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CIÊNCIA DO SOLO

TESE

Caracterização e Eficiência Simbiótica de Bactérias Isoladas de Nódulos de Feijão-mungo [*Vigna radiata* (L.) Wilczek]

Vinício Oliosi Favero

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CARACTERIZAÇÃO E EFICIÊNCIA SIMBIÓTICA DE BACTÉRIAS ISOLADAS DE NÓDULOS DE FEIJÃO-MUNGO [*Vigna radiata* (L.) Wilczek]

VINÍCIO OLIOSI FAVERO

Sob a Orientação do Pesquisador
Segundo Sacramento Urquiaga Caballero

e Co-orientação dos Pesquisadores
Norma Gouvêa Rumjanek e **Gustavo Ribeiro Xavier**

Tese submetida como requisito parcial para obtenção do grau de **Doutor**, no Programa de Pós-Graduação em Agronomia – Ciência do Solo, Área de Concentração em Biologia do Solo.

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BIOGRAFIA

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RESUMO GERAL

FAVERO, Vinício Oliosi. **Caracterização e eficiência simbiótica de bactérias isoladas de nódulos de feijão-mungo [*Vigna radiata* (L.) Wilczek].** 2022. 118f. Tese (Doutorado em Agronomia, Ciência do Solo). Instituto de Agronomia, Universidade Federal Rural do Rio de Janeiro, Seropédica-RJ, 2022.

O feijão-mungo é uma leguminosa de origem asiática com grande importância mundial, principalmente em países em desenvolvimento. Seu cultivo comercial no Brasil tem se expandido nos últimos anos, visando atender ao mercado internacional, e isso tem despertado para a necessidade de estudos relacionado ao seu cultivo no país, e dentre estes, os relacionados à fixação biológica de nitrogênio. Nesse sentido, objetivou-se com este estudo, avaliar a nodulação do feijão-mungo com rizóbios nativos de solos brasileiros, isolar os rizóbios associados, caracterizá-los e avaliá-los quanto à capacidade de nodulação e eficiência simbiótica. Para isso, no Capítulo I, foi avaliada a nodulação de dois genótipos de feijão-mungo por rizóbios nativos em dez solos brasileiros, além do isolamento das bactérias presentes nos nódulos, seguido de caracterização morfogenética e avaliação da capacidade de nodulação. De forma geral, as plantas cultivadas em amostras dos solos da região Sudeste apresentaram maior nodulação e crescimento comparadas àquelas cultivadas nas amostras da região Centro-Oeste. A partir dos nódulos, foram obtidas 101 bactérias, as quais foram agrupadas aos seguintes gêneros: *Bradyrhizobium* (66), *Rhizobium* (19), *Mesorhizobium* (4), *Ensifer* (3), *Leifsonia* (3), *Bacillus* (3), *Agrobacterium* (1), *Mycolicibacterium* (1) e *Kaistia* (1). Isolados de *Bradyrhizobium* foram os únicos capazes de nodular o feijão-mungo, sendo aqueles oriundos de solos da região Sudeste os mais eficientes; já quanto ao grupo filogenético, de forma geral, isolados próximos à espécie de *Bradyrhizobium yuanmingense* se mostraram mais eficientes. No Capítulo II, foi caracterizado o microbioma dos nódulos de dois genótipos de feijão-mungo cultivados em amostras de dez solos brasileiros, utilizando-se a técnica de sequenciamento do gene 16S rRNA por NGS (*Next-Generation Sequencing*) Illumina MiSeq. A OTU0001 (*Operational Taxonomic Units*) pertencente ao gênero *Bradyrhizobium* representou mais de 99% das sequências recuperadas. *Pseudomonas* foi o gênero não-rizobiano mais abundante, e esteve presente apenas em nódulos da cultivar MGS Esmeralda, revelando uma diferença de especificidade entre genótipos. No Capítulo III, foi avaliada a inoculação de 31 isolados de *Bradyrhizobium* em comparação aos rizóbios nativos em feijão-mungo cultivado em vaso com solo, incluindo a avaliação da aplicação de doses de N na semeadura. A inoculação dos isolados resultou em incrementos de até 79% em massa de nódulos, de 66% em massa de parte aérea e de 55% no N acumulado oriundo da fixação biológica de N, comparados ao tratamento sem inoculação; no entanto, as plantas inoculadas tiveram menor crescimento que o tratamento com N fertilizante (160 kg ha^{-1} de N). Quando sob aplicação de N na semeadura, houve incrementos no desenvolvimento das plantas, mas com redução na nodulação. No Capítulo IV, avaliou-se a inoculação cruzada do feijão-mungo com estirpes elite de *Bradyrhizobium* usadas em inoculantes comerciais para soja e feijão-caupi no Brasil, além da comparação com isolados obtidos de nódulos de feijão-mungo. A estirpe SEMIA 587 (*B. elkanii*) recomendada para soja, e as estirpes UFLA 3-84 (*B. viridifuturi*), BR 3267 (*B. yuanmingense*) e INPA 3-11B (*B. elkanii*) recomendadas para feijão-caupi, foram capazes de nodular o feijão-mungo. A SEMIA 587, a UFLA 3-84 e os isolados de feijão-mungo apresentaram maior eficiência em nodulação e crescimento das plantas, e portanto, apresentam potencial para inoculação do feijão-mungo no Brasil.

Palavras-Chave: *Vigna radiata*. Seleção de rizóbios eficientes. Fixação biológica de nitrogênio.

GENERAL ABSTRACT

FAVERO, Vinício Oliosi. **Characterization and symbiotic efficiency of bacteria isolated from mung bean [*Vigna radiata* (L.) Wilczek] nodules.** 2022. 118p. Thesis (Doctorate in Agronomy, Soil Science). Instituto de Agronomia, Universidade Federal Rural do Rio de Janeiro, Seropédica-RJ, 2022.

Mung bean is a legume of Asian origin with great worldwide importance, mainly in developing countries. Its commercial cultivation in Brazil has expanded in recent years, aiming to meet the international market, and this has awakened the need for studies related to its cultivation in the country, and among these, those related to biological nitrogen fixation. In this sense, the objective of this study was to evaluate the nodulation of mung bean with rhizobia native to Brazilian soils, isolate the associated rhizobia, characterize them and evaluate them in terms of nodulation capacity and symbiotic efficiency. In Chapter I, the nodulation of two mung bean genotypes by native rhizobia in ten Brazilian soils was evaluated, in addition to the isolation of bacteria present in the nodules, followed by morphogenetic characterization and evaluation of the nodulation capacity. In general, plants grown in soil samples from the Southeast region showed higher nodulation and growth compared to those grown in samples from the Midwest region. From the nodules, 101 bacteria were obtained: *Bradyrhizobium* (66), *Rhizobium* (19), *Mesorhizobium* (4), *Ensifer* (3), *Leifsonia* (3), *Bacillus* (3), *Agrobacterium* (1), *Mycolicibacterium* (1) and *Kaistia* (1). *Bradyrhizobium* isolates were the only ones capable of nodulating mung bean, and those from soils in the Southeast region were the most efficient; as for the phylogenetic group, in general, isolates close to the *Bradyrhizobium yuanmingense* specie were more efficient. In Chapter II, the microbiome characterization of the nodules of two mung bean genotypes cultivated in samples of ten Brazilian soils was performed, using the 16S rRNA gene sequencing technique by NGS (Next-Generation Sequencing) Illumina MiSeq. OTU0001 (Operational Taxonomic Units) belonging to the *Bradyrhizobium* genus represented more than 99% of the recovered sequences. *Pseudomonas* was the most abundant non-rhizobia genera, and was present only in nodules of the MGS Esmeralda cultivar, revealing a difference in specificity between genotypes. In Chapter III, the inoculation of 31 *Bradyrhizobium* isolates compared to native rhizobia in mung bean grown in pots with soil was evaluated, including the evaluation of the application of N rates at sowing. The inoculation of the isolates resulted in increments of up to 79% in nodule weight, 66% in shoot dry weight, and of 55% in the accumulated N from the FBN compared to the treatment without inoculation, however, they had less growth than treatment with N fertilizer (160 kg ha^{-1} of N). When under application of N doses at sowing, there were increases in plant development, but with a reduction in nodulation. In Chapter IV, the inoculation potential of mung bean was verified through cross-inoculation with elite Bradyrhizobium strains used in commercial inoculants for soybean and cowpea in Brazil, in addition to a comparison with isolates obtained from mung bean nodules. The strain SEMIA 587 (*B. elkanii*) recommended for soybean, and the strains UFLA 3-84 (*B. viridifuturi*), BR 3267 (*B. yuanmingense*), and INPA 3-11B (*B. elkanii*) recommended for cowpea were able to to nodulate the mung bean. SEMIA 587, UFLA 3-84, and mung bean isolates showed greater efficiency in nodulation and plant growth, and therefore, may contribute to mung bean inoculation in Brazil.

Key words: *Vigna radiata*. Selection of efficient rhizobia. Biological nitrogen fixation.

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1 INTRODUÇÃO GERAL

O feijão-mungo [*Vigna radiata* (L.) Wilczek], também conhecido no Brasil como feijão-mungo-verde, mungo-verde ou feijão-moyashi (VIEIRA; VIEIRA; VIEIRA, 2001), é uma leguminosa originária da Índia (FULLER, 2007; LAMBRIDES; GODWIN, 2007; ZUKOVSKIJ, 1962), e muito consumida em toda a Ásia. É conhecido por produzir grãos com elevado teor de proteínas e de carboidratos, além de possuir boas características nutricionais (DU et al., 2018; YI-SHEN; SHUAI; FITZGERALD, 2018). Seu ciclo curto (NAIR et al., 2012) e a boa adaptação às elevadas temperaturas e ao estresse hídrico (HANUMANTHARAO; NAIR; NAYYAR, 2016; SHARMA et al., 2016) são características que favorecem o seu cultivo em regiões tropicais.

O feijão-mungo, assim como a maioria das leguminosas de uso agrícola, é capaz de fixar o nitrogênio atmosférico através da associação com rizóbios (HAYAT et al., 2008; HERRIDGE et al., 2005), o que pode reduzir os custos relacionados à aplicação de fertilizantes nitrogenados. É capaz de nodular com diferentes gêneros de rizóbios (YANG et al., 2008; ZHANG et al., 2008), sendo um hospedeiro com baixa especificidade simbiótica. Estudos conduzidos na Ásia mostram que isolados pertencentes ao gênero *Bradyrhizobium* (APPUNU et al., 2009; RISAL et al., 2012; YANG et al., 2008; ZHANG et al., 2008), *Ensifer* (PANDYA; KUMAR; RAJKUMAR, 2013; YANG et al., 2008; ZHANG et al., 2008), *Rhizobium* (YANG et al., 2008; ZHANG et al., 2008) e *Mesorhizobium* (YANG et al., 2008) são capazes de nodular feijão-mungo. Em estudos de isolamento, o gênero *Bradyrhizobium* foi considerado o mais predominante (YANG et al., 2008; ZHANG et al., 2008), no entanto, com análise de sequenciamento, verificou-se que pode haver uma codominância ou dominância dos gêneros *Bradyrhizobium* e *Ensifer*, a depender do solo de cultivo (HAKIM et al., 2018, 2020). Em um destes estudos, o gênero *Ensifer* representou de 4 até 99% das sequências bacterinas presentes nos nódulos de feijão-mungo (HAKIM et al., 2020), demonstrando que pode haver grandes diferenças nas comunidades rizobianas de nódulos de feijão-mungo.

Apesar do feijão-mungo associar-se a diferentes gêneros rizobianos, testes de eficiência em vaso com solo e campo, em sua grande maioria, são realizados com estirpes de *Bradyrhizobium* (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; DELIĆ et al., 2011; TARIQ et al., 2012). O efeito de sua inoculação tem sido positivo em diversos países (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; DELIĆ et al., 2011; HAKIM et al., 2020; TARIQ et al., 2012), entretanto, não é incomum haver baixos incrementos na nodulação e no desenvolvimento do feijão-mungo (HERRIDGE et al., 2005; MATHU et al., 2012). Nesse sentido, revela-se a importância do estudo de genótipos de feijão-mungo mais eficientes quanto à fixação biológica de nitrogênio (FBN) e da seleção de estirpes de *Bradyrhizobium* mais competitivas e eficazes, de modo a aumentar os efeitos da inoculação.

A produção do feijão-mungo está concentrada nos países asiáticos, sobretudo na Índia, mas é cultivado em diversos países, incluindo outros continentes. No Brasil, o feijão-mungo vem sendo cultivado há décadas em baixa escala comercial, no entanto, nos últimos anos, seu cultivo se expandiu para áreas tecnificadas do Cerrado brasileiro. Esta expansão se deve à demanda do mercado internacional, e em grande parte por países asiáticos, sobretudo Índia e China. No Cerrado brasileiro, o cultivo de feijão-mungo tem sido cultivado após a soja, sendo uma opção de diversificação aos cultivos de milho, feijão-caupi e feijão-comum. O ciclo curto, adaptação ao estresse hídrico, baixo custo de produção e qualidade dos grãos são algumas das vantagens relacionadas ao seu cultivo nestas áreas.

Dada a expansão do cultivo de feijão-mungo e sua inserção nas áreas agrícolas do Cerrado brasileiro, verifica-se que são necessários estudos relacionados à cultura no Brasil.

Diversos estudos foram realizados para avaliação do desempenho agronômico de genótipos de feijão-mungo em condições brasileiras (DUQUE; PESSANHA, 1990; SAYÃO; BRIOSO; DUQUE, 1991; VIEIRA et al., 2011; VIEIRA; NISHIHARA, 1992; VIEIRA; OLIVEIRA; VIEIRA, 2003; VIEIRA; PINTO; VIANA, 2005), e inclui a recomendação de cultivares (VIEIRA et al., 2002, 2008). No entanto, estudos relacionados à FBN ainda são incipientes, e foram iniciados nos últimos anos, com o isolamento de microrganismos (SILVA et al., 2021) e testes de nodulação com estirpes de rizóbios recomendadas para outras leguminosas (SANTOS, 2020). Portanto, é necessário o isolamento e a caracterização das bactérias associadas ao feijão-mungo cultivado no Brasil, bem como seleção quanto à eficiência para inoculação.

Nesse sentido, objetivou-se com esta tese, avaliar a nodulação do feijão-mungo com rizóbios nativos de solos brasileiros, isolar os rizóbios associados e caracterizá-los, bem como avaliá-los quanto à eficiência simbiótica em condições axênicas e em vaso com solo. Para isso, o objetivo geral foi dividido nos seguintes objetivos específicos:

a) Avaliar a nodulação do feijão-mungo por rizóbios nativos de solos brasileiros, bem como a sua relação com o desenvolvimento das plantas;
b) Obter e caracterizar morfologicamente e geneticamente isolados bacterianos de nódulos de feijão-mungo cultivados em amostras de diferentes solos brasileiros;
c) Caracterizar os microbiomas dos nódulos de feijão-mungo cultivados em amostras de diferentes solos brasileiros, utilizando-se a técnica de sequenciamento por NGS (*Next-Generation Sequencing*) Illumina MiSeq a partir do gene 16S rRNA;
d) Avaliar a eficiência simbiótica dos isolados através da inoculação de plantas de feijão-mungo em condição axênica, bem como a sua influência no desenvolvimento das plantas;
e) Avaliar a eficiência simbiótica dos isolados comparados aos rizóbios nativos através da inoculação do feijão-mungo cultivado em vaso com solo, e seu efeito na contribuição da FBN e no desenvolvimento das plantas;
f) Avaliar o efeito da aplicação de doses de N no plantio associadas à inoculação sob a nodulação e o desenvolvimento do feijão-mungo cultivado em vaso com solo;
g) Avaliar a inoculação cruzada de estirpes de *Bradyrhizobium* utilizadas em inoculantes comerciais para soja e feijão-caupi no Brasil, e compará-las com isolados obtidos de nódulos de feijão-mungo.

Para o atendimento dos objetivos da tese e uma melhor organização dos ensaios experimentais, a tese foi dividida em quatro capítulos.

No Capítulo I (Characterization and nodulation capacity of native bacteria isolated from mung bean nodules used as a trap plant in Brazilian tropical soils) foi avaliada a nodulação de dois genótipos de feijão-mungo em dez amostras de solos brasileiros, e o efeito desta nodulação no desenvolvimento das plantas. A partir deste ensaio, foram isoladas e caracterizadas morfologicamente e geneticamente bactérias presentes nos nódulos, as quais foram avaliadas em condições axênicas quanto à capacidade de nodulação e o efeito no desenvolvimento das plantas de feijão-mungo.

No Capítulo II (*Bradyrhizobium* as the only rhizobial inhabitant of mung bean (*Vigna radiata*) nodules in tropical soils: a strategy based on microbiome for improving biological nitrogen fixation using bio-products) foi realizada a caracterização do microbioma de nódulos de feijão-mungo cultivados no Brasil. Para isso, foi realizado o sequenciamento de uma porção do gene 16S rRNA utilizando o método de NGS Illumina MiSeq a partir do DNA total extraído dos nódulos de dois genótipos de feijão-mungo cultivados em dez amostras de solos brasileiros.

No Capítulo III (*Bradyrhizobium* strains from Brazilian tropical soils promotes increases in nodulation, growth and nitrogen fixation in mung bean) foi avaliada a inoculação

de isolados de *Bradyrhizobium* em plantas de feijão-mungo cultivadas em vaso com solo, a fim de compará-los com a população de rizóbios nativos, incluindo a avaliação da contribuição da FBN. Além disso, foram testadas doses de N associadas à inoculação de *Bradyrhizobium*, visando maximizar o desenvolvimento do feijão-mungo inoculado.

No Capítulo IV (Cross-inoculation of elite commercial *Bradyrhizobium* strains from cowpea and soybean in mung bean and comparison with mung bean isolates) foi avaliada a inoculação do feijão-mungo com estirpes elite de *Bradyrhizobium* usadas em inoculantes comerciais para soja e feijão-caupi no Brasil, além da comparação com isolados obtidos de nódulos de feijão-mungo cultivado em solos brasileiros.

2 REVISÃO DE LITERATURA

2.1 Cultura do Feijão-Mungo

O feijão-mungo é uma planta herbácea, dicotiledônea, pertencente à ordem Fabales, família Leguminosae (Fabaceae), tribo Phaseoleae, gênero *Vigna*, subgênero *Ceratotropis*, espécie *radiata* e subespécie *radiata* [*Vigna radiata* (L.) Wilczek] (LAMBRIDES; GODWIN, 2007). No Brasil, é também denominado como feijão-mungo-verde, mungo-verde ou feijão-moyashi (VIEIRA; VIEIRA; VIEIRA, 2001). É uma leguminosa originária da Índia (FULLER, 2007; LAMBRIDES; GODWIN, 2007; ZUKOVSKIJ, 1962) e muito consumida em toda a Ásia. O continente asiático é um grande produtor de feijão-mungo, com aproximadamente 6 milhões de hectares, principalmente nas regiões Sul e Sudeste, e cada vez mais difundido para outros continentes (NAIR et al., 2012). A produtividade média de feijão-mungo na Ásia entre os anos de 1980-2000 foi de 689 kg ha^{-1} (LAMBRIDES; GODWIN, 2007; WEINBERGER, 2003).

O feijão-mungo tem seu cultivo voltado para a produção de grãos secos, que podem ser consumidos cozidos, na forma de farinhas e amidos, ou na forma de brotos, cujo mesmo é utilizado em um prato típico conhecido como moyashi (TANG et al., 2014; VIEIRA; VIEIRA; VIEIRA, 2001). Seus grãos têm alto valor nutricional e proteico (DU et al., 2018; YI-SHEN; SHUAI; FITZGERALD, 2018), tendo de 20 a 24% de proteína, e de 50 a 63% de carboidratos (LAMBRIDES; GODWIN, 2007; TANG et al., 2014). Seus grãos variam quanto ao tamanho e peso em função do genótipo da planta e das condições de cultivo, mas, geralmente, 100 sementes possuem massa inferior a 8 g (LAMBRIDES; GODWIN, 2007; VIEIRA et al., 2008; VIEIRA; OLIVEIRA; VIEIRA, 2003). Seu consumo está associado a diversos benefícios à saúde (HAI-JUN; JIA; YAO, 2012; HOU et al., 2019; KRUAWAN; TONGYONK; KANGSADALAMPAI, 2012; TANG et al., 2014), principalmente quando consumido na forma de broto, já que o processo de germinação melhora sua qualidade nutricional (EL-ADAWY et al., 2003).

O feijão-mungo é uma leguminosa anual, de porte ereto para a maioria das linhagens, e bem adaptada às condições de clima tropical e subtropical (DUQUE; PESSANHA, 1990), e sua altura varia de 0,3 a 1,5 m (VIEIRA; OLIVEIRA; VIEIRA, 2003). A cultura requer temperatura mínima média em torno de 20 a 22 °C, e ideal de 28 a 30 °C (VIEIRA; VIEIRA; VIEIRA, 2001). No Brasil, atingiu-se a produtividade de 2550 kg ha^{-1} com a cultivar MGS Esmeralda no período primavera-verão em Prudente de Morais-MG (VIEIRA et al., 2008). É uma cultura adaptada a diferentes condições ambientais, como baixa umidade do solo e temperaturas mais elevadas (HANUMANTHARAO; NAIR; NAYYAR, 2016; SHARMA et al., 2016).

Feijão-mungo é uma espécie de ciclo curto, com colheita entre 60 e 70 dias (NAIR et al., 2012; NALAMPANG, 1992). No Brasil, a floração pode iniciar entre 28 e 33 dias após a emergência e o aparecimento das primeiras vagens maduras entre 56 e 60 dias, e podem variar em função do genótipo da planta e das condições edafoclimáticas (VIEIRA et al., 2002, 2008). O feijão-mungo possui floração indeterminada, podendo durar até algumas semanas (NALAMPANG, 1992), o que resulta em baixa uniformidade na maturação dos grãos, sendo comumente necessário realizar mais de uma colheita (VIEIRA et al., 2002). Estudos de melhoramento genético quanto à homogeneidade da floração e ao uso de produtos dessecantes podem contribuir para a realização da colheita mecanizada, o que poderá resultar na redução de custos e aumento da qualidade dos grãos.

O feijão-mungo foi introduzido no Brasil há décadas, e têm sido cultivado em baixa escala comercial, como uma opção de comercialização no mercado local (BARRADAS; SAYÃO; DUQUE, 1989). Desde então, genótipos de feijão-mungo foram selecionados quanto à adaptação às condições brasileiras (DUQUE; PESSANHA, 1990; DUQUE; SOUTO;

ABBOUD, 1987; SAYÃO; BRIOSO; DUQUE, 1991; VIEIRA et al., 2002, 2008, 2011; VIEIRA; NISHIHARA, 1992; VIEIRA; OLIVEIRA; VIEIRA, 2003; VIEIRA; PINTO; VIANA, 2005; VIEIRA; VIEIRA; ANDRADE, 1992), tendo sido realizado o registro de cultivares (Cultivar Web, http://sistemas.agricultura.gov.br/snpc/cultivarweb/cultivares_registradas.php) (Tabela 1). A maioria das linhagens introduzidas no Brasil são oriundas do Asian Vegetable Research and Development Center (AVRDC), o qual possui um germoplasma de feijão-mungo com mais de 6700 acessos (SCHAFLEITNER et al., 2015).

Tabela 1. Genótipos de feijão-mungo registrado no Ministério da Agricultura, Pecuária e Abastecimento, conforme Cultivar Web*

Denominação	Mantenedor	Nº de registro	Data de Registro
BRS Esperança	Empresa Brasileira de Pesquisa Agropecuária - Embrapa	50575	27/04/2022
CF17M	Cerrado Futuro Biotech Ltda	50818	18/02/2022
IAC VR211	Instituto Agronômico de Campinas- IAC	49692	22/10/2021
VR 53**	Instituto Agronômico de Campinas- IAC	47860	27/05/2021
FMV65	Lodea Consultoria e Comercio de Sementes Ltda e APROFIR - Associação dos produtores de feijão, trigo e irrigantes de Mato Grosso	46001	19/11/2020
Camaleão	Empresa de Pesquisa Agropecuária de Minas Gerais - EPAMIG e Empresa Brasileira de Pesquisa Agropecuária - Embrapa	36829	13/11/2018
MGS Esmeralda	Empresa de Pesquisa Agropecuária de Minas Gerais - EPAMIG	22096	06/03/2008
Ouro Verde MG 2	Empresa de Pesquisa Agropecuária de Minas Gerais - EPAMIG	10390	02/08/2001
Moyashi	Agristar do Brasil Ltda	09765	21/03/2001
Ouro Verde	Empresa de Pesquisa Agropecuária de Minas Gerais - EPAMIG	06444	29/09/2000
Mungo	Agristar do Brasil Ltda	04956	07/06/2000

*Cultivar Web (http://sistemas.agricultura.gov.br/snpc/cultivarweb/cultivares_registradas.php): acesso em 17/08/2022. **Genótipo registrado como material experimental / pré-comercial.

O feijão-mungo tem sido considerado uma boa opção para o sistema produtivo do Cerrado brasileiro, dada seu baixo custo, boa adaptabilidade e produtividade. Seu ciclo curto e boa adaptação às condições climáticas permitem que seu plantio seja realizado em áreas sem irrigação, obtendo um rápido retorno do investimento realizado. É uma nova opção de cultura a ser plantada após a soja, principalmente em caso ter havido atrasos que tenham resultado no impedimento ao plantio do milho ou feijão-comum, dado o regime pluviométrico do Cerrado brasileiro. Como a demanda pelo mercado nacional é baixa, há uma tendência de que os plantios sejam feitos através de contratos futuros visando a exportação, objetivando diminuir riscos inerentes à comercialização.

2.2 Fixação Biológica de Nitrogênio

O nitrogênio é um elemento essencial às plantas, e requerido em grandes quantidades (MALAVOLTA, 1980). É abundante na atmosfera, ocupando cerca de 78% do volume do ar atmosférico, com predominância para a forma N_2 (N≡N) (STEVENSON; COLE, 1999). No solo, o nitrogênio está disponível em diversas formas, incluindo amônio, nitrato, aminoácidos, peptídeos e formas insolúveis (WILLIAMS; MILLER, 2001). As espécies vegetais assimilam o nitrogênio nas formas inorgânicas, como nitrato (NO_3^-) e amônio (NH_4^+), contudo, variam quanto à sua preferência (FERNANDES, 2006; WILLIAMS; MILLER, 2001). Apesar de sua abundância na atmosfera, o nitrogênio geralmente possui baixa concentração de formas

assimiláveis no solo, o que por sua vez, limita o crescimento vegetal em cultivos agrícolas (HUNGRIA; CAMPO; MENDES, 2007), sendo importante a introdução de N via fertilização (MALAVOLTA, 1980). A utilização de fertilizantes nitrogenados solúveis é considerada importante para o suprimento de nitrogênio na agricultura (JENKINSON, 2001; SMIL, 2004), no entanto, sua produção através do sistema Haber-Bosch possui custo elevado e acarreta a liberação de gazes de efeito estufa (CREWS; PEOPLES, 2004; RAZON, 2015).

A FBN é uma importante fonte de entrada de nitrogênio na natureza (CREWS; PEOPLES, 2004; HERRIDGE; PEOPLES; BODDEY, 2008). É um processo biológico realizado por alguns microrganismos procariotos, os quais possuem o complexo enzimático da nitrogenase (ANDERSON; RITTLE; PETERS, 2013; YANDULOV; SCHROCK, 2003). Este complexo possibilita que estes organismos sejam capazes de quebrar a tripla ligação do N_2 levando à produção de amônia (NH_3) (CASSINI; FRANCO, 2006; NEVES; RUMJANEK, 1997). A nitrogenase converte o N_2 à amônia utilizando energia na forma de adenosina trifosfato (ATP), sob elevado custo energético (TAIZ; ZEIGER, 2004). Em algumas plantas da família *Fabaceae*, a FBN ocorre em associação simbiótica no interior de estruturas especializadas formadas em suas raízes, denominadas de nódulos (MASSON-BOIVIN; SACHS, 2018; SPRENT, 2008). Nessa associação, a planta fornece carboidratos provenientes da fotossíntese e nutrientes necessários ao crescimento da bactéria, que por sua vez, disponibiliza N na forma de amônia (TAIZ; ZEIGER, 2004).

A contribuição da FBN para o suprimento de nitrogênio é variável em função da espécie vegetal e da cultivar utilizada, e dos microrganismos envolvidos (HERRIDGE; PEOPLES; BODDEY, 2008). A quantificação da contribuição da FBN pode ser feita através de diferentes metodologias, dentre elas, pelo aumento no acúmulo de nitrogênio na planta, atividade da redução de acetileno no sistema radicular (SCHÖLLHORN; BURRIS, 1967) e análise isotópica dos tecidos vegetais (BODDEY et al., 2000). A soja é um exemplo de espécie considerada eficiente na FBN, sendo capaz de obter até 95% do nitrogênio requerido para seu desenvolvimento (HERRIDGE; PEOPLES; BODDEY, 2008). No Brasil, dado o caso de sucesso na inoculação da cultura da soja, estima-se que a FBN em seu cultivo tenha gerado uma economia de US$ 25 bilhões para uma área de 25 milhões de hectares cultivados na safra 2012/2013 (HUNGRIA; MENDES, 2015), além dos benefícios ambientais advindos da redução do uso de fertilizantes nitrogenados.

Ao visar a sustentabilidade dos sistemas agrícolas, as práticas que promovem e maximizam a FBN são consideradas importantes ecologicamente (CREWS; PEOPLES, 2004). O fornecimento de nitrogênio para as lavouras via fixação biológica diminui ou mesmo dispensa o uso de fertilizantes nitrogenados no cultivo de diversas leguminosas de interesse agrícola (HUNGRIA; NOGUEIRA; ARAUJO, 2015; SILVA JÚNIOR et al., 2018). Nesse sentido, é importante que as culturas agrícolas, a exemplo do feijão-mungo, sejam estudadas visando otimizar a FBN, como forma de reduzir a aplicação de fertilizantes nitrogenados.

O feijão-mungo é capaz de fixar o nitrogênio atmosférico através da associação com rizóbios nativos do solo (HAYAT et al., 2008; HERRIDGE et al., 2005). A contribuição da FBN para o seu desenvolvimento tem sido muito variável, com valores entre 9 e 78% em estudos conduzidos na Oceania e na América do Norte, cujas avaliações resultaram em valores médios de aproximadamente 30% (DIATTA et al., 2020; HERRIDGE et al., 2005). No Brasil, estudos recentes verificaram uma contribuição um pouco maior, com valores que variaram entre 35 e 66%, tendo havido um incremento de até 90% no nitrogênio acumulado derivado da FBN em função da inoculação com rizóbio (SANTOS, 2020). Nesse sentido, verifica-se a necessidade de intensificar os estudos relacionados à inoculação de rizóbios em feijão-mungo no Brasil, visando otimizar a FBN.

2.3 Bactérias Presentes em Nódulos de Feijão-Mungo

A eficiência da FBN está diretamente relacionada aos microrganismos associados e à planta hospedeira, e a relação simbiótica entre ambos, definida como especificidade (XAVIER et al., 2006). O feijão-mungo é considerado um hospedeiro de baixa especificidade simbiótica, dada a capacidade de associação com rizóbios de várias espécies de diferentes gêneros rizobianos (YANG et al., 2008; ZHANG et al., 2008). Em estudos conduzidos na Ásia, bactérias dos gêneros *Bradyrhizobium* (APPUNU et al., 2009; RISAL et al., 2012; YANG et al., 2008; ZHANG et al., 2008), *Ensifer* (PANDYA; KUMAR; RAJKUMAR, 2013; YANG et al., 2008; ZHANG et al., 2008), *Rhizobium* (YANG et al., 2008; ZHANG et al., 2008) e *Mesorhizobium* (YANG et al., 2008) isoladas de nódulos de feijão-mungo foram capazes de induzir a nodulação. Destes gêneros, o *Bradyrhizobium* é comumente o mais encontrado, e portanto, é considerado o simbionte preferido na associação com feijão-mungo (YANG et al., 2008; ZHANG et al., 2008).

Estudos utilizando técnicas independentes de cultivo mostram diferentes cenários para a diversidade simbiótica de feijão-mungo (HAKIM et al., 2018, 2020). Na Ásia, mostrou-se que pode ocorrer a codominância ou a dominância dos gêneros *Bradyrhizobium* e *Ensifer* em nódulos de feijão-mungo, a depender do solo de cultivo (HAKIM et al., 2018, 2020), e chegaram a representar até 94 e 99,9% do total de sequências recuperadas (HAKIM et al., 2020). Estas diferenças no microbioma de nódulos de feijão-mungo mostram que as características do solo de cultivo influenciam diretamente na relação planta-rizóbio. Dentre estas características, verificou-se uma relação positiva entre o pH do solo e a abundância de *Ensifer* em nódulos de feijão-mungo (HAKIM et al., 2020).

Como dito anteriormente, bactérias dos gêneros *Rhizobium* e *Mesorhizobium* são comumente isoladas de nódulos de feijão-mungo, e são capazes de induzir a nodulação (YANG et al., 2008; ZHANG et al., 2008). No entanto, em estudos usando técnicas independentes de cultivo, *Rhizobium* e *Mesorhizobium* corresponderam a apenas 2,06 e 0,06% do total de sequências recuperadas de nódulos de feijão-mungo, respectivamente, enquanto *Bradyrhizobium* e *Ensifer* corresponderam a 32,05 e 35,84% (HAKIM et al., 2018). Em outro estudo, não foram recuperadas sequências de *Mesorhizobium*, e as de *Rhizobium* estavam em baixa abundância, enquanto a soma de *Bradyrhizobium* e *Ensifer* corresponderam a no mínimo 94% do total de sequências recuperadas dos nódulos de feijão-mungo (HAKIM et al., 2020). Estes resultados sugerem que *Bradyrhizobium* e *Ensifer* parecem ser simbiontes mais abundantes em nódulos de feijão-mungo, a depender do solo de cultivo, e que *Rhizobium* e *Mesorhizobium* geralmente estão em baixa proporção ou as vezes não estão presentes.

Até pouco tempo, acreditava-se que os nódulos de leguminosas eram habitados apenas por rizóbios, variando de acordo com a característica da planta quanto à especificidade simbiótica. As comunidades microbianas de nódulos de leguminosas não são formadas apenas por rizóbios, e vários estudos já mostraram que diversos gêneros de bactérias não-rizobianas habitam os nódulos e formam complexas comunidades microbianas (ASERSE et al., 2013; CARDOSO et al., 2018; DE MEYER et al., 2015; LEITE et al., 2017; MARTÍNEZ-HIDALGO; HIRSCH, 2017; TRABELSI; CHIHAOUI; MHAMDI, 2017; ZHANG et al., 2018). Com feijão-mungo não é diferente, e portanto, sequências de diferentes gêneros de bactérias não-rizobianas já foram recuperadas de seus nódulos (HAKIM et al., 2018, 2020).

A estrutura da comunidade microbiana dos nódulos de leguminosas e o papel da maioria das bactérias não-rizobianas na relação simbiótica planta-rizóbio ainda são pouco compreendidos. A avaliação da composição dessas comunidades dentro do nódulo é uma ferramenta interessante, no sentido que permite selecionar microrganismos que podem melhorar o crescimento das plantas. Além disso, esse conhecimento pode auxiliar no desenvolvimento de produtos biológicos com múltiplos organismos, com o objetivo de

aumentar ainda mais os benefícios da inoculação, seja pela promoção da FBN (ANDREWS; ANDREWS, 2017; DE MEYER et al., 2015), atividade de biocontrole (BERG et al., 2017), promoção de crescimento (TARIQ et al., 2014), entre outros.

Dada a elevada contribuição da FBN para a cultura do feijão-mungo e a importância da maximização desse processo em sistemas agrícolas, é fundamental o isolamento e a caracterização das bactérias associadas aos nódulos de feijão-mungo cultivado em solos brasileiros. Estes são os primeiros passos para seleção de bactérias promissoras para a inoculação de feijão-mungo no Brasil.

2.4 Inoculação de Feijão-Mungo no Brasil

A agricultura brasileira é baseada em uma elevada diversidade de espécies vegetais, incluindo leguminosas de grãos, forrageiras, de adubação verde e arbóreas. Dada a elevada importância das leguminosas, estudos já foram realizados quanto à diversidade de bactérias associadas aos seus nódulos, com uma ampla lista de microrganismos autorizados junto ao Ministério da Agricultura, Pecuária e Abastecimento (MAPA) para o uso em inoculantes comerciais (BRASIL, 2011).

Para o feijão-mungo, os estudos dos rizóbios associados aos seus nódulos ainda são incipientes no Brasil. A partir da informação de que estirpes de *Bradyrhizobium*, *Ensifer*, *Rhizobium* e *Mesorhizobium* são capazes de nodular o feijão-mungo (YANG et al., 2008; ZHANG et al., 2008), foram realizados testes de inoculação cruzada com estirpes elite usadas em inoculantes comerciais no Brasil quanto ao potencial para inoculação do feijão-mungo. Esta estratégia visa facilitar o processo de recomendação de uma estirpe para uso em inoculante comercial para o feijão-mungo. Nesse sentido, foi realizado um estudo para avaliação de estirpes de *Bradyrhizobium* spp. usadas em inoculantes comerciais para soja e feijão-caupi, e de *Rhizobium* spp. para o feijão-comum quanto à capacidade de nodulação no feijão-mungo cultivar Camaleão (SANTOS, 2020). Os resultados mostraram que as estirpes de *Bradyrhizobium* spp. SEMIA 587 autorizada para a soja, e UFLA 3-84 e INPA 3-11B autorizadas para feijão-caupi foram capazes de nodular o feijão-mungo, e resultaram em boa nodulação (média de 49 e 72 nódulos por planta). Ao contrário, as estirpes BR 29, BR 3267 e BR 3262 de *Bradyrhizobium* spp. resultaram em uma nodulação ineficiente (média de 1 a 3 nódulos por planta); já as estirpes CPAC 15 e CPAC 7 de *Bradyrhizobium* spp., e as estirpes CIAT 899, PRF 81 e H 12 de *Rhizobium* spp. não foram capazes de nodular o feijão-mungo.

