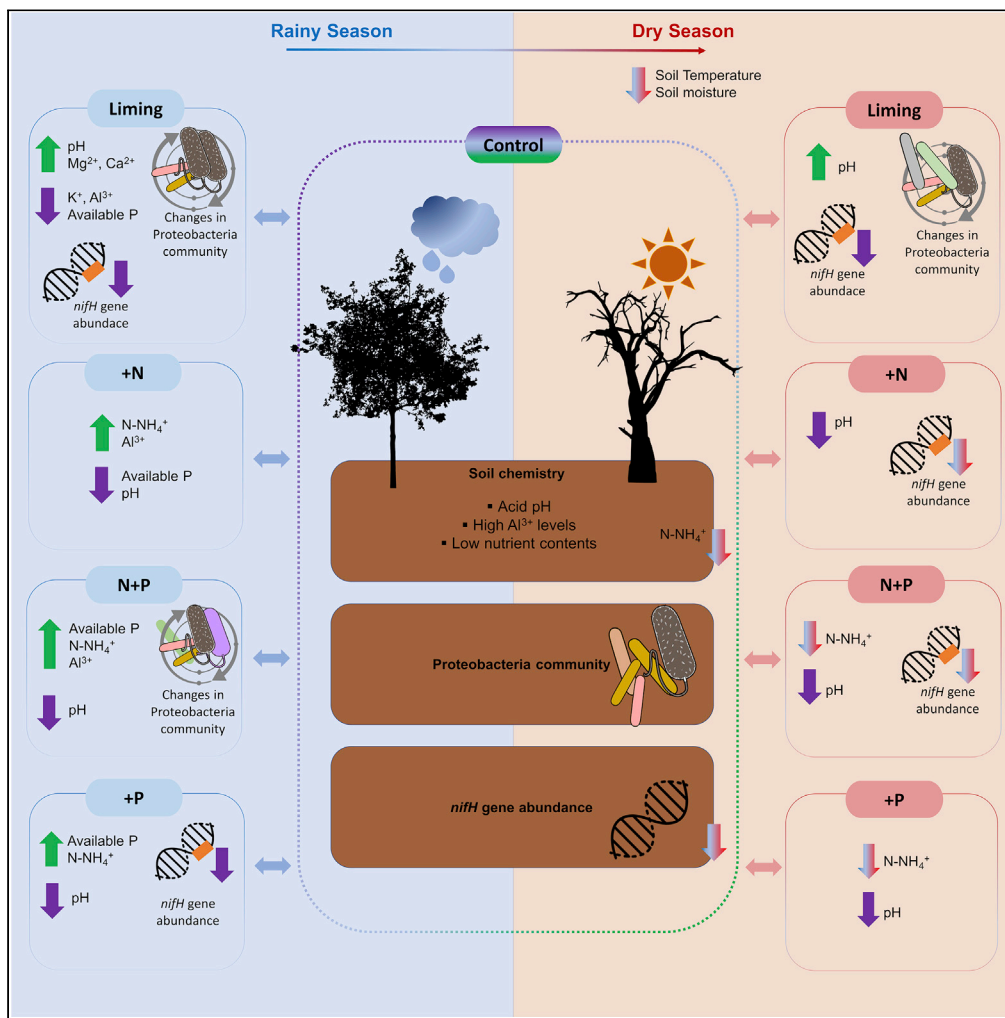## Article

Seasonal and long-term effects of nutrient additions and liming on the nifH gene in cerrado soils under native vegetation

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Highlights

First assessment of nutrient addition effects on nifH gene abundance in Cerrado soils

Increases in soil pH and nutrient contents negatively affected nifH gene abundance

nifH gene relative abundance was positively affected by precipitation's seasonality

Reduction in potential BNF suggests that eutrophication can induce long-term changes

Article

Seasonal and long-term effects of nutrient additions and liming on the nifH gene in cerrado soils under native vegetation

Rafaella Silveira,[1,3,*] Thiago de Roure Bandeira de Mello,[2] Maria Regina Silveira Sartori,[2] Gabriel Sérgio Costa Alves,[1] Fernando Campos de Assis Fonseca,[1] Carla Simone Vizzotto,[1] Ricardo Henrique Krüger,[1] and Mercedes Maria da Cunha Bustamante[2,*]

SUMMARY

Biological nitrogen fixation (BNF) represents the main input source of N in tropical savannas. BNF could be particularly important for Brazilian savannas (known as Cerrado) that show a highly conservative N cycle. We evaluated the effects of seasonal precipitation and nutrient additions on the *nifH* gene abundance in soils from a long-term fertilization experiment in a Cerrado's native area. The experiment consists of five treatments: (1) control, (2) liming, (3) nitrogen (N), (4) nitrogen + phosphorus (NP), and (5) phosphorus (P) additions. The *nifH* gene sequence was related to Bradyrhizobium members. Seasonal effects on N-fixing potential were observed by a decrease in the *nifH* relative abundance from rainy to dry season in control, N, and NP treatments. A significant reduction in *nifH* abundance was found in the liming treatment in both seasons. The findings evidenced the multiple factors controlling the potential N-fixing by free-living diazotrophs in these nutrient-limited and seasonally dry ecosystems.

INTRODUCTION

Over the last decades, the scientific community's attention has increased toward the substantial role of tropical regions in many biogeochemical cycles, especially on the N budget. The nitrogen cycle is a complex set of processes (Box 1, Figure 1) vastly mediated by soil microorganisms (Swift et al., 1998; Galloway et al., 2004; Reed et al., 2010; Hietz et al., 2011; Pajares and Bohannan, 2016). The majority of studies focus on forest ecosystems (Hietz et al., 2011; Homeier et al., 2017; Figueiredo et al., 2019; Brookshire et al., 2019). However, tropical savannas could also play a significant role in critical processes of the N cycle (Bustamante et al., 2006), such as in the biological nitrogen fixation (BNF), considered as the main source of N in these areas with estimated inputs ranging from 16 to 44 kg N ha^{-1} year^{-1} (Cleveland et al., 1999).

The Brazilian Cerrado is the second largest biome in South America, covering about 24% of the national territory and predominates in central Brazil (Ribeiro and Walter, 2008). It is one of the most heterogeneous and biodiverse tropical savannas worldwide, known to support a highly complex vegetation structure that evolved under interactions between water and nutrient availability (Myers et al., 2000; Reatto et al., 2008; Silva et al., 2008; Haridasan 2008). The soil microbial communities in this biome have been studied in terms of composition, diversity, and richness (Quirino et al., 2009; Viana et al., 2011; Araujo et al., 2012; Rampelotto et al., 2013; Pereira de Castro et al., 2016). However, information on the role of microorganisms in nitrogen cycling remains scarce. In particular, information on the impact of nutrient enrichment in soil microbiome from natural areas has been poorly discussed.

