PATRÍCIA DE FREITAS COSTA

DIVERSIDADE E EFICIÊNCIA SIMBIÓTICA DE BACTÉRIAS FIXADORAS DE NITROGÊNIO ISOLADAS DO QUADRILÁTERO FERRÍFERO E CAPTURADAS POR SIRATRO (*Macroptilium atropurpureum*) E CAUPI (*Vigna unguiculata*)

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutora.

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DIVERSITY AND SYMBIOTIC EFFICIENCY OF NITROGEN-FIXING BACTERIA ISOLATED FROM QUADRILÁTERO FERRÍFERO AND CAPTURED BY SIRATRO SIRATRO (*Macroptilium atropurpureum*) AND COWPEA (*Vigna unguiculata*)

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RESUMO

Objetivando avaliar a ocorrência, a diversidade genética, a diversidade fenotípica e a eficiência simbiótica de bactérias fixadoras de nitrogênio que nodulam leguminosas (BFNNL) de cinco áreas com diferentes coberturas vegetais e características de solo, na região do Quadrilátero Ferrífero (Minas Gerais – Brasil), foram coletados solos de quatro pontos em áreas de canga, cerrado, mata, capim e eucalipto. O acesso às BFNNL e o estudo da eficiência simbiótica foram realizados em casa de vegetação, utilizando duas espécies de leguminosas: siratro (*Macroptilium atropurpureum*) e caupi (*Vigna unguiculata*). Foram obtidas estirpes de BFNNL de todas as cinco áreas avaliadas. O caupi se mostrou mais efetivo na captura, uma vez que capturou estirpes de todos os pontos, em todas as áreas, e apresentou maior densidade de BFNNL que o siratro. A comunidade de BFNNL presente na área de capim foi mais eficiente que das demais áreas utilizando siratro como planta isca e a área de canga apresentou menor eficiência das comunidades ao utilizar caupi. Um grande número de estirpes obtidas tanto pelo caupi quanto pelo siratro se mostraram eficientes na fixação biológica de N_2, revelando potencial biotecnológico. Os atributos edáficos pH, saturação de bases e teor de alumínio foram os que mais estiveram relacionados aos atributos biológicos. A eficiência relativa e matéria seca da parte aérea das comunidades de BFNNL foram ótimos indicadores das mudanças na vegetação. A diversidade fenotípica considerou características como tempo de crescimento, alteração do pH do meio de cultura, tamanho, forma e cor da colônia. A caracterização genética foi obtida por meio do sequenciamento parcial do gene 16S rRNA aliado à técnica de BOX-PCR. A área de mata foi a mais diversa e a de canga foi a menos diversa. Alguns gêneros de BFNNL como *Bradyrhizobium* e *Rhizobium* foram capturados por ambas as espécies vegetais, porém outros gêneros foram capturados apenas pelo siratro ou apenas pelo caupi, evidenciando a importância do uso de mais de uma espécie vegetal em estudos de diversidade. O presente trabalho é o primeiro relato sobre diversidade e eficiência simbiótica de estirpes de BFNNL em ambiente de canga.

Palavras-chave: Rizóbio. Fixação biológica de nitrogênio. Diversidade. Canga.

ABSTRACT

With the objective of evaluating the occurrence, genetic diversity, phenotypical diversity and symbiotic efficiency of nitrogen fixating Leguminosae nodulating bacteria (NFLNB) of five areas with different vegetation cover and soil characteristics, in the region of the Iron Quadrangle (Minas Gerais – Brazil), we collected soil from four sampling points in Canga, Cerrado, Forest, grass and eucalyptus areas. The access to NFLNB and the study on symbiotic efficiency was conducted in greenhouse using two leguminous plants: siratro (*Macroptilium atropurpureum*) and caupi (*Vigna unguiculata*). We obtained strains of NFLNB from all five evaluated areas. The caupi was more effective in the capture, since it captures strains from all points, in all areas, and presented the highest NFLBN density than siratro. The NFLNB community present in the grass area was more efficient than those from the other areas using siratro as bait plant, and the canga area presented lowest efficiency of the communities using caupi. A large number of strains obtained by both the caupi and siratro were efficient in the biological fixation of N_2, revealing the biotechnological potential. The edaphic attributes pH, base saturation and content of aluminum were the most related to the biological attributes. Relative efficiency and shoot dry matter of the NFLBN communities were optimum indicators of the vegetation changes. For phenotypical diversity, we considered characteristics such as growth time, changes of pH of the culture medium, and size, shape and color of the colonies. Genetic characterization was obtained by means of partial sequencing of the 16S rRNA gene allied to the BOX-PCR technique. The forest and canga areas were more and less diverse, respectively. Some NFLBN genera such as *Bradyrhizobium* and *Rhizobium*, were captured only be the sitratro ou only by the caupi, demonstrating the importance of using more than one plant species in diversity studies. The present work is the main report on diversity and symbiotic efficiency of NFLBN strains in canga environment.

Keywords: Rhizobium. Biologic fixation of Nitrogen. Diversity. Canga.

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PRIMEIRA PARTE

1 INTRODUÇÃO

A mineração é uma das principais atividades econômicas brasileira e, apesar dos benefícios, gera impactos consideráveis no ambiente. O Quadrilátero Ferrífero, localizado na região centro-sudeste do Brasil, é uma das regiões que mais contribui para a extração mineral no país. Nessa região encontra-se o maior percentual de áreas de canga do país, sendo este um tipo de ambiente com características peculiares como solos rasos e ácidos e grande taxa de endemismos em relação à fauna e flora. Apesar da peculiaridade, a canga é um ambiente pouco estudado, não havendo relatos na literatura sobre grupos de microrganismos como as bactérias fixadoras de nitrogênio (BFN).

O impacto causado pela mineração muitas vezes é irreversível. Ambientes degradados possuem solos pouco férteis, o que dificulta o processo de recuperação, e um dos nutrientes mais limitantes é o nitrogênio. Nesse sentido, a utilização de BFN e leguminosas pode ser uma alternativa de grande interesse para aumentar a fertilidade do solo.

As BFN são procariontes que possuem a capacidade de fixar o nitrogênio atmosférico (N_2) em uma forma assimilável para os organismos vivos (NH_3). Esse aporte de nitrogênio no solo favorece, principalmente, o crescimento vegetal de leguminosas que estão associadas às BFN. Apesar de os estudos sobre a fixação biológica de nitrogênio ter crescido bastante nas últimas décadas, muito ainda deve ser elucidado. Considerando que conhecemos apenas cerca de 1% de toda diversidade microbiana, uma parcela considerável de microrganismos ainda deve ser identificada e avaliada.

Dessa maneira, estudos que visam à compreensão entre a relação desses microrganismos e o ambiente são de fundamental importância. É preciso conhecer melhor a ocorrência, diversidade, função e a relação microrganismo-

planta para que o potencial biotecnológico desse grupo de microrganismos seja satisfatoriamente explorado.

Assim, o objetivo do presente trabalho foi avaliar a ocorrência, diversidade genética e fenotípica e a eficiência simbiótica de BFNNL em cinco áreas com diferentes coberturas vegetais na região do Quadrilátero Ferrífero (Sabará – MG).

2 REFERENCIAL TEÓRICO

2.1 Canga e o Quadrilátero Ferrífero

As cangas são ambientes ricos em rochas com alto teor de ferro (compostas por até 90% de óxidos de ferro) e possuem características peculiares como solos ácidos, rasos e com reduzidos índices de fertilidade (VICENT; MEGURO, 2008). Constituem um dos sistemas brasileiros menos conhecidos, embora esteja entre os mais ameaçados, principalmente, devido à intensa atividade mineradora associada aos seus afloramentos de ferro (SKIRYCZ et al., 2014). Apesar de apresentar alta taxa de endemismo de plantas e invertebrados (GIULIETTI et al., 2009; JACOBI et al., 2007), os estudos sobre diversidade da microbiota do solo nesse ambiente são bastante escassos. Considerando grupos microbianos específicos, como bactérias fixadoras de nitrogênio, não foram encontrados estudos até o momento.

Áreas de canga estão presentes, principalmente, no Quadrilátero Ferrífero (MG), além de ocorrerem em Carajás (PA), Caetité (BA) e Morraria de Urucum (MS) (JACOBI; CARMO, 2008). O Quadrilátero Ferrífero está situado na região centro-sudeste do estado de Minas Gerais e é uma área de transição entre Mata Atlântica e Cerrado.

Grande parte do minério de ferro produzido no Brasil tem origem no Quadrilátero Ferrífero e sua extração ocasiona impactos ambientais bastante danosos ou até mesmo irreversíveis. A principal causa de perda de habitat e degradação deve-se à abertura de cavas para a extração do minério (CARMO; JACOBI, 2013).

A obrigação de recompor uma área atingida pela mineração está prevista no decreto nº 97.632, de 10 de abril de 1989, que criou a obrigatoriedade da apresentação do Plano de Recuperação de Áreas Degradadas (PRAD) pelos empreendimentos que se destinam à exploração de recursos, porém, esse decreto

não é claro na forma como deve ser feita a recuperação da área (LIMA; FLORES; COSTA, 2006).

Uma das principais falhas nos projetos de recuperação ambiental é o desconhecimento das características dos ecossistemas atingidos (LIMA; FLORES; COSTA, 2006). O levantamento da fauna e da flora deve ser um dos primeiros passos em um processo que vise à conservação ou recuperação de uma área. Nesse processo pouca atenção é dada à microbiota do solo, o que é um erro visto que esta é a base fundamental envolvida nos ciclos biogeoquímicos de diversos elementos na natureza.

Os microrganismos são os responsáveis pela decomposição da matéria orgânica presente no solo. Esse processo possibilita a ciclagem de nutrientes como nitrogênio, fósforo, cálcio, magnésio, entre outros. O teor de alguns nutrientes no solo muitas vezes pode ser baixo para sustentar a biota desse ambiente, principalmente para suprir as necessidades fisiológicas de espécies vegetais. Alguns microrganismos possuem a capacidade de aumentar a fertilidade do solo seja auxiliando as plantas na absorção de nutrientes, seja na fixação de nutrientes importantes, como no caso de bactérias fixadoras de nitrogênio.

2.2 Fixação Biológica de Nitrogênio

O nitrogênio é considerado um macronutriente importante para a biossíntese de diversas moléculas orgânicas nos seres vivos, como, proteínas, enzimas, ácidos nucleicos, entre outras; sendo crucial para diversos processos biológicos.

Esse elemento constitui cerca de 78% dos gases presentes na atmosfera, sendo este, o principal compartimento onde é encontrado. Contudo, o nitrogênio gasoso só pode ser assimilado por alguns microrganismos que possuem o complexo enzimático enzima nitrogenase, capaz de converter o N_2 atmosférico

em amônia (NH_3), que é uma das formas assimiláveis de nitrogênio. Esse processo é denominado de fixação biológica de nitrogênio e é realizado por um grupo específico de bactérias, chamadas de bactérias fixadoras de nitrogênio (BFN) (MOREIRA; SIQUEIRA, 2006).

A fixação biológica de nitrogênio (FBN) é, portanto, responsável pela disponibilização desse elemento para os organismos do solo, favorecendo, principalmente, o crescimento vegetal. Os fertilizantes industriais nitrogenados além de terem um custo elevado para produção são ambientalmente prejudiciais, uma vez que a lixiviação desses compostos compromete a qualidade de solos e corpos hídricos. Dessa maneira, a FBN é uma alternativa efetiva, de menor custo e ambientalmente mais segura, que tem sido utilizada nas últimas décadas em substituição aos fertilizantes químicos (OLDROYD; DIXON, 2014).

No solo existem representantes de BFN de vida livre, associativos e simbiontes. Os simbiontes possuem relação mutualística, principalmente, com plantas da família *Leguminosae*, e produzem estruturas características dessa relação nas raízes e, eventualmente, no caule das plantas hospedeiras. Essas estruturas são denominadas de nódulos, os quais comportam as bactérias fixadoras de nitrogênio que nodulam leguminosas (BFNNL). As BFN associativas podem ser encontradas em plantas de diversas famílias, porém seu estudo concentra-se na associação com plantas da família *Poaceae* (*Gramineae*). As BFN associativas não formam estruturas como os nódulos em plantas, sendo o estudo dessa relação bastante distinto das BFNNL.

As BFNNL são comumente denominadas de rizóbio. Este nome originou-se da primeira espécie de bactéria fixadora de nitrogênio descrita – *Rhizobium leguminosarum*, de onde também se derivou o nome da primeira família *Rhizobiaceae*. Porém, a descoberta de novas espécies em outras famílias tornou esse nome inapropriado. Assim, algumas denominações podem ser

encontradas na literatura, sendo utilizado no presente trabalho o termo bactérias fixadoras de nitrogênio que nodulam leguminosas (BFNNL).

A utilização de BFNNL em culturas de leguminosas tem sido amplamente estudada. No Brasil, atualmente, a fixação biológica de nitrogênio supre totalmente as necessidades de N no cultivo de soja (MOREIRA; SIQUEIRA, 2006). Outras culturas ainda não são totalmente supridas com o nitrogênio fixado biologicamente, mas estudos têm avançado muito, principalmente em culturas de feijão comum (LAMMEL et al., 2013; SOARES et al., 2006b; YAGI et al., 2015) e feijão caupi (COSTA et al., 2014; SOARES et al., 2006a; ZILLI et al., 2006).

