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Diversity and Efficiency of Rhizobia Communities from Iron Mining Areas Using Cowpea as a Trap Plant

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ABSTRACT: Mining is an important economic activity. However, its impact on environment must be accessed, mainly on relevant processes for their sustainability. The objective of this study was to evaluate the diversity and efficiency of symbiotic nitrogen fixing bacterial communities in soils under different types of vegetation in the *Quadrilátero Ferrífero*: ironstone outcrops, Atlantic Forest, neotropical savanna, and a rehabilitated area revegetated with grass. Suspensions of soil samples collected under each type of vegetation were made in a saline solution to capture rhizobia communities that were then inoculated on cowpea [*Vigna unguiculata* (L.) Walp.], which was used as a trap plant. The symbiotic efficiency of the communities was evaluated in a greenhouse experiment and the data obtained were correlated to the chemical and physical properties of the soils under each type of vegetation. At the end of the experiment, the bacteria present in the nodules were isolated to evaluate their diversity. The highest numbers of nodules occurred in the treatment inoculated with soil samples from rehabilitated area revegetated with grass and neotropical savanna vegetation, and the lowest numbers were observed in the treatment inoculated with soil samples from ironstone outcrops and Atlantic Forest. In relation to root dry matter, the treatment inoculated with soil samples from Neotropical savanah was superior to those inoculated with soil samples from the other areas; already, in relation to the shoot dry matter, no significant difference among the treatments was observed. The soil properties with the greatest influence on the microbial communities were Al^{3+} content, considered as high in the Atlantic Forest and neotropical savanna vegetation, as intermediate in the iron outcrops, and as very low in the rehabilitated area revegetated with grass; organic matter, considered as very high in the ironstone outcrops and neotropical savanna, as high in the Atlantic Forest, and as low in the rehabilitated area revegetated with grass; and the pH, with intermediate acidity level in the rehabilitated area revegetated with grass, high level of acidity in the iron outcrops and neotropical savanna, and very high acidity in the Atlantic Forest. After isolation of the nodules, 380 bacterial strains were obtained and separated into 27 groups by cultural characterization analysis. Genetic diversity was evaluated by the 16S rRNA gene partial sequencing of 156 strains, which identified some bacteria belonging to nitrogen-fixing Leguminosae nodulating bacterial genera (*Rhizobium*, *Bradyrhizobium, Burkholderia*, *and Cupriavidus*), some representative of associative bacteria (*Bacillus*, *Paenibacillus*, *Herbaspirillum*, *Pseudomonas*, and *Agrobacterium*), and other genera (*Brevibacillus*, *Novosphingobium*, *Chitinophaga*, *Dyella*, *Acinetobacter*, and *Stenotrophomonas*). The highest genetic diversity of bacteria was found in the rehabilitated area revegetated with grass indicated that it was effective in soil rehabilitation

Keywords: biological indicators, biological N_2 fixation, ironstone outcrops.

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INTRODUCTION

The *Quadrilátero Ferrífero* is in the central area of the state of Minas Gerais, Brazil, and stands out in the national and world scene for its importance as an iron ore producing region. The landscape of this environment is currently composed of fragments of Brazilian environmental hotspots, the Brazilian neotropical savanna (*Cerrado*) and the Atlantic Forest, which have been intensely transformed by human activities, with emphasis on urbanization and mining, generating impacts such as soil removal and consequent loss of vegetation cover (Jacobi and Carmo, 2008). Soils in this region are mainly ferruginous and generally of low fertility, acidic, and shallow; this has a considerable effect on the vegetation cover, which is composed of plants adapted to these peculiar conditions in the areas of ironstone outcrops (Costa, 2007; Carvalho Filho et al., 2010).

Knowing and evaluating the functions of microorganisms native to these environments is important for selecting strains with biotechnological potential for in situ bioremediation and which can be used as inoculants of species used in revegetation of degraded areas. These microorganisms have an important role in nutrient cycling and in improving nutrient availability, which favors the establishment of plants. They also show potential as indicators of environment changes, such as in pH, Al^{3+} contents, and organic matter, which are the factors that most influence the occurrence of microorganisms in the soil (Powlson et al., 1987; Siqueira et al., 1994; Lauber et al., 2008; Jesus et al., 2009).

Most bacteria found in the soil depend on interactions with plants; this interaction favors plant growth by enabling inorganic phosphate solubilization, biological N_2 fixation, production of plant hormones, and production of antifungal compounds, among others factors (Lim et al., 1991; Vessey, 2003; Hara and Oliveira, 2005; Marra et al., 2012; Costa et al., 2013; Rufini et al., 2014; Panizzon et al., 2016). In the case of N_2-fixing Leguminosae-nodulating bacteria (NFLNB), isolated strains represent essential genetic resources for the selection of strains with biotechnological potential, including revegetation of degraded areas.

Cowpea [*Vigna unguiculata* (L.) Walp] has been used as a trap plant in studies that evaluate the diversity of NFLNB due to its ability to establish symbiosis with different genera of bacteria, such as *Bradyrhizobium*, *Rhizobium*, and *Mesorhizobium* (Melloni et al., 2006; Guimarães et al., 2012; Costa et al., 2013; Jaramillo et al., 2013;). Identification and selection of NFLNB that establish symbiosis with cowpea and other legumes is important to reduce the use of N fertilizers, which promotes the economic and environmental sustainability of agriculture (Lacerda et al., 2004; Soares et al., 2006; Sousa and Moreira, 2011).

The hypothesis of this work is that diversity and efficiency of rhizobia communities differ significantly in soils under different types of vegetation under influence of mining activities. Thus, the aim of this study was to evaluate the symbiotic, genetic, and phenotypic diversity of cowpea-nodulating rhizobia communities from soils associated with ironstone outcrops, neotropical savanna, Atlantic Forest vegetation, and a rehabilitated area revegetated with grass in the Quadrilátero Ferrífero of Minas Gerais, Brazil, and to verify the influence of soil physical and chemical properties on the microbiota.

MATERIALS AND METHODS

Study areas

The collection area was in the municipalities of Nova Lima, in the Technology Center of Ferroso - Miguelão, and in Brumadinho, in the Córrego do Feijão Mine, which belong to Vale S/A. The vegetation of the study site was identified as follows: neotropical savanna, ironstone outcrops, Atlantic Forest, and a rehabilitated area revegetated with grass (Figure 1). According to the Forest Inventory of Minas Gerais of 2009, the vegetation present in this area is called *Campo Rupestre* (in this study described as ironstone outcrops) and Atlantic Forest. The inventory presents no information on the neotropical savanna area.