Nitrogen is the main component of proteins and nucleic acids, and therefore, it is considered as one of the most critical elements for the existence of life on Earth. The N turnover comprises a series of complex and dynamic reactions strongly dependent on environmental oxidizing or reducing conditions. These reactions (Box 1, Figure 1) are typically divided into BNF, nitrification, denitrification, anaerobic ammonia oxidation (*anammox*), and ammonification processes (Canfield et al., 2010; Stein and Klotz, 2016) catalyzed by specific microbial enzymes (Correa-Galeote et al., 2014). In Cerrado soils, the most substantial fraction of inorganic N in the soil is N-NH$_4^+$, and the N cycle stages that occur under anaerobic conditions little contribute

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> **Box 1. The nitrogen cycle: a complex set of reactions performed almost exclusively by microbial enzymes**
>
> Nitrogen is a highly dynamic element in nature, occurring in different chemical forms strongly influenced by environmental oxidative and reductive conditions. The N biogeochemical cycle (Figure 1) involves a high number of chemical transformations catalyzed by a wide variety of microbial enzymes (Correa-Galeote et al., 2014). The N cycle integrates dissimilative, assimilative, and decomposition processes (Levy-Booth et al., 2014). Briefly, N-fixing microbial communities that express the nitrogenase enzyme, encoded by the *nifH* gene, reduce the atmospheric N_2 to NH_4^+ in a dissimilative process. The available NH_4^+ can pass through two metabolic routes: (1) the dissimilatory pathway in which NH_4^+ is transformed into hydroxylamine (NH_2OH) by the activity of the enzyme ammonia monooxygenase (encoded by the *amoA* gene) present in ammonium oxidizing bacteria and archaea or (2) the immobilization by plants and microorganisms in an assimilative process of N-NH_4^+. In the dissimilative processes, the NH_2OH is quickly oxidized to NO_2^- by microorganisms that express the *hao* gene, which encodes a hydrazine oxidase associated with nitrification (includes the nitritation and nitratation) and also with the *anammox* process. Following the nitrification's metabolic pathway, the NO_2^- can be oxidized to NO_3^- by a nitrate oxidoreductase synthesized by microbial communities that express *nxr* genes. Plants or microorganisms assimilate the NO_3^- or it can be directly reduced to NH_4^+ by the dissimilative nitrate reduction to ammonium (DNRA) or even return to NO_2^- in another dissimilative reduction process when the *napA*, *narG*, or *nasA* genes are expressed. These genes encode nitrate reductases involved in the initial stage of denitrification. The NO_2^- (from the dissimilative reduction process or generated in the nitritation stage) can return to NH_4^+, be directly reduced to gaseous forms, or pass through the *anammox* process before being reduced to N_2. Nitrite reductases encoded by the *nirA* gene catalyze the reduction of NO_2^- to NH_4^+. The reduction of NO_2^- to the gaseous form NO involves nitrite reductases encoded by genes such as *nirS* and *nirK*. The *norB* genes encode nitric oxide reductases that catalyze the NO reduction to N_2O, and the *nosZ* gene encodes nitrous oxide reductases that catalyze the N_2O conversion to N_2. In the *anammox* process, either NH_4^+ or NO_2^- which have undergone the partial nitrification process or resulting from the dissimilative reduction of NO_3^- can be converted to hydrazine (N_2H_4) and then to N_2 from the activity of a hydrazine oxidoreductase, encoded by *hzo* gene. In assimilative pathways, the N-NH_4^+ or N-NO_3^- are immobilized by microbes and plants. The element N can return to the cycle through decomposition. The organic nitrogen compounds (N_{org}) are degraded during the litter decomposition and can be immobilized again by plants or microorganisms. The N_{org} could also be mineralized into NH_4^+, continuing the cycle. The N_{org} mineralization or ammonification can be catalyzed by several other enzymes, such as chitinase, encoded by the *chiA* gene.

to the N balance (Nardoto and Bustamante, 2003). For example, net nitrification in native Cerrado soils is often undetectable (Nardoto and Bustamante, 2003), and emissions of NOx and N_2O are very low, suggesting a highly conservative N cycle (Pinto 2002; Fernandes Cruvinel et al., 2011). Thus, BNF can represent the most critical N metabolism process in Cerrado soils, as observed for many terrestrial ecosystems, where this process represents about 97% of natural N inputs (Vitousek et al., 2002; Galloway et al., 2004).

In BNF, N_2 is assimilated and transformed only by a select group of microorganisms that can be plant symbionts or free-living diazotrophs making the understanding of the microbial ecology involved essential (Reed et al., 2010; Pajares and Bohannan, 2016). These microorganisms are capable of expressing the nitrogenase enzyme codified by *nif* genes. The *nifH* gene has been accessed with molecular techniques for studies on microbial communities' potential to fix atmospheric N (Gaby and Buckley, 2011, 2012; Pajares and Bohannan, 2016). This gene encodes the iron-protein subunit of the nitrogenase enzyme complex in bacteria and Archaea (Zehr et al., 2003), whose role is to catalyze the reduction of N_2 to NH_4^+ in the BNF, a high energy-demanding process (Zehr et al., 2003; Shridhar, 2012). From this process, reactive N forms required for macromolecule biosynthesis are made available for plant uptake. Since the *nifH* gene is widely distributed between bacteria and Archaea with conserved sequences in both domains (Zehr et al., 2003), it may be considered as a useful biological marker to infer the ecological role and potential of the microbial community in N-fixing (Gaby and Buckley, 2012). Proteobacteria members and some other taxonomic lineages within Actinobacteria, Cyanobacteria, and Firmicutes contain the *nifH* gene (Mirza et al., 2014; Dahal et al., 2017). These bacterial groups and specially Proteobacteria are highly abundant in Cerrado soils (Quirino et al., 2009; Souza et al., 2016; Pereira de Castro et al., 2016).

Cerrado's soil microbiome is sensitive to soil chemical characteristics and management (Souza et al., 2016; Silva et al., 2019). It also responds to the marked seasonality that regulates the water availability in the biome (Pereira de Castro et al., 2016). Likewise, BNF could be affected by environmental changes promoted by liming and nutrient additions into soils (both standard practices in agricultural areas in the Cerrado) since the content as well as the ratios between nutrients can affect the function of these organisms
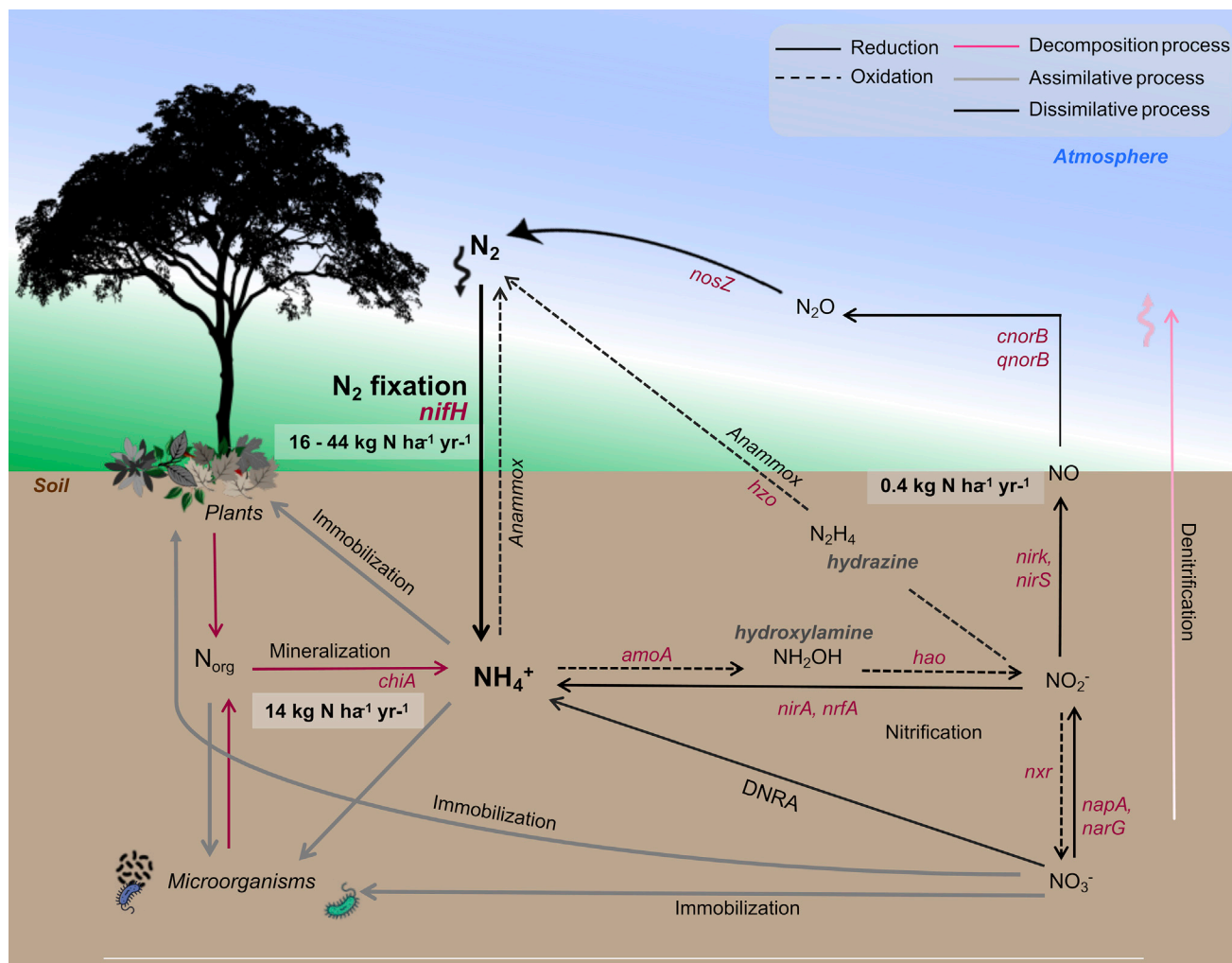
Figure 1. Nitrogen cycle
Schematic representation of major pathways in the N cycle occurring in the atmosphere-soil interface. The microbial genes involved in each step are indicated. The values in the figure represent the fluxes in the N budget measured for the cerrado *sensu stricto* described in a review by Bustamante et al. (2006). *Anammox*, anaerobic ammonia oxidation; DNRA, dissimilative nitrate reduction to ammonium; N_{org}, organic nitrogen. Assimilative processes are indicated by gray arrows. Dissimilative processes are indicated by solid dark arrows. Decomposition is indicated by colored solid arrows. Reduction and oxidation reactions are represented by solid and dashed arrows, respectively.

