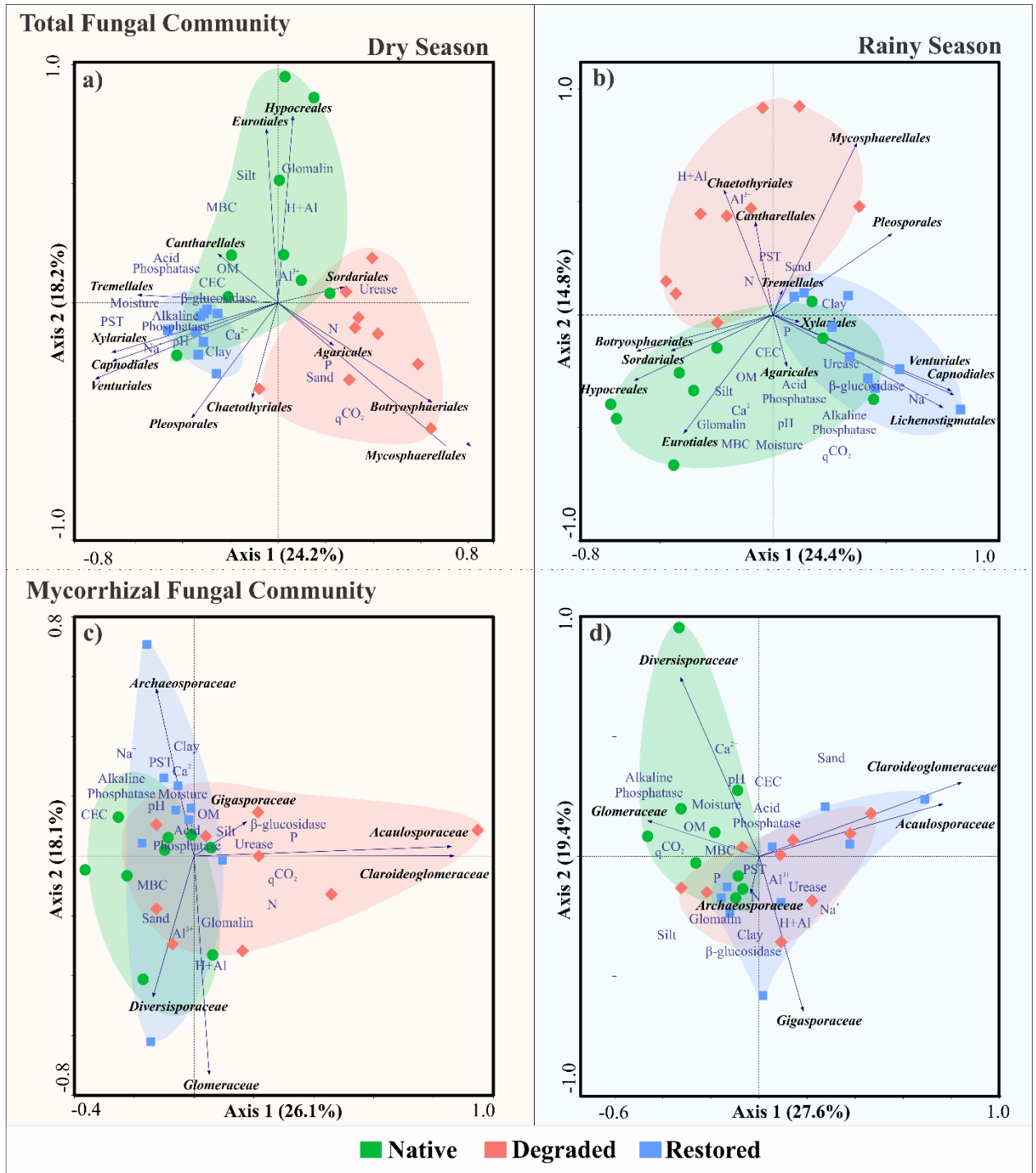


Figure 6 - Redundancy Analysis (RDA) of soil chemical, physical, and biological attributes against fungal communities structure. Total fungal community in the dry season (a) and the rainy season (b). Mycorrhizal fungal community in the dry season (c) and the rainy season (d).

We plotted relative abundance graphs for our treatments with the five most abundant orders of total fungal community and the families of mycorrhizal fungal communities for each treatment and in the two seasons (dry and rainy) (**Figure 7**). The order Pleosporales was dominant for all treatments, but in native in the dry period, the order Hypocreales (~21%) was the most abundant, followed by Pleosporales (~20%) and Capnodiiales (~19%). In degraded,

the orders Mycosphaerellales (~20%), Botryosphaeriales (~5%), and Agaricales (~3%) were the three most abundant after Pleosporales (~35%) in the dry season, while in the rainy season, Chaetothyriales (~9%), Sordariales (~7%) and Hypocreales (5%) were the most abundant after Pleosporales (~35%). In restored, Capnodiales (~15%), Venturiales (~7%), Xylariales (~4%), and Mycosphaerellales (~3%) were the most abundant orders after Pleosporales (~40%) in the dry season, while in the rainy season, Capnodiales (~19%), Lichenostigmatales (~18%) and Venturiales were the most abundant after Pleosporales (30%).

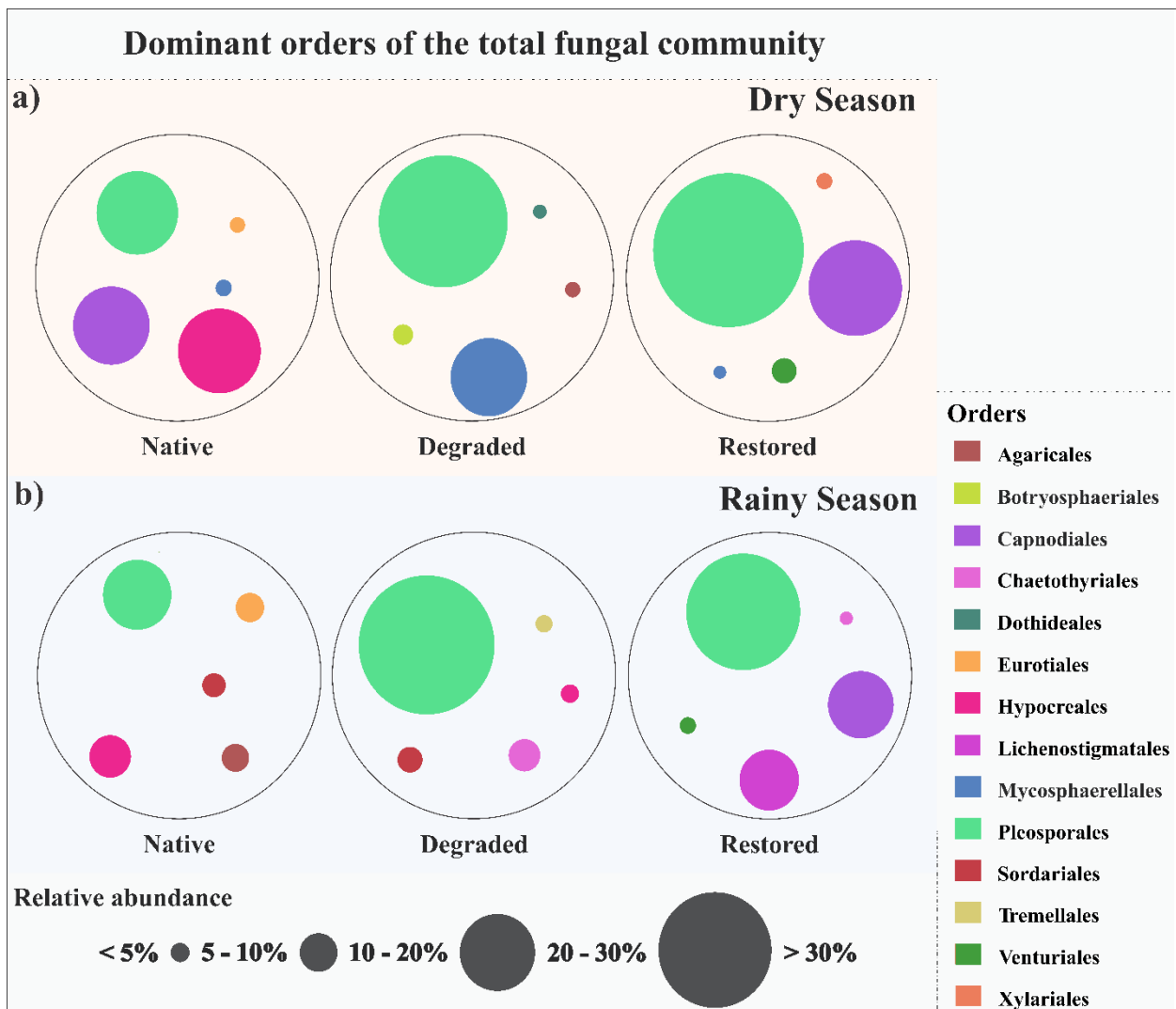


Figure 7 - Five dominant orders of total fungal community within each treatment (Native, Degraded, and Restored) in the dry season (a) and the rainy season (b).