O desenvolvimento de inoculantes eficientes é dependente de estudos aprofundados desse grupo de bactérias, principalmente sobre sua diversidade e atividade metabólica. O Brasil, por ser um país de grande extensão territorial, apresenta características bastante divergentes de região para região, como: formação e composição do solo, clima, temperatura, entre outras; que afetam diretamente a comunidade microbiana e suas relações no sistema solo-planta. Portanto, o estudo da diversidade da microbiota do solo, em especial de BFNNL, é crucial para o manejo e conservação do ambiente e deve estar relacionado às características de cada região. Além disso, estima-se que grande parte da diversidade de BFNNL ainda é desconhecida (MOREIRA; HUISING; BIGNELL, 2010; MOREIRA; SIQUEIRA, 2006), justificando a ampliação dos estudos sobre esses microrganismos.

2.3 Diversidade de BFNNL

Estudos sobre a diversidade de comunidades de BFNNL dependem do isolamento dos nódulos radiculares, ou caulinares, das leguminosas hospedeiras. Os nódulos podem ser coletados de leguminosas encontradas em campo ou de plantas iscas cultivadas em laboratório.

Para o cultivo em laboratório, as plantas são cultivadas em substrato contendo diluições do solo nativo em que se pretende avaliar a diversidade. Os cultivos com plantas iscas (cultivo armadilha) fornecem informações sobre a especificidade entre as estirpes bacterianas e as leguminosas, já que algumas estirpes são capazes de estabelecer simbiose com diferentes espécies de leguminosas ao passo que outras têm preferências por determinados gêneros (ORMEÑO-ORRILLO et al., 2011). O inverso também ocorre, existindo espécies leguminosas consideradas promíscuas, que nodulam com uma ampla variedade de BFNNL, como siratro (*Macroptilium atropurpureum*) (LIMA et al., 2009), o feijão comum (*Phaseolus vulgaris*) (MELLONI et al., 2006) e o feijão caupi (GUIMARÃES et al., 2012; JARAMILLO et al., 2013); e plantas que parecem nodular com apenas alguns gêneros, como é o caso da *Sesbania virgata* que nodula apenas com *Azorhizobium doebereinerae* (MOREIRA; SIQUEIRA, 2006).

A diversidade genética e a relação filogenética de diferentes estirpes de BFNNL podem ser avaliadas utilizando técnicas moleculares, como o sequenciamento completo ou parcial do gene 16S rRNA (SILVA et al., 2012). Porém, alguns gêneros de BFNNL como o *Bradyrhizobium* possuem sequências gênicas altamente conservadas, dificultando sua caracterização apenas pelo sequenciamento do 16S rRNA (WILLEMS; COOPMAN; GILLIS, 2001). Dessa maneira, é interessante que diferentes técnicas moleculares estejam aliadas, aumentando o poder de resolução. A interação entre diferentes técnicas moleculares tem facilitado os estudos filogenéticos desses microrganismos e permitido a identificação de novas espécies (BORGES et al., 2016; GUIMARÃES et al., 2012).

A técnica de BOX-PCR é uma variação do tradicional PCR, que utiliza primer específico para amplificar regiões conservadas e repetitivas do DNA cromossômico bacteriano. Existem três famílias de sequências repetitivas: REP

(Repetitive element palindromic) (35 – 40 pb), ERIC (Enterobacterial repetitive intergenic consensus sequence) (124 – 127 pb) e BOX (154 pb). A amplificação das sequências produz um padrão de bandas semelhante a um fingerprinting. Os primers de BOX geram um fingerprinting robusto e produz um padrão de fragmentos mais complexo. Os primers para REP geram menor complexidade, mas ainda permitem a diferenciação dos isolados. Já os primers para ERIC são mais sensíveis às condições não desejáveis da PCR como presença de contaminantes na amostra de DNA e gera padrões de bandeamento altamente discriminatórios (VERSALOVIC et al., 1994).

Para avaliar a diversidade de BFNNL pode-se utilizar, também, análises morfológicas das estirpes, onde são avaliadas características como tempo de crescimento, coloração, formato, tamanho da colônia, produção de goma, entre outras. A caracterização morfológica é uma ferramenta que permite separar as estirpes analisadas em grupos, para posterior caracterização genética e é fundamental para identificar a posição taxonômica dos isolados (MAÂTALLAH et al., 2002). A filogenia desse grupo microbiano também pode ser estudada através de análises de genes funcionais. Um desses genes é o nifH que codifica a Fe-proteína do complexo nitrogenase, a qual é altamente conservada em microrganismos fixadores de N, e essencial para a redução do nitrogênio (ZEHR, 2003).

A diversidade microbiana está atrelada às características edáficas como cobertura vegetal, temperatura e umidade do solo (JESUS et al., 2009; MOREIRA; SIQUEIRA, 2006). Esta pode ainda ser reflexo de perturbações ambientais como extração de minério (CHAER et al., 2011), agricultura (KÖBERL et al., 2016) e pastagem (ORMEÑO-ORRILLO et al., 2011).

2.4 Índices de diversidade

As medidas de diversidade geralmente consideram o número de espécies e a abundância dessas espécies em uma determinada área. De acordo com Magurran (1987), existem três tipos de medidas principais: índices de riqueza de espécies, modelos de abundância de espécies e índices que consideram a abundância proporcional de espécies. Entre os índices que relacionam riqueza de espécies com a abundância, os mais conhecidos e utilizados são os índices de Shannon e Simpson (HUGHES et al., 2001). Estes índices têm sido utilizados em trabalhos sobre diversidade de bactérias fixadoras de nitrogênio (MCINNES et al., 2004; ORMEÑO-ORRILLO et al., 2011, VARGAS et al., 2007).

2.5 Fatores que interferem na simbiose BFNNL e leguminosas

A fixação biológica de nitrogênio (FBN) é um processo que pode ser afetado por diversos fatores bióticos e abióticos. As características do solo como pH, umidade, temperatura, manejo e concentração de nutrientes são as mais representativas (HUNGRIA; VARGAS, 2000; MOREIRA; SIQUEIRA 2006). Em termos bióticos, as bactérias fixadoras de nitrogênio podem encontrar ambientes com pouca disponibilidade de fonte de carbono e competir com outros microrganismos por essas fontes.

Uma característica bastante interessante que limita o desenvolvimento das BFNNL é a concentração de nitrogênio combinado no solo. Assim, solos que contenham altas concentrações deste elemento, como aqueles que têm adição de fertilizantes industriais, mostram-se pouco responsivos na inoculação de BFNNL (MAGALHÃES et al., 1982). Nesses casos, a nodulação não chega a ocorrer, pois a planta evita o gasto energético de contribuir com fotossintatos para as BFN, uma vez que elas não sentem a necessidade de utilizar o nitrogênio fixado biologicamente.

Outro fator que afeta a simbiose e a diversidade de BFNNL é o tipo de vegetação e o tipo de uso que se faz do solo. Solos impactados pela mineração, agricultura, pastagem ou pelo cultivo possuem microbiota distinta de solos de ambientes com menor atividade antrópica, como florestas primárias (DEPRET et al., 2004; LIMA et al., 2009).

A substituição de florestas pela agricultura ou pastagem, por exemplo, tem causado impacto na diversidade de BFN, como mostrado por Ormeño-Orrilo et al. (2011). Nesse estudo, áreas em reabilitação apresentaram maior diversidade de *Bradyrhizobium* que áreas utilizadas para cultivo de feijão e áreas de pastagem. Gerhing et al. (2005) em estudo sobre fixação biológica na Amazônia Central relataram que a contribuição das leguminosas e, consequentemente, a fixação biológica de nitrogênio em floresta secundária é maior que em floresta primária, sendo esse processo mais acentuado em áreas em regeneração do que em florestas clímax.

Jesus et al. (2005) mostraram a influência de diversos usos da terra na diversidade de BFNNL em três sistemas de uso do solo na Amazônia Ocidental. Nesse estudo os autores relatam que poucos isolados foram obtidos de solo sob floresta, enquanto que um maior número de isolados foi obtido nas áreas de cultivo de mandioca e de pupunheira. Lima et al. (2009) avaliaram as comunidades de BFNNL em diversos sistemas de uso do solo da região Amazônica. Os autores utilizaram o siratro como planta isca para acessar a diversidade de BFNNL nas áreas estudadas, e, observaram que amostras de solo sob agrofloresta e agricultura induziram a formação de um maior número de nódulos na planta isca que solos sob floresta. Além disso, a alta diversidade obtida nos solos sob sistemas de uso de agrofloresta e agricultura sugere uma alta resiliência desse grupo microbiano mesmo em sistemas distintos de uso do solo.

A extração mineral também é uma atividade que impacta drasticamente o ambiente como um todo. Estudos mostram o quanto a diversidade, densidade e atividade metabólica de microrganismos são afetadas por essa atividade (CHAER et al., 2011; FARRELL et al., 2010; TRINDADE; GRAZZIOTTI; TÓTOLA, 2000). Além de altas concentrações de metais pesados nessas áreas, a retirada da cobertura vegetal altera as condições físicas, químicas e biológicas do solo, pela ausência da contribuição da rizosfera nesses ambientes. Melloni et al. (2006) relatam o quanto o impacto da extração mineral pode afetar na diversidade de BFNNL. Nesse estudo, os autores reforçam que ambientes em recuperação contêm uma diversidade de BFNNL superior ao ambiente impactado pela extração de bauxita, principalmente, quando na recuperação da área são utilizadas leguminosas para revegetação.

Microrganismos são sensíveis a mudanças ambientais, assim, mudanças na atividade microbiana, na estrutura e função de comunidades podem servir como indicador de impactos antropogênicos e predizer sobre o sucesso de planos de recuperação de ambientes degradados (ZILLI et al., 2003; WINDING; HUND-RINKEB; RUTGERSC, 2005).

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 - BIOLOGICAL NITROGEN FIXATION BY BACTERIAL COMMUNITIES UNDER DIFFERENT VEGETATION TYPES IN AN IRON MINING REGION

NORMAS DA REVISTA APLLIED SOIL ECOLOGY

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Abstract

Biological nitrogen fixation is an important process for the structure and functioning of soil communities, and it is carried out by specific groups of bacteria that can live freely or form symbiosis with plants. Studies on communities of symbiotic N_2-fixing bacteria can indicate the degree of recovery and/or disturbance of an environment, and they provide access to genetic resources with potential application in biotechnological processes. But this kind of information is scarce for natural and managed ecosystems subjected to mining activities. In this work, we evaluated the occurrence and the symbiotic efficiency of nitrogen-fixing Leguminosae nodulating bacteria (NFLNB) communities in 'canga' environments and other adjacent vegetation [grass, forest, Cerrado (Brazilian Savannah), and Eucalyptus] in the Quadrilátero Ferrífero, MG - Brazil. Canga, a peculiar habitat found mainly in the Quadrilátero Ferrífero, is poorly studied in relation to its diversity, especially for soil biota, with no study on NFLNB being currently available. Grass (*Melinis minutiflora*) was planted in soil deposition sites removed for the opening of iron mining pit. For the capture of the NFLNB communities in the five vegetation types, two promiscuous plant species were used as trap - siratro (*Macroptilium atropurpureum*) and cowpea (*Vigna unguiculata*).

Symbiotic efficiency of NFLNB communities was evaluated in greenhouse and related to the chemical and physical soil attributes through principal component analysis. The use of cowpea as trap plant promoted NFLNB capture at all sampling points, in all kinds of vegetation. Nodulation in cowpea did not vary significantly among vegetation types. On the other hand, when cowpea was used as trap plant, NFLNB density was higher than that found with siratro in all vegetation types. The latter, in turn, was less efficient in the capturing NFLNB because nodulation did not occur at all the evaluated points. When siratro was used as trap plant, NFLNB communities of the grass area induced higher number of nodules and were more efficient compared to the other vegetation types. The relative efficiency of NFLNB community of canga was the lowest among all vegetation types for cowpea, and equivalent to the treatment with low concentration of mineral nitrogen. The soil attributes more strongly related to biological attributes were pH, base saturation and aluminum content. Relative efficiency and shoot dry matter were good biological indicators of NFLNB community variation among vegetation types.

Keywords: Symbiotic efficiency; nitrogen fixing bacteria; canga; trap plant; *Macroptilium atropurpureum; Vigna unguiculata*

1. INTRODUCTION

The use of Nitrogen-fixing Leguminosae nodulating bacteria (NFLNB) is widespread in Brazil, especially for soybean production, in which the inoculation with NFLNB completely meets the nitrogen demand by the plant, replacing nitrogen fertilizers, and reducing the economic and environmental costs associated with N fertilizers (Moreira, 2006). However, considering the complexity of the soil and its micro habitats, as well as the climate diversity in Brazil, studies on NFLNB interactions with biotic and abiotic factors of the ecosystem are still necessary to understand the ecology of these organisms, and to pave the way for their biotechnological applications, not only in soybean but also in other species of agriculture or forestry interest (Alves et al., 2003).

Vegetation cover type is believed to influence the diversity and symbiotic efficiency of NFLNB because these microorganisms can be affected by chemical, physical and biological attributes of the soil, all of them directly affected by the vegetation (Jesus et al., 2005; Lima et al., 2009; Pule-Meulenberg et al., 2010). Several studies have evaluated the

diversity and the efficiency of NFLNB in different environments, in both controlled (greenhouse) and field conditions, using cowpea (*Vigna unguiculata*) and siratro (*Macroptilium atropurpureum*) as trap plant, due to their promiscuity, with several evidences of the biotechnological potential of native symbiont bacteria (Bromfiel et al., 1990; Guimarães et al., 2012; Jaramillo et al., 2013; Lima et al., 2009; Ormeño-Orrillo et al., 2012; Rufini et al. 2014).

Generally, plants considered promiscuous nodulate with different NFLNB genera, such as cowpea and siratro. However, there is evidence of greater affinity of promiscuous plant species to some NFLNB groups, as shown by Ormeño-Orrilo et al. (2012). These authors, evaluating areas of primary forest, secondary forest, pasture, and agriculture (mainly corn and beans fields), captured distinct groups of *Bradyrhizobium* for each plant species (siratro and cowpea). This affinity to certain NFLNB groups can also influence the symbiotic efficiency achieved by the plant species.