(e.g., Vitousek et al., 2013; Weisany et al., 2013). A previous study showed that fertilization with N and N combined with P altered other N metabolism process in Cerrado soils under native vegetation (Jacobson et al., 2011). There was an increase in NOx emissions when only the N was added into the soils. On the other hand, NOx emissions were lower when the N fertilizer was added together with P (Jacobson et al., 2011). These results indicate a greater immobilization of N in the biomass and reinforce the co-limitation of the N cycling in Cerrado soils by N and P (Jacobson et al., 2011). Also, the seasonal distribution of rainfall determines the microbial activity in Cerrado soils with an $N-NO_3^-$ accumulation for short periods after the first rain events and subsequent immobilization (Nardoto and Bustamante, 2003). However, evidence of ecological determinants of microbial groups' potential fixation activity in the Cerrado soils under native vegetation is quite scarce. To understand the potential impacts of nutrient additions and liming under N_2 fixation in Cerrado soils, we investigated how it could affect the abundance of *nifH* genes in soils from a long-term fertilization experiment in a natural area of Cerrado. We expected that increase in mineral nitrogen availability would affect N-fixing free-living microorganisms as it occurs in symbiotic associations. Thus, we hypothesized that the addition of nitrogen and nitrogen combined with phosphorus would reduce the abundance of the *nifH* gene of free-living N-fixers. The effect would be more evident with N

Table 1. Soil properties

Variable	Rainy					Dry				
	Control	Ca	N	NP	P	Control	Ca	N	NP	P
Moisture (%)	37.83	38.30	36.20	37.10	38.90	25.83	20.13	24.13	25.17	25.50
	±3.25	±1.53	±1.68	±1.17	±0.15	±0.41	±1.56	±0.96	±1.23	±0.72
Temperature (°C)	22.07	21.25	22.75	21.63	22.63	18.17	19.06	18.37	18.47	18.17
	±0.42	±0.26	±1.30	±0.90	±0.81	±0.38	±0.19	±0.79	±0.65	±0.16
pH (H_2O)	4.67	6.27	3.76	3.73	4.08	4.00	6.11	3.64	3.95	3.73
	±0.21	±0.11	±0.07	±0.11	±0.08	±0.09	±0.09	±0.04	±0.30	±0.08
$N-NO_3^-$ (mg/kg)	2.27	2.22	2.18	1.64	2.60	1.61	1.73	0.88	1.04	1.37
	±0.25	±0.62	±0.61	±0.54	±0.61	±0.14	±0.30	±0.18	±0.72	±0.26
$N-NH_4^+$ (mg/kg)	25.13	24.37	43.78	96.43	37.04	9.02	8.32	6.58	8.44	6.68
	±8.95	±7.13	±15.02	±66.24	±25.21	±1.16	±1.35	±0.41	±2.17	±0.19
TN (%)	0.19	0.17	0.18	0.20	0.19	–	–	–	–	–
	±0.02	±0.01	±0.02	±0.02	±0.02					
TC (%)	3.66	3.46	3.40	3.64	3.65	–	–	–	–	–
	±0.60	±0.15	±0.49	±0.36	±0.37					
C:N	18.85	20.42	18.65	18.54	19.02	–	–	–	–	–
	±0.87	±0.28	±0.38	±0.29	±0.41					
P (available, mg/dm³)	1.70	0.65	1.28	6.98	6.60	–	–	–	–	–
	±0.36	±0.33	±0.13	±2.86	±2.67					
K (mg/dm³)	28.75	10.75	22.75	28.50	26.75	–	–	–	–	–
	±6.18	±1.89	±3.40	±3.32	±2.50					
Mg^{2+} ($cmol_c/dm^3$)	0.11	2.73	0.08	0.13	0.10	–	–	–	–	–
	±0.03	±0.15	±0.01	±0.08	±0.02					
Ca^{2+} ($cmol_c/dm^3$)	0.19	4.69	0.15	0.40	0.30	–	–	–	–	–
	±0.05	±0.20	±0.02	±0.17	±0.06					
Al^{3+} ($cmol_c/dm^3$)	0.94	0.00	1.38	1.46	1.07	–	–	–	–	–
	±0.17	±0.00	±0.15	±0.14	±0.24					

Soils (0-10 cm depth) were collected in the rainy and dry seasons of 2018 in a long-term fertilization experiment in a typical cerrado area located at Reserva Ecológica do IBGE, Brasília, Brazil. Results are expressed as mean values ±standard error. Control, untreated control; Ca, liming; N, nitrogen addition; NP, nitrogen and phosphorus addition; P, phosphorus addition; TC, total carbon; TN, total nitrogen.

and P combined supplementation due to a reduction of phosphorus limitation which would favor nitrogen assimilation. Additionally, we hypothesized that increased soil pH in response to liming could increase *nifH* gene abundance.

RESULTS

Soil parameters

Soil moisture (37.6% and 24.3%) and temperature (22.2°C and 18.4°C) decreased from the rainy to the dry season (Table 1). The inorganic N content ($N-NO_3^-$ and $N-NH_4^+$) also showed marked seasonal variation, decreasing concentrations from rainy to the dry season in all treatments (Table 1). The soil pH showed less seasonal variation but was strongly affected by the treatments. In comparison with the control treatment, there was an increase in soil pH in the liming treatment (control vs. liming—rainy: 4.67 vs. 6.11; dry: 4.00 vs. 6.27), N (rainy: 3.76; dry: 3.64), NP (rainy: 3.73; dry: 3.95), and P treatments (rainy: 3.73; dry: 3.95) (Table 1).

Other soil chemical characteristics such as total carbon (TC), total nitrogen (TN), and concentrations of available P, K^+, Mg^{2+}, Ca^{2+}, and Al^{3+} were determined during the rainy season (Table 1). TC varied between 3.66% in control and 3.40% in N treatment; the maximum TN percentage was 0.20% in NP, and the minimum was 0.17% in liming treatment, mean values of the CN ratio varied between 20.42 in liming and 18.54 in N

plots. We observed almost three-fold reduction in available P content in liming plots and an increased about four-fold in the NP and P treatment (control: 1.70 vs. liming: 0.65; N: 1.28; NP: 6.98; P: 6.60 mg/dm^3). The K content decreased approximately three-fold in the liming treatment compared to control plots (control: 28.75 vs. liming: 10.75 mg/dm^3), while Mg^{2+} (control: 0.11 vs. liming: 4.69 cmol$_c$/dm^3) and Ca^{2+} (control: 0.19 vs. liming: 4.69 cmol$_c$/dm^3) increased about 25-fold in the liming treatment (Table 1). The Al^{3+} contents were zero in the liming treatment (control: 0.94 vs. liming: 0.00 cmol$_c$/dm^3) but increased in the NP treatment (1.46 cmol$_c$/dm^3) compared with control plots (Table 1).