The Glomeraceae family was the one with the highest relative abundance in all treatments (Native, degraded, and restorative) (**Figure 8**), with greater abundance in the dry season ($p < 0.05$) (**Figure 9 – d**). In the dry season, Cloroideoglomeraceae (7%) was the second most abundant in degraded areas, followed by Acaulosporaceae and Gigasporaceae,

which presented an abundance close to 1%. In the rainy season, Gigasporaceae (~19%), Acaulosporaceae (~13%), and Claroideoglomeraceae (~11%) were the most abundant after Glomeraceae (~53%). In native areas in dry season, Claroideoglomeraceae and Diversisporaceae presented abundances lower than 1%. In the rainy season, Acaulosporaceae (~13%), Diversisporaceae (~12%), and Gigasporaceae (~2.5%) were the most abundant families after Glomeraceae (~49). In the degraded areas, Archaeosporaceae (~5%), Claroideoglomeraceae (~3%), and Diversisporaceae (>1%) were the most abundant families after Glomeraceae (~77%) in the dry season, while Gigasporaceae (~20%), Acaulosporaceae (~14%), and Claroideoglomeraceae (~13%), were the most abundant in the rainy season, also after Glomeraceae (~37%).

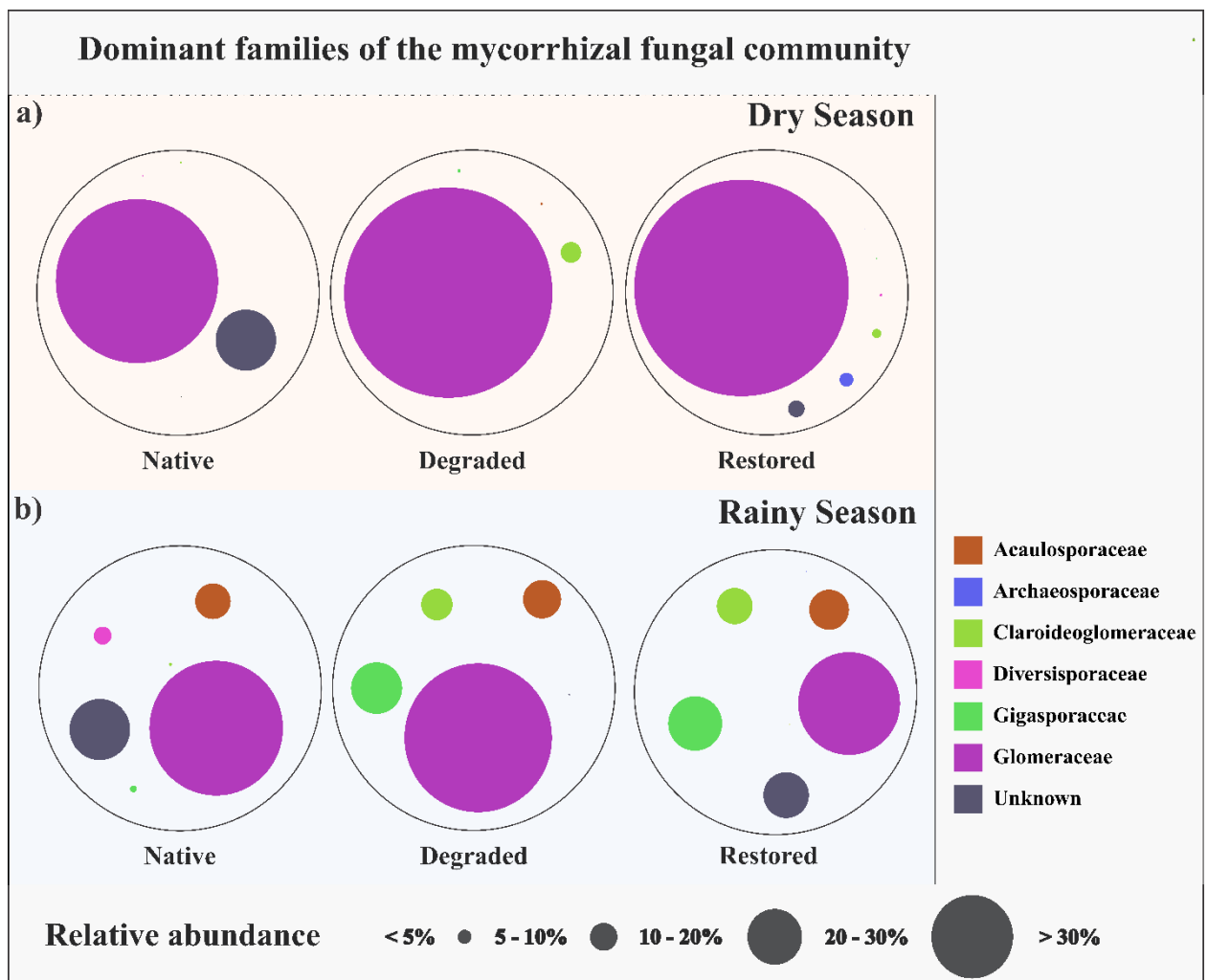


Figure 8 - Five dominant orders of mycorrhizal fungal community within each treatment (Native, Degraded, and Restored) in the dry season (a) and the rainy season (b). β -glucosidase.

A differential abundance analysis based on log₂ changes of Order level taxonomics showed that 14 orders in the total fungal community differed between treatments (**Figure 9** –

a), and 14 differed between dry and rainy seasons (**Figure 9 – b**). In the AMF community, four families differed between treatments (**Figure 9 – c**), and three varied between the dry and rainy seasons (**Figure 9 – d**). In the total fungal community, Eurotiales, Mortierellales, and Trichosporonales differed positively for Natives detected as degraded. Lichenostigmatales, Orbiliales, and Xylariales were more abundant in restoration than in degraded areas. The orders Capnodiales, Helotiales, and Trichosporonales were more abundant in both native and restorative when detected in degraded conditions. The order Mycosphaerellales was higher in degraded than restored and did not differ from native. Archaeosporales, Botryosphaeriales, Corticiales, Magnaporthales, and Polyporales were more abundant in degraded detected at native and restored. In the mycorrhizal fungal community, the abundance of Acaulosporaceae, Claroideoglomeraceae, and Gigasporaceae was higher in degraded compared to native areas, and there was no difference in reference to restored. As for seasonality, the families Acaulosporaceae and Gigasporaceae were higher in the rainy season, and only Glomeraceae showed a higher value for the dry season.