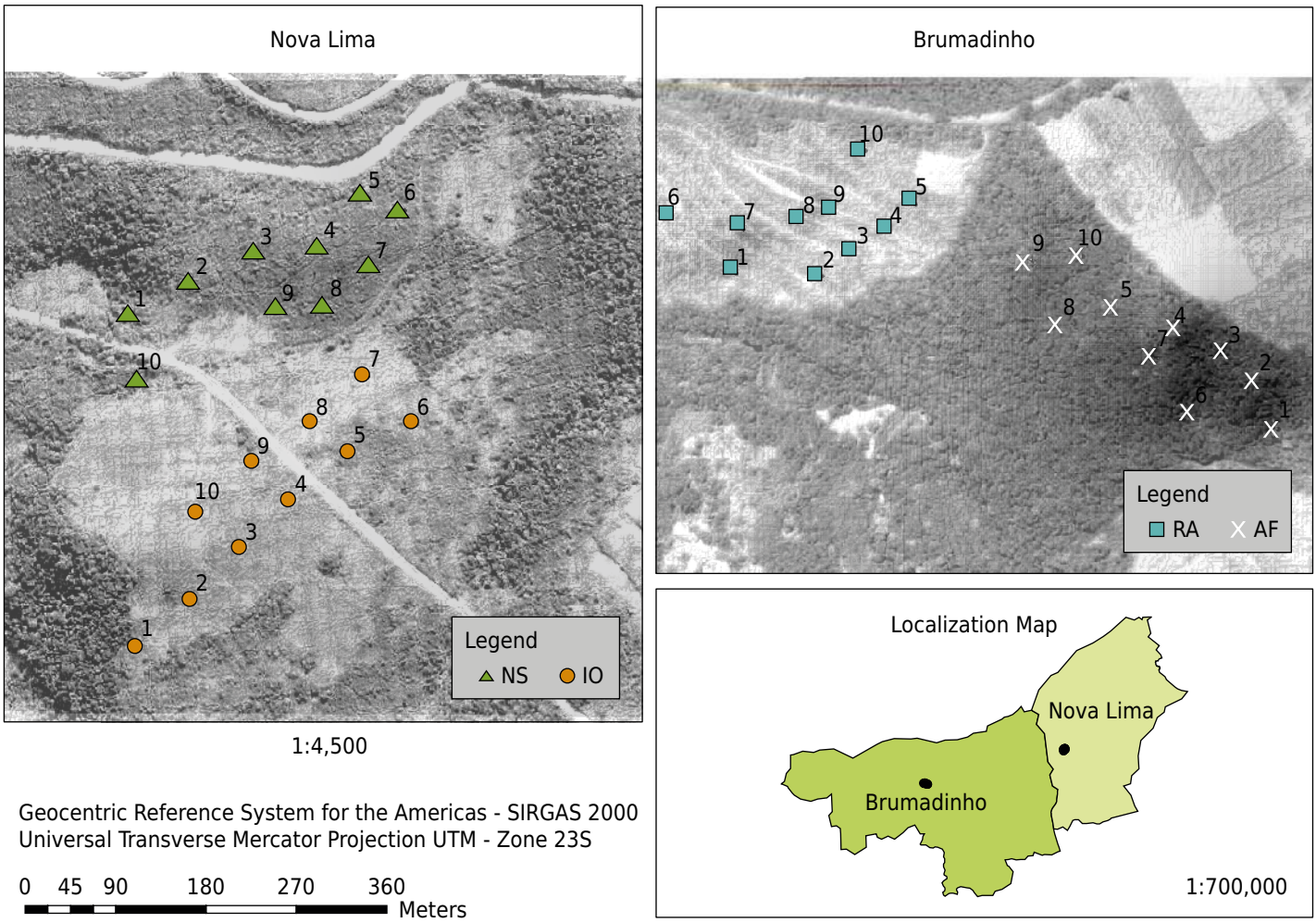

Figure 1. Map showing the collection sites in the municipalities of Brumadinho, MG and Nova Lima, MG in the neotropical savanna (NS), ironstone outcrops (IO), Atlantic Forest (AF) and rehabilitated area revegetated with grass (RA).

The rehabilitated area revegetated with grass had had Atlantic Forest vegetation, which was removed to establish an ore deposit near the railroad loading area. When the mining area was closed, a recovery project was carried out by planting tree species; however, they did not survive to successive fires, and the planted grass (*Panicum maximum* Jacq) spread throughout the area. To date, the predominant species are *Brachiaria decumbens* (Brachiaria), *Melinis minutiflora* (molasses grass), and *Panicum maximum* Jacq. (Guinea grass).

Sampling and physical-chemical characterization of soil

Soil samples were collected from August 9 to 15, 2015. For soil sampling, two transects at approximately 50 m distance were drawn in each type of vegetation. On each transect, five points were georeferenced, at approximately 50 m distance, resulting in 10 points per type of vegetation. Five subsamples were collected from each point, five meters apart from each other, at the 0.00-0.20 m depth, resulting in a composite sample from each of the 40 georeferenced points. Samples were deposited in sterile plastic bags and polystyrene boxes and taken to the laboratory, where they were stored at 4 °C in a cold chamber until use

Capture and efficiency of bacterial communities using cowpea as a trap plant

The experiment was carried out from October to December 2015 under greenhouse conditions and consisted of eight treatments: inoculations with soil suspensions from each type of vegetation, two positive controls [inoculation with strains approved by MAPA as inoculants for cowpea: UFLA 03-84 (*Bradyrhizobium sp.*) and INPA 03-11B (*Bradyrhizobium elkanii*)],

and two negative controls without inoculation [with high (HN) and low (LN) mineral N concentration]. The experimental design was completely randomized, with three replications.

Seeds were disinfested and planted in longneck bottles containing Hoagland and Arnon (1950) nutrient solution, following the method described by Florentino et al. (2009). Seeds were pre-germinated on moist sterile filter paper that was folded, wrapped in aluminum foil, and stored in a growth chamber at 28 °C until radicle emission. Soil samples from each point were resuspended in 0.85 % NaCl solution (Moreira et al., 2010) at a 1:1 ratio, and 1 mL of the suspension was inoculated on each seedling. A nutrient solution with 5.25 mg L^{-1} of N was used in the inoculated treatments and in the control without inoculation and with low mineral N concentration. A nutrient solution with 52.5 mg L^{-1} of N was used in the control without inoculation and with high mineral N concentration.

At 30 days after planting, plants were harvested and the following traits were evaluated: number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), and relative efficiency (RE). For determination of NN, nodules were collected from the roots and counted. Three nodules per plant were selected for isolation. The other nodules were placed in glass jars; shoot and roots were placed in paper bags and kept in a forced air circulation oven at 60 °C until constant weight for determination of NDM, RDM, and SDM. The relative efficiency of each treatment was calculated by the following formula:

$$RE = (SDM\ inoculated/SDM\ with\ N) \times 100 \quad \text{Eq. 1}$$

where RE is the relative efficiency, and SDM is the shoot dry matter.

Data on number of nodules, nodule dry matter, shoot dry matter, root dry matter, and relative efficiency were subjected to analysis of variance (Anova) using the statistical analysis software SISVAR 5.6 (Ferreira, 2011). The NN and NDM data were transformed to square root of (x + 1). The effects of treatments were compared by the Scott-Knott test at 5 % significance (Scott and Knott, 1974).

Relationship between physicochemical properties and biological variables

Chemical and physical properties of soils and biological variables were correlated by Principal Component Analysis (PCA) using the R software (R Development Core Team, 2011).

Isolation and cultural characterization of bacterial strains

Three nodules per plant were collected for isolation of bacterial strains. Nodules were surface disinfected in ethyl alcohol (92.8 %) for 30 s and 3 % H_2O_2 for 3 min, and washed six times with sterilized distilled water. The reagents and the water used in the nodule disinfection process were changed for each treatment, and the last wash water was plated to evaluate the effectiveness of the disinfection process. Nodules were then macerated and scattered in the form of streaks on plates with culture medium 79 (Fred and Waksman, 1928) with bromothymol blue, in order to obtain isolated bacterial colonies. The plates were stored in an incubator at a constant temperature of 28 °C, and colonies in the culture media were evaluated for 10 days or more. Colonies were evaluated according to the following parameters: border (whole, wavy, filamentous, lobed, or jagged), color (white, creamy, yellow, or pink), change in pH of culture medium (alkalinization, neutralization, and acidification), time for growth of colony (1-3 days - rapid growth, 4-5 days - intermediate growth, 6-10 days slow growth, 10 days or more - very slow growth), shape (punctate, circular, or irregular), consistency (viscous, dry, aqueous, gummy, or butyric), diameter (mm), elevation (flat, lens, convex, drop-like, umbilicate, or umbonate), surface (smooth, rough, or papillose), optic details (transparent or opaque), production of exopolysaccharides (very low, low, moderate, or abundant), and dye absorption. The isolates obtained were stored in culture medium 79 in sterile distilled water at room temperature and 20 % glycerol at -80 °C.

16S rRNA gene partial sequencing

For genetic identification of the isolates, 16S rRNA gene partial sequencing was performed. Genomic DNA was extracted by the Alkaline Lysis Method (Niemann et al., 1997). Amplification of the 16S rRNA gene followed the procedures described by Guimarães et al. (2012) through use of the primers 27F (GAGTTTGACCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Lane, 1991). The Polymerase Chain Reaction (PCR) products were sent to the Macrogen laboratory in South Korea for purification and sequencing. To evaluate the quality of the sequences obtained, the software BioNumerics 7.1 was used (AppliedMaths, Austin, TX, USA). The sequences were subsequently subjected to the BLASTn (Bethesda, MD, USA) for comparison with similar sequences already deposited in the GenBank, National Center for Biotechnology Information (NCBI). The genetic diversity in different vegetation types was also evaluated by the Shannon index, which considers both the richness and abundance of species. For calculation purposes, the different species found in each type of vegetation were considered.

RESULTS

Chemical and physical analysis of soils

The interpretation of soil chemical analysis was performed based on the recommendations of the Soil Fertility Commission of the State of Minas Gerais (Alvarez et al., 1999) (Table 1). Potassium content in the Atlantic Forest (75.60 mg dm^{-3}), neotropical savanna (72.60 mg dm^{-3}), and rehabilitated area revegetated with grass (88.20 mg dm^{-3}) was classified as intermediate. In ironstone outcrops, low K^+ content (56.80 mg dm^{-3}) was observed, classified as intermediate. Phosphorus content was very low and statistically similar among the different types of vegetation [low in ironstone outcrops (1.59 mg dm^{-3}), rehabilitated area revegetated with grass (1.66 mg dm^{-3}), neotropical savanna (1.36 mg dm^{-3}), and Atlantic Forest (2.15 mg dm^{-3})]. Sulfur content in all types of vegetation was classified as very good, and the rehabilitated area revegetated with grass (45.14 mg dm^{-3}) and neotropical savanna (36.29 mg dm^{-3}) exhibited the highest concentrations. Manganese content was considered as high in all types of vegetation, reaching the highest values in the neotropical savanna (112.29 mg dm^{-3}), rehabilitated area revegetated with grass (104.00 mg dm^{-3}), and ironstone outcrops (88.88 mg dm^{-3}).