Taxonomy characterization of *nifH* gene and 16S rRNA taxonomic links

The BLAST nucleotide alignment result showed that the *nifH* gene sequence recovered from three cloned fragments was classified in the Proteobacteria cluster, comprising sequences most closely related to *Bradyrhizobium* members (over 93% identical; see Table S1).

From the previously filtered 16S rRNA database (Silveira et al., 2020), we analyzed a total of 612,745 good-quality sequences representing the Proteobacteria group. The result corresponded to 3,863 taxa comprising three classes, 71 orders, 122 families, and 236 genera. The taxonomy tree based on this data set showed a substantial difference in the abundance of Proteobacteria members between the NP treatment and untreated control during the rainy season (Figure 2A). Proteobacteria abundance in P treatment also differed from N and liming treatments and in liming compared with NP treatment (Figure 2A). During the dry season, Proteobacteria member abundances differed mainly in the comparisons between liming versus all treatments, including the control plots, with lesser differences in the other pairwise comparisons (Figure 2A). Taxonomic groups inside Proteobacteria phylum comprised the genera *Rhodoplanes*, *Methylovirgula*, *Rhodomicrobium*, *Roseiarcus*, *Pedomicrobium*, *Rhizobium*, *Bradyrhizobium*, and others affiliated to Alphaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria classes represented in Figure 2A.

The principal coordinate analysis (PCoA) ordination based on Bray-Curtis dissimilarity shown a clear difference in Proteobacteria community distribution among liming samples and all the other treatments, including control (Figure 2B). The N and NP treatments also differed in Proteobacteria community distribution compared with control. The first two PCoA axes explain approximately 60% of data variability (Figure 2B).

Seasonal and nutrient addition effects on *nifH* gene abundance

The relative abundance of the *nifH* gene was affected by the seasonality of precipitation (p < 0.001) (Figure 3). Relative abundance of the *nifH* gene counted over four-fold higher during the rainy season in the control plot (p < 0.001) and more than two-fold higher in the N (p < 0.01) and NP (p < 0.001) plots during the same sampling period compared with the dry season.

During the rainy season, the *nifH* gene relative abundance decreased approximately ten- and two-fold in the liming (p < 0.001) and P (p < 0.05) treatments compared to control plots, respectively (Figure 3). Also, the *nifH* gene abundance was about six-fold lower in the liming treatment compared to N and NP treatments (p < 0.001) and about four-fold lower than the relative counts in P treatment (p < 0.001). There were no differences in *nifH* relative abundance in the N and NP treatments compared to control (p > 0.05). In the dry season, the relative abundance of *nifH* decreased about four-fold in liming treatment compared to control (p < 0.001). Again, the liming treatment showed lower *nifH* abundance than N, NP, and P treatments (between four and five-fold lower; p < 0.001). There were no differences between these last three treatments and control plots (p > 0.05). The *nifH* gene relative abundance was positively correlated with soil moisture (0.48, p < 0.01) and with the N-NH$_4^+$ content (0.38, p < 0.05) (Figure 4).

DISCUSSION

The soil microbial component constitutes the base for terrestrial ecosystem functioning. Here, we report a comprehensive *nifH* gene evaluation as a proxy to depict the interactive effects between precipitation and the long-term nutrient addition on N-fixing activity by diazotrophic soil community. Firstly, the *nifH* cloned fragment's phylogenetic characterization indicated the sequence taxonomy identity related to *Bradyrhizobium* members. The genus *Bradyrhizobium* comprises a group of N-fixing rhizobia in Proteobacteria that could be called generalists with versatile metabolism and ecological relationships. For example,
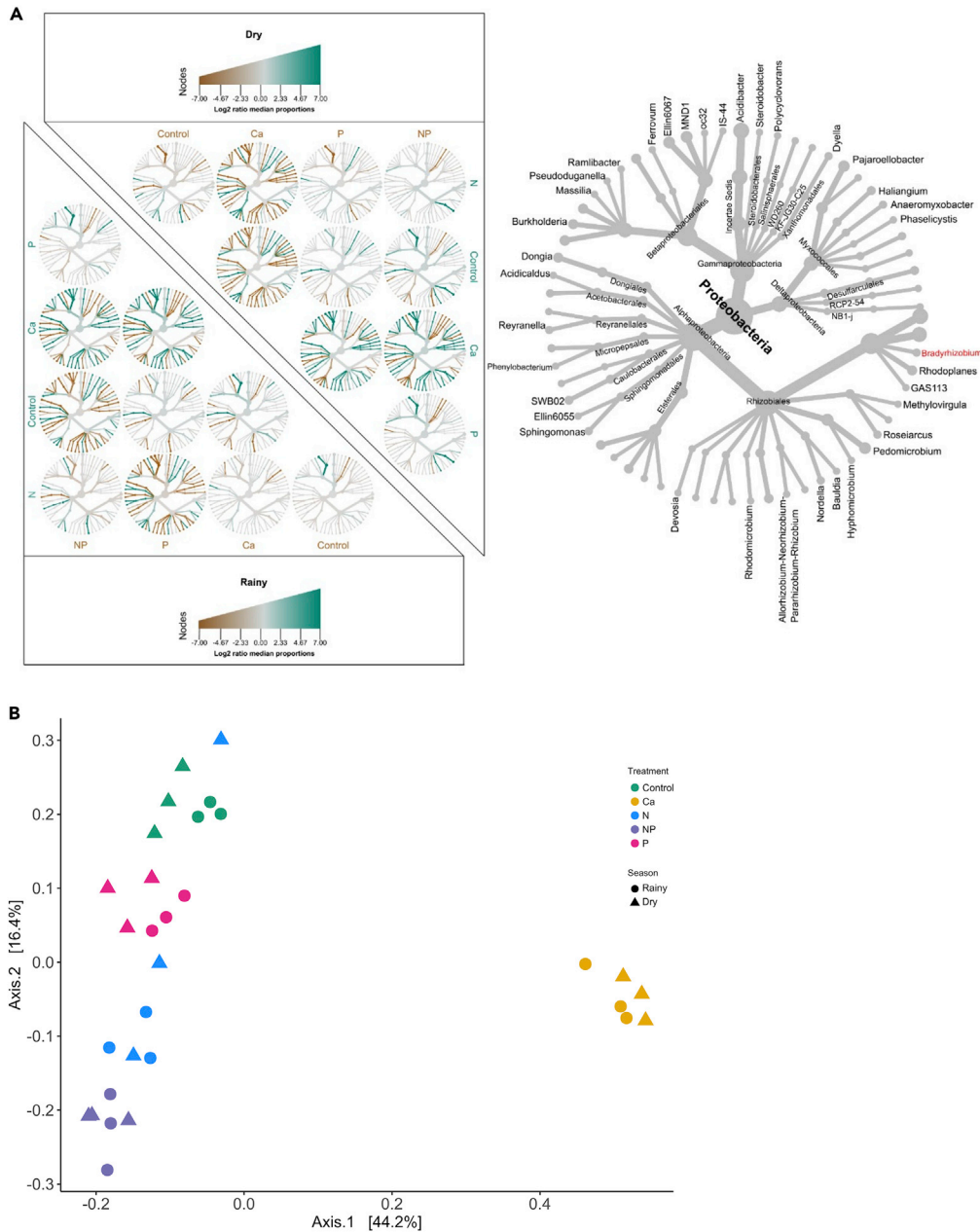
Figure 2. Proteobacteria community

