UNIVERSIDADE DO VALE DO TAQUARI - UNIVATES
PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU*
EM BIOTECNOLOGIA

# AVALIAÇÃO DE RIZÓBIOS NO INCREMENTO DA PRODUÇÃO DE FEIJÃO (*Phaseolus vulgaris* L.) E NO BIOCONTROLE DO FUNGO *Macrophomina phaseolina*

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia (PPGBiotec) da Universidade do Vale do Taquari – Univates, como parte dos requisitos para obtenção do grau de Doutor em Biotecnologia, na Área de Concentração Biotecnologia Agroalimentar.

Orientador: Dr. Raul Antonio Sperotto
Coorientadora: Dra. Camille Eichelberger Granada

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A Banca examinadora abaixo aprova a Tese apresentada ao Programa de Pós-Graduação em Biotecnologia, da Universidade do Vale do Taquari - Univates, como parte da exigência para a obtenção do grau de Doutor em Biotecnologia, na área de concentração Biotecnologia Agroalimentar:

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Lajeado, 14 de fevereiro de 2020

*A minha amada esposa Camila,*

*Ao meu querido filho Gustavo,*

*A minha sogra Nair Castro de Castro* (*In memoriam*).

**DEDICO**

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# SUMÁRIO

# LISTA DE ABREVIAÇÕES

AIA – Ácido indol acético

BPCP – Bactérias promotoras do crescimento de plantas

DAE – Dias após a emergência

FBN – Fixação biológica de nitrogênio

GC-MS – Cromatografia gasosa associada à espectrometria de massas

HCN – Ácido cianídrico

IAs – Inoculantes agrícolas

$N_2$ – Gás nitrogênio

NF – fatores nod, do inglês *nod factros*

$NH_3^+$ – Amônia

$NH_4^+$ – Amônio

$NO_2^-$ – Nitrito

$NO_3^-$ – Nitrato

# RESUMO

O cultivo de feijão comum (*Phaseolus vulgaris* L.) é de importância econômica, nutricional e cultural em diversos países. Esta espécie leguminosa pode estabelecer relações simbióticas com bactérias nodulíferas, popularmente conhecidas como rizóbios, resultando na Fixação Biológica do Nitrogênio (FBN). Além de desempenhar um papel fundamental na FBN, este grupo de microrganismos pode reduzir a incidência de doenças causadas por fitopatógenos em várias culturas. Nesse sentido, este trabalho objetivou avaliar, a campo, a resposta do feijoeiro comum à inoculação de rizóbios nativos, estudando seus efeitos sobre indicadores de crescimento e nutrição dessa planta, e também estudar a capacidade dessas bactérias no biocontrole das principais doenças da podridão das raízes do feijão comum. A resposta de *Rhizobium* sp. no desenvolvimento de *M. phaseolina* MP 53 foi inicialmente avaliada *in vitro.* Para a avaliação a campo, duas espécies de rizóbios (SEMIA4107 e SEMIA4108), previamente selecionadas em função da sua resposta em experimentos em casa de vegetação, foram avaliadas frente à estirpe recomendada comercialmente para o feijoeiro comum (SEMIA 4088 - *Rhizobium tropici*). O delineamento experimental empregado foi em blocos ao acaso, totalizando quatro repetições, sendo avaliados cinco tratamentos: ausência de fertilizante nitrogenado (-N); adição de N-mineral (+N); inoculante líquido contendo a estirpe recomendada (SEMIA 4088); tratamentos contendo os inoculantes com os isolados SEMIA4108 e SEMIA4107. As avaliações agronômicas deram-se a partir de 45 e 90 dias após a emergência das plantas (DAE). As plantas inoculadas com os rizóbios SEMIA 4107 apresentaram uma produtividade de grãos ligeiramente maior do que o controle nitrogenado (+N). O desenvolvimento de plantas inoculadas foi diferente do padrão observado para o controle nitrogenado (+N), porém, nenhuma perda de produtividade foi detectada. Para a avaliação do antagonismo *in vitro* contra o *M. phaseolina* MP53, isolados de rizóbios foram obtidos através do método de planta-isca, utilizando-se plantas de feijão comum. Após a avaliação do antagonismo, amostras do meio líquido foram filtradas e submetidas a rota-evaporação para posterior identificação dos metabólitos pela técnica de cromatografia gasosa associada à espectrometria de massas (GC-MS). Entre as 40 estirpes de rizóbios isoladas, somente os isolados R26, L5 e VC28 tiveram efeito inibitório *in vitro* contra o fitopatógeno, com redução de aproximadamente 50% na massa micelial. Os metabólitos identificados por GC-MS já foram caracterizados na literatura como antimicrobianos e podem estar relacionados à atividade de antagonismo observada. Dessa forma, esse trabalho demonstrou dois resultados impactantes na busca por uma agricultura mais sustentável. Primeiro, a utilização de rizóbios pode ser uma alternativa à produção do feijão comum, tanto pela sua utilização como inoculante na FBN em substituição ao uso tradicional de adubos nitrogenados, quanto pela diversificação na busca por novas estirpes adaptadas a essa cultura. Além disso, essas bactérias possuem potencial para o biocontrole das principais doenças da podridão das raízes do feijão comum.