The extraction of iron ore, very common in the Quadrilátero Ferrífero region, irreversibly impacts the exploited environment. In addition to the removal of vegetation cover and soil disturbance, there is the production of a barren material that is stockpile, covered with vegetation, which are

usually grasses. Areas which suffer from mineral extraction should be further studied in relation to the diversity of micro and macro organisms, since this knowledge is critical to the success of recovery plans of degraded areas.

The Quadrilátero Ferrífero is an area of approximately 7000 km^2, located in the central-south region of the state of Minas Gerais. It has great economic importance to Brazil, since much of the iron ore mined in the country originates from this region. It is a transition area between the Atlantic Forest and the Cerrado (which consists of a mosaic of vegetation types, highlighting the rocky field), at altitudes greater than 900 m, on rocky outcrops, in cangas (Carmo et al., 2013).

Cangas are composed of up to 90% iron oxides, and contains shallow acidic soils with low fertility. Because of their peculiar characteristics, they present high endemism of plants and invertebrates (Jacobi et al., 2007; Skirycz et al., 2014). Canga environment is one of the least known ecosystems in areas under the influence of mining. Little is known about the biodiversity of this environment, with most of the current research in this habitat being focused on fauna (Skirycz et al., 2014). Despite the importance of the Quadrilátero Ferrífero from an economic and

environmental point of view, and the influence of vegetation cover type on microbial communities, there are no studies that address NFLNB in canga or in other vegetation types subjected to management resulting from mining in this area. The few studies targeting microbiota in the iron mining area have been carried out in other countries and are more general, which consider a wide range of microbial groups (Hong et al, 2015; Xing et al, 2015).

This work aimed to answer the following questions: are there NFLNB communities in 'canga' environments? How are NFLNB communities changed by soil deposition from iron pit mining and grass planting? What physical and chemical attributes of the soil are more related to these communities in the different vegetation types? What trap species is more efficient in the capture of NFLNB communities in those mining-impacted environments? Thus, we evaluated the ability of nodulation and nitrogen fixation by NFLNB communities captured in soils under five vegetation types (cerrado, canga, forest, eucalyptus and grass), using two kinds of promiscuous trap plants - siratro and cowpea.

2. MATERIALS AND METHODS

2.1 Characteristics of the studied area

The study was carried out in an area of the Quadrilátero Ferrífero, with five vegetation types, with approximately 716.47 ha, in the municipality of Sabará, located in the central-southeastern Minas Gerais - Brazil. This region is located in the Serra da Piedade and is composed of a geosystem of ferruginous rocks, predominantly on Itabirite, constituents of the iron formations Cauê and Gandarela (Itabira Group, Minas Supergroup). The area covers a deactivated iron ore mining pit, called Córrego do Meio, and its surroundings (Figure 1). The climate is typically tropical, with short dry season (June to September), and wet season, marked by rainfall concentrated in the hot season. The average annual temperature is 20.8º C.

In the studied area, there is cerrado native vegetation with dense rainforest fragments and typical canga vegetation (region of rocky outcrops) at the higher points, and introduced vegetation composed of eucalyptus and molasses grass (*Melinis minutiflora*).

2.2 Sampling and physical and chemical characterization of the soil

Samplings were carried out in September 2013, following the methodology described by Moreira et al. (2010). In each vegetation type,

four sampling points were randomly chosen, totaling twenty points along the five vegetation types. At every point, 12 subsamples were collected to form a composite sample for physical, chemical, and biological analysis. Samples simply were arranged in two concentric circles around a georeferenced central point, in a radius of 3 and 6 m, respectively, at a depth of 0-20 cm.

From the collected soil samples, approximately 200g were used in microbiological analysis of presence and efficiency of NFLNB communities; whereas about 400 g were used in the physical and chemical characterization of the soil, carried out by the Soil Science Department of the Federal University of Lavras (UFLA). Table 1 shows the evaluated parameters.

The chemical and physical attributes of the soil were evaluated by analysis of variance (ANOVA) using the SISVAR version 5.6 software (Ferreira, 2011), and using the Scott-Knott test for grouping the means (Scott and Knott, 1974), at 5% significance. In addition, these variables were subjected to principal component analysis (PCA), using the R software (R Development Core Team, 2011).

2.3 Capture and symbiotic efficiency of NFLNB communities

Two experiments were performer in a greenhouse at the Laboratory of Biology, Microbiology and Soil Biological Processes (UFLA) to obtain NFLNB strains and to evaluate their symbiotic efficiency. Siratro (*Macroptilium atropurpureum*) was used as trap plant in the first experiment, whereas cowpea (*Vigna unguiculata*) was used in the second experiment.

In both cases, cultivation was performer in 250 cm^3 plastic tubes, containing a mixture of sand and vermiculite (1:1 - v:v), sterilized by moist heat autoclaving (1 atm, 127°C, 1h). Pre-germinated seeds were planted in plastic tubes. To supply nutrients for the plants, the nutrient solution of Hoagland and Arnon (1950) was used according to the need of the plant, usually at every 48 hours. The capture experiment using siratro occurred between the months of October and November, 2013, under uncontrolled temperature varying from 23 to 41°C, in a greenhouse. The capture experiment using cowpea occurred between the months of April and May 2014, under uncontrolled temperature ranging from 22 to 38°C.

For inoculation of the tubes, 10 g of soil samples were resuspended in 90 mL of saline sterile solution (0.85%), inoculated (1.0 mL per tube) in the plastic tubes, in which two siratro or cowpea seeds were planted.

After the appearance of the first true leaves, plants were thinned, leaving one plant per tube. Two negative controls were included without inoculation: one receiving nutrient solution containing low concentration of mineral nitrogen (5.25 $mg.L^{-1}$) (N-) with the following composition of stock solutions added to 4 liters of water: 0.4 ml of 236.16 g · $liter^{-1}$ $CaN_2O_6 \cdot 4H_2O$; 0.1 ml of 115.03 g · $liter^{-1}$ $NH_4H_2PO_4$; 0.6 ml of 101.11 g · $liter^{-1}$ KNO_3; 2.0 ml of 246.9 g · $liter^{-1}$ $MgSO_4 \cdot 7H_2O$; 3.0 ml of 87.13 g · $liter^{-1}K_2SO_4$; 10 ml of 12.6 g · $liter^{-1}$ $CaH_4P_2O_8 \cdot H_2O$; 200 ml of 1.72 g · $liter^{-1}$ $CaSO_4 \cdot 2H_2O$; 1 ml of 10 g · $liter^{-1}$ $FeCl_3$; and 1 ml of micronutrients (2.86 mg · $liter^{-1}$ H_3BO_3; 2.03 mg · $liter^{-1}$ $MnSO_4 \cdot 4H_2O$; 0.22 mg ·$liter^{-1}$ $ZnSO_4 \cdot 7H_2O$; 0.08 mg · $liter^{-1}$ $CuSO_4 \cdot 5H_2O$; and 0.09 mg ·$liter^{-1}$ Na_2MoO_4 H_2O), and another containing high concentration of mineral nitrogen (52.5 $mg.L^{-1}$) (N+) with the following composition of stock solutions added to 4 liters of water: 4 ml of 236.16 g · $liter^{-1}$ $CaN_2O_6 \cdot 4H_2O$; 1 ml of 115.03 g · $liter^{-1}$ $NH_4H_2PO_4$; 6 ml of 101.11 g · $liter^{-1}$ KNO_3; 2.0 ml of 246.9 g · $liter^{-1}$ $MgSO_4 \cdot 7H_2O$; 1 ml of 10 g $liter^{-1}$ $FeCl_3$; and 1 ml of micronutrients. In the positive controls, the reference strains UFLA 04-0212 (ST212) and SEMIA 656 were used for siratro, and INPA 03-11B (*Bradyrhizobium elkanii*) and UFLA 03-84

(*Bradyrhizobium sp.)* were used for cowpea. These strains have already been considered effective in previous studies (Florentino et al., 2009, Soares et al., 2006). Treatments receiving soil inoculum and reference strains received Hoagland and Arnon nutrient solution with low concentration of mineral N.

All treatments were carried out in triplicate, and distributed in a completely randomized design (CRD). Fifty five days after cultivation, both siratro and cowpea plants were harvested for evaluation of nodulation and for the isolation of NFLNB strains.

Shoots, roots and nodules were separated and dried in forced circulation oven (60°C). Shoot dry matter was used to evaluate the relative efficiency (RE) of biological nitrogen fixation, following the formula:

$RE = (SDM_{inoculated} / SDM_{with\ N}) \times 100$

In which: RE: relative efficiency; SDM: shoot dry matter

The dry matter of shoots (SDM), roots (RDM) and nodules (NDM), the number of nodules (NN) and relative efficiency (RE) were evaluated by the analysis of variance (ANOVA) using the SISVAR version 5.6 (Ferreira, 2011) software. After verifying the effect of the treatments, the

Scott-Knott test was used for grouping the means (Scott and Knott, 1974), at 5% significance level.

2.4 Multivariate analysis of chemical, physical and biological soil attributes

The physical, chemical and biological attributes of the collected soils were evaluated and related by means of principal component analysis and of correlation analysis (Pearson correlation coefficient), using the R software (R Development Core Team, 2011).

3. RESULTS

3.1 Physical and chemical characterization of the soil

As shown in Table 1, in relation to the chemical attributes of the soil, all areas presented low pH. However, the grass area showed the most moderate value among the evaluated areas, with mean pH of 5.95. In all the other areas, the soils were more acidic, with pH values lower than 5,0. Moreover, the grass area also showed the lowest aluminum concentration, whereas the eucalyptus area presented the highest aluminum concentration ($p < 0.05$). On the other hand, the grass area presented the lowest values for organic matter, with the canga area having the highest organic matter content. In general, with regards to soil fertility, the grass area showed more suitable properties like pH, Al and V, and was distinct from the other vegetation types ($p < 0.05$).

3.2 Capture and symbiotic efficiency of NFLNB communities

The two trap species used in this study differ from each other regarding their ability to capture NFLNB strains along the five studied vegetation types. When cowpea was used as trap plant, nodulation was always observed in plants inoculated with soil from any sampling point and any vegetation type, despite of having been inoculated with soil samples stored six months more than those used to inoculate siratro. In the experiment with siratro, there was nodulation of 100% of the plants inoculated with soil from the grass area, 67% with soil from the forest, 58% with soil from cerrado, 50% with the soil of eucalyptus, and 17% with soil from canga. In both experiments, negative controls (low and high nitrogen concentrations) did not nodulate while the positive controls nodulated, indicating, respectively, absence of contamination and existence of appropriate conditions for nodulation.

Table 2 shows the symbiotic efficiency parameters of NFLNB communities from each studied vegetation type, using siratro and cowpea as trap plants. When using siratro as trap plant, plants inoculated with soil from grass presented values of number of nodules (NN), nodules dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM) and relative efficiency (RE) statistically similar ($p < 0.05$) to those found for

plants inoculated with the reference strain (UFLA 04-212), but higher ($p < 0.05$) than those observed in plants inoculated with soil from forest, cerrado, canga, and eucalyptus. Regarding their ability to promote siratro growth, NFLNB communities from grass were as efficient as the treatment with high nitrogen concentration ($p > 0.05$).

In the experiment with cowpea, forest, cerrado, eucalyptus, and grass showed SDM and RE similar ($p > 0.05$) to those obtained with the reference strains UFLA 03-84 and INPA 03-11B, but lower ($p < 0.05$) than those observed for the treatment with high mineral nitrogen concentration. For NFLNB communities from canga, RE value was lower ($p < 0.05$) than that obtained in other areas, and similar ($p > 0.05$) to the control with low mineral nitrogen concentration.

3.3. Relationship between chemical, physical and biological attributes of the soil under different vegetation types

Figure 2 lists soil chemical and physical attributes of the areas along with biological variables (NN, NDM, SDM, RDM and RE) obtained from NFLNB communities captured with siratro (2A) and cowpea (2B). Correlations between these variables and the principal components are listed in Tables S1 and S2 of the supplementary material.

The principal component analysis (PCA) reveals that the biological variables are not correlated with the soil physical attributes (sand, clay, and silt content), but they are influenced by chemical attributes. Biological attributes (SDM, NN, NDM and RE) are inversely correlated with the aluminum content, and directly related with the base saturation index (V) and pH when siratro is used as trap plant. For cowpea, biological attributes (SDM and RE) are directly correlated with pH and base saturation index, and inversely correlated with acidity potential, cation exchange capacity at 7.0 pH (T), and organic matter content.

Table 1 shows that grass is the vegetation type with the lowest aluminum content, highest base saturation index, and highest pH, differing from the others ($p < 0.05$). Grass is also the vegetation type with the best biological attributes when siratro was used as trap plant.

The correlation analysis (Pearson coefficient) among biological attributes showed that NN and NDM are correlated with RE when siratro is used as trap plant ($\rho = 0.94$); however, this correlation was not observed when cowpea was used as trap plant ($\rho = 0.32$ between NN and RE; $\rho = 0.27$ between NDM and RE).

4. DISCUSSION

The soil, being a heterogeneous, dynamic, and complex environment, shapes its microbial communities directly or indirectly through its chemical, physical, and biological attributes (Moreira, 2006). Among these, certain chemical attributes such as pH and aluminum content (Jesus et al., 2009; Lima et al., 2009), as well as the organic matter and micronutrients content (Mn, Zn, Fe, B, etc.), are the main attributes influencing soil microbiota (Li et al., 2015; Moreira, 2006).