A capacidade de nodulação do feijão-mungo com rizóbios isolados de outras leguminosas já foi avaliada anteriormente, e não é incomum que algumas estirpes de *Bradyrhizobium* possuam baixa eficiência na nodulação ou mesmo não consigam nodular o feijão-mungo (NGUYEN et al., 2017; WU et al., 2020). Em alguns casos, genes que codificam proteínas de secreção do Tipo III (T3SS) limitaram esta associação simbiótica (NGUYEN et al., 2017; OKAZAKI et al., 2009; PIROMYOU et al., 2019; SONGWATTANA et al., 2017), entretanto, depende do genótipo da planta (OKAZAKI et al., 2009; PIROMYOU et al., 2019). Nesse sentido, é possível que a ineficiência ou a incapacidade de nodulação do feijão-mungo por algumas estirpes elites de *Bradyrhizobium* usadas em soja e feijão-caupi (SANTOS, 2020) esteja relacionada a estas características gênicas, podendo haver ainda diferenças caso sejam utilizados outros cultivares de feijão-mungo. A partir disso, é importante que sejam realizados estudos da capacidade de nodulação destas estirpes com outros genótipos de feijão-mungo, incluindo ainda a comparação da eficiência com rizóbios isolados de seus nódulos.

3 CAPÍTULO I

CHARACTERIZATION AND NODULATION CAPACITY OF NATIVE BACTERIA ISOLATED FROM MUNG BEAN NODULES USED AS A TRAP PLANT IN BRAZILIAN TROPICAL SOILS

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3.1 RESUMO

O feijão-mungo [*Vigna radiata* (L.) Wilczek] é uma leguminosa de origem asiática com grande importância mundial, principalmente em países em desenvolvimento. É conhecido por sua associação promíscua com rizóbios do solo, que fornecem nitrogênio por meio da fixação biológica de nitrogênio (FBN). Na perspectiva de aumentar a produtividade, esforços estão em andamento para identificar bactérias que formem associações simbióticas mais eficientes com o feijão-mungo. Nesse sentido, avaliou-se a nodulação de duas cultivares de feijão-mungo com rizóbios nativos de 10 amostras de solos tropicais brasileiros e realizamos o isolamento de cepas bacterianas presentes nos nódulos. Esses isolados foram caracterizados morfologicamente e agrupados com base no sequenciamento do gene 16S rRNA. Os isolados também foram avaliados quanto à capacidade de nodulação e eficiência da FBN em feijão-mungo. Observou-se diferenças no desenvolvimento da planta de feijão-mungo dependendo do solo, para ambas cultivares. Em geral, plantas cultivadas em amostras de solo da região da Mata Atlântica apresentaram maior nodulação e massa seca da parte aérea em relação às amostras do Cerrado brasileiro (savana tropical). A partir dos nódulos, foram isoladas 101 cepas bacterianas, que foram classificadas de acordo com o gene 16S rRNA em nove gêneros: *Bradyrhizobium* (66), *Rhizobium* (19), *Mesorhizobium* (4), *Ensifer* (3), *Leifsonia* (3), *Bacillus* (3), *Agrobacterium* (1), *Mycolicibacterium* (1) e *Kaistia* (1). *Bradyrhizobium* foi o gênero mais abundante, e seus isolados formaram 12 grupos filogenéticos. Avaliações de inoculação revelaram que apenas isolados de *Bradyrhizobium* induziram a formação de nódulos em feijão-mungo, com a eficiência da FBN dos isolados diferindo em função do grupo filogenético e solo de origem. Plantas inoculadas com isolados agrupados com *Bradyrhizobium yuanmingense* B071 apresentaram maiores incrementos na massa seca de parte aérea. Considerando a origem do solo, observou-se que plantas inoculadas com *Bradyrhizobium* isoladas de amostras de solo do estado de Mato Grosso apresentaram menores incrementos na nodulação e massa seca de parte aérea. Esses resultados sugerem que o *Bradyrhizobium* é o simbionte predominante nos nódulos do feijão-mungo cultivado em solos tropicais brasileiros, e seus isolados apresentam potencial para inoculação do feijão-mungo, principalmente quando cultivados em solos que apresentam baixa nodulação por rizóbios nativos.

Palavras-chave: Feijão-mungo. Rizóbios nativos do solo. Isolamento. 16S rRNA. *Bradyrhizobium*. Inoculação.

3.2 ABSTRACT

Mung bean [*Vigna radiata* (L.) Wilczek] is a legume of Asian origin with great importance worldwide, particularly in developing countries. It is known for its promiscuous association with soil rhizobia, which provides nitrogen via biological nitrogen fixation (BNF). From the prospective of increasing productivity, efforts are ongoing to identify bacteria that form more efficient symbiotic associations with mung bean. In this regard, we evaluated the nodulation of two mung bean cultivars with native rhizobia from 10 samples of tropical Brazilian soil and performed the isolation of bacterial strains present in the nodules. These isolates were characterized morphologically, and grouped based on sequencing of the 16S rRNA gene. The isolates were also evaluated for nodulation capacity in mung bean and BNF efficiency. We found differences in mung bean plant development depending on the soil for both cultivars. In general, the plants cultivated in soil samples from the Atlantic Forest region showed greater nodulation and shoot dry weight compared to the Brazilian Cerrado (tropical savanna). On the basis of an evaluation of nodulation by native rhizobia, we isolated 101 bacterial strains, which were classified according to 16S rRNA gene into nine genera: *Bradyrhizobium* (66), *Rhizobium* (19), *Mesorhizobium* (4), *Ensifer* (3), *Leifsonia* (3), *Bacillus* (3), *Agrobacterium* (1), *Mycolicibacterium* (1), and *Kaistia* (1). *Bradyrhizobium* was the most abundant genus, and its isolates formed 12 phylogenetic groups. Inoculation assessments revealed that only *Bradyrhizobium* isolates induced nodule formation in mung bean, with the BNF efficiency of isolates differing depending on phylogenetic group and soil of origin. Plants inoculated with isolates grouped with *Bradyrhizobium yuanmingense* B071 were found to show greater increases in shoot dry weight. Considering the soil origin, we observed that plants inoculated with *Bradyrhizobium* isolated from Mato Grosso state soil samples showed smaller increments in nodulation and shoot dry weight. These results suggest that the *Bradyrhizobium* is the predominant symbiont in the nodules of mung bean grown in Brazilian tropical soils, and its isolates have the potential for mung bean inoculation, especially when grown in soils that provide low nodulation by native rhizobia.

Keywords: Mung bean. Native soil rhizobia. Isolation. 16S rRNA. *Bradyrhizobium*. Inoculation.

3.3 INTRODUCTION

Grain legumes are an important foods worldwide, particularly in tropical developing countries (BRUINSMA, 2009). Among the pulses, mung bean [*Vigna radiata* (L.) Wilczek] is widely grown in Asia, and is noted for its short cycle (NAIR et al., 2012), good adaptation to high temperatures and water stress (HANUMANTHARAO; NAIR; NAYYAR, 2016; SHARMA et al., 2016), and production of grains with good nutritional characteristics (DU et al., 2018; YI-SHEN; SHUAI; FITZGERALD, 2018).

Mung bean has the capacity to fix atmospheric nitrogen via its association with the rhizobia (HAYAT et al., 2008; HERRIDGE et al., 2005), which can possibly increase the grain. However, the contribution of biological nitrogen fixation (BNF) to the development of mung bean has been found to show considerable variability, with values ranging between 17 and 80% (HAYAT et al., 2008), with an average of approximately 63% (HERRIDGE; PEOPLES; BODDEY, 2008).

Notably, mung bean is characterized by low symbiotic specificity, being able to associate symbiotically with bacteria of different genera (YANG et al., 2008; ZHANG et al., 2008). Studies conducted in Asia using the isolation technique show that bacteria in the genera *Bradyrhizobium* (APPUNU et al., 2009; RISAL et al., 2012; YANG et al., 2008; ZHANG et al., 2008), *Ensifer* (PANDYA; KUMAR; RAJKUMAR, 2013; YANG et al., 2008; ZHANG et al., 2008), *Rhizobium* (YANG et al., 2008; ZHANG et al., 2008), and *Mesorhizobium* (YANG et al., 2008) can induce nodule formation in mung bean. The genus *Bradyrhizobium* have in some cases been shown to be the most abundant symbiont (YANG et al., 2008; ZHANG et al., 2008), however, recent studies have revealed that there may be codominance or dominance of the *Bradyrhizobium* and *Ensifer* genera depending on the soil (HAKIM et al., 2018, 2020). The *Ensifer* genus can represent 4 to 99% of the rhizobial sequences in mung bean nodules analyzed using sequencing techniques (HAKIM et al., 2020).

Non-rhizobial bacteria (NRB) have already been identified in the nodules of several legume species (DE MEYER et al., 2015; LEITE et al., 2017; MARTÍNEZ-HIDALGO; HIRSCH, 2017; ZHANG et al., 2018), including mung bean (HAKIM et al., 2018, 2020). Although these bacteria do not induce nodule formation, there is a consensus that they can co-inhabit nodules (PANDYA; KUMAR; RAJKUMAR, 2013; ZGADZAJ et al., 2015), and some studies have already reported the beneficial effects of NRBs with respect to plant growth promotion (RÍOS-RUIZ et al., 2019; VERMA; MISHRA; ARORA, 2019; VYAS, 2018). Some NRB strains have already been isolated from mung bean nodules, such as *Bacillus* and *Agrobacterium* genera (TARIQ et al., 2012), even though their role, if any, in the symbiotic plant/rhizobia relationship is still poorly understood.

Recently, mung bean crop production has expanded in Brazil, mainly in the Cerrado (tropical savanna) areas, with the aim to export the grain to other countries. To date, however, associated studies have focused primarily on mung bean genotype selection (DUQUE; PESSANHA, 1990; VIEIRA et al., 2002, 2008), and consequently, little information has accumulated with respect to nodulation and associations with soil bacteria. Accordingly, in this study, we sought to investigate mung bean nodulation by native rhizobia derived from different tropical soils in Brazil, to isolate and characterize bacterial strains associated with nodules, and to evaluate their ability to induce nodulation in mung bean.

3.4 MATERIAL AND METHODS

3.4.1 Mung bean nodulation in tropical soils

Mung bean plants were grown in modified Leonard jars (VINCENT, 1970), using soil samples as inoculant, with methodology similar to that of Marra et al. (2012). An experiment was carried out in a randomized block design with seven replications, in a 2 × 10 factorial scheme (genotype × soil). 'MGS Esmeralda' (VIEIRA et al., 2008) and Camaleão cultivars were grown using 10 soil samples. The soil samples were collected in the Cerrado and Atlantic Forest regions from areas where mung bean and/or other legumes have been cultivated (Table 2). At each site, ten simple subsamples of soil were collected from depths between 0 and 20 cm. These were homogenized and sieved through a 4 mm mesh to obtain composite samples. After collection in the field, the soil samples MW_MT-I, MW_MT-II, MW_MT-III, SE_MG-I, and SE_MG-II were transported in thermally insulated boxes, and subsequently stored under refrigeration at approximately 22 °C. The remaining samples were collected and stored under refrigeration on the same day. For all samples, the interval between collection and sowing mung bean seeds was less than eight days. The three collection areas in the Midwest Region of the Brazilian Cerrado (MW_MT-I, MW_MT-II, and MW_MT-III) were under intensive grain cultivation, such as a soybean–corn–common bean. The Southeast (SE_RJ-I) area of the Atlantic Forest region is cultivated based on a conventional family farming system; SE_RJ-II, SE_RJ-III, and SE_RJ-V were experimental fields under organic farming systems; and SE_MG-I, SE_MG-II, and SE_RJ-IV were areas of conventional cultivation.

Leonard jars were filled with approximately 0.6 L of substrate composed of gravel and vermiculite (2:1 v v^{-1}), and then sterilized in autoclave. The seeds used were superficially disinfested by immersion in 70% ethanol and hydrogen peroxide (35%) for one and three min, respectively, followed by ten washes in sterile distilled water (VINCENT, 1970). A layer of soil sample (0.1 L) was added over the substrate already sterilized on each jar (Appendix 1), and five surface-sterilized seeds were sowed on this layer. After thinning, two plants remained, and a layer of sterile sand was added over the soil sample. Norris' nutrient solution (NORRIS; DATE, 1976) devoid of N and sterilized in an autoclave was applied, with a weekly 300 mL replacement for each pot. In the first week, a nutrient solution with half the ionic strength was used in order to avoid possible salt stress in the initial plant development. Plants were collected 35 days after emergence, and the roots were separated from the aerial parts at the point of cotyledon insertion, and nodules were detached from the roots to obtain the number and determination of fresh weights. Fresh nodules were used to isolate bacterial strains. The shoots were dried to a constant weight at 65 °C.

Mung bean biomass data were subjected to a two-way analysis of variance (plant genotype and soil) and the averages were compared using the Scott–Knott test at 5% probability. Data analyses were conducted using the ExpDes.pt package (v. 1.2.0) (FERREIRA et al., 2013), and the plotting using ggplot2 (v. 3.3.0) (WICKHAM, 2016) and cowplot (v. 1.0.0) (WILKE, 2016), both in the R environment (v. 4.0.2) (R CORE TEAM, 2020). The number and fresh weight of the nodules were transformed using $(x)^{0.5}$. Correlations between the chemical fertility of the soil sample and the shoot dry weight of mung bean and nodulation by native rhizobia were verified based on principal component analysis using the factoextra package (v. 1.0.7) (KASSAMBARA; MUNDT, 2020) in the R environment (v. 4.0.2) (R CORE TEAM, 2020).

Table 2. Identification, location, and fertility analysis of soil material samples used for planting mung bean in Leonard jars

Soil sample†	Latitude and longitude	‡pH	Al^{3+}	Ca^{2+}	Mg^{2+}	H+Al	P	K^{+}	C
			-------- $cmol_c\ dm^{-3}$ --------				---- $mg\ L^{-1}$ ----		%
MW_MT-I	15°14'05.8"S 53°58'51.1"W	6.45	0.00	3.50	1.56	3.48	104.37	136.98	1.35
MW_MT-II	15°13'37.9"S 53°58'48.1"W	5.55	0.00	2.95	0.92	5.97	115.33	270.40	1.40
MW_MT-III	15°23'33.5"S 54°26'46.7"W	4.30	0.34	2.88	0.77	11.09	214.78	190.34	1.85
SE_MG-I	20°24'07.57"S 42°49'05.08"W	4.61	0.33	1.24	0.36	5.97	74.47	149.39	0.70
SE_MG-II	21°14'36.74"S 43° 9'30.55"W	5.98	0.00	4.04	1.03	4.65	71.09	216.39	1.31
SE_RJ-I	22°38'4.61"S 42°48'40.40"W	4.44	2.35	1.29	0.39	14.98	138.17	49.00	3.64
SE_RJ-II	22°20'52.36"S 43°25'2.24"W	5.74	0.00	1.70	0.24	3.17	92.15	80.42	0.44
SE_RJ-III	22°20'54.84"S 43°25'2.27"W	5.91	0.00	3.32	1.26	4.32	161.48	224.70	1.27
SE_RJ-IV	22°45'22.27"S 43°40'2.03"W	5.73	0.00	1.90	0.49	3.66	24.03	85.89	0.68
SE_RJ-V	22°45'16.36"S 43°40'28.04"W	6.51	0.00	3.40	0.73	2.84	155.59	139.96	0.86

† Soil origin: SE = Southeast, MW = Midwest, RJ = Rio de Janeiro, MG = Minas Gerais and MT = Mato Grosso
‡Measured in water. Analysis methods: pH = Potentiometric, Aluminum = Titration, Ca = Atomic absorption, Mg = Atomic absorption, H+Al = Titration, P = Colorimetric, K = Flame photometry, Carbon = Walkey & Black.

3.4.2 Isolation of bacterial strains from nodules, morphological characterization, and sequencing of the 16S rRNA gene

For the purposes of isolating bacteria from mung bean nodules, we used four replicate plants, from each one of which, five nodules were randomly selected, with a total of 400 nodules. The nodules were superficially disinfested by immersion in 70% ethanol and sodium hypochlorite (4-6%) for one and five min, respectively, followed by ten washes in sterile distilled water. The disinfested nodules were crushed in a Petri dish containing yeast mannitol agar (YMA) culture medium (FRED; WAKSMAN, 1928) with bromothymol blue pH indicator. After purification, the growth time of the isolates was defined as that when the first isolated colony appeared. Morphological characterization of the isolates was performed after an interval twice that of the initial growth time, based on the traits mentioned by Hungria et al. (2001) and Martins (1996). The isolates were preserved in the ultra-freezer (-80 °C) using YM medium (FRED; WAKSMAN, 1928) with glycerol (30% v v^{-1}).

16S rRNA gene amplification was performed using bacterial cells suspended in DNA-free water as template (2.0 μL of suspension with optical density of approximately 2.0 at 600 nm), using a Gotaq DNA polymerase kit (Promega, U.S), and the primers 27F and 1492R (FURUSHITA et al., 2003). The PCR products were sequenced using the same two primers and a Big Dye™ Terminator Cycle Sequencing Ready Reaction v. 3.1 kit (Applied Biosystems) in an automatic DNA sequencer (ABI 3500; Applied Biosystems), according to the manufacturer's recommendations. The sequences were analyzed and assembled using BioNumerics 7.5 (Applied Maths, Belgium) and verified via Basic Local Alignment Search Tool (BLAST) analyses (ALTSCHUL et al., 1997). The sequences have been deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers shown in Table S1. Alignment and phylogenetic analysis were performed using MEGA 7

software (KUMAR; STECHER; TAMURA, 2016). Phylogenetic trees were constructed based on the maximum likelihood (ML) method using the Tamura 3-parameter + G model (TAMURA, 1992). This model was selected as the best model tool available in MEGA 7. Bootstrap values are shown when the relationships represented were observed in at least 50% of the 500 replicates.

3.4.3 Nodulation test by non-*Bradyrhizobium* isolates

Plants of the 'MGS Esmeralda' mung bean cultivar (VIEIRA et al., 2008) were grown in bottles (VINCENT, 1970) in a greenhouse to evaluate the nodule formation capacity of 35 isolates belonging to the genera *Rhizobium*, *Mesorhizobium*, *Ensifer*, *Leifsonia*, *Bacillus*, *Kaistia*, *Mycolicibacterium*, and *Agrobacterium*. In addition to the treatments inoculated with these isolates, absolute (without inoculation or nitrogen application) and positive (inoculation with a nodulating *Bradyrhizobium* BR 14533 isolate, which had already been identified as a symbiont of mung bean in our previous studies) controls were assessed. Assessment were carried out based on a completely randomized design with each treatment comprising three replicates.

The seeds were superficially disinfested as previously described, pre-germinated for three days in a Petri dish with filter paper moistened with distilled water, and transplanted into long-neck jars containing 250 mL of Norris' nutrient solution (NORRIS; DATE, 1976) devoid of N and sterilized in an autoclave.

For inoculum production, the isolates were grown in YM culture medium (FRED; WAKSMAN, 1928), with agitation of 150 rpm at 28 ºC for three days, and showed an optical density at 600 nm ($O.D._{600}$) greater than 1.0, suggesting at least 10^8 cell mL^{-1}. Two days after transplanting, the seedlings were inoculated with 1.0 mL aliquots of each isolate inoculum. The plants were grown for 30 days after transplanting to check for the presence or absence of nodules.

3.4.4 Nodulation test by *Bradyrhizobium* isolates

Sixty-six *Bradyrhizobium* isolates were used to inoculate plants of the 'MGS Esmeralda' mung bean cultivar (VIEIRA et al., 2008), grown in Leonard jars (VINCENT, 1970) in a greenhouse. In addition to the inoculation treatments using isolates, absolute (without inoculation or nitrogen application) and nitrogen controls (without inoculation and with nitrogen application) were assessed. A randomized block design with four replications was adopted. Leonard jars were filled with gravel and vermiculite (2:1 v v^{-1}) and sterilized in an autoclave. For the production of inoculum, the isolates were grown in YM culture medium (FRED; WAKSMAN, 1928) for six days at 28 °C with agitation at 150 rpm, after which time, all liquid cultures had an optical density at 600 nm ($O.D._{600}$) greater than 0.8, indicating a concentration of at least 10^8 cell mL^{-1}. Prior to planting, mung bean seeds were superficially disinfested as previously described. Five seeds were sown in each jar, and an inoculum aliquot (0.250 mL) was used to inoculate each seed at the time of sowing. After emergence, thinning was performed, and two plants per jar remained. Nutritive solution was applied as described in section 3.4.1. For the nitrogen control treatment, 60, 120, 120, 180, and 180 mg of ammonium nitrate per plant was applied at weeks 1, 2, 3, 4 and 5, respectively, based on a N dosage of 80 kg ha^{-1} and in a final stand of 350.000 plants ha^{-1}. The plants were collected using the same method as described in section 3.4.1.

The data obtained were subjected to an analysis of variance, with means being compared using the Scott–Knott test at 5% probability. For data analysis, we used the ExpDes.pt package (v. 1.2.0) (FERREIRA et al., 2013), and the ggplot2 (v. 3.3.0) (WICKHAM, 2016) and cowplot

(v. 1.0.0) (WILKE, 2016) for plotting in the R environment (v. 4.0.2) (R CORE TEAM, 2020). The nodule count data were transformed using $(x + 1)^{0.5}$, whereas the root and shoot dry weight data were transformed using $(x)^{0.5}$. Comparisons between phylogenetic groups and soils of origin with respect to isolate efficiency based on shoot dry weight were performed with a permutational multivariate analysis of variance (PERMANOVA) using the "adonis" function of the vegan package (v. 2.5-6) (OKSANEN et al., 2019), the Euclidean similarity index with 999 permutations, and by paired comparisons using the "adonis.pair" function of the EcolUtils R package (SALAZAR, 2018), with correction using the false discovery rate method (BENJAMINI; HOCHBERG, 1995). Analyses were conducted in the R environment (v. 3.5.3) (R CORE TEAM, 2019). Correlations between nodulation and the shoot dry weight of mung bean by native rhizobia, and of mung bean inoculated with *Bradyrhizobium* isolates, and the chemical fertility data of soil samples were verified based on principal component analysis, as described in section 3.4.1.

3.5 RESULTS

3.5.1 Mung bean nodulation by native rhizobia

We found that the nodulation of mung bean by native rhizobia isolated from 10 Brazilian soils differed depending on the locale of soil collection (Figure 1a, b and c). In terms of nodule numbers, we detected no significant interaction between the soil of origin and plant genotype ($p = 0.13$), nor any differences between the two genotypes ($p = 0.06$) (Figure 1b). Mung bean plants grown in the SE_RJ-V soil had the highest number of nodules (Figure 1a), and plants grown in Mato Grosso state soils tended to produce a lower number of nodules compared with those grown in Southeast region soils. On average, plants grown in Mato Grosso soils and SE_RJ-V soils were characterized by development of 7 and 64 nodules per plant, respectively, which represents an 800% difference.

With respect to nodule fresh weight (Figure 1c), we found a significant interaction between the soil of origin and plant genotype ($p < 0.01$). The nodules of plants grown in all Southeast region soils were found to have higher average nodule weights compared with those grown in Mato Grosso state soils, regardless of mung bean genotype. The only exception in this latter regard being the difference between plants grown in MW_MT-III soil, in which the cultivar 'Esmeralda' showed a higher average nodule weight. With regards to shoot dry weight (Figure 1d), there was significant interaction between the factors ($p \leq 0.05$). The SE_MG-II and SE_RJ-V soils presented higher averages, independent of genotype. Mato Grosso soils (MW_MT-I, MW_MT-II and MW_MT-III) and SE_RJ-I soil had the lowest averages for the 'Camaleão' cultivar; for 'Esmeralda', the MW_MT-I, MW_MT-II, and SE_RJ-I soils presented lower averages.

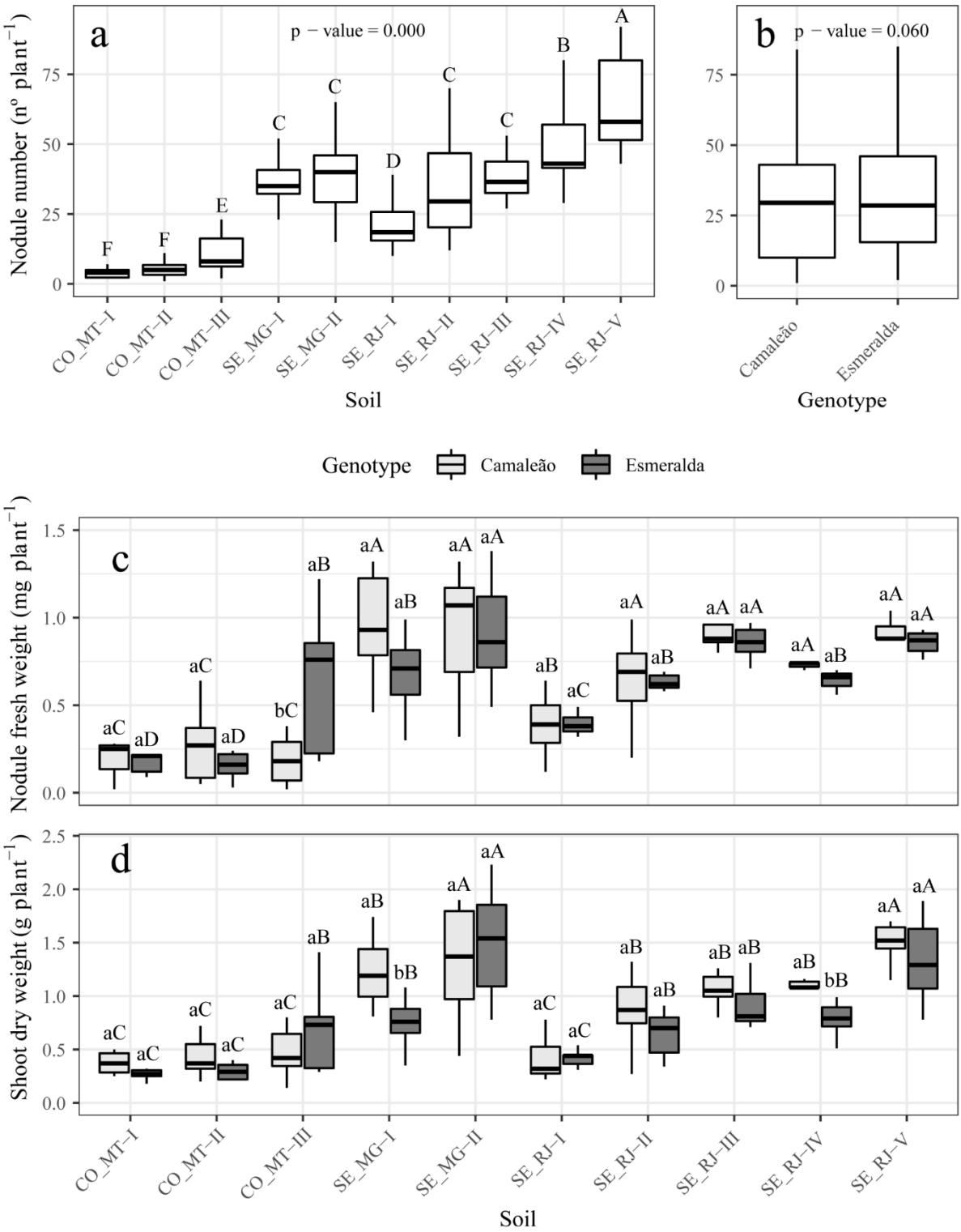


Figure 1. Boxplot of nodule number (nº plant^{-1}) for soil (a) and genotype (b), fresh nodule weight (mg plant^{-1}) (c) and shoot dry weight (g plant^{-1}) (d) of mung bean plants grown in Leonard jars using soil material as an inoculum. Plants collected 35 days after emergence. Distinctive letters, lowercase between genotypes and uppercase between soils, indicate statistical difference by the Scott–Knott test at 5% probability.

The results of principal component analysis indicated that pH, calcium, and magnesium contents of the soil samples showed positive correlations with the nodulation and shoot dry weight of mung bean grown in Leonard jars (Figure 2). However, this does not fully explain, since the MW_MT-I sample has a high pH, but resulted in low nodulation and shoot dry weight. Accordingly, it can be assumed that in addition to the aforementioned variables, other factors intrinsic to the soil may be associated with the observed differences in mung bean development.

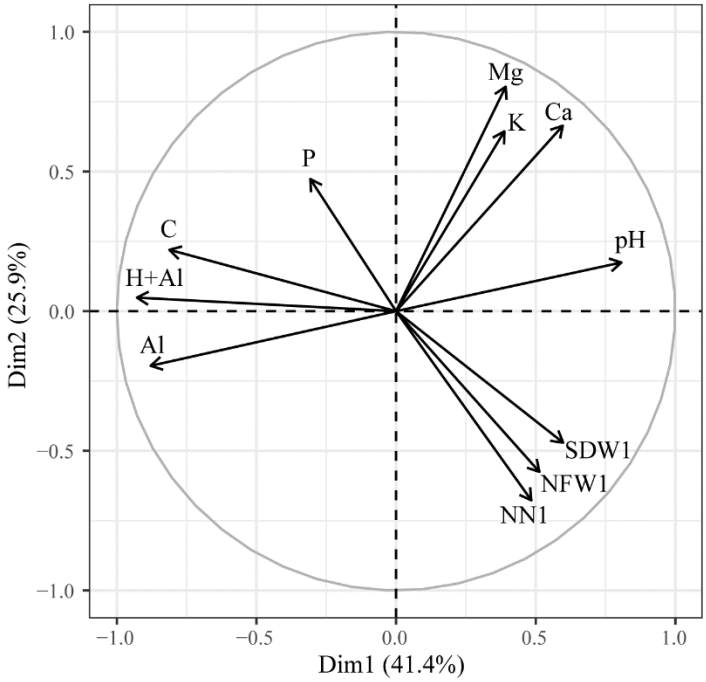


Figure 2. Principal component analysis (PCA) between nodule number (NN1), nodule fresh weight (NFW1), and shoot dry weight (SDW1) of mung bean grown in modified Leonard jars containing soil sample as inoculant and the chemical fertility data of soil samples presented in Table 2: pH, Al^{+3} (aluminum), Ca^{2+} (calcium), Mg^{2+} (magnesium), H+Al (potential acidity), P (phosphorus), K^{+} (potassium), and C (carbon).

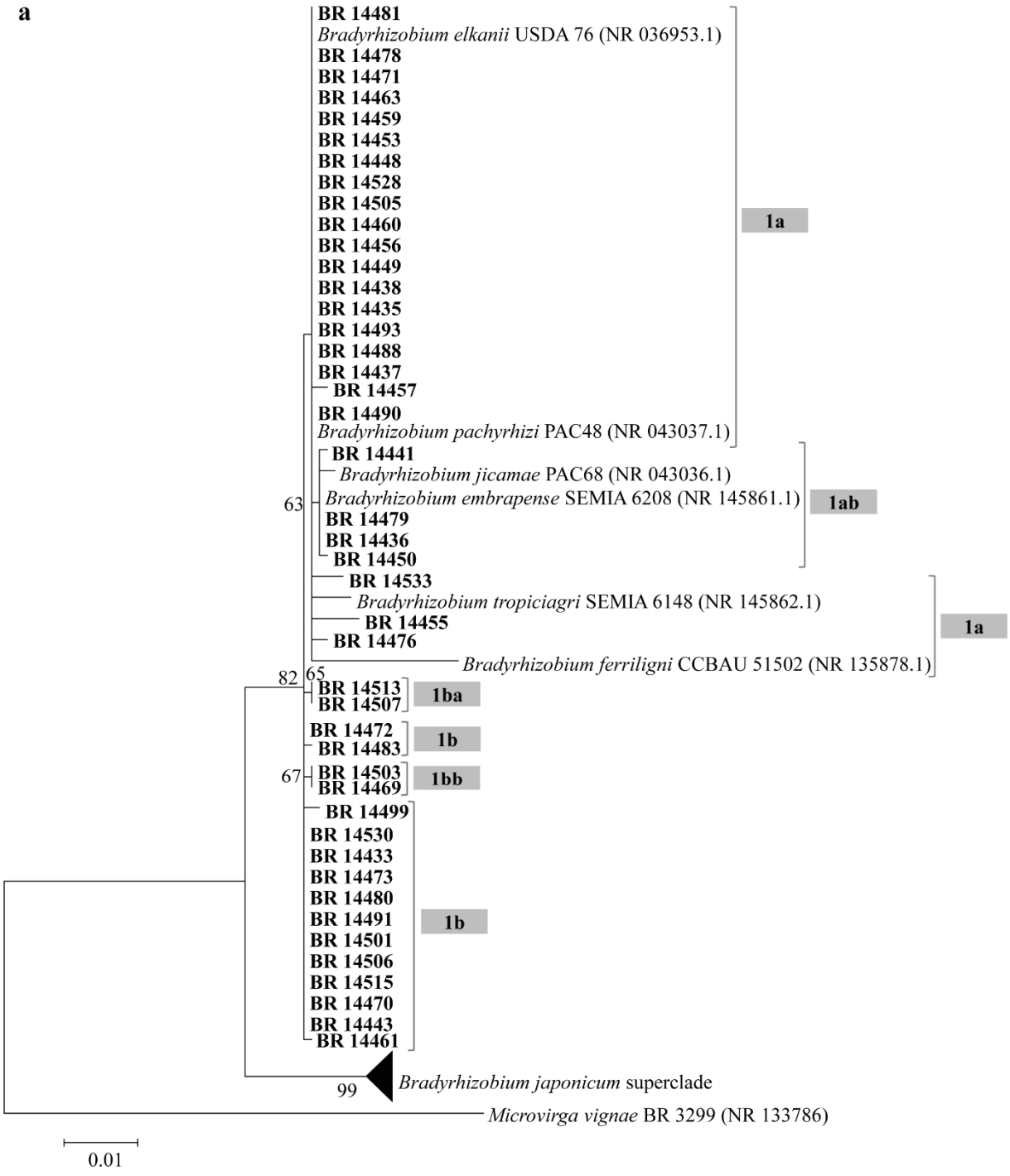
3.5.2 Morphological characterization and sequencing of the 16S rRNA gene

In isolation, morphological characteristics were used to decrease the redundancy within each genotype and soil isolate; therefore, only 101 bacterial strains were isolated and morphologically characterized (Appendix 2). No differences were observed between the two mung bean genotypes, which resulted in a selection of morphotypes within the different soil samples. In general, mung bean nodule isolates tended to be slow growing (67/101), and among this group, all 67 isolates increased the medium pH, 41 produced copious amounts of mucus, and 61 were characterized by white colonies.

A BLAST search against type strain materials indicated that the isolates are affiliated with the genera *Bradyrhizobium* (66 isolates), *Rhizobium* (19), *Mesorhizobium* (4), *Ensifer* (3), *Leifsonia* (3), *Bacillus* (3), *Agrobacterium* (1), *Mycolicibacterium* (1), and *Kaistia* (1) (Appendix 2, Figure 3a, b and Figure 4). These genera belong to the phyla *Proteobacteria* (*Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, *Ensifer*, *Agrobacterium*, and *Kaistia*), *Firmicutes* (*Bacillus*), and *Actinobacteria* (*Leifsonia* and *Mycolicibacterium*), accounting for 94, 4, and 3 isolates, respectively. In total, 91% of the isolates are classified in a group collectively referred to as rhizobia (*Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, and *Ensifer*),

which, in both temperate and tropical regions, are considered the most important with respect to legume nodulation.