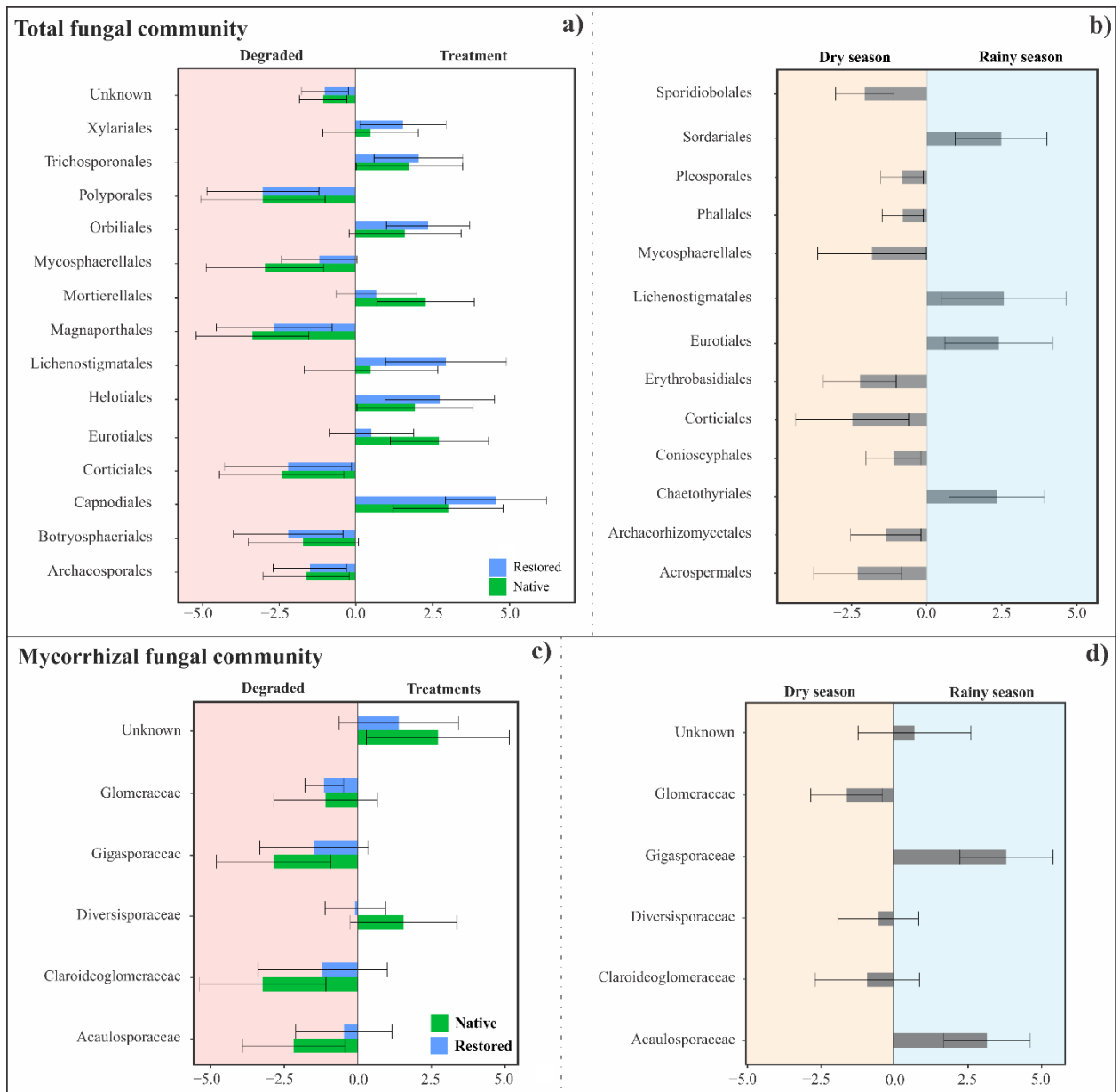


Figure 9 - Differential analysis based on log₂ changes of Order level taxonomic ranks in the land variable treatment (Native and Restored) and season (Rainy) in reference to the degraded and dry season, respectively, for total fungal community and Mycorrhizal fungal community. β -glucosidase.

Discussion

Soil microorganisms are essential in soil ecosystem functions (Mendes et al 2017). There is a growing understanding of the importance of soil microbe diversity and structure as indicators of soil function and quality (Mendes and Tsai, 2018; Huang et al., 2019; Sun et al., 2022). Several critical ecosystem processes are driven by soil microorganisms, including the cycling of essential elements and nutrients (such as carbon, nitrogen, phosphorus, and sulfur), litter decomposition, regulating plant growth, and influencing the coexistence and diversity of plant species (Teste et al., 2017; Cotta et al., 2019). However, we still need more understanding of modulation in soil fungal communities across the desertification process,

especially in Brazilian drylands (Caatinga biome), South America's most extensive tropical dry forest (Souza et al., 2021). Although soil enzymes have been measured in degraded areas affected by desertification, it is known that there is a harmful impact on soil microorganisms and their biochemical activity. Therefore, the fungal community in native, degraded, and restored soils from Brazilian Caatinga was evaluated in this study. Consequently, we proposed the hypothesis that degradation by overgrazing and restoration by fencing changed the status of fungal communities (total fungi and arbuscular mycorrhizal fungi [AMF]), decreasing and increasing diversity parameters, respectively.

Vegetation removal or soil disturbance affects biodiversity and ecosystem functioning and reduces soil fertility, decreasing soil resources for microbial life, that is, organic matter and nutrient availability (Zhu et al., 2020). Reestablishment of vegetation, contributed to an increase in the values of TOC, P, CE, MBC, and β -glucosidase, indicators of improved soil quality/health regarding fertility and biology grassland experiment in Jena, Germany (Prommer et al., 2020). In contrast, overgrazing increased the values of Al^{3+} , Na^+ , and soil density, which are indicators of soil degradation, since these parameters are indicators of acidity and salinity (Nawaz et al., 2013). Oliveira et al. (2019) reported that overgrazing increases soil density, contributing to soil compaction.

In general, the values of C-, N-, and P-acquiring enzymes were higher in native and restored soils, and it could be related to the permanent presence of vegetation in native (e.g., herbaceous plants, stunted trees, and bushes) and restored (grazing exclusion with secondary vegetation) soils (Oliveira-Filho, 2019). Previous studies have reported that the presence of plants contributed to higher values of C-, N-, and P-acquiring enzymes (Piotrowska-Długosz et al., 2022; Medeiros et al., 2023). In contrast, degraded soils, which does not present plants neither litter, presented the lowest values of C-, N-, and P-acquiring enzymes. In addition, the degraded soil presents lowest content of organic C and nutrients, such as N and P, which contributes to decrease the activity of enzymes. As comparison, the degraded soil showed a reduction of ~31% and ~43% in organic C, compared to the restored and native soils (Pereira et al., 2021). Therefore, the lowest activity of enzymes found in degraded soil can be related to this decreased availability of organic sources and nutrients (Ge et al., 2010; Araujo et al., 2022).

Despite differences in soil moisture between the dry and rainy seasons, the highest values were recorded in the area under native forest, followed by the restored area. Moisture is a well-known factor that affects microbial activity and C dynamics (Qu et al., 2021). Thus, higher soil in water content in native and grazing-exclusion creates a more conducive

environment for microbial growth and activity when compared with degraded sites. The greater proportion of soil surface covered by vegetation in these areas results in the interception of solar rays and a reduction in evaporation rate. Thus, strategies that regulate soil moisture (e.g., grazing-exclusion), can benefit the soil microbial community, and enhance biological indicators, such as enzyme activity (Gupta et al., 2022; Silatsa et al., 2017; Thapa et al., 2021). In the rainy season, native areas showed higher abundance based on Shannon's diversity index, and restored had intermediate results ($p < 0.05$). Similar results were reported for the soil bacterial community (Pereira et al., 2021).

Pleosporales was the most abundant order in all treatments in both seasons (dry and rainy). In degraded and restored areas, the relative abundance of this order was around 35%. In contrast, in areas of native vegetation, even though it was the most abundant order, it did not exceed a relative abundance of 20%. Pleosporales is one of the most oversized orders within Dothideomycetes (Ascomycota) and includes species that are widespread worldwide, primarily as soil saprotrophs, but can also inhabit plant residues (Phookamsak et al., 2014). Certain groups within this category contain plant endophytes that play a crucial role in the ecosystem. These include a variety of dark septate endophytes, as documented by studies such as Knapp et al. (2015). According to Knapp et al.'s (2012) research, Pleosporales can be found in plant species in North American semi-arid grasslands.