The optimum pH for NFLNB growth is between 6.0 to 7.0, according to Jordan (1984), although some species are able to tolerate, or even prefer pH values around 5.0 (Rufini et al., 2011; Soares et al., 2014). Low pH associated with high aluminum concentration has been reported as one of the main problems of the Brazilian soils, and may compromise the microorganisms present in the soil (Lima et al. 2009; Moreira, 2006). When considering the soil chemical attributes (pH, Al, V) that most affected the biological attributes in this study, for both siratro and cowpea, the grass was the vegetation type under which those chemical attributes were the most favorable probably, reflecting in higher RE of the communities captured with siratro.

Regarding NFLNB capture by trap plants, cowpea was more effective than siratro because there was nodulation of 100% of the trap plants. The number of nodules can be considered as an indirect indication of NFLNB density, since very high density of these bacteria provide greater infection of plant roots, increasing the number of nodules when compared to a soil with low NFLNB density (Lima et al., 2009). Therefore, it can be inferred that cowpea captured a higher NFLNB density as compared to siratro. Similar data are presented by Ormeño-Orrilo et al. (2012), who compared the NFLNB captured using cowpea and siratro as trap plants. The authors obtained a greater number of strains with cowpea when compared with siratro, and also found difference in NFLNB diversity captured by these plant species.

Considering the efficiency of biological nitrogen fixation by NFLNB communities, the greatest efficiency of the grass when siratro was used may also be related to the higher NFLNB density (NN) obtained in this area, since there was positive correlation between these variables. On the other hand, when using cowpea as trap plant, there was no correlation between NN or NDM and RE. Therefore, considering the data in this

work, only the soil chemical attributes influenced RE when using this plant species.

Biological nitrogen fixation can be considered as an indirect indicator of disturbance of an area because many studies have shown that, in balanced environments, such as forests, where nitrogen is not a limiting nutrient in the soil, the frequency of nodulation and the efficiency of biological nitrogen fixation by NFLNB is lower (Jaramillo et al., 2013; Lima et al., 2009). On the other hand, environments that suffered some kind of environmental impact usually have high nitrogen fixation activity by NFLNB, since these environments are in recovery process (Melloni et al., 2004; Melloni et al., 2006), which also explains the higher efficiency of the community in the grass.

The soil which had grass as vegetation, in general, showed more desirable chemical attributes (lower acidity, lower aluminum content, higher base saturation index), being distinct from the others areas in relation to the soil chemical attributes. These attributes possibly made the NFLNB community of this area be more abundant and efficient in biological nitrogen fixation.

Although NFLNB communities from the other areas did not show high values for relative efficiency and other biological attributes, this type of study is significant because it demonstrates the presence of NFLNB communities in these environments, especially for being the first report on the occurrence of NFLNB in ¨canga¨ environments. Moreover, these communities can be composed of strains with variable efficiency, which diversity will be further studied after their isolation. Structures like spores that confers better microbial survival in adverse soil conditions are absent in both alfa and beta rhizobia. Despite of that, results of this work show a high saprophytic competence of rhizobia communities, since they were found in highly modified anthropogenic soils by mining activities, as well as, rock outcrops like ¨canga¨.

The species used as trap plants in this study have considerable agronomic value. Cowpea has stood out as a culture of great nutritional value and important income source, especially in northeastern Brazil (Costa et al., 2015; Oliveira et al., 2013). Siratro, in turn, is most commonly used as green manure, improving the quality of degraded soils (Nascimento et al., 2003). Thus, study on native NFLNB communities able to increase the production of these two plant species is very

important because it can reveal strains with biotechnological potential for the production of inoculants.

5. CONCLUSIONS

Cowpea was more efficient than siratro in capturing NFLNB at all sampling points, and captured higher NFLNB density in all vegetation types, including "canga" rock outcrops. On the other hand, siratro was able to discriminate NFLNB communities among areas with regards to their relative efficiency (RE), while cowpea showed no difference in RE among vegetation types in this study.

Biological attributes were influenced by different soil chemical attributes depending on the plant species used as trap. For siratro, the base saturation index, the aluminum concentration and the pH were the soil attributes that most influenced the biological attributes of NFLNB communities. On the other hand, for cowpea, in addition to those attributes, cation exchange capacity at 7.0 pH, and organic matter were strongly related to biological variables. Relative efficiency and SDM were the biological attributes that better discriminates vegetation types in the Quadrilátero Ferrífero.

The grass area stands out in relation to the other vegetation types, and is directly related with base saturation and pH, and inversely related with

Al. In addition, it is directly related with the main biological attributes, indicating the importance of biological N_2 fixation in areas under rehabilitation process.

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FIGURES CAPTION

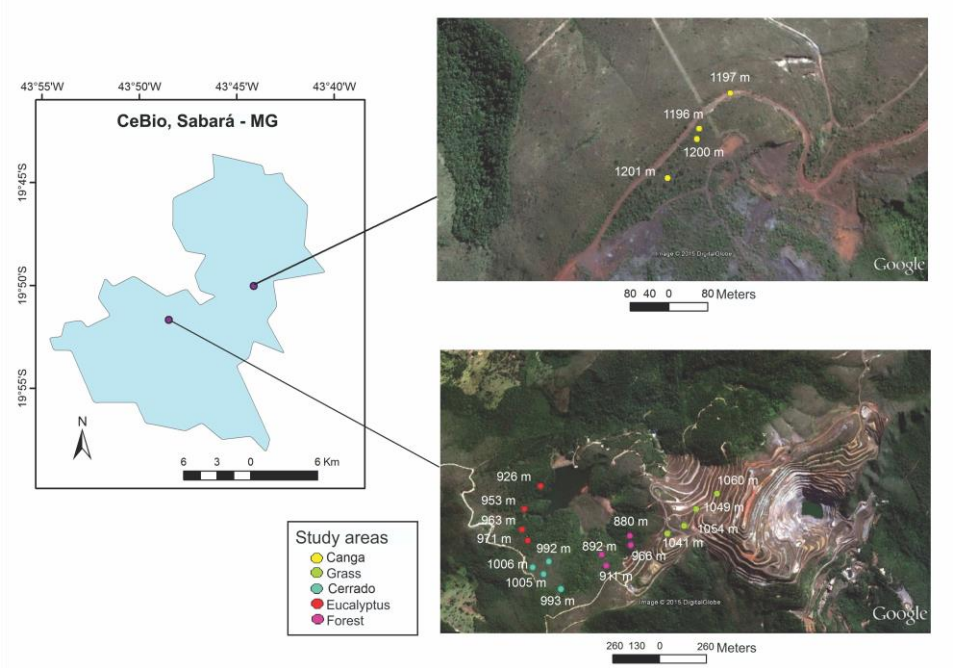

Fig. 1.

Studied area, evaluated for the presence and efficiency of nitrogen-fixing leguminosae nodulating bacteria communities, located in the municipality of Sabará - MG, with illustration of the sampling points and of the altitude of each point.

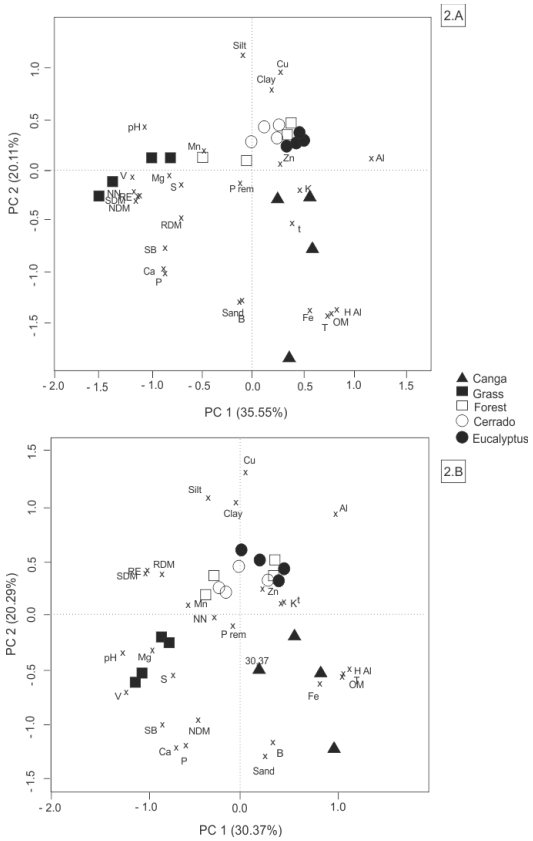

Fig. 2.

Principal Component Analysis (PCA) relating chemical and physical attributes of the soil to the biological variables (SDM – shoot dry matter, RDM - root dry matter, NDM - nodules dry matter, NN - number of nodules and RE - relative efficiency) of areas with different vegetation types in the Quadrilátero Ferrífero. 2.A – experiment with siratro. 2.B - experiment with cowpea. Soil chemical variables: H + Al - acidity potential, SB - sum of exchangeable bases, t - effective cation exchange capacity, T - cation exchange capacity at 7.0 pH, V - base saturation index, m - aluminum saturation index, OM - organic matter, P rem - remaining phosphorus.

Table 1.

Physical and chemical attributes of the soil under five vegetation types in the Quadrilátero Ferrífero - MG, represented by the mean of the four replications for each area.

Vegetation type	O.M [1] dag.Kg^{-1}	pH [1]	Fe [2]	K [2]	P [2]	Ca [1]	Mg [1]	Al [1]	H + Al [4]	Mn [2]	S [3]
			----------mg.dm^{-3}----------			----------cmol.dm^{-3}----------				----mg.dm^{-3}----	
Forest	4.77 b	4.65 b	79.5 a	68.5 a	1.93 b	0.57 a	0.42 a	1.75 b	9.86 b	67.8 a	11.15 b
Cerrado	3.46 b	4.95 b	102.7 a	48 b	1.49 b	0.47 a	0.15 b	1.35 b	6.56 b	22.4 b	11.11 b
Eucalyptus	5.09 b	4.72 b	122.6 a	38 b	1.93 b	0.12 a	0.15 b	2.6 a	12.17 b	18.2 b	17.46 b
Canga	9.57 a	4.5 b	236.7 a	56 a	3.81 b	0.77 a	0.15 b	1.55 b	22.54 a	17.0 b	14.44 b
Grass	1.32 c	5.95 a	43.3 a	32.5 b	6.65 a	1.15 a	0.42 a	0.10 c	1.49 b	41.9 a	28.93 a
Vegetation type	Zn [2]	B [1]	Cu [2]	P-rem [2]	V [1]	SB [1]	T [1]	t [1]	Sand [1]	Silt [1]	Clay [1]
	-------mg.dm^{-3}-------			mg.L^{-1}	%	-------cmolc.dm^{-3}-------			---------dag.K^{-1}---------		
Forest	2.34 a	0.15 c	3.02 a	17.83 a	10.54 b	1.17 a	11.03 b	2.92 a	27.2 c	42.2 a	30.5 a
Cerrado	0.88 a	0.08 c	2.1 a	20.86 a	10.99 b	0.77 a	7.33 b	2.12 a	35.7 c	36.7 a	27.5 a
Eucalyptus	0.76 a	0.12 c	2.76 a	12.64 a	2.95 b	0.37 a	12.54 b	2.97 a	30.2 c	34.0 a	35.7 a
Canga	1.8 a	0.25 a	0.69 b	14.65 a	5.29 b	1.07 a	23.61 a	2.62 a	62.0 a	17.5 b	20.5 a
Grass	0.61 a	0.18 b	0.73 b	17.16 a	48.93 a	1.65 a	3.15 b	1.75 a	46.7 b	32.7 a	20.5 a

† Means followed by the same letter in the column do not differ by the Scott Knott test at 5% probability. OM - organic matter, P rem - remaining phosphorus, T - cation exchange capacity at pH 7.0, t - effective cation exchange capacity, SB - sum of exchangeable bases, V - base saturation index, H + Al – acidity potential.

‡ Attributes analyzed by: 1. Vettori (1969). 2. Mehlich (1953). 3. Richards (1954). 4. Baker (1986).

Table 2.
Number of nodules (NN), nodules dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), and relative efficiency (RE) of NFLNB communities in soil under different vegetations in the Quadrilátero Ferrífero, Sabará (MG), captured with siratro or cowpea, in relation to controls.

Vegetation type	Siratro					Vegetation type	Cowpea				
	NN*	NDM* mg plant^{-1}	SDM* ----g.plant^{-1}----	RDM*	RE* %		NN*	NDM* ------ g plant^{-1} ------	SDM*	RDM*	RE* %
Forest	11 b	8.7 b	0.16 b	0.14 b	29.4 b	Forest	60 a	0.06 b	1.27 b	0.30 a	60.40 b
Cerrado	9 b	6.3 b	0.12 b	0.12 b	22.6 b	Cerrado	78 a	0.06 b	1.18 b	0.31 a	56.11 b
Canga	4 b	3.5 b	0.13 b	0.13 b	23.5 b	Canga	49 a	0.07 b	0.83 c	0.23 a	39.6 c
Eucalyptus	5 b	3.3 b	0.14 b	0.12 b	26.7 b	Eucalyptus	57 a	0.05 b	1.28 b	0.33 a	61.21 b
Grass	36 a	25.0 a	0.28 a	0.15 a	51.0 a	Grass	64 a	0.08 b	1.46 b	0.36 a	69.52 b
SEMIA 656	35 a	16.0 b	0.25 a	0.11 b	46.0 a	03-84	88 a	0.07 b	1.27 b	0.29 a	60.58 b
JFLA 04-212	54 a	38.0 a	0.37 a	0.20 a	69.6 a	03-11B	73 a	0.11 a	1.29 b	0.36 a	61.41 b
N-	0 b	0 b	0.11 b	0.12 b	19.9 b	-N	0 b	0 c	0.87 b	0.25 a	39.01 c
N+	0 b	0 b	0.54 a	0.18 a	100 a	+N	0 b	0 c	2.09 a	0.64 a	100 a

* Means (n=4) followed by the same letter in the column do not differ by the Scott Knott test at 5% de probability.