Bradyrhizobium isolates were more abundant in our data, and among the 66 isolates of this genus, 44 (67%) were found to group in the *Bradyrhizobium elkanii* superclade (Figure 3a), whereas the remaining 22 (33%) clustered with members of the *B. japonicum* superclade (Figure 3b). Within the *B. elkanii* superclade (Figure 3a), the isolates were categorized into five subgroups (number of isolates): 1a (22), 1ab (4), 1ba (2), 1b (14), and 1bb (2); the most abundant group, 1a, was grouped with *B. elkanii*. In the *B. japonicum* superclade (Figure 3b), seven subgroups were formed (number of isolates): 2a (2), 2baaa (2), 2baab (2), 2baac (1), 2bab (3), 2bba (3), and 2bbb (9); the isolates of 2bbb, the most abundant subgroup, were phylogenetically related to *B. yuanmingense*. *Bradyrhizobium* isolates separation into different subgroups within the aforementioned superclades indicates that a diverse range of species in this genus occur in nodules of mung bean grown in Brazil. Moreover, subgroups of isolates without representative strains indicate the occurrence of currently undescribed bacterial species.


a
BR 14481
Bradyrhizobium elkanii USDA 76 (NR 036953.1)
BR 14478
BR 14471
BR 14463
BR 14459
BR 14453
BR 14448
BR 14528
BR 14505
BR 14460
BR 14456
BR 14449
BR 14438
BR 14435
BR 14493
BR 14488
BR 14437
BR 14457
BR 14490
Bradyrhizobium pachyrhizi PAC48 (NR 043037.1)
1a
BR 14441
Bradyrhizobium jicamae PAC68 (NR 043036.1)
63
Bradyrhizobium embrapense SEMIA 6208 (NR 145861.1)
BR 14479
BR 14436
BR 14450
1ab
BR 14533
Bradyrhizobium tropiciagri SEMIA 6148 (NR 145862.1)
BR 14455
BR 14476
Bradyrhizobium ferriligni CCBAU 51502 (NR 135878.1)
1a
82
65
BR 14513
BR 14507
1ba
BR 14472
BR 14483
1b
67
BR 14503
BR 14469
1bb
BR 14499
BR 14530
BR 14433
BR 14473
BR 14480
BR 14491
BR 14501
BR 14506
BR 14515
BR 14470
BR 14443
BR 14461
1b
99
Bradyrhizobium japonicum superclade
Microvirga vignae BR 3299 (NR 133786)
0.01

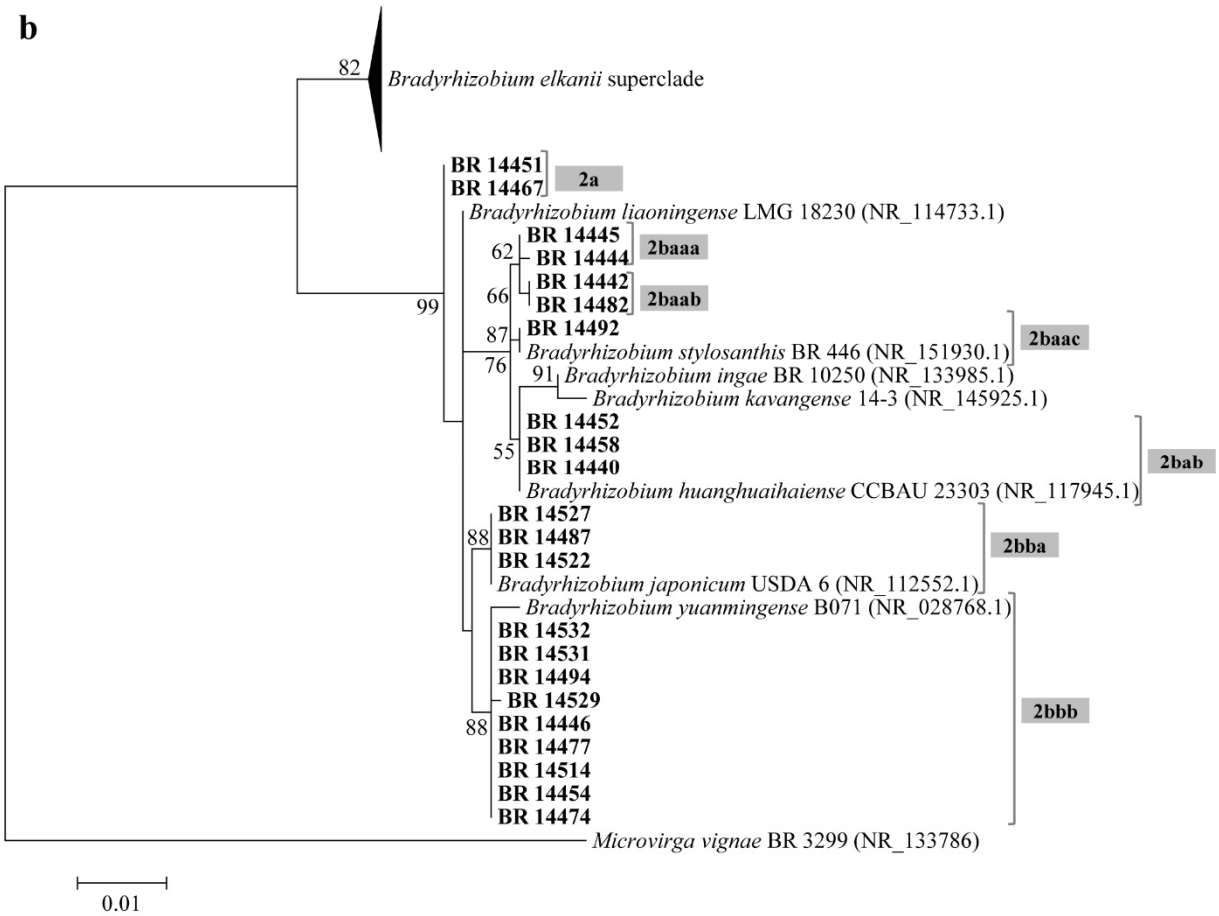


Figure 3. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences estimated from 952 base pair positions: isolates grouped in the *Bradyrhizobium elkanii* (a) and *Bradyrhizobium japonicum* (b) superclades. Bootstrap values are shown when the relationships represented have been observed in at least 50% of 500 replicates. Phylogenetic groups with a bootstrap value of at least 50% of replicates are identified by a gray text box. The tree was obtained using the Tamura 3-parameter + G model. The scale bar represents 0.01% of base pair substitutions. Isolates obtained from mung bean nodules are highlighted in bold. The *Microvirga vignae* strain 'BR 3299' was used as outgroup.

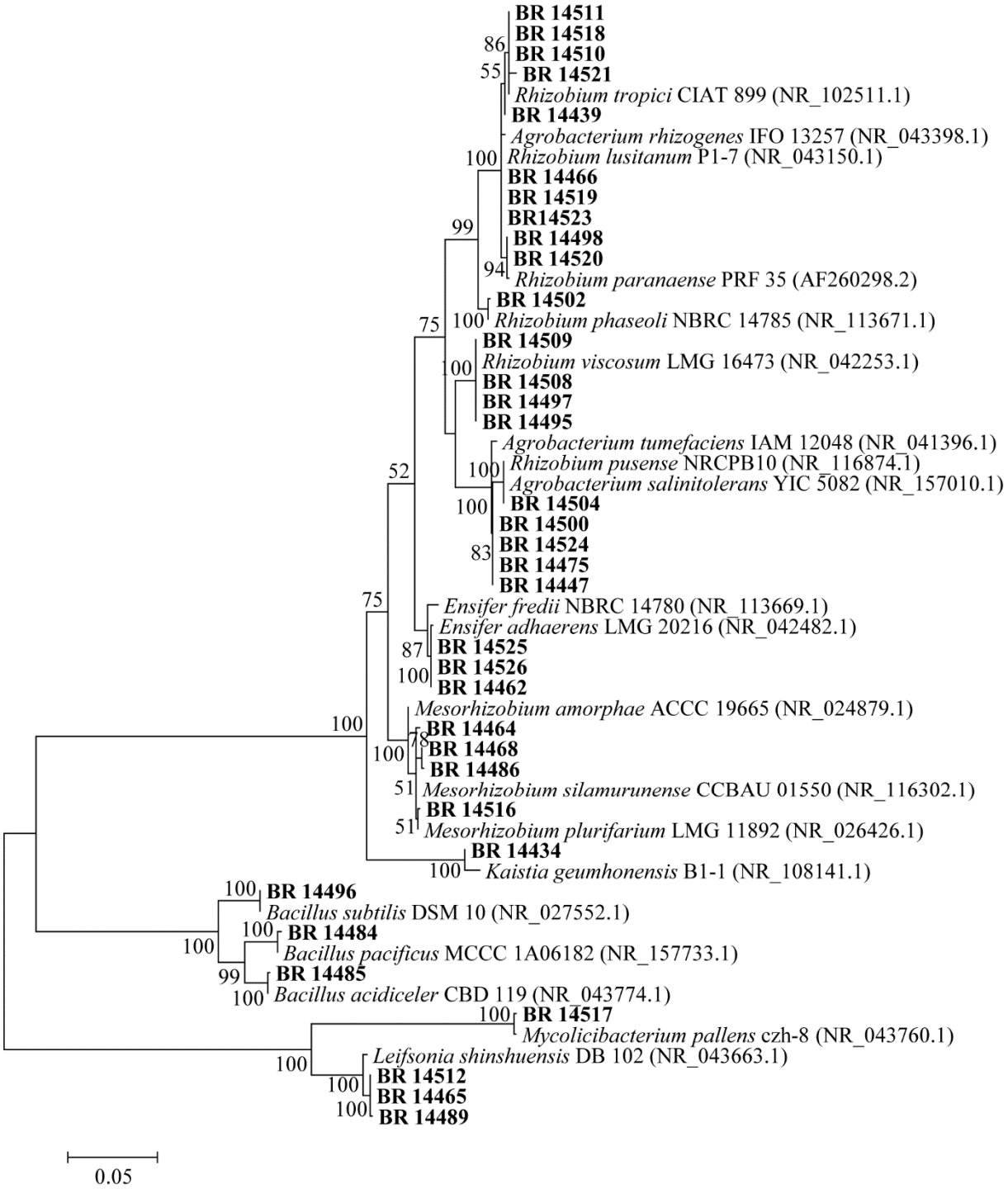


Figure 4. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences estimated from 1026 base pair positions for isolates other than those from the genus *Bradyrhizobium*. Bootstrap values are shown when the relationships represented have been observed in at least 50% of 500 replicates. The tree was obtained using the Kimura 2-parameter + G + I model. The scale bar represents 0.05% of base pair substitutions. Isolates obtained from mung bean nodules are highlighted in bold.

3.5.3 Nodulation test

Given that we did not observe any marked morphological differences between the isolates obtained from the two mung bean genotypes, we chose to evaluate nodulation capacity

using only the 'Esmeralda' cultivar, because it is the most studied and widespread cultivar in Brazil. In our tests, the isolates of *Rhizobium*, *Mesorhizobium*, *Ensifer*, *Bacillus*, *Agrobacterium*, *Leifsonia*, *Mycolicibacterium*, and *Kaistia* did not induce nodule formation under axenic conditions, whereas positive control plants, inoculated with a *Bradyrhizobium* BR 14533 isolate, showed abundant nodulation in all replicates.

With the exception of BR 14487, all *Bradyrhizobium* isolates were found to induce nodule formation in mung bean (Table 3). A subsidiary cross-inoculation evaluation similarly revealed that isolate BR 14487 was also unable to induce nodulation in cowpea (*Vigna unguiculata* (L.) Walp) and siratro (*Macroptilium atropurpureum*) (Appendix 3). The treatments whose isolates formed nodules had an average of 60 nodules per plant, which corresponded on average to 99 mg of nodules. The highest number and weight of nodules were obtained in mung bean inoculated with isolate BR 14477, which induced the production of 144 nodules per plant with a dry weight of 183 mg. Compared with the averages of the other isolates, these values represent differences of 90 and 87% in the number and weight of nodules, respectively.

With respect to the root and shoot dry weights of inoculated plants, we observed that those subjected to treatment with nitrogen application showed higher averages (Tabel 3). Plants in group of six inoculation treatments had the highest average root dry weights, which corresponded to an average 58% (0.52/0.9 g) of the nitrogen control weight and an increase of 273% (0.52/0.14 g) compared to the absolute control weight. Regarding shoot dry weight, plants in 62 inoculation treatments (94%) showed averages higher than the absolute control and were divided into five groups. Plants in a group of eight inoculation treatments had higher average shoot dry weights, corresponding to an average 50% (1.58/3.16 g) of the nitrogen control weight and an increase of 700% (1.58/0.18 g) compared with the absolute control weight. A second group of plants, receiving 31 treatments, with slightly lower averages, showed increases of more than 500% (1.12/0.18 g) compared with the absolute control weight. These data indicate that the nodules that developed in response to inoculation with these *Bradyrhizobium* isolates contributed to promoting the growth of mung bean plants.

Our findings also indicated that the observed variation in shoot dry weight could be attributed to differences in phylogenetic groups based on 16S rRNA sequencing (Figure 5a) and soil origin (Figure 5b). Specifically, we observed that nodulation efficiency varied within the *B. elkanii* (subgroup 1) and *B. japonicum* (subgroup 2) superclades. These differences in shoot dry weight related to phylogenetic group and isolate soil of origin were confirmed based on PERMANOVA ($p < 0.01$).

On the basis of paired comparisons, we also established that plants inoculated with isolates in the 2bbb group, which are phylogenetically related to *B. yuanmingense* B071, were characterized by higher average shoot dry weights (Figure 5a and Appendix 5), and similar results were obtained for the group 2bab and 2baab isolates. In contrast, plants inoculated with isolates from groups 2bba and 1ba were found to be characterized by the lowest average shoot dry weights. The phylogenetic group 2bba is phylogenetically close to *B. japonicum* USDA 6, whereas the 1ba isolate within the *B. elkanii* superclade and has yet to be affiliated with a described species.

Table 3. Number and nodule dry weight, root, and shoot of mung bean inoculated with *Bradyrhizobium* spp. isolates. Plants collected 35 days after emergence (continue)

Isolate	Soil origin	Phylogenetic group	Nodule number (nº plant^{-1})	Nodule dry weight (mg plant^{-1})	Root dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})
BR 14477	SE_RJ-I	2bbb	144 a	183 a	0.58 b	1.86 b
BR 14446	SE_RJ-V	2bbb	112 b	138 b	0.49 b	1.73 b
BR 14529	SE_RJ-I	2bbb	63 d	123 b	0.52 b	1.56 b
BR 14531	SE_RJ-V	2bbb	83 c	136 b	0.52 b	1.56 b
BR 14532	SE_MG-II	2bbb	63 d	128 b	0.53 b	1.53 b
BR 14458	SE_RJ-III	2bab	54 d	105 c	0.49 b	1.49 b
BR 14455	SE_RJ-I	1a	41 e	112 b	0.47 c	1.47 b
BR 14454	SE_RJ-V	2bbb	103 b	137 b	0.45 c	1.44 b
BR 14482	SE_RJ-IV	2baab	101 b	136 b	0.43 c	1.34 c
BR 14442	SE_RJ-V	2baab	79 c	123 b	0.40 c	1.28 c
BR 14436	SE_RJ-I	1ab	37 e	95 c	0.40 c	1.27 c
BR 14438	SE_RJ-II	1a	66 d	120 b	0.44 c	1.26 c
BR 14472	SE_RJ-III	1b	79 c	114 b	0.42 c	1.26 c
BR 14452	SE_RJ-III	2bab	92 c	119 b	0.44 c	1.25 c
BR 14481	SE_RJ-IV	1a	81 c	107 c	0.41 c	1.24 c
BR 14440	SE_RJ-III	2bab	82 c	112 b	0.42 c	1.21 c
BR 14469	SE_RJ-I	1bb	51 d	139 b	0.41 c	1.19 c
BR 14437	SE_RJ-II	1a	51 d	93 c	0.44 c	1.17 c
BR 14514	SE_MG-II	2bbb	69 d	104 c	0.43 c	1.15 c
BR 14474	SE_RJ-V	2bbb	78 c	114 b	0.38 c	1.14 c
BR 14449	SE_RJ-I	1a	57 d	112 b	0.37 c	1.13 c
BR 14450	SE_RJ-I	1ab	36 e	90 c	0.42 c	1.12 c
BR 14493	SE_MG-I	1a	60 d	88 c	0.38 c	1.10 c
BR 14494	SE_MG-II	2bbb	66 d	102 c	0.39 c	1.10 c
BR 14479	SE_RJ-I	1ab	37 e	99 c	0.35 c	1.10 c
BR 14435	SE_RJ-I	1a	45 e	112 b	0.35 c	1.10 c
BR 14444	SE_RJ-IV	2baaa	78 c	121 b	0.38 c	1.10 c
BR 14445	SE_RJ-IV	2baaa	67 d	114 b	0.38 c	1.10 c
BR 14483	SE_RJ-II	1b	44 e	99 c	0.38 c	1.07 c
BR 14480	SE_RJ-I	1b	51 d	111 b	0.36 c	1.06 c
BR 14476	SE_RJ-II	1a	54 d	95 c	0.36 c	1.05 c
BR 14478	SE_RJ-III	1a	42 e	91 c	0.37 c	1.03 c
BR 14461	SE_RJ-V	1b	60 d	96 c	0.34 c	1.03 c
BR 14506	SE_MG-I	1b	72 c	119 b	0.36 c	1.00 c
BR 14470	SE_RJ-I	1b	57 d	122 b	0.36 c	1.00 c
BR 14456	SE_RJ-I	1a	47 e	122 b	0.33 c	0.97 c
BR 14471	SE_RJ-I	1a	54 d	127 b	0.36 c	0.96 c
BR 14457	SE_RJ-III	1a	66 d	96 c	0.31 d	0.93 c
BR 14433	SE_RJ-I	1b	60 d	121 b	0.34 c	0.91 c
BR 14443	SE_RJ-IV	1b	66 d	113 b	0.29 d	0.86 d
BR 14463	SE_RJ-II	1a	104 b	93 c	0.27 d	0.85 d
BR 14473	SE_RJ-V	1b	59 d	91 c	0.31 d	0.84 d
BR 14505	SE_MG-I	1a	49 e	91 c	0.31 d	0.82 d
BR 14459	SE_RJ-I	1a	52 d	101 c	0.27 d	0.81 d
BR 14515	SE_MG-II	1b	50 d	94 c	0.31 d	0.79 d
BR 14448	SE_RJ-I	1a	57 d	90 c	0.31 d	0.79 d
BR 14467	SE_RJ-III	2a	58 d	89 c	0.26 d	0.78 d
BR 14528	MW_MT-II	1a	62 d	106 c	0.35 c	0.77 d
BR 14530	SE_MG-II	1b	52 d	85 c	0.29 d	0.77 d
BR 14491	MW_MT-I	1b	45 e	106 c	0.33 c	0.76 d
BR 14490	MW_MT-I	1a	66 d	99 c	0.28 d	0.74 d
BR 14503	SE_MG-II	1bb	41 e	75 c	0.27 d	0.74 d
BR 14451	SE_RJ-III	2a	51 d	79 c	0.23 e	0.70 d
BR 14488	MW_MT-III	1a	67 d	89 c	0.31 d	0.69 d

Table 3. Continuation.

Isolate	Soil origin	Phylogenetic group	Nodule number (n^o plant^{-1})	Nodule dry weight (mg plant^{-1})	Root dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})
BR 14460	SE_RJ-II	1a	75 c	79 c	0.24 d	0.61 e
BR 14492	SE_MG-I	2baac	57 d	74 c	0.22 e	0.58 e
BR 14453	SE_RJ-III	1a	24 f	65 d	0.22 e	0.56 e
BR 14501	SE_MG-I	1b	61 d	71 c	0.26 d	0.55 e
BR 14499	MW_MT-I	1b	34 e	59 d	0.27 d	0.52 e
BR 14533	SE_RJ-III	1a	45 e	70 c	0.20 e	0.41 f
BR 14441	SE_RJ-III	1ab	54 d	53 d	0.17 e	0.40 f
BR 14507	MW_MT-II	1ba	17 f	35 e	0.20 e	0.37 f
BR 14527	MW_MT-II	2bba	47 e	44 e	0.21 e	0.34 f
BR 14522	MW_MT-I	2bba	25 f	29 e	0.19 e	0.27 g
BR 14513	MW_MT-I	1ba	3 g	17 f	0.16 e	0.22 g
BR 14487	MW_MT-II	2bba	0 g	0 f	0.16 e	0.19 g
Absolute control			0 g	0 f	0.14 e	0.18 g
Nitrogen control			0 g	0 f	0.90 a	3.16 a
Coefficient of variation (%)			13.93	17.69	9.26	9.35

Averages followed by distinct letters differ by Scott-Knott test at 5% probability.

With respect to the soil of origin, we found that in general, plants inoculated with isolates obtained from Mato Grosso state soils showed lower shoot dry weights than did those inoculated with isolates obtained from other soils (Figure 5b and Appendix 5). Plants inoculated with SE_RJ-V soil isolates showed higher shoot dry weights, although these values did not differ significantly from those of plants inoculated with isolates from SE_RJ-I and SE_RJ-IV soils. The distribution of the points within phylogenetic groups and soils of origins (Figure 5a and b) indicates that there is a difference between the isolates within each group and soil; however, selection of the most efficient isolates should take into account the average shoot dry weights obtained in each treatment, and not only the phylogenetic group or soil of origin.

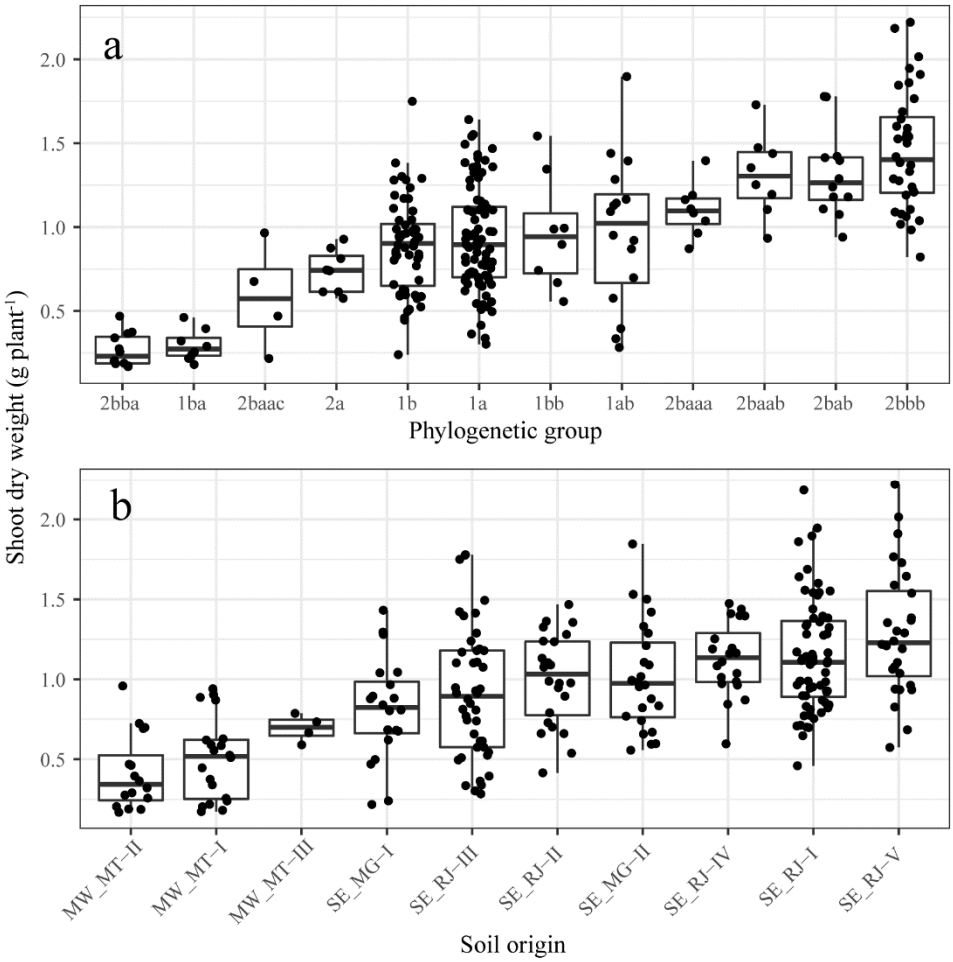


Figure 5. Boxplot for the shoot dry weights of mung bean plants inoculated with *Bradyrhizobium* isolates and grouped according to phylogenetic group (a) and soil of origin (b).

Principal component analysis revealed that the efficiency of *Bradyrhizobium* isolates in terms of nodulation and shoot dry weight is correlated with these two parameters of mung bean grown under the effect of the native rhizobia population (Figure 6). This suggests that the efficient rhizobia present in soil samples may have been successfully isolated in our study.

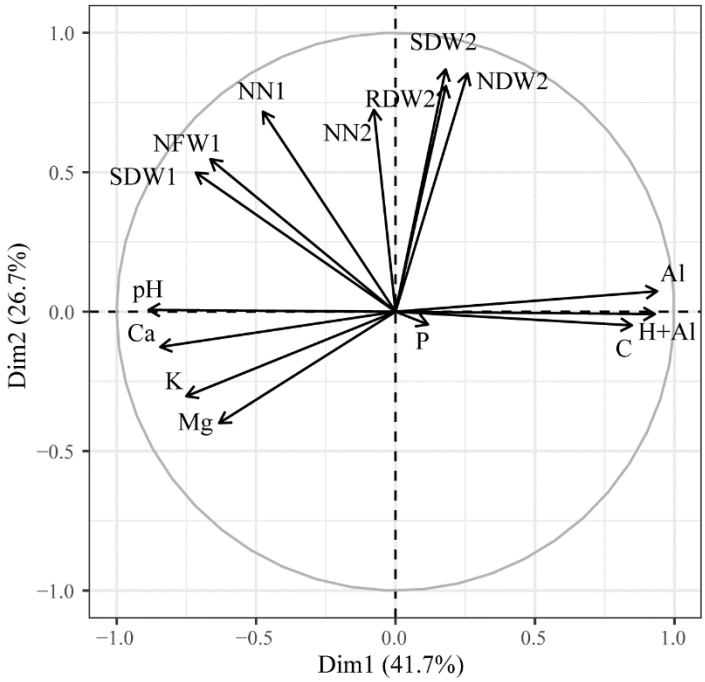


Figure 6. Principal component analysis (PCA) between nodule number (NN1), nodule fresh weight (NFW1) and shoot dry weight (SDW1) of mung bean grown in modified Leonard jars containing soil sample as inoculant, and nodule number (NN2), nodule dry weight (NDW2), and shoot dry weight (SDW2) of mung bean inoculated with *Bradyrhizobium* isolates. In addition, the chemical fertility data of soil samples is presented in Table 2: pH, Al^{+3} (aluminum), Ca^{2+} (calcium), Mg^{2+} (magnesium), H+Al (potential acidity), P (phosphorus), K^{+} (potassium), and C (carbon).

3.6 DISCUSSION

Mung bean has recently been introduced as a commercial crop in the Brazilian Cerrado and, similar to cowpea, the cultivated area is increasing annually. This growth in production can be attributed, at least in part, to the fact that these pulses fit well into rotations with other main crops, such as soybean, and there is also the potential to export the grains to other countries, given the existing demand.

Nitrogen represents an important factor in the production costs of most crops, and considering that Brazil imports more than 80% of the nitrogen fertilizer used, facilitating BNF is a practice warranting considerably greater attention. In this regard, it is important to note that Brazil has already been successful in exploiting BNF in soybean, cowpea, and others crops (HUNGRIA; NOGUEIRA; ARAUJO, 2013, 2015; SILVA JÚNIOR et al., 2018).

In the present study, we used the modified Leonard jar as an alternative trap host method to obtain nitrogen-fixing nodules in mung bean plant. For this, we add a layer of soil sample in a Leonard jar to serve as the bacterial inoculum (Appendix 1), ensuring a sufficient nutrient supply via the nutrient solution. The use of soil samples or suspensions in systems containing nutrient solution has already been successfully carried out for other legumes (CASTRO et al., 2017; FLORENTINO et al., 2009; MARRA et al., 2012). In addition to providing a source of bacteria, the main advantage of this system is that it enables an evaluation of the capacity of soil-established rhizobia to fix nitrogen, given that this element is the only nutrient missing from the provided nutrient source. We can thus assume that the differences observed in plant growth are attributable to the activity of soil-borne bacteria and not soil nutrient deficiencies. Indeed, a primary purpose of the present study was to minimize any effects of soil nutrients during nodule formation and colonization, and therefore meeting this requirement necessitated a low soil/substrate ratio. Conversely, if we had used soil-filled pots, soil chemical parameters would have determined plant growth to a larger extent, and thereby indirectly influenced nodule colonization.

The low nodulation observed in mung bean plants grown in some soils samples, particular those derived from Mato Grosso state (Figure 1a and c), is conceivably related to the low concentrations of native rhizobia with a capacity to nodulate this plant. The higher nodulation and shoot dry weight observed for plants grown in SE_MG-II and SE_RJ-V soil samples indicate that the rhizobial populations in these soils are more effective with respect to promoting nodulation and plant growth (Figure 1a, c and d). Native populations of rhizobia are influenced to varying extents by the chemical characteristics of soils, and in this regard, a positive relationship between soil pH and the diversity/abundance of soil bacterial communities is commonly observed (ANDREW et al., 2012; KARIMI et al., 2018; ROUSK et al., 2010). However, based on the findings of the present study, we suspect that other factors intrinsic to the soil may have contributed to the observed differences in nodulation. High plant diversity and cultivation history appear to play an important role in soil diversity (BERG; SMALLA, 2009; HARTMANN et al., 2009; SMALLA et al., 2001). For example, the high nodulation observed in plants grown in the SE_RJ-V soil sample may be related to the prevailing management system. The area from which this soil was obtained has been farmed under an integrated system of agroecological production since 1993, entailing the cultivation of an extensive range of crops, including green fertilizers, grain legumes, and vegetables, in the absence of any application of fertilizers or pesticides (NEVES et al., 2005). In contrast, Cerrado soils (MW_MT-I, MW_MT-II, and MW_MT-III), which we found to be associated with a low frequency of nodulation, were obtained from areas subjected to intensive grain cultivation, characterized by inputs of several different agrochemicals. These differences thus tend to indicate that in addition to the chemical characteristics of the soil, native rhizobial populations are influenced by other factors, such as the management of these areas.

It has been established that the development of mung bean shoot is positively correlated with the weight and nodule number (SOUZA et al., 2008; ZILLI et al., 2006), and consequently, a low frequency of nodulation or inefficient rhizobia can affect plant growth and reduced grain yield (BHUIYAN; MIAN; ISLAM, 2008; HERRIDGE et al., 2005; MATHU et al., 2012). In this sense, the inoculation of legumes with appropriate rhizobia can promote considerable increases in nodulation and grain yield in agricultural soils with inherently low native rhizobial populations (SINGLETON; TAVARES, 1986; THIES; SINGLETON; BOHLOOL, 1991). Thus, given the low nodulation of mung bean cultivated in the three soil samples obtained from Mato Grosso state, located in the Brazilian Cerrado, suggests that inoculation with efficient rhizobial strains would promote increase in the grain yield of mung bean cultivated in these areas, and also demonstrate the necessity for inoculation to promote satisfactory plant nodulation.

The rhizobia genera isolated in this study, including *Bradyrhizobium* (APPUNU et al., 2009; RISAL et al., 2012; YANG et al., 2008; ZHANG et al., 2008), *Ensifer* (PANDYA; KUMAR; RAJKUMAR, 2013; YANG et al., 2008; ZHANG et al., 2008), *Rhizobium* (YANG et al., 2008; ZHANG et al., 2008), and *Mesorhizobium* (YANG et al., 2008), have also been obtained from the nodules of mung bean grown in Asia. Among these, *Bradyrhizobium* is commonly the most isolated genus of mung bean nodules (RISAL et al., 2012; YANG et al., 2008; ZHANG et al., 2008), which is consistent with the findings of the present study. The grouping of isolates with respect to different *Bradyrhizobium* species reinforces the previously observed low symbiotic specificity of mung bean for the species in this genus (APPUNU et al., 2009; YANG et al., 2008), as has also been reported for *Vigna unguiculata* (MOHAMMED; JAISWAL; DAKORA, 2018; NDUNGU et al., 2018; TAMPAKAKI et al., 2017; XAVIER et al., 2006) and *Vigna mungo* (APPUNU et al., 2009). Moreover, we speculate that the greater abundance of isolates belonging to the *B. elkanii* superclade may be related to their wide distribution in tropical and subtropical regions (MENNA; HUNGRIA, 2011). Different results were found in Nepal, where 92% of the *Bradyrhizobium* isolates have been grouped with *B. japonicum,* with only 8% showing an affiliation with *B. elkanii* (RISAL et al., 2012).

Nevertheless, despite previous reports indicating the predominance of the genus *Bradyrhizobium* (RISAL et al., 2012; YANG et al., 2008; ZHANG et al., 2008), the findings of some studies have indicated the codominance or dominance of either *Bradyrhizobium* or *Ensifer* based on nodule total DNA sequencing, and that the relative proportions of these genera vary depending on the cultivated soil (HAKIM et al., 2018, 2020). In contrast, we found that only 3% of the isolates were grouped into the *Ensifer* genus (Figure 4 and Appendix 2), and in general, other isolation studies have found proportional representations of up to only 7% (YANG et al., 2008; ZHANG et al., 2008). It is, however, conceivable that such differences are attributable to biases of isolation techniques and/or sequencing methods, or are even inherent to the soil conditions in which the plants are grown. Considering the genus *Rhizobium*, a smaller proportion is common in mung bean nodule isolates, with up to 7% of the isolates (YANG et al., 2008; ZHANG et al., 2008), whereas in the present study, we found that 18% of isolates were derived from this genus, thereby indicating that there is a higher abundance of *Rhizobium* in the nodules of mung bean cultivated in tropical Brazilian soils.

There is also a prevailing consensus that in addition to rhizobia, several other bacterial genera inhabit legume nodules (DE MEYER et al., 2015; HAKIM et al., 2020; LEITE et al., 2017; MARTÍNEZ-HIDALGO; HIRSCH, 2017; ZHANG et al., 2018). These bacteria have been shown to invade root tissues during the formation of the infection cord induced by symbiont (PANDYA; KUMAR; RAJKUMAR, 2013; ZGADZAJ et al., 2015). This would account for our observation of several NRB genera (*Leifsonia*, *Bacillus*, *Agrobacterium*, *Mycolicibacterium*, and *Kaistia*) in the mung bean plants examined in the present study (Figure 4 and Appendix 2). Given their rapid growth characteristic (with the exception of

Mycolicibacterium, according to our data), these bacteria can often be isolated more readily than those with slow growth (Appendix 2).

Of the NRB genera identified herein, *Leifsonia* was found to be the most abundant (Figure 4 and Appendix 2), and strains of bacteria within this genus have previously been isolated from the nodules of cowpea (LEITE, 2015) and common bean (CARDOSO; HUNGRIA; ANDRADE, 2012) grown in Brazil; however, their nodulation capacity has yet to be verified. Certain strains of *Leifsonia* have been shown to have the potential to promote plant growth (KANG et al., 2014; PASSARI et al., 2015), whereas others can cause plant diseases, such as knuckle rickets in sugarcane caused by *Leifsonia xyli* subsp. *xyli* (BRUMBLEY et al., 2006). The isolates obtained in the present study were found to group with *L. shinshuensis*, which is characterized by phosphate solubilizing, and nitrogen fixation, and production of the hormone indole-3-acetic acid (IAA) (LIAQAT; ELTEM, 2016). No studies were found indicating that *L. shinshuensis* is pathogenic to plants.

Bacteria in the genus *Bacillus* have previously been isolated from the nodules of mung bean (TARIQ et al., 2012), as well as from other legumes (APPUNU et al., 2018; ARIF et al., 2017; BAI et al., 2002; DAHMANI et al., 2020; KORIR et al., 2017). Although these strains did not induce nodule formation, a stimulating effect was found when co-inoculated with nodulating rhizobia strains (APPUNU et al., 2018; ARIF et al., 2017; BAI et al., 2002; DAHMANI et al., 2020; KORIR et al., 2017; TARIQ et al., 2012).

Some strains of *Agrobacterium* have also been isolated from mung bean nodules (TARIQ et al., 2012), as well as from other legumes (DELAMUTA et al., 2020; LIU et al., 2010; WANG et al., 2006) and plants in other families (ARAÚJO; MACCHERONI; AZEVEDO, 2009; LIAQAT; ELTEM, 2016; RASHID; CHARLES; GLICK, 2012), and are thus considered to be common endophytic bacteria in a range of plant species. Despite their isolation from legume nodules, it is not common to observe nodulation promoted by *Agrobacterium* (LAJUDIE et al., 1999; LIU et al., 2010; TARIQ et al., 2012; WANG et al., 2006). Strains of this genus can promote plant growth (SHAHAROONA; ARSHAD; ZAHIR, 2006; TARIQ et al., 2012), under *in vitro* conditions, some strains can produces IAA, solubilizes phosphate, fixes nitrogen, and produces siderophores (LIAQAT; ELTEM, 2016; TARIQ et al., 2012). The taxonomy of the genus *Agrobacterium* has sometimes proved to be controversial, and some strains have been reclassified as *Rhizobium* (FARRAND; BERKUM; OGER, 2003; KUYKENDALL et al., 2001), however, the separation between the two genera is still accepted, and more complete phylogenetic studies have been conducted (LAJUDIE et al., 2019). Very recently, *Agrobacterium fabacearum* was described based on several strains isolated from nodules of soybean and common bean; however, this species appears to be incapable of inducing plant nodulation (DELAMUTA et al., 2020).

A further genus identified in the present study, *Mycolicibacterium*, emerged after a proposal to subdivide the genus *Mycobacterium* (GUPTA; LO; SON, 2018). Although there are no previous reports of *Mycolicibacterium* being isolated from mung bean nodules, it has previously been isolated from soybean roots (LIU et al., 2019a) and potato (BARRIO-DUQUE et al., 2019), and there are reports of its occurrence in soil (D'ERRICO et al., 2020; KRANZ; WHITMAN, 2019). Currently, although little is known regarding the relationships between the bacteria in this genus and plants, some species are known to degrade hydrocarbons, among which is *Mycolicibacterium pallens* (D'ERRICO et al., 2020; HENNESSEE et al., 2009; STELIGA et al., 2020), with which the isolate identified in the present study was grouped. Even though most of the species of this genus are saprophytic and considered non-pathogenic to humans (GUPTA; LO; SON, 2018), some are known to cause infections (RIPOLL et al., 2009).

Similarly, there have to date been no reports of *Kaistia* bacteria isolated from mung bean nodules; however, they have previously been isolated from the nodules of acacias (HOQUE; BROADHURST; THRALL, 2011) and clover (ARONE et al., 2014). Along with the genera

Ensifer and *Rhizobium*, *Kaistia* is classified within the family *Rhizobiaceae* (IM et al., 2004), and there are indications that strains of *Kaistia* have a suppressive effect on pathogens (FUJIWARA et al., 2016). Accordingly, the potential beneficial effects of these strains in plants warrant further study.