Zinc contents were classified as high in the ironstone outcrops (3.29 mg dm^{-3}) and neotropical savanna (3.13 mg dm^{-3}), and as good in the rehabilitated area revegetated with grass (1.60 mg dm^{-3}) and in the Atlantic Forest (1.92 mg dm^{-3}). Boron content was classified as low in the ironstone outcrops (0.26 mg dm^{-3}), neotropical savanna (0.20 mg dm^{-3}), and Atlantic Forest (0.20 mg dm^{-3}), and as very low in the rehabilitated area revegetated with grass (0.15 mg dm^{-3}). Cupper content was considered as high in the rehabilitated area revegetated with grass (2.14 mg dm^{-3}), as low in the ironstone outcrops, and as good in the neotropical savanna and Atlantic Forest. Magnesium content was considered as low in all types of vegetation, and no difference was observed ($p<0.05$) between the vegetation areas. Calcium content was considered as low in the rehabilitated area revegetated with grass (0.75 $cmol_c$ dm^{-3}), neotropical savanna (0.91 $cmol_c$ dm^{-3}), and Atlantic Forest (0.99 $cmol_c$ dm^{-3}), and as intermediate in the ironstone outcrops (1.28 $cmol_c$ dm^{-3}). The soils collected have loam texture in the ironstone outcrops and rehabilitated area revegetated with grass, and clayey texture in the neotropical savanna and Atlantic Forest.

Soil under the rehabilitated area revegetated with grass had a pH(H_2O) 5.60, indicating an intermediate level of acidity. Soils of the ironstone outcrops and neotropical savanna had mean pH 4.72 and 4.97, respectively, indicating high acidity. In the Atlantic Forest environment, the mean pH value indicates high acidity soil (4.21). Exchangeable acidity was considered as high in the neotropical savanna (1.56 $cmol_c$ dm^{-3}) and in the Atlantic Forest (1.90 $cmol_c$ dm^{-3}). In the ironstone outcrop soil, the exchangeable acidity value was classified as intermediate (0.85 $cmol_c$ dm^{-3}).

Table 1. Chemical and physical properties of the soils collected in ironstone outcrops, rehabilitated area revegetated with grass, neotropical savanna, and Atlantic Forest vegetation areas at the Ferrous Technology Center - CTF Miguelão and in the Córrego do Feijão Mine, Vale S/A

Property	Ironstone outcrops	Rehabilitated area revegetated with grass	Neotropical savanna	Atlantic Forest
pH(H_2O)	4.72 b	5.60 a	4.97 b	4.21 c
K (mg dm^{-3})(2)	56.80 b	88.20 a	72.60 a	75.60 a
P (mg dm^{-3})(2)	1.59 b	1.66 b	1.36 b	2.15 a
Ca^{2+} ($cmol_c$ dm^{-3})(1)	1.28 a	0.75 a	0.91 a	0.99 a
Mg^{2+} ($cmol_c$ dm^{-3})(1)	0.24 a	0.30 a	0.38 a	0.45 a
Al^{3+} ($cmol_c$ dm^{-3})(1)	0.85 b	0.09 c	1.56 a	1.90 a
H+Al ($cmol_c$ dm^{-3})(4)	12.64 a	1.94 b	15.46 a	12.26 a
SB ($cmol_c$ dm^{-3})	1.66 a	1.27 a	1.47 a	1.63 a
t ($cmol_c$ dm^{-3})	2.51 a	1.36 b	3.03 a	3.53 a
T ($cmol_c$ dm^{-3})	14.31 a	3.21 b	16.94 a	13.89 a
V (%)	13.56 b	40.73 a	12.72 b	13.64 b
m (%)	33.78 b	6.76 c	46.13 b	59.30 a
OM (dag kg^{-1})(1)	7.58 a	1.38 c	8.30 a	4.94 b
rem-P (mg L^{-1})	12.71 a	11.03 a	4.56 b	11.02 a
Zn (mg dm^{-3})(2)	3.29 a	1.60 b	3.13 a	1.92 b
Fe (mg dm^{-3}) (2)	403.7 a	150.8 b	134.5 b	124.7 b
Mn (mg dm^{-3}) (2)	88.88 a	104.00 a	112.29 a	40.76 b
Cu (mg dm^{-3}) (2)	0.57 b	2.14 a	0.79 b	0.80 b
B (mg dm^{-3})(1)	0.26 a	0.15 a	0.20 a	0.20 a
S (mg dm^{-3})(3)	26.58 b	45.14 a	36.29 a	29.06 b
Clay (g kg^{-1})(1)	214 c	249 c	376 b	456 a
Silt (g kg^{-1})(1)	174 a	262 a	243 a	188 a
Sand (g kg^{-1})(1)	612 a	489 b	381 c	356 c

Means followed by the same letter in the line do not differ by the Scott-Knott test at 5 % probability. Values evaluated based on the recommendations of the Soil Fertility Commission of the State of Minas Gerais (Alvarez et al., 1999). pH in water, soil:solution 1:2.5; Ca^{2+}, Mg^{2+}, and Al^{3+}: extractor 1 mol L^{-1} KCl; H+Al: extractor SMP; SB: sum of bases; T: cation exchange capacity at pH 7.0; t: effective cation exchange capacity; V: bases saturation; m: aluminum saturation; Rem-P: remaining phosphorus; OM: organic matter, oxidation with 5 mol L^{-1} $Na_2Cr_2O_7 4N+H_2SO_4$; P, K, Fe, Zn, Mn, Cu: Mehlich-1 extractor; S: extractor Monocalcium Phosphate Acetic Acid; B: hot water extractor. Properties analyzed according to the methods proposed by: (1) Vettori (1969); (2) Mehlich (1953); (3) Richards (1954); and (4) Shoemaker et al. (1961).

Soil under the rehabilitated area revegetated with grass had a low mean value for exchangeable acidity (0.09 $cmol_c$ dm^{-3}). The mean values of potential acidity found in the ironstone outcrops, neotropical savanna, and Atlantic Forest vegetation were classified as very high (12.64, 15.46, and 12.26 $cmol_c$ dm^{-3}, respectively). This promotes high potential CEC, which indicates that the soil has the ability to retain more nutrients, despite the low pH. In the rehabilitated area revegetated with grass, the potential acidity value was 1.94 $cmol_c$ dm^{-3}, which was considered as low. Soils under the ironstone outcrops and neotropical savanna vegetation had very high organic matter, with values of 7.58 and 8.30 dag kg^{-1}, respectively. The soil of the Atlantic Forest vegetation had a value of 4.94 dag kg^{-1}, which is classified as high. The rehabilitated area revegetated with grass had the lowest value, 1.38 dag kg^{-1}. The ironstone outcrops and neotropical savanna had the highest values for organic matter (7.58 and 8.30 dag kg^{-1}, respectively), followed by the Atlantic Forest (4.94 dag kg^{-1}) and rehabilitated area revegetated with grass (1.38 dag kg^{-1}).

Soils of all the environments had high Fe contents, especially the ironstone outcrops vegetation area, with 403.7 mg dm^{-3}. The other areas had very close values, ranging from 124.7 to 150.8 mg dm^{-3}. In all types of vegetation, Fe content in the soil was high, especially in the ironstone outcrops, due to the type of soil and rocks, characterized by the presence of a lateritic crust, which limits plant growth and development; thus, many species already known as endemic to the area with these characteristics prevail in this area.

The soils of the Quadrilátero Ferrífero are mostly derived from itabirite (a metamorphic BIF), which explains their high iron concentration. The critical level for iron is 45 mg dm^{-3} (Alvarez et al., 1999). In this study, chemical analysis showed that the lowest value was 124.7 mg dm^{-3}. This result corroborates the soil characterization made by Carvalho Filho et al. (2010) in this region, which describes the soils of the inner face of the Serra da Moeda as shallow and generally very stony that are derived from itabirite, with great iron concentration.