SUPPLEMENTARY MATERIAL

Table S1.
Principal component analysis of physical, chemical and biological attributes of soils under different vegetation types in the Quadrilátero Ferrífero, in an experiment with siratro.

Variance components	PC 1	PC2
Eigenvalues	9.5980	5.4303
% of variance	35.55	20.11
Cumulative %	**35.55**	**55.66**
Variables	Correlation with the principal components	
pH	**-0.7825**	0.2304
K	0.33370	-0.0796
P	-0.6309	-0.5196
Ca	-0.6344	-0.4850
Mg	-0.6244	-0.0171
Acidity Potential (H + Al)	0.5369	-0.6869
Al	**0.7055**	0.0425
Cation exchange capacity at pH 7 (T)	0.4876	**-0.7211**
Effective cation exchange capacity (t)	0.2723	-0.2757
Sum of exchangeable bases (SB)	-0.6428	-0.3799
Base saturation index (V)	**-0.8783**	-0.0210
Removable P	-0.0614	-0.0596
Organic matter (M.O)	0.5120	**-0.7038**
Zn	0.1738	0.0305
Fe	0.3832	-0.6969
Mn	-0.3799	0.1134
Cu	0.2130	0.4799
B	-0.0554	-0.6657
S	-0.5347	-0.0565
Clay	0.1608	0.4082
Silt	-0.0530	0.6137
Sand	-0.0707	-0.6715
Shoot dry matter (SDM)	**-0.8016**	-0.1081
Root dry matter (RDM)	-0.4922	-0.2380
Number of nodules (NN)	**-0.8443**	-0.0890
Nodules dry matter (NDM)	**-0.8122**	-0.1352
Relative efficiency (RE)	**-0.8008**	-0.1076

† PC - principal components. Values in bold greater than or equal to 0.7 indicate strong association for the interpretation of the main components.

Table S2.
Principal component analysis of physical, chemical and biological attributes of soils under different vegetation types in the Quadrilátero Ferrífero, in an experiment with cowpea.

Variance components	PC 1	PC2
Eigenvalues	8.2000	5.6685
% of variance	30.37	20.29
Cumulative %	30.37	51.37
Variables	Correlation with the principal components	
pH	**-0.8092**	-0.2031
K	0.2814	0.0408
P	-0.3544	-0.6489
Ca	-0.4189	-0.6580
Mg	-0.6022	-0.1784
Acidity Potential (H + Al)	**0.7678**	-0.3168
Al	0.6278	0.4527
Cation exchange capacity at pH 7 (T)	**0.7331**	-0.3631
Effective cation exchange capacity (t)	0.3233	0.0543
Sum of exchangeable bases (SB)	-0.4771	-0.5530
Base saturation index (V)	**-0.7959**	-0.3570
Removable P	-0.0413	-0.0662
Organic matter (M.O)	**0.7352**	**-0.3572**
Zn	0.1212	0.0962
Fe	0.5886	-0.4192
Mn	-0.4126	0.0516
Cu	0.0235	**0.7026**
B	0.2467	-0.6408
S	-0.4584	-0.3076
Clay	-0.0181	0.5413
Silt	-0.2990	0.6205
Sand	0.2085	**-0.7633**
Shoot dry matter (SDM)	**-0.7212**	0.2088
Root dry matter (RDM)	-0.6048	0.1911
Number of nodules (NN)	-0.1954	-0.0136
Nodules dry matter (NDM)	-0.2656	-0.4679
Relative efficiency (RE)	**-0.7211**	0.2087

† PC - principal components. Values in bold greater than or equal to 0.7 indicate strong association for the interpretation of the main components.

ARTIGO 2 - DIVERSITY AND SYMBIOTIC EFFICIENCY OF NITROGEN-FIXING BACTERIA CAPTURED BY SIRATRO AND COWPEA FROM SOILS UNDER DIFFERENT VEGETATION TYPES, IN QUADRILÁTERO FERRÍFERO – MG, BRAZIL

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(VERSÃO PRELIMINAR)

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ABSTRACT

The edaphic characteristics of the soil, as well as the type of vegetation cover, are factors that influence microbial diversity and functionality. The Quadrilátero Ferrífero is the main area of occurrence of 'cangas' in Brazil, which are rocky outcrops with high Fe content. In general, this area is characterized by soils with low pH and few nutrients availability, in addition to intense Fe mining activity. There are few studies on the microbial diversity of this environment, with no reports in the literature regarding the occurrence and diversity of nitrogen-fixing bacteria. This study aimed to assess the phenotypic and genetic diversity and the symbiotic efficiency of nitrogen-fixing *Leguminosae* nodulating bacteria (NFLNB) in 'canga', in sites, revegetated with grass (*Melinis minutiflora*) which received soil from the opening of mining pits, and other adjacent vegetation (forest, cerrado, and eucalyptus), located in the Quadrilátero Ferrífero, Sabará - MG. It was also assessed the effectiveness of promiscuous species siratro and cowpea in the access to NFLNB diversity. For phenotypic characterization, it was assessed: time of growth, size, shape and color of colonies, and pH change in culture medium 79. For genetic characterization of the NFLNB strains, it was used 16S rRNA gene partial sequencing together with the BOX-PCR. The latter allowed assessing the intraspecific diversity, enabling better discrimination among strains. Among the evaluated areas, the grass and the 'canga' were the most and the least diverse vegetation types, respectively. Some NFLNB genera, such as *Bradyrhizobium* and *Rhizobium*, were captured by both plant species. However, other genera were captured only by siratro or only by cowpea, evidencing the

importance of using more than one plant species in studies on diversity. A large number of strains obtained by both cowpea and siratro proved to be effective in N_2 biological fixation, revealing their biotechnological potential. This study is the first report on diversity and symbiotic efficiency of NFLNB strains in 'canga' environment.

Keywords: trap plant species, mining, BOX-PCR, 16S rRNA, 'canga'.

1. INTRODUCTION

The exploitation of natural resources may result in serious environmental problems, such as ecosystem unbalance, loss of biotic diversity, and consequently loss of essential ecosystem services for the maintenance of the human species (Farrell *et al.*, 2010). Thus, it is important that the planning of any mining activity be linked to a subsequent recovery project in the area to be exploited (Szwedzicki, 2001). The management effectiveness and the measurability of the recovery of degraded areas depend on the knowledge of the organisms present in the soil and their interactions with the environment (Chaer et al., 2011).

The Quadrilátero Ferrífero region stands out for the mining activity, being an important source of natural resources for the country. This region holds the largest area of 'canga' in Brazil, approximately 100 km^2. 'Cangas' are outcrops which were formed millions of years ago, resulting from the weathering of underlying banded iron formations (BIF), and are one of the least studied ecosystems of Minas Gerais, although it is among the most threatened environments, mainly due to the intense mining activity associated with its iron outcrops (Skirycz et al., 2014). There are few studies on the diversity of this environment, and the available ones are mostly about the vegetation, since 'canga' holds several rare species (Giulietti et al., 2009; Jacobi et al., 2012). This lack of studies is especially noticeable for soil microbiota.

The soil, which is diverse in living organisms, such as small invertebrates, plants and microorganisms, is one of the components of the ecosystem mostly affected by mineral extraction. The microorganisms

present the soil are crucial for nutrient cycling and for the mineralization of the organic matter found in this environment (Moreira, 2006). Particularly, nitrogen-fixing bacteria (NFB) are naturally present in soils; they have important role in plant nutrition, and are key components in the nitrogen cycle, which is one of the most limiting nutrient for the revegetation of areas disturbed by mining (Chaer et al., 2011).

Studies on legume-rhizobia symbiosis have considerably increased in recent decades; however, there is much more information to be assessed regarding this relationship, such as the specificity between legumes and several NFLNB genera, in addition to the environmental influence, such as vegetation cover and edaphic characteristics of the soil. It is estimated that most part of the NFLNB biodiversity is still unknown (Moreira *et al.*, 2010), especially those found in under-explored environments, such as 'cangas'.

Studies on the diversity and phylogenetic relationship of NFLNB can be carried out using molecular techniques, such as 16S rRNA gene full or partial sequencing (Guimarães et al., 2012; Silva *et al.*, 2012; Jaramillo et al., 2013). However, the sequencing of this gene does not always provide enough resolution to discriminate inter or intraspecies level, such as the bacteria of the *Bradyrhizobium* genus (Willems *et. al.*, 2001). In some cases, i.e., for some genera, it is possible to reach the species level in an identification by 16S rRNA gene sequencing. However, usually, the most reliable is to confirm only the genus. Therefore, it is necessary that the 16S rRNA gene sequencing is combined with other techniques, such as the BOX-PCR, which presents high intraspecific resolution (Versalovic et

al., 1994; Guimarães *et al.*, 2012; Jaramillo et al., 2013), in order to provide more precise information on the genetic diversity of strains.

Microorganisms diversity can be influenced by several factors, such as physical and chemical characteristics of the soil (Jesus *et al.*, 2009), human activity on the environment, such as mining (Nóbrega *et al.*, 2004; Melloni *et al.*, 2006), vegetation type (Lima et al., 2009), as well as temperature and rainfall, among others. Knowing the diversity and functionality of the microorganisms found in the soil is the first step to assess the quality of an environment. These organisms have the potential to be used as indicators of environmental quality for the monitoring of areas under recovery (Trindade *et al.*, 2000; Silveira *et al.*, 2004). Furthermore, microorganisms isolation can be a way of accessing relevant genetic resources for the implementation and the success of environmental recovery plans.

This study aims to assess the phenotypic and genetic diversity and the symbiotic efficiency of nitrogen-fixing leguminosae nodulating bacteria (NFLNB) in 'canga' soils and in different types of adjacent vegetation (grass, forest, cerrado and eucalyptus) in the Quadrilátero Ferrífero, Sabará-MG. In addition, it was assessed the efficiency of the promiscuous species siratro (*Macroptilium atropurpureum*) and cowpea (*Vigna unguiculata*) regarding the access to diversity and effciency of these bacteria.

2. MATERIALS AND METHODS

2.1 Characteristics of the study area

The study was carried out in the Quadrilátero Ferrífero area, using five vegetation types, with approximately 716.47 hectares, in the

municipality of Sabará, located in the central-southeastern state of Minas Gerais - Brazil. This region is located in the Serra da Piedade, in the Quadrilátero Ferrífero, and is composed of a geosystem of ferruginous rocks, predominantly itabirito, constituents of the iron formations Cauê and Gandarela (Itabira Group, Minas Supergroup). The area named CeBio covers a deactivated iron ore mining pit, known as Córrego do Meio, and its surroundings (Figure 1). The climate is typically tropical, with short dry season (June to September), and sharp wet season, marked by rainfall concentrated in the hot season. The average annual temperature is 20.8º C.

In the study area, there were cerrado native vegetation with fragments of the Atlantic Forest and typical 'canga' vegetation (region of rocky outcrops rich in Fe) in higher altitutes, and an introduced vegetation composed of eucalyptus and molasses grass (*Melinis minutiflora*), and the latter is used in revegetation of the soil removed by the opening of mining pits and deposited in other sites. Four points per vegetation type were sampled (Figure 1).

The chemical and physical characteristics of the soil of the assessed areas have been described and presented in previous work (Costa et al, submitted).

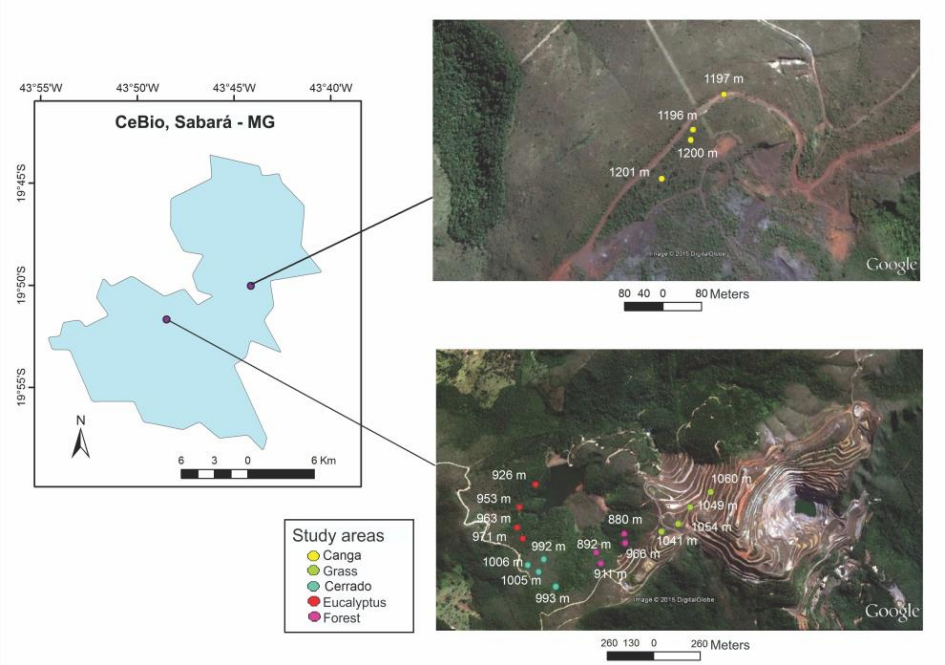

Figure 1. Study area, assessed for phenotypic and genotypic diversity, and for efficiency of nitrogen-fixing nodulating bacteria in the Quadrilátero Ferrífero - MG, with illustration of the collection points and of the altitude of each point.