**Palavras-chave:** fixação biológica de nitrogênio, contaminação ambiental, rizóbios nativos, biocontrole, *Macrophomina phaseolina,* metabólitos antimicrobianos.

# ABSTRACT

Common bean (*Phaseolus vulgaris* L.) cultivation is of economic, nutritional and cultural importance in several countries. This legume species can establish symbiotic relationships with noduliferous bacteria, popularly known as rhizobia, resulting in Biological Nitrogen Fixation (BNF). In addition to playing a key role in BNF, this group of microorganisms can reduce the incidence of diseases caused by phytopathogens in various crops. Thus, this work aimed to evaluate the response of common bean to the inoculation of native rhizobia, studying its effects on growth and nutrition indicators of this plant and also to study the capacity of these bacteria in the biocontrol of the main rot diseases in common bean plants. The response of *Rhizobium* sp. on development of *M. phaseolina* MP 53 was evaluated *in vitro*. For field tests, two rhizobia species, SEMIA4107 and SEMIA4108, previously selected for their response in greenhouse experiments, were evaluated against the commercially recommended strain for common bean, SEMIA 4088 - *Rhizobium tropici*). The experimental design was randomized blocks, totaling four replications, and five treatments were evaluated: absence of nitrogen fertilizer (-N); addition of N-mineral (+ N); liquid inoculant containing the recommended strain (SEMIA 4088); treatments containing inoculants with SEMIA4108 and SEMIA4107 isolates. Agronomic evaluations took place 45 and 90 days after plant emergence (DAE). Plants inoculated with SEMIA 4107 rhizobia showed slightly higher grain yield than the nitrogen control (+ N). The development of inoculated plants was different from the standard observed for nitrogen control (+ N), but no yield losses were detected. For the evaluation of *in vitro* antagonism against *M. phaseolina* MP53, rhizobia isolates were obtained by the bait plant method using common bean plants. After the evaluation of the antagonism, samples of the liquid medium were filtered and subjected to rotary evaporation, for subsequent identification of the metabolites by gas chromatography mass spectrometry-associated (GC-MS) technique. Among the 40 isolated rhizobia strains, only the isolates R26, L5 and VC28 presented an *in vitro* inhibitory effect against phytopathogen, with a reduction of approximately 50% in mycelial mass. The metabolites identified by GC-MS have already been characterized as antimicrobials, and may be related to the antagonism activity observed. Thus, this work showed two important results in the search for a more sustainable agriculture. First, the use of rhizobia may be an alternative to common bean production, both for its use as an inoculant in BNF as a substitute for the traditional use of nitrogenous fertilizers, as well as for the diversification in the search for new strains adapted to this crop. In addition, these bacteria have the potential for biocontrol of major common bean root rot diseases.



**Keywords:** biological nitrogen fixation, environmental contamination, indigenous *Rhizobium*, biocontrol, *Macrophomina phaseolina*, antimicrobial metabolites.