In our nodulation test, isolates of the genera *Rhizobium*, *Mesorhizobium*, *Ensifer*, *Leifsonia*, *Bacillus*, *Agrobacterium*, *Mycolicibacterium*, and *Kaistia* did not nodulate mung bean. Contrasting observation have, however, been made in Asia (India, China, and Pakistan), where strains of *Rhizobium* (HAMEED et al., 2004; YANG et al., 2008; ZHANG et al., 2008), *Mesorhizobium* (LU et al., 2009; YANG et al., 2008), and *Ensifer* (HAKIM et al., 2018; PANDYA; KUMAR; RAJKUMAR, 2013; YANG et al., 2008; ZHANG et al., 2008) have been shown to be effective in nodulating mung bean. We did not find any studies reporting that bacteria in the genera *Rhizobium*, *Mesorhizobium*, and *Ensifer* were incapable of nodulating mung bean. There are reports that strains of *Bacillus* and *Agrobacterium* do not induce nodulation in their original hosts (LAJUDIE et al., 1999; LIU et al., 2010; TARIQ et al., 2012; WANG et al., 2006), and there have been no reports to date on *Leifsonia* strains being isolated from mung bean nodules, and strains of *Mycolicibacterium* and *Kaistia* being isolated from the nodules of any legumes. Collectively, our findings with respect to the isolation of strains of *Rhizobium*, *Mesorhizobium*, *Ensifer*, *Leifsonia*, *Bacillus*, *Agrobacterium*, *Mycolicibacterium*, and *Kaistia* from mung bean nodules and their inability to induce nodulation, indicate that the bacteria in these genera are co-inhabitants of the nodules of mung bean grown in tropical Brazilian soils.

Among the isolates obtained in this study, only those phylogenetically related to the genus *Bradyrhizobium* were able to nodulate mung bean (Table 3). Native strains of this genus have been found to nodulate mung bean in several countries, including China (YANG et al., 2008; ZHANG et al., 2008), India (APPUNU et al., 2009), Pakistan (HAKIM et al., 2018; HAMEED et al., 2004; TARIQ et al., 2012), and Nepal (RISAL et al., 2012). Among the 66 isolates of this genus identified in the present study, only BR 14487 failed to induce nodulation under the experimental conditions we employed (Table 3); however, there are no reports that strains of *Bradyrhizobium* have the capacity to nodulate mung bean This tends to contrast with previous observations indicating that all examined strains of *Bradyrhizobium* have the capacity to nodulate mung bean. We found that the BR 14487 isolate was also incapable of nodulating cowpea and siratro (Appendix 3), two species known to have low specificity with respect to native rhizobial symbionts, and which are commonly used as bait plants (CASTRO et al., 2017; GUIMARÃES et al., 2012; LIMA et al., 2009; SILVA et al., 2012). Accordingly, these observations would tend to indicate that this isolate is either an inefficient or incapable of nodulating legumes. Our phylogenetic analysis based on 16S rRNA indicated that this isolate groups with the USDA 6 strain of *B. japonicum*, together with the isolates BR 14527 and BR 14522, forming the 2bba group (Figure 3b). The treatments inoculated with these isolates had low nodulations and increases in shoot dry weight (Figure 5a). These results suggest that this group has a low efficiency in nodulation and BNF in mung bean.

The phylogenetic group 2bbb, which is close to *B. yuanmingense* B071 showed higher performance with respect to host plant shoot dry weight (Figure 3b and Figure 5a). Previously, phylogenetic analysis based on the *nifD* gene has indicated that strains close to *B. yuanmingense* are more efficient symbionts than are the *B. elkanii* C-11 and *B. japonicum* USDA 6 strain in nitrogen-fixing in mung bean (RISAL et al., 2012). Consistently, the most efficient strains have also been shown to be those that group with *B. yuanmingense* in 16S rRNA and ITS-based phylogenies (RISAL et al., 2012). Conversely, strains of *B. elkanii* and *B. japonicum* have been reported to be more efficient than *B. yuanmingense* in nitrogen-fixing in soybean (RISAL et al., 2010). The findings of these studies are thus consistent with phylogenetic differences within

Bradyrhizobium with respect to nodulation, with strains of *B. yuanmingense* appearing to be more efficient in BNF in mung bean.

With regards to isolate's soil of origin, observed differences in the shoot dry weight of inoculated plants corroborate the data obtained for nodulation by native rhizobia and plant development (Figure 1a, c, Figure 5b and Figure 6), indicating that the modified Leonard jars using soil sample as inoculant allows the selection of efficient rhizobia. These results revealed that strains of *Bradyrhizobium* spp. isolated from Mato Grosso state soils in Cerrado region tend to have low efficiency with respect to nodulation and BNF in mung bean, when compared with isolates obtained from the Atlantic Forest region. Accordingly, it is suggested that *Bradyrhizobium* strains isolated from soil samples from the Atlantic Forest region may be promising candidates for the inoculation of mung bean in tropical Brazilian soils, mainly for the Cerrado region, thereby contributing to the promotion of BNF and plant growth.

3.7 CONCLUSIONS

The finding of this study demonstrated that the nodulation of mung bean with native rhizobia is variable in soils from Brazil; soils from the Cerrado from Mato Grosso State showed low nodulation and, consequently, low plant development. Isolation of bacteria from mung bean nodules revealed a predominance of the genus *Bradyrhizobium*, and the isolates were categorized into 12 phylogenetic groups based on 16S rRNA gene sequencing. In contrast to the findings of previous studies, isolates of *Rhizobium*, *Mesorhizobium*, and *Ensifer* did not induce nodulation in mung bean. With a single exception, all *Bradyrhizobium* isolates were observed to induce nodulation; however, differences were observed with respect to their effects on plant development, and appear to be related to phylogenetic grouping and the soil of origin. Isolates that promoted the highest shoot dry weights formed a phylogenetic group close to *B. yuanmingense* B071. With respect to the soil of origin, we established that plants inoculated with *Bradyrhizobium* isolates originating from Mato Grosso state soils (Brazilian Cerrado) were characterized by lower shoot dry weight, which may be related to the low nodulation of mung bean grown in these soil samples. Our results indicate that selection of *Bradyrhizobium* isolates should be performed for the inoculation of mung bean in tropical Brazilian soils.

4 CAPÍTULO II

Bradyrhizobium AS THE ONLY RHIZOBIAL INHABITANT OF MUNG BEAN (*Vigna Radiata*) NODULES IN TROPICAL SOILS: A STRATEGY BASED ON MICROBIOME FOR IMPROVING BIOLOGICAL NITROGEN FIXATION USING BIO-PRODUCTS

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4.1 RESUMO

O feijão-mungo apresenta potencial de cultivo em condições tropicais devido à elevada adaptabilidade. Além disso, sua capacidade de se beneficiar da fixação biológica de nitrogênio (FBN) através da associação com rizóbios diminui a dependência da aplicação de fertilizantes nitrogenados. As comunidades microbianas do solo são influenciadas por fatores biogeográficos e pelas propriedades do solo, e representam uma fonte de rizobactérias capazes de estimular o crescimento das plantas. Nesse sentido, objetivou-se com este estudo, investigar as comunidades microbianas de nódulos de feijão-mungo cultivado em solos brasileiros, como forma de apoiar a seleção de bactérias benéficas em interação com plantas de feijão-mungo cultivadas em solos tropicais, como parte de um programa de inoculação de sementes para aumentar a produtividade com base na FBN e outros mecanismos. Para isso, dois genótipos de feijão-mungo (Camaleão e Esmeralda) foram cultivados em 10 amostras de solo, e o microbioma dos nódulos foi caracterizado por NGS (*next-generation sequencing*) Illumina MiSeq usando o gene 16S rRNA. Mais de 99% das sequências bacterianas presentes nos nódulos mostraram semelhança com o gênero *Bradyrhizobium*, o único rizóbio presente em nódulos de feijão-mungo em nosso estudo. Uma maior diversidade bacteriana foi associada ao genótipo Esmeralda cultivado nas amostras de solo coletadas em áreas do agronegócio (MW_MT-I, II ou III); ambos os genótipos apresentaram elevada diversidade bacteriana quando cultivados na amostra de solo do agroecossistema orgânico (SE_RJ-V). Além disso, as OTUs próximas a *Bradyrhizobium elkanii* dominaram em todas as amostras de solo, exceto na amostra do agroecossistema orgânico, onde apenas *B. japonicum* esteve presente. A comunidade bacteriana de nódulos de feijão-mungo foi influenciada principalmente pelo pH do solo, e pelos teores de K, Ca e P. Além disso, também foi detectada uma diferença relativa aos genótipos quanto à colonização dos nódulos por sequências agrupadas em uma OTU próxima ao gênero *Pseudomonas*. Embora estejam em baixa abundância, em torno de 0,1% do total, as sequências da OTU agrupada à *Pseudomonas* foram recuperadas apenas de nódulos do genótipo Esmeralda, sugerindo uma característica diferente quanto à especificidade entre macro e microsimbiontes. A análise do microbioma orientará as próximas etapas no desenvolvimento de um inoculante para feijão-mungo, composto por uma cepa eficiente de *Bradyrhizobium* de forma isolada ou coinoculada com uma cepa de *Pseudomonas*. Diante dos resultados alcançados, a avaliação de parâmetros de ecologia microbiana é uma potente ferramenta capaz de acelerar o processo de desenvolvimento de inoculantes e melhorar os benefícios à cultura pelos microrganismos do solo.

Palavras-chave: Feijão-mungo. Microbioma. Nódulo. Rizóbio nativo. *Bradyrhizobium*. Simbiontes. *Pseudomonas*. Fixação biológica de nitrogênio.

4.2 ABSTRACT

The mung bean has a great potential under tropical conditions given its high content of grain protein. Additionally, its ability to benefit from biological nitrogen fixation (BNF) through association with native rhizobia inhabiting nodule microbiome provides most of the nitrogen dependence on fertilizers. Soil microbial communities which are influenced by biogeographical factors and soil properties, represent a source of rhizobacteria capable of stimulating plant growth. The objective of this study is to support selection of beneficial bacteria that form positive interactions with mung bean plants cultivated in tropical soils, as part of a seed inoculation program for increasing grain yield based on the BNF and other mechanisms. Two mung bean genotypes (Camaleão and Esmeralda) were cultivated in ten soil samples. Nodule microbiome was characterized by next-generation sequencing using Illumina MiSeq 16S rRNA. More than 99% of nodule sequences showed similarity with *Bradyrhizobium* genus, the only rhizobial present in nodules in our study. Higher bacterial diversity of soil samples collected in agribusiness areas (MW_MT-I, II or III) was associated with Esmeralda genotype, while an organic agroecosystem soil sample (SE_RJ-V) showed the highest bacterial diversity independent of genotype. Furthermore, OTUs close to *Bradyrhizobium elkanii have* dominated in all soil samples, except in the sample from the organic agroecosystem, where *B. japonicum* was also present. Bacterial community of mung bean nodules is mainly influenced by soil pH. Besides a difference on nodule colonization by OTU sequences close to the *Pseudomonas* genus regarding the two genotypes was detected too. Although representing a small rate, around 0.1% of the total, *Pseudomonas* OTUs were only retrieved from nodules of Esmeralda genotype, suggesting a different trait regarding specificity between macro- and micro-symbionts. The microbiome analysis will guide the next steps in the development of an inoculant for mung bean aiming to promote plant growth and grain yield, composed either by an efficient *Bradyrhizobium* strain on its own or co-inoculated with a *Pseudomonas* strain. Considering the results achieved, the assessment of microbial ecology parameters is a potent coadjuvant capable to accelerate the inoculant development process and to improve the benefits to the crop by soil microorganisms.

Keywords: Mung bean. Microbiome. Nodule Native rhizobia. *Bradyrhizobium* Symbionts. *Pseudomonas*. Biological nitrogen fixation.

4.3 INTRODUCTION

Mung bean [*Vigna radiata* (L.) Wilczek] is a widely cultivated crop on the Asian continent. It has good adaptability to tropical climate conditions and its grain has high nutritional value (DU et al., 2018; YI-SHEN; SHUAI; FITZGERALD, 2018). Mung bean was introduced in Brazil several decades ago, being characterized as a small-scale crop with low national consumption (BARRADAS; SAYÃO; DUQUE, 1989; DUQUE; SOUTO; ABBOUD, 1987). However, the cultivated area has been increasing in recent years, aiming to meet the export demand of Asian countries, especially India. Nowadays mung bean has been cultivated in agribusiness areas of the Brazilian Cerrado in succession to soybean and corn. The low implantation cost, short cycle, good temperature adaptation and water regime contribute to its development and grain yield increasing its acceptance by farmers (HANUMANTHARAO; NAIR; NAYYAR, 2016; NAIR et al., 2012; SHARMA et al., 2016).

Mung bean benefits from biological nitrogen fixation (BNF) through association with native rhizobia (HERRIDGE et al., 2005), which decreases the demand for the nitrogen fertilizer application. Overall, mung bean shows low specificity towards *Bradyrhizobium* genus (RISAL et al., 2012) and it benefits from seed inoculation with efficient selected elite strains (DELIĆ et al., 2011). Phylogeny and symbiotic efficiency studies show that organisms belonging to the *Bradyrhizobium* genus are the most important micro-symbiont for this species (APPUNU et al., 2009; RISAL et al., 2012; YANG et al., 2008; ZHANG et al., 2008). In addition to *Bradyrhizobium*, other genera belonging to the large group of rhizobia are also reported as mung bean symbionts, despite being less studied: *Ensifer* (= *Sinorhizobium*) (HAKIM et al., 2018, 2020), *Rhizobium* (YANG et al., 2008; ZHANG et al., 2008) and *Mesorhizobium* (LU et al., 2009).

Sequencing 16S rRNA gene amplicons from root nodules, Hakim et al. (2018) showed a codominance of *Bradyrhizobium* and *Ensifer* genera as micro-symbionts of mung bean cultivated in Pakistan. However, a subsequent study identified a dominance of up to 94 and 99% of sequences belonging to *Bradyrhizobium* and *Ensifer* genera, respectively, depending on the soil (HAKIM et al., 2020). This difference in the mung bean nodule microbiome shows that soil characteristics directly influence the plant-microorganism relationship.

Studies on microbial community of legume root nodules have shown the presence of several non-rhizobial bacteria (NRB) genera (HAKIM et al., 2018; LEITE et al., 2009, 2017; MARTÍNEZ-HIDALGO; HIRSCH, 2017). Until recently, it was thought that legume nodules were only inhabited by rhizobia, according to the plant trait regarding host specificity. However, recent studies have shown a different picture where nodules from several legume species have a great diversity of microorganisms (ASERSE et al., 2013; CARDOSO et al., 2018; DE MEYER et al., 2015; LEITE et al., 2017; MARTÍNEZ-HIDALGO; HIRSCH, 2017; TRABELSI; CHIHAOUI; MHAMDI, 2017; ZHANG et al., 2018). Hence, the microbial community structure and the role of most NRB in the plant/rhizobia symbiotic relationship are still poorly understood. The evaluation of nodule microbial community composition is a potent tool which enables selecting beneficial microorganisms to improve plant development. Furthermore, this knowledge can assist in the development of multi-organism biological products aiming to increase grain yield. Several studies have focused on the possible benefits to cultivated plant by atmospheric N fixation (ANDREWS; ANDREWS, 2017; DE MEYER et al., 2015), biocontrol activity (BERG et al., 2017), or plant growth promotion (TARIQ et al., 2014), among others.

There are currently no studies related to BNF and nodule NRB for mung bean under agroecosystem conditions in Brazil. Therefore, we used the 16S rRNA Illumina MiSeq sequencing to investigate bacterial mung bean root nodule microbiomes, aiming to characterize the composition of rhizobia and NRB in different soils in Brazil, and to verify differences in

the nodule communities between two mung bean genotypes. Our study aims to support the selection of beneficial microorganisms for mung bean plants as part of a seed inoculation program for increasing grain yield based on BNF and other bacterial functions.

4.4 MATERIAL AND METHODS

4.4.1 Plant cultivation

Mung bean plants were grown using Leonard jars (VINCENT, 1970) maintained in a greenhouse located in Seropédica, RJ, Brazil. An experiment was conducted in a factorial scheme (soil × genotype) and a randomized block design with three replications: ten soil samples from different regions of Brazil and two mung bean genotypes, MGS Esmeralda (VIEIRA et al., 2008) and the Camaleão cultivars. Ten soil samples were collected in agricultural areas located in the Midwest and Southeast Brazilian regions, previously cultivated with mung bean and/or other legumes (Figure 7 and Table 4). Ten simple samples were collected with an auger at 0-20 cm depth, homogenized and sieved (< 4 mm) to obtain a composite sample. Two mung bean cultivars registered at the Brazilian Ministry of Agriculture, Livestock and Supply were used: MGS Esmeralda (registry 22096) and Camaleão (registry 36829). MGS Esmeralda was developed by Asian Vegetable Research and Development Center (Shanhua, Taiwan), as a result of crossing between the lines VC 1973A and VC 2768A (VIEIRA et al., 2008). Camaleão is a recently released cultivar in 2018, developed by Minas Gerais Agricultural Research Agency, EPAMIG. Seeds of both cultivars are available at EPAMIG (asagro@epamig.br).

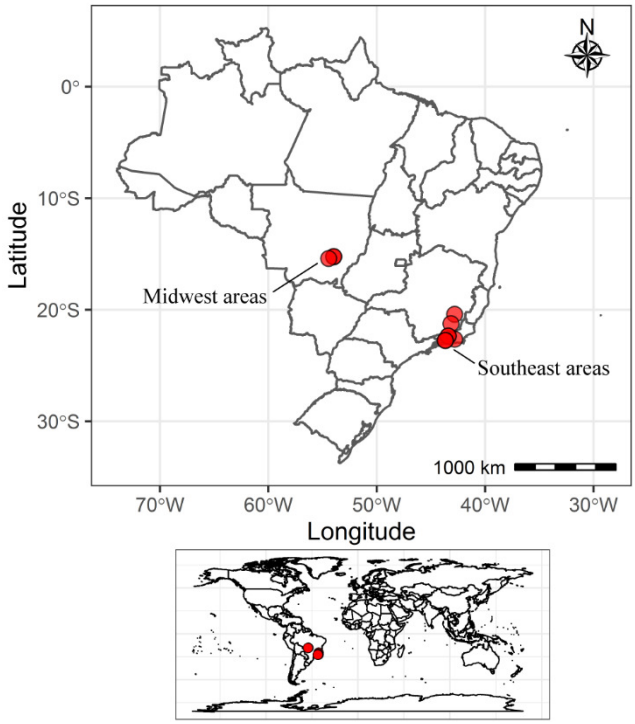

Figure 7. Map of Brazil with the locations of soil sample collection sites.

Seven and three soil samples were collected from areas under conventional and organic management, respectively. The three Midwest areas (MW_MT-I, MW-MT-II and MW_MT-III), the two Minas Gerais state areas (SE_MG-I and SE_MG-II) and two areas from Rio de Janeiro state (SE_RJ-I and SE_RJ-IV) were under conventional management, while the three areas under organic management were located in Rio de Janeiro state (SE_RJ-II, SE_RJ-III and SE_RJ-V). Six areas belong to experimental fields located in research and educational governmental centers used for field trials with different agricultural crops (SE_MG-I, SE_MG-

II, SE_RJ-II, SE_RJ-III, SE_RJ-IV and SE_RJ-V). The Midwest areas are typical of intensive agriculture located in the Brazilian Cerrado region (MW_MT-I, MW-MT-II and MW_MT-III), while SE_RJ-I is characterized as a family farming.

Leonard jars were adapted so that the soil was used as an inoculum. The vessels were filled with approximately 600 cm^3 of substrate composed of sterilized gravel and vermiculite (2:1 v v^{-1}). A layer of soil material (100 cm^3) was added on the surface of the sterilized substrate. Seeds were sown directly in the soil layer and then a final layer of sterilized sand was added to the top. The seeds used were superficially disinfested by immersion in 70% ethanol and hydrogen peroxide for one and three minutes, respectively, followed by ten washes in sterile distilled water (VINCENT, 1970).

Five seeds per jar were sown and then thinned to two plants per jar. Next, 300 mL of Norris' nutrient solution devoid of N and sterilized in an autoclave was applied, weekly into each jar (NORRIS; DATE, 1976). In the first week, a nutrient solution with half the ionic strength was used. Plants were collected at 35 days after emergence.

Table 4. Soil sample identification, location, cultivation history, precipitation, and fertility analysis of soil material used for planting mung bean in Leonard jars

Soil sample*	Latitude and longitude	Cultivation history	Annual average rainfall**	pH	Al^{3+}	Ca^{2+}	Mg^{2+}	P	K^{+}	C
			mm		------- $cmol_c\ dm^{-3}$ -------			---- $mg\ L^{-1}$ ----		%
MW_MT-I	15°14'05.8"S 53°58'51.1"W	Soybean, corn and sorghum	1794	6.45	0.00	3.50	1.56	104.37	136.98	1.35
MW_MT-II	15°13'37.9"S 53°58'48.1"W	Mung bean, soybean, cowpea and corn	1794	5.55	0.00	2.95	0.92	115.33	270.40	1.40
MW_MT-III	15°23'33.5"S 54°26'46.7"W	Mung bean, soybean, cowpea and corn	1794	4.30	0.34	2.88	0.77	214.78	190.34	1.85
SE_MG-I	20°24'07.57"S 42°49'05.08"W	Mung bean	1269	4.61	0.33	1.24	0.36	74.47	149.39	0.70
SE_MG-II	21°14'36.74"S 43° 9'30.55"W	Common bean	1391	5.98	0.00	4.04	1.03	71.09	216.39	1.31
SE_RJ-I	22°38'4.61"S 42°48'40.40"W	Mung bean, common bean and cowpea	1100	4.44	2.35	1.29	0.39	138.17	49.00	3.64
SE_RJ-II	22°20'52.36"S 43°25'2.24"W	Common bean and cowpea	1192	5.74	0.00	1.70	0.24	92.15	80.42	0.44
SE_RJ-III	22°20'54.84"S 43°25'2.27"W	Azuki and mung bean	1192	5.91	0.00	3.32	1.26	161.48	224.70	1.27
SE_RJ-IV	22°45'22.27"S 43°40'2.03"W	Common bean and cowpea	1100	5.73	0.00	1.90	0.49	24.03	85.89	0.68
SE_RJ-V	22°45'16.36"S 43°40'28.04"W	Mung bean, soybean, peanut and other vegetables	1100	6.51	0.00	3.40	0.73	155.59	139.96	0.86

*Soil origin: SE = Southeast, MW = Midwest, RJ = Rio de Janeiro, MG = Minas Gerais and MT = Mato Grosso. **Annual average rainfall for the region based on data from the last ten years (Source: National Institute of Meteorology - INMET). Al^{+3} (aluminum), Ca^{2+} (calcium), Mg^{2+} (magnesium), P (phosphorus), K^{+} (potassium) and C (carbon). Analysis methods: pH = Potentiometric, Aluminum = Titration, Ca = Atomic absorption, Mg = Atomic absorption, H+Al = Titration, P = Colorimetric, K = Flame photometry, Carbon = Walkey & Black.

4.4.2 DNA extraction from nodules

Plants were collected, nodules were detached from the roots and kept in a super freezer (-80 °C). For extraction, nodules were superficially disinfested by soaking in 70% ethanol for one minute and in sodium hypochlorite (4-6%) for five minutes, followed by eight washes in sterile distilled water. The disinfestation procedure was performed in 15 mL tubes using approximately 2 mL of reagent or water at each step added and removed by an automatic pipette. Homogenization was carried out in a bench vortex for 15 seconds in each step. After disinfestation, 500 mg of nodules were macerated in liquid nitrogen, followed by DNA extraction using the Fast DNA Spin Kit for Soil (MObio) according to the manufacturer's instructions.

4.4.3 16S rRNA gene amplification

Nodule DNA was purified and subjected to next-generation sequencing (NGS) with Illumina MiSeq library preparation by Macrogen (Korea, Seoul). The V3-V4 region of the 16S rDNA gene was amplified using 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGTATCTAATCC) (HERLEMANN et al., 2011) primers with amplification of approximately 440 base pairs (bp).

4.4.4 Bioinformatics and data analysis

The sequences were analyzed with Mothur software (v. 1.44.2) (SCHLOSS et al., 2009). The forward and reverse sequences were grouped into contigs using the make.contigs command and processed using screem.seqs to remove ambiguous sequences and those that had more than eight homopolymers. Sequences under 440 and over 443 bp were excluded. They were then aligned using the Silva ribosomal RNA gene database (v. 138) (QUAST et al., 2013). Misaligned strings and non-informative columns were removed using the screen.seqs and filter.seqs commands. Rare sequences were grouped with the abundant sequences using the pre.cluster command with a difference threshold of 4 bp. Chimeric sequences were then removed using the chimera.vsearch command (ROGNES et al., 2016). Classification was performed using the Ribosomal Database Project (COLE et al., 2009) to gender level, with 80% bootstrap. Mitochondria, chloroplast, archaea, eukaryote, and unknown domain sequences were eliminated using the remove.lineage command. Single sequence operational taxonomic units (OTUs) were removed and samples were randomly subsampled to the smallest sample size. Taxonomy and distribution data from OTUs were exported and used in other programs. We filter OTUs based on abundance, and OTUs with less than forty sequences have been removed.

Rarefaction curves were generated by Mothur and plotting in the R environment (R CORE TEAM, 2020). Number of observed OTUs, Chao1 estimator, diversity by the Shannon index and Shannon evenness were also generated by Mothur, and analyzed for significant differences through analysis of variance (anava) in the R environment (v. 4.0.2) (R CORE TEAM, 2020) using the ExpDes.pt package (v. 1.2.0) (FERREIRA et al., 2013). Betadiversity data were generated using the phyloseq package (v. 1.32.0) (MCMURDIE; HOLMES, 2013), and analyzed for significant difference through permutational multivariate analysis of variance (PERMANOVA) using the "adonis" function and for dispersion of variances with the "betadisper", both with 999 permutations and using the vegan package (v. 2.5-6) (OKSANEN et al., 2019) in the R environment (R CORE TEAM, 2020). For plotting, we use the ggplot2 (v. 3.3.0) (WICKHAM, 2016) and cowplot (v. 1.0.0) (WILKE, 2016) packages.

After filtering the less abundant OTUs, we carry out an alignment and phylogenetic analyze of the most abundant sequences of the classified OTUs. For this, we use the MEGA 7

software (KUMAR; STECHER; TAMURA, 2016). The phylogenetic tree was built using the Maximum likelihood (ML) method using the Kimura 2-parameter + G model (KIMURA, 1980). This model was chosen based on the best model tool available in the MEGA 7 software. The bootstrap values were shown when the relationships represented were observed in at least 50% of the 1000 replicates.

4.5 RESULTS

4.5.1 Characteristics of amplicon libraries

A total of 4,301,345 raw tags stemming from 16S rRNA gene amplification were obtained. After read-quality filtering and perform a 97% sequence similarity using Mothur with Silva ribosomal RNA gene database, 1,182,393 sequences were acquired, corresponding to 19,706 sequences per sample (range = 19,559 to 19,777), spread along 1,094 OTUs.

Next, a second sequence filtering aiming at eliminating low abundance and low variance OTUs yields 1,177,815 sequences and a mean value per sample of 19,630 (range = 19,451 to 19,727). This last step removed 4,578 sequences, approximately 0.39% from the total, but brought down OTU number to 17, reflecting a high level of rare sequences scattered through the samples obtained from the original data. The 17 OTUs range from a minimum of 42 to a maximum of 1,175,646 sequences. On average 10 ± 2 OTUs were detected per sample.

Besides the high rare sequence amount, a unique OTU present in all samples accounted for more than 99.8% suggesting a framework of ecological dominance on nodule microbiome. The methodology used for filtering our data produced satisfactory rarefaction curves (Figure 8) and estimated coverage values greater than 99.9% (GOOD, 1953), which revealed that OTU libraries were sufficiently large to capture the most common OTUs and, that the probability to find a new OTU is nearly zero (coverage deficit).

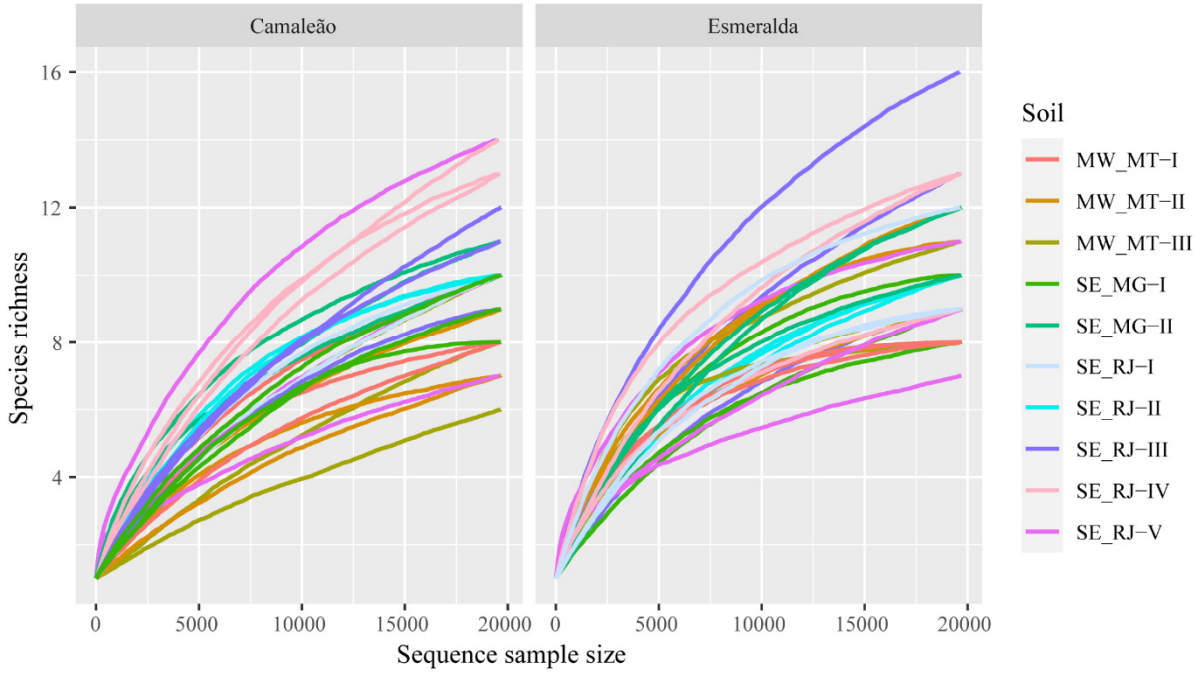

Figure 8. Rarefaction curves for species richness at 97% similarity as a function of sample size for Camaleão and Esmeralda mung bean genotypes and ten soil samples.

4.5.2 Bacterial community richness and diversity

There was not a significant interaction between soil samples and mung bean genotypes for number of OTUs and richness index Chao1 (Figure 9). A greater number of OTUs was recovered from nodules of mung bean cultivated on sterile substrate mixed with soil samples collected at SE_MG-II, SE_RJ-III and SE_RJ-IV in comparison to other soil samples (p = 0.002) (Figure 9a). SE_RJ-III and SE_RJ-IV soil samples also promoted high values for Chao1

index (p = 0.013) (Figure 9c). Mung bean genotypes on its own were not capable to influence observed OTUs and Chao1 index (Figure 9b and d).

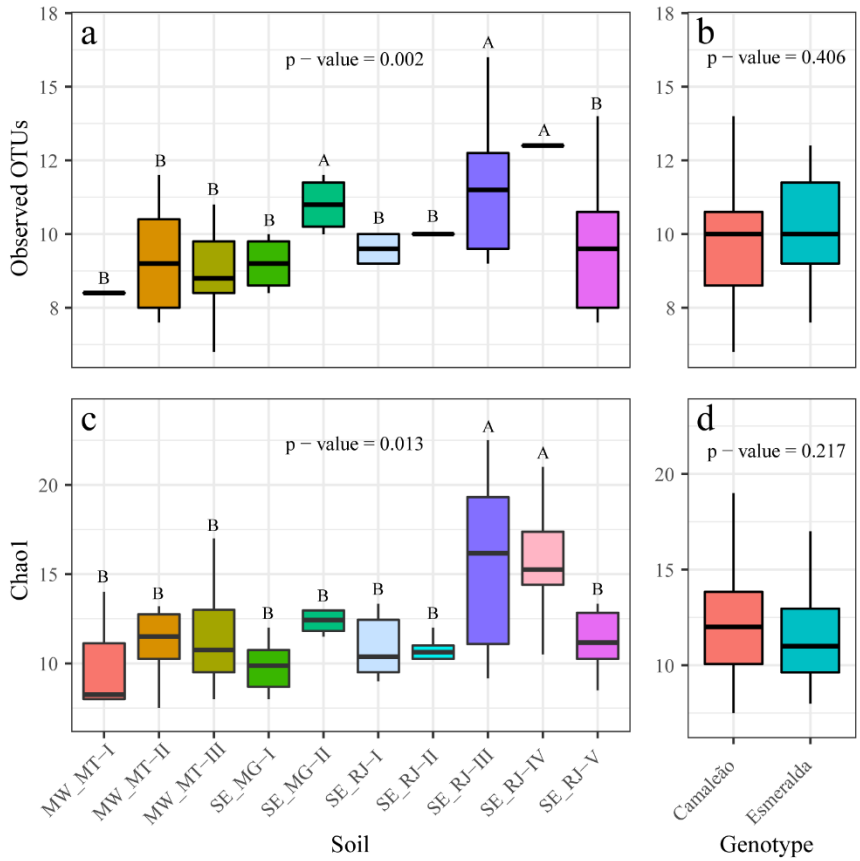

Figure 9. Number of observed OTUs (a and b) and estimated richness by Chao1 (c and d) of bacterial communities from nodules of Camaleão and Esmeralda mung bean genotypes inoculated with ten different soils. p - values are based on anava. Distinctive letters indicate statistical difference by the Scott–Knott test at 5% probability.

Community Shannon´s diversity and evenness showed interaction between mung bean genotypes and the origin of soil samples, at a probability of 0.035 and 0.008, respectively (Figure 10a and b). Nodule microbiome from plants grown on MW-MT-I, MW-MT-II and MW-MT-III soil samples revealed values for Esmeralda genotype that were about twice the values observed for Camaleão considering both diversity and evenness. Diversity of remaining genotypes were not affected by soil origin.

Soil sample from SE_RJ-V area showed higher diversity and evenness indices when compared to other soil samples (Figure 10a and b). This area is an organically managed area where diverse plant cultivation practice may be associated to high nodule microbiome diversity, although it is not capable to stimulate a high OTU number nor a Chao1 richness index (Figure 9a and c). Diversity and evenness indices deal with both diversity and abundance, but according to our data the result is related mainly to abundance.

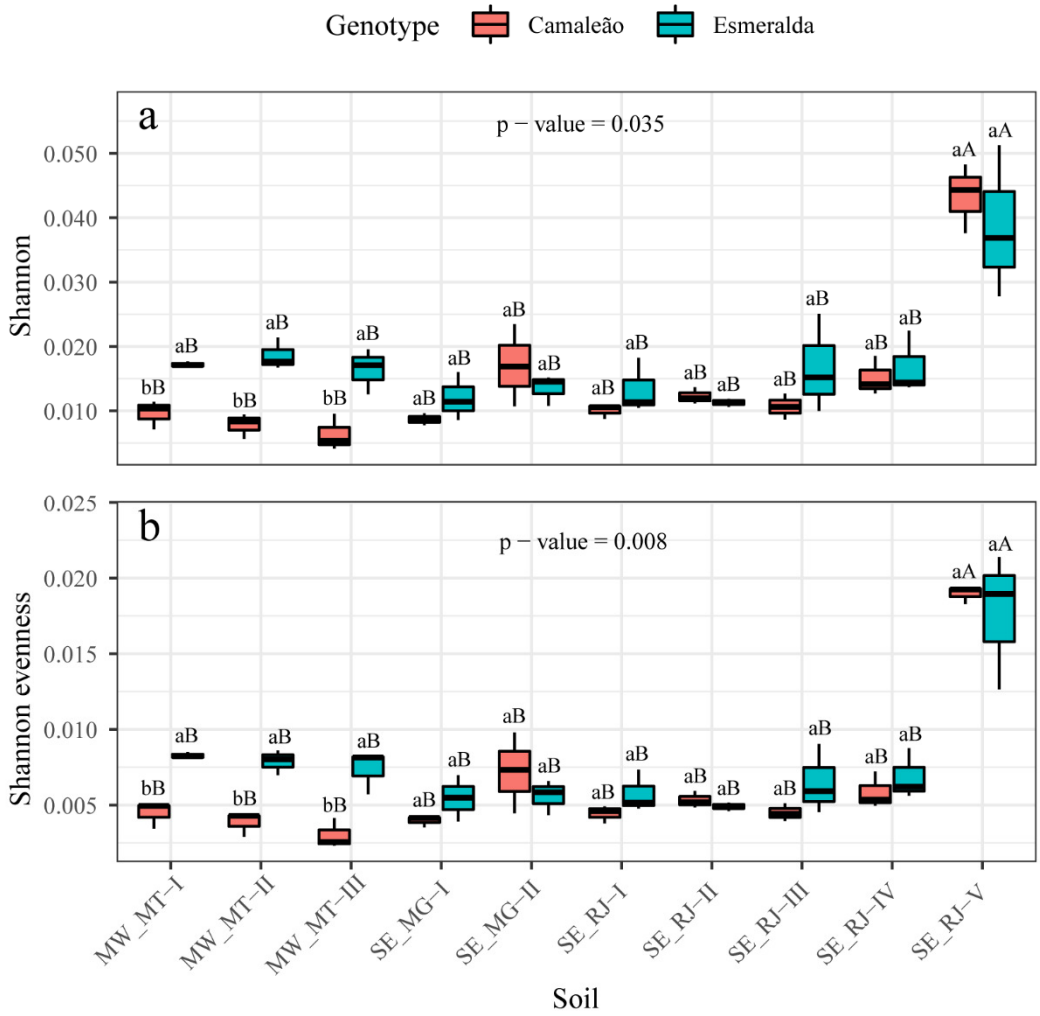

Figure 10. Shannon's diversity (a) and evenness (b) of bacterial communities from nodules of Camaleão and Esmeralda mung bean genotypes inoculated with ten different soils. *p* - values are based on anava. Distinctive letters, lowercase between genotypes and uppercase between soils, indicate statistical difference by the Scott–Knott test at 5% probability.

