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Species of associative N_2-fixing bacteria in phytophysiognomies of the *Quadrilátero ferrífero*, MG, Brazil

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Resumo

A mineração é essencial para o desenvolvimento socioeconômico de um país, porém gera impactos ambientais severos e efeitos negativos à biodiversidade edáfica. Muitos métodos são utilizados para a reabilitação de áreas degradadas sendo o principal a revegetação com espécies nativas, podendo ser potencializada se combinada com inoculação de microrganismos promotores de crescimento vegetal. Portanto, levantamentos e estudos da diversidade destes microrganismos, como as bactérias fixadoras de N_2 associativas (BFN-associativas), representam o primeiro passo para seu posterior uso em reabilitação de áreas degradadas. O objetivo do trabalho foi isolar, caracterizar e identificar espécies de BFN-associativas, no período seco e no úmido, em amostras de solo sob quatro fitofisionomias no Quadrilátero Ferrífero: Mata Atlântica, Cerrado, Canga e "Capim", uma área revegetada com gramíneas após a mineração de ferro. As estirpes foram isoladas em meios de cultura NFb e FAM. Após caracterização cultural foi realizado o sequenciamento parcial do gene 16S rDNA e comparação com sequências depositadas no GenBank. Foram obtidas 37 estirpes em todas as fitofisionomias exceto Mata Atlântica. Os meios de cultura permitiram o crescimento bacteriano típico de diversas fixadoras de N_2. *Gluconacetobacter diazotrophicus*, considerada obrigatoriamente endofítica, foi a espécie com maior frequência de isolamento do solo. Houve maior densidade e diversidade na fitofisionomia Capim, indicando que o processo de recuperação é efetivo com relação às BFN.

Palavras chave: *Gluconacetobacter diazotrophicus, Paraburkholderia*, *Azospirillum*, bactérias promotoras de crescimento vegetal, mineração de ferro

Abstract

Mining is essential for the socioeconomic development of a country; however, it generates severe environmental impacts and negative effects on edaphic biodiversity. Many methods are used for the rehabilitation of degraded areas; especially revegetation with native species. This method can be enhanced if combined with inoculation with plant growth-promoting microorganisms. Thus, surveys and studies of the diversity of these microorganisms, such as associative N_2-fixing bacteria, are the first step for their subsequent use in rehabilitation of degraded areas. The objective of this study was to isolate, characterize, and identify associative N_2-fixing bacteria in dry and rainy seasons in soil samples from four phytophysiognomies in the *Quadrilátero Ferrífero* ("Iron Quadrangle" mining region): Atlantic Forest, *Cerrado* (neotropical savanna), *Canga* (ironstone outcrops), and an area revegetated with grasses after iron mining. The isolates were obtained by using NFb and FAM as culture media. After culture characterization, partial sequencing of the 16S rDNA gene was carried out for comparison with sequences available in GenBank. A total of 37 strains were obtained from all the phytophysiognomies examined, except for Atlantic Forest. The culture media allowed growth typical of diverse N2-fixing bacteria. *Gluconacetobacter diazotrophicus*, usually considered obligate endophytic, was the species of highest frequency among the strains isolated from soil. Higher density and diversity were found in the grass phytophysiognomy, suggesting the effectiveness of the rehabilitation processes regarding N2-fixing bacteria.

Key words: *Gluconacetobacter diazotrophicus*, *Paraburkholderia*, *Azospirillum,* plant growth-promoting bacteria, iron mining

INTRODUCTION

Nitrogen-fixing bacteria transform atmospheric N_2 into ammonia for their own metabolism and also make it available to plants. The contribution of nitrogen to the soil-plant system arising from associations is less efficient than from symbiotic relationships. Nevertheless, associations are also linked to plant growth promotion through production of hormones (IAA), antibiotics, phosphate solubilization, and pathogen-antagonistic action, among other effects (MOREIRA and SIQUEIRA, 2006).

Diverse human interventions in the soil for agricultural purposes, and especially for mining, modify its physical and chemical properties and, consequently, its biological properties, thus affecting the edaphic microbial community. The presence or absence of plant cover, as well as the type of plant cover, the soil type, and other factors affect both the diversity and the density of symbiotic N_2-fixing bacteria in the soil (CASTRO et al., 2017).