Hierarchical taxonomy of Proteobacteria (A) and principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity (B). Members of Proteobacteria were recovered from the 16S rRNA gene in the soil samples (0–10 cm depth) collected in the rainy and dry seasons of 2018. Soils were collected from a typical cerrado area where the long-term fertilization experiment was installed, in the Reserva Ecológica do IBGE, Brasília, Brazil. The gray tree on the right represents a key for the unlabeled trees. Each of the smaller trees represents a comparison between the treatments in the columns and rows. Node size represents the relative proportions for that taxon. A taxon colored brown is more abundant in the treatment in the column, and a taxon colored green is more abundant in the treatment of the row. Control, untreated control; Ca, liming; N, nitrogen addition; NP, nitrogen and phosphorus addition; P, phosphorus addition.

they can include plant-interactive or free-living microorganism species (Kahindi et al., 1997; van Elsas et al., 2019). In the present study, we evaluated *nifH* DNA sequences from bulk soil and predicted the *nifH* gene sequence to belong to the putative non-symbiont diazotrophic community. Previous studies in tropical rainforest soils in Costa Rica (Reed et al., 2010) and in Amazon (Mirza et al., 2014) also have reported
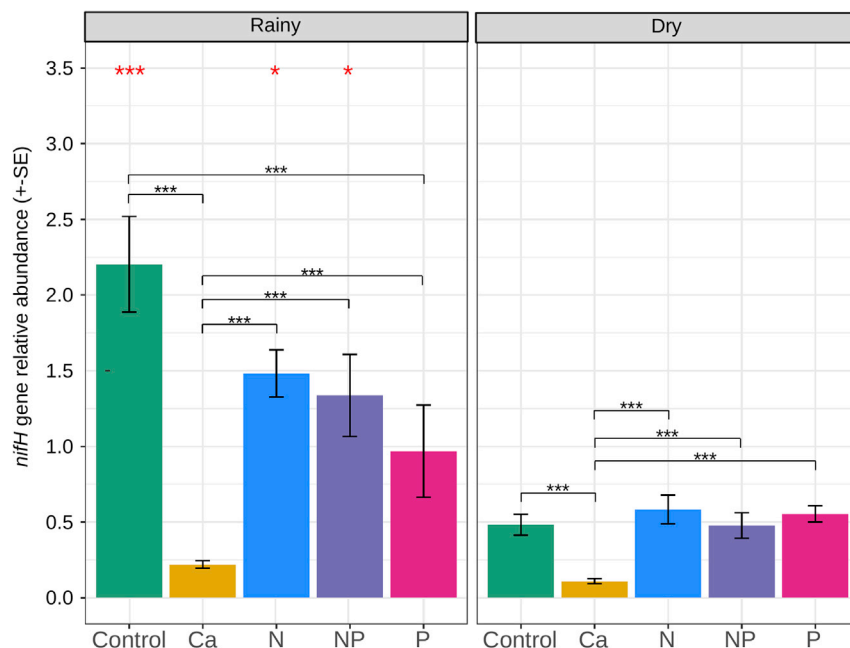
Figure 3. *nifH* gene abundance

Relative abundance of *nifH* gene in the cerrado' soil samples based on calibrated normalized relative quantities (CNRQ values) generated in the qbase + software. The relative abundance of *nifH* gene was measured from soils (0–10 cm depth) collected in the rainy and dry seasons of 2018 in a long-term fertilization experiment in a typical cerrado area, located at Reserva Ecológica do IBGE, Brasília, Brazil. Control, untreated control; Ca, liming; N, nitrogen addition; NP, nitrogen and phosphorus addition; P, phosphorus addition. Bar plots and error bars represent the average and standard errors between technical replicates, respectively. Red asterisks indicate significant differences in *nifH* relative abundance between rainy and dry seasons for the same treatment. Black asterisks indicate significant differences in *nifH* relative abundance among treatments. *** = p < 0.01; * = p < 0.05.

nifH clone sequences associated to free-living or associative Alphaproteobacteria, including the genera *Gluconacetobacter*, *Azospirillum*, *Burkholderia*, *Bradyrhizobium*, and others.

The Proteobacteria community taxonomic diversity data set has shown differences in Proteobacteria member abundance and distribution among treatments in the rainy and dry seasons. It could suggest the potential effects of nutrient addition, promoting shifts in Proteobacteria assembly or in diazotrophic microorganisms affiliated to other taxonomic groups. These changes could also be associated with the decrease in *nifH* gene abundance (presumably negative effect in N_2 fixation) in the treated plots, revealing the potential reduction in the microbial N-fixing process promoted by nutrient additions.

In general, *nifH* abundance was relatively smaller in all nutrient addition plots, with more pronounced differences in the comparisons with liming treatment. The unexpected significant decrease in *nifH* gene copy numbers in liming plots could be related to the combined changes in soil chemical parameters (Han et al., 2019) since environmental conditions strictly control the nitrogenase enzyme (Poly et al., 2001; Huergo et al., 2012; Han et al., 2019). The major environmental change promoted by liming into soils was the pH increase. Furthermore, there was also an increase in nutrient contents such as exchangeable Ca^{2+} and Mg^{2+} and concomitant decreasing in K^+, available P levels, and Al^{3+} immobilization. A previous soil evaluation indicated a reduction of approximately three-fold in Fe contents in the liming treatment, even after nine years without new additions in our study area (unpublished data). Iron represents an essential element to the nitrogenase proteins complex (Zehr et al., 2003; Gaby and Buckley, 2011), which could be extensively demanded by diazotrophs (Mills et al., 2004; Larson et al., 2018). Although studies including the interactive effects among environmental controls on free-living N-fixing are scarce, similar results were observed in a long-term acidic Ultisol fertilization experiment located in a subtropical monsoon climate region from China (Lin et al., 2018). The researchers found a substantial decrease in *nifH* gene abundance in plots under NPK plus lime fertilization, associated with the increase in soil pH. The authors also indicated shifts in
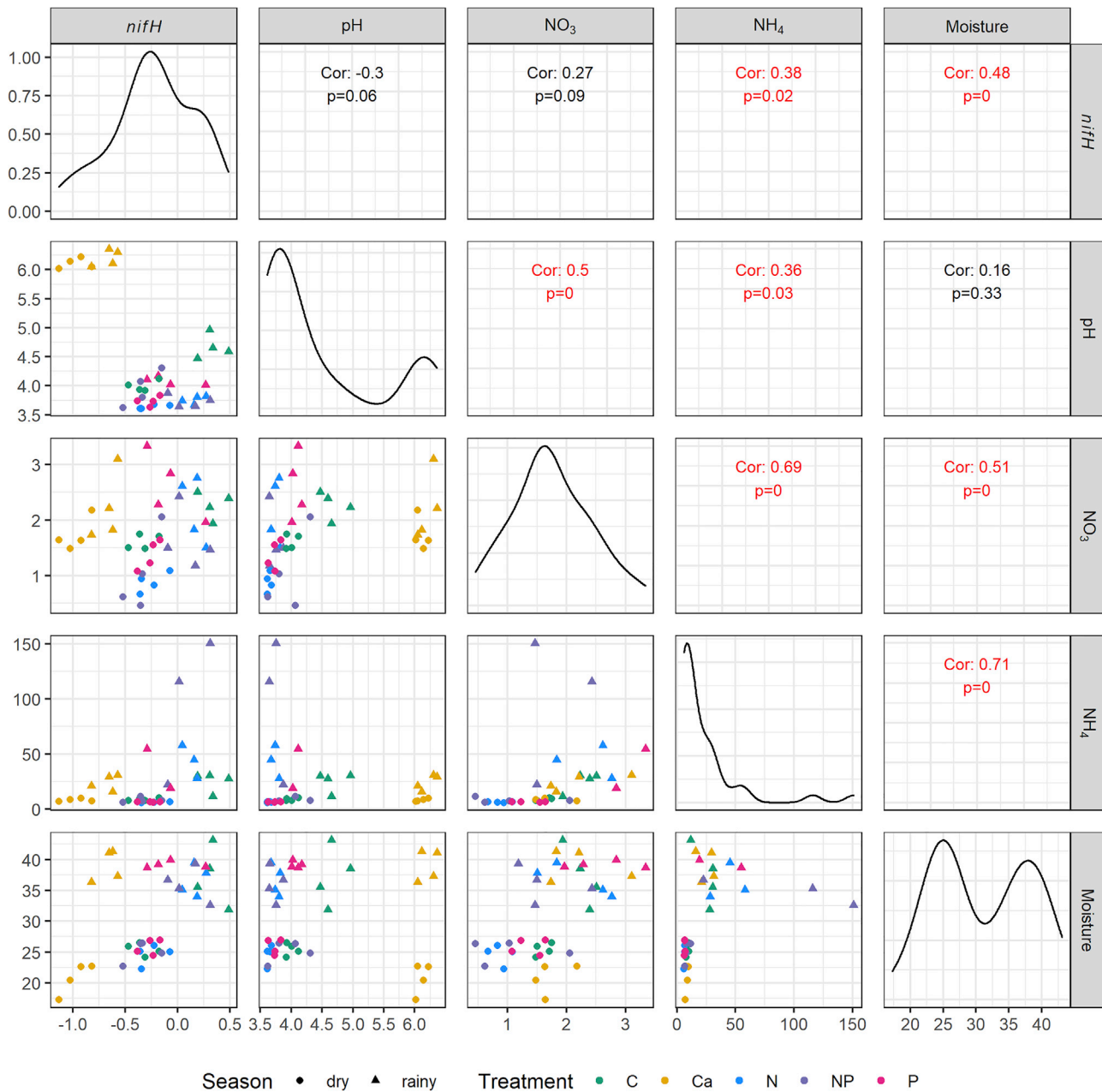
Figure 4. Soil parameters and *nifH* relative abundance