4.5.3 Mung bean genotypes and soil origins on nodule bacterial community composition

The beta diversity evaluation through PERMANOVA analysis showed a significant difference among soils (p = 0.001) (Figure 11a) and between genotypes (p = 0.002) (Figure 11b). Principal Coordinate Analysis (PCoA) explains a large part of the data variability, corresponding to 94.9% in the two axes. Regardless of the soil type, distribution of OTUs was homogeneous, except for nodule communities retrieved from SE_RJ-V soil sample, which was completely separated from the others (p = 0.001) (Figure 11a). Although Esmeralda genotype had a larger diversity than Camaleão, considering OTUs composition, soil origin influences the latter more than the former as it moves away from the central core, especially when cultivated in SE_RJ-V soil sample. Ten Camaleão bacterial communities are outside the intersection area, where most samples are concentrated. In contrast, just five Esmeralda bacterial communities are found outside the central core. Under these study conditions, both plant genotypes and soil origin seem to affect bacterial community diversity inhabitanting mung bean nodule.

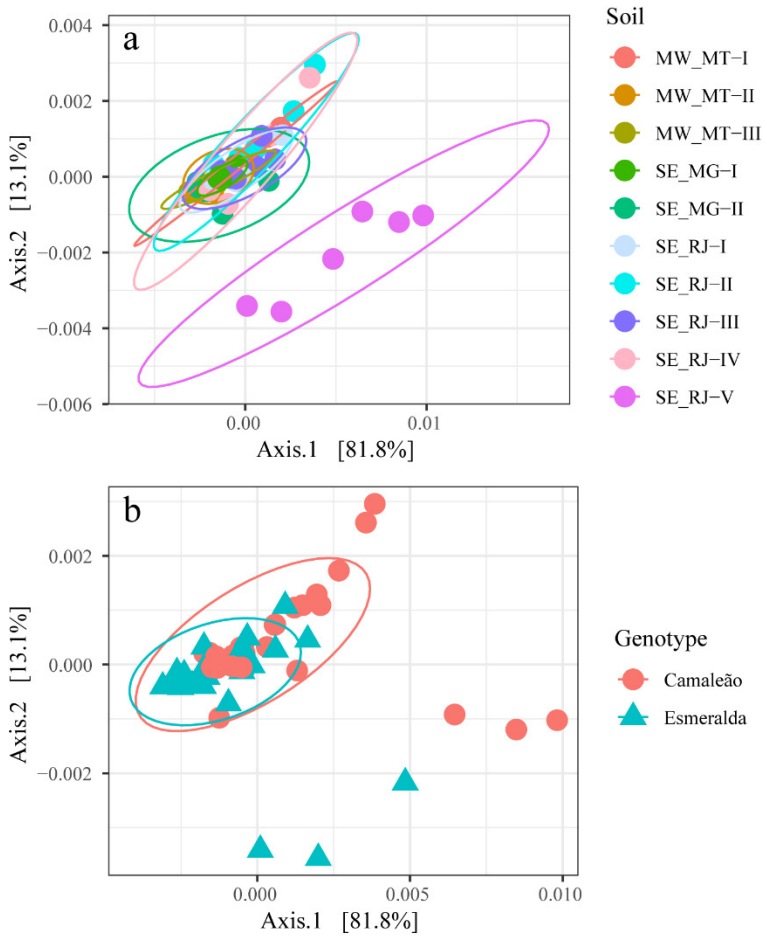

Figure 11. Principal coordinates analysis (PCoA) of Bray-Curtis distances and the Permutational MANOVA (PERMANOVA) according to nodule bacterial communities estimated by 16S rRNA gene sequencing. Samples coded for 10 soil samples (p = 0.001) (a), and from genotypes Camaleão and Esmeralda of *Vigna radiata* (p = 0.002) (b) at the OTU level.

A Canonical analysis of principal coordinates (CAP) between nodule bacterial communities and chemical fertility variables of soil samples explained 39.8% of the variance considering the two axes, and an anova analysis determined the significance level (p = 0.001) (Figure 12). From this analysis, pH had a greater influence on the bacterial community of mung bean nodules from Camaleão genotype when grown in SE_RJ-V soil sample, whereas, Esmeralda nodule community in this soil sample is related to K, Ca and P concentrations (Figure 12). In general, the points referring to the SE_RJ-V soil are more distant from the others, and presented a higher pH value, around 6.51, while the other soils, an average of 5.41.

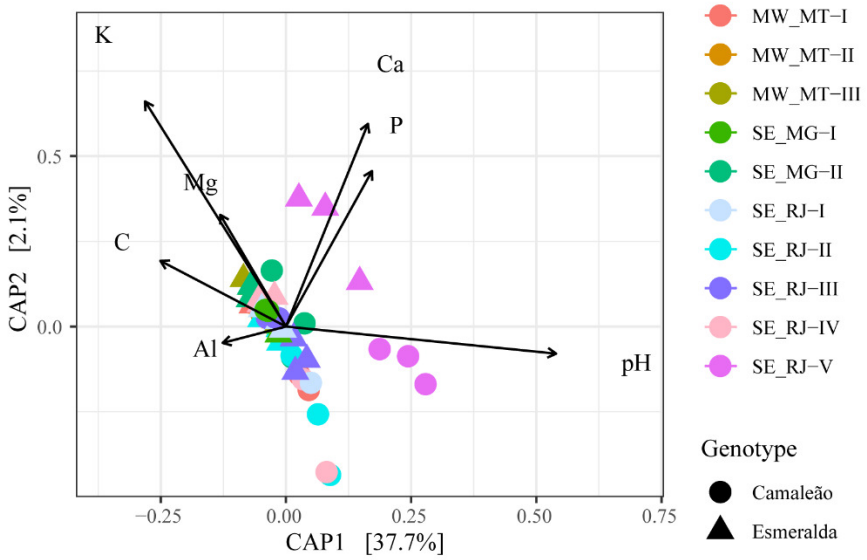

Figure 12. Canonical analysis of principal coordinates (CAP) of Bray-Curtis distances and the Permutational ANOVA between nodule bacterial communities (10 soil samples x 2 mung bean genotypes) estimated by 16S rRNA gene sequencing and the chemical data of soil samples presented in Table 3: pH, Al^{3+} (aluminum), Ca^{2+} (calcium), Mg^{2+} (magnesium), P (phosphorus), K^{+} (potassium) and C (carbon).

4.5.4 Characterization of bacterial taxa present in mung bean nodules and phylogenetic analysis

Upon filtration, the representative OTU_S retrieved from mung bean nodules belong only to *Alphaproteobacteria* and *Gammaproteobacteria* classes. The *Bradyrhizobium* genus was prevalent regardless of soil samples or mung bean genotypes, and two OTUs (OTU0001 and OTU0002) were associated to this genus (Figure 13a). OTU0001 corresponded to more than 99% of the sequences of nodule bacteria for all samples. Therefore, for better visualization of less abundant groups, we also present the data of relative distribution without OTU0001 (Figure 13b). In this case, *Bradyrhizobium* OTU0002 was characteristic of soil sample SE_RJ-V for both genotypes. These data corroborate the differences already observed for the alpha and beta diversity analyses (Figure 10a, b and Figure 11a). No sequences representing other rhizobial genera were found.

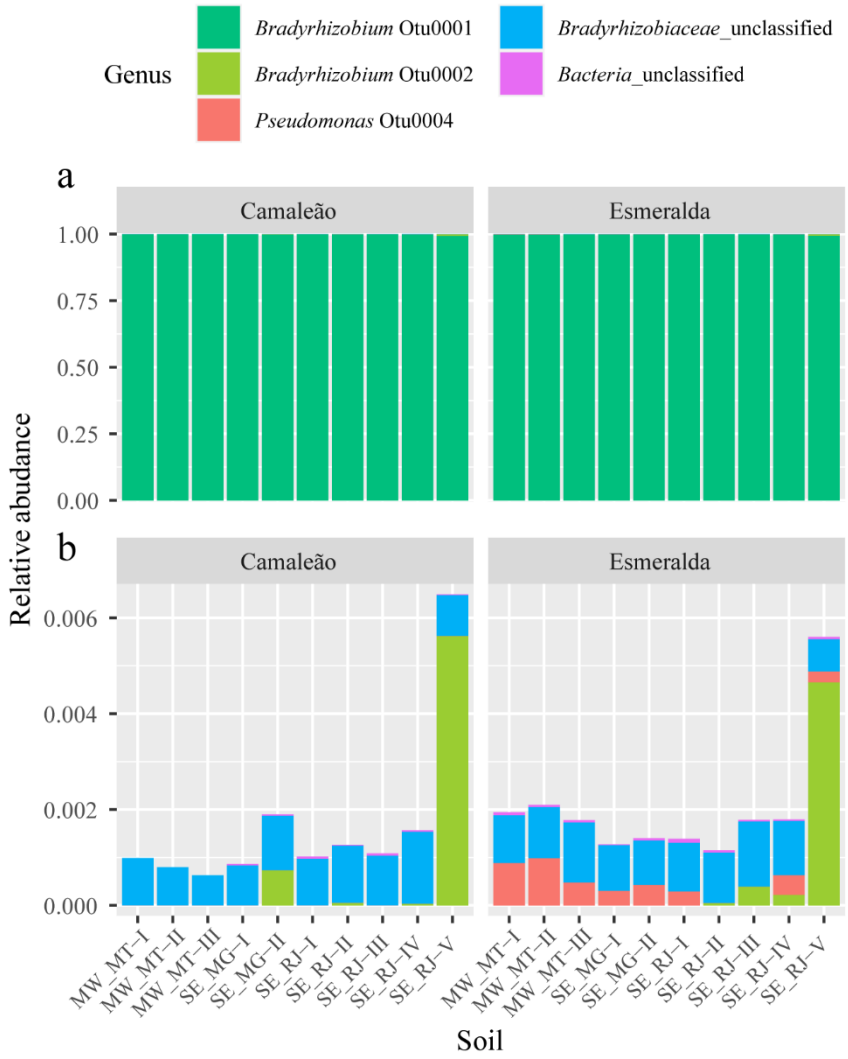

Figure 13. Relative abundance of sequences based on the 16S rRNA gene from nodules of Camaleão and Esmeralda mung bean genotypes cultivated in ten different soil samples: relative abundance for all OTUs (a); and, for low abundance OTUs, after removal of OTU0001 (*Bradyrhizobium*) (b).

After filtering the less abundant OTUs, we found only one NRB in nodule communities. The OTU0004 was classified as a *Pseudomonas* and it was recovered only from Esmeralda genotype nodules cultivated in eight soil samples (Figure 13b). This OTU corresponded approximately to 0.1% of the total sequences analyzed for the Esmeralda genotype. Only nodules from SE_RJ-I and SE_RJ-II soils were not colonized by *Pseudomonas*. As pointed out before, these results suggest a difference in the specificity trait between mung bean genotypes, as only Esmeralda genotype allows nodule occupation by *Pseudomonas* strains. Moreover, the data shows differences in soil bacterial communities regarding the *Pseudomonas* genus, since it was not present in Esmeralda nodules from plants grown in two out of ten evaluated soils.

OTU0001 and OTU0002 related to *Bradyrhizobium* genus and OTU0004 related to *Pseudomonas* genus had 5770, 587 and 111 representative sequences, respectively. We performed a phylogenetic analysis using the ten most abundant sequences from OTU0001 and five from each OTU0002 and OTU0004, which represented 98.8% of the total sequences of our data after filtering. The other OTUs were not classified at the genus level, therefore, were not

included. As for the representative sequences of *Pseudomonas*, none of the sequences were related to the described species (Figure 14).

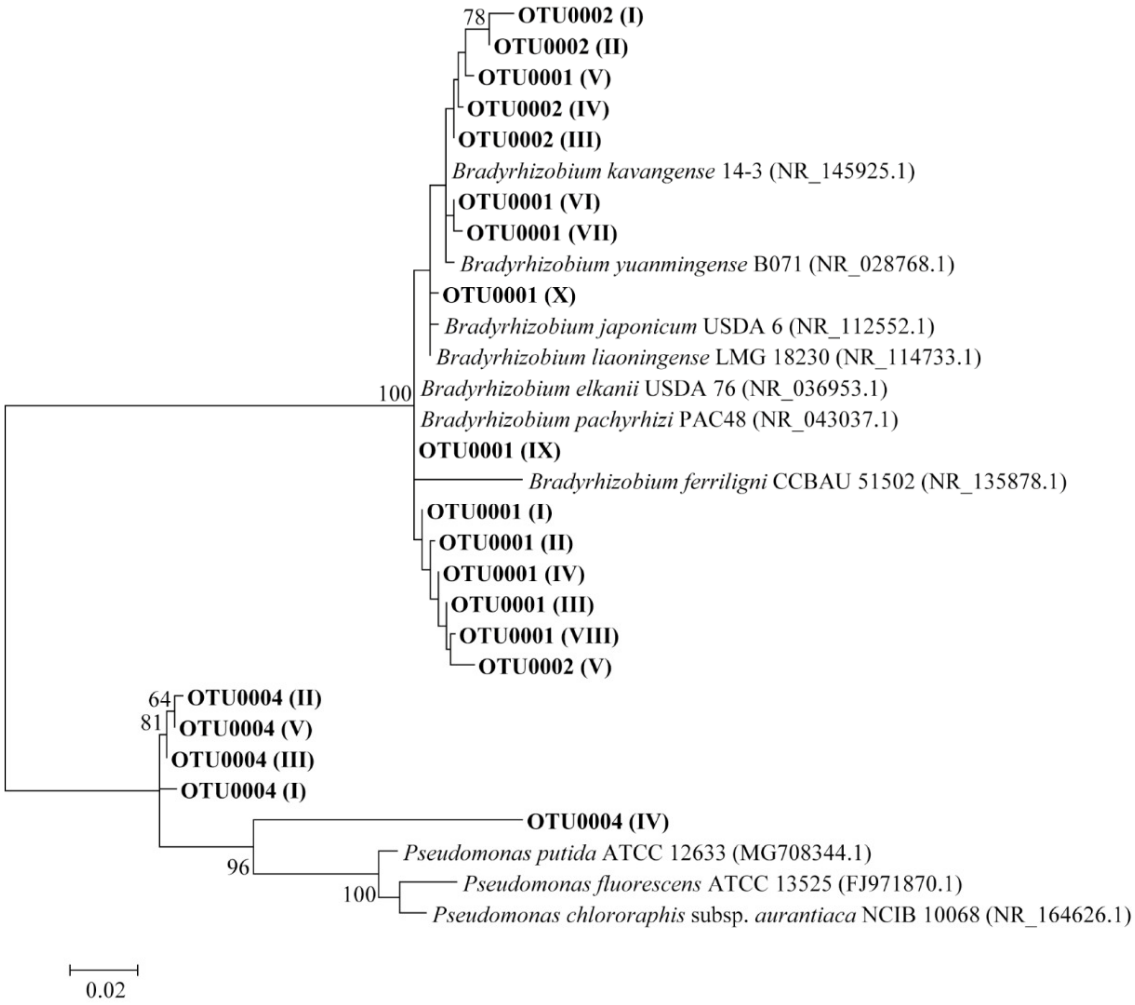

Figure 14. Maximum likelihood phylogenetic tree for OTUs classified, based on 16S rRNA gene sequences. Tree estimated through 441 base pair positions. The ten most abundant sequences from OTU0001 and five from OTU0002 and OTU0004 were used. Letters in Roman numerals indicate order of abundance of the strings within their respective OTUs. Bootstrap values are shown when the relationships represented have been observed in at least 50% of 1000 replicates. The tree was obtained using the Kimura 2-parameter + G model.

The representative sequences of OTU0001 were divided between the *Bradyrhizobium japonicum* and *B. elkanii* superclades, while most of the OTU0002 sequences were grouped within *B. japonicum* (Figure 14). Besides that, all sequences associated to nodules from SE_RJ-V soil sample belong to *B. japonicum* (Figure 15), while, in the remaining soil samples, the vast majority of sequences is affiliated to *B. elkanii*. Furthermore, in this soil, all the sequences phylogenetically evaluated either from OTU0001 or OTU0002 were grouped with *B. japonicum*, regardless of the mung bean genotype (Figure 15).

Figure 15. Distribution of the ten most abundant sequences of OTU0001 and five of OTU0002 belonging to the *Bradyrhizobium* genus, based on the 16S rRNA gene from nodules of Camaleão and Esmeralda mung bean genotypes cultivated in ten different soil samples.

4.6 DISCUSSION

As a general pattern, nodules from Esmeralda genotype presented greater bacterial diversity than nodules from Camaleão, especially when associated to certain soil samples tested in the present study. Domestication of plant species is recognized to cause a strong decrease on genetic diversity of modern crop cultivars (PÉREZ-JARAMILLO; MENDES; RAAIJMAKERS, 2016). Therefore, germplasms submitted to an intense selection process through several crossing of varieties aiming to improve stability of a desirable trait tend to be more restrictive regarding microbial associations (GEPTS, 2004; GROSS; OLSEN, 2010; KIM et al., 2014; MUTCH; YOUNG, 2004; PÉREZ-JARAMILLO; MENDES; RAAIJMAKERS, 2016). Although plant traits capable to drive microbiome assembly and functions are largely unknown, plant breeding is understood as an opportunity to shape efficient colonization with elite strains, contributing to promote an increase in plant biomass and grain yield, or improve resistance to pests and disease (BERG et al., 2017; MENDES; GARBEVA; RAAIJMAKERS, 2013; PARTIDA-MARTÍNEZ; HEIL, 2011). The low bacterial diversity determined on nodule microbiome of Camaleão genotype may suggest that as the breeding program proceeds, specificity towards the micro-symbionts increases and may be the reason why it is not colonized by *Pseudomonas* strains.

Considering soil origin influence, a greater alpha and beta bacterial diversity was found in nodules of plants cultivated in SE_RJ-V soil sample. A Canonical analysis of principal coordinates related the microbial community present in Camaleão nodules mainly to pH values, whereas Esmeralda nodule community is related to K, Ca and P concentrations (Figure 12). It is known that soil microbial communities are influenced by soil properties (FIERER; JACKSON, 2006; SANTOYO et al., 2017). For example, a greater abundance of the *Ensifer* genus was found in mung bean nodules grown in higher pH soils (HAKIM et al., 2020), and a positive relationship between microbial diversity with soil pH (ANDREW et al., 2012; ROUSK et al., 2010).

Besides that, plant genotype due to its physiological traits might interact in a specific way with soil attributes and that may result on distinct nodule microbial communities. Nevertheless, it must be emphasized that microbial communities from the remain nine soil samples did not differentiate between them (Figure 11). These data suggest that microbial community distribution is influenced by other factors than soil chemistry, such as the area crop history and management type. High crop diversity is a main feature associated to soil sample from SE_RJ-V area where organic agriculture follows the principle of plant diversification through crop rotation, green manure, crop alley and agroforestry as standard practices. SE_RJ-V area is an integrated system for agroecological production which has been implemented since 1993, aiming to establish a high diversity environment free from synthetic fertilizer and pesticide application attained by the constant cultivation of several and diverse green manure fertilizers, grain legumes, vegetable, and fruit species (NEVES et al., 2005). Soils from organic farming systems are reported to have greater microbial diversity due to reduced use of agrochemicals (HARTMANN et al., 2015; RAMIREZ; CRAINE; FIERER, 2012; WANG et al., 2016, 2017; WEESE et al., 2015; XIA et al., 2015). Furthermore, high diversity above ground tends to boost below ground diversity (BERG; SMALLA, 2009; HARTMANN et al., 2009; OFEK et al., 2014; SMALLA et al., 2001; VAN DER PUTTEN; KLIRONOMOS; WARDLE, 2007), which may explain the highest diversity found in the nodule microbiome, since soil is regarded to be the main source of nodule bacteria (MARQUEZ-SANTACRUZ et al., 2010).

The observed prevalence of *B. japonicum* needs to be further investigated in this area, but it is possible that high diversity might also exert some influence on nodule microbiome.

This high discrimation among the two superclades, brings a new knowledge about nodule bacteria and may be part of a strategy to improve BNF contribution for mung bean.

Nodule microbiome analysis of rhizobial populations shows the *Bradyrhizobium* genus as the predominant mung bean micro-symbiont in Brazilian tropical soils independent from both plant genotypes and whether soil samples present (or do not) a history of mung bean cultivation. A predominance of a rhizobial genus in legume nodules is commonly reported (HAKIM et al., 2020; ROCHA et al., 2020; SHARAF et al., 2019; ZHENG et al., 2020). Isolation of diazotrophic strains from mung bean nodules cultivated in different soils worldwide have shown a predominance of *Bradyrhizobium* strains (APPUNU et al., 2009; RISAL et al., 2012; YANG et al., 2008; ZHANG et al., 2008). However, *Ensifer* strains have also been reported to be isolated from mung bean nodules (PANDYA; KUMAR; RAJKUMAR, 2013; YANG et al., 2008; ZHANG et al., 2008). In another study, a microbiome characterization of mung bean nodules from an experimental field area in Pakistan using pyrosequencing recently showed a co-dominance of strains from both *Bradyrhizobium* and *Ensifer* (HAKIM et al., 2018). Illumina sequencing was used for analyzing nodule bacterial communities in mung bean cultivated in four major cropping areas of Pakistan, the results identified bacterial nodule communities dominated by either *Bradyrhizobium* or *Ensifer* strains, depending on the edaphoclimatic conditions (HAKIM et al., 2020). *Ensifer* sequences corresponded to 99% of the total nodule rhizobial sequences in mung bean cultivated in a desert soil, while *Bradyrhizobium* sequences amounted to up to 94% under milder conditions. In contrast to data from the studies performed in Pakistan, we did not find OTUs related to *Ensifer* in the present study, suggesting that this genus is either not present or it is not capable of colonizing mung bean cultivated in tropical soil conditions.

An evaluation of nodule microbiome of cowpea cultivated in Brazilian soils also did not detect sequences of the *Ensifer* genus (LEITE et al., 2017). However, results from rhizobial isolation using specific cultural medium reveal that cowpea displays a low symbiotic specificity towards the micro-symbiont, and therefore it is able to nodulate with a broad range of different rhizobial species: *Bradyrhizobium*, *Rhizobium*, *Ensifer* and *Mesorhizobium* (SILVA et al., 2012; TULU et al., 2018; ZHANG et al., 2007). The low specificity trait has led cowpea to be used as a trap plant in several studies for isolating rhizobia from soil (CASTRO et al., 2017; GUIMARÃES et al., 2012; SILVA et al., 2012). Controversial results obtained from different techniques are not uncommon and may be either caused by the use of rich nutrient culture media which may favor some bacterial groups, which is nevertheless a minor nodule occupant; or inherent differences due to amplification efficiency of each sequence. This may explain why some rhizobium strains isolated from nodules display poor or no capacity as a micro-symbiont (LU et al., 2009; YANG et al., 2008; ZHANG et al., 2008; ZILLI et al., 1999). The lack of *Ensifer* sequences in both cowpea and mung bean nodules analyzed by culture-independent methods suggests that the genus may have limited symbiotic ability under tropical edaphoclimatic conditions.

Presence of *Ensifer* genus from the nodule microbiome of mung bean cultivated in Pakistan may be related to characteristics of local soils. OTUs belonging to the *Ensifer* genus have been found in mung bean nodules grown in alkaline soils with a pH higher than 7.8 (HAKIM et al., 2020). Furthermore, the *Ensifer* genus was only found to be dominant in desert soil (HAKIM et al., 2020). The soils used in our study have acidic characteristics, with pH between 4.3 and 6.5, and whose place of origin have mean annual precipitation varying from 1,100 to 1,794 mm. Microbial communities are mainly influenced by soil characteristics such as pH (ANDREW et al., 2012; FIERER; JACKSON, 2006; LAUBER et al., 2009; ROUSK et al., 2010), temperature (BRAKER; SCHWARZ; CONRAD, 2010; GARCIA-PICHEL et al., 2013; ZHOU et al., 2016) and rainfall (CHEN et al., 2015). The data from both Pakistan and

Brazil suggest that the *Ensifer* genus is mainly capable to form symbiosis when mung bean is cultivated in arid alkaline soils.

Rhizobium and *Mesorhizobium* strains isolated from mung bean nodules using cultural media were capable of forming nodules under controlled conditions (LU et al., 2009; YANG et al., 2008; ZHANG et al., 2008). However, we did not detect sequences of these genera in our study. A small percentage of sequences belonging to *Rhizobium* and *Mesorhizobium* genera was found in mung bean nodules grown in Pakistan, corresponding to 2.06 and 0.06% for *Rhizobium* and *Mesorhizobium*, respectively (HAKIM et al., 2018). In another study, *Mesorhizobium* OTUs were not found, while *Rhizobium* corresponded to only 0.8% of the total sequences analyzed (HAKIM et al., 2020). In a study conducted in Venezuela, *Rhizobium* strains have been isolated from mung bean nodules (RAMÍREZ et al., 2020). In this sense, the absence of OTUs from *Ensifer*, *Rhizobium* and *Mesorhizobium* genera in our study could be related to either a PCR bias caused by the low concentration of these organisms, or a possible allocation of these OTUs as unclassified groups (LEE et al., 2012; VAN DIJK; JASZCZYSZYN; THERMES, 2014). In conclusion, although these genera are able to form nodules in mung bean, they may not be considered as a main micro-symbiont for the crop. It is possible that some of these strains are like a nodule NRB, which nevertheless does not have a clear role.

The *Pseudomonas* genus was the most abundant NRB observed on the nodule bacterial populations evaluated, except for the SE_RJ-II, SE_RJ-III soil samples and Camaleão genotype. These results suggest that bacterial nodule diversity is influenced by the soil and regulated by the plant, which implies that specificity towards the micro-symbionts may be genotype dependent (HARTMANN et al., 2009; LEITE et al., 2017; LIU et al., 2019b; WAGNER et al., 2016). Furthermore, *Pseudomonas* sequences may have arised from Esmeralda seeds, but since this has been observed in eight out of ten soil samples, we might consider that other intrinsic factors are influencing the pattern. Plant tissue endophytes may originate either from environmental infection (horizontal transmission), or be vertically transmitted via seed or vegetative propagation (JOHNSTON-MONJE; RAIZADA, 2011; YAN et al., 2019). Legume nodule colonization by symbiotic bacteria is a consequence of a complex genetic mechanism which has been well described, while colonization mechanisms by NRB are still unclear, although it appears the plant plays an important role (GAGE, 2020; WANG; LIU; ZHU, 2018). In comparing plant and soil origin influences on nodule colonization by NRB, plant genotypes possess more favorable traits related to nodule occupancy by microbial communities (MURESU et al., 2008; PANDYA; KUMAR; RAJKUMAR, 2013; REGUS et al., 2017; SACHS; QUIDES; WENDLANDT, 2018; WESTHOEK et al., 2017; ZGADZAJ et al., 2015). Nodulation factors involved in plant-microorganism chemotaxis may lead to a selection of associated organisms (KOBAYASHI; BROUGHTON, 2008; WANG et al., 2012). *Pseudomonas* strains have already been found in mung bean nodules grown in Pakistan (HAKIM et al., 2018, 2020), as well as in other legume species (ASERSE et al., 2013; CARDOSO et al., 2018; DE MEYER et al., 2015; HOQUE; BROADHURST; THRALL, 2011; KUKLINSKY-SOBRAL et al., 2004; LEITE et al., 2017; OLIVEIRA-LONGATTI et al., 2014).

In addition to *Pseudomonas*, another 16 NRB genera were shown by in our unfiltered data (Table with raw data is available in the Supplementary Material section available at https://doi.org/10.3389/fpls.2020.602645). Except for *Pseudomonas* strains, the presence of NRB genera sequences does not seem to be important, considering the low numbers and the inconsistency among replicates. The under representativeness of these taxa may be related to a low soil bacterial abundance or to inherent difficulties during the amplification reaction by the NGS method.

In summary, the knowledge acquired from our results should support the development of new inoculants for mung bean under tropical condition. To this end, two premises were used: soil samples from agriculture areas with a legume cultivation history were used as a seed inoculant; and, conditions where plant were grown would favor BNF, consisting of a reduced amount of soil and a nutrient solution devoid of N. The dominance of *Bradyrhizobium* strains inhabiting mung bean nodules cultivated on different soil samples, regardless of plant genotype, suggests that this is the main nodule micro-symbiont for the crop in Brazilian tropical soil areas evaluated. Additionally, the bacterial communities showed the ability to form reddish nodules, which is an indicative of the presence of leghemoglobin and an active nitrogenase (LARRAINZAR et al., 2020; OTT et al., 2005; SINGH; VARMA, 2017).

The bacterial community strategy used in our study was capable to provide the identification of a pattern that may guide the development of a new rhizobial inoculant for mung bean capable of increasing BNF and grain yield. Technological implementation will require isolating and selecting efficient strains for the crop, which will be our next step continuing this work. In addition, co-inoculation with *Pseudomonas* strains will also be evaluated. A greater richness of beneficial microorganisms colonizing nodules is thought to contribute to plant growth, as well as to improve resistance to pathogens. From the results, we suggest that an efficient *Bradyrhizobium* strain on its own or co-inoculated with *Pseudomonas* strains, in this case, dependent on the plant genotype, could promote mung bean growth and improve grain yield, thereby resulting in a better cost/benefit ratio taking into account the agriculture production.

5 CAPÍTULO III

Bradyrhizobium STRAINS FROM BRAZILIAN TROPICAL SOILS PROMOTE INCREASES IN NODULATION, GROWTH AND NITROGEN FIXATION IN MUNG BEAN

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5.1 RESUMO

O feijão-mungo [*Vigna radiata* (L.) Wilczek] é uma leguminosa de origem asiática cultivada em diversos países, inclusive no Brasil, onde seu cultivo comercial tem se expandido nos últimos anos. Tem uma associação promíscua com rizóbios para fixação do N_2, mas a nodulação em solos brasileiros tem sido altamente variável. Para obter rendimentos satisfatórios com baixo custo de produção, estudos estão sendo realizados para selecionar rizóbios nativos de solos brasileiros que resultem em associações simbióticas eficientes com feijão-mungo. No Brasil, um estudo anterior indicou que apenas estirpes de *Bradyrhizobium* foram capazes de nodular feijão-mungo em condições axênicas. Nesse sentido, objetivou-se com este estudo avaliar o efeito de 31 estirpes de *Bradyrhizobium* e níveis crescentes de fertilizantes nitrogenados na nodulação, crescimento das plantas e fixação de N_2. As estirpes de *Bradyrhizobium* resultaram em aumentos de até 71, 79, 43, 66, 40 e 55% no número de nódulos e biomassa de nódulos, raízes e de parte aérea, N acumulado e contribuição da fixação biológica de nitrogênio (FBN), respectivamente, em comparação com o controle não inoculado, no entanto, não houve aumento no rendimento de grãos. Em comparação com a testemunha com fertilizante nitrogenado, as plantas inoculadas apresentaram menor biomassa e conteúdo de N acumulado, apesar da alta contribuição da FBN. De modo geral, as estirpes agrupadas filogeneticamente ao super clado de *Bradyrhizobium japonicum*, principalmente aquelas próximas a *B. yuanmingense* B071, apresentaram maior eficiência em relação às do super clado de *B. elkanii*. A aplicação de fertilizante nitrogenado aumentou a biomassa de raiz e de parte aérea, porém reduziu a nodulação. Estirpes de *Bradyrhizobium* isoladas de solos tropicais brasileiros apresentaram potencial para inoculação de feijão-mungo, porém, a contribuição do FBN não foi suficiente para atender a demanda de N da planta.

Palavras-chave: Feijão-mungo. *Vigna radiata*. Estirpes de *Bradyrhizobium*. Inoculação. Fixação biológica de nitrogênio. Doses starter de N.

5.2 ABSTRACT

Mung bean [*Vigna radiata* (L.) Wilczek] is a legume of Asian origin cultivated in several countries, including Brazil, where its commercial cultivation has expanded in recent years. It has a promiscuous N_2-fixing association with rhizobia, but nodulation in Brazilian soils has been highly variable. To achive satisfactory yields with low production costs, studies are being carried out to select rhizobia from soils in Brazil that result in efficient symbiotic associations with mung bean. Previous studies indicate that only *Bradyrhizobium* strains were able to nodulate mung bean in Brazilian soils. The objective of this study was to evaluate the effect of 31 *Bradyrhizobium* strains and increasing levels of N fertilizer on nodulation, plant growth and N_2 fixation. *Bradyrhizobium* strains resulted in increases of up to 71, 79, 43, 66, 40 and 55%, respectively, in nodule number and nodule, root and shoot biomasses, accumulated N and biological nitrogen fixation (BNF) contribution compared to the uninoculated control, however, there was no increase in grain yield. Compared to the control with N fertilizer, inoculated plants had lower biomass and accumulated N, despite the high BNF contribution. In general, strains of *Bradyrhizobium japonicum* superclade, especially those close to *B. yuanmingense* B071, showed greater efficiency compared to those of the superclade of *B. elkanii*. The application of N fertilizer increased root and shoot biomasses, however, significantly reduced nodulation. *Bradyrhizobium* strains isolated from Brazilian tropical soils showed potential for mung bean inoculation, however, the BNF contribution was not sufficient to meet the plant's N demand.

Keywords: Mung bean. *Vigna radiata*. *Bradyrhizobium* strains. Inoculation. Biological nitrogen fixation. Starter doses of N.

5.3 INTRODUCTION

Legume grains are important sources of protein to feed the population around the world, especially in developing countries (CHENG et al., 2019). Mung bean [*Vigna radiata* (L.) Wilczek] is a legume widely cultivated in Asia (LAMBRIDES; GODWIN, 2007) and is noted for its short growth cycle (NAIR et al., 2012), adaptation to high temperature and water stress (HANUMANTHARAO; NAIR; NAYYAR, 2016; SHARMA et al., 2016), and grain production with good nutritional characteristics (DU et al., 2018; YI-SHEN; SHUAI; FITZGERALD, 2018).

Mung bean is able to biologically fix atmospheric nitrogen through association with bacteria from the rhizobium group (HAYAT et al., 2008; HERRIDGE et al., 2005). However, a low population density of native or inefficient rhizobia can result in low yields. In this sense, studies have been carried out to verify the efficiency of bacterial strains in biological nitrogen fixation (BNF) in mung bean and possible increases in productivity through the inoculation technology.

Studies carried out in several countries have shown that mung bean can nodulate with bacteria of the genera *Bradyrhizobium* (YANG et al., 2008; ZHANG et al., 2008), *Ensifer* (HAKIM; IMRAN; MIRZA, 2021; ZHANG et al., 2008), *Rhizobium* (YANG et al., 2008; ZHANG et al., 2008) and *Mesorhizobium* (YANG et al., 2008). Owing to this diversity of micro-symbionts, mung bean is known as a legume with low symbiotic specificity. However, tests conducted in pot and field experiments have mostly used strains of *Bradyrhizobium* (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; DELIĆ et al., 2011; TARIQ et al., 2012). In Pakistan, studies of mung bean nodules indicate that the genera *Bradyrhizobium* and *Ensifer* may cohabit mung bean nodules (HAKIM et al., 2018). *Ensifer* strains were also studied in inoculation trials in the field in Pakistan and one strain showed a similar performance to *Bradyrhizobium* (HAKIM et al., 2020). However, in Brazilian tropical soils, it was found that more than 99% of bacterial sequences from mung bean nodules belong to the genus *Bradyrhizobium* (FAVERO et al., 2021b), and it is the only genus able to nodulate the plant under axenic condition (FAVERO et al., 2021a).

Bradyrhizobium strains have been commonly used for mung bean inoculation in several countries, with a positive effect on plant development when compared to the soil native rhizobia population (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; DELIĆ et al., 2011; HAKIM et al., 2020; TARIQ et al., 2012). However, it is common to have a low increase in plant nodulation and development compared to uninoculated control (HERRIDGE et al., 2005; MATHU et al., 2012), and sometimes it is important to apply nitrogen doses associated with inoculation to improve mung bean grain yield (DELIĆ et al., 2011). These results suggest that screening of symbiotically efficient mung bean genotypes and competitive and effective *Bradyrhizobium* strains in promoting BNF should be carried out. The inoculation effect is believed to be greater in soils with a low population of native rhizobia (HERRIDGE et al., 2005). In Brazilian tropical soils, mung bean nodulation with native rhizobia has been variable, being lower in Brazilian Cerrado (tropical savanna) soils compared those of the Atlantic Forest (FAVERO et al., 2021a). In this sense, it is suggested that efficient strain selection studies should be carried out for mung bean inoculation in these areas.

In Brazil, the mung bean has been studied for decades in the evaluation of genotypes (DUQUE; PESSANHA, 1990; SAYÃO; BRIOSO; DUQUE, 1991; VIEIRA et al., 2002, 2008; VIEIRA; NISHIHARA, 1992), but there are no studies on the inoculation of *Bradyrhizobium* strains under non-axenic conditions. The first bacterial isolates from mung bean nodules were obtained in recent years (FAVERO et al., 2021a; SILVA et al., 2021). *Bradyrhizobium* strains isolated from nodules of mung bean cultivated in Brazil have been reported as important symbionts in studies under axenic conditions, having verified differences in efficiency in

relation to their phylogenetic group and the soil of origin (FAVERO et al., 2021a). Therefore, tests are needed to compare the most promising isolates in the presence of the native rhizobia population. To this purpose, we conducted an experiment with these most promising *Bradyrhizobium* isolates, with the objective to evaluate them for nodulation and their effect on mung bean development compared to native rhizobia, as well as on biological nitrogen fixation. Additionally, we carried out a second experiment to verify the effect of *Bradyrhizobium* inoculation associated with the application of N fertilizer as possible starter doses of nitrogen on mung bean nodulation and growth.