2.2 Origin of strains and phenotypic characterization

Strains have been captured from canga, grass, forest, cerrado and eucalyptus, using siratro and cowpea as trap species, in a previous study (Costa et al, submitted). Seventy-seven strains were isolated from siratro nodules, and 118 strains were isolated from cowpea nodules.

Rhizobia was isolated from three nodules of each plant. Nodules were disinfected with etilic alcohol (95%) for 30s followed by imersion in H_2O_2 for 3 min and six succesive washings in sterilized distilled water.

Then, they were crushed and streaked in petri dishes containg ¨79¨ solid medium (Fred e Waksman, 1928) (pH 6,8), with bromothimol blue. Cultures were incubated at oven at 28ºC for at least 15 days, and evaluated daily during this period.

For phenotypic characterization, strains were cultivated in petri dishes in medium 79 (Fred and Waksman, 1928), and had the following characteristics assessed: time of growth, according to the colony appearance (fast- 3 days, intermediate – 4 to 5 days, and slow - more than 6 days); culture medium pH change (acid, neutral or alkaline); size, shape and color of the colonies. Strains were divided into different phenotypic groups according to these characteristics.

All strains were preserved using four methods: glass tubes containing solid medium 79 (slants), maintained at room temperature; microtubes containing liquid medium 79 plus 20% glycerol (w/v), preserved in a freezer at -80 °C; microtubes containing sterile water and maintained at room temperature; lyophilization, maintained in a climatized room at 21 °C. These strains belong to the collection of the Department of Soil Biology, Microbiology and Biological Processes of the Federal University of Lavras.

2.3.3 Identification of NFLNB strains

The partial sequencing of the 16S rRNA gene was carried out for strains identification. Thus, strains were previously grown in solid medium 79 (Fred and Waksman, 1928) at 28 °C, for 5-6 days (slow- and intermediate-growth bacteria), or for 3 days (fast-growth bacteria), and had the genomic DNA extracted by the alkaline lysis method, according to Niemann et al. (1997). For the 16S rRNA gene amplification, it was

used the pairs of primers 27F (AGAGTTTGACCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Lane, 1991), following the methodology described by Guimarães et al. (2012).

Sequences had their quality visually verified by the BioNumerics 7.1 software (Applied Maths, Sint-Martens – Latem, Belgium), and were subsequently submitted to the BLAST (Basic Local Alignment Search Tool) for comparison with the sequences deposited in the GenBank (National Center for Biotechnology Information).

2.3.4 Genetic Diversity by the BOX-PCR

Strains identified by 16S rRNA gene partial sequencing as belonging to the *Bradyrhizobium* or *Rhizobium* genera had their genetic diversity assessed by the BOX-PCR technique. Strains which did not belong to these two genera (*Bosea, Panebacillus, Methylobacterium, Dyella, Leifsonia* and *Tumebacillus*) were not included in the analysis, since the 16S rRNA gene sequencing allowed distinguishing them from the others.

The strains assessed by the BOX-PCR were grown in medium 79 at 6.8 pH, and after the growth of isolated colonies, one or two colonies were transferred to a microtube containing 1 mL sterile ultrapure water. The mixture was homogenized, heated at 95 °C for 10 minutes, and then the tubes were transferred to ice for thermal shock, in order to finalize the process of DNA extraction.

PCR reaction (25 µl) was carried out with the following volumes (µl): 9.45 sterile ultrapure water; 1.25 dNTPs (100 mM); 5.0 Gitschier buffer (5X) (Rademaker et al., 1997); 0.4 BSA (20 mg.mL^{-1}); 2.5 DMSO (100%); 1.0 of 0.3 µg. µL-1 primer BOX 5´-

CTACGGCAAGGCGACGCTGACG- 3´ (Versalovic et al., 1994); 0.4 *Taq* DNA polymerase; and 5.0 DNA. Amplification consisted of the following steps: initial denaturation cycle at 95 °C for 7 min; 35 cycles at 94 °C for 1 minute; 1 min at 53 °C and 8 min at 65 ° C; a final extension cycle at 65 °C for 16 min. The amplified fragments were separated by electrophoresis at 90 V in agarose gel at 1.5%, in 0.5X TAE buffer for 15 hours at room temperature. It was used 1kb plus DNA Ladder (InvitrogenTM) as marker. Finally, the gel was stained with ethidium bromide and photographed.

Genetic diversity of strains was analyzed by the presence or absence of polymorphic bands in the gel. Data were grouped by the UPGMA algorithm, using the Jaccard coefficient, with the aid of the BioNumerics 7.1 software (Applied Maths, Sint-Martens-Latem).

Genetic diversity in the different vegetation types was also assessed by calculating the Shannon index, which considers both the richness and abundance of species. This ratio is expressed by $H' = - \Sigma (P \times lnP)$, in which P is the relative abundance of each species and ln is the natural logarithm (Magurran, 1988), which was used to calculate the relative number of species - exp (H') that compares the local genetic diversity (γ diversity) in the assessed areas. For calculation purposes, it was considered the different genotypic groups instead of the species.

2.3.5 Authentication and symbiotic efficiency of strains

Strains were authenticated to verify the nodulation ability in the plant species of origin, as well as their symbiotic efficiency. Therefore, two experiments were carried out in greenhouse, one with siratro, from August to October 2014 (minimum temperature of 20°C, and maximum

temperature of 37°C), and another with cowpea, from October to November (minimum temperature of 25°C, and maximum temperature of 43°C). Experiments were carried out in tubes containing sand and vermiculite in a ratio of 1:1 (v:v), sterilized by moist heat autoclaving (1 atm, 127 °C, 1h). Pre-germinated seeds were planted in tubes. For plant nutrition inoculated with studied strains and reference strains, it was used the nutrient solution of Hoagland and Arnon (Hoagland and Arnon, 1950) with low concentration of mineral nitrogen N- (5.25 $mg.L^{-1}$), according the plant need, usually every 48 hours. For inoculation, 1mL aliquot of the culture medium 79 (liquid) containing each of the strains in pure culture was used in each tube. It was used two seeds of each plant species per tube, and after the appearance of the first true leaves, plants were thinned, leaving only one plant per tube. Two negative control treatments were included (uninoculated): one with the addition of high concentration of mineral nitrogen N+ (52.5 $mg.L^{-1}$), with the following composition of stock solutions added to 4 liters of water: 4 ml of 236.16 $g.liter^{-1}$ CaN_2O_6 $4H_2O$; 1 ml of 115.03 $g.liter^{-1}$ $NH_4H_2PO_4$; 6 ml of 101.11 $g.liter^{-1}$ KNO_3; 2.0 ml of 246.9 $g.liter^{-1}$ $MgSO_4$ $7H_2O$; 1 ml of 10 $g.liter^{-1}$ $FeCl_3$; and 1 ml of micronutrients (2.86 $mg.liter^{-1}$ H_3BO_3; 2.03 $mg.liter^{-1}$ $MnSO_4$ $4H_2O$; 0.22 $mg.liter^{-1}$ $ZnSO_4$ $7H_2O$; 0.08 $mg.liter^{-1}$ $CuSO_4$ $5H_2O$; and 0.09 $mg.liter^{-1}$ Na_2MoO_4 H_2O), and one with low concentration of mineral nitrogen N- (5.25 $mg.L^{-1}$), with the following composition of stock solutions added to 4 liters of water: 0.4 ml of 236.16 $g.liter^{-1}$ CaN_2O_6 $4H_2O$; 0.1 ml of 115.03 $g.liter^{-1}$ $NH_4H_2PO_4$; 0.6 ml of 101.11 $g.liter^{-1}$ KNO_3; 2.0 ml of 246.9 $g \cdot liter^{-1}$ $MgSO_4$ $7H_2O$; 3.0 ml of 87.13 $g.liter^{-1}$ K_2SO_4; 10 ml of 12.6 $g.liter^{-1}$ $CaH_4P_2O_8 \cdot H_2O$; 200 ml of 1.72 $g.liter^{-1}$

$CaSO_4$ $2H_2O$; 1 ml of 10 g.liter^{-1} $FeCl_3$; and 1 ml of micronutrients. Positive controls were inoculated with the reference strains UFLA 04-0212 ($ST_2$12) and SEMIA 656 for siratro, and INPA 03-11B (*Bradyrhizobium elkanii*) and UFLA 03-84 (*Bradyrhizobium sp.)* for cowpea, which have already been considered effective in previous studies (Florentino *et al.,* 2009, Soares *et al.,* 2006).

Nodulation ability was assessed by the presence or absence of nodules at the end of the experiment (50 days for siratro, and 40 days for cowpea). All treatments were assessed in triplicate and distributed in Completely Randomized Design (CRD). At the end of the cultivation, plants were collected, and shoots were separated and dried in a forced air circulation oven. Shoot dry matter was used to assess the relative efficiency (RE) of biological nitrogen fixation, following the formula:

$$RE = (SDM_{inoculated} / SDM_{N+}) \times 100$$

Where RE= relative efficiency; SDM= shoot dry matter, inoculated- at treatment inoculated with the tested strain r N+- at treatment with high mineral N concentration

Relative efficiency of the strains was assessed by analysis of variance (ANOVA) using the SISVAR version 5.6 software (Ferreira, 2011). After the assessment of the effect of treatments, the Scott-Knott test (Scott and Knott, 1974) was carried out for means clustering at 5% significance.

3. RESULTS

3.1 Phenotypic characterization of NFLNB

When assessing the phenotypic characteristics of the strains, it was possible to form 15 distinct groups to determine the phenotypic diversity of the vegetation types. The 77 strains captured by siratro were separated

into 10 phenotypic groups, of which only two (2.6%) showed fast growth and acidified the culture medium. Cowpea captured strains of all phenotypic groups. It was found that 13 strains (11%) presented fast growth and acidified the culture medium; two strains presented fast growth and neutralized the pH of the medium (1.7%); while the rest (103 strains, 87.3%) presented slow growth and alkalized the culture medium (Table 1).

Table 1. Phenotypic groups formed based on the phenotypic characteristics* of strains isolated from soils with different vegetation types in the Quadrilátero Ferrífero, MG.

Phenotypic groups	Phenotypic Characteristics					Strains captured by siratro	Strains captured by cowpea
	pH Change	Time of growth	Color	Size	Shape		
1	Alkaline	Slow	Cream	Big	Irregular	17	27
2	Alkaline	Slow	Cream	Punctiform	Round	2	2
3	Alkaline	Slow	Cream	Medium	Irregular	21	36
4	Alkaline	Slow	Cream	Medium	Round	7	2
5	Alkaline	Slow	Cream	Small	Irregular	4	16
6	Alkaline	Slow	Cream	Small	Round	22	14
7	Alkaline	Slow	Red	Small	Round	0	5
8	Alkaline	Slow	Yellow	Small	Round	1	1
9	Acid	Fast	Cream	Medium	Irregular	0	1
10	Acid	Fast	Cream	Medium	Round	0	5
11	Acid	Fast	Yellow	Big	Irregular	0	1
12	Acid	Fast	Yellow	Big	Round	1	4
13	Acid	Fast	Yellow	Small	Round	0	1
14	Acid	Intermediate	Yellow	Punctiform	Round	1	1
15	Neutral	Fast	Yellow	Medium	Irregular	1	2

* Time of growth, according to the colony appearance (fast - 3 days, intermediate – 4 to 5 days, and slow - more than 6 days); culture medium pH change (acid, neutral or alkaline); size (Punctiform – less than 2 mm; small – 2 to 3 mm, medium – 4 to 6 mm; big – bigger than 7 mm), shape and color of the colonies.

Considering both plant species, the different vegetation types presented a very similar number of phenotypic groups. The forest and the eucalyptus environments presented the highest number of similar phenotypic groups (9 groups), followed by the grass and the cerrado (8 groups), and finally the 'canga' (7 groups) (Table 2 and 3)

3.2 Identification of NFLNB strains

For genetic identification, it was selected representative strains of each phenotypic group, and of all vegetation types, being 65 strains from siratro (3 of 'canga', 14 of eucalyptus, 4 of cerrado, 18 of forest, and 26 of grass), and 46 strains from cowpea (7 of 'canga', 6 of eucalyptus, 13 of cerrado, 6 of forest, and 14 of grass).

The partial sequencing of the 16S rRNA gene of strains captured with siratro identified five genera: *Bradyrhizobium, Rhizobium, Bosea, Leifsonia* and *Tumebacillus*. Among the strains captured with cowpea, the following genera were identified: *Bradyrhizobium, Rhizobium, Methylobacterium, Paenibacillus* and *Dyella*. Tables 2 and 3 list the strains captured with siratro and cowpea, respectively, with their origin and genetic identification.

Table 2. Genetic identification and relative efficiency (RE) of strains obtained in the capture experiment from different vegetation types, using siratro as bait plant, based on 16S rRNA gene partial sequencing, compared with the NCBI database.