Several studies performed in Brazil have reported the effects of different soil cover conditions in mining areas on associative N_2-fixing microorganisms. Melloni et al. (2004) and Nobrega et al. (2004) found low density of N_2-fixing bacteria in areas under rehabilitation after decades of bauxite mining in comparison with agricultural systems. Nevertheless, there was wide phenotypic diversity among the isolates from the area. This high diversity, according to the authors, would stem from the resilience of the microorganisms in the soil from the prior plant cover, for that soil was used for rehabilitation of the mined areas. In addition, these microorganisms could have been introduced through replanting of seedlings or from the seeds used in planting. Grasses, leguminous plants, native tree species, and eucalyptus were used in these studies. In a study conducted in areas contaminated with heavy metals, Moreira et al. (2008) evaluated the density of associative N_2-fixing microorganisms, as well as their phenotypic and genetic characterization and observed that the densities found were similar to those registered in agricultural soils reported in the literature by other authors. Therefore, although there may have been a decline in the population densities of the associative N_2-fixing bacteria due to changes arising from soil use, the persistence of diverse isolates indicates the capacity of these microorganisms to resist the stress conditions imposed by the environment. This information reinforces the importance of this group for use in association with plants in recovery of degraded areas.

Mining companies are obliged to mitigate the socioeconomic and environmental damage generated in their areas of activity. For that reason, they make use of legal tools that complement the factors linked to environmental impacts, such as the Avaliação de Impacto Ambiental – AIA (Environmental Impact Evaluation) and Estudos de Impacto Ambiental – EIA (Environmental Impact Studies), among others (BARBIERI, 2007). Evaluation of associative N_2-fixing bacteria in agricultural areas due to their potential in promoting plant growth has had promising results in increasing yield (BOLÍVAR-ANILLO et al., 2016). Thus, this same technique may be viable in soils that have been degraded and later revegetated. A survey of the microbial communities in these areas under mining and not under mining is necessary. Microbial communities in the areas not under mining are already adapted to the local edaphic and climatic conditions. Studies on associative N_2-fixing bacteria in iron-mining areas were not found.

The aim of this study was to isolate, characterize, and identify associative N_2-fixing bacteria in four phytophysiognomies: *Canga* (ironstone outcrops), Grass (revegetated grass areas), *Cerrado* (neotropical savanna), and Atlantic Forest – under the effects of iron mining in the *Quadrilátero Ferrífero* ("Iron Quadrangle" mining region).

MATERIALS AND METHODS

The N_2-fixing bacteria were isolated from soil samples collected in an iron mining area of the company Vale S/A in the Quadrilátero Ferrífero. The phytophysiognomies are in the state of Minas Gerais in the municipality of Nova Lima in the Iron Technology Center (Centro de Tecnologia de Ferrosos – Miguelão) and in the municipality of Brumadinho in the Córrego do Feijão Mine. Local climate is characterized as Humid Subtropical (Cwa - Köppen-Geiger). The chemical and physical characteristics of the soil samples are shown in Table 1.

Table 1. Chemical and physical analysis of soils collected in Canga (ironstone outcrops), Grass (a rehabilitated area revegetated with grass), Cerrado (neotropical savanna), and Atlantic Forest vegetation areas at the Iron Technology Center (Centro de Tecnologia de Ferrosos – CTF Miguelão) and in the Córrego do Feijão Mine, Vale S/A (CASTRO et al., 2017)

Area	pH	[2]K	[2]P	[1]Ca^{2+}	[1]Mg^{2+}	[1]Al^{3+}	[4]H+Al	SB	t	T	V
		--mg dm^{-3}--		----------$cmol_c.dm^{-3}$----------							%
Canga	4.72	56.80	1.59	1.28	0,24	0,85	12,64	1.66	2.51	14.31	13.56
Grass	5.60	88.20	1.66	0.75	0.30	0.09	1.94	1.27	1.36	3.21	40.73
Cerrado	4.97	72.60	1.36	0.91	0.38	1.56	15.46	1.47	3.03	16.94	12.72
Atlantic Forest	4.21	75.60	2.15	0.99	0.45	1.90	12.26	1.63	3.53	13.89	13.64