5.4 MATERIAL AND METHODS

5.4.1 Inoculation of *Bradyrhizobium* strains isolated from Brazilian tropical soils

a) Experimental design

An experiment was carried out in a greenhouse with mung bean [*Vigna radiata* (L.) Wilczek] MGS Esmeralda cultivar (VIEIRA et al., 2008) grown in pots containing 3 kg of soil. A randomized block design in a factorial scheme 33 x 2 was adopted (31 *Bradyrhizobium* strains + two controls, and two harvests, respectively), with 4 replications. The strains used for inoculation were selected from a study of bacterial isolates from mung bean nodules grown in Brazilian tropical soils and that performed better in the nodulation under axenic conditions, and which are deposited at the "Johanna Döbereiner Biological Resources Centre" at Embrapa Agrobiologia (FAVERO et al., 2021a, Appendix 6). In addition to the inoculated treatments, absolute (without inoculation or nitrogen application) and nitrogen (without inoculation and with nitrogen application) controls were added. Two harvests were made: 33 days after emergence (DAE) (beginning of flowering) to assess nodulation, plant development and the contribution of BNF using the ^{15}N natural abundance technique, and another 60 DAE (dry pods) to assess grain production. Pots were added to each block where sorghum [*Sorghum bicolor* (L.) Moench cultivar BRS 802], *Brachiaria brizantha* (cultivar Marandu) and non-nodulating common bean (*Phaseolus vulgaris* L. cultivar Norh 54) were grown separately to be used as reference plants[1] to estimate the contribution of BNF by the technique of natural abundance of ^{15}N.

b) Soil material collection

Soil (a Planosol – Alfisols) was taken to depth of 0-20 cm from a fallow area with no history of legume cultivation in the last ten years, located in Seropédica, Rio de Janeiro, Brazil (22°44'56.7"S, 43°40'00.9"W). Chemical analysis[2] (NOGUEIRA; SOUZA, 2005) of the soil was: pH, 5.0; Al^{3+}, 0.14 $cmol_c\ dm^{-3}$; Ca^{2+}, 0.63 $cmol_c\ dm^{-3}$; Mg^{2+}, 0.16 $cmol_c\ dm^{-3}$; H+Al, 2.06 $cmol_c\ dm^{-3}$; P, 5.49 mg dm^{-3}; K^+, 16.40 mg dm^{-3}; oxidisable C, 0.21%; and total N, 0.04%. The pH was increased by adding the equivalent of 1,500 kg ha^{-1} of dolomitic limestone (0.75 g kg^{-1} of soil), and fertilized with 100, 60 and 50 kg ha^{-1} (50, 30 and 25 mg kg^{-1} of soil, respectively) of P_2O_5, K_2O and fritted trace elements (FTE) (S, 3.9%; B, 1.8%; Mn, 2%; Cu, 0.8%; Zn, 9%), respectively. The equivalent of 5,000 kg ha^{-1} of dry and ground maize straw (4 mm sieve) (2.5 g kg^{-1} of soil) (with 0.57 and 38.3% of nitrogen and carbon, respectively) was added in order to immobilize part of the available mineral nitrogen, according to the recommendation of Brasil (2011). Application of limestone, fertilizers and straw per pot was carried out considering 2,000,000 kg^{-1} ha of soil to a depth of 20 cm.

c) Quantification of the population of native rhizobia

The population of native soil rhizobia able to nodulate mung bean was quantified using the most probable number (MPN) of infection in plants method, with cultivation in long neck bottles (VINCENT, 1970). Seeds of mung bean MGS Esmeralda cultivar were superficially

[1] $\delta^{15}N$ of soil obtained by reference plants: sorghum = 4.67, *Brachiaria brizantha* = 4.33, and non-nodulating common bean = 3.60.

[2] Analysis methods: pH = Potentiometric, Aluminum = Titration, Ca = Atomic absorption, Mg = Atomic absorption, H+Al = Titration, P = Colorimetric, K = Flame photometry, Carbon = Walkey & Black, N = Kjedahl.

disinfected using immersion in ethanol (70%) for one minute and in hydrogen peroxide for three minutes, followed by ten washes in sterile distilled water. The seeds were pre-germinated in Petri dishes with moistened paper in a growth chamber at 28 °C. The seedlings were transplanted into long neck bottles containing 300 mL of sterile, nitrogen-free nutrient solution (NORRIS; DATE, 1976). Twenty-four hours after transplanting, the seedlings were inoculated with 1 mL of a suspension of the serial decimal dilution of the soil sample, at concentrations from 10^{-1} to 10^{-8}, in triplicate. To make the suspension, 10 g of soil was initially used in 90 mL of saline solution (NaCl 0.85%). Subsequently, the moisture of the soil sample was determined by the greenhouse method to carry out the correction. A negative (uninoculated) and a positive control (inoculated with *Bradyrhizobium* BR 14454) were added. Plants were collected 30 days after inoculation to verify the presence or absence of nodules. Data were interpreted using the MPN table (ANDRADE; HAMAKAWA, 1994).

d) Culture conditions of isolates, seed inoculation and plant cultivation

The strains were grown in yeast mannitol culture medium (FRED; WAKSMAN, 1928) under agitation of 150 rpm at 28 °C for 6 days until they reached optical density at 600 nm above 0.8, indicating a concentration above 10^8 cells mL^{-1}.

The seeds were surface disinfected as described for the MPN assay, and five seeds were sown in each pot, and each one received 1 mL of inoculum according to its respective treatment. After emergence, two plants were retained in each pot. The nitrogen treatment received applications of 120, 240, 240, 360 and 360 mg of NH_4NO_3 per plant, at 2, 9, 16, 23 and 30 DAE, based on a dosage equivalent to 160 kg N ha^{-1} and a final stand of 350,000 plants ha^{-1}.

e) Variables and data analysis

At 33 DAE, the shoot tissue was cut at the insertion point of the cotyledon, and the roots were cut into two parts, in order to separate nodules on the first 5 cm of the lateral root ("crown") and those lower on the roots. The nodules were detached from both parts of the roots separately, allowing an assessment of the pattern of distribution of the nodules in the root between the treatments used. Nodules, roots and shoots were dried to a constant weight at 65 ºC. A second harvest was taken at 60 DAE, at the mature pod stage. The shoots, roots and grain were collected and dried, as described above.

After drying, the shoot tissue samples from the first harvest 33 DAE were ground in a Wiley mill (< 1 mm) and subsequently to a fine powder with a roller mill (ARNOLD; SCHEPERS, 2004). Aliquots containing 30 to 50 µg N were analyzed for ^{15}N abundance using an automated continuous flow isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to the output of a Costech Total C and N Analyzer (model ECS-4010) in the "John Day Stable Isotope Laboratory" of Embrapa Agrobiologia.

The percentage of N derived from the atmosphere (%Ndfa) was estimated for the shoot tissue using the method of Shearer & Kohl (1986):

$$\%Ndfa = 100 \times \frac{\delta^{15}\text{Nref} - \delta^{15}\text{Nmung bean}}{\delta^{15}\text{Nref} - \text{B}}$$

where δ^{15}Nref and δ^{15}Nmung bean are the ^{15}N abundance values of the reference plants and the mung bean plants, respectively, and 'B' value is the ^{15}N abundance of mung bean when grown with BNF as the only N source. The B value of -2.5‰ for mung bean shoots was obtained from Unkovich et al. (2008).

Data were subjected to analysis of variance to verify the significant difference between treatments, and the assumptions of normality and homogeneity of the residuals were verified using the Shapiro-Wilk and Bartlett tests, respectively. Variables with significant differences indicated by the F test of the analysis of variance had the treatment means grouped using the Scott-Knott test at 5% probability. The nitrogen control plants did not nodulate and therefore were not included in the analysis of variance. To fulfill the assumptions described above, the data on the nodule number and the % nodule dry weight at the first 5 cm from the root were transformed by $\lambda = -0.4$ and $\lambda = 2.05$, respectively, using the Box-Cox (SAKIA, 1992) through the MASS package (v. 7.3-53.1) (VENABLES; RIPLEY, 2002). Means were plotted using the ggplot2 package (v. 3.3.0) (WICKHAM, 2016). The correlation between the variables was verified for the inoculated treatments through a principal component analysis using the factoextra package (v. 1.0.7) (KASSAMBARA; MUNDT, 2020). All statistical analyzes and data graphs were performed in the R environment (v. 4.0.5) (R CORE TEAM, 2021).

5.4.2 *Bradyrhizobium* inoculation associated with the application of fertilizer N doses

A second experiment was carried out in a greenhouse with the mung bean MGS Esmeralda cultivar (VIEIRA et al., 2008) grown in pots containing 3 kg of soil. A randomized block design in a 7 × 2 factorial scheme was adopted, for N doses and harvests, respectively, with 5 replications. Six N doses associated with the inoculation of *Bradyrhizobium* BR 14454 strain were used: 0, 15, 30, 45, 60 and 75 kg N ha^{-1} (0, 28.9, 57.8, 86.7, 115.6 and 144.5 mg N kg^{-1} of soil, respectively) in the form of NH_4NO_3, based on 350,000 plants ha^{-1}. For inoculation, we chose the BR 14454 strain due to the promising results based on the data from the first experiment. Additionally, an absolute control without inoculation and without the application of N fertilizer was adopted. Harvests were made 17 and 38 DAE, allowing to verify the effect of N doses on nodulation and biomass in an early and late manner

Soil material from Planosol was used, as described above, and for this collection it had the following chemical characteristics (NOGUEIRA; SOUZA, 2005), as described above: pH, 5.8; Al^{3+}, 0.00 $cmol_c$ dm^{-3}; Ca^{2+}, 0.61 $cmol_c$ dm^{-3}; Mg^{2+}, 0.22 $cmol_c$ dm^{-3}; H+Al, 0.74 $cmol_c$ dm^{-3}; P, 6.03 mg dm^{-3}; K^+, 27.14 mg dm^{-3}; oxidisable C, 0.25%; and total N, 0.04%. Liming, fertilization, quantification of the population of native soil rhizobia able to nodulate mung bean and strain growth were also performed as described above. On the day of planting, N doses were incorporated into the soil, and the steps of seed disinfection, sowing, inoculation and plant thinning were carried out as described for the previous experiment.

Samples were collected at 17 and 38 DAE, and the variables number and dry weight of nodule, dry weight of root and shoot were obtained and analyzed as described above. Root dry weight and shoot were transformed by $\log(x + 1)$ to fulfill the assumptions of normality and homogeneity of the residuals. Variables with significant differences indicated by the F test of the analysis of variance had the treatment means compared using the Tukey test at 5% probability. In addition, nitrogen doses were submitted to regression analysis for comparison of N doses. All analyses were performed using the R environment (v. 4.0.5) (R CORE TEAM, 2021).

5.5 RESULTS

5.5.1 Inoculation of *Bradyrhizobium* strains isolated from Brazilian tropical soils

For the *Bradyrhizobium* strain inoculation assay, the quantification of native soil rhizobia able to nodulate mung bean using MPN indicated the presence of 3.5×10^2 rhizobia cells g $soil^{-1}$. In general, the mung bean plants responded well to the inoculation of *Bradyrhizobium* strains regarding nodulation, but the addition of N (NH_4NO_3), equivalent to 160 kg N ha^{-1} totally inhibited nodulation (Table 5). Thirteen inoculated treatments resulted in plants with a higher nodule number than the control without nitrogen addition. These treatments resulted in an average increase of 46% in the number of nodules and reached up to 71% (Table 5).

The nodule pattern distribution showed that all inoculated treatments resulted in a higher nodule number in the upper part of the root (Tabel 5). The inoculated treatments presented between 44 and 91% of the nodules in the upper part of the root, while the control without inoculation presented 32%. These results suggest that inoculation with *Bradyrhizobium* strains resulted in a change in the distribution pattern of root nodules, and that inoculated strains had preferentially colonized the upper part of the root.

Twelve inoculated treatments resulted in greater nodule dry weight than the absolute control (Tabel 5). The average increase in nodule dry weight was 52% with a maximum of 79%. At least 25 *Bradyrhizobium* strains resulted in higher nodule weight in the upper part of the root when compared to the uninoculated control. Eight strains had more than 85% of the nodule dry weight in the upper part of the root, while the absolute control had 35%. These results showed a similar trend as nodule number and suggest that the inoculation of specific strains resulted in increases in mung bean nodulation.

The root and shoot dry weight were higher for 12 inoculated treatments in comparison to the absolute control and corresponded to an average increase of 27 and 49%, respectively (Tabel 5), and the other strains showed similar values to the uninoculated control. Of those 12 strains with the greatest increases in root and shoot biomass, nine had higher nodule dry weight than the absolute control and three were similar. Compared to nitrogen control, all inoculated treatments had lower values for root and shoot dry weight. The group of inoculated treatments that had the highest root and shoot weight represented only 63 and 60% of the weight of the nitrogen control for root and shoot, respectively. For the shoot nitrogen accumulation, 17 treatments showed a mean 23% higher than the absolute control.

Table 5. Means and standard errors of nodule number, percentage of nodule number in the crown position, nodule dry weight and percentage of nodule dry weight of crown position of mung bean plants cultivated in pots with soil and inoculated with different *Bradyrhizobium* strains, in addition to absolute control. Plants collected 33 days after emergence

Treatment	Nodule number	Percentage of nodule number in the crown position	Nodule dry weight	Percentage of nodule dry weight in the crown position
	plant^{-1}	%	mg plant^{-1}	%
BR 14474	119 ± 10 a	77 ± 1 a	227 ± 18 a	85 ± 2 a
BR 14532	117 ± 17 a	83 ± 5 a	210 ± 17 a	89 ± 4 a
BR 14531	122 ± 8 a	77 ± 1 a	206 ± 11 a	85 ± 3 a
BR 14446	100 ± 7 a	85 ± 3 a	205 ± 32 a	92 ± 2 a
BR 14458	112 ± 2 a	82 ± 1 a	196 ± 13 a	89 ± 1 a
BR 14442	107 ± 3 a	82 ± 5 a	190 ± 9 a	87 ± 4 a
BR 14440	133 ± 1 a	77 ± 2 a	187 ± 3 a	80 ± 2 b
BR 14454	120 ± 13 a	65 ± 10 b	187 ± 16 a	77 ± 10 b
BR 14438	91 ± 9 b	65 ± 2 b	182 ± 13 a	82 ± 2 b
BR 14477	96 ± 3 b	74 ± 4 a	180 ± 22 a	81 ± 3 b
BR 14482	113 ± 9 a	65 ± 2 b	176 ± 6 a	76 ± 2 b
BR 14452	99 ± 6 a	79 ± 3 a	175 ± 6 a	86 ± 2 a
BR 14514	108 ± 6 a	75 ± 2 a	164 ± 6 b	80 ± 3 b
BR 14494	94 ± 9 b	73 ± 5 a	163 ± 3 b	81 ± 3 b
BR 14444	86 ± 7 b	64 ± 6 b	162 ± 15 b	72 ± 5 c
BR 14472	104 ± 12 a	58 ± 7 b	158 ± 13 b	67 ± 10 c
BR 14479	87 ± 10 b	57 ± 4 b	156 ± 12 b	70 ± 5 c
BR 14529	96 ± 12 b	91 ± 2 a	156 ± 20 b	94 ± 1 a
BR 14455	84 ± 2 b	45 ± 5 c	155 ± 6 b	54 ± 8 d
BR 14450	85 ± 3 b	60 ± 6 b	152 ± 15 b	69 ± 7 c
BR 14435	82 ± 7 b	58 ± 2 b	146 ± 11 b	65 ± 1 c
BR 14493	96 ± 2 b	63 ± 5 b	146 ± 1 b	70 ± 7 c
BR 14483	91 ± 1 b	50 ± 3 c	145 ± 6 b	58 ± 3 d
BR 14445	76 ± 6 b	55 ± 5 b	144 ± 7 b	60 ± 6 c
BR 14476	97 ± 4 b	56 ± 6 b	141 ± 5 b	60 ± 7 c
BR 14480	121 ± 11 a	51 ± 5 c	139 ± 10 b	41 ± 6 d
BR 14469	93 ± 14 b	46 ± 1 c	132 ± 14 b	53 ± 6 d
BR 14481	86 ± 12 b	62 ± 5 b	131 ± 19 b	64 ± 5 c
BR 14436	67 ± 4 b	44 ± 4 c	130 ± 9 b	57 ± 6 d
BR 14437	89 ± 3 b	67 ± 3 b	129 ± 6 b	78 ± 3 b
BR 14449	90 ± 5 b	52 ± 2 c	129 ± 12 b	48 ± 4 d
Absolute control	78 ± 6 b	32 ± 4 d	127 ± 4 b	35 ± 5 d
Nitrogen control	0 ± 0	-	0 ± 0	-
Coefficient of variation (%)	16.5	13.0	15.0	14.0

Means followed by the same letters in the same column are not statistically different (Scott-Knott test at $p<0.05$)

In evaluating the contribution of BNF, the contribution of N_2 fixation ranged from 70 to 89% (Table 6).

Of the 31 inoculated treatments, 22 treatments showed a significantly higher proportion of N derived from N_2 fixation than the absolute control. For nitrogen content supplied via BNF, 14 inoculated treatments showed increases in comparison to the uninoculated control, with a mean increase of 40% and a maximum of 55% (Table 6). These results indicate a high contribution of BNF to mung bean in the conditions of this study, including the control. However, although native rhizobia resulted in high N_2 fixation, 14 inoculated strains still resulted in increases in nitrogen content via BNF from 26 to 55% compared to the control. For grain dry weight, the inoculated treatments showed lower means than the nitrogen control,

indicating that the nitrogen made available via BNF in the inoculated treatments was insufficient to meet the demand of the mung bean plant. Although we have found a high contribution from BNF, reaching up to 89%, the amount of nitrogen from BNF was not sufficient to enhance plant growth or grain production, compared to treatment that received N-fertilizer. Alternatives must be evaluated in order to increase biomass, grain yield and N content when using the inoculation technology.

Table 6. Means and standard errors of root dry weight, shoot dry weight, N accumulated in the shoot, N derived from BNF, N accumulated derived from BNF at 33 days after emergence, and grain dry weight at 60 days after emergence of mung bean plants cultivated in pots of soil and inoculated with different *Bradyrhizobium* strains, in addition to absolute and nitrogen controls

Treatment	Root dry weight	Shoot dry weight	N accumulated in shoot	N derived from BNF	N accumulated from BNF	Grain dry weight
	g plant^{-1}	g plant^{-1}	mg plant^{-1}	%	mg plant^{-1}	g plant^{-1}
BR 14531	1.51 ± 0.10 b	2.91 ± 0.02 b	87 ± 5 b	70 ± 5 b	61 ± 6 a	3.10 ± 0.16 b
BR 14532	1.33 ± 0.06 b	2.81 ± 0.14 b	74 ± 6 b	71 ± 7 b	54 ± 9 b	3.08 ± 0.17 b
BR 14477	1.47 ± 0.08 b	2.79 ± 0.11 b	87 ± 6 b	74 ± 5 b	64 ± 5 a	3.16 ± 0.15 b
BR 14454	1.31 ± 0.06 b	2.75 ± 0.11 b	80 ± 7 b	83 ± 2 a	66 ± 4 a	3.10 ± 0.21 b
BR 14494	1.28 ± 0.14 b	2.60 ± 0.13 b	78 ± 8 b	84 ± 4 a	67 ± 10 a	3.26 ± 0.07 b
BR 14514	1.26 ± 0.12 b	2.60 ± 0.24 b	79 ± 9 b	89 ± 2 a	70 ± 9 a	3.18 ± 0.04 b
BR 14446	1.19 ± 0.05 b	2.58 ± 0.12 b	70 ± 6 b	73 ± 3 b	51 ± 5 b	3.00 ± 0.08 b
BR 14474	1.27 ± 0.12 b	2.51 ± 0.19 b	73 ± 5 b	80 ± 3 a	58 ± 2 a	3.04 ± 0.09 b
BR 14482	1.41 ± 0.10 b	2.51 ± 0.35 b	81 ± 11 b	84 ± 3 a	68 ± 8 a	3.01 ± 0.10 b
BR 14458	1.52 ± 0.16 b	2.47 ± 0.12 b	81 ± 10 b	86 ± 2 a	70 ± 9 a	3.25 ± 0.11 b
BR 14452	1.33 ± 0.14 b	2.44 ± 0.26 b	76 ± 7 b	85 ± 3 a	64 ± 7 a	3.22 ± 0.10 b
BR 14529	1.28 ± 0.11 b	2.40 ± 0.22 b	61 ± 5 c	73 ± 5 b	45 ± 5 b	2.69 ± 0.16 b
BR 14442	1.07 ± 0.12 c	2.21 ± 0.35 c	69 ± 14 b	89 ± 1 a	62 ± 13 a	3.21 ± 0.1 b
BR 14440	0.93 ± 0.03 c	2.09 ± 0.13 c	63 ± 10 c	79 ± 3 a	51 ± 10 b	3.07 ± 0.08 b
BR 14444	0.90 ± 0.12 c	2.07 ± 0.30 c	70 ± 13 b	87 ± 1 a	60 ± 10 a	3.16 ± 0.10 b
BR 14483	1.10 ± 0.13 c	2.04 ± 0.31 c	70 ± 12 b	83 ± 0 a	58 ± 10 a	3.33 ± 0.07 b
BR 14455	0.99 ± 0.12 c	2.02 ± 0.29 c	70 ± 10 b	80 ± 3 a	57 ± 10 a	3.12 ± 0.12 b
BR 14493	0.99 ± 0.19 c	2.01 ± 0.34 c	72 ± 16 b	73 ± 3 b	53 ± 13 b	3.19 ± 0.04 b
BR 14438	0.96 ± 0.07 c	1.99 ± 0.09 c	66 ± 6 c	82 ± 4 a	55 ± 7 b	3.30 ± 0.05 b
BR 14472	0.94 ± 0.16 c	1.95 ± 0.29 c	68 ± 11 c	86 ± 3 a	59 ± 10 a	3.20 ± 0.29 b
BR 14445	1.08 ± 0.11 c	1.87 ± 0.28 c	70 ± 12 b	75 ± 4 b	54 ± 12 b	3.18 ± 0.08 b
BR 14479	0.91 ± 0.02 c	1.82 ± 0.32 c	61 ± 10 c	87 ± 1 a	53 ± 8 b	2.99 ± 0.03 b
BR 14481	1.09 ± 0.26 c	1.81 ± 0.42 c	62 ± 15 c	80 ± 1 a	50 ± 12 b	3.13 ± 0.08 b
BR 14435	1.12 ± 0.19 c	1.79 ± 0.20 c	63 ± 7 c	82 ± 1 a	51 ± 5 b	3.10 ± 0.08 b
BR 14476	0.79 ± 0.13 c	1.79 ± 0.34 c	61 ± 12 c	80 ± 3 a	49 ± 10 b	3.10 ± 0.08 b
BR 14437	0.75 ± 0.12 c	1.78 ± 0.35 c	58 ± 12 c	78 ± 1 b	45 ± 10 b	3.02 ± 0.04 b
BR 14450	0.92 ± 0.10 c	1.76 ± 0.22 c	64 ± 11 c	81 ± 3 a	52 ± 10 b	3.09 ± 0.13 b
BR 14449	0.93 ± 0.20 c	1.74 ± 0.27 c	61 ± 12 c	81 ± 5 a	51 ± 11 b	2.98 ± 0.26 b
BR 14436	0.75 ± 0.16 c	1.61 ± 0.32 c	58 ± 14 c	76 ± 2 b	45 ± 11 b	3.35 ± 0.14 b
BR 14469	0.74 ± 0.18 c	1.57 ± 0.27 c	57 ± 11 c	84 ± 3 a	48 ± 10 b	3.10 ± 0.14 b
BR 14480	0.81 ± 0.12 c	1.53 ± 0.23 c	55 ± 10 c	84 ± 3 a	46 ± 10 b	3.05 ± 0.07 b
Absolute control	1.06 ± 0.07 c	1.75 ± 0.23 c	62 ± 10 c	72 ± 0 b	45 ± 7 b	3.01 ± 0.09 b
Nitrogen control	2.15 ± 0.07 a	4.37 ± 0.37 a	213 ± 15 a	-	-	4.09 ± 0.16 a
Coefficient of variation (%)	20.5	15.4	13.9	6.9	16.4	8.0

Means followed by the same letters in the same column are not statistically different (Scott-Knott test at $p<0.05$)

By evaluating all the variables described through a principal component analysis for inoculated treatments, there is correlation between the variables of nodulation, root and shoot dry weight, and accumulated N (Figure 16). We added to the principal component analysis

information about the phylogenetic groups of *Bradyrhizobium* strains, as described by Favero et al. (2021b). The BNF contribution and of grain dry weight had a weaker relationship with the nodulation and resulted in a smaller contribution in the ordering analysis, which may be related to little difference between treatments for these variables. As they are more closely related to the main axis of ordination, it is suggested that for selection purposes, at least 14 strains are indicated for use in further studies of inoculation efficiency for mung bean, as they had a positive relationship with these variables.

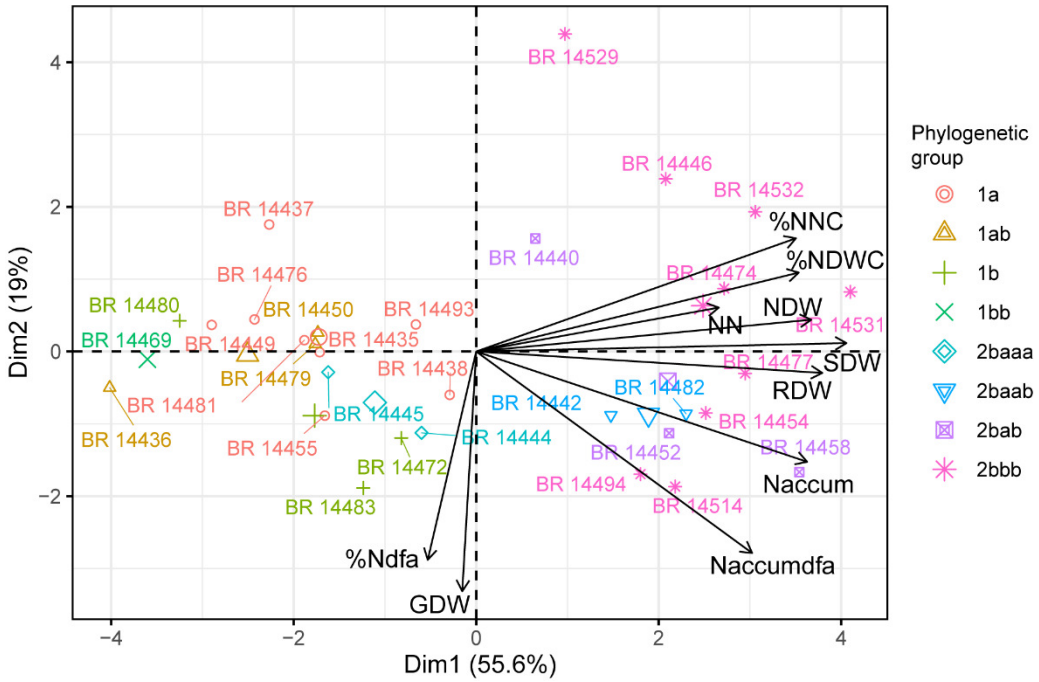

Figure 16. Principal component analysis between nodule number (NN), nodule number in the crown (%NNC), nodule dry weight (NDW), nodule dry weight in the crown (%NDWC), root dry weight (RDW), shoot dry weight (SDW), N accumulated in shoot (Naccum), N derived from BNF (%Ndfa), biologically fixed N accumulated (Naccumdfa) and grain dry weight (GDW) of mung bean plants cultivated in pots with soil and inoculated with *Bradyrhizobium* strains. The phylogenetic groups of these strains are based on divisions between the superclades of *B. elkanii* and *B. japonicum* for groups starting with 1 and 2, respectively, according to data published by Favero et al. (2021a). The smaller symbols represent each individual (strain) and the larger ones represent the centroid of phylogenetic group.

In principal component analysis, the phylogenetic groups are based on divisions between *B. elkanii* and *B. japonicum* superclades for groups starting with 1 and 2, respectively. In general, the greater grouping of *B. japonicum* superclade strains to the variables of nodulation, plant development and accumulated N suggests that strains close to this clade are more efficient for mung bean inoculation. Of this superclade, the 2bbb phylogenetic group close to *B. yuanmingense* B071 has nine strains and all have a high relationship with the variables described above. Three other phylogenetic groups within the *B. japonicum* superclade are also related to the variables described, being 2bab which is close to *B. huanghuaihaiense* CCBAU23303, and 2baab and 2baaa which did not form cluster with the species already described.

5.5.2 *Bradyrhizobium* inoculation associated with the application of fertilizer N doses

For the *Bradyrhizobium* inoculation experiment associated with the application of N fertilizer as starter doses of N, the quantification of native soil rhizobia able to nodulate mung bean using MPN indicated the presence of 2.4×10^4 rhizobia cells g soil^{-1}. To evaluate the effectiveness of inoculation of the *Bradyrhizobium* strain, we compared the treatment without the application of N (dose 0) with the absolute control. We found a difference in the evaluation of 38 DAE for the number and dry weight of nodule (Figure 17a and b), with an increase of 57 and 16%, respectively. The means were 14 and 8% higher for dry root and aerial part, respectively, and were insufficient to have a significant difference (Fig. 2c and d), and may be related to a higher density of native rhizobia compared to the first experiment.

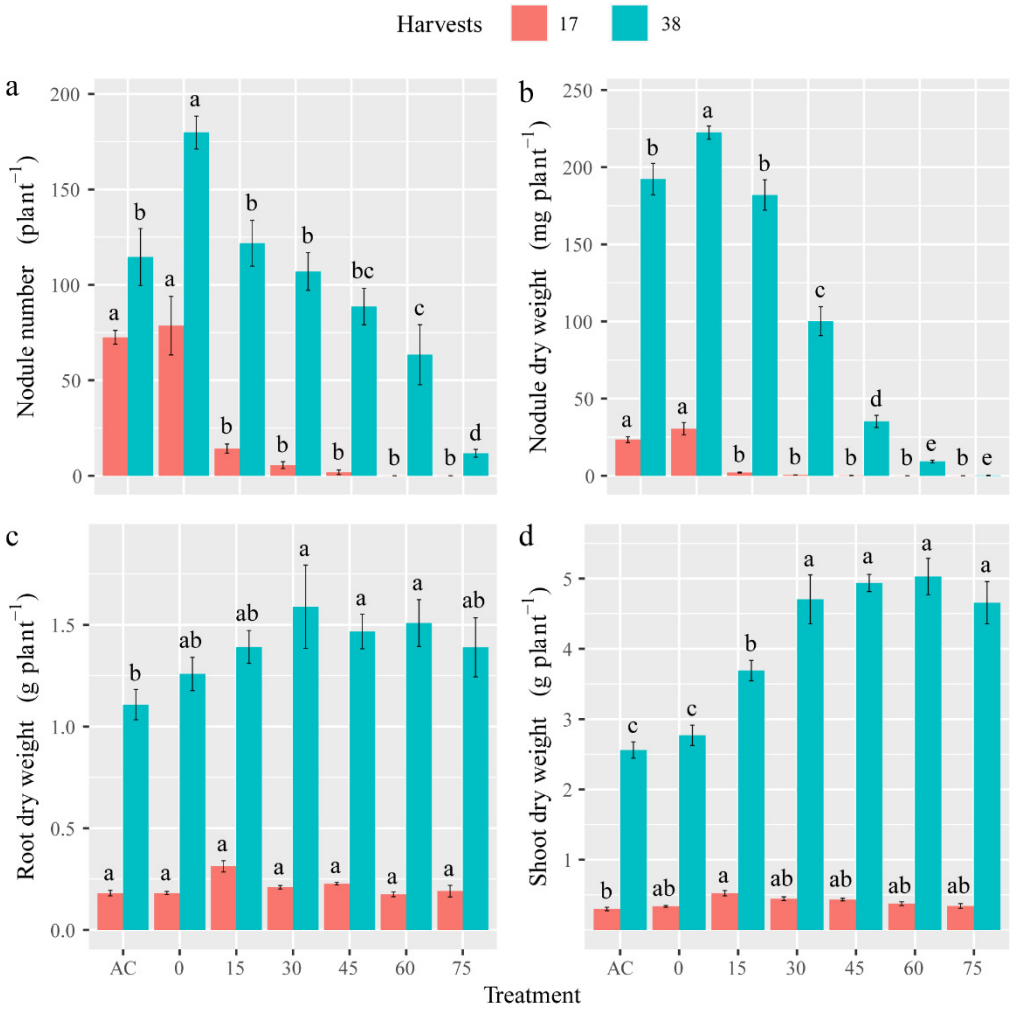

Figure 17. Nodule number (a), nodule dry weight (b), root (c) and shoot (d) of mung bean plants harvested at 17 and 38 days after emergence, cultivated in pot with soil under the application of N doses (0, 15, 30, 45, 60 and 75 kg ha^{-1}) associated with *Bradyrhizobium* strain inoculation, in addition to the absolute control (AC). Bars (means) topped by the same letters for the same harvest are not statistically different by Tukey test at 5% probability. Vertical lines on bars represent standard errors. Coefficient of variation (%): nodule number = 31.13, nodule dry weight = 19.06, root dry weight = 23.93 and shoot dry weight = 15.43.

The application of nitrogen doses significantly reduced the nodulation at both evaluations (Figure 17a, b and Appendix 7). The application of a dose corresponding to 15 kg ha^{-1} of N was sufficient to result in a reduction of 32 and 18% in the number and nodule dry weight, respectively, at the evaluation of 38 DAE. In the evaluation at 17 DAE, the reduction was even greater, of 82 and 93% for the number and nodule dry weight, respectively. These results suggest that the inhibitory effect of N doses on nodulation is greater in the initial phase, that is, nodulation is reduced and delayed as a function of N application.

Nitrogen application had a positive effect on root dry weight at harvest at 38 DAE (Figure 17c and Appendix 7). The doses of 15 and 30 kg ha^{-1} increased 11 and 26% in relation to the treatment without N, respectively. For shoot dry weight, there was a positive relationship as a function of N doses (Figure 17d and Appendix 7). The doses of 15 and 30 kg ha^{-1} increased by 33 and 70% in relation to the treatment without the application of N.

5.6 DISCUSSION

Mung bean was introduced decades ago in Brazil but has only recently been cultivated on a commercial scale in the Brazilian Cerrado. In these areas, mung bean has been used for rotation with soybean and maize crops, benefiting from their short cycle, demand for export of their grains and low production costs. Brazil has already been successful in exploiting BNF in soybean, common bean, cowpea and other crops (HUNGRIA; NOGUEIRA; ARAUJO, 2015; SILVA JÚNIOR et al., 2018; ZILLI et al., 2021) and in order to promote inoculation technology for mung bean, studies are being carried out aiming at the isolation, characterization and selection of efficient rhizobia associated with mung bean grown in Brazil (FAVERO et al., 2021a; SILVA et al., 2021). In a previous study, *Bradyrhizobium* strains were isolated from mung bean nodules grown in soil samples from Brazil, and pre-selected for nodulation capacity under axenic conditions (FAVERO et al., 2021a).

In present study, the inoculation of some strains of *Bradyrhizobium* increased the nodulation, biomass and nitrogen fixation when compared to the population of native rhizobia in the soil. Inoculation of mung bean with *Bradyrhizobium* strains has provided increases in mung bean development when compared to the soil population of native rhizobia in several countries (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; DELIĆ et al., 2011; HAKIM et al., 2020; TARIQ et al., 2012). However, it is common to have low increments with the use of inoculation (HERRIDGE et al., 2005; MATHU et al., 2012). In our study, the inoculation of some *Bradyrhizobium* strains resulted in increases in nodulation of up to 71 and 79% in the nodule number and nodule dry weight, respectively. Differences in mung bean nodulation due to *Bradyrhizobium* inoculation are common and may vary as a function of the strain used (CHRISTOPHER et al., 2018; MATHU et al., 2012), the plant genotype (ISLAM et al., 2006) and nodulation capacity of native soil rhizobia (FAVERO et al., 2021a; HERRIDGE et al., 2005; MATHU et al., 2012). Significant effects of inoculation on mung bean nodulation were found in studies carried out in several countries, and differences were found ranging from 53 to 344 and from 53 to 194% in nodule number and nodule dry weight, respectively (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; HAKIM et al., 2020; ISLAM et al., 2006; TARIQ et al., 2012).

In our study, inoculation resulted in increases of up to 43, 66 and 40% for dry weight of the root, shoot and N accumulated in the shoot, respectively. However, they showed lower means than the nitrogen control with a dose corresponding to 160 kg ha^{-1} of N. In other studies, mung bean biomass and nodulation responses to inoculation has also been variable with increments ranging from 28 to 675% (CHRISTOPHER et al., 2018; ISLAM et al., 2006) and 11 at 350% (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; DELIĆ et al., 2011; ISLAM et al., 2006) for root and shoot dry weight, respectively.

In a large study of mung bean development in commercially grown areas in Australia, BNF contributed from 9 to 78%, averaging only 27% (HERRIDGE et al., 2005). In our study, the BNF contribution was between 70 and 89%, with an average of 76%, which suggests a high contribution, even including the treatment without inoculation. In this sense, data associated with low biomass and N accumulation of inoculated treatments compared to the nitrogen control suggest that other factors may have limited the development of the inoculated plants given high energy demand for BNF performance, such as light and nutrients, or even by pot size, and its need more studies to verify the ability of N supply by BNF to mung bean.

In our study, increments in grain dry weight due to *Bradyrhizobium* inoculation were not observed. In field experiments conducted in other countries, the inoculation of *Bradyrhizobium* strains has provided increases of 11 to 53% in mung bean yield, and was associated with increased nodulation (BHUIYAN; MIAN, 2007; HAKIM et al., 2020; TARIQ et al., 2012) and in shoot dry weight (BHUIYAN; MIAN, 2007; DELIĆ et al., 2011). A

principal component analysis indicated that nodulation, root and shoot dry weight, and accumulated N from our study are positively correlated, therefore, the effect of inoculation on grain dry weight may have been limited by other factors, the example, by the size of the pot. In this sense, field experiments should be carried out to verify the effect of inoculation on mung bean yield.