With the Differential analysis of Order level taxonomic ranks in the land variable treatment, 14 orders showed differences between treatments. Most of these orders are characterized as rare, scarce in number, and frequently encountered. However, Botryosphaeriales, Capnodiales, Eurotiales, Lichenostigmatales, Mycosphaerellales, and Xylariales represent the orders that differed between treatments and are not characterized as rare species. Between the dry and rainy seasons, Chaetothyriales, Eurotiales, Lichenostigmatales, Mycosphaerellales, Pleosporales, and Sordariales were the orders that showed significant differences and were not characterized as rare groups. These are the orders that distanced our treatments the most. With the PCoA based on Bray-Curtis distances and the RDA analysis, it was possible to notice a distancing of the communities in the degraded areas compared to those in native and restored areas.

The Capnodiales order is was the second dominant in the restored areas and the third most in the native areas in the dry season (**Figure 7 – a**), and it was also more abundant ($p < 0.05$) in these two areas compared to degraded areas (**Figure 9 – a**). The dominance of Capnodiales is a very interesting result, as it is probably due to the scarcity of more competitive decomposers, such as Basidiomycetes. Capnodiales are generally associated with

leaf surfaces and are especially abundant in areas where plant-sucking insects produce honeydew (Chomnunti et al., 2014), this order has also been found as dominant fungi on rock surfaces and other highly exposed environments (Ruibal et al., 2005). Some species of this order are commonly known as black sooty molds as they have melanized spores and hyphae (Fröhlich-Nowoisky et al., 2009). Spores melanization confers resistance to microbial attack and abiotic degradation by UV radiation, thus improving viability in long-range dispersal (Wyatt et al., 2013). The predominance of Capnodiales in soils of a tropical dry forest such as the Caatinga may be due to the resilience of melanized spores in comparison with other groups of fungi, as some climatic characteristics of this environment, such as water stagnation and high levels of salts in the soil, can be considered a stressor for some microbial groups.

The order Hypocreales, one of the most abundant in native and restorative species, has high agronomic importance. The genus *Trichoderma*, one of the most studied for biological control (Gangaraj et al., 2023), is present in this order. On the other hand, the genus *Fusarium*, which includes many pathogenic species that cause a wide range of plant diseases that lead to major economic losses worldwide, also belongs to this order (Nikitin et al., 2023). Botryosphaeriales, Eurotiales, Lichenostigmatales, Mycosphaerellales, and Xylariales, orders that showed higher abundance and significant differences between treatments, also have pathogens of economic importance. However, we still do not have a technology to relate based only on genomic sequencing data, the presence of a pathogens in the soil. Organisms that cause plant diseases are extremely specific. In addition, for example, in Pleosporales, there are several pathogens, but from the aerial part of the plants, in which the inoculum in the soil alone cannot transmit the disease (Mugambi and Huhndor, 2009). Another important point is whether these soils are suppressive or conducive to certain diseases has yet to be known. Further studies of microbial ecology and plant microbiome would be necessary for more accurate information related to the interactions of benefic and phytopathogenic microorganisms in this gradient of soil desertification.

We evaluated the AMF community in soils degraded by overgrazing and restored by grazing exclusion (20 years) in the semiarid region of Brazil. Our results thus support the beneficial role of grazing exclusion in recovering semiarid soils in the face of desertification. More significantly, AMF can make plants more resilient to abiotic stresses brought on by climate change events, which can reduce plant removal and improve adaptability, dispersal, and survival rates, making this particular group of microorganisms essential to the soil ecosystem's health. (Ferlian et al., 2021; Genre et al., 2020). Interestingly, despite the soil degraded properties found in soil under overgrazing, studies report that AMF spore abundance

is not affected by degradation (Silva et al 2022). In addition, they are the most abundant fungal structures of infectious propagules in the soil (Bueno and Moora, 2019).

Glomeraceae, Gigasporaceae, and Acaulosporaceae showed differences between treatments and seasons, while Claroideoglomeraceae showed only between treatments. Our study showed that Glomeraceae is the most abundant family in all treatments and seasons (**Figure 8**), agreeing with Carrillo-Saucedo et al. (2018), who, based on the classical taxonomy of spores of arbuscular mycorrhizal fungi, showed Glomeraceae family as the dominant in a tropical dry forest in the state of Jalisco, Mexico. Our previous study with classical taxonomy of spore also showed Glomeraceae as the most abundant family in Native, restored, and the second most abundant in degraded areas (Silva et al., 2022). Species of Glomeraceae and Acaulosporaceae have been identified in areas contaminated by metals due to their greater ability to sporulate and colonize plants (Moreira et al., 2015). Thus, members of Acaulosporaceae and Glomeraceae should be included as keystone species to mitigate climatic changes in drylands soils.

Mycorrhizal fungal community diversity was found to be higher in the soil under grazing exclusion, agreeing with Silva et al. (2022), probably due to the presence of legume species, such as *Mimosa tenuiflora* (Oliveira Filho et al., 2019) that develop mutualistic interactions with AMF (Souza et al., 2016). Total fungal community diversity was higher under native areas; this is probably due to higher MO, moisture and fertility of the soil. The redundancy analysis clearly separated both communities between soil under overgrazing and grazing exclusion, and this suggests that the process of restoration by grazing exclusion can effectively change the structure of the fungal community.

With our study, it was possible to notice that the soil desertification in the Caatinga biome seriously affects the diversity and composition of soil fungal communities. On the other hand, grazing exclusion has been shown to present potential in recovering the diversity and composition of soil fungal communities, especially for mycorrhizal, by altering soil chemistry and biological parameters and the relationships among dominant fungal taxa.

Conclusions

In this study, we investigated the fungal communities in native, degraded, and restored soils of the Brazilian Caatinga biome. Pleosporales is the dominant order of fungal community for all areas present in the caatinga biome, regardless of the level of degradation. Our findings suggest that degraded soils have changed these communities and decreased the soil quality/health parameters. Total fungal and AMF communities in soils from Brazilian

semiarid are sensitive to both processes of desertification and restoration. The Glomeraceae and Acaulospora families are key groups that should be further studied in these climate conditions. Glomeraceae appears as the most dominant family in all areas and more strongly in the dry season. The process of desertification, by overgrazing, contributes to degrading soil chemical, physical, and biological properties, and changing the structure, diversity, and composition of fungal communities. Also, the grazing-exclusion, as an ecological practice to restore semiarid soils, present the potential to improve soil properties, and community under ongoing climate changes globally. Thus, grazing-exclusion, in long-term, can be a good strategy to restore degraded soils in the Brazilian semiarid.

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3. FUNCTIONAL GENES RELATED TO NITROGEN AND PHOSPHORUS CYCLING IN DEGRADED AND RESTORED AREAS FROM BRAZILIAN DRYLANDS

Abstract

The practice of grazing exclusion has been applied to soil conservation, particularly in semiarid regions such as the Caatinga biome. However, it is unclear whether grazing exclusion reduces the negative effects of overgrazing on functional genes related to nutrient cycles such as nitrogen and phosphorus. The decrease in soil functional capacity caused by degradation is mainly due to the loss of ecological processes carried out by microbial communities. Therefore, evaluating the ecological functions of soil microbial communities is essential to understand the long-term impacts of anthropogenic actions on soil systems. However, there is still a lack of information on how degradation can alter ecological processes, such as nitrogen (N) and phosphorus (P) cycling. This study evaluated the impact of long-term overgrazing and grazing exclusion on the soil microbial functional pools related to N and P cycling compared to native Caatinga soil. The results revealed differences ($p < 0.05$) in the abundance of the genes *phoD*, *amoA* (AOA and AOB), *nifH*, *nirK*, *nirS*, and *nosZ* between the three areas. The disturbances caused by the degradation promoted an imbalance in the N cycle, indicating that degraded soils are not a receptive environment to the accumulation of N in the soil. There was a reduction ($p < 0.05$) of the enzyme alkaline phosphatase and the abundance of gene *phoD*, reducing the potential for P mineralization. The results showed that the restoration improved the soil properties, highlighting the potential of grazing exclusion as a restoration strategy for degraded soils in the Caatinga biome. This study provided important insights into the impacts of degradation on soil microbial functions and the potential of grazing exclusion as a restoration strategy.