Capture and symbiotic efficiency of bacterial communities using cowpea as a trap plant

In the controls without inoculation with low and high N concentration, the nodulation was negative and reference strains nodulated normally, indicating that there was no contamination and that the experimental conditions were favorable to symbiosis. In relation to number of nodules, the nodulation ability of the bacterial communities obtained from inoculations prepared from soil suspensions from rehabilitated area revegetated with grass and from neotropical savanna vegetation areas was higher than that from Atlantic Forest and from ironstone outcrops (Table 2). The rehabilitated area revegetated with grass had the highest value for nodule dry matter, followed by neotropical savanna. The area of neotropical savanna stood out for root dry matter, surpassing the other areas. The values of shoot dry matter and relative efficiency did not differ statistically between the areas. Nodules were observed in all treatments; the rehabilitated area revegetated with grass had the highest value, followed by neotropical savanna, Atlantic Forest, and ironstone outcrops.

Relationship between soil physicochemical properties and biological variables

Results of the principal component analysis between soil physicochemical properties and biological variables explained 48 % of the total variance (PC1: 33 % and PC2: 15 %). These results, together with the correlation matrix, allowed better understanding of the relationship between the physicochemical properties of the soils collected in the different environments and the bacterial communities (Figure 2). The physicochemical properties of the soils, together with shoot dry matter, nodule dry matter, root dry matter, number of nodules, and relative efficiency, were correlated. Analysis of PC1 shows that number of nodules and nodule dry matter are directly correlated with pH, base saturation, Mn, and Cu and inversely correlated with exchangeable acidity, potential acidity, effective CEC, CEC at pH 7, Al saturation, and organic matter (Table 3).

By analyzing the spatial distribution of the points in the PCA, it can be inferred that the soil under the rehabilitated area revegetated with grass is moving closer to neotropical savanna conditions, which indicates possible recovery. The soil under ironstone outcrops is

Table 2. Shoot dry matter (SDM), root dry matter (RDM), number of nodules (NN), nodule dry matter (NDM), and relative efficiency (RE) in plants inoculated with soil solutions under ironstone outcrops, rehabilitated area revegetated with grass, neotropical savanna, and Atlantic Forest of the Ferrous Technology Center - CTF Miguelão and in the Córrego do Feijão Mine, Vale S/A

Treatment	SDM	RDM	NN	NDM	RE
	g per plant			mg per plant	%
Neotropical savanna	0.68 a	0.22 a	4.24 a	1.01 b	36.80 a
Ironstone outcrops	0.70 a	0.16 b	1.31 b	1.00 c	37.86 a
Rehabilitated area revegetated with grass	0.74 a	0.18 b	4.74 a	1.02 a	40.12 a
Atlantic Forest	0.78 a	0.16 b	2.04 b	1.00 c	42.02 a
CV (%)	12.36	11.65	37.22	0.47	12.36

Means followed by the same letter in the columns do not statistically differ by the Scott-Knott test at 5 % probability.

isolated, possibly due to physicochemical conditions, and is very different from the other ecosystems analyzed in the study, especially in relation to Fe contents. Iron contents were high in all environments, but were even higher in this area. The points of Atlantic Forest and neotropical savanna overlap, and the same characteristics may influence the soil conditions.

Isolation and cultural characterization of plant growth promoting bacteria

A total of 380 bacterial strains grown in solid culture medium were obtained from the experimental isolation of cowpea nodules. The rehabilitated area revegetated with grass and the neotropical savanna exhibited 161 and 125 isolates from the 10 collection sites, respectively. The ironstone outcrops exhibited 29 isolates, and only sites 5, 6, and 7 exhibited nodules. The Atlantic Forest exhibited 65 isolates, and only sites 1, 2, 5, and 9 did not exhibit nodulation.

Analysis of growth rate, change in pH of the culture medium, and production of mucus, showed that 27 culture groups were formed: fast/alkaline/scarce (FALS), fast/neutral/abundant (FNAB), fast/alkaline/moderate (FALM), fast/alkaline/little (FALL), fast/neutral/scarce (FNS), fast/neutral/moderate (FNM), fast/acid/moderate (FAM), fast/neutral/little (FNL), fast/acid/little (FAL), slow/alkaline/abundant (SALAB), slow/alkaline/moderate (SALM), slow/alkaline/little (SALL), slow/neutral/scarce (SNS), slow/neutral/moderate (SNM), slow/neutral/little (SNL), slow/acid/moderate (SAM), slow/acid/little (SAL), very slow/alkaline/little (VSALL), very slow/neutral/little (VSNL), very slow/acid/abundant (VSAAB), very slow/acid/moderate

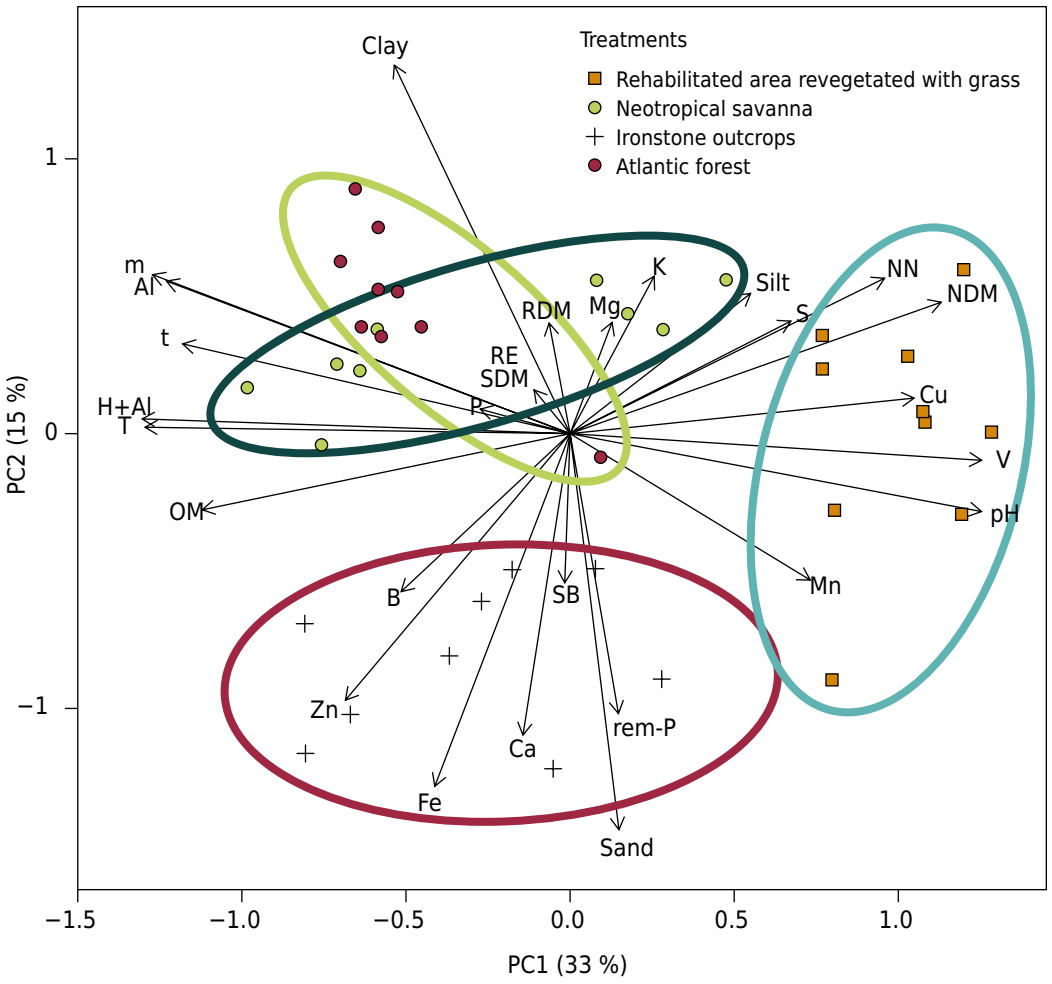

Figure 2. Principal component analysis (PCA) relating soil physical and chemical properties and biological variables (SDM: shoot dry matter, RDM: root dry matter, NDM: nodule dry matter, NN: number of nodules, and RE: relative efficiency) of the different types of vegetation in the *Quadrilátero Ferrífero*. Soil chemical properties: H+Al: potential acidity, SB: sum of bases, t: effective CEC, OM: organic matter, rem-P: remaining phosphorus, V: base saturation, m: aluminum saturation, and T: CEC pH 7.