	m	OM	Rem-P	[2]Zn	[2]Fe	[2]Mn	[2]Cu	[1]B	[3]S	Clay	Silt	Sand
	%	$dag kg^{-1}$	$mg L^{-1}$	----------$mg dm^{-3}$----------						g kg^{-1}		
Canga	33.8	7.58	12.71	3.29	403.7	88.9	0.57	0.26	26.58	214	174	612
Grass	6.76	1.38	11.03	1.60	150.8	104.0	2.14	0.15	45.14	249	262	489
Cerrado	46.1	8.30	4.56	3.13	134.5	112.3	0.79	0.20	36.29	376	243	381
Atlantic Forest	59.3	4.94	11.02	1.92	124.7	40.76	0.80	0.20	29.06	456	188	356

pH in water, soil:solution 1:2.5; Ca^{2+}, Mg^{2+}, Al^{3+}: 1 mol L^{-1} KCl extractant; H+Al: SMP extractant; SB: sum of bases; T: cation exchange capacity at pH 7.0; t: effective cation exchange capacity; V: base 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 extractant; S: monocalcium phosphate - acetic acid extractant; B: hot water extractant. Properties analyzed according to the methods proposed by [1] Vettori (1969); [2] Mehlich (1953); [3] Richards (1954); and [4] Shoemaker et al. (1961).

Two soil samplings were carried out – the first on August 10-15, 2015, in the dry season, and the second on January 11-15, 2016, in the rainy season. The phytophysiognomies of the samples were characterized as Cerrado "stricto sensu", Canga, Atlantic Forest, and Grass (revegetated grassland), for a total of four different phytophysiognomies. The vegetation of the Canga and Atlantic Forest areas were described according to the forest inventory, Inventário Florestal de Minas Gerais (2009). Only the Grass phytophysiognomy, which represented an area rehabilitated after iron mining, was an area under the impact of mining activity. Mining residues had been stored in the Grass area, and currently the area has a plant cover predominantly of grasses, such as signalgrass (*Brachiaria decumbens)*, molasses grass *(Melinis minutiflora)* and panicgrass (*Panicum maximum* Jacq) (CASTRO et al., 2017). This phytophysiognomy underwent a rehabilitation process before planting, which consisted of soil tillage with liming. The four phytophysiognomies were divided into two transects at a 50-meter distance from each other and, in each transect, five points were marked off and georeferenced at a distance of five meters from each other, constituting 10 points per transect (CASTRO et al., 2017) (Figure 1). Samples were collected from the 0-20 cm layer, transported in thermal containers, and placed in cold storage at 4ºC until analysis.

Two semi-solid culture media – NFb (DÖBEREINER et al., 1995) and FAM (MAGALHÃES and DÖBEREINER, 1984) – were used to isolate the N_2-fixing bacteria, both for the samples collected in the dry season and in the rainy season. The strain BR11001^T (A*zospirillum brasilense*) was used as a positive control for validation of the medium regarding film formation, indicating the presence of N_2-fixing bacteria.

Ten grams of soil from each point were suspended in salt solution (0.85% NaCl), obtaining dilutions of 10^{-1} for rapid preliminary evaluation in the dry season, which aimed at a semi-quantitative evaluation. Later evaluation aimed at density by the most probable number (MPN) method, both in the rainy season and in the dry season. For that purpose, ten grams of soil from each point were suspended in salt solution (0.85% NaCl) and successively diluted to a 10^{-4} dilution. In both evaluations (semi-quantitative and density), a 0.1 mL aliquot of suspension in each dilution was inoculated in containers with 15 mL of each of the two semi-solid media, with five replications in preliminary evaluation in the dry season and three replications in density evaluation in the dry and rainy seasons. Soon after inoculation, the containers were incubated in a growth chamber at 30°C for 14 days. Evaluation of the growth of N_2-fixing bacteria in the containers was performed visually, observing the presence or absence of a film on the surface of the culture medium (DÖBEREINER et al., 1995).

After the incubation period, a portion of the film was removed on the surface of the positive containers and re-inoculated on the respective semi-solid NFb or FAM medium. These cultures were incubated so as to confirm film formation. After confirmation, the cultures were chopped up into dishes containing NFb and FAM solid media with the addition of 0.2 g of yeast extract and incubated for five days at 30ºC in BOD. After being chopped up several times into dishes with NFb and FAM solid media, pure cultures were obtained. Semi-quantitative evaluations were made, observing the number of containers with films for collection of soil in the dry season. In the second collection of soil, in the rainy season, and once more in the soil collected in the dry season, the MPN (most

probable number) was calculated (MACGRADY, 1992). The isolates were chopped up into potato dextrose agar medium for confirmation of purity, culture characterization, and storage. Density analysis was performed by the Sisvar 5.6, Build 86 program, with the Scott-Knott test at .05%, transforming the data to log $(x+1)^{0.5}$.