Keywords: Soil functions, Microbial ecology, N and P cycles, Drylands.

Introduction

As the largest seasonally dry tropical forest found in South America, the Caatinga biome takes up 70% (0.85/1.2 million km²) of the Brazilian semiarid (Souza et al., 2021). The Caatinga is considered an important hotspot of biodiversity owing to the presence of endemic plants and animal species (Leal et al., 2005; Pereira et al., 2021; Silva et al., 2022). This biome presents soils with high vulnerability to desertification due to their environmental conditions, such as geology and climate, limiting the suitability for different land uses. According to CGEE (2016), approximately 70,000 km² of the Brazilian semiarid are undergoing a process of desertification, leading to the loss of soil biodiversity and unbalanced ecosystem functions.

Overgrazing is an important and unsuitable practice that has accelerated the desertification in the Caatinga biome, and it has decreased the diversity and changed the structure of soil microbial communities (Pereira et al., 2021; 2022). Grazing exclusion has been shown to be effective in restoring soil properties, mainly microbial status and diversity,

in the long term (Oliveira Filho et al., 2019; Pereira et al., 2021). Worldwide, grazing exclusion has been reported to be an effective practice method in restoring soil properties (Xun et al., 2018; Liu et al., 2020, Byrnes et al., 2018).

Therefore, studies have shown that overgrazing has negatively affected the soil microbial communities while grazing exclusion could reduce these negative effects and restore microbial properties (Pereira et al., 2021; 2022). This restoration in soil microbial communities is essential given that microorganisms perform several ecosystem functions related to the cycling of nutrients, such as N and P, essential to plant productivity (Naylor et al., 2022). For example, N is a structural component of amino acids, proteins, and enzymes (Trovato et al., 2021), essential for plant growth. Regarding P in soils, this element is critical in major biological systems associated with energy storage, cell replication, and protein synthesis (Wu et al., 2019). Although important to soil and plants, both nutrients' dynamic and availability depend on soil organic matter (SOM) and microbial communities. Thus, these features are concerning since the process of desertification has depleted the soil organic matter content (Oliveira Filho et al., 2019; Silva et al., 2022).

The transformation of organic nutrients from SOM into bioavailable forms to plants involves several microbial genes and their expressed enzymes (Meng et al., 2022). For example, the *phoD* gene is related to the mineralization of organic P, being a sensitive biomarker to investigate the biological function of the alkaline phosphatase (Zheng et al., 2021; Rodriguez et al., 2023). Regarding N cycling, different processes are carried out by the microbial community and modulated by several functional genes that can be used as markers for the presence of certain process steps, such as biological nitrogen fixation (*nifH*), ammonia oxidation (*amoA*) for archaea (AOA) and bacteria (AOB), nitrate reduction (*nirS* and *nirK*), and nitrous oxide reduction (*nosZ*) (Wallenstein and Vitgalys, 2005).

Although the above microbial functional genes have been well-studied in several soil and environmental conditions (Philippot et al., 2013; Ding et al., 2023; Chen et al., 2021; Li et al., 2022)), studies assessing the effect of overgrazing and grazing-exclusion on the microbial functional genes in soils from Caatinga tropical dry forest have yet to be reported. Here, we assessed the dynamics of *amoA*, *nifH*, *nirK*, *nirS*, *nosZ*, and *phoD* genes in soils under desertification (overgrazing) and restoration (grazing-exclusion) in the Brazilian semiarid region. We hypothesized that the desertification process reduces the abundance of genes related to N and P cycles while grazing exclusion could recover. To address this hypothesis, we analyzed functional genes using quantitative PCR with DNA extracted from soils collected in areas under desertification (overgrazing), restoration (twenty years of grazing

exclusion), and native forests in the Brazilian drylands region.

Material and Methods

Study site

The study was conducted in Irauçuba, Ceará, Brazil (3°44' 46"S e 39°47'00"W). The yearly precipitation is approximately 454 mm (FUNCEME; 2022), mainly concentrated between January and May (**Figure 1 – Chapter 1**). The climate in this region is hot semiarid (BSh) according to Köppen's classification system, with mean temperature ranging from 26°C to 28°C (Alvares et al., 2013). The soil was classified as Planosols accordingly to WRB/FAO (Oliveira Filho et al., 2019).

The use of natural pastures is a frequent practice by farmers in this region. In 2000, grazing-exclusion systems were implemented in 50 m x 50 m (2,500 m²) areas, separated by fences to avoid animal grazing. To avoid edge effects, we sampled in an area of 40 m x 40 m.

We collected 27 soil samples in the dry season (Oct/2021) and 27 in the rainy season (Apr/2022) in three different scenarios, as follows: native Caatinga vegetation (Native), 20 years of grazing exclusion by fencing (Restored) and areas of advanced desertification by overgrazing (Degraded). These scenarios were repeated in three large areas, separated by ~ 2 km. At each sampling point, we collected nine sub-samples, which were homogenized to create a composite sample. Thus, we analyzed 54 samples (three areas, three scenarios, three composite replicates, and two seasons). The experimental design was detailed extensively in Pereira et al. (2021) and Silva et al. (2022).

Soil chemical and physical characterization

The extraction methods for the nitrogen forms (total N, NH₄⁺, NO₃⁻) were extracted by the steam distillation method proposed by Kjeldahl (Nelson and Sommers, 1982; Vezzani et al., 2001; Freitas et al., 2013). Phosphorus (P) was extracted through the ion exchange resin following van Raij et al. (2001). The pH was measured in a CaCl₂ solution (0.01 mol L⁻¹). Total organic carbon (TOC) was extracted by performing carbon oxidation in organic form with potassium dichromate (K₂Cr₂O₇) and determined by colorimetry. The NH₄⁺ and NO₃⁻ fractions were determined following the aerobic incubation method proposed by Hart et al. (1994). Soil (Aluminum [Al₃⁺], calcium [Ca₂⁺], and magnesium [Mg₂⁺]) was extracted using KCl solution (1 mol L⁻¹), while sodium (Na⁺) was extracted by Mehlich⁻¹ solution (EMBRAPA, 2009). Electrical conductivity (EC) was measured using an electrical conductivity meter. Cation exchange capacity (CEC) was determined following the method

Raij et al. (2001) described for tropical soils. A pipette method was used to measure the clay fraction (Gee and Bauder, 1986), while sieving was used to measure the sand fraction, and the silt fraction was determined by subtracting the total mass from the sand and clay fractions. A gravimetric method with oven drying was used to determine soil moisture, in which wet soil samples were weighed and then dried in an oven at 105°C until they reached a constant mass. The samples were weighed again in the following step, and the mass difference represented the water mass. The results of soil physicochemical characteristics can be found in Table 1, chapter 1.

Alkaline phosphatase activity and microbial biomass carbon

The alkaline phosphatase (Alk, EC 3.1.3.1) activity was determined following the procedures described in Tabatabai (1994). Assays were conducted in triplicate by incubating 1 g of fresh soil with *p*-nitrophenol phosphate (PNP). Samples were incubated at 37°C for 1 h⁻¹, and the reaction was stopped by adding CaCl₂ (0.5 M) and NaOH (0.5 M). The extracts were filtered in Whatman's paper (n° 2), and the resulting color intensities were measured at 410 nm. Values are expressed as μmol of PNP produced per g of soil (dry weight equivalent) per 1 h. Enzyme activities were calculated using a standard curve developed with a *p*-nitrophenol solution.

Microbial biomass carbon (MBC) was determined by the chloroform fumigation-extraction method. 10 g of soil from each sample was fumigated with chloroform (99.8%) and digested with potassium dichromate for 30 min at 100 °C. Extracts were determined by titration using iron sulfate and ammoniacal ferrous sulfate (Brookes et al., 1985; Vance et al., 1987).