Table 3. Principal component analysis of physical, chemical, and biological properties of soils under different vegetation types in the *Quadrilátero Ferrífero* environments

Variable	PC1	PC2
	Correlation with the principal components	
Shoot dry matter (SDM)	-0.11	0.16
Root dry matter (RDM)	-0.06	0.40
Number of nodules (NN)	**0.96**	0.57
Nodule dry matter (NDM)	**1.13**	0.48
Relative efficiency (RE)	-0.11	0.16
pH(H_2O)	**1.25**	-0.28
K	0.26	0.57
P	-0.27	0.09
Ca^{2+}	-0.14	**-1.10**
Mg^{2+}	0.13	0.40
Al^{3+}	**-1.23**	0.56
Potential acidity (H+Al)	**-1.30**	0.05
Sum of bases (SB)	-0.02	-0.54
Effective CEC (t)	**-1.18**	0.33
CEC pH 7 (T)	**-1.29**	0.02
Base saturation (V)	**1.25**	-0.10
Al saturation (m)	**-1.27**	0.58
Organic matter (OM)	**-1.12**	-0.28
Remaining P (rem-P)	0.15	**-1.02**
Zn	-0.68	**-0.97**
Fe	-0.41	**-1.28**
Mn	**0.73**	-0.53
Cu	**1.05**	0.13
B	-0.51	-0.57
S	0.67	0.41
Clay	-0.54	**1.34**
Silt	0.55	0.51
Sand	0.15	**-1.44**
Explained variance (%)		
Individual	33	15
Accumulated	33	48

PC: Principal component. The values in bold which are greater than or equal to 0.7 indicate strong correlation for interpretation of the behavior of the principal components.

(VSAM), very slow/acid/little (VSAL), intermediate/alkaline/moderate (IALM), intermediate/alkaline/little (IALL), intermediate/neutral/abundant (INAB), intermediate/acid/little (IAL), and intermediate/neutral/little (INL).

Cultural groups which had the greatest number of representatives had a slow growth rate; they alkalinized the medium and produced little mucus (SALL, 23 %); they had intermediate growing time and maintained the characteristics of the medium neutral and with little production of mucus (INL, 13 %); and fast growing, which alkalinize the medium and had little mucus production (FALL, 12 %) (Figure 3), with the highest cultural diversity observed in the neotropical savanna and Atlantic Forest (18 groups), followed by the rehabilitated area revegetated with grass (15 groups) and ironstone outcrops (9 groups). Of the 380 isolates, 35 % showed slow growth time, 3 % very slow, 29 % intermediate, and 33 % fast. Regarding pH changes in the culture medium, 53 % alkalized, 12 % acidified, and 35 % retained the neutral medium. Mucus production by the isolates was also evaluated, and it was abundant in 4 %, moderate in 15 %, little in 80 %, and scarce in 1 %.

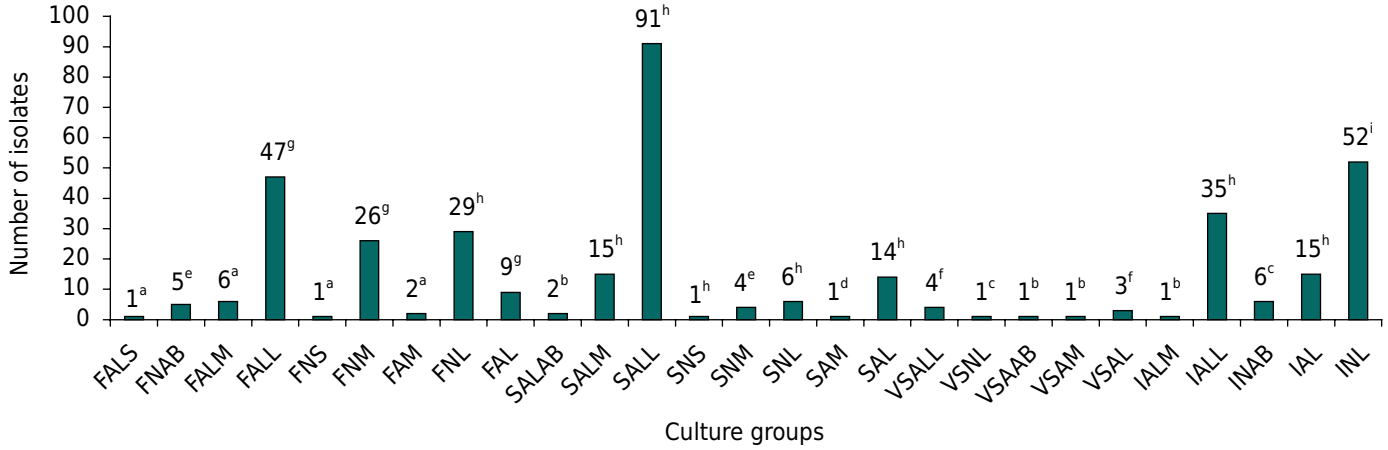

Figure 3. Number of soil isolates from different areas distributed in 27 culture groups based on growth rate, change in pH of the culture medium, and mucus production. Fast alkaline scarce (FALS), fast neutral abundant (FNAB), fast alkaline moderate (FALM), fast alkaline little (FALL), fast neutral scarce (FNS), fast neutral moderate (FNM), fast acid moderate (FAM), fast neutral little (FNL), fast acid little (FAL), slow alkaline abundant (SALAB), slow alkaline moderate (SALM), slow alkaline little (SALL), slow neutral scarce (SNS), slow neutral moderate (SNM), slow neutral little (SNL), slow acid moderate (SAM), slow acid little (SAL), very slow alkaline little (VSALL), very slow neutral little (VSNL), very slow acid abundant (VSAAB), very slow acid moderate (VSAM), very slow acid little (VSAL), intermediate alkaline moderate (IALM) intermediate alkaline little (IALL), intermediate neutral abundant (INAB), intermediate acid little (IAL), and intermediate neutral little (INL). Bacteria isolated from areas: [a] neotropical savanna; [b] Atlantic Forest; [c] Rehabilitated area revegetated with grass; [d] Ironstone outcrops; [e] Rehabilitated area revegetated with grass and neotropical savanna; [f] Neotropical savanna and Atlantic Forest; [g] Rehabilitated area revegetated with grass, neotropical savanna and Atlantic Forest; [h] Rehabilitated area revegetated with grass, neotropical savanna, Atlantic Forest, and ironstone outcrops; and [i] Rehabilitated area revegetated with grass, ironstone outcrops, and Atlantic Forest.

16S rRNA gene partial sequencing

The 16S RNAr gene partial sequencing was performed for 156 of the 380 strains isolated from nodules in the experiment. These strains have representatives in 18 of the 27 cultural groups formed (FNL, IALL, INL, FNAB, FNM, SALL, INAB, SNL, SALM, FALL, FALM, SAL, FAL, SALAB, IAL, SAM, SNS, and FAM). The analyzed sequences ranged from 320 to 1420 base pairs, with 98 % to 100 % similarity with the sequences of strains that have already been deposited in the NCBI GenBank. The table 4 shows the species already known as N_2 fixing nodulating bacteria. In contrast, table 5 shows the species and genera that have not yet been proven to be N_2 fixing nodulating bacteria. Strains belonging to genera which constitute both bacterial types were included in table 4. The sequences determined in this study have been deposited in GenBank under accession numbers MF495721 to MF495861. The most frequent genera were *Burkholderia* (Moulin et al., 2001), *Rhizobium* (Frank, 1889), and *Bradyrhizobium* (Jordan, 1982), representing 60 % of the isolates present in the soils of all types of vegetation. *Bradyrhizobium* was not found only in ironstone outcrops.

From soil under ironstone outcrops soil, 18 strains were sequenced. Seventy two percent of these strains belong to the genus *Burkholderia*, comprising associative species such as *B. acidipaludis*, and legume symbiont species such as *B. nodosa* (Chen et al., 2007), which is also considered to be a free-living N_2 fixing bacterium. Only one representative of the genus *Chitinophaga* and one of the genus *Rhizobium* were identified, and species of the genus *Paenebacillus*. From soil under the neotropical savanna, 54 strains were sequenced, and the genus *Burkholderia* (55.5 %) prevailed, with representatives of *B. nodosa, B. sabiae*, and *B. tropica*. In addition to this genus, the genera *Rhizobium* and *Bacillus* were also identified. Of the 15 isolates sequenced from soil under the Atlantic Forest, most of them belonged to the genus *Paenebacillus*, followed by *Bradyrhizobium*, with three representatives; the others are distributed among the genera *Rhizobium, Brevibacillus, Chitinophaga, Acinetobacter, Novosphingobium*, and *Burkholderia*, with only one representative in each genus. Of the isolates obtained from the soil under rehabilitated area revegetated with grass, 69 strains were sequenced, most of them belonging to the genus *Rhizobium* (28.9 %). In this environment, the following bacterial genera were identified: *Burkholderia*,

Table 4. Identification of rhizobia strains isolated from cowpea nodules based on 16S rRNA gene sequencing