# 1 INTRODUÇÃO

Os desafios que a agricultura deverá enfrentar nas próximas décadas são muitos. O aumento esperado de 30% na população global, intensificando a concorrência por recursos cada vez mais escassos em terra, água e energia, além da ameaça frente às mudanças climáticas, são fatores a serem equacionados (FAO, 2014; HUNTER et al., 2017). Com uma população que tende a alcançar 9,3 bilhões em 2050, as estimativas são de que a produção agrícola precisará aumentar dos atuais 8,4 bilhões de toneladas para quase 13,5 bilhões de toneladas por ano. Entretanto, acompanhar essa taxa de produção implica na ampla difusão de fertilizantes, pesticidas e regimes de irrigação, o que certamente aumentará o impacto da agricultura na qualidade da água, aquíferos, vida selvagem e clima (FAO, 2014).

Os fertilizantes nitrogenados, embora representem a forma assimilada com maior rapidez pelas plantas, apresentam custo elevado e diversos impactos ambientais estão associados ao seu uso, como a eutrofização de águas superficiais, a contaminação de águas subterrâneas, a poluição atmosférica e a perda da qualidade do solo, trazendo desafios para a agricultura sustentável moderna (CHEN et al., 2018). O uso indiscriminado de agrotóxicos promove a poluição em um nível global, causando impactos ambientais como a adaptação de pragas e sua resistência, perda de fertilidade e erosão do solo, diminuição da biodiversidade, desertificação, além de impactos na saúde pública, atingindo diferentes grupos populacionais, como trabalhadores em diversos ramos de atividades, moradores do entorno de fábricas e fazendas, além de todos nós, que consumimos alimentos contaminados (CARNEIRO et al., 2015; OLIVARES et al., 2017; BOMBARDI, 2017).

Uma alternativa à demanda crescente de adubos artificiais, especialmente aos adubos nitrogenados, bem como ao uso de agrotóxicos, é o uso de rizóbios que realizam a fixação

biológica do nitrogênio (FBN) em associação com algumas espécies de plantas, como as leguminosas (HUNGRIA et al., 2013; VOLPIANO et al, 2018). Essas bactérias possuem a capacidade de fixar o nitrogênio atmosférico, disponibilizando-o para a planta em troca de fontes de carbono necessárias à sua sobrevivência, em um processo de simbiose. Além de desempenharem um papel importante na aquisição de nutrientes (BEEBE et al. 2013), os rizóbios constituem uma alternativa interessante para controlar a disseminação de doenças de plantas (DESHWAL et al., 2003; DAS et al., 2017). Nesse caso, o efeito dessas bactérias se deve à ação antagônica que esses microrganismos realizam contra diversos fitopatógenos fúngicos e bacterianos, estando o potencial de biocontrole atribuído, principalmente, aos seus metabólitos secundários bioativos (SINGH et al., 2019).

Nesse sentido, a associação simbiótica entre rizóbios e leguminosas pode ser estratégica, visando contribuir para a nutrição e o biocontrole de doenças em plantas, sendo explorada tecnologicamente na forma de inoculantes agrícolas (IAs), que são preparados com microrganismos selecionados em função de sua eficiência às culturas da soja, ervilha, fava, lentilha, feijão, entre outras. Dentre às muitas espécies leguminosas, o feijão-comum (*Phaseolus vulgaris* L.) está entre os alimentos mais consumidos no mundo, sendo considerado uma fonte de proteína adicional em países menos desenvolvidos, até mesmo como substituta da proteína animal (BROUGTHON., et al 2003).

Sendo assim, neste trabalho pode-se elucidar a versatilidade dos rizóbios enquanto promotores de crescimento em feijão-comum, especialmente pela FBN, e como agentes de biocontrole dos principais fungos fitopatogênicos que atacam essa cultura. Para tanto, o trabalho foi dividido em dois capítulos. No Capítulo I avaliou-se, através de experimentos a campo, a resposta do feijoeiro comum à inoculação de rizóbios nativos, previamente isolados e caracterizados molecularmente, estudando seus efeitos sobre indicadores de crescimento e

nutrição dessas plantas. Essa etapa contou com a elaboração de um artigo original que está em revisão. No Capítulo II, a capacidade de inibição por isolados de rizóbios frente a fungos fitopatogênicos causadores da podridão das raízes de feijão-comum foi investigada através da elaboração de um artigo de revisão publicado e, posteriormente, a inibição *in vitro* do desenvolvimento do fitopatógeno *Macrophomina phaseolina* MP53 por *Rhizobium* sp. foi avaliada e apresentada em um artigo original em fase final de preparação.