For culture characterization, the isolates were incubated for 4 days in a growth chamber at 30°C. The characteristics of the colonies evaluated were days of appearance, mean diameter (mm), shape (punctiform, circular, and irregular), margin (entire, undulate, lobate, serrated, and filamentous), surface (smooth, rough, and papillated), gum production (scarce, little, moderate, and abundant), elevation (flat, lenticular, convex, drop-like, umbonate, and umbilicate), consistency of growth matter (dry, aqueous, gummy, viscous, and butyric), optical details (transparent, translucent, opaque, and shiny), and color (white, pink, light pink, cream, dark cream).

Genetic identification was made by partial sequencing of the 16S rRNA gene in strains representing all the phenotypic groups of the cultures obtained. The alkaline lysis method was used for extraction of genomic DNA from each strain (NIEMANN et al., 1997). For amplification of the 16S rRNA gene, a mixture was prepared for each polymerase chain reaction (PCR), with a final volume of 50 µL, containing 4.5 µL DNA, 5 µL 10X KCl buffer, 4 µL MgCl2 (2.5 mM), 5 µL dNTP Mix (2 mM), 1 µL of each primer [27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') at 10 mmol L^{-1}] (LANE, 1991), 0.4 µL of Taq DNA polymerase (5U μL^{-1}), and ultrapure sterile water. The amplification reaction was performed in an Eppendorf Mastercycler thermal cycler under the following conditions: initial denaturation at 94°C for 5 min, 35 denaturation cycles (94°C for 40 s), annealing (55°C for 40 s), extension (72°C for 90 s), and a final extension of 72°C for 7 min. An aliquot of each reaction of amplified products was analyzed in agarose gel (1% w/v) with the addition of the stain SYBR Safe (Invitrogen) under UV light. The -1 Kb DNA Ladder marker was used in the gel to verify amplification of the gene. Purification of the PCR products and the DNA sequencing (with the primers 27F and 1492R) were performed by the laboratory WemSeq Biotecnologia (Curitiba, PR, Brazil), and the quality of the sequences was analyzed with the BioNumerics 7.5 software. The sequences that were valid and of quality were submitted to BLASTn (Bethesda, MD, USA) and compared to type strains in the GenBank (National Center for Biotechnology Information) database to obtain the most similar accession.

RESULTS AND DISCUSSION

In preliminary evaluation in the dry season, 4% (2/50) of the samples of the Grass phytophysiognomy were positive, i.e., with film formation confirmed, in the FAM culture medium, and 6% (3/50) in the NFb medium (Table 2). In the Canga phytophysiognomy, 8% (4/50) of the samples were positive in NFb medium, and there was no film formation in the FAM medium. In the Cerrado and Atlantic Forest phytophysiognomies, there was no film formation in either of the culture media.

Table 2. Frequency of containers with culture media (FAM and NFb) manifesting positive for growth typical of N_2-fixing bacteria in soil samples from different phytophysiognomies in the dry season (semi-quantitative evaluation).

Area	FAM	NFb
	%	
Grass	4 a	6 a
Canga	8 a	0 b
Cerrado	0 a	0 b
Atlantic Forest	0 a	0 b
Coefficient of Variation (%)	6.92	4.94

Both in the dry season and in the rainy season in the Grass area, N_2-fixing bacteria were detected in the NFb and FAM media, and in the Canga, only in the FAM (Table 3). In the Cerrado, there was only growth in the FAM medium in the rainy season, and no growth was detected in the Atlantic Forest in either season or medium. The phytophysiognomy with greatest density was Grass, with significant difference from the others for growth in FAM and NFb media in the rainy season, though without significant difference in the dry season (Table 3).

Table 3. Density of associative N_2-fixing bacteria in the dry and rainy seasons obtained by the most probable number method.