UFLA Code	Origin	Cultural groups[1]	NPB[2]	Most similar sequences found in the GenBank		
				Specie or genus	SI[3]	Accession
					%	
UFLA 03-597	Ironstone outcrops	FNL	1304[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-598	Ironstone outcrops	FNL	1273[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-599	Ironstone outcrops	FNL	1337[C]	*Burkholderia nodosa*	99	AY773198
UFLA 03-600	Ironstone outcrops	IALL	1295[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-602	Ironstone outcrops	INL	1295[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-603	Ironstone outcrops	INL	1308[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-605	Ironstone outcrops	INL	1296[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-606	Ironstone outcrops	INL	1329[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-607	Ironstone outcrops	INL	1264[C]	*Rhizobium miluonense*	99	GU120632
UFLA 03-608	Ironstone outcrops	INL	1267[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-595	Ironstone outcrops	INL	1278[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-604	Ironstone outcrops	INL	1364[F]	*Burkholderia nodosa*	99	AM284972
UFLA 03-513	Grass	FNAB	1302[C]	*Burkholderia lata*	100	AM905038
UFLA 03-516	Grass	FNAB	1280[C]	*Rhizobium miluonense*	99	NR044063
UFLA 03-521	Grass	FNL	1204[C]	*Rhizobium* sp.	99	KR232948
UFLA 03-522	Grass	FNL	1279[C]	*Burkholderia* sp.	99	JQ518349
UFLA 03-525	Grass	FNL	1384[C]	*Burkholderia* sp.	99	JQ518344
UFLA 03-530	Grass	FNM	1267[C]	*Rhizobium* sp.	99	KM979037
UFLA 03-531	Grass	FNM	1317[C]	*Burkholderia* sp.	99	JQ518349
UFLA 03-536	Grass	FNM	1245[C]	*Rhizobium* sp.	99	KR232948
UFLA 03-537	Grass	FNM	1261[C]	*Rhizobium* sp.	100	KR232948
UFLA 03-538	Grass	FNM	1349[C]	*Rhizobium miluonense*	99	JN896360
UFLA 03-540	Grass	FNM	1223[C]	*Rhizobium* sp.	99	KJ128395
UFLA 03-541	Grass	FNM	1232[C]	*Rhizobium tropici*	99	KT962907
UFLA 03-547	Grass	FNM	1236[C]	*Rhizobium miluonense*	99	GU120632
UFLA 03-549	Grass	FNM	1222[C]	*Rhizobium miluonense*	99	GU120632
UFLA 03-550	Grass	FNM	1243[C]	*Rhizobium miluonense*	99	GU120632
UFLA 03-554	Grass	SALL	1150[C]	*Bradyrhizobium paxllaeri*	100	NR133708
UFLA 03-571	Grass	INL	1391[C]	*Burkholderia* sp.	99	JQ518351
UFLA 03-577	Grass	INAB	1323[C]	*Burkholderia caribensis*	99	CP013103
UFLA 03-578	Grass	INAB	1292[C]	*Rhizobium* sp.	99	FJ025129
UFLA 03-579	Grass	INAB	1353[C]	*Rhizobium* sp.	98	KM979037
UFLA 03-580	Grass	INAB	1236[C]	*Rhizobium tropici*	99	KP687377
UFLA 03-581	Grass	INAB	1261[C]	*Rhizobium miluonense*	100	GU120632
UFLA 03-587	Grass	INL	1209[C]	*Rhizobium miluonense*	100	GU120632
UFLA 03-539	Grass	FNM	465[F]	*Rhizobium multihospitium*	100	JN896359
UFLA 03-544	Grass	FNM	564[F]	*Rhizobium alamii*	99	GU552885
UFLA 03-545	Grass	FNM	712[F]	*Bradyrhizobium* sp. *AM 7*	99	KF927053
UFLA 03-671	Grass	SNL	456[R]	*Bradyrhizobium* sp.	99	KX527927.1
UFLA 03-646	Grass	SALL	661[F]	*Bradyrhizobium* sp.	99	KJ658704.1
UFLA 03-584	Grass	SALM	528[F]	*Bradyrhizobium* sp.	99	JX316045.1
UFLA 03-660	Grass	SALL	575[F]	*Bradyrhizobium* sp.	99	KJ658645.1
UFLA 03-649	Grass	SALL	578[F]	*Bradyrhizobium* sp.	100	KY548146.1
UFLA 03-661	Grass	SALL	637[F]	*Bradyrhizobium* sp.	100	JX316045.1
UFLA 03-662	Grass	SALL	485[F]	*Bradyrhizobium* sp.	100	JX316045.1
UFLA 03-663	Grass	SALL	454[F]	*Bradyrhizobium* sp.	100	KY548146.1
UFLA 03-665	Grass	SALM	699[F]	*Bradyrhizobium* sp.	100	HQ698303.1
UFLA 03-546	Grass	FNM	581[F]	*Rhizobium multihospitium*	100	EF035077

Continue

Table 4. Identification of rhizobia strains isolated from cowpea nodules based on 16S rRNA gene sequencing. Continuation

UFLA Code	Origin	Cultural groups[1]	NPB[2]	Most similar sequences found in the GenBank		
				Specie or genus	SI[3]	Accession
UFLA 03-636	Grass	FNM	1262[C]	*Rhizobium miluonense*	100	GU120632
UFLA 03-675	Grass	IALL	1394[C]	*Cupriavidus* sp.	99	AB542369.1
UFLA 03-484	Neotropical savanna	FNL	575[F]	*Rhizobium multihospitium*	99	EF035077
UFLA 03-461	Neotropical savanna	FALL	1314[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-463	Neotropical savanna	FALL	1306[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-464	Neotropical savanna	FALL	1351[C]	*Burkholderia nodosa*	100	AM284971
UFLA 03-465	Neotropical savanna	FALL	1316[C]	*Burkholderia nodosa*	99	AY773198
UFLA 03-466	Neotropical savanna	FALL	1348[C]	*Rhizobium miluonense*	99	KF979146
UFLA 03-467	Neotropical savanna	FALL	1341[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-470	Neotropical savanna	FALL	1366[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-471	Neotropical savanna	FALL	1333[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-473	Neotropical savanna	FALL	1304[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-474	Neotropical savanna	FALL	1311[C]	*Burkholderia nodosa*	99	AM284970
UFLA 03-476	Neotropical savanna	FALL	1369[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-477	Neotropical savanna	FNL	1361[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-485	Neotropical savanna	FNL	1381[C]	*Burkholderia nodosa*	99	AM284971
UFLA 03-491	Neotropical savanna	IALL	1295[C]	*Burkholderia nodosa*	99	AY773192
UFLA 03-506	Neotropical savanna	FALL	701[R]	*Paraburkholderia sabiae*	93	KT390903
UFLA 03-703	Neotropical savanna	FALM	1330[C]	*Bradyrhizobium elkanii*	100	KX396582.1
UFLA 03-704	Neotropical savanna	SAL	1191[C]	*Bradyrhizobium* sp.	99	LC095718.1
UFLA 03-507	Neotropical savanna	FALL	1273[C]	*Burkholderia* sp.	99	KU844030.1
UFLA 03-679	Neotropical savanna	FALL	1293[C]	*Burkholderia nodosa*	99	AY773192.1
UFLA 03-683	Neotropical savanna	SALL	1253[C]	*Bradyrhizobium elkanii*	100	KX396582.1
UFLA 03-506	Neotropical savanna	FALL	1389[C]	*Burkholderia nodosa*	99	AY773192.1
UFLA 03-696	Neotropical savanna	FALL	1285[C]	*Burkholderia nodosa*	99	AY773192.1
UFLA 03-497	Neotropical savanna	SALM	1371[C]	*Burkholderia nodosa*	99	AY773192.1
UFLA 03-593	Neotropical savanna	IALL	1395[C]	*Burkholderia nodosa*	99	AM284970.1
UFLA 03-698	Neotropical savanna	FALM	1375[C]	*Burkholderia* sp.	98	AB366339.1
UFLA 03-699	Neotropical savanna	FNL	1337[C]	*Burkholderia nodosa*	99	AY773192.1
UFLA 03-689	Neotropical savanna	SALL	1251[C]	*Bradyrhizobium embrapense*	100	NR_145861.1
UFLA 03-454	Neotropical savanna	FALM	1393[C]	*Burkholderia nodosa*	99	AM284971.1
UFLA 03-500	Neotropical savanna	SALL	1203[C]	*Bradyrhizobium* sp	99	LC095713.1
UFLA 03-508	Neotropical savanna	SALL	1272[C]	*Bradyrhizobium* sp	99	KF933595.1
UFLA 03-680	Neotropical savanna	SALL	1218[C]	*Bradyrhizobium* sp	99	EF158574.2
UFLA 03-691	Neotropical savanna	FAL	1383[C]	*Burkholderia nodosa*	99	AY773192.1
UFLA 03-692	Neotropical savanna	FAL	1279[C]	*Burkholderia nodosa*	99	AY773192.1
UFLA 03-694	Neotropical savanna	FALM	1231[C]	*Burkholderia* sp	99	FN543647.1
UFLA 03-672	Grass	SALL	683[F]	*Bradyrhizobium* sp.	99	LC095679.1
UFLA 03-684	Neotropical savanna	SALL	475[F]	*Bradyrhizobium* sp.	100	KY548146.1
UFLA 03-502	Neotropical savanna	SALL	582[F]	*Bradyrhizobium* sp.	99	KY548146.1
UFLA 03-678	Neotropical savanna	SALL	577[F]	*Bradyrhizobium* sp.	100	KY548146.1
UFLA 03-690	Neotropical savanna	FALL	812[R]	*Burkholderia nodosa*	100	AY773198.1
UFLA 03-637	Atlantic Forest	SALL	575[F]	*Bradyrhizobium* sp.	99	KY548146.1
UFLA 03-626	Atlantic Forest	SALAB	575[F]	*Bradyrhizobium* sp.	100	KY548146.1
UFLA 03-629	Atlantic Forest	SALL	507[F]	*Bradyrhizobium elkanii*	100	KP744139
UFLA 03-609	Atlantic Forest	FNM	965[F]	*Rhizobium milluonense*	99	KF979146