Spearman's correlation between soil parameters and *nifH* gene relative abundance in soils (0–10 cm depth) from a typical cerrado area where the long-term fertilization experiment was installed in the Reserva Ecológica do IBGE, Brasília, Brazil. Significant correlations and their respective p values are highlighted in red. Control, untreated control; Ca, liming; N, nitrogen addition; NP, nitrogen and phosphorus addition; P, phosphorus addition.

microbial community composition, replacing the dominant *Bradyrhizobium* genus by *Azohydromonas* in the treatment submitted to lime addition (Lin et al., 2018).

The *nifH* gene abundance was also reduced in P treatment during the rainy season. In this treatment, there was an increase in the P available content and a slight reduction in soil pH. The importance of P availability to N_2 fixation is recognized in several studies indicated by Bustamante et al. (2006) in a review of the nitrogen cycle in tropical and temperate savannas. The available P contents are described as a limiting factor to N_2 fixation in P-deficient soils in the tropics (Bustamante et al., 2006; Van Langenhove et al., 2019) and could

be critical to activate genes for the nitrogenase synthesis (Stock et al., 1990). However, although the increase in P availability is usually described to increase N_2 fixation, the free-living diazotrophs could perform BNF over a broader range of phosphorus supply or accessibility than symbionts (Smercina et al., 2019), suggesting other controls to *nifH* abundance for this group.

In a review about the pivotal players controlling nitrogenase activity, Huergo et al. (2012) highlight the evolved mechanisms in diazotrophs to shut down N_2 fixation when $N-NH_4^+$ is available in the environment to avoid energy waste during the N_2 reduction in BNF process. Similarly, the $N-NH_4^+$ was indicated as one of the main drivers of the *nifH* abundance variation across different agricultural soils, where lower gene copy numbers were related to increased $N-NH_4^+$ levels (Pereira e Silva et al., 2013). Here, the *nifH* relative abundance was negatively affected in plots with higher $N-NH_4^+$ levels and also in the liming treatment, where $N-NH_4^+$ showed a minor variation compared with control. Thus, the contradictory low positive correlation found between *nifH* abundance and $N-NH_4^+$ in our study could not reflect a linear relationship because the variability in $N-NH_4^+$ contents in the soil was more pronounced between seasons due to changes in soil moisture. This result reinforces the understanding that gene abundance could be determined by seasonal and long-term interactive effects in soil chemistry and biological components, such as interactions between soil moisture and $N-NH_4^+$ contents and the pervasive long-term alterations in pH and nutrient availability.

The N cycling in Cerrado ecosystems is conservative (Bustamante et al., 2009). The N conservation mechanisms in these ecosystems could be also associated with the low abundance and activity of genes involved in the nitrification process such as *amoA*, in native Cerrado soils (Catão et al., 2017). However, a previous study in our experimental area showed that the N addition into soils resulted in a rise in NOx emissions to the atmosphere (Jacobson et al., 2011). Our results point to a reduction in potential BNF, indicating that eutrophication can induce long-term changes.

The seasonal dynamic of non-symbiotic N-fixing in Cerrado soils is poorly understood. Our analysis indicated a strong seasonal influence on *nifH* abundances. Reducing *nifH* gene abundance in control, N, and NP plots was observed in the dry season. The seasonal modulation in *nifH* abundance may be associated with a high decrease in the soil moisture and temperature during the dry season. It could promote greater oxygen gas diffusion across soil aggregates (Tipping, 2004), increasing aerobic conditions representing a critical inhibitor of nitrogenase activity (Fay, 1992; Norman and Friesen, 2017). Also, aerobic conditions demand substantial energetic resource investments to nitrogenase protection in free-living soil diazotrophs (Norman and Friesen, 2017). Some diazotroph bacteria could even cease nitrogenase production in the presence of high oxygen levels (Bruijn, 2015; Hill, 1988; Reed et al., 2010), which could contribute to explain the decrease in trend in *nifH* abundance during the dry season.

Limitations of the study

It is essential to highlight that the observed *nifH* gene abundance does not indicate the N_2 fixation rate level but represents a proxy to explore the potential N_2 fixation by free-living diazotrophs. Thus, further evaluations on *nifH* sequencing, *nifH* gene expression based on RNA reverse transcription, and isotope technology will be necessary to infer N-fixing rates to specific diazotrophs taxa and changes in the microbial functions in response to environmental changes promoted by precipitation seasonality and by nutrient additions and liming.

CONCLUSIONS

Overall, the increase in nutrient availability and soil pH negatively affected the *nifH* gene abundance. In contrast, the higher moisture levels in the rainy period had seemed to contribute to the highest *nifH* relative proportions. Changes in Proteobacteria community distribution among treatments could also be related to variation in the observed *nifH* abundance since Proteobacteria harbor some important N-fixer groups. Our study is a pioneer for providing the first assessment of the effects of nutrient addition on *nifH* gene abundance in typical acid soils of Cerrado. Cerrado is a critical Brazilian biome both for agriculture and biodiversity conservation. It is currently strongly threatened by rapid and intensive land use changes and associated impacts. Understanding the potential functionality of soil microbial community in nutrient cycles, such as N cycle, represents a crucial advance to support future studies that could implement new approaches to soil preservation and sustainable use.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102349.

AUTHOR CONTRIBUTIONS

Conceptualization, M.M.C.B., R.H.K., and R.S.; methodology, M.M.C.B., M.R.S.S., G.S.C.A., and F.C.A.F.; validation, G.S.C.A. and R.H.K.; formal analysis, R.S. and T.R.B.M; investigation, R.S., G.S.C.A., and F.C.A.F.; writing – original draft, R.S.; writing – review & editing, M.M.C.B., R.H.K., M.R.S.S., C.S.V., and T.R.B.M.; funding acquisition, M.M.C.B. and R.H.K.; supervision, M.M.C.B. and R.H.K.; project administration, M.M.C.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

Seasonal and long-term effects of nutrient

additions and liming on the nifH gene

in cerrado soils under native vegetation

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Transparent Methods

Experimental area

The study was conducted in a long-term nutrient addition experiment (Kozovits et al., 2007) (Figure S1) in the Roncador Ecological Reserve of the Brazilian Institute of Geography and Statistics (Reserva Ecológica do IBGE, Recor IBGE), in Brasília, Federal District, Brazil (15º56'S, 47º53'W). The study area is a typical cerrado, a vegetation type characterized by continuous herbaceous layer associated with a shrubby-arboreal layer (Ribeiro and Walter, 2008). The soil in the area is a Red Latosol according to Brazilian Soil Taxonomy System (Santos et al., 2018), deep, clayey, acidic, with high levels of aluminum and low cation exchange capacity (Motta et al., 2002; Reatto et al., 2008; Ribeiro and Walter, 2008). According to the Köppen classification, the climate is categorized as AW, with a well-defined rainy and dry seasons (October to April, May to September, respectively). The wet period comprises about 90% of the annual rainfall, with an average of around 1,500 mm and warmer temperatures (Silva et al., 2008).