Code	Vegetation type	Origin point	RE (%)[1]	Phenotypic group	pb	Similar sequence found in the GenBank		
						Genetic identification of the most similar accession	N. of the most similar accession	Similarity (%)
UFLA 04-481	Eucalyptus	E1	99.24 a	1	563[R]	*Bradyrhizobium sp.*	KJ532470.1	100
UFLA 04-511	Eucalyptus	E1	33.76 c	12	528[R]	*Rhizobium sp.*	AB809379.1	100
UFLA 04-470	Eucalyptus	E1	115.9 a	3	634[R]	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-461	Eucalyptus	E1	92.74 a	3	647[R]	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-464	Eucalyptus	E1	120.4 a	1	775[R]	*Bradyrhizobium sp.*	KF114655.1	99
UFLA 04-480	Eucalyptus	E1	95.56 a	1	555[R]	*Bradyrhizobium sp.*	KF114645.1	100
UFLA 04-482	Eucalyptus	E1	99.89 a	3	937[R]	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-467	Eucalyptus	E2	101.8 a	4	667[R]	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-473	Eucalyptus	E4	81.06 b	3	711[R]	*Bradyrhizobium sp.*	JQ771204.1	99
UFLA 04-503	Eucalyptus	E4	101.8 a	1	586[R]	*Bradyrhizobium sp.*	HG940529.1	99
UFLA 04-483	Eucalyptus	E4	80.73 b	1	668[R]	*Bradyrhizobium sp.*	HG940529.1	100
UFLA 04-457	Eucalyptus	E4	91.02 a	1	231[R]	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 04-522	Eucalyptus	E4	80.08 b	2	680[R]	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-485	Eucalyptus	E4	82.79 b	3	670[R]	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-458	Forest	M6	111.2 a	1	621[R]	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-465	Forest	M8	109.9 a	1	471[R]	*Bradyrhizobium sp.*	KF927050.1	100

Code	Vegetation type	Origin point	RE (%)[1]	Phenotypic group	pb	Similar sequence found in the GenBank		
						Genetic identification of the most similar accession	N. of the most similar accession	Similarity (%)
UFLA 04-489	Forest	M8	107.2 a	6	663 R	*Bradyrhizobium sp.*	KF114652.1	99
UFLA 04-507	Forest	M8	68.39 b	3	430 F	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 04-469	Forest	M8	93.61 a	3	672 R	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-495	Forest	M8	100.7 a	6	727 R	*Bradyrhizobium sp.*	AB681387.1	99
UFLA 04-491	Forest	M8	69.91 b	3	263 R	*Bradyrhizobium sp.*	JN697683.1	100
UFLA 04-466	Forest	M8	95.45 a	5	631 R	*Bradyrhizobium sp.*	KF927050.1	99
UFLA 04-513	Forest	M8	39.18 c	14	939 R	*Leifsonia sp.*	HM587751.1	100
UFLA 04-506	Forest	M8	72.62 b	3	617 R	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-475	Forest	M8	83.98 b	3	669 R	*Bradyrhizobium sp.*	KF114651.1	99
UFLA 04-519	Forest	M8	86.9 a	3	611 R	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-490	Forest	M8	81.82 b	3	467 R	*Bradyrhizobium sp.*	JQ911631.1	100
UFLA 04-487	Forest	M9	98.59 a	1	435 R	*Bradyrhizobium sp.*	FJ390902.1	100
UFLA 04-523	Forest	M9	113.2 a	6	937 R	*Bradyrhizobium sp.*	KF114652.1	100
UFLA 04-524	Forest	M9	94.91 a	2	584 R	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-509	Forest	M9	101.9 a	1	724 R	*Bradyrhizobium sp.*	KF114655.1	99
UFLA 04-488	Forest	M9	69.91 b	8	674 R	*Bosea sp.*	DQ440826.1	100
UFLA 04-451*	Cerrado	CE1	Nd	15	452 R	*Tumebacillus flagellatus*	NR_109600.1	99
UFLA 04-460	Cerrado	CE1	75.0 b	6	615 R	*Bradyrhizobium sp.*	KF114645.1	100
UFLA 04-474	Cerrado	CE1	78.25 b	3	665 R	*Bradyrhizobium sp.*	KF802582.1	99
UFLA 04-525	Cerrado	CE1	65.69 b	3	723 R	*Bradyrhizobium sp.*	KF114655.1	100

Code	Vegetation type	Origin point	RE (%)[1]	Phenotypic group	pb	Similar sequence found in the GenBank		
						Genetic identification of the most similar accession	N. of the most similar accession	Similarity (%)
UFLA 04-516	Grass	C2	82.03 b	3	793 [R]	*Bradyrhizobium sp.*	GU433465.1	100
UFLA 04-512	Grass	C2	88.96 a	6	1016 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-478	Grass	C2	109.2 a	6	299 [R]	*Bradyrhizobium sp.*	KJ818094.1	100
UFLA 04-453	Grass	C2	97.51 a	4	405 [R]	*Bradyrhizobium sp.*	CP007569.1	99
UFLA 04-456	Grass	C2	86.9 a	4	723 [R]	*Bradyrhizobium sp.*	FJ390933.1	99
UFLA 04-455	Grass	C2	84.09 b	4	684 [R]	*Bradyrhizobium sp.*	AB681387.1	99
UFLA 04-471	Grass	C3	89.17 a	6	561 [R]	*Bradyrhizobium sp.*	CP007569.1	99
UFLA 04-515	Grass	C3	92.96 a	6	806 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-463	Grass	C3	95.67 a	1	578 [R]	*Bradyrhizobium sp.*	KF943790.1	100
UFLA 04-459	Grass	C3	99.89 a	6	952 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-505	Grass	C3	104.9 a	6	611 [R]	*Bradyrhizobium sp.*	CP007569.1	99
UFLA 04-510	Grass	C3	106.6 a	4	681 [R]	*Bradyrhizobium sp.*	AB681387.1	99
UFLA 04-462	Grass	C3	93.39 a	4	799 [R]	*Bradyrhizobium sp.*	AB681387.1	99
UFLA 04-500	Grass	C4	108 a	3	356 [R]	*Bradyrhizobium sp.*	KJ818095.1	100
UFLA 04-517	Grass	C4	78.79 b	6	920 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-499	Grass	C4	75.97 b	4	771 [R]	*Bradyrhizobium sp.*	AY904735.1	99
UFLA 04-518	Grass	C4	99.46 a	6	947 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-479	Grass	C4	107.3 a	6	711 [R]	*Bradyrhizobium sp.*	FJ390933.1	99
UFLA 04-493	Grass	C5	98.48 a	6	944 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-514	Grass	C5	88.09 a	6	160 [F]	*Bradyrhizobium sp.*	HQ641251.1	100

Code	Vegetation type	Origin point	RE (%)[1]	Phenotypic group	pb	Similar sequence found in the GenBank		
						Genetic identification of the most similar accession	N. of the most similar accession	Similarity (%)
UFLA 04-497	Grass	C5	88.31 a	1	503 [R]	*Bradyrhizobium sp.*	JQ911631.1	100
UFLA 04-498	Grass	C5	92.42 a	6	842 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-496	Grass	C5	95.34 a	6	580 [R]	*Bradyrhizobium sp.*	KC508845.1	100
UFLA 04-502	Grass	C5	92.53 a	6	719 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-494	Grass	C5	79.76 b	6	850 [R]	*Bradyrhizobium sp.*	AB681387.1	99
UFLA 04-492	Grass	C5	90.36 a	6	688 [R]	*Bradyrhizobium sp.*	JF266642.1	99
UFLA 04-447	Canga	CA2	112.0 a	1	166 [R]	*Bradyrhizobium sp.*	KJ818095.1	100
UFLA 04-521	Canga	CA2	114.6 a	1	474 [R]	*Bradyrhizobium sp.*	KF114655.1	100
UFLA 04-452	Canga	CA4	85.82 a	1	327 [R]	*Bradyrhizobium sp.*	KF927050.1	100
N+	Control	-	100 a	-	-	-	-	-
N-	Control	-	28.03 c	-	-	-	-	-
SEMIA 656	Control	-	100.8 a	-	-	-	-	-
UFLA 04-212	Control	-	105 a	-	-	-	-	-

* Not authenticated strains. Nd – not determined

[1] Means followed by the same letter in the column do not differ by the Scott Knott test at 5% probability.

[F] – Forward sequence.

[R] – Reverse sequence.

Table 3. Genetic identification and relative efficiency (RE) of strains obtained in the capture experiment from different vegetation types, using cowpea as bait plant, based on 16S rRNA gene partial sequencing, compared with the NCBI database.

Code	Vegetation type	Points of origin	RE (%)	Phenotypic group	pb	Similar sequence found in the GenBank		
						Genetic identification of the most similar accession	N. of the most similar accession	Similarity (%)
UFLA 03-374	Eucalyptus	E2	76.13 b	1	473 [F]	*Bradyrhizobium sp.*	KC113605.1	100
UFLA 03-369	Eucalyptus	E3	72.57 b	1	567 [R]	*Bradyrhizobium sp.*	KF114645.1	99
UFLA 03-407	Eucalyptus	E3	75.64 b	3	512 [F]	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 03-446*	Eucalyptus	E3	Nd	5	713 [R]	*Bradyrhizobium sp.*	KF114635.1	99
UFLA 03-390	Eucalyptus	E4	79.24 b	3	605 [F]	*Bradyrhizobium sp.*	KJ532472.1	100
UFLA 03-448*	Eucalyptus	E4	Nd	1	501 [R]	*Bradyrhizobium sp.*	KF943790.1	100
UFLA 03-433	Forest	M6	48.89 d	1	793 [R]	*Bradyrhizobium sp.*	KF114645.1	100
UFLA 03-432	Forest	M6	77.6 b	3	583 [F]	*Bradyrhizobium sp.*	KJ818095.1	100
UFLA 03-425	Forest	M6	75.73 b	7	524 [F]	*Methylobacterium sp.*	AB220096.1	100
UFLA 03-423*	Forest	M8	Nd	3	766 [R]	*Bradyrhizobium sp.*	KF114645.1	100
UFLA 03-426	Forest	M9	65.77 c	1	646 [F]	*Bradyrhizobium sp.*	KF114634.1	100
UFLA 03-397	Forest	M9	78.71 b	3	754 [F]	*Bradyrhizobium sp.*	KC113622.1	100
UFLA 03-371	Cerrado	CE1	80.93 b	1	548 [R]	*Bradyrhizobium sp.*	KJ532470.1	100
UFLA 03-406	Cerrado	CE1	67.37 c	1	522 [F]	*Bradyrhizobium sp.*	KF114636.1	100
UFLA 03-358	Cerrado	CE1	61.51 c	10	585 [F]	*Rhizobium sp.*	KJ880017.1	99
UFLA 03-355	Cerrado	CE1	67.55 c	10	651 [R]	*Rhizobium sp.*	KJ748400.1	99

Code	Vegetation type	Points of origin	RE (%)	Phenotypic group	pb	Similar sequence found in the GenBank		
						Genetic identification of the most similar accession	N. of the most similar accession	Similarity (%)
UFLA 03-417	Cerrado	CE2	72.66 b	1	381 R	*Bradyrhizobium sp.*	KJ532470.1	100
UFLA 03-419	Cerrado	CE2	54.6 d	3	512 F	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 03-398	Cerrado	CE2	59.24 c	1	454 F	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 03-363	Cerrado	CE2	66.4 c	3	627 F	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 03-428	Cerrado	CE2	52.71 d	7	535 F	*Methylobacterium sp.*	AM989026.1	100
UFLA 03-380	Cerrado	CE4	74.44 b	3	445 R	*Bradyrhizobium sp.*	KF943791.1	100
UFLA 03-383	Cerrado	CE4	48.93 d	11	615 F	*Rhizobium sp.*	KF773128.1	100
UFLA 03-401	Cerrado	CE4	58.8 c	3	495 F	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 03-418	Cerrado	CE4	73.91 b	5	856 R	*Bradyrhizobium sp.*	KC508879.1	100
UFLA 03-450*	Grass	C3	Nd	13	742 F	*Paenibacillus sp.*	EU621909.1	99
UFLA 03-376	Grass	C3	67.51 c	6	502 F	*Bradyrhizobium sp.*	KC508845.1	100
UFLA 03-365	Grass	C3	89.6 a	6	465 F	*Bradyrhizobium sp.*	KF927052.1	100
UFLA 03-402	Grass	C3	64.71 c	6	503 F	*Bradyrhizobium sp.*	JN578802.2	100
UFLA 03-436*	Grass	C3	Nd	9	540 F	*Dyella sp.*	JX909049.1	99
UFLA 03-356	Grass	C3	76.27 b	12	498 F	*Rhizobium sp.*	KJ831227.2	100
UFLA 03-349	Grass	C3	68.18 c	12	460 R	*Rhizobium sp.*	LM655398.1	100
UFLA 03-370	Grass	C3	71.73 b	1	508 F	*Bradyrhizobium sp.*	KC113622.1	100
UFLA 03-368	Grass	C4	54.66 d	6	628 R	*Bradyrhizobium sp.*	CP007569.1	99
UFLA 03-364	Grass	C4	74.53 b	3	679 R	*Bradyrhizobium sp.*	KF114645.1	100
UFLA 03-431	Grass	C4	63.86 c	6	539 F	*Bradyrhizobium sp.*	KC508845.1	100

Code	Vegetation type	Points of origin	RE (%)	Phenotypic group	pb	Similar sequence found in the GenBank		
						Genetic identification of the most similar accession	N. of the most similar accession	Similarity (%)
UFLA 03-386	Grass	C4	63.37 c	6	505 [F]	*Bradyrhizobium sp.*	JN578802.1	100
UFLA 03-399	Grass	C4	74.8 b	4	273 [F]	*Bradyrhizobium sp.*	KF927052.1	100
UFLA 03-357	Grass	C5	53.95 d	12	506 [F]	*Rhizobium sp.*	FJ183429.1	99
UFLA 03-456	Canga	CA1	82.13 b	12	535 [F]	*Rhizobium sp.*	JQ771199.1	100
UFLA 03-434	Canga	CA1	56.13 d	5	387 [F]	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 03-387*	Canga	CA1	Nd	5	375 [R]	*Bradyrhizobium sp.*	KJ532472.1	100
UFLA 03-394	Canga	CA1	50.97 d	1	468 [F]	*Bradyrhizobium sp.*	KC113620.1	100
UFLA 03-410	Canga	CA4	83.95 b	10	495 [R]	*Rhizobium sp.*	EF061100.1	99
UFLA 03-435	Canga	CA6	59.51 c	3	450 [F]	*Bradyrhizobium sp.*	KF927050.1	100
UFLA 03-385	Canga	CA6	77.24 b	3	571 [F]	*Bradyrhizobium sp.*	KJ818095.1	100
N+	Control	-	100 a	-	-	-	-	-
N-	Control	-	44.57 d	-	-	-	-	-
INPA 03-11B	Control	-	73.02 b	-	-	-	-	-
UFLA 03-84	Control	-	67.46 c	-	-	-	-	-

*Not authenticated strains. Nd – not determined.