[1] Phenotypic groups formed from the growth rate, change in pH of the culture medium, and mucus production; FNAB: fast/neutral/ abundant, FALL: fast/alkaline/little, FNM: fast/neutral/moderate, FNL: fast/neutral/little, FAL: fast/acid/little, SALL: slow/alkaline/ little, SAL: slow/acid/little, IALL: intermediate/alkaline/little, INAB: intermediate/neutral/abundant, INL: intermediate/neutral/little, SNL: slow/neutral/little, SALM: slow/alkaline/ moderate, FALM: fast/alkaline/moderate, and SALAB: slow/alkaline/abundant. [2] NPB: number of pair of bases. [3] SI: percentage of similarity in the GenBank. [C]: Contig, [F]: Forward, and [R]: Reverse.

Table 5. Identification of bacterial strains isolated from cowpea nodules based on 16S rRNA gene sequencing

UFLA Code	Origin	Cultural groups[1]	NPB[2]	Most similar sequences found in the GenBank		
				Specie or genus	SI[3]	Accession
					%	
UFLA 03-601	Ironstone outcrops	IALL	1384[C]	*Burkholderia acidipaludis*	98	AB513181
UFLA 03-646	Ironstone outcrops	SAL	555[F]	*Paenebacillus cineris*	100	KF979149.1
UFLA 03-645	Ironstone outcrops	IAL	588[F]	*Paenebacillus cineris*	100	KF979149.1
UFLA 03-644	Ironstone outcrops	IAL	595[F]	*Paenebacillus cineris*	100	KF979149.1
UFLA 03-640	Ironstone outcrops	SAM	325[R]	*Paenebacillus* sp.	100	KX396552.1
UFLA 03-643	Ironstone outcrops	SNL	547[R]	*Chitinophaga sancti*	98	NR_040917.1
UFLA 03-515	Grass	FNAB	1272[C]	*Agrobacterium tumefaciens*	99	JX110605
UFLA 03-517	Grass	FNL	1273[C]	*Herbaspirillum huttiense*	99	GU433469
UFLA 03-519	Grass	FNL	1391[C]	*Bacteroidetes bacterium*	99	FJ786046
UFLA 03-523	Grass	FNL	1355[C]	*Bacillus subtilis*	99	KP192484
UFLA 03-524	Grass	FNL	1279[C]	*Pseudomonas fuscovaginae*	99	KP197056
UFLA 03-527	Grass	FNL	1365[C]	*Terriglobus* sp.	99	AY587229
UFLA 03-528	Grass	FNL	1370[C]	*Burkholderia kururiensis*	99	KP974790
UFLA 03-529	Grass	FNM	1289[C]	*Pseudomonas koreensis*	99	KC790283
UFLA 03-532	Grass	FNM	1305[C]	*Burkholderia metallica*	100	NR042636
UFLA 03-535	Grass	FNM	1274[C]	*Agrobacterium tumefaciens*	99	KJ921039
UFLA 03-548	Grass	FNM	1312[C]	*Burkholderia kururiensis*	99	EF178438
UFLA 03-551	Grass	FAL	1298[C]	*Paenibacillus polymyxa*	99	GU120632
UFLA 03-552	Grass	FAL	1300[C]	*Paenibacillus polymyxa*	99	EU882855
UFLA 03-565	Grass	INL	1294[C]	*Bacteroidetes bacterium*	99	FJ786046
UFLA 03-567	Grass	INL	1279[C]	*Pseudomonas koreensis*	99	KC790283
UFLA 03-568	Grass	INL	1289[C]	*Burkholderia kururiensis*	99	EF178438
UFLA 03-569	Grass	INL	1384[C]	*Burkholderia kururiensis*	99	KP974790
UFLA 03-570	Grass	INL	1322[C]	*Brevibacillus centrosporus*	99	NR112211
UFLA 03-573	Grass	FAL	1335[C]	*Burkholderia gladioli*	99	GQ337697
UFLA 03-594	Grass	INL	1256[C]	*Bacteroidetes bacterium*	99	FJ786046
UFLA 03-566	Grass	INL	1027[R]	*Burkholderia kururiensis*	100	EF178438
UFLA 03-588	Grass	SALL	1247[C]	*Dyella* sp.	99	KU296959.1
UFLA 03-673	Grass	INL	1418[C]	*Paenebacillus cineris*	99	NR_042189.1
UFLA 03-656	Grass	IAL	1285[C]	*Enterobacter* sp.	99	KR189750.1
UFLA 03-528	Grass	FNL	1389[C]	*Burkholderia Kururiensis*	99	AB568319.1
UFLA 03-659	Grass	IALL	1282[C]	*Bacteroidetes bacterium*	98	FJ786045.1
UFLA 03-655	Grass	SALL	1416[C]	*Paenebacillus cineris*	99	NR_042189.1
UFLA 03-669	Grass	FNL	422[F]	*Pseudomonas* sp	99	KJ831451.1
UFLA 03-707	Grass	SALL	437[F]	*Paenibacillus cineris*	99	KF979149.1
UFLA 03-670	Grass	INL	517[F]	*Paenebacillus* sp	99	KX418985.1
UFLA 03-612	Atlantic Forest	IALL	1297[C]	*Burkholderia kururiensis*	98	AB568319
UFLA 03-613	Atlantic Forest	IALL	1240[C]	*Novosphingobium aromaticivorans*	99	HF930753
UFLA 03-617	Atlantic Forest	IAL	1305[C]	*Paenibacillus polymyxa*	99	JN084141
UFLA 03-622	Atlantic Forest	INL	1281[C]	*Chitinophaga filiformis*	99	NR040909
UFLA 03-633	Atlantic Forest	SAL	1416[C]	*Paenibacillus cineris*	99	KF979149
UFLA 03-614	Atlantic Forest	IALL	1082[C]	*Brevibacillus nitrificans*	99	NR112926
UFLA 03-630	Atlantic Forest	SALL	1406[C]	*Paenibacillus cineris*	99	KF979149
UFLA 03-638	Atlantic Forest	SAL	629[F]	*Paenibacillus cineris*	100	KF979149.1
UFLA 03-706	Atlantic Forest	IAL	507[R]	*Paenebacillus* sp.	99	KT281432.1
UFLA 03-639	Atlantic Forest	FALF	1397[C]	*Acinetobacter* sp	99	HM629404.1
UFLA 03-635	Atlantic Forest	SNS	1406[C]	*Paenebacillus cineris*	99	NR_042189.1
UFLA 03-462	Neotropical savanna	FALL	1280[C]	*Brevibacillus Centrosporus*	99	NR_112211.1
UFLA 03-676	Neotropical savanna	SAL	1254[C]	*Paenibacillus relictisesami*	97	NR_133806.1
UFLA 03-702	Neotropical savanna	FAL	1407[C]	*Paenibacillus favisporus*	99	NR_029071.1
UFLA 03-486	Neotropical savanna	FNL	603[F]	*Bacillus* sp. *GM-1-2*	100	KT957627
UFLA 03-507	Neotropical savanna	FALL	759[R]	*Paraburkholderia tropica*	99	KP974788
UFLA 03-695	Neotropical savanna	FALL	1420[C]	*Paenibacillus cineris*	99	EF178439.1
UFLA 03-681	Neotropical savanna	SALL	1416[C]	*Paenibacillus rhizosphaerae*	99	NR_043166.1
UFLA 03-693	Neotropical savanna	FAM	1399[C]	*Bacillus* sp	100	KJ733993.1
UFLA 03-688	Neotropical savanna	SNL	467[F]	*Brevibacillus nitrificans*	99	KM894191.1
UFLA 03-677	Neotropical savanna	FALL	612[F]	*Brevibacillus centrosporus*	99	DQ339677.1
UFLA 03-705	Neotropical savanna	FAL	580[F]	*Bacillus subtilis*	100	KJ870194.1
UFLA 03-493	Neotropical savanna	IALL	719[R]	*Paraburkholderia hiiakae*	99	JF763857.1
UFLA 03-687	Neotropical savanna	FALL	491[R]	*Burkholderia tropica*	99	KP974788
UFLA 03-682	Neotropical savanna	SALL	690[R]	*Stenotrophomonas* sp.	99	KP729429.1
UFLA 03-479	Neotropical savanna	FALL	532[R]	*Brevibacillus nitrificans*	100	KM8941911