Area	Most probable number - MPN			
	Dry season		Rainy season	
	FAM medium(1)	NFb medium(2)	FAM medium(1)	NFb med.(2)
Canga	2.8 $X10^1$ a	0 a	0.5 $X10^1$ b	0 b
Grass	3.3 $X10^1$ a	0.7 $X10^1$ a	2.9 $X10^1$ a	1.5 $X10^1$ a
Cerrado	0 a	0 a	0.8 $X10^1$ b	0 b
Atlantic Forest	0 a	0 a	0 b	0 b
CV (%)	120.02	53.81	70.98	74.71

(1)MPN – Mean of the most probable number of cells per gram from 3 soil samples in FAM culture medium for each area; (2)MPN – Mean of the most probable number of cells per gram from 3 soil samples in NFb culture medium for each area; CV: Coefficient of variation.

Compared to other studies that evaluated the density of associative N_2-fixing bacteria in different land use systems with similar edaphic and climatic characteristics, all the phytophysiognomies can be considered to be of low density. However, the samples under the effect of grasses had greater density, with values similar to those obtained by other authors (MAGALHÃES and DÖBEREINER,

1984; MELLONI, 2004; SILVA et al., 2011; SILVA and MELLONI, 2011). Silva et al. (2011) report a tendency for greater species diversity of associative N_2-fixing bacteria isolated in land use systems in the Amazon region for the pasture land use system with predominance of grasses. Silva and Melloni (2011) also obtained greater density of associative N_2-fixing bacteria in grass areas, similar to the results obtained in this study. The lack of studies regarding nutrient cycling in areas under the effects of mining leads us to establish two hypotheses. Greater density in the grass area is likely not due only to the distinct and favorable rhizosphere effect of grasses (BULGARELLI et al., 2013). Another possibility is that greater deficiency of N in the soil caused by the greater effect of leaching leads to greater demand for N by plants, which stimulate N_2-fixing bacteria.

In contrast, edaphic microbial communities are also affected by the soil physical-chemical attributes, such as acidity and aluminum content, which are considered to be high in all the phytophysiognomies (Table 1). These characteristics may have negatively affected the microbiota in all the phytophysiognomies, except for the Grass phytophysiognomy, which exhibited lower values for these attributes in relation to the others. These results corroborate those found by Castro et al. (2017) in evaluation of rhizobia in relation to the physical-chemical attributes of the same phytophysiognomies under study here. Palmer and Young (2000) report that soil bacterial diversity may be affected by clay content, pH, and organic matter. Organic matter exhibited very high values in Canga and Cerrado, high in Atlantic Forest, and low in grasslands, and thus, restrictions of N may have stimulated N_2-fixing bacteria.

The appearance of the colonies in potato medium and the characteristics of the 37 strains varied in relation to the parameters observed in the following manner: “circular, irregular, and punctiform” in relation to shape; “lenticular, convex, umbonate, umbilicate, and pulvinate” for elevation; “whole and undulate” for margins; “smooth and rough” for surface; “shiny and opaque” for optical details; and “white, cream, and pink” for color. In general, growth occurred in three days, conferring rapid growth to all the strains, and the mean diameter was 0.54 cm.

The greatest phenotypic diversity was also obtained in the Grass phytophysiognomy. According to Moreira and Siqueira (2006), non-symbiotic species related to nitrogen fixation occur in the soil, abundantly in the rhizosphere and endophytically in plant species, an occurrence more related to grasses. Some of the more commonly found species belong to the genera *Azospirillum*, *Gluconacetobacter*, *Herbaspirilum*, and *Burkholderia./Paraburkholderia* The soil collected in the grassland area is predominantly covered with signalgrass (*Brachiaria decumbes*), molasses grass (*Melinis minutiflora*), and panicgrass (*Panicum maximum*) used for replanting (CASTRO et al., 2017). Before planting, this phytophysiognomy passed through a rehabilitation process that consisted of soil tillage with liming. Baldani et al. (1999) report that introducing grasses in degraded areas for their rehabilitation can be advantageous, since the influence of non-symbiotic bacteria that are free-living and/or endophytic is directly related to the associated vegetation. Revegetation with grasses in the phytophysiognomy made a positive contribution to increasing the density of these bacteria in the soil and, consequently, a larger number of strains were acquired. This corroborates Silva et al. (2011) and Magalhães and Döbereiner (1984), who observed greater density of diazotrophic microorganisms associated with pasture, in contrast with the smaller number of strains in the other phytophysiognomies, such as forests, from which strains were not obtained.