[1] Means followed by the same letter in the column do not differ by the Scott Knott test at 5% probability.

[F] – Forward sequence.

[R] – Reverse sequence.

Bradyrhizobium and *Rhizobium* are widely recognized and are described in the literature as NFLNB. They are commonly found in studies on biological nitrogen fixation (Masson-Boivin et al., 2009). On the other hand, there is not enough information in the literature reporting *Dyella* and *Tumebacillus* (which were not authenticated in this work) as NFLNB. Therefore, these genera were not considered as NFLNB when quantifying the genetic diversity in each area. Although *Paenibacillus* has not been authenticated, it has already demonstrated nitrogen-fixation ability in previous studies (Costa et al., 2012; Kwak et al., 2015). *Methylobacterium* is described as nitrogen-fixing nodulating bacteria (Sy et al., 2001). *Bosea*, which is described as endophytic with N_2-fixation ability (Andam et al., 2007), and *Leifsonia*, which is not commonly reported as nitrogen-fixing leguminosae nodulating bacteria, presented positive nodulation in this study. However, they were not considered in the NFLNB diversity indices (Table 4 and 5). *Leifsonia* and *Bosea* genera were obtained from nodules which also contained *Bradyrhizobium* strains, thus, they are indeed nodule endophytes.

Table 4 lists the vegetation types studied with NFLNB genera identified by *the16S rRNA* gene partial sequencing. Considering only this technique, the highest number of genera was found in cerrado. In the 'canga', int the grass and in the eucalyptus vegetation, only *Bradyrhizobium* and *Rhizobium* were identified, and in the forest vegetation, it was identified *Bradyrhizobium* and *Methylobacterium* genera.

Table 4. Occurrence of NFLNB genera in the Quadrilátero Ferrífero - MG, in areas with different vegetation covers.

Vegetation type	*Bradyrhizobium*	*Rhizobium*	*Methylobacterium*
Grass	X	X	
Canga	X	X	
Eucalyptus	X	X	
Cerrado	X	X	X
Forest	X		X

3.3 Genetic diversity by the BOX-PCR

Groups formed by the BOX-PCR, considering 70% similarity, presented genotypic groups formed by strains derived from each vegetation type (Figure 2). Genetic diversity was determined using the Shannon index, which considered the number of genotypic groups of each vegetation type (Table 5). To obtain the number of groups, it was considered the information obtained from the BOX-PCR for *Bradyrhizobium* and *Rhizobium* genera, and of the *16S rRNA* gene partial sequencing for the other genera.

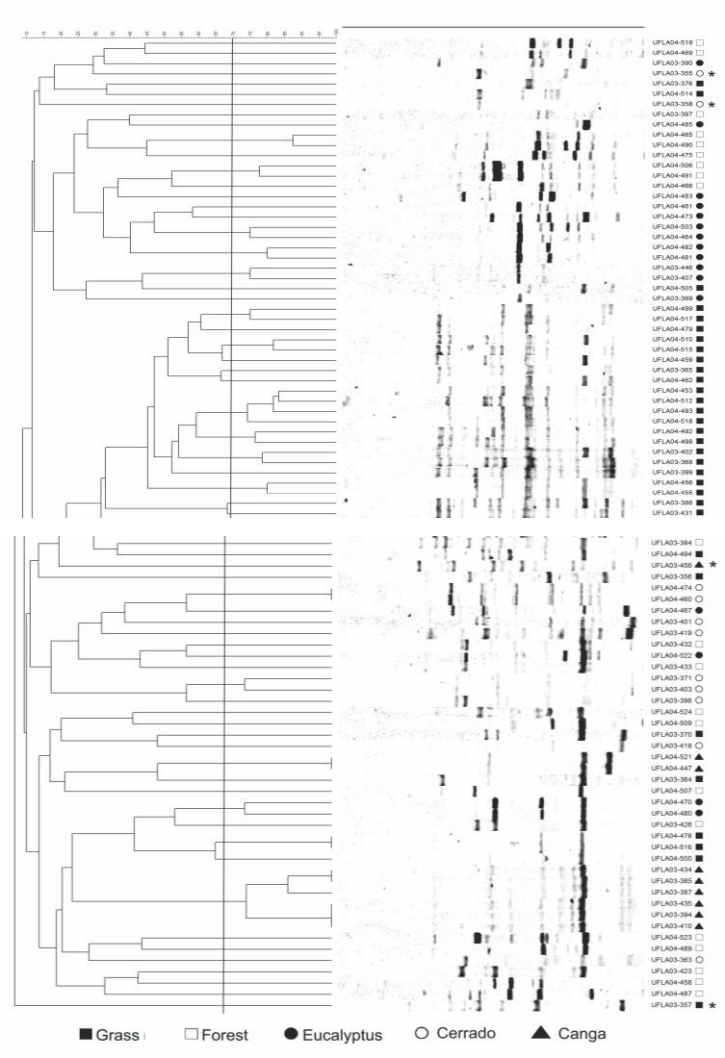

Figure 2. Dendrogram of genetic similarity (based on the BOX-PCR) of nitrogen-fixing nodulating bacteria captured using cowpea and siratro as trap plant, in different vegetation types in the Quadrilátero Ferrífero - MG. Strains whose codes are followed by an asterisk in the dendrogram belong to the *Rhizobium* genus, and the others belong to the *Bradyrhizobium* genus.

Table 5. NFLNB ɣ Diversity represented by the Shannon index and by the relative number of genotypes in areas with different vegetation types in the Quadrilátero Ferrífero - MG, using siratro and cowpea as bait plant.

Vegetation type	Genotypic group[1]	Shannon Index H'	Relative number of genotypes
Canga	3	0,84	2,33
Cerrado	11	2,25	9,52
Eucalyptus	12	2,42	11,31
Forest	22	2,87	23,36
Grass	24	2,97	21,68

[1.] Genotypic groups obtained by the BOX-PCR and by the 16S rRNA gene partial sequencing

Thus, genetic diversity among the vegetation types, considering the genotypes obtained both with cowpea and siratro, followed the order: Grass> Forest> Eucalyptus> Cerrado> Canga.

3.4 Authentication and symbiotic efficiency of strains

Of the 77 strains captured with siratro, 65 were subjected to authentication regarding the nodulation ability, the other 12 strains were not recovered after storage. Of the strains captured with cowpea, 46 representatives of the cultural groups (Table 1) were selected for the authentication test and for the assessment of the symbiotic efficiency, except for the phenotypic group number 9 and 13. These groups are represented by only one strain (UFLA 03-436 – *Dyella sp.* and UFLA 03-450 – *Paenibacillus* sp, respectively), and plants died before the end of the experiment. Moreover, plants inoculated with strains UFLA 03-446, UFLA 03-448, UFLA 03-423 and UFLA 03-387 (*Bradyrhizobium*) also

died during the experiment, which hindered the assessment of symbiotic efficiency and authentication. Thus, 40 strains captured with cowpea were assessed regarding authentication and symbiotic efficiency. Strains of the *Bosea* and *Leifsonia* genera, which are not recognized as nodulating strains, also nodulated.

Controls with high and low nitrogen concentration did not nodulate, indicating that the experiment was properly carried out, and there was no contamination.

The symbiotic efficiency NFLNB strains is listed in Tables 2 and 3. Of the strains captured with siratro, 68.6% had RE similar to the N + control and to the reference strains, and all of them belong to the *Bradyrhizobium* genus. The other genera were represented by the strains UFLA 04-511 (*Rhizobium*) and UFLA 04-513 (*Leifsonia*), which were inefficient in biological N_2 fixation. However, UFLA 04-488 (*Bosea*), which was efficient, presented RE lower than that of the N + controls and of the reference strains ($p < 0.05$). Of the 3.1% inefficient strains (similar to the N- control), none belong to the *Bradyrhizobium* genus.

Of the strains captured with cowpea, only UFLA 03-365 (*Bradyrhizobium*), which was captured in the grass, presented RE similar to the N + control; 45% had RE similar to the INPA 03-11B reference strain and belong to *Bradyrhizobium, Rhizobium* and *Methylobacterium* genera; 32.5% were inefficient, and belong to the *Bradyrhizobium* and *Rhizobium* genera; and 20% were inefficient, similar to the N- control, and belong to the *Bradyrhizobium, Rhizobium* and *Methylobacterium* genera.

4. DISCUSSION

Both phenotypic and genetic diversity were distinct when considering the plant species used as trap plant. Siratro captured mostly slow-growth strains and which alkalized the culture medium, typical of the *Bradyrhizobium* genus. On the other hand, cowpea captured both slow and fast-growth strains which acidified the culture medium, typical of other genera, such as *Rhizobium* (Moreira and Pereira, 2001).

In relation to genetic diversity, the composition of NFLNB communities differed when considering the two species used in the capture, since some genera were identified in a particular vegetation type using cowpea as trap plant, but were not identified when using siratro. These phenotypic and genetic characterization data are in accordance with the literature, which provides more accurate information regarding NFLNB diversity by using more than one species as trap plant (Moreira et al., 2008; Lima et al., 2009; Moreira et al., 2010; Guimarães et al., 2012; Jaramillo et al., 2013; Ormeño-Orrillo et al., 2012).

Siratro and cowpea are considered promiscuous for presenting nodulation with a great variety of NFLNB genera. Nonetheless, these species present selectivity for some genera (Ormeño-Orrilo et al., 2012). This seems to happen with siratro, since most of the strains captured by this plant belong to the *Bradyrhizobium* genus, while cowpea, although presenting predominance of the *Bradyrhizobium* genus, also captured more strains of other genera. The occurrence of different NFLNB genera in the same area, using cowpea and siratro as trap plant, was also

observed by Guimarães et al. (2012), Jaramillo et al. (2013) and Lima et al. (2009).

Melloni *et al.* (2006) reported the impact of bauxite mining on NFLNB diversity. The authors highlight that environments under recovery contain greater NFLNB diversity than environments impacted by bauxite extraction, especially when legume species are used in the recovery. The same was observed by Nóbrega et al. (2004) in areas under recovery. The grass, after receiving the soil of the opening of the mining pit, can be considered an environment under recovery. As reported by Melloni et al. (2006) and Nóbrega et al. (2004), we found that this environment stood out for presenting high NFLNB diversity, which was very close to that found in the forest.

Lima *et al.* (2009) assessed the morphological and genetic diversity of NFLNB captured with siratro as trap plant in different land use systems (LUS) in the Amazon region. Comparing the diversity obtained by morphological and genetic analyses, the authors noted that morphology can be used as indicative of the genetic diversity of an area, without overestimating it. The same was observed by Smit et al. (2001). Furthermore, the morphological analysis is a convenient and inexpensive technique compared with the genetic analysis.

When comparing the phenotypic diversity with the genetic diversity, considering only the *16S rRNA* gene partial sequencing, it is observed that both analyses had similar results, indicating the forest as the most diverse, and ‘canga’ as the least diverse. By using the BOX-PCR technique, the profile of the genetic diversity of the vegetation types was similar, confirming the greatest diversity of NFLNB in the forest, and lowest

diversity in the ‘canga’ environment. These results confirm that different techniques should be used together for diversity studies in order to increase the resolution power, and thus provide more reliable results (Zhang et al., 2007).

Although ‘canga’ presented lower NFLNB diversity, this type of study is significant for being the first report on the occurrence of NFLNB in ‘canga’ vegetation in the Quadrilátero Ferrífero.

Many studies on diversity using siratro and/or cowpea as trap plant found *Bradyrhizobium* as dominant genus (Melloni *et al.,* 2006; Lima *et. al.,* 2009; Guimarães *et al.,* 2012). The same was found in this study, since 85% of the sequenced bacteria belong to this genus.

Regarding the symbiotic efficiency of the assessed strains, siratro captured more efficient strains than cowpea, and most of these strains belong to the *Bradyrhizobium* genus. In addition, a great number of strains, both captured by siratro and cowpea, presented RE similar to that of the reference strains. These strains may be of agronomic and commercial interest, especially if they are used in areas with similar soil characteristics.

Studies which relate different vegetation types with the diversity of key groups of microorganisms, such as NFLNB, are important for indicating the effect of each vegetation type on diversity, and for enabling the understanding of the community structure, which will be important for biodiversity conservation studies. Moreover, the study on the diversity of native NFLNB is crucial, since it can reveal sources of essential

genetic resources for selection of strains adapted to different environmental conditions.

5. CONCLUSIONS

The most diverse vegetation type in NFLNB was the grass, followed by the forest. 'Canga', despite having lower NFLNB diversity, had most of the strains with high efficiency of biological nitrogen fixation, proving to be a source of genetic resources with biotechnological potential.

NFLNB communities were sensitive indicators of change in the vegetation type and management in the Quadrilátero Ferrífero.

Cowpea and siratro have distinct promiscuity regarding symbiosis with NFLNB genera. In relation to efficiency, the strains captured by cowpea varied from inefficient and very efficient. Siratro mostly captured very efficient strains.

In NFLNB diversity studies, it is essential to use more than one species as bait plant in order to ensure greater access to the bacterial species present in a given area. Similarly, it is recommended the combination of techniques for morphological and genetic analysis, since they are complementary.

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