[1] Phenotypic groups formed from the growth rate, change in pH of the culture medium, and mucus production; FNAB: fast/neutral/ abundant, FALL: fast/alkaline/ little, FNM: fast/neutral/moderate, FNL: fast/neutral/little, FAL: fast/acid/little, SALL: slow/alkaline/ little, SAL: slow/acid/little, IALL: intermediate/alkaline/little, INAB: intermediate/neutral/abundant, INL: intermediate/neutral/little, SNL: slow/neutral/little, SALM: slow/alkaline/moderate, FALM: fast/alkaline/moderate, and SALAB: slow/alkaline/abundant. [2] NPB: number of pair of bases. [3] SI: percentage of similarity in the GenBank. [C]: Contig, [F]: Forward, and [R]: Reverse.

Bradyrhizobium, Cupriavidus, Agrobacterium, Herbaspirillum, Bacillus, Pseudomonas, Terriglobus, Paenebacillus, Dyella, Enterobacter, and *Brevibacillus*.

The genetic diversity of bacteria isolated from nodules was much higher in the soil under rehabilitated area revegetated with grass, followed by that under the Atlantic forest, neotropical savanna, and ironstone outcrops (Table 6).

DISCUSSION

From the soil collected, cowpea captured NFLNB species/genera among others that promote plant growth. According to Moreira and Siqueira (2006), nodulation can be influenced by a factor such as temperature, which can affect several stages in infection, formation, and function of nodules in the case of plant symbioses with NFLNB. During the experiment, the temperature in the greenhouse reached 46 °C, which may have negatively affected nodulation in some treatments. Soil chemical properties may also have affected nodulation of some treatments, especially those related to acidity and low concentration of nutrients, which may negatively affect microbiota (Moreira, 2006; Jesus et al., 2009; Lima et al., 2009). From the 16S rRNA gene partial sequencing, free-living, associative, and symbiotic N_2-fixing bacteria that act as plant growth promoters were identified, in addition to other genera commonly isolated from nodules.

The diversity of NFLNB in soils of the Quadrilátero Ferrífero of Minas Gerais was evaluated by Costa (2016) that observed the influence of soil chemical properties on the microbiota, corroborating the results obtained in this study. Principal component analysis (PCA) showed that soil chemical properties, such as pH, Al^{3+} content, and base saturation, provided more favorable conditions under the rehabilitated area revegetated with grass, possibly due to the influence of soil tillage. Among the soil properties, factors related to acidity, such as Al^{3+} content and pH, are those that most directly influence microbial communities, which possibly favored the higher nodulation rate in the treatments inoculated with soil from the rehabilitated area revegetated with grass.

The genus *Burkholderia* can benefit plant growth in several ways, such as in siderophore production and phosphate solubilization (Vial et al., 2007; Collavino et al., 2010; Marra et al., 2011; Mathew et al., 2014). This genus represented 46 % of the sequenced isolates, occurring in all types of vegetation. The selection of strains, such as those of the genus *Burkholderia*, which are able to adapt to certain soil conditions, can improve yield under field conditions and reduce the use of N fertilizers and, consequently, agricultural costs (Alves et al., 2016). Reis Jr et al. (2010) analyzed the nodulation and biological N_2 fixation of *Mimosa* species in the neotropical savanna and *Caatinga* biomes in Brazil. The authors suggest that *Burkholderia* spp. prefer soils with high acidity, which was corroborated in the present study since this genus occurred more frequently under ironstone outcrops.

Some researchers have suggested the name *Paraburkholderia* as a new name for part of the species in the genus *Burkholderia*. This genus occurred more frequently in a study carried out by Dall'Agnol et al. (2016), and, according to the authors, this may be associated with the properties of neotropical savanna soils, such as their pH, high Al contents, and low fertility.

Table 6. Diversity of rhizobia and other bacteria isolated from nodules formed in cowpea after inoculation by suspensions of soil from different vegetation types of the *Quadrilátero Ferrífero,* MG, Brazil

Vegetation type	Shannon Index H'	Relative number of genotypes
Ironstone outcrops	1.24	3.46
Neotropical savanna	2.21	9.13
Atlantic Forest	2.25	9.44
Rehabilitated area revegetated with grass	2.95	19.07

The altitude may also favor the predominance of this genus, since it influences humidity and/or temperature (Bontemps et al., 2010). Moreover, Dall'Agnol et al. (2016) state that the presence of *Burkholderia* (*Paraburkholderia*) in these environments did not indicate a preference of these genera for acidic conditions, but a tolerance to these conditions, which may represent an important role of these bacteria in maintenance of the ecosystem in these environments, characterized by acid soils with high Al saturation and low N content.

The genus *Bradyrhizobium* was identified among the isolates obtained from the plants inoculated with bacteria from soils from the rehabilitated area revegetated with grass, Atlantic Forest, and neotropical savanna. *Rhizobium* was the most frequent genus under the rehabilitated area revegetated with grass; however, it was also identified in other types of vegetation. Bacteria belonging to these genera are known to be N_2 fixers, and they form symbiosis with different leguminous plants, which is of agronomic importance because it benefits plant development (Zahran, 1999; Moreira and Siqueira, 2006). Bacteria belonging to the genus *Herbaspirillum* usually occur in grasses (Baldani et al., 1996; Olivares et al., 1997) and were identified under the rehabilitated area revegetated with grass. In addition, the genus *Brevibacillus* was identified, which occurs in grasses, and it also acts as a plant growth promoter (Nakamura, 1991; Shida et al., 1996; Lima, 2009).

Nodule endophytic but non-symbiotic bacteria belonging to the genera *Agrobacterium, Pseudomonas*, and *Terriglobus* also occurred in this area (Bai et al., 2002; Mhamdi et al., 2005; Wang et al., 2006; Kan et al., 2007; Li et al., 2008; Muresu et al., 2008). The genus *Bacillus* was identified under neotropical savanna and the rehabilitated area revegetated with grass, and includes the plant growth promoting rhizobacteria, commonly found in the rhizosphere of plants (Araujo, 2008; Jaramillo, 2010). Some representatives of this genus, in addition to endophytes, have been reported to nodulate siratro and cowpea, together with representatives of the genus *Paenibacillus*, found in the rehabilitated area revegetated with grass and the Atlantic Forest, but this still needs to be proven (Halverson and Handelsman, 1991; Siddiqui and Mahmood, 1999; McSpadden Gardener, 2004; Silva et al., 2007; Li et al., 2008; Marra et al., 2012; Costa et al., 2013; Jaramillo et al., 2013).

The genus *Novosphingobium* (formerly *Sphingomonas*) was isolated under the Atlantic Forest. Members of this genus are able to degrade polycyclic aromatic hydrocarbons and are frequently isolated from petroleum-contaminated soils; they are important for in situ bioremediation (Balkwill et al., 1997; Zhou et al., 2016). The genus *Chitinophaga* was also found under the Atlantic Forest vegetation. Representatives of this genus have already been isolated from the soil and rhizosphere of the plants (Kämpfer et al., 2006; Kim and Jung, 2007; Lee et al., 2007, 2009; Weon et al., 2009; Chung et al., 2012; Li et al., 2013). Even in the rhizosphere of leguminous plants, there may be other N_2 fixers, and the rhizosphere of grasses may have a representative number of NFLNB, which was observed in this study, especially in the rehabilitated area revegetated with grass.

The high genetic diversity of bacteria found in the rehabilitated area revegetated with grass was a good indicator of success in its rehabilitation.

CONCLUSIONS

The chemical properties of the soils that influenced biological properties were pH, sum of bases, and aluminum content.

Burkholderia, *Rhizobium*, and *Bradyrhizobium* were the most common genera.

The presence of soils with high levels of acidity may have favored high occurrence of the genus *Burkholderia.*

The greatest genetic diversity among vegetation types was found in the rehabilitated area revegetated with grass.

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