Canga was the phytophysiognomy with the second largest number of strains obtained. Shrubs, adapted to the particular conditions, and grasses occur in this environment (COSTA, 2007), whose soil has the greatest content of iron (Table 1). Strains were not obtained from the native Atlantic Forest area, which was also observed by Melloni et al. (2004) when they studied the density and the diversity of diazotrophic bacteria in a bauxite mining area under rehabilitation and adjacent phytophysiognomies.

The partial sequences of the 16S rDNA of the 37 strains, most with 1140 and 1300 base pairs, had similarity from 98% to 100% with sequences deposited in the GenBank. All the strains were identified up to genus, and some up to species level, with a total of 9 genera (*Rhizobium*, *Azospirillum*, *Paraburkholderia*, *Burkholderia*, *Caulobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebisiella*, *Mesorhizobium* and *Agrobacterium*) and 10 species (*Agrobacterium rhizogenes*, *Azospirillum formonsense, A. brasilense*, *Paraburkolderia caribensis, Paraburkholderia cledonica*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum hiltneri*, *Klebisiella pneumoniae*, *Rhizobium miluonense and Agrobacterium tumefasciens*) (Table 4). The identification only at the genus level was due to the fact that more than one type of strain of known species has the same similarity, indicating that the high conservation of the 16S rRNA gene limits the discrimination of species in these genera. Additional analyzes are being carried out to identify the species of these strains. *Burkholderia* and *Gluconacetobacter* were the most ubiquitous genera: they were isolated in two of the four phytophysiognomies under study.

The strains UFLA209, UFLA210, UFLA211, UFLA212, UFLA213, UFLA 214, UFLA 215, UFLA 216, UFLA 217, UFLA 218, UFLA224, UFLA225, UFLA228, UFLA229, UFLA230, UFLA231, UFLA232, UFLA233, UFLA238 to UFLA243 and UFLA245 belong to the N2-fixing genera *Rhizobium*, *Azospirillum*, *Paraburkholderia Burkholderia, Gluconacetobacter,, Herbaspirilum*, *Klebsiella* and *Mesorhizobium*, all of them coming from the Grass phytophysiognomy.

The *Rhizobium* genus, isolated in Fam and NFb media and the Grass phytophysiognomy (UFLA 209, 210, 241, 243, and 245) corroborate the study of Castro et al. (2017), who studied the same phytophysiognomies and obtained species of this genus. This genus has diazotrophic species that form nodules with leguminous plants and are able to supply nitrogen by symbiosis with some plants of this family (MOREIRA, 2010). The fact of being symbionts does not impede isolating them from the soil because this genus is present in the edaphic environment before establishing symbiosis with legumes, and it may also promote growth of non-legume plants, as shown by Mishra et al. (2006) and Filho et al. (2016) in rice.

Although the NFb and FAM media favor the growth of *Azospirillum* spp., only three strains belong to this genus [*A. formosense* (UFLA 211 and UFLA 212) and *A. brasilense* (UFLA 213)], were isolated in NFb medium. The *Burkholderia* and *Paraburkholderia* genera had eight representatives, six of them identified only to the genus level (UFLA 215, UFLA 217, UFLA 218, UFLA 219, UFLA220 and UFLA 221) and 2 species [*P. caribensis* (UFLA 214), and *B. caledonica* (UFLA 216)]. These genera are widely discussed in studies on diversity of associative diazotrophic bacteria, with potential for nitrogen fixation, growth promotion, and tolerance to some metals (MOREIRA et al., 2008; SUN et al., 2010; BOLÍVAR-ANILLO et al., 2016). It is mainly associated with grasses such as wheat, rice, and sugarcane, promoting growth and the possibility of reducing the use of fertilizers in these crops. Association with grasses is also characteristic of the genera *Azospirillum*, *Gluconacetobacter* and *Herbaspirilum* (BERTALAN et al., 2009; SABINO et al., 2012; MOREIRA et al., 2016).