Quantitative real-time PCR

Genomic soil DNA was extracted from 0.25 g of soil (fresh weight) using the DNeasy PowerSoil Pro Kit (QIAGEN), following the manufacturer's instructions. Quantitative PCR (qPCR) was performed to enumerate copies of functional genes involved in soil phosphorus and nitrogen cycling (*phoD*, *amoA* (AOA), *amoA* (AOB), *nifH*, *nirK*, *nirS* and *nosZ* (Table 1). All reactions were run in duplicate 20 μL reactions comprising 10 μL of 2x Ssoadvanced Universal SYBER Green Supermix (Bio-Rad, USA), 1 μL of a forward/reverse primer mixture (10 pmol μL⁻¹ each) of each primer (Table 1), 2 μL of genomic soil DNA (5 ng μL⁻¹), and 6 μL of nuclease-free sterile water. The protocol for *phoD* was as follows: 3 min at 95 °C, followed by 45 cycles of 10 s melting at 95 °C and 30 s anneal and elongation at 58 °C. The

protocol for all the nitrogen genes was as follows: 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s with an additional 15 s at 80°C. PCR-grade water (no template) was used as a negative control. To verify the specificity of the qPCR, a melting curve procedure followed by a gel-based analysis of amplification products were performed for each functional gene. Standard curves (10^1 to 10^9 copies) were generated with plasmid DNA containing partial fragments of each gene; *nifH*, *amoA* (AOA), *amoA* (AOB), *nirK*, *nirS*, and *nosZ*. Reactions were performed on a 96-well PCR plate using the CFX Connect™ Real-Time System (BIO-RAD).

Table 1 - Characterization of forward and reverse primer pairs of phosphorus and nitrogen genes.

Target gene	Primer	Sequence (5' - 3')	Size (bp)	Reference
Alkaline phosphatase	ALPS-F730 ALPS-R1101	CAGTGGGACGACCACGAGGT GAGGCCGATCGGCATGTTCG	370	Sakurai et al. (2008)
Ammonia oxidation Archaea	Crenamo A23f Crenamo A616r	ATGGTCTGGCTWAGACG GCCATCCATCTGTATGTCCA	~500	Tourna et al (2008)
Ammonia oxidation Bacteria	amoA 1f amoA 2r	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	Rotthauwe et al (1997)
Nitrogen biological fixation	nifH - PolF nifH - PolR	TGCGAYCCSAARGCBGACTC ATSGCCATCATYTCRCCGGA	<500	Poly et al (2001)
Nitrate reduction	nirK 876 nirK 1040	ATYGGCGGVCA YGGCGA GCCTCGATCAGRTTRTGTT	165	Henry et al (2004)
Nitrate reduction	nirS Cd3aF nirS R3cd	AACGYSAAGGARACSGG GASTTCGGRTGSGTCTTSAYGAA	< 500	Throback et al (2004)
Oxide reduction	nosZ1F nosZ1R	WCSYTGTTTCMTCGACAGCCAG ATGTTCGATCARCTGVKCRTTYTC	453	Kloos et al. (2001)

Statistical analyses

Data were tested for residuals normality and homoscedasticity using the Shapiro–Wilk's and the Breusch–Pagan tests, respectively. Once data were observed to meet the Nested ANOVA criteria, post-hoc tests were utilized as Tukey's test (5%) to compare mean groups. A redundancy analysis (RDA) triplots were used to compare soil physicochemical and biological parameters to functional genes. The data analyses were performed in the RStudio

(version 3.6.3) and Canoco® software (v. 4.5) for Windows.

Results

The abundance of functional genes varied significantly across the three areas ($p < 0.05$). Specifically, the *phoD* abundance and alkaline phosphatase activity were significantly higher ($p < 0.05$) in native as compared to both degraded and restored areas (**Figure 2A**). However, the values of phosphatase found in the restored area were similar to those observed in the native area (phosphatase and or alkaline phosphatase activity, be consistent in the manuscript). In addition, the results showed a high correlation between *phoD* copy number and phosphatase activity (**Figure 2B**).

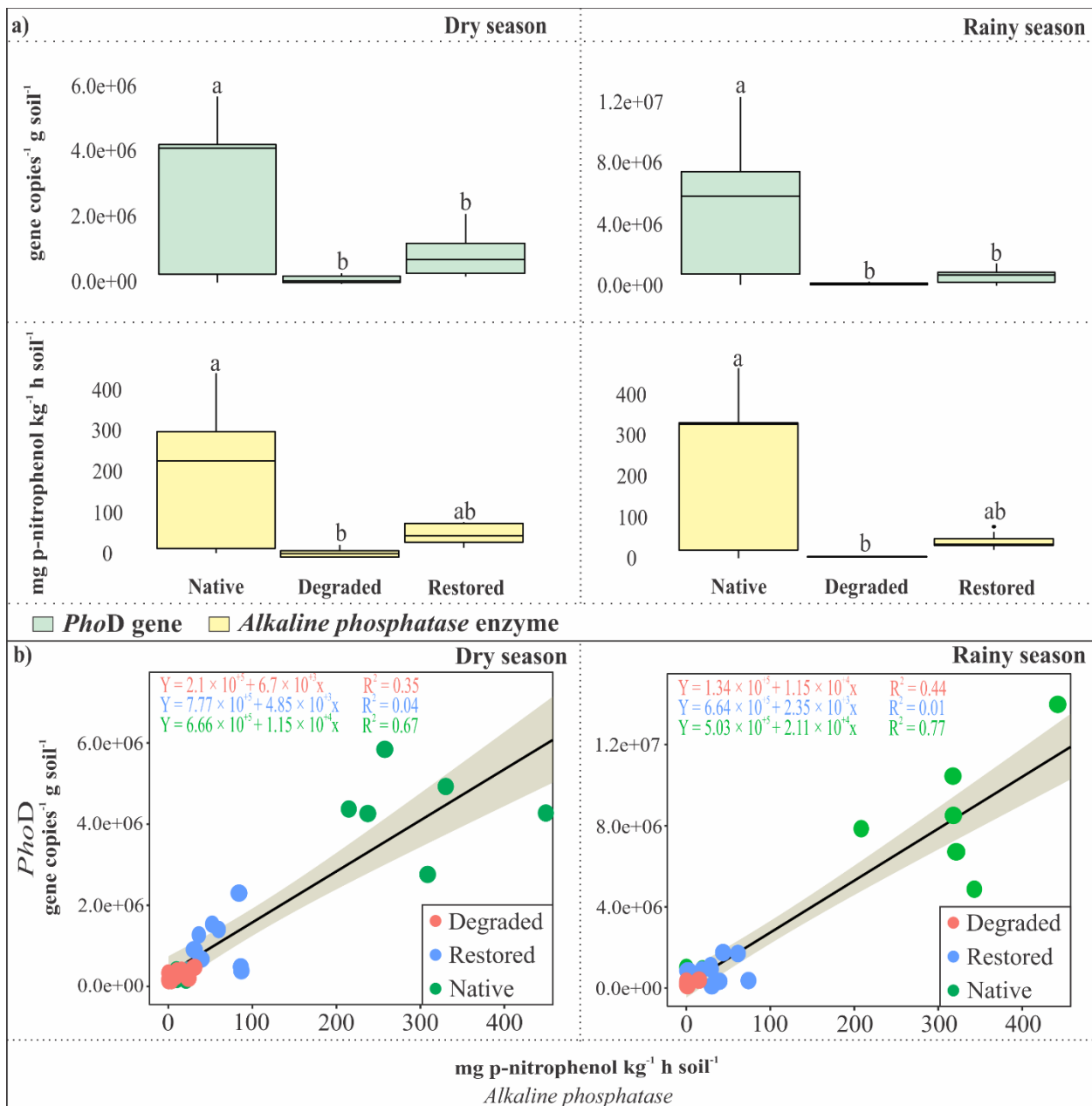


Figure 1 – Differences in *phoD* gene abundance and alkaline phosphatase activity of a desertification gradient in

dry and rainy season (A). Seasonality of *phoD* gene abundance and alkaline phosphatase activity (B). Linear regression analyses between *phoD* gene and alkaline phosphatase in dry and rainy season. (C).