# 2 OBJETIVOS

## 2.1 Objetivo geral

Avaliar a resposta do feijoeiro comum à inoculação de rizóbios nativos, além de avaliar a capacidade desses rizóbios de atuarem como agentes de biocontrole dos principais fungos fitopatogênicos causadores da podridão das raízes do feijão-comum (*Phaseolus vulgaris* L.).

## 2.2 Objetivos específicos

- Avaliar a eficiência de dois novos isolados de *Rhizobium* sp. no incremento da produção de feijão-comum (*Phaseolus vulgaris* L.) através de experimentos a campo;
- Elaborar um artigo de revisão sobre o biocontrole de rizóbios nas principais doenças da podridão das raízes em feijão-comum, causadas pelos fitopatógenos *Rhizoctonia solani*, *Fusarium oxysporum*, *Fusarium solani* e *Macrophomina phaseolina*;
- Avaliar *in vitro* a capacidade de inibição do fitopatógeno *Macrophomina phaseolina* MP 53 por *Rhizobium* sp.;
- Identificar os metabólitos gerados durante o antagonismo entre *Rhizobium sp.* e *Macrophomina phaseolina* MP 53.

# 3 REFERENCIAL TEÓRICO

## 3.1 Rizóbios na produção de feijão-comum

O cultivo de feijão-comum (*Phaseolus vulgaris* L.) é extremamente importante em diversos países, sendo a principal fonte de proteína para milhões de pessoas ao redor do mundo. O Brasil é o terceiro maior produtor de feijão do mundo, produzindo 3,03 milhões de toneladas em 2,8 milhões de hectares (FAO, 2017). O Brasil é também um dos maiores consumidores do mundo, com aproximadamente 17 kg por pessoa ao ano (CONAB, 2018).

Para atender a demanda dessa grande produção, altas quantidades de nitrogênio são necessárias para adubação (HUNGRIA et al., 2013; GOPALAKRISHNAN et al., 2015). Contudo, o uso de adubos nitrogenados eleva os custos de produção e promove diversos impactos ambientais em razão de perdas durante sua aplicação. Consequentemente, podem causar a eutrofização de águas superficiais, contaminação de águas subterrâneas, perda de qualidade do solo e a emissão de gases de efeito estufa, trazendo desafios para a agricultura sustentável moderna (CHEN et al., 2018).

Mesmo sendo um dos maiores produtores e consumidores de grãos de feijão, o Brasil apresenta um rendimento médio nacional que está entre os mais baixos do mundo, estimado em apenas 982 kg/ha na safra de 2018 (HUNGRIA et al., 2013; CONAB, 2019). Fatores associados à baixa adoção de tecnologias pelo agricultor e o cultivo em solos degradados vêm contribuindo para esse cenário, e melhorias consideráveis, de baixo custo, podem ser obtidas com a adoção da prática de fixação biológica do nitrogênio (FBN) (HUNGRIA et al., 2013).

A FBN é realizada principalmente por bactérias gram negativas pertencentes ao gênero *Rhizobium*. Esses microrganismos são capazes de fixar o nitrogênio atmosférico ($N_2$) e

convertê-lo em amônia assimilável à planta, sendo explorados tecnologicamente na forma de inoculantes agrícolas (IAs). Cabe ressaltar que o sucesso da inoculação do feijoeiro com estirpes altamente eficientes de rizóbio está associado à habilidade competitiva de tais estirpes e a adaptação às condições ambientais (PELEGRIN et al., 2009). No entanto, em *P. vulgaris*, a ineficiência dos inoculantes está vinculada à promiscuidade que é característica da espécie, e que permite a nodulação com diversas espécies de bactérias nativas. Ainda, a FBN no feijoeiro pode ser prejudicada devido ao curto ciclo da cultura (MARTÍNEZ-ROMERO, 2003; KASCHUK et al., 2006; OLVEIRA et al., 2011).