In the principal components analysis, in addition to verify the correlation between the variables described, we used information from the phylogenetic groups of these strains, carried out by Favero et al. (2021a), and we identified that the phylogenetic groups of the *B. japonicum* superclade seem to be more efficient for mung bean inoculation when compared to the *B. elkanii* superclade groups. In general, the 2bbb phylogenetic group, with nine strains close to *B. yuanmingense* B071 obtained from nodules of mung bean cultivated in Brazil, showed high values for nodulation, plant development and N accumulation than other phylogenetic groups. In the earlier study of Favero et al. (2021a), the 2bbb phylogenetic group was also the one with the highest efficiency, and therefore, its strains were selected for studies under non-axenic conditions. Previously, in a study carried out in Nepal, strains close to *B. yuanmingense* were found to be more efficient symbionts than *B. elkanii* C-11 and *B. japonicum* USDA 6 strains concerning in promoting nitrogen fixation in mung bean (RISAL et al., 2012). Recently, a study carried out in Pakistan indicated that *B. yuanmingense* B071 is predominant among mung bean isolates of the genus *Bradyrhizobium* (HAKIM; IMRAN; MIRZA, 2021). The results of these studies confirm differences in efficiency for mung bean inoculation with regard to phylogenetic groups, indicating that strains close to *B. yuanmingense* are more efficient. These results should be confirmed in field studies, as well as verifying the reasons for this greater efficiency.

In general, treatments inoculated with *Bradyrhizobium* resulted in increases in mung bean nodulation, development and BNF activity compared to the uninoculated control. However, these increments were not enough to correspond to the mung bean development under the application of a dose corresponding to 160 kg ha^{-1} of N. In this sense, ways to optimize the mung bean growth under *Bradyrhizobium* inoculation should be evaluated. The application of initial doses of N associated with inoculation with rhizobia can optimize the growth of inoculated legumes (DABA; HAILE, 2000; GEBREHANA; DAGNAW, 2020; MENDES; HUNGRIA; VARGAS, 2003; RAHMAN et al., 2018), however, depending on the plant species, nodulation inhibition might be observed (MENDES; HUNGRIA; VARGAS, 2003; ZHANG; MAÁCE; SMITH, 2000). In our study, the increases observed for root and shoot dry weight suggest that BNF has not been able to supply the N demand by the plant, however, the application of N doses resulted in a reduction in nodulation, even with a low application of just 15 kg ha^{-1}. In commercial crops in Australia, the contribution of BNF in mung bean has been found to have a negative relationship with soil nitrate concentration (HERRIDGE et al., 2005), an inhibition of BNF has already been observed in other legumes (LORENZO et al., 1990; MATAMOROS et al., 1999). In this sense, it is suggested that N applications have resulted in a smaller contribution from BNF, however, it is necessary to also test other nitrogen sources, such as urea.

Depending on the *Bradyrhizobium* strain used for inoculation, the application of 20 kg ha^{-1} of N as potassium ammonium nitrate (KAN, 27% N) can result in increases of up to 11% in mung bean yield (DELIĆ et al., 2011). Also, without nodulation evaluation and without rhizobia inoculation, the application of 25 and 50 kg ha^{-1} of N as urea (46% N) increased productivity by 10 and 16%, respectively (AKBARI; BARANI; AHMADI, 2008). In this sense, evaluations with mung bean under field conditions must be carried out in order to investigate the effect of N doses associated with *Bradyrhizobium* inoculation on BNF and yield.

5.7 CONCLUSIONS

The results of this study showed that *Bradyrhizobium* strains isolated from Brazilian tropical soils can increase up to 71 and 79% in the number and nodule dry weight, respectively. In root dry weight, shoot and N accumulated in the shoot from the BNF, the increments can reach 43, 66 and 55%, respectively. The strains grouped with *B. japonicum* superclade showed greater potential for inoculation with mung bean, especially those grouped with *B. yuanmingense*. The application of N doses resulted in increases in mung bean development, but significantly reduced nodulation. Although there were high proportional contributions from BNF, reaching up to 89%, the amount of N derived from BNF was insufficient to rival bean fertilized with N-fertilizer. This shows the necessity for field inoculation studies including the evaluation of different N rates of N fertilizer application to determine whether mung bean relying mainly on BNF can gives yields as high as those experienced with N fertilizer application.

6 CAPÍTULO IV

CROSS-INOCULATION OF ELITE COMMERCIAL *Bradyrhizobium* STRAINS FROM COWPEA AND SOYBEAN IN MUNG BEAN AND COMPARISON WITH MUNG BEAN ISOLATES

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6.1 RESUMO

O objetivo deste estudo foi avaliar a eficiência da nodulação e fixação de N_2 de estirpes *Bradyrhizobium* recomendadas para feijão-caupi e soja quando utilizadas como inoculantes para feijão-mungo em comparação com isolados de *Bradyrhizobium* obtidos de nódulos de feijão-mungo. Este estudo contribuirá para o processo de recomendação de um inoculante para feijão-mungo, e determinará se algum inoculante comercial existente é eficiente na nodulação desta cultura. Para isso, foi instalado um experimento em casa de vegetação sob condições axênicas para avaliação da inoculação de oito estirpes elite utilizadas em inoculantes comerciais, e outro em vaso com solo não estéril, a fim de compará-las com 13 estirpes de *Bradyrhizobium* isoladas de nódulos de feijão-mungo. Os resultados revelaram que a estirpe SEMIA 587 (*Bradyrhizobium elkanii*) usada como inoculante comercial para soja, e as estirpes UFLA 3-84 (*B. viridifuturi*), BR 3267 (*B. yuanmingense*) e INPA 03-11B (*B. elkanii*) usadas para feijão-caupi podem nodular eficientemente o feijão-mungo. Por outro lado, a estirpe BR 3262 (*B. pachyrhizi*) usada para inoculação do feijão-caupi, e a CPAC 15 (*B. japonicum*), CPAC 7 (*B. diazoefficiens*) e BR 29 (29 W; *B. elkanii*) usadas para soja não conseguiram nodular o feijão-mungo. Embora o feijão-mungo seja considerado uma leguminosa promíscua, esses resultados sugerem a existência de incompatibilidade simbiótica com algumas estirpes de *Bradyrhizobium*. A eficiência das estirpes elite SEMIA 587 (*B. elkanii*) e UFLA 3-84 (*B. viridifuturi*) em termos de aumento da nodulação e crescimento das plantas foi semelhante às estirpes de *Bradyrhizobium* isoladas de nódulos de feijão mungo, portanto, deve ser avaliada sob condições de campo para verificar sua contribuição para a fixação biológica de nitrogênio em feijão-mungo.

Palavras-chave: Feijão-mungo. *Vigna radiata* (L.) Wilczek. Inoculação cruzada. Estirpes de *Bradyrhizobium*. Fixação biológica de nitrogênio.

6.2 ABSTRACT

The objective of this study was to evaluate the efficiency of nodulation and N_2 fixation of strains *Bradyrhizobium* recommended for cowpea and soybean when used as inoculants for mung bean in comparison with *Bradyrhizobium* isolates obtained from mung bean nodules. This study will contribute to the process of recommending an inoculant for mung bean, and determine if any existing commercial inoculant is efficient in nodulation of this crop. An experiment was conducted in a greenhouse under axenic conditions for the cross-inoculation of eight elite strains used in commercial inoculants. Subsequently the efficiency of these strains was examined in a pot experiment with non-sterile soil and compared with that of the 13 *Bradyrhizobium* isolates. Results revealed that the SEMIA 587 (*Bradyrhizobium elkanii*) strain used as commercial inoculants for soybean and the UFLA 3-84 (*B. viridifuturi*), BR 3267 (*B. yuanmingense*), and INPA 03-11B (*B. elkanii*) strains used for cowpea could efficiently nodulate mung bean. Conversely, BR 3262 (*B. pachyrhizi*) for cowpea and CPAC 15 (*B. japonicum*), CPAC 7 (*B. diazoefficiens*), and BR 29 (29 W; *B. elkanii*) for soybean could not achieve the same outcome. Although mung bean is considered a promiscuous legume, these results suggest the existence of symbiotic incompatibility with some *Bradyrhizobium* strains. The efficiency of the elite SEMIA 587 (*B. elkanii*) and UFLA 3-84 (*B. viridifuturi*) strains in terms of increased nodulation and plant growth was similar to those of *Bradyrhizobium* strains isolated from mung bean nodules and, therefore, should be evaluated under field conditions to verify their contribution to biological nitrogen fixation in mung bean.

Keywords: Mung bean. *Vigna radiata* (L.) Wilczek. Cross-inoculation. *Bradyrhizobium* Strains. Biological nitrogen fixation.

6.3 INTRODUCTION

Mung bean [*Vigna radiata* (L.) Wilczek] is an Asian legume (LAMBRIDES; GODWIN, 2007) with a short cycle (NAIR et al., 2012), and it has good adaptability to tropical climatic conditions (HANUMANTHARAO; NAIR; NAYYAR, 2016; SHARMA et al., 2016). Its grains are rich in carbohydrates and proteins (DU et al., 2018; LAMBRIDES; GODWIN, 2007), and it is an important crop in several countries and widely consumed in Asia. In Brazil, mung bean has been cultivated for decades, but on a small scale commercially. In recent years, its cultivation has expanded to agribusiness areas to produce grains mainly for international markets, and it has been considered a good option for inclusion in soybean or maize production cycles.

Mung bean benefits from biological nitrogen fixation (BNF) through its association with soil rhizobia (Hayat et al. 2008; Herridge et al. 2005), reducing N fertilizer requirement. In Brazil, few studies have focused on BNF in mung bean. The first findings showed that *Bradyrhizobium* elite strains from commercial inoculants for soybean and cowpea in Brazil can be effective in nodulating the Camaleão mung bean cultivar (SANTOS, 2020). It has also been observed that nodulation of mung bean in Brazilian tropical soils varies with soil type, resulting in low crop yields in some soils (FAVERO et al., 2021a). This suggests that there is a need for the introduction of efficient rhizobia strains to ensure optimum nodulation. A study to search for efficient rhizobia showed that *Bradyrhizobium* accounts for more than 99% of the bacteria sequenced in nodules of mung bean grown in Brazilian soils (FAVERO et al., 2021b). However, other rhizobia genera have been isolated from mung bean nodules in Brazilian soils (FAVERO et al., 2021a; SILVA et al., 2021), but only *Bradyrhizobium* isolates were able to forms nodules on mung bean (FAVERO et al., 2021a). In an experiment with mung bean grown in pots with non-sterile soil, inoculation of these isolates increased nodulation, growth, and accumulated N derived from BNF by over 50%, even where BNF values were high for non-inoculated controls (average of 72%) (FAVERO et al., 2022).

Mung bean is known for its promiscuity, that is, with ability to nodulate with several rhizobia genera (*Bradyrhizobium*, *Ensifer*, *Rhizobium*, and *Mesorhizobium*) (Yang et al. 2008; Zhang et al. 2008), which is important for the selection of efficient strains. As in Brazil, *Bradyrhizobium* genus has more importance in the symbiotic relationship with mung bean in others countries, and its low specificity associated with several *Bradyrhizobium* species (ZHANG et al., 2008), which may contribute to the selection of efficient strains. However, some studies have shown that some *Bradyrhizobium* strains do not nodulate mung bean (NGUYEN et al., 2017; OKAZAKI et al., 2009; PIROMYOU et al., 2019; SONGWATTANA et al., 2017; WU et al., 2020). In some cases, genes encoding the type III secretion system (T3SS) limit or completely inhibit the symbiotic association (NGUYEN et al., 2017; OKAZAKI et al., 2009; PIROMYOU et al., 2019; SONGWATTANA et al., 2017); however, incompatibility may differ depending on plant genotype (OKAZAKI et al., 2009; PIROMYOU et al., 2019).

Cross-inoculation of rhizobial strains in different legume species is frequently evaluated (GUIMARAES et al., 2016; ZILLI et al., 2011), and it may be possible that elite *Bradyrhizobium* strains from commercial inoculants for other crops can contribute to mung bean inoculation in Brazil. For example, commercial soybean inoculants are efficient in nodulating cowpea, and some have similar efficiency to that of strains authorized for cowpea inoculation (ZILLI et al., 2011). However, cases of incompatibility are also common, for exemple, the *Bradyrhizobium* strain CB1015 which is widely used as a commercial inoculant for cowpea, mung bean, and adzuki bean in Australia (BULLARD; ROUGHLEY; PULSFORD, 2005) is ineffective for pigeon pea (BROCKWELL et al., 1991). These findings indicate the need for studies on the symbiotic specificity of each legume species.

A cross-inoculation trial using elite *Bradyrhizobium* strains from commercial inoculants for soybean and cowpea in Brazil showed that these strains could be effective in nodulating the Camaleão mung bean cultivar (SANTOS, 2020). However, the efficiency of these strains should be verified in other genotypes, as well as the comparison with rhizobia isolated from mung bean nodules. For this reason the general objective of this study was to evaluate the effectiveness of the inoculation of mung bean with *Bradyrhizobium* strains from Brazilian tropical soils. The specific objectives were: (1) to evaluate elite *Bradyrhizobium* strains from commercial inoculants of soybean and cowpea in Brazil regarding the ability to nodulate the MGS Esmeralda mung bean cultivar in axenic conditions, and (2) to evaluate the effect of elite *Bradyrhizobium* strains inoculation of commercial soybean and cowpea inoculants on mung bean plants grown in a pots assay using non-sterile soil, comparing them with *Bradyrhizobium* isolates obtained from mung bean nodules.

6.4 MATERIAL AND METHODS

6.4.1 Mung bean inoculation with elite *Bradyrhizobium* strains under axenic condition

a) Experimental design

A greenhouse experiment was conducted to evaluate the ability of elite *Bradyrhizobium* strains to form nodules on the cultivar MGS Esmeralda of mung bean (VIEIRA et al., 2008) grown in long neck bottles (VINCENT, 1970). The experiment was arranged in a completely randomized design with three replications. The elite strains authorized for use as commercial inoculants for cowpea and soybean in Brazil were evaluated (Table 7) (Brasil 2011). Each elite strain was inoculated onto its original host legume species using the BRS Guariba cowpea and BRS 7780IPRO soybean cultivars as positive controls. The positive control for mung bean was the treatment inoculated with the BR 14454 isolate, a strain known to be efficient for mung bean nodulation (FAVERO et al., 2021a). Absolute controls (without inoculation) were included for cowpea, soybean, and mung bean. The MGS Esmeralda, cowpea BRS Guariba, and soybean BRS 7780IPRO used in this study are cultivars registered with the Brazilian Ministry of Agriculture, Livestock and Supply under numbers 22096, 14768, and 33475, respectively https://sistemas.agricultura.gov.br/snpc/cultivarweb/cultivares_registradas.php. Mung bean MGS Esmeralda cultivar was developed by the Asian Vegetable Research and Development Center (Shanhua, Taiwan) by crossing the VC 1973A and VC 2768A lines (VIEIRA et al., 2008).

Table 7. Elite *Bradyrhizobium* strains used in commercial inoculants in Brazil for cowpea and soybean in Brazil, according to Brasil (2011)

Strain	Recomended legume[a]	Taxonomy	Reference
UFLA 3-84 (= SEMIA 6461, BR 3302)	Cowpea [*Vigna unguiculata* (L.) Walp]	*B. viridifuturi*	Costa et al. (2019); Lacerda et al. (2004)
BR 3267 (= SEMIA 6462)		*B. yuanmingense*	Leite et al. (2018); Martins et al. (2003)
INPA 3-11B (= SEMIA 6463, BR 3301)		*B. elkanii*	Guimarães et al. (2015); Lacerda et al. (2004)
BR 3262 (= SEMIA 6464)		*B. pachyrhizi*	Leite et al. (2018); Zilli; Xavier; Rumjanek (2008)
CPAC 15 (= SEMIA 5079, BR 86, DF 24, CNPSo 7)	Soybean [*Glycine max* (L.) Merrill]	*B. japonicum*	Siqueira et al. (2014); Vargas et al. (1992)
CPAC 7 (= SEMIA 5080, BR 85, CNPSo 6)		*B. diazoefficiens*	Siqueira et al. (2014); Vargas et al. (1992)
SEMIA 587 (= BR 96)		*B. elkanii*	Peres (1979); Souza et al. (2012)
BR 29 (= 29 W, SEMIA 5019)		*B. elkanii*	Peres (1979)

[a]INPA 3-11B strain was isolated from *Centrosema* sp.; however, it was efficient for cowpea inoculation.

b) Seed disinfection and strain inoculation

Cowpea, soybean, and mung bean seeds were disinfected by immersing them in ethanol (70%) and hydrogen peroxide (35%) for 1 and 3 min, respectively, and subsequently washed 10 times in sterile distilled water. Disinfected mung bean seeds were pre-germinated in a Petri dish with moistened paper for 24 h. Cowpea and soybean seeds were pre-germinated under the same conditions as mung bean; however, they were incubated for 72 h. Next, the seeds were transplanted into long neck bottles containing 300 mL of sterile nitrogen-free Norris nutrient solution (YATES et al., 2016).

The elite *Bradyrhizobium* strains and the BR 14454 isolate were provided by Johanna Döbereiner Biological Resource Center at Embrapa Agrobiologia, Brazil, and grown in yeast mannitol (YM) culture medium (HUNGRIA et al., 2016) under agitation at 150 rpm at 28 °C for 6 days, until an optical density of >0.8 at 600 nm, indicating a concentration of at least 10^8 cell mL^{-1}. Next, the seedlings were inoculated with 1 mL of the bacterial suspension grown in YM approximately 48 h after the transplantation of pre-germinated seeds into the long neck bottles. The plants were collected 30 days after inoculation to verify the presence of nodules.

6.4.2 Mung bean inoculation with elite *Bradyrhizobium* strains and mung bean isolates in non-sterile soil

a) Experimental design

A second experiment was performed in a greenhouse to determine the effects of inoculating the MGS Esmeralda mung bean cultivar (VIEIRA et al., 2008) grown in pots containing 3 kg of soil with *Bradyrhizobium* strains and mung bean isolates. The experiment was arranged in a randomized block design with four replications. The treatments comprised inoculation with 21 *Bradyrhizobium* strains: 8 elite strains used in commercial inoculants of cowpea and soybean (Table 7) (Brasil 2011) and 13 isolates from mung bean nodules (FAVERO et al., 2021a). An absolute control (without inoculation) was also included.

b) Soil material

Soil (a Planosol – Alfisol) was collected from an area located in Seropédica, Rio de Janeiro, Brazil (22°44′56.7″ S, 43°40′00.9″ W), where legumes had not been grown for the last 10 years. The soil was sampled from a depth of 0–20 cm, sieved in a 4 mm mesh, and homogenized. The soil chemical characterization was performed according to the standard methods by Nogueira and Souza (2005), using a homogeneous sample of the soil material used. The results of the analyses were as follows: pH, 5.81; Al^{3+}, 0.00 $cmol_c$ dm^{-3}; Ca^{2+}, 0.61 $cmol_c$ dm^{-3}; Mg^{2+}, 0.22 $cmol_c$ dm^{-3}; H+Al, 0.74 $cmol_c$ dm^{-3}; P, 6.03 mg dm^{-3}; K^+, 27.14 mg dm^{-3}; C, 0.25%; and total N, 0.04%. pH was increased by adding dolomitic limestone (0.75 g kg^{-1} soil), and 50, 30, and 25 mg kg^{-1} soil were fertilized with P_2O_5, K_2O, and fritted trace elements (S, 3.9%; B, 1.8%; Mn, 2%; Cu, 0.8%; and Zn, 9%), respectively. Additionally, dry and ground maize straw was added (2.5 g kg^{-1} soil) to immobilize some of the available mineral N, as recommended by Brasil (2011).

c) Soil rhizobia quantification

To determine the soil rhizobia population in the soil capable of nodulating mung bean an experiment was peformed in long neck bottles (VINCENT, 1970) using the most probable number (MPN) method (O'Hara et al. 2016). First, MGS Esmeralda (VIEIRA et al., 2008) seeds

were disinfected, pre-germinated, and transplanted into long neck bottles containing Norris nutrient solution as described above. Plants were inoculated 48 h after transplanting using 1 mL of suspensions of serial decimal dilutions of soil in triplicate. Additionally, a control without inoculation and a positive control inoculated with the *Bradyrhizobium* strain BR 14454 were included. Soil moisture was determined using the oven-drying method to correct the number of rhizobia. The plants were collected 30 days after inoculation to verify the presence of nodules, and the data were interpreted using the MPN table (O'HARA et al., 2016).

d) Seed disinfection and strain inoculation

For inoculation, the strains were grown, and the seeds were disinfected, as described above. Five seeds were sown per pot, and each pot was inoculated with 1 mL of a bacterial suspension containing the *Bradyrhizobium* strain. After emergence, plants were thinned to two plants per pot.

e) Harvest and measurements

The plants were collected 30 days after emergence. The roots were cut into two parts to separate the nodules of the first 5 cm of the lateral root ("crown") and those of the lower part of the root to evaluate the effect of treatments on the distribution pattern of nodules, as adopted by Favero et al. (2022). The nodules, roots, and shoots were dried in an oven with forced air circulation at 65 °C until a constant weight was obtained.

f) Statistical analyses

Data were subjected to analysis of variance after being verified for the assumptions of normality and homogeneity of variances of the residuals, using the Shapiro–Wilk and Bartlett tests, respectively. For the adequacy of the assumptions, the nodule number (%) and nodule dry weight of the first 5 cm of the root were transformed by $\lambda = 2.6$ and $\lambda = 3.4$, respectively, as indicated by Box–Cox (SAKIA, 1992) in the MASS package (VENABLES; RIPLEY, 2002). The means were grouped using the Scott–Knott test at 5% probability. The correlation between the variables was verified through principal component analysis using the factoextra package (KASSAMBARA; MUNDT, 2020). Data were analyzed using R environment (R CORE TEAM, 2021).

6.5 RESULTS AND DISCUSSION

The nodulation test under axenic conditions showed that the recommended cowpea strains UFLA 3-84 (*B. viridifuturi*), BR 3267 (*B. yuanmingense*), and INPA 3-11B (*B. elkanii*) and the recommended soybean strain SEMIA 587 (*B. elkanii*) nodulated mung bean (Table 8). Additionally, all strains were able to nodulate their original host. However, the cowpea strain BR 3262 (*B. pachyrhizi*) and the soybean strains CPAC 15 (*B. japonicum*), CPAC 7 (*B. diazoefficiens*), and BR 29 (*B. elkanii*) did not nodulate the MGS Esmeralda cultivar (Table 8). These results suggest a close symbiotic relationship between some *Bradyrhizobium* strains and mung bean, although it is considered a promiscuous legume (YANG et al., 2008; ZHANG et al., 2008).

Table 8. Evaluation of elite *Bradyrhizobium* strains used in commercial inoculants of cowpea and soybean for the nodulation capacity of the MGS Esmeralda mung bean cultivar under axenic conditions. Plants collected 30 days after emergence

Strain	Original host	Nodulation effectiveness					
		Mung bean			Original host		
		RI	R2	R3	RI	R2	R3
UFLA 3-84	Cowpea	+	+	+	+	+	+
BR 3267	Cowpea	+	+	+	+	+	+
INPA 3-11B	Cowpea	+	+	+	+	+	+
BR 3262	Cowpea	-	-	-	+	+	+
CPAC 15	Soybean	-	-	-	+	+	+
CPAC 7	Soybean	-	-	-	+	+	+
SEMIA 587	Soybean	+	+	+	+	+	+
BR 29	Soybean	-	-	-	+	+	+

+ and - indicate positive and negative results for nodulation, respectively. Absolute control (without inoculation) mung bean, cowpea and soybean plants did not form nodules, and positive control mung bean plants (inoculated with strain BR 14454) formed nodules.

BR 3267, BR 3262, and BR 29 inefficiently nodulate the mung bean cultivar Camaleão, and CPAC 15 and CPAC 7 do not nodulate this legume (SANTOS, 2020). Therefore, the ability to form an efficient symbiosis differs depending on the plant genotype as in our study BR 3262 and BR 29 did not form nodules with MGS Esmeralda cultivar. The elite *Bradyrhizobium* strain SEMIA 587, recommended for soybean inoculation in Brazil, is efficient in cowpea nodulation; however, CPAC 7 and CPAC 15 are inefficient (ZILLI et al., 2011). These results suggest similar symbiotic patterns between mung bean and cowpea.

Despite mung bean being associated with different *Bradyrhizobium* species, some *Bradyrhizobium* strains isolated from other legumes are incompatible with mung bean and this is related to the presence of the type III secretion system - T3SS in *B. elkanii* USDA 61 (NGUYEN et al., 2017; OKAZAKI et al., 2009) and *B. diazoefficiens* USDA110 (PIROMYOU et al., 2019); they are also associated with different proteins in *Bradyrhizobium guangxiense* CCBAU 53363 (WU et al., 2020). In some cases, *B. elkanii* USDA 61 and *B. diazoefficiens* USDA110 could not form nodules on mung bean. However, symbiotic specificity may vary depending on plant genotype (OKAZAKI et al., 2009; PIROMYOU et al., 2019).

The strains BR 3262, CPAC 15, CPAC 7, and BR 29 which did not nodulate mung bean in our study belong to *B. pachyrhizi*, *B. japonicum*, *B. diazoefficiens*, and *B. elkanii*, respectively. However, for these species, only one *B. elkanii* and *B. diazoefficiens* strains were

were reported to be unable to mung bean nodulate (OKAZAKI et al., 2009; PIROMYOU et al., 2019).

When the *Bradyrhizobium* inoculation efficiency experiment was performed to quantify the native rhizobia capable of nodulating mung bean using MPN, there were 2.4×10^4 rhizobia cells per gram of soil. Seven mung bean isolates and BR 29 increased the nodule number by up to 50% compared with the control without inoculation (Figure 18a). Furthermore, all strains that could nodulate mung bean under axenic conditions resulted in a greater nodule number in the upper part of the root; only BR 29, BR 3262, and CPAC 7 strains did not differ from the absolute control (Figure 18c). However, the *B. elkanii* strain BR 29 increased nodule number only in the lower part of the root although it could not nodulate mung bean under axenic conditions (Table 8). The nodulation in the upper part of the root for UFLA 3-84 (*B. viridifuturi*) and SEMIA 587 (*B. elkanii*) was greater than that of the other elite strains capable of nodulating mung bean, and can be considered efficient in mung bean nodulation.

Furthermore, 16 strains increased nodule dry weight by up to 57% compared with that of the absolute control (Figure 18b). A previous study has shown that inoculation of *Bradyrhizobium* isolates obtained from mung bean nodules increased mung bean nodule number and dry weight, respectively, by up to 71 and 79% in Brazilian soils (FAVERO et al., 2022). In other countries, inoculation with *Bradyrhizobium* strains positively affected mung bean nodulation (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; HAKIM et al., 2020; ISLAM et al., 2006; TARIQ et al., 2012).

Among the strains capable of nodulating mung bean under axenic conditions, BR 14458 and INPA 3-11B had nodule dry weight that were not significantly different from that of the absolute control (Figure 18b). As for the nodulation pattern based on nodule weight, all strains capable of nodulating mung bean (Table 8), exhibited greater nodulation in the upper part of the root (Figure 18d). For the nodule number, BR 29 increased the nodule dry weight; however, the nodule distribution pattern did not change.

In general, the root and shoot dry weights increased by a similar proportion in the treatments (Figure 18e and f). Among the strains used in commercial inoculants for cowpea and soybean, the UFLA 3-84 of *B. viridifuturi* and SEMIA 587 of *B. elkanii* increased the shoot dry weight by amounts similar to mung bean isolates. Although BR 3267 of *B. yuanmingense* and INPA 3-11B of *B. elkanii* are able to nodulate under axenic conditions (Table 8), and have increased nodulation in the upper part of the root, these strains did not result in increases in root and shoot dry weight (Figure 18e and f). UFLA 3-84 and SEMIA 587 have been reported to efficiently nodulate the Camaleão mung bean cultivar under axenic conditions (SANTOS, 2020). In Brazil, the SEMIA 587 strain, recommended for soybean, increased nodulation and growth in cowpea, performing similar to strains authorized for commercial inoculants for cowpea (ZILLI et al., 2011).

Among the strains capable of nodulating mung bean, BR 3267 and INPA 3-11B had no significant effect on root dry weight. Similary, BR 3267, INPA 3-11B, and BR 14458 inoculation had no significant effect on shoot dry weight. On the other hand, all strains isolated from mung bean nodules increased the shoot dry weight of mung bean, except BR 14458. Therefore, the BR 3267 (*B. yuanmingense*) and INPA 3-11B (*B. elkanii*) of commercial cowpea inoculants are inefficient for mung bean inoculation even though they can nodulate mung bean.

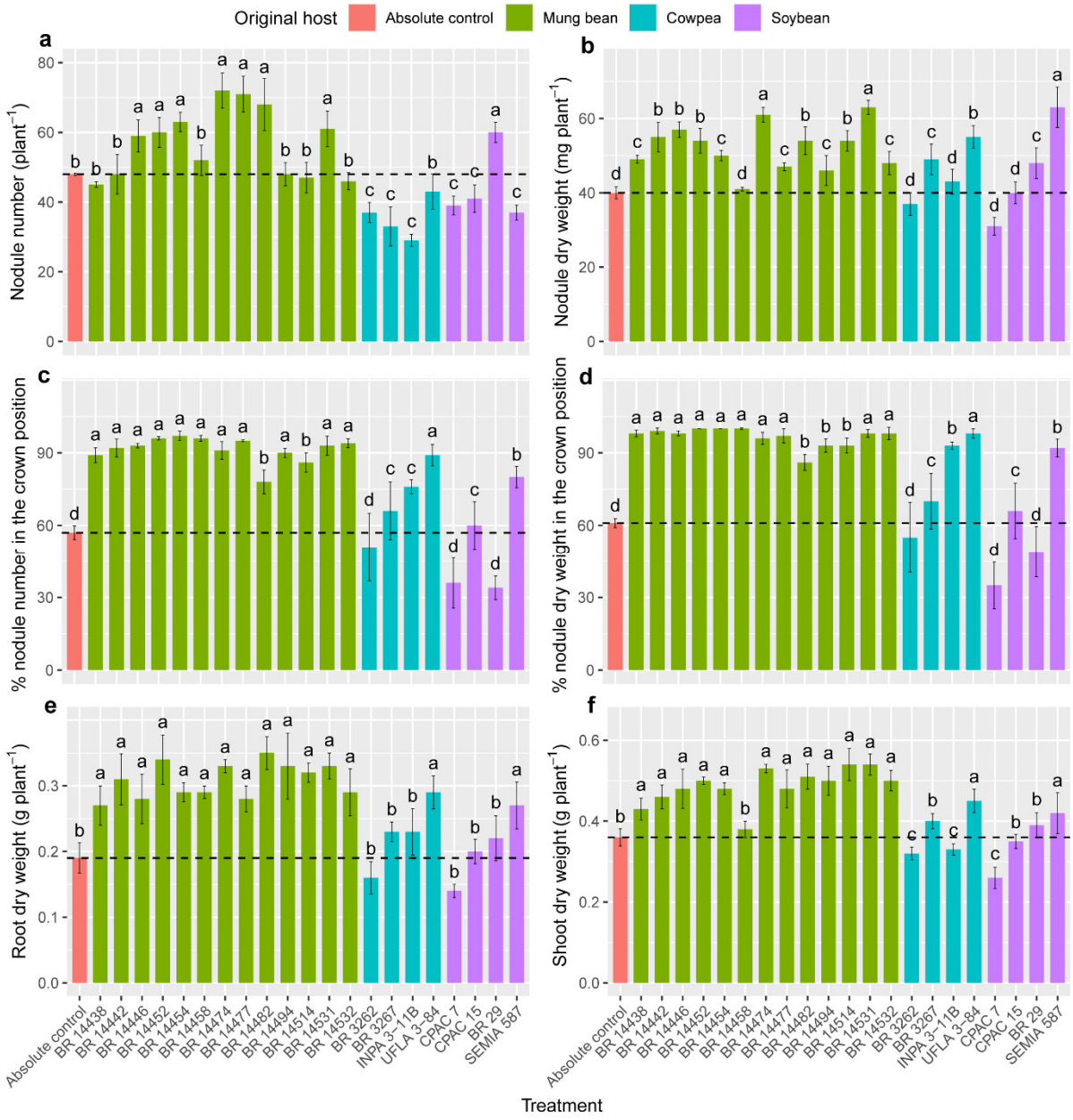

Figure 18. Nodule number (a), nodule dry weight (b), percentage of nodule number in the crown position (c), percentage of nodule dry weight of crown position (d), root dry weight (e), and shoot dry weight (f) of the MGS Esmeralda mung bean cultivar grown in pots with soil and inoculated with different *Bradyrhizobium* strains, in addition to the absolute control. Plants collected 30 days after emergence. Original host means the species from which the strain came. Bars (means) topped by the same letters are not statistically different by Scott–Knott test at 5% probability. Vertical lines on bars represent standard errors. Horizontal lines represents the mean of absolute control. Coefficient of variation (%): nodule number = 16.56, nodule dry weight = 12.29, percentage of nodule number in the crown position = 13.42, percentage of nodule dry weight of crown position = 12.52, root dry weight = 19.67, and shoot dry weight = 13.60.

The root and shoot dry weight as a function of inoculation increased by up to 84 and 50%, respectively. A previous study evaluated the inoculation of *Bradyrhizobium* isolates obtained from mung bean nodules and recorded an increase in root and shoot dry weight of up

to 43 and 66%, respectively (FAVERO et al., 2022). In other countries, the inoculation of *Bradyrhizobium* strains has enhanced mung bean growth (BHUIYAN; MIAN, 2007; CHRISTOPHER et al., 2018; DELIĆ et al., 2011; ISLAM et al., 2006); however, it is not uncommon to have a low increase in plant growth as a function of inoculation (MATHU et al., 2012).

Principal component analysis showed a correlation between nodulation and plant growth variables (Figure 19). Generally, treatments inoculated with strains isolated from mung bean nodules were more related to these variables, including those inoculated with SEMIA 587 and UFLA 3-84 strains, indicating that these strains may be more efficient for mung bean inoculation. Contrary, CPAC 7, CPAC 15, BR 29, and BR 3262 strains that could not nodulate mung bean were closer to absolute control. On the other hand, BR 3267 and INPA 3-11B strains showed intermediate values; therefore, despite being able to nodulate, they have low mung bean inoculation efficiency. In this sense, our results suggest that SEMIA 587, UFLA 3-84, and isolates from mung bean nodules may be used for mung bean inoculation and should be evaluated in future field studies.

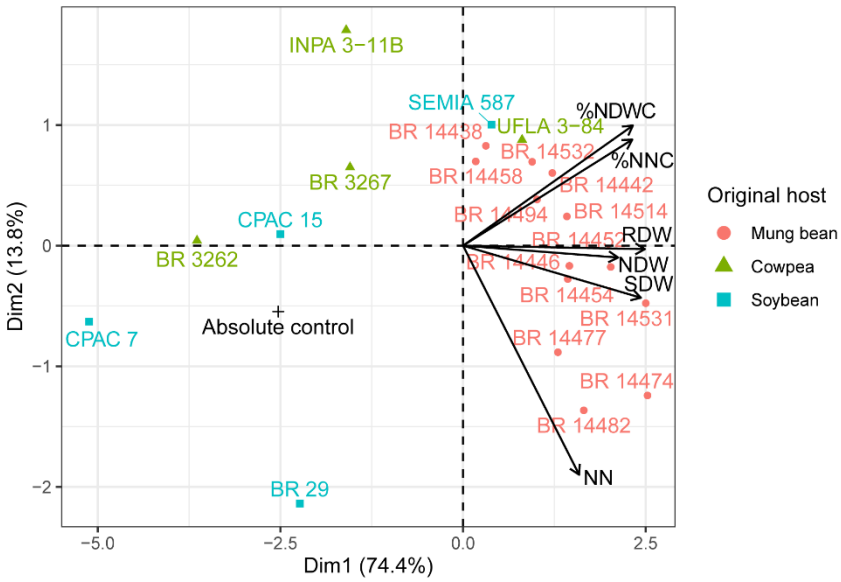

Figure 19. Principal component analysis between nodule number (NN), percentage of nodule number in the crown position (%NNC), nodule dry weight (NDW), percentage of nodule dry weight in the crown position (%NDWC), root dry weight (RDW), and shoot dry weight (SDW) of mung bean plants cultivated in pots with soil and inoculated with *Bradyrhizobium* strains, in addition, absolute control.

6.6 CONCLUSIONS

The SEMIA 587 (*Bradyrhizobium elkanii*) strain recommended for soybean, and the UFLA 3-84 (*B. viridifuturi*), BR 3267 (*B. yuanmingense*), and INPA 3-11B (*B. elkanii*) strains recommended for cowpea, are capable of nodulating mung bean. Conversely, the BR 3262 (*B. pachyrhizi*) of cowpea; and the CPAC 15 (*B. japonicum*), CPAC 7 (*B. diazoefficiens*), and BR 29 (*B. elkanii*) recommended for soybean are not capable of nodulating mung bean. SEMIA 587 (*B. elkanii*), UFLA 3-84 (*B. viridifuturi*), and isolates from mung bean should be evaluated under field conditions to verify their contribution to biological nitrogen fixation in mung bean.