In the dry season, the copy number of *nifH* and *nirK* genes was higher ($p < 0.05$) in both native and restored areas as compared to degraded areas (**Figure 2**). In contrast, the copy number of archaeal *amoA*, *nirS*, and *nosZ* genes did not vary among different areas (**Figure 3**). The exception bacterial *amoA* was higher ($p < 0.05$) in the native area than in others. In the rainy season, archaeal *amoA*, *amoA* (AOB), *nirK*, and *nosZ* copy numbers were higher in native than degraded and restored areas, while *nifH* did not vary between areas. Interestingly, *nirS* gene abundance was higher in the degraded area than in other treatments.

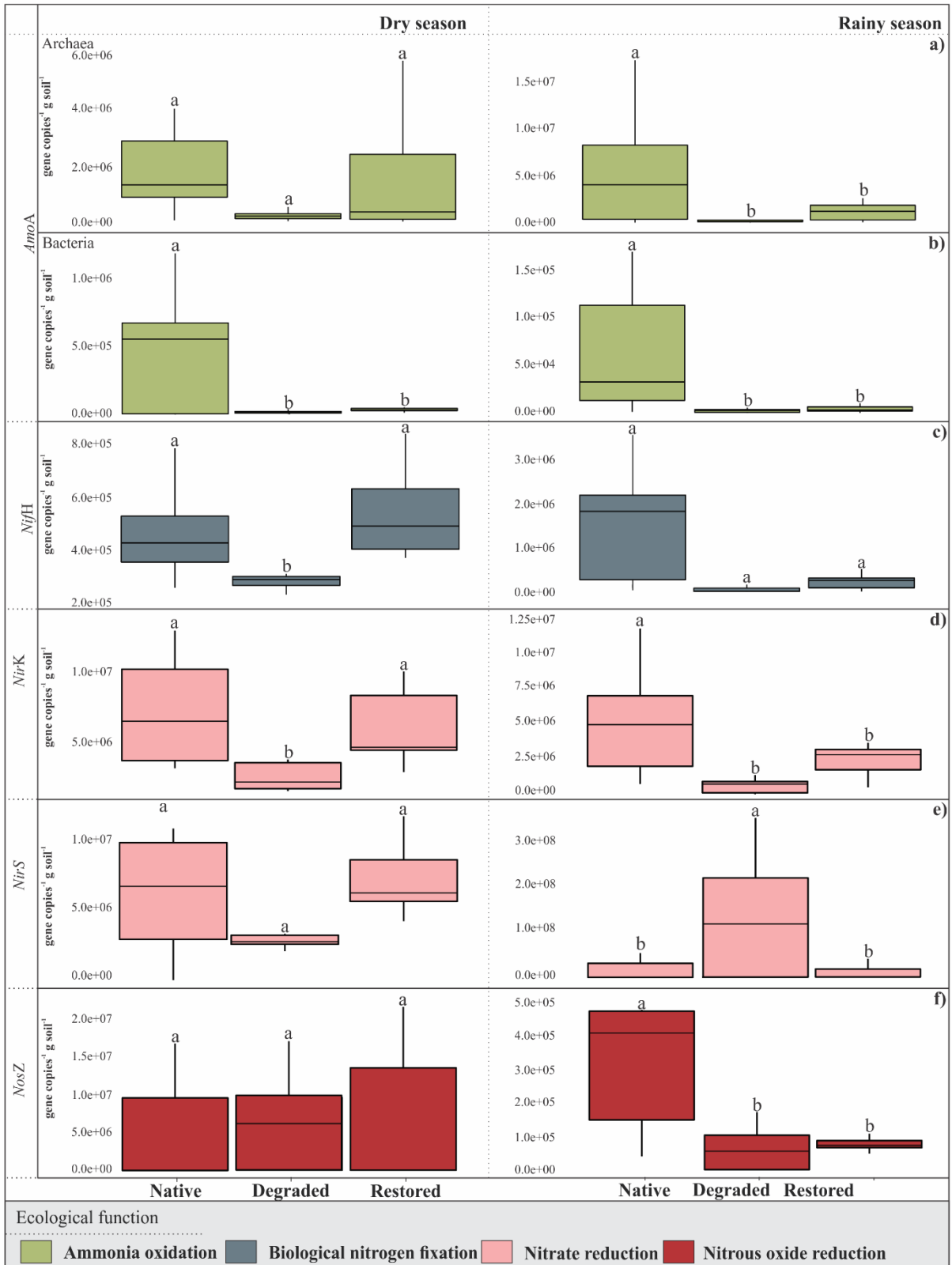


Figure 2 - Differences in nitrogen cycling genes (*amoA* [archaea, bacteria], *nifH*, *nirK*, *nirS* and *nosZ*) abundance in dry and rainy season.

RDA axes 1 explained 48.1% and 71.1% of the dry and rainy seasons variation, respectively (**Figure 3**). Both RDA separated the native and degraded areas and showed the

restored area in the transition. In both seasons, the majority of chemical and biological properties were clustered with the native area, highlighting total N, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, phosphatase, *phoD*, *amoA* (AOB and AOA), *nifH*, and *nirK*. In contrast, *nosZ* and *nirS* clustered with degraded areas in the dry and rainy seasons, respectively.

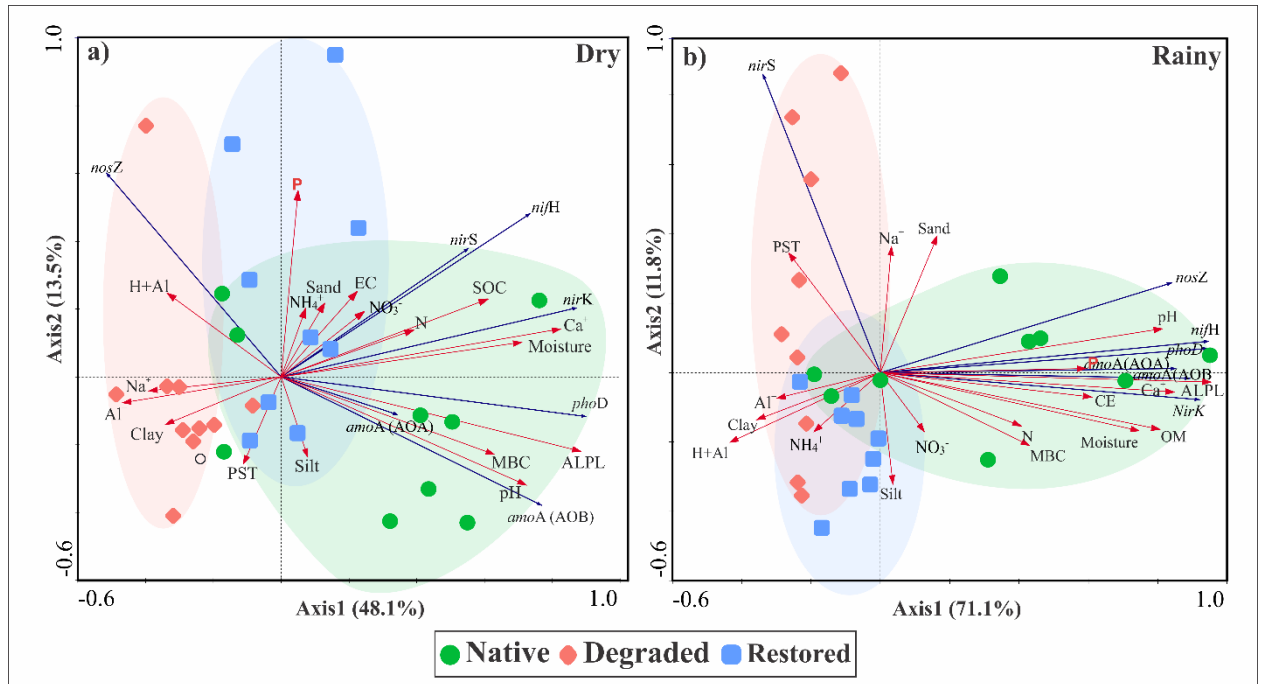


Figure 3 - Redundancy analysis (RDA), based on the correlation of soil physicochemical and biological parameters to functional genes in a gradient of desertification. a) Dry season; b) Rainy season. Data are shown for three different land use types, native, degraded and restored; red vectors for the soil chemical, physical parameters, and alkaline phosphatase activity, blue vectors for microbial genes (nitrogen and phosphorus cycling).