Estudos realizados em casa de vegetação demonstraram que a inoculação de novos isolados de rizóbios em plantas de feijão elimina a necessidade da adubação nitrogenada (STAJKOVIC et al., 2011; FAGERIA et al., 2014; de SOUZA et al., 2015). Em contrapartida, a falta de respostas consistentes à inoculação e a baixa nodulação em experimentos a campo têm sido frequentemente relatadas em todo o mundo (HUNGRIA et al., 2003). Outros fatores relacionados à acidez do solo, baixo pH e concentrações elevadas de Al tóxico também podem limitar o processo de infecção das raízes, a formação de nódulos e a assimilação do N pela planta (PELEGRIN et al., 2009; FERREIRA et al., 2012).

### 3.2 A fixação biológica de nitrogênio (FBN) e a infecção da planta

Apesar de o nitrogênio terrestre estar amplamente distribuído na atmosfera na forma de gás nitrogênio ($N_2$), correspondendo a aproximadamente 80% do ar atmosférico, esta molécula, por apresentar uma tripla ligação entre os dois átomos, é bastante estável, não sendo assimilável pelos eucariotos (GUREVITCH et al., 2009). No entanto, uma pequena parcela do $N_2$ atmosférico pode sofrer clivagem da tripla ligação por descargas elétricas, com posterior

oxidação e carregamento ao solo pelas chuvas, podendo então ser aproveitado pelas plantas (DAVIDSON et al., 1991).

Geralmente, o processo de assimilação do nitrogênio nas plantas se dá pela absorção do nitrato ($NO_3^-$) pelas raízes, conversão em nitrito ($NO_2^-$), e posteriormente em amônio ($NH_4^+$), que ainda é convertido em nitrogênio-amida da glutamina, envolvendo um significativo gasto energético. Um processo equivalente pode ainda ocorrer com a assimilação de nitrogênio-amida após absorção direta de amônio (FAÇANHA et al., 2013), quando o mesmo estiver disponível no solo. Entretanto, devido a fatores climáticos, geológicos e biológicos, nos solos tropicais geralmente estas formas assimiláveis de N estão disponíveis em quantidades muito pequenas (ELKAN, 1992).

Durante o seu ciclo biogeoquímico, a maioria das transformações sofridas pelo N, tais como oxidação de amônia em nitrato, redução de nitrato à nitrito, síntese de compostos amoniacais e nitratos, amonificação da matéria orgânica e desnitrificação, ocorrem pela ação de microrganismos, e entre estas transformações está a FBN (GUREVITCH et al., 2009). Neste processo, microrganismos clivam enzimaticamente o $N_2$, convertendo-o em amônia.

De Bruijn e colaboradores (2015) citam dois tipos de FBN: a fixação simbiótica, em que o microrganismo utiliza subprodutos da planta para a manutenção do seu metabolismo, podendo em troca fornecer o nitrogênio necessário para planta através da FBN; e a fixação assimbiótica, em que o microrganismo tem vida livre e obtém do ambiente os recursos necessários para seu desenvolvimento. Neste último caso, a assimilação do N fixado pela planta se dá após o rompimento da célula microbiana.

As enzimas responsáveis pela redução do $N_2$ em $NH_3$ são denominadas nitrogenases, sendo formadas por duas subunidades: uma dinitrogenase redutase, e outra dinitrogenase propriamente dita. A subunidade dinitrogenase redutase, contendo centros Fe-S, tem função de

transferência de elétrons, enquanto a dinitrogenase, geralmente com um centro Fe-Mo, tem função de quebra da tripla ligação da molécula de $N_2$ (BURGESS e LOWE, 1996).

A FBN em leguminosas se dá pela interação entre o microrganismo fixador e a planta, formando nódulos na raiz, e consequentemente dispondo de um ambiente microaerófilo, propício à ação das nitrogenases que podem ser inativadas pela presença do oxigênio ($O_2$) (SEEFELDT et al., 2009; OLDROY, 2013). Na agricultura, a simbiose entre leguminosas e bactérias fixadoras de nitrogênio do gênero *Rhizobium* é considerada a mais importante (HUNGRIA et al., 2013).