The experiment started in 1998 with biannual fertilizer additions applied manually on the litter layer at the transition period between rainy and dry season until 2006 (Jacobson et al., 2011). The experiment is composed of five treatments consisting of **1)** Control: without nutrient addition; **2)** Liming treatment (Ca): addition of 4 t ha^{-1} $year^{-1}$ of 60% dolomitic limestone (CaO+MgO) + 40% agricultural plaster ($CaSO_4.2H_2O$); **3)** N treatment: addition of 100 kg ha^{-1} $year^{-1}$ of nitrogen in the form of ammonium sulfate (($NH_4)_2SO_4$); **4)** P treatment: addition of 100 kg ha^{-1} $year^{-1}$ of simple superphosphate 20% - $Ca(H_2PO_4)_2$ + $CaSO_4.2H_2O$; and **5)** NP treatment: addition of 100 kg ha^{-1} $year^{-1}$ of ammonium sulfate together with 100 kg ha^{-1} $year^{-1}$ simple superphosphate 20%. Each treatment consists of four 15 x 15 m plots randomly distributed with a minimum distance of 10 m between them. Each plot is subdivided into nine subplots (5 x 5 m) (Figure S1). After 11 years without any chemical fertilizer addition, we resumed the experimental additions with applications in November 2017 and April 2018. We applied the liming treatment only in November 2017 with half of the doses previously described because of previous applications' residual effects. The soil sampling was done in March (rainy season) and August 2018 (dry season), representing four months after the first soil nutrient supplementation and four months after the second nutrient application.

Soil sampling

Soil samples (0-10 cm depth) were randomly collected in five different subplots per plot during the rainy (March) and dry (August) seasons of 2018. The five samples per plot were composed (avoiding roots) into a single representative sample of each of the four plots per treatment. Subsamples for molecular analyses were immediately frozen into liquid nitrogen in the field and then, stored in -80°C until downstream procedures. For the chemical analysis, the composed samples were sieved in a 2 mm mesh.
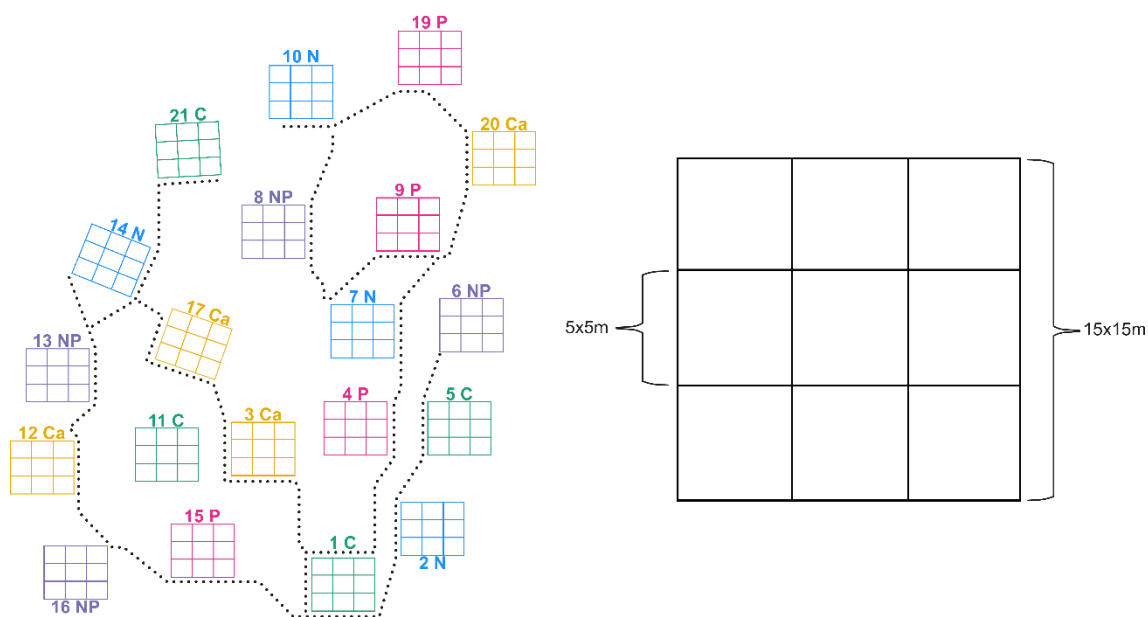
Figure S1 Nutrient additions experiment. Diagram of the experimental plots' distribution in the study area in a typical cerrado located at Reserva Ecológica do IBGE, Brasília, Brazil. Each treatment consists of four 15 x 15 m plots randomly distributed with a minimum distance of 10 m between them. Each plot is subdivided into nine subplots (5 x 5m). Treatments: C - control, Ca - liming, N - nitrogen, NP - nitrogen plus phosphorus, and P - phosphorus.

Soil chemical and physical properties

Soil collected in the rainy season was analyzed for Ca^{2+}, Mg^{2+}, Al^{3+}, K^+, available P (Mehlich I), and pH (water at 1:2.5) according to Empresa Brasileira de Pesquisa Agropecuária protocols (Embrapa, 1997). Total carbon (TC) and nitrogen (TN) contents were determined by combustion of samples under continuous helium flow, in an elemental analyzer (Carlo Erba, CHN - 1110) coupled to the mass spectrometer Thermo Finnigan Delta Plus. For soils from rainy and dry seasons, the $N-NO_3^-$ determination was carried out directly by UV-absorption at 218 nm, 228 nm, 254 nm, and 280 nm (Meier 1991). The $N-NH_4^+$ content was determined by colorimetric reaction with the Nessler reagent at 425 nm. The soil moisture was determined by oven drying samples at 105°C to constant weight. The soil temperature at 10 cm was evaluated *in situ* using Digi-Sense® thermometer.

DNA extraction, PCR, cloning and sequencing

Metagenomic DNA was extracted in duplicates from 0.5g of soil with the FastDNA® Spin kit (MP Biomedicals), following the manufacturer's protocol. An additional purification step was performed using PowerClean® DNA Clean-Up kit (Mo Bio Laboratories, Inc.). The DNA quality and quantity were evaluated in a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometra. The 16S rRNA and *nifH* genes were amplified in a first PCR round using the primer set 515F (5'-GTGYCAGCMGCCGCGGTAA-3') / 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al., 2015; Parada et al., 2016) and nifH-F (5'-AAAGGYGGWATCGGYAARTCCACCAC-3') / nifH-R (5'-

TTGTTSGCSGCRTACATSGCCATCAT-3') (Rosch et al., 2002), respectively. For both genes, the amplification was performed in a 20 µl PCR reaction mixture containing 1X PCR buffer, 2.0 mM MgCl2, 0.2 mM dNTP mix, 0.2 mM of each oligonucleotide primer, 0.5U Platinum™ Taq DNA Polymerase (ThermoFisher Scientific, Waltham, MA, USA), and 50 ng of DNA template. PCR conditions were as follows: 3 min at 95°C, followed by 30 cycles at 95°C for 60 s, annealing at 55°C (16S rRNA gene) or 63°C (*nifH*) for 60s, 72°C for 60 s, and the final extension at 72°C for 7 min.