Discussion

Nowadays, soil degradation has been recognized as an important threat to soil biodiversity, mainly to microbial communities (Tibbett et al., 2020). Indeed, several previous studies have reported losses of important microbial groups, decreases in soil biodiversity, and changes in the composition of microbial communities (Araujo et al., 2016; Pereira et al., 2021; Zheng et al., 2023). Due to soil degradation in microbial communities, soil functioning can also be altered. Efforts of soil restoration could ameliorate these negative effects on the microbial communities and the soil functioning. This study assessed genes related to ammonia oxidation, biological nitrogen fixation (BNF), nitrate and nitrous oxide reduction, and phosphate mineralization to address the hypothesis that soil degradation reduces. In contrast, soil restoration could recover microbial functions within the N and P cycles, partially depending on seasonality. Corroborating with this hypothesis, we observed that the abundance

of some functional genes decreased with soil degradation while presenting a recovery in their abundance after 20 years of soil restoration, in the dry season, including *nifH*, and *nirK*, which are two important steps for biological nitrogen fixation and removal in the ecosystem.

The simultaneous increase in *nifH* and *nirK* can be explained by increasing microbes containing both genes, like *Bradyrhizobium japonicum* (Sánchez et al., 2010). In the rainy season, however, the soil restoration did not ameliorate the negative impact of degradation on any functional genes. The exception was the *nirS* copy number higher in the degraded area. Therefore, our results showed an effect of seasons, probably influenced by soil moisture, on the responses of functional genes to soil degradation and restoration.

Regardless of the seasons, our results suggest important consequences for particular soil processes, such as ammonia oxidation and BNF, being severely reduced by degradation areas regardless of the season and in addition to genes abundance, the levels of total N, NH_4^+ -N, NO_3^- -N were lower in the degraded in the two seasons. Indeed, ammonium oxidation is considered a limiting step in the nitrification process and is catalyzed by ammonia monooxygenase encoded by the *amoA* gene (Gao et al., 2016). The restoration process showed a small recovery in *nifH*, which controls the BNF process. This is important because BNF is one of the few ways nitrogen enters the soils, especially in forest environments (Sardar et al., 2023). The observed reductions in the copy numbers of the bacterial and archaeal *amoA* and *nifH* promoted by degradation may be a direct consequence of losses related to important microbial groups carrying these particular functions (Pereira et al., 2021), such as ammonia-oxidizing bacteria and diazotrophs. For instance, previous studies report that ammonia-oxidizing bacteria are highly sensitive to soil disturbance, such as high salinity and contamination (Araujo et al., 2016; Guo et al., 2020).

This study is the first to report ammonia-oxidizing organisms' losses in Brazilian dryland's degraded soils. Previous studies have shown a decrease in *nifH* copy number and diazotrophs in soils under degradation in which ecosystem in China (Che et al., 2017; Zhang et al., 2022). Our results showed an increased abundance of the *nirS* gene in soils under degradation, and it may indicate losses of N from the soil to the atmosphere as gases NO, N_2O and N_2 since the *nirS* gene encodes nitrate reduction (denitrification) (Gineyts and Niboyet, 2023). Denitrification is an important process responsible for the losses of N from the soil, bringing environmental issues to the atmosphere (Ding et al., 2021) and considering the high abundance of the *nirS* and *nirK* genes and the lowest ($p < 0.05$) NO_3^- levels found in degraded. We can infer that denitrification is intensified in these areas, decreasing the pool of N in the soil and favouring nitrogen losses in gaseous forms.

Regarding the P cycle, we quantified the copies of *phoD* in our three different soils, which was identified as the most frequent alkaline phosphatase gene in the microbial community (Tan et al., 2013). Our results showed a significant positive relationship between *phoD* abundance and alkaline phosphatase activity, as Fraser et al. (2015) observed in a long-term study under distinct land management. As reported in previous studies, land degradation led to a significant negative effect on soil bacterial community (Araujo et al., 2016; Pereira et al., 2021), which is important to P turnover since bacteria are responsible for excretes phosphatases that catalyze the hydrolysis of ester-phosphate bonds (Fraser et al., 2015).

Finally, we applied the RDA to identify the correlation between chemical and biological properties to the abundance of the functional genes (Garizi et al., 2011). This analysis showed that the native area was separated from the degraded area, showing the restored area in the transition. This indicates that the chemical and biological properties found in the restored area were closer to those of the native area, which suggests that the restoration process can improve the soil properties (Farrell et al., 2020). Interestingly, the functional variables, such as phosphatase, *phoD*, *amoA* (AOB and AOA), *nifH*, and *nirK*, were more associated with the native area and suggest maintaining soil functionality in the native area. In contrast, *nosZ* and *nirS* clustered with the degraded area, which could suggest these genes as indicators of environmental stress or degradation.

Conclusions

In this study, we investigated the abundance of functional genes related to the nitrogen (N) and phosphorus (P) cycles in native, degraded, and restored soils of the Brazilian Caatinga biome. Our findings suggest that degraded soils have lower copies of the quantified genes, indicating a microbiological limitation in N and P. These results have important implications for restoring degraded soils in this biome, highlighting the need to prioritize approaches to improve soil microbial functions. Our study also demonstrated that long-term grazing exclusion might effectively restore the ecological functions of N and P cycles in the Caatinga biome. This finding is particularly relevant for managing and conserving this unique and fragile ecosystem, as overgrazing has been identified as a major driver of soil degradation in the region. This study provided important insights about key functional genes involved in N and P cycles and demonstrated the potential of grazing exclusion for restoring soil ecological functions.

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3. FINAL REMARKS

Northeast Brazil is home to a wide range of biomes, but over 70% of its territory belongs to the Caatinga biome. Although the Northeast region was the basis of Brazil's economy in the mid-eighteenth century, it has a growing GDP, average human development indices, large agricultural production areas and urban centers with millions of inhabitants. The region is currently facing serious problems of social inequality, internal areas of the semi-arid region have thousands of people in extreme poverty, as in the region of the Irauçuba Desertification Center, which was our study center for this research. This type of environment is expected to become even more severe with climate change, challenging productivity in these areas.

Desertification is a devastating ecological process, in addition to the environmental and ecological damage portrayed in this study, it makes agriculture unfeasible, which is the main means of survival for a large part of the population that lives there. Studies like this are essential to increase our database and open doors to more targeted studies related to sustainable agriculture in dry lands and recovery of areas affected by desertification. The characterization of microbial communities is undoubtedly one of the first steps to invest in technologies that enable soil recovery mechanisms. Suppose we can visualize what we have, what we've lost, and what's recoverable. In this case, we took an important step towards building a theoretical foundation that seeks an efficient strategy to combat the desertification of the sertão, valuing the permanence of its people and providing decent living conditions.

Therefore, there is a clear need for intensive studies in the setting up of experimental tests that consider multiple regions and establish relationships between them, mainly using previous knowledge, as is the case of the Acaulosporaceae and Glomeraceae FMA families, which are presented as key groups in desertified areas. . We must know the functional attributes of native plants, soil organisms and their relationships so that we can make appropriate predictions of how dry forest ecosystems such as the Caatinga will respond to management changes, environmental changes and climate events in the coming decades. We need to be prepared with ecological approaches to the conservation of the Caatinga and efficient and sustainable management.