O processo de formação do nódulo nas leguminosas ocorre após a sinalização da planta por meio da liberação de flavonoides e isoflavinas, que exercem atração quimiotrófica sobre bactérias simbiontes presentes no solo (OLDROY, 2013). Esta sinalização somente será disparada quando a planta se deparar com quantidade insuficiente de N assimilável no solo (SAGOLSHEMCHA et al., 2011). As bactérias, por sua vez, migram por quimiotaxia até a rizosfera, onde se proliferam e iniciam a produção de oligossacarídeos de lipoquitina, também conhecidos como fatores Nod (NF, do inglês *Nod Factors*) (MARKS et al., 2013; OLIVEIRA et al., 2013), codificados pelos genes nod. Os NF são responsáveis por sinalizar ao hospedeiro a presença da simbionte, e então, preparar a planta para a infecção por parte da bactéria (OLDROYD, 2013).

Durante o contato, as células dos pelos radiculares liberam NFs, promovendo seus enrolamentos (FAGAN et al., 2007). Um canal de infecção é criado pela planta no eixo do pelo radicular, e por ele dá-se a infecção das raízes pela bactéria. Cabe ressaltar que somente infectam a planta as bactérias capazes de escapar das respostas imunes e que este processo está relacionado com a especificidade bactéria-planta e com a disponibilidade de N no solo (OLDROYD, 2013).

A infecção e formação dos nódulos por rizóbios pode ser afetada negativamente por outros grupos presentes no solo, tais como bacteriófagos (HALMILLAWEWA et al., 2015), protozoários (GURIJALA e ALEXANDER 1990), fungos (ANGLE et al., 1981), além de outros grupos de rizóbios (MOAWAD et al., 2004) e fatores físicos ou químicos do solo, como disponibilidade de nutrientes (FERREIRA et al., 2012; COSTA et al., 2013), porosidade, umidade e temperatura (ORR et al., 2011; WOLF e ROHRS, 2001; MONKS et al., 2012).

### 3.3 Tecnologia de inoculantes agrícolas

Inoculantes agrícolas (IAs) são preparados contendo microrganismos ativos capazes de promover melhorias na qualidade e produtividade de culturas agrícolas (SIQUEIRA et al., 2014), substituindo totalmente ou parcialmente os fertilizantes convencionais (PELEGRIN et al., 2009), atuando como promotores de crescimento (YUAN et al., 2010; OSÓRIO FILHO et al., 2014) e indutores de resistência/tolerância a estresses bióticos/abióticos (APARICIO-FABRE et al., 2013; AHLUWALIA et al., 2014; RODRIGUEZ-LÓPEZ et al., 2014).

A indicação das estirpes recomendadas para a produção de inoculantes no Brasil é o resultado da reunião da Rede de Laboratórios para a Recomendação, Padronização e Difusão de Tecnologia de Inoculantes Microbianos de Interesse Agrícola (RELARE), e o controle de qualidade do produto final é regulado pela Instrução Normativa nº 13 do Ministério da Agricultura, Pecuária e Abastecimento (MAPA) (BRASIL, 2011).

Diversos levantamentos em diferentes ecossistemas têm demonstrado que microrganismos com potencial para fixação do N também possuem outros efeitos benéficos às plantas, como solubilização de minerais (CARDOSO et al., 2012; FERREIRA et al., 2012) e promoção de crescimento (COSTA et al., 2014). Estirpes com potencial fixador já reconhecido

ainda não foram recomendadas, apontando para a necessidade de atualizações nas recomendações de uso (FERREIRA et al., 2012; DALL'AGNOL et al., 2014).

No Brasil, os IAs mais populares são compostos de rizóbios utilizados para FBN no cultivo de soja (*Glycine max* (L.) Merr.), sendo responsáveis por uma expressiva economia no consumo de adubos nitrogenados. Nesse caso, a tecnologia de inoculação está muito avançada e pode inspirar o aprimoramento da tecnologia para outras plantas, como o feijão-comum (HUNGRIA et al., 2013).

Conforme Mendonça et al. (2017), a inoculação de rizóbios em algumas leguminosas de importância agronômica dispensa a aplicação de adubos nitrogenados nos solos, tendo como resultado a redução do custo de produção. Entretanto, antes de se utilizar comercialmente uma estirpe de bactéria como IA é necessário uma série de estudos que avaliem sua capacidade em estabelecer simbiose, seu potencial em fixar nitrogênio em associação com a leguminosa de interesse e, sobretudo, sua classificação taxonômica e relações evolutivas (NAMKELEJA et al., 2016; SOUSA et al., 2018).