The *Caulobater* genus can be found in diverse types of environments, including the soil. The UFLA 222 and UFLA 223 strains isolated in FAM medium coming from the Grass phytophysiognomy (Table 4) belong to this genus, with 100% similarity with the strains deposited in GenBank through homology of the 16S rRNA gene. Some of the characteristics observed in reference to the white color and growth pH from 6 to 6.8 in the medium used, both in isolation and in culture characterization of these strains, are similar to those of the strain 7F14T isolated from the rhizosphere soil under the watermelon crop (SUN et al. 2017), where it formed a phylogenetic lineage within the *Caulobacter* genus. This genus is also related to pyrene degradation in soils contaminated with polycyclic aromatic hydrocarbons (PAHs) (JONES et al., 2008).

The species *Gluconacetobacter diazotrophicus*, and *Gluconacetobacter* sp. were isolated in the Grass and Canga phytophysiognomies in the FAM medium. All *G.* sp. had 100% similarity with type strains of *G. diazotrophicus* and of other G. species. They represent 14 of the 37 strains, and has biotechnological characteristics related to nitrogen fixation, secretion of organic acids, and promotion of plant growth (BERTALAN et al., 2009). It should be noted that *G. diazotrophicus* has been considered an obligate endophyte (DÖBEREINER et al., 1995), but it was isolated from soil in this study.

UFLA 240, isolated in NFb medium in the Grass phytophysiognomy (Table 4) belonging to the family *Enterobacteriaceae* and to the genus *Klebisiella*, is commonly isolated from the soil. This genus has high production of siderophores in rhizosphere soils, and may be related to plant growth promotion (MOREIRA et al., 2016). The species *Klebisiella pneumoniae* isolated from a soil under eucalyptus growing is able to solubilize phosphorus (MASSENSSINI et al., 2016). According to Torres (2016) and Pereira (2016), although this species is able to fix atmospheric nitrogen, it may be an opportunistic pathogen in human beings, linked to hospital infections.

UFLA244 belongs to the genus *Agrobacterium*, commonly found in soils. This is a genus of great biotechnological and phytoremediation importance. *Agrobacterium rhizogenes*, the species identified for the strain UFLA 209, can induce what is known as “hairy root” (MOREIRA and SIQUEIRA, 2006). Strains of *Agrobacterium* are used in genetic engineering, such as tissue culture for production of secondary metabolites and recombinant proteins and in studies of plant metabolism (RON et al., 2014).

CONCLUSIONS

Both media, NFb and FAM, allowed growth of *Azospirillum* spp., as is already well known in the literature, but they also made it possible to isolate other genera and species of N_2-fixing bacteria, including *Rhizobium* and *Mesorhizobium*, which are symbiotic genera. The greatest density and, consequently, largest number of strains were obtained in the Grass phytophysiognomy in the dry season in the FAM medium, corroborating the positive effect of associated vegetation. *Gluconacetobacter* was isolated most frequently.

The bacterial genera obtained in this isolation can be used in future studies of biotechnological and environmental interest.

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Table 4. Origin (culture medium, season, and phytophysiognomy) and identification of isolates coming from phytophysiognomies in the Quadrilátero Ferrífero through comparison with sequences of accessions deposited in the GenBank.