The 16S rRNA and *nifH* PCR products were excised from 0.8% agarose gels and purified using PureLink Quick Gel Extraction kit (Thermo Fisher Scientific; Waltham, MA). After quantification on nanodrop, 100 ng of the purified 16S rRNA and *nifH* amplicons were cloned into a pGEM-T Easy cloning vector (Promega; Madison, WI) according to manufacturer's protocols and transformed into *E. coli* DH5α competent cells by heatshock method. Transformant colonies were inoculated into selective LB medium (100 µg/uL, ampicillin; 20 mg/mL, X-GAL and 100 mM/mL, IPTG) and incubated at 37 °C for 16 hours. Plasmid extraction was performed by alkaline lysis method (Sambrook, 2001) and the presence of the insert checked by digestion with *Eco*RI restriction enzyme (New England Biolabs, Ipswich, MA). Three positive clones for each gene were sequenced in reverse and forward strand using the Sanger method to confirm gene sequence and annotation.

After genes sequence confirmation, we performed the design and synthesis of non-degenerate 16S (16S-R1F: 5'-GTGTAGCGGTGAAATGCGTAGATAT-3'; 16S-R1R: 5'-ATCCTGTTTGCTCCCCACG-3', ~108 bp) and *nifH* (nifH-1F: 5'- TGTTGGCGGCATACATGGC-3', nifH-1R: 5'- TCACCTCGATCAACTTCCTGG-3', ~130 bp) primers to reduce possible bias in the qPCR results (Gaby and Buckley, 2017). The new primers design and Tm calculation were conducted in the Geneious software 10.1 (Biomatters Ltd., Auckland, New Zealand) based on genes sequence generated by Sanger sequencing.

Taxonomic links

The obtained *nifH* gene sequences were analyzed for quality using the PHRED algorithm (Ewing et al., 1998), using the Electropherogram quality analysis tool available on the Embrapa website: http://asparagin.cenargen.embrapa.br/phph/. The cloned sequences that showed a PHRED value greater than 20 with at least 350 nucleotides was selected. The taxonomic identity of *nifH* cloned fragments was conducted based on the ten significant alignments (> 200 bp; see Table S1) optimized for highly similar sequences on BLAST based on the Nucleotide Collection (nt) Database (Morgulis et al., 2008; Zhang et al., 2000).

We have filtered out our previous taxonomic dataset based on Illumina sequencing of the 16S rRNA gene (see Silveira et al., 2020, data available in NCBI Sequence Read Archive Bioproject PRJNA647807) to generate a heat-tree of the taxonomic diversity of the Proteobacteria. The Proteobacteria representation choice was considered because this phylum includes a large number of N-fixing bacteria, and is highly abundant in Cerrado soils. Also, the most representative *nifH* gene sequence in the present study was annotated inside this group

(see Results section). From the Proteobacteria dataset, we computed a hierarchical heat tree with taxonomic diversity (class, order, family, and genera) based on the log 2 ratio of the median proportions using the metacoder package in R (Foster et al., 2017). The comparisons were carried out among treatments of nutrient additions for each season. To evaluate Proteobacteria community distribution among samples, we carried out out a Principal Coordinates Analysis (PCoA) based on the Bray-Curtis dissimilarities using the phyloseq package in R (McMurdie and Holmes, 2013).

Standard curve for *nifH* and 16S quantification

External standard curves for absolute quantification of *nifH* and 16S copy numbers were generated from dilution series of cloned fragments containing known quantities of DNA copies (between 3.0 x 10² and 3.0 x 10⁹). As the vector and PCR insert sequences were known, the copy numbers of *nifH* and 16S were derived directly from the concentration of extracted plasmid DNA, using the following equation:

$$m = [n] \, [1.096 \, x \, 10 - 21 \, g \, / \, bp]$$

Where n =plasmid size (with insert); m =mass. A regression equation was developed plotting the Log10 values of plasmid copy numbers against CT values to obtain the slope (b) and y-intercept (a) and generate the correlation between copy numbers and CT values. The absolute copy number (CN) in each sample was estimated by using the following regression equation:

$$CN = antilog10 \, (Ct - a) \, / \, b$$

Quantitative PCR (qPCR)

The quantitative PCR (qPCR) method was used to quantify the *nifH* gene abundance (biological N fixation potential) from soil samples (0-10 cm) using the primers set *nifH*-1F/*nifH*1-R. The 16S rRNA gene abundance was estimated in samples using the primer set 16S-R1F/16S-R1R. The qPCR was performed in a StepOnePlus™ Real-Time PCR Systems (Life Technologies). qPCR assays were carried out in triplicates contained 5 µl of 1X Power SYBR™ Green PCR Master Mix (Applied Biosystems), 0.2 mM of each oligonucleotide primer, 50 ng of DNA template. PCR-grade water was used as a negative control. The PCR conditions for both, *nifH* and 16S rRNA genes were as follows: 2 min at 50°C, 2 min at 95°C, followed by 40 cycles at 95°C for 3 s and annealing at 60°C for 30 s. The melt curve protocol was 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. Product specificity was confirmed by melt curve.

Copy number calculations

The calibrated normalized relative quantities (CNRQ values) were generated in the qbase+ software version: 3.2. Relative quantities calculations were corrected for PCR efficiency. For each treatment, amounts of the target gene (*nifH*) and the reference gene (16S) were

assessed in the two seasons. The relative copy number (Q) of *nifH* vs. 16S gene was calculated using the following equation:

$$Q_R \; = \; \frac{N_{nifH}^{R}}{N_{16S}^{R}} \quad \text{and} \quad Q_D \; = \; \frac{N_{nifH}^{D}}{N_{16S}^{D}}$$

Where Q_R is the ratio of *nifH* vs. 16S copy numbers in the rainy season; Q_D is the ratio of *nifH* vs. 16S copy numbers in the dry season; N_{nifH}^{R} is the absolute copy number of *nifH* in the rainy season; N_{nifH}^{D} is the absolute copy number of *nifH* in dry season; N_{16S}^{R} is the absolute copy number of 16S in the rainy season; and N_{16S}^{D} is the absolute copy number of 16S in the dry season.

Statistical Analysis

Seasonal and treatment effects on relative quantities of *nifH* gene were evaluated by two-way ANOVA followed by the Tukey-Kramer HSD test for multiple comparisons ($p < 0.05$). Significant differences in gene relative counts were determined using the log-transformed data. Spearman's correlation was used to test the association between soil parameters and the relative quantities of *nifH* gene. All the statistical analysis was performed in R software.

Table S1 *nifH* sequence annotation. The ten significant alignments (> 200 bp) on BLAST *nifH* cloned fragment based on the Nucleotide collection (nt) database. Related to Figure 2.

Description	Query Cover (%)	Per. Ident (%)	Accession
Bradyrhizobium sp. strain BRUESC756 nitrogenase reductase (nifH) gene, partial cds	91	96.43	KY548259.1
Bradyrhizobium sp. strain BRUESC765 nitrogenase reductase (nifH) gene, partial cds	97	94.39	KY548268.1
Bradyrhizobium sp. strain BRUESC978 nitrogenase reductase (nifH) gene, partial cds	93	95.56	KY548244.1
Bradyrhizobium sp. strain BRUESC781 nitrogenase reductase (nifH) gene, partial cds	90	96.39	KY548261.1
Bradyrhizobium sp. strain BRUESC757 nitrogenase reductase (nifH) gene, partial cds	90	96.37	KY548260.1
Bradyrhizobium sp. strain BRUESC771 nitrogenase reductase (nifH) gene, partial cds	97	94.16	KY548272.1
Bradyrhizobium sp. strain BRUESC1001 nitrogenase reductase (nifH) gene, partial cds	90	96.13	KY548246.1
Uncultured Bradyrhizobium sp. clone SF-2-8 nitrogenase (nifH) gene, partial cds	98	93.58	KM052355.1
Uncultured bacterium clone ISA7508 NifH (nifH) gene, partial cds	96	93.91	KP069513.1
Uncultured bacterium clone ISA5107 NifH (nifH) gene, partial cds	96	93.91	KP069507.1

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