7 CONCLUSÕES GERAIS

Os resultados deste estudo demonstram que a nodulação do feijão-mungo com rizóbios nativos é variável em solos brasileiros: em solos do Cerrado Matogrossense, as plantas apresentam baixa nodulação e, consequentemente, menor desenvolvimento, comparado aos solos da região Sudeste. O isolamento de bactérias a partir de nódulos revelou uma predominância do gênero *Bradyrhizobium*, e que mostraram alta eficiência na nodulação do feijão-mungo em condição axênica. Em contraste com outros estudos, isolados de *Rhizobium*, *Mesorhizobium* e *Ensifer* não foram capazes de nodular o feijão-mungo. Diferenças foram observadas em relação aos efeitos da inoculação de isolados de *Bradyrhizobium* no desenvolvimento das plantas, e parecem estar relacionadas ao agrupamento filogenético e ao solo de origem. Os isolados que promoveram os maiores valores de massa seca de parte aérea formaram um grupo filogenético próximo ao *Bradyrhizobium yuanmingense* B071. Com relação ao solo de origem, plantas inoculadas com isolados de *Bradyrhizobium* originários de solos do estado de Mato Grosso (Cerrado brasileiro) foram caracterizadas por menor massa seca de parte aérea, o que pode ter relação com a baixa nodulação do feijão-mungo cultivado nessas amostras de solo.

O sequenciamento do gene 16S rRNA do microbioma de nódulos mostrou que mais de 99% das sequências bacterianas presentes nos nódulos de feijão-mungo pertencem ao gênero *Bradyrhizobium*, e este foi o único gênero rizobiano detectado em nódulos de feijão-mungo através desta metodologia. Sequências próximas a *B. elkanii* dominaram em todos os nódulos, exceto em plantas cultivadas na amostra de solo do Sistema Integrado de Produção Agroecológica - SIPA, onde apenas *B. japonicum* esteve presente. A comunidade bacteriana de nódulos de feijão-mungo foi influenciada principalmente pelo pH do solo, e pelos teores de K, Ca e P. Além disso, também foi detectada uma diferença relativa aos genótipos quanto à colonização dos nódulos por sequências agrupadas ao gênero *Pseudomonas*. Embora representem uma taxa pequena, em torno de 0,1% do total, as sequências da OTU agrupada à *Pseudomonas* foram recuperadas apenas de nódulos do genótipo MGS Esmeralda, sugerindo uma característica diferente quanto à especificidade entre macro e microsimbiontes.

Em um experimento em vaso com solo não estéril, a inoculação das estirpes de *Bradyrhizobium* isoladas de feijão-mungo resultaram em incrementos de até 71 e 79% no número e na massa seca de nódulos, respectivamente. Em massa seca de raiz, de parte aérea e no N acumulado na parte aérea derivado da FBN, os incrementos foram de até 43, 66 e 55%, respectivamente. As estirpes agrupadas ao super clado de *B. japonicum* apresentaram maior potencial de inoculação com feijão-mungo, principalmente aquelas agrupadas com *B. yuanmingense*. Embora tenha havido alta contribuição da FBN, chegando até 89%, a quantidade de N derivada da FBN foi insuficiente para equivaler ao crescimento do feijão-mungo com N-fertilizante. Sob aplicação de doses *starter* de N, houve aumento no desenvolvimento do feijão-mungo, mas com reduções significativas na nodulação.

Sob inoculação cruzada, a estirpe SEMIA 587 (*B. elkanii*) recomendada para soja, e as estirpes UFLA 3-84 (*B. viridifuturi*), BR 3267 (*B. yuanmingense*) e INPA 3-11B (*B. elkanii*) recomendadas para feijão-caupi, foram capazes de nodular o feijão-mungo. Por outro lado, a estirpe BR 3262 (*B. pachyrhizi*) de feijão-caupi, e as estirpes CPAC 15 (*B. japonicum*), CPAC 7 (*B. diazoefficiens*) e BR 29 (*B. elkanii*) de soja não foram capazes de nodular o feijão-mungo. A eficiência das estirpes elite SEMIA 587 (*B. elkanii*) e UFLA 3-84 (*B. viridifuturi*) em termos de aumento da nodulação e crescimento das plantas foi semelhante às estirpes de *Bradyrhizobium* isoladas de nódulos de feijão mungo, portanto, deve ser avaliada sob condições de campo para verificar sua contribuição para a fixação biológica de nitrogênio em feijão-mungo.

8 CONSIDERAÇÕES FINAIS

No Brasil, há décadas, o feijão-mungo vem sendo objeto de estudos, no entanto, antes do início desta tese não haviam estudos relacionados aos rizóbios associados ao feijão-mungo no país e o seu potencial quanto à FBN. Nesse sentido, os resultados desta tese são importantes para os primeiros achados sobre a FBN em feijão-mungo em solos tropicais brasileiros.

Os resultados encontrados neste estudo demonstram que o feijão-mungo apresenta nodulação variável em solos brasileiros, com baixa nodulação em solos do Cerrado Matogrossense. Os gêneros *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium* e *Ensifer* foram isolados de nódulos de feijão-mungo, no entanto, apenas isolados de *Bradyrhizobium* foram capazes de nodular. Esta incapacidade do isolados de *Rhizobium*, *Mesorhizobium* e *Ensifer* nodular o feijão-mungo ainda não havia sido relatada em estudos conduzidos em outros países, o que demonstra uma diferença quanto aos rizóbios associados ao feijão-mungo cultivado em solos brasileiros.

Através da análise das comunidades microbianas de nódulos de feijão-mungo pelo sequenciamento do gene 16S rRNA usando o *Next-Generation Sequencing* (NGS) Illumina MiSeq, identificou-se que mais de 99% das sequências recuperadas pertencem ao gênero *Bradyrhizobium*, sendo este o único rizóbio presentes em nódulos de feijão-mungo cultivado em solos brasileiros. Apesar de terem sido isoladas bactérias pertencentes a *Rhizobium*, *Mesorhizobium* e *Ensifer*, estes gêneros não foram detectados na análise de microbioma, revelando uma diferença na análise da diversidade dos rizóbios dos nódulos em função do método de isolamento em meio de cultura e do sequenciamento por NGS. Esta diferença pode estar relacionada ao fato dos isolados de *Rhizobium*, *Mesorhizobium* e *Ensifer* apresentarem crescimento rápido e colônias com muito muco, o que facilita o seu isolamento em meio de cultura.

Ainda sobre as comunidades bacterianas dos nódulos de feijão-mungo, *Pseudomonas* foi o gênero não-rizobiano mais abundante, no entanto, esteve presente apenas em nódulos do cultivar MGS Esmeralda, revelando uma diferença na especificidade entre genótipos. Estes achados indicam que são necessários estudos visando o isolamento de *Pseudomonas*, seguido de teste de coinoculação com estirpes de *Bradyrhizobium* eficientes utilizando a cultivar MGS Esmeralda.

Sob condições de cultivo em vaso com solo não-estéril, identificou-se que o feijão-mungo apresenta elevada contribuição da FBN, com valores entre 70 e 89%. Embora tenha havido alta contribuição da FBN, a quantidade de N derivada do FBN foi insuficiente para equivaler ao crescimento do feijão-mungo com N-fertilizante (160 kg ha^{-1} de N). As respostas de inoculação devem ser verificadas em campo, incluindo testes com outras cultivares, com a finalidade de identificar estirpes e cultivares mais eficientes quanto à FBN.

Sob estudo de inoculação cruzada, as estirpes SEMIA 587 (*B. elkanii*) recomendada para soja, e a UFLA 3-84 (*B. viridifuturi*) recomendada para feijão-caupi, foram capazes de nodular eficientemente o feijão-mungo, e apresentaram eficiência similar às estirpes de *Bradyrhizobium* isoladas de nódulos de feijão-mungo. Nesse sentido, sugere-se que estas estirpes podem facilitar o processo de recomendação de uma estirpe para compor um inoculante comercial para inoculação do feijão-mungo no Brasil e em outros países, a depender da disponibilidade destas estirpes no mercado local. Por outro lado, a incapacidade de nodular o feijão-mungo pelas estirpes BR 3262 (*B. pachyrhizi*) de feijão-caupi, CPAC 15 (*B. japonicum*), CPAC 7 (*B. diazoefficiens*) e BR 29 (*B. elkanii*) de soja, demonstram uma maior especificidade simbiótica do feijão-mungo com estas estirpes, as quais inclusive são conhecidas por nodularem o feijão-caupi. Estes resultados também podem contribuir para futuros estudos relacionados à especificidade simbiótica do feijão-mungo, bem como para a busca de estirpes mais eficientes.

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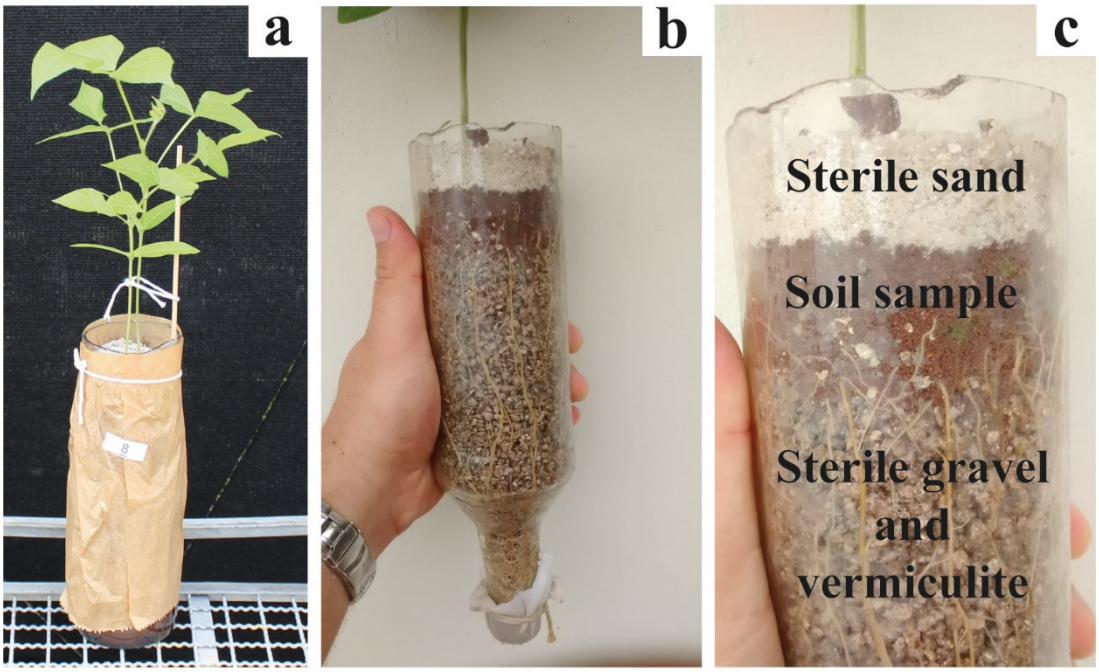
10 APÊNDICE



Appendix 1. Adaptation of the Leonard jars for mung bean plant growth using sterile substrate and the soil sample as an inoculant: details of the plants grown in a modified Leonard jar (a), and substrate (sterile gravel and vermiculite), non-sterile soil sample and sterilized sand (b and C) used in layers.

Appendix 2. Morphological characteristics and identification of isolates obtained from mung bean nodules using 16S rRNA. The plants were cultivated in different Brazilian tropical soils (continue)

Isolate	Soil origin	Genus	16S accession number	GT	pH	AM	CC	CD	CS	CE	CT
BR 14433	SE_RJ-I	*Bradyrhizobium* sp.	MT904884	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14434	SE_RJ-I	*Kaistia* sp.	MT904885	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14435	SE_RJ-I	*Bradyrhizobium* sp.	MT904886	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14436	SE_RJ-I	*Bradyrhizobium* sp.	MT904887	S	Alk	M	W	2 ≤ ≥ 4	NC	E	NT
BR 14437	SE_RJ-II	*Bradyrhizobium* sp.	MT904888	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14438	SE_RJ-II	*Bradyrhizobium* sp.	MT904889	S	Alk	F	W	< 2	C	F	NT
BR 14439	SE_RJ-III	*Agrobacterium* sp.	MT904890	F	Aci	M	Y	> 4	C	E	NT
BR 14440	SE_RJ-III	*Bradyrhizobium* sp.	MT904891	S	Alk	M	Y	< 2	C	E	NT
BR 14441	SE_RJ-III	*Bradyrhizobium* sp.	MT904892	S	Alk	M	W	2 ≤ ≥ 4	NC	F	NT
BR 14442	SE_RJ-V	*Bradyrhizobium* sp.	MT904893	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14443	SE_RJ-IV	*Bradyrhizobium* sp.	MT904894	S	Alk	F	W	< 2	C	F	NT
BR 14444	SE_RJ-IV	*Bradyrhizobium* sp.	MT904895	S	Alk	M	W	2 ≤ ≥ 4	C	E	T
BR 14445	SE_RJ-IV	*Bradyrhizobium* sp.	MT904896	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14446	SE_RJ-V	*Bradyrhizobium* sp.	MT904897	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14447	SE_RJ-V	*Rhizobium* sp.	MT904898	F	Aci	M	Y	2 ≤ ≥ 4	C	E	T
BR 14448	SE_RJ-I	*Bradyrhizobium* sp.	MT904899	S	Alk	M	W	< 2	C	E	NT
BR 14449	SE_RJ-I	*Bradyrhizobium* sp.	MT904900	S	Alk	M	W	> 4	C	E	NT
BR 14450	SE_RJ-I	*Bradyrhizobium* sp.	MT904901	S	Alk	M	W	> 4	C	E	NT
BR 14451	SE_RJ-III	*Bradyrhizobium* sp.	MT904902	S	Alk	M	Y	< 2	C	E	NT
BR 14452	SE_RJ-III	*Bradyrhizobium* sp.	MT904903	S	Alk	F	Y	< 2	C	E	NT
BR 14453	SE_RJ-III	*Bradyrhizobium* sp.	MT904904	S	Alk	M	W	2 ≤ ≥ 4	NC	E	T
BR 14454	SE_RJ-V	*Bradyrhizobium* sp.	MT904905	S	Alk	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14455	SE_RJ-I	*Bradyrhizobium* sp.	MT904906	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14456	SE_RJ-I	*Bradyrhizobium* sp.	MT904907	S	Alk	M	W	< 2	C	E	NT
BR 14457	SE_RJ-III	*Bradyrhizobium* sp.	MT904908	S	Alk	F	W	< 2	C	F	NT
BR 14458	SE_RJ-III	*Bradyrhizobium* sp.	MT904909	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14459	SE_RJ-I	*Bradyrhizobium* sp.	MT904910	S	Alk	F	W	< 2	C	F	NT
BR 14460	SE_RJ-II	*Bradyrhizobium* sp.	MT904911	S	Alk	F	W	< 2	C	F	NT
BR 14461	SE_RJ-V	*Bradyrhizobium* sp.	MT904912	S	Alk	F	W	< 2	C	F	NT
BR 14462	SE_RJ-II	*Ensifer* sp.	MT904913	F	Aci	M	Y	> 4	C	E	NT
BR 14463	SE_RJ-II	*Bradyrhizobium* sp.	MT904914	S	Alk	F	W	< 2	C	F	NT
BR 14464	SE_RJ-II	Mesorhizobium sp.	MT904915	F	Aci	M	Y	> 4	C	E	T
BR 14465	SE_RJ-V	*Leifsonia* sp.	MT904916	F	Aci	F	W	< 2	C	F	T
BR 14466	SE_RJ-V	*Rhizobium* sp.	MT904917	F	Aci	M	Y	> 4	C	E	NT
BR 14467	SE_RJ-III	*Bradyrhizobium* sp.	MT904918	S	Alk	F	W	< 2	C	F	NT
BR 14468	SE_RJ-III	Mesorhizobium sp.	MT904919	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14469	SE_RJ-I	*Bradyrhizobium* sp.	MT904920	S	Alk	M	W	< 2	C	E	NT
BR 14470	SE_RJ-I	*Bradyrhizobium* sp.	MT904921	S	Alk	M	W	< 2	C	E	NT
BR 14471	SE_RJ-I	*Bradyrhizobium* sp.	MT904922	S	Alk	M	W	2 ≤ ≥ 4	NC	E	T
BR 14472	SE_RJ-III	*Bradyrhizobium* sp.	MT904923	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14473	SE_RJ-V	*Bradyrhizobium* sp.	MT904924	S	Alk	M	W	> 4	C	E	NT
BR 14474	SE_RJ-V	*Bradyrhizobium* sp.	MT904925	S	Alk	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14475	SE_RJ-II	*Rhizobium* sp.	MT904926	F	Aci	M	Y	2 ≤ ≥ 4	C	E	T
BR 14476	SE_RJ-II	*Bradyrhizobium* sp.	MT904927	S	Alk	F	W	< 2	C	F	NT
BR 14477	SE_RJ-I	*Bradyrhizobium* sp.	MT904928	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14478	SE_RJ-III	*Bradyrhizobium* sp.	MT904929	S	Alk	M	W	< 2	C	E	NT
BR 14479	SE_RJ-I	*Bradyrhizobium* sp.	MT904930	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14480	SE_RJ-I	*Bradyrhizobium* sp.	MT904931	S	Alk	M	W	2 ≤ ≥ 4	NC	E	T
BR 14481	SE_RJ-IV	*Bradyrhizobium* sp.	MT904932	S	Alk	F	W	< 2	C	F	NT
BR 14482	SE_RJ-IV	*Bradyrhizobium* sp.	MT904933	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14483	SE_RJ-II	*Bradyrhizobium* sp.	MT904934	S	Alk	F	W	< 2	C	F	NT
BR 14484	SE_RJ-II	*Bacillus* sp.	MT904935	F	Alk	F	W	> 4	NC	F	T
BR 14485	SE_RJ-II	*Bacillus* sp.	MT904936	F	Aci	F	Y	2 ≤ ≥ 4	NC	F	T
BR 14486	SE_MG-II	Mesorhizobium sp.	MT904937	F	Aci	M	Y	2 ≤ ≥ 4	C	E	T
BR 14487	MW_MT-II	*Bradyrhizobium* sp.	MT904938	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14488	MW_MT-III	*Bradyrhizobium* sp.	MT904939	S	Alk	F	W	< 2	C	F	NT
BR 14489	SE_MG-I	*Leifsonia* sp.	MT904940	F	Aci	M	Y	< 2	C	F	T
BR 14490	MW_MT-I	*Bradyrhizobium* sp.	MT904941	S	Alk	F	W	< 2	C	F	T

Appendix 2. Continuation

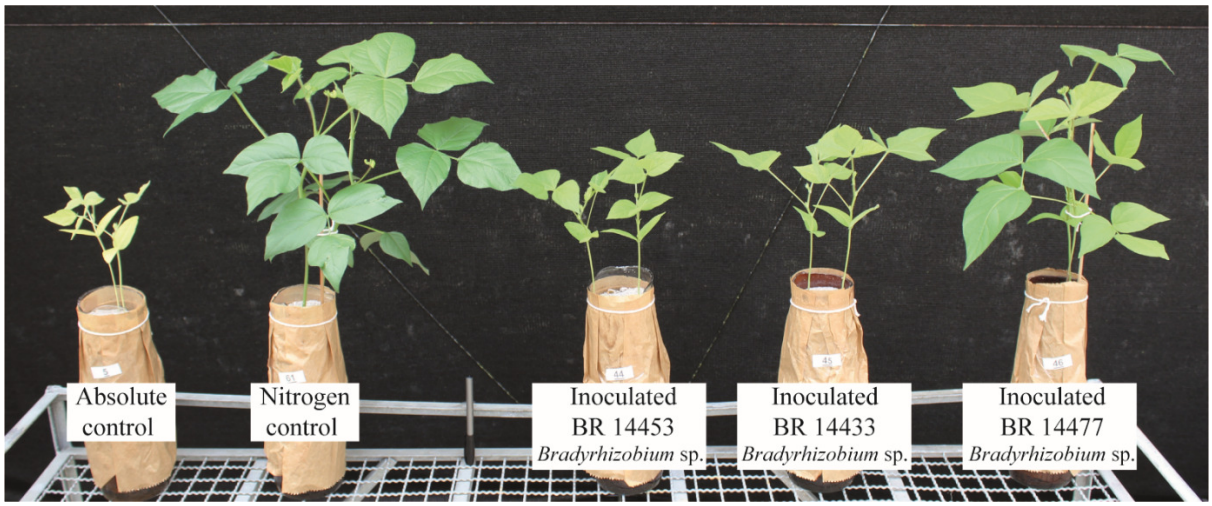
Isolate	Soil origin	Genus	16S accession number	GT	pH	AM	CC	CD	CS	CE	CT
BR 14491	MW_MT-I	*Bradyrhizobium* sp.	MT904942	S	Alk	F	W	< 2	C	F	NT
BR 14492	SE_MG-I	*Bradyrhizobium* sp.	MT904943	S	Alk	F	W	< 2	C	F	NT
BR 14493	SE_MG-I	*Bradyrhizobium* sp.	MT904944	S	Alk	F	W	< 2	C	F	NT
BR 14494	SE_MG-II	*Bradyrhizobium* sp.	MT904945	S	Alk	M	W	< 2	C	E	NT
BR 14495	SE_MG-II	*Rhizobium* sp.	MT904946	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14496	SE_MG-II	*Bacillus* sp.	MT904947	F	Aci	F	Y	> 4	NC	F	NT
BR 14497	SE_MG-II	*Rhizobium* sp.	MT904948	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14498	SE_MG-II	*Rhizobium* sp.	MT904949	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14499	MW_MT-I	*Bradyrhizobium* sp.	MT904950	S	Alk	F	W	< 2	C	F	NT
BR 14500	MW_MT-III	*Rhizobium* sp.	MT904951	F	Aci	M	Y	2 ≤ ≥ 4	C	E	T
BR 14501	SE_MG-I	*Bradyrhizobium* sp.	MT904952	S	Alk	F	W	< 2	C	F	NT
BR 14502	MW_MT-I	*Rhizobium* sp.	MT904953	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14503	SE_MG-II	*Bradyrhizobium* sp.	MT904954	S	Alk	F	W	< 2	C	F	NT
BR 14504	MW_MT-I	*Rhizobium* sp.	MT904955	F	Aci	M	Y	2 ≤ ≥ 4	C	E	T
BR 14505	SE_MG-I	*Bradyrhizobium* sp.	MT904956	S	Alk	F	W	< 2	C	F	NT
BR 14506	SE_MG-I	*Bradyrhizobium* sp.	MT904957	S	Alk	F	W	< 2	C	F	NT
BR 14507	MW_MT-II	*Bradyrhizobium* sp.	MT904958	S	Alk	F	W	< 2	C	F	NT
BR 14508	MW_MT-II	*Rhizobium* sp.	MT904959	F	Aci	M	Y	> 4	C	E	NT
BR 14509	MW_MT-III	*Rhizobium* sp.	MT904960	F	Aci	M	Y	> 4	C	E	NT
BR 14510	MW_MT-III	*Rhizobium* sp.	MT904961	F	Aci	M	Y	> 4	C	E	NT
BR 14511	MW_MT-III	*Rhizobium* sp.	MT904962	F	Aci	M	Y	> 4	C	E	T
BR 14512	MW_MT-III	*Leifsonia* sp.	MT904963	F	Aci	F	Y	< 2	C	F	T
BR 14513	MW_MT-I	*Bradyrhizobium* sp.	MT904964	S	Alk	F	W	< 2	C	F	NT
BR 14514	SE_MG-II	*Bradyrhizobium* sp.	MT904965	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14515	SE_MG-II	*Bradyrhizobium* sp.	MT904966	S	Alk	F	W	< 2	C	F	NT
BR 14516	SE_MG-II	Mesorhizobium sp.	MT904967	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14517	SE_MG-II	*Mycolicibacterium* sp.	MT904968	S	Alk	M	Y	< 2	C	E	NT
BR 14518	SE_MG-I	*Rhizobium* sp.	MT904969	F	Aci	M	Y	> 4	C	E	NT
BR 14519	SE_MG-I	*Rhizobium* sp.	MT904970	F	Aci	M	Y	> 4	C	E	NT
BR 14520	MW_MT-III	*Rhizobium* sp.	MT904971	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14521	MW_MT-III	*Rhizobium* sp.	MT904972	F	Aci	M	Y	2 ≤ ≥ 4	C	E	T
BR 14522	MW_MT-I	*Bradyrhizobium* sp.	MT904973	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14523	SE_MG-II	*Rhizobium* sp.	MT904974	F	Aci	M	Y	2 ≤ ≥ 4	C	E	T
BR 14524	MW_MT-II	*Rhizobium* sp.	MT904975	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14525	MW_MT-II	*Ensifer* sp.	MT904976	F	Aci	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14526	MW_MT-II	*Ensifer* sp.	MT904977	F	Aci	M	W	2 ≤ ≥ 4	C	F	NT
BR 14527	MW_MT-II	*Bradyrhizobium* sp.	MT904978	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14528	MW_MT-II	*Bradyrhizobium* sp.	MT904979	S	Alk	F	W	< 2	C	F	NT
BR 14529	SE_RJ-I	*Bradyrhizobium* sp.	MT904980	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14530	SE_MG-II	*Bradyrhizobium* sp.	MT904981	S	Alk	M	W	> 4	C	E	T
BR 14531	SE_RJ-V	*Bradyrhizobium* sp.	MT904982	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14532	SE_MG-II	*Bradyrhizobium* sp.	MT904983	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14533	SE_RJ-III	*Bradyrhizobium* sp.	MT904984	S	Alk	M	W	> 4	C	E	T

GT – growth time (F – fast, S – slow); pH (Alk – alkaline, Aci – acid); AM – abundance of mucus (F – few, M – many); CC – colony color (W – white, Y – yellow); CD – colony diameter in mm; CS – colony shape (C – circular, NC – non-circular); CE – colony elevation (P – flat, E – elevated); CT – colony transparency (T – transparent, NT – non-transparent).

Appendix 3. Nodulation test of the BR 14487 strain of the genus *Bradyrhizobium* with mung bean, cowpea and siratro cultivated under axenic conditions †

Legume specie	Inoculated strain or treatment	Result §		
		R1	R2	R3
Mung bean [*Vigna radiata* (L.) Wilczek]	BR 14487	-	-	-
	Negative control	-	-	-
	Positive control (BR 14454)	+	+	+
Cowpea [*Vigna unguiculata* (L.) Walp]	BR 14487	-	-	-
	Negative control	-	-	-
	Positive control (BR 3267) ‡	+	+	+
Siratro (*Macroptilium atropurpureum*)	BR 14487	-	-	-
	Negative control	-	-	-
	Positive control (soil sample)	+	+	+

† Test conducted on long neck bottles, similar to that described in section 3.4.3.
‡ *Bradyrhizobium* strain cowpea nodulating (LEITE et al., 2017; http://dx.doi.org/10.1016/j.bjm.2017.01.007).
§ + and - indicate positive and negative nodulation, respectively.



Appendix 4. Mung bean MGS Esmeralda cultivar in Leonard jar under axenic conditions to compare the effect of *Bradyrhizobium* strains inoculation isolated from mung bean nodules cultivated in Brazilian soils.

Appendix 5. Paired comparisons by permutational multivariate analysis of variance (PERMANOVA) for the levels of phylogenetic groups and soil origin of mung bean isolates based on the shoot dry weights of the inoculated treatments (continue)

Paired comparisons			
Phylogenetic group	p-value corrected	Soil origin	p-value corrected
1a <-> 1ab	0.659	MW_MT-I <-> MW_MT-II	0.364
1a <-> 1b	0.420	MW_MT-I <-> MW_MT-III	0.186
1a <-> 1ba	0.002 *	MW_MT-I <-> SE_MG-I	0.009 *
1a <-> 1bb	0.766	MW_MT-I <-> SE_MG-II	0.003 *
1a <-> 2a	0.123	MW_MT-I <-> SE_RJ-I	0.003 *
1a <-> 2baaa	0.160	MW_MT-I <-> SE_RJ-II	0.003 *
1a <-> 2baab	0.002 *	MW_MT-I <-> SE_RJ-III	0.003 *
1a <-> 2baac	0.061	MW_MT-I <-> SE_RJ-IV	0.003 *
1a <-> 2bab	0.002 *	MW_MT-I <-> SE_RJ-V	0.003 *
1a <-> 2bba	0.002 *	MW_MT-II <-> MW_MT-III	0.066
1a <-> 2bbb	0.002 *	MW_MT-II <-> SE_MG-I	0.003 *
1ab <-> 1b	0.401	MW_MT-II <-> SE_MG-II	0.003 *
1ab <-> 1ba	0.002 *	MW_MT-II <-> SE_RJ-I	0.003 *
1ab <-> 1bb	0.970	MW_MT-II <-> SE_RJ-II	0.003 *
1ab <-> 2a	0.175	MW_MT-II <-> SE_RJ-III	0.003 *
1ab <-> 2baaa	0.468	MW_MT-II <-> SE_RJ-IV	0.003 *
1ab <-> 2baab	0.082	MW_MT-II <-> SE_RJ-V	0.003 *
1ab <-> 2baac	0.167	MW_MT-III <-> SE_MG-I	0.500
1ab <-> 2bab	0.059	MW_MT-III <-> SE_MG-II	0.093
1ab <-> 2bba	0.002 *	MW_MT-III <-> SE_RJ-I	0.027 *
1ab <-> 2bbb	0.004 *	MW_MT-III <-> SE_RJ-II	0.093
1b <-> 1ba	0.002 *	MW_MT-III <-> SE_RJ-III	0.346
1b <-> 1bb	0.486	MW_MT-III <-> SE_RJ-IV	0.005 *
1b <-> 2a	0.175	MW_MT-III <-> SE_RJ-V	0.014 *
1b <-> 2baaa	0.061	SE_MG-I <-> SE_MG-II	0.083
1b <-> 2baab	0.002 *	SE_MG-I <-> SE_RJ-I	0.003 *
1b <-> 2baac	0.066	SE_MG-I <-> SE_RJ-II	0.075
1b <-> 2bab	0.002 *	SE_MG-I <-> SE_RJ-III	0.364
1b <-> 2bba	0.002 *	SE_MG-I <-> SE_RJ-IV	0.003 *
1b <-> 2bbb	0.002 *	SE_MG-I <-> SE_RJ-V	0.003 *
1ba <-> 1bb	0.002 *	SE_MG-II <-> SE_RJ-I	0.164
1ba <-> 2a	0.002 *	SE_MG-II <-> SE_RJ-II	0.887
1ba <-> 2baaa	0.002 *	SE_MG-II <-> SE_RJ-III	0.318
1ba <-> 2baab	0.002 *	SE_MG-II <-> SE_RJ-IV	0.234
1ba <-> 2baac	0.045 *	SE_MG-II <-> SE_RJ-V	0.039 *
1ba <-> 2bab	0.002 *	SE_RJ-I <-> SE_RJ-II	0.129
1ba <-> 2bba	0.571	SE_RJ-I <-> SE_RJ-III	0.005 *
1ba <-> 2bbb	0.002 *	SE_RJ-I <-> SE_RJ-IV	0.882
1bb <-> 2a	0.141	SE_RJ-I <-> SE_RJ-V	0.117
1bb <-> 2baaa	0.360	SE_RJ-II <-> SE_RJ-III	0.364
1bb <-> 2baab	0.061	SE_RJ-II <-> SE_RJ-IV	0.163

Appendix 5. Continuation

Paired comparisons			
Phylogenetic group	p-value corrected	Soil origin	p-value corrected
1bb <-> 2baac	0.141	SE_RJ-II <-> SE_RJ-V	0.014 *
1bb <-> 2bab	0.027 *	SE_RJ-III <-> SE_RJ-IV	0.065
1bb <-> 2bba	0.002 *	SE_RJ-III <-> SE_RJ-V	0.003 *
1bb <-> 2bbb	0.004 *	SE_RJ-IV <-> SE_RJ-V	0.151
2a <-> 2baaa	0.002 *		
2a <-> 2baab	0.002 *		
2a <-> 2baac	0.296		
2a <-> 2bab	0.002 *		
2a <-> 2bba	0.002 *		
2a <-> 2bbb	0.002 *		
2baaa <-> 2baab	0.090		
2baaa <-> 2baac	0.013 *		
2baaa <-> 2bab	0.084		
2baaa <-> 2bba	0.002 *		
2baaa <-> 2bbb	0.013 *		
2baab <-> 2baac	0.012 *		
2baab <-> 2bab	0.970		
2baab <-> 2bba	0.002 *		
2baab <-> 2bbb	0.353		
2baac <-> 2bab	0.002 *		
2baac <-> 2bba	0.015 *		
2baac <-> 2bbb	0.002 *		
2bab <-> 2bba	0.002 *		
2bab <-> 2bbb	0.296		
2bba <-> 2bbb	0.002 *		

* indicates significant difference at $p = 0.05$.

Appendix 6. Soil origin, 16S accession number in the NCBI and morphological characteristics of *Bradyrhizobium* strains evaluated in our study. Strains isolated from mung bean nodules cultivated in Brazilian tropical soils and described by Favero et al. (2021a) (Chapter I) †

Strain	Soil origin ‡	16S accession number in NCBI	GT	pH	AM	CC	CD	CS	CE	CT
BR 14435	22°38'4.61"S, 42°48'40.40"W	MT904886	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14436	22°38'4.61"S, 42°48'40.40"W	MT904887	S	Alk	M	W	2 ≤ ≥ 4	NC	E	NT
BR 14437	22°20'52.36"S, 43°25'2.24"W	MT904888	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14438	22°20'52.36"S, 43°25'2.24"W	MT904889	S	Alk	F	W	< 2	C	F	NT
BR 14440	22°20'54.84"S, 43°25'2.27"W	MT904891	S	Alk	M	Y	< 2	C	E	NT
BR 14442	22°45'16.36"S, 43°40'28.04"W	MT904893	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14444	22°45'22.27"S, 43°40'2.03"W	MT904895	S	Alk	M	W	2 ≤ ≥ 4	C	E	T
BR 14445	22°45'22.27"S, 43°40'2.03"W	MT904896	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14446	22°45'16.36"S, 43°40'28.04"W	MT904897	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14449	22°38'4.61"S, 42°48'40.40"W	MT904900	S	Alk	M	W	> 4	C	E	NT
BR 14450	22°38'4.61"S, 42°48'40.40"W	MT904901	S	Alk	M	W	> 4	C	E	NT
BR 14452	22°20'54.84"S, 43°25'2.27"W	MT904903	S	Alk	F	Y	< 2	C	E	NT
BR 14454	22°45'16.36"S, 43°40'28.04"W	MT904905	S	Alk	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14455	22°38'4.61"S, 42°48'40.40"W	MT904906	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14458	22°20'54.84"S, 43°25'2.27"W	MT904909	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14469	22°38'4.61"S, 42°48'40.40"W	MT904920	S	Alk	M	W	< 2	C	E	NT
BR 14472	22°20'54.84"S, 43°25'2.27"W	MT904923	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14474	22°45'16.36"S, 43°40'28.04"W	MT904925	S	Alk	M	Y	2 ≤ ≥ 4	C	E	NT
BR 14476	22°20'52.36"S, 43°25'2.24"W	MT904927	S	Alk	F	W	< 2	C	F	NT
BR 14477	22°38'4.61"S, 42°48'40.40"W	MT904928	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14479	22°38'4.61"S, 42°48'40.40"W	MT904930	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14480	22°38'4.61"S, 42°48'40.40"W	MT904931	S	Alk	M	W	2 ≤ ≥ 4	NC	E	T
BR 14481	22°45'22.27"S, 43°40'2.03"W	MT904932	S	Alk	F	W	< 2	C	F	NT
BR 14482	22°45'22.27"S, 43°40'2.03"W	MT904933	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14483	22°20'52.36"S, 43°25'2.24"W	MT904934	S	Alk	F	W	< 2	C	F	NT
BR 14493	20°24'07.57"S, 42°49'05.08"W	MT904944	S	Alk	F	W	< 2	C	F	NT
BR 14494	21°14'36.74"S, 43° 9'30.55"W	MT904945	S	Alk	M	W	< 2	C	E	NT
BR 14514	21°14'36.74"S, 43° 9'30.55"W	MT904965	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14529	22°38'4.61"S, 42°48'40.40"W	MT904980	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14531	22°45'16.36"S, 43°40'28.04"W	MT904982	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT
BR 14532	21°14'36.74"S, 43° 9'30.55"W	MT904983	S	Alk	M	W	2 ≤ ≥ 4	C	E	NT

† Modified from Favero et al. (2021a) (https://doi.org/10.1016/j.apsoil.2021.104041) (Chapter I).

‡ Latitude and longitude, respectively.

GT – growth time (F – fast, S – slow); pH (Alk – alkaline, Aci – acid); AM – abundance of mucus (F – few, M – many); CC – colony color (W – white, Y – yellow); CD – colony diameter in mm; CS – colony shape (C – circular, NC – non-circular); CE – colony elevation (P – flat, E – elevated); CT – colony transparency (T – transparent, NT – non-transparent).

Appendix 7. Regression equations for nodule number, nodule dry weight, root dry weight and shoot dry weight for mung bean plants harvested at 17 and 38 days after emergence, cultivated in pots with soil in response to N doses (0, 15, 30, 45, 60 and 75 kg ha^{-1}) associated with *Bradyrhizobium* strain inoculation

Harvests (DAE)	Regression equations	Determination coefficient	Significance
	Nodule number ($plant^{-1}$)		
17	$y = 0.0277x^2 - 2.9155x + 68.888$	0.89	**
38	$y = -1.9692x + 169.24$	0.95	**
	Nodule dry weight (mg $plant^{-1}$)		
17	$y = 0.0117x^2 - 1.1809x + 25.668$	0.83	**
38	$y = -3.2271x + 212.56$	0.94	**
	Root dry weight (g $plant^{-1}$)		
17	$y = -3E\text{-}05x^2 + 0.0016x + 0.2186$	0.26	NS
38	$y = -0.0001x^2 + 0.0129x + 1.2586$	0.82	*
	Shoot dry weight (g $plant^{-1}$)		
17	$y = -8E\text{-}05x^2 + 0.0052x + 0.3781$	0.58	*
38	$y = -0.0008x^2 + 0.0866x + 2.7159$	0.99	**

* and ** indicates that the parameters were significant at $p = 0.05$ and $p = 0.01$, respectively. NS = not significant. DAS = days after emergence.