Strain	Medium (1)	Season 2)	Origin (3)	Species/genus	Identification based on the accession of greatest similarity found in the Genbank			GenBank accession numbers (NCBI)
					NBP (5)	%	NCBI acession no	
UFLA209	FAM	Wet	Grass	*Rhizobium* sp.	622^{R}	99.5	JX292484.1	MT071941
UFLA210	NFb	Wet	Grass	*Rhizobium* sp.	1148^{C}	100	MH819518.1	MT071900
UFLA211	NFb	Dry	Grass	*Azospirillum formosense*	877^{R}	99.9	NR_117483.1	MT071942
UFLA212	NFb	Dry	Grass	*Azospirillum formosense*	881^{R}	99.8	NR_117483.1	MT071943
UFLA213	NFb	Dry	Grass	*Azospirillum brasilense*	1190^{C}	99	NR_042845.1	MT071901
UFLA214	FAM	Wet	Grass	*Paraburkholderia caribensis*	1256^{C}	99.7	NR_026462.1	MT071902
UFLA215	FAM	Wet	Grass	*Paraburkholderia* sp	734^{R}	99.9	CP013103.1	MT071944
UFLA216	FAM	Wet	Grass	*Paraburkholderia caledonica*	1304^{C}	99.6	NR_025057.1	MT071903
UFLA217	FAM	Wet	Grass	*Burkholderia* sp.	1194^{C}	100	MF495750.1	MT071904
UFLA218	FAM	Wet	Grass	*Burkholderia* sp.	1203^{C}	99.5	EU677416.1	MT071905
UFLA219	FAM	Wet	Cerrado	*Burkholderia* sp.	812^{R}	99.9	EU677416.1	MT071945
UFLA220	FAM	Wet	Canga	*Paraburkholderia* sp.	658^{R}	100	MN239497.1	MT071946
UFLA221	FAM	Wet	Cerrado	*Burkholderia* sp.	540^{F}	100	MG859635.1	MT071792
UFLA222	FAM	Wet	Grass	*Caulobacter* sp.	1146^{C}	100	KP165519.1	MT071906
UFLA223	FAM	Wet	Grass	*Caulobacter sp.*	1128^{C}	100	KP165519.1	MT071907
UFLA224	FAM	Dry	Grass	*Gluconacetobacter diazotrophicus*	1191^{C}	98	NR074284.1	MT071908
UFLA225	FAM	Dry	Grass	*Gluconacetobacter* sp.	1248^{C}	100	AF127408.1	MT071909
UFLA226	FAM	Dry	Canga	*Gluconacetobacter* sp.	1296^{C}	100	AF127408.1	MT071910
UFLA227	FAM	Dry	Canga	*Gluconacetobacter* sp.	1246^{C}	100	AF127408.1	MT071911
UFLA228	FAM	Dry	Grass	*Gluconacetobacter* sp.	1148^{C}	100	AF127408.1	MT071912
UFLA229	FAM	Dry	Grass	*Gluconacetobacter* sp.	873^{R}	100	NR074284.1	MT071947
UFLA230	FAM	Dry	Grass	*Gluconacetobacter* sp.	1255^{C}	100	AF127408.1	MT071913
UFLA231	FAM	Dry	Grass	*Gluconacetobacter* sp.	513^{R}	100	AF127408.1	MT071948
UFLA232	FAM	Dry	Grass	*Gluconacetobacter* sp.	1251^{C}	100	NR114382.1	MT071914
UFLA233	FAM	Dry	Grass	*Gluconacetobacter* sp.	1232^{C}	100	AF127408.1	MT071915
UFLA234	FAM	Dry	Canga	*Gluconacetobacter* sp.	1189^{C}	100	AF127408.1	MT071916
UFLA235	FAM	Dry	Canga	*Gluconacetobacter* sp.	1151^{C}	100	AF127408.1	MT071917
UFLA236	FAM	Dry	Canga	*Gluconacetobacter* sp.	1193^{C}	100	NR074284.1	MT071918
UFLA237	FAM	Dry	Canga	*Gluconacetobacter* sp.	1161^{C}	100	NR074284.1	MT071919
UFLA238	FAM	Wet	Grass	*Gluconacetobacter* sp.	751^{R}	100	NR074284.1	MT071949
UFLA239	FAM	Wet	Grass	*Herbaspirillum hiltneri*	1199^{C}	99.9	NR_043582.1	MT071920
UFLA240	NFb	Dry	Grass	*Klebsiella* sp.	725^{F}	100	KR189748.1	MT071793
UFLA241	NFb	Dry	Grass	*Rhizobium miluonense*	1243^{C}	99	NR_044063.1	MT071921

UFLA242	NFb	Dry	Grass	*Mesorhizobium* sp.	639[R]	99.4	LC040873.1	MT071950
UFLA243	NFb	Wet	Grass	*Rhizobium* sp.	1207[C]	99.9	MH665723.1	MT071922
UFLA244	NFb	Wet	Grass	*Agrobacterium tumefaciens*	1190[C]	99.7	AE007870.2	MT071923
UFLA245	NFb	Wet	Grass	*Rhizobium* sp.	487[R]	99.8	MN437571.1	MT071951

*The isolation of strains occurred only in the dilution 10^{-1}. [(1)]Culture medium used for isolation; [(2)] Collection season of the soil samples; [(3)] Phytophysiognomy; [(4)] NBP – number of base pairs obtained in partial sequencing of the 16S gene; [C] – sequences obtained by "Contigs"; [R] – sequences obtained by "Reverse"; [F] – sequences obtained by Forward.

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