### 3.3.1 Bactérias recomendadas para o cultivo de feijão-comum no Brasil

As estirpes de rizóbios indicadas pela RELARE e que constam na Instrução Normativa nº 13 do MAPA são a SEMIA 4077, recomendada pela Embrapa Cerrados, a SEMIA 4080, recomendada pela Embrapa Soja/IAPAR, e a SEMIA 4088, recomenda pela Embrapa Soja/Embrapa Cerrados. Todas pertencem à espécie *Rhizobium tropici* e foram recomendadas para feijoeiro após testes à campo (BRASIL, 2011).

### 3.4 Rizóbios no biocontrole de fungos fitopatogênicos do feijão-comum

O interesse no controle biológico tem aumentado em função da preocupação do público em geral quanto ao uso de agroquímicos no ambiente, buscando-se assim, alternativas para o controle de doenças de plantas (WHIPPS, 2004). No Brasil, um dos motivos para essa preocupação refere-se à utilização massiva de agrotóxicos atrelada ao avanço da agricultura e produção agropecuária voltadas para a conversão em *commodities* e agroenergia, fazendo com que o país consuma cerca de 20% de todo agrotóxico produzido no mundo (PALAEZ et al., 2015; BOMBARDI, 2017).

Além disso, as mudanças recentes no padrão de práticas agrícolas motivadas pelo cultivo natural (orgânico) em detrimento ao uso de pesticidas trouxe à tona a importância da utilização de microrganismos capazes de realizarem esse tipo de função (SINGH et al., 2019). Nesse sentido, o controle biológico de patógenos presentes no solo é uma abordagem sustentável, pois contribui para um ambiente equilibrado entre plantas, patógenos, agentes de controle biológico e comunidades microbianas na rizosfera (GAO et al., 2012). Essa interação pode ocorrer em função da produção de antibióticos e/ou compostos químicos por parte do agente de controle, que inibem o desenvolvimento do microrganismo patogênico (DAS et al., 2017).

As bactérias promotoras de crescimento de plantas (BPCP) são residentes epífitas ou endofíticas, não patogênicas, que atuam diretamente promovendo o crescimento, ou indiretamente como agentes de controle biológico de doenças de plantas, sendo os gêneros *Pseudomonas*, *Bacillus*, *Burkholderia*, *Streptomyces*, *Rhizobium*, *Bradyrhizobium*, *Acetobacter* e *Herbaspirilum*, e as espécies A*grobacterium radiobacter* e *Enterobacter cloacae* consideradas as principais BPCP empregadas na agricultura (MARIANO et al., 2013).

Neste contexto, os microrganismos do gênero *Rhizobium* vem ganhando destaque como agentes de biocontrole por inibir o desenvolvimento de diversos fungos fitopatogênicos pertencentes a diferentes gêneros como *Fusarium*, *Rhizoctonia*, *Sclerotium* e *Macrophomina* (DAS et al., 2017). Conforme Singh e colaboradores (2019), os mecanismos associados ao controle biológico de fitopatógenos por rizóbios consistem na produção de antibióticos, sideróforos, ácido cianídrico (HCN), enzimas, além de solubilização de fosfato, competição e indução dos mecanismos de defesa das plantas, enquanto que a atividade antagônica está relacionada à produção de metabólitos secundários como HCN, sideróforos, rizobiotoxinas, enzimas líticas, produção de ácido indol acético (AIA) e solubilização de fosfato.

Os fungos fitopatogênicos possuem grande importância econômica pois acometem culturas em desenvolvimento no campo ou ainda na fase de pós-colheita (PRESTI et al., 2015). Alguns autores estimam que a produção agrícola mundial sofra perdas aproximadas de 10% pela ação desses fitopatógenos a cada ano e ainda consideram que as mudanças climáticas possam favorecer essa situação, intensificando esse problema (OERKE et al., 2006; PRESTI et al., 2015).

No cultivo do feijão-comum, as doenças fúngicas são consideradas a principal causa da baixa produtividade em todo o mundo, causando perdas de 80 a 100% em rendimento (SINGH e SCHWARTZ, 2010). Dentre as diversas doenças que incidem na cultura do feijão, as podridões radiculares causadas por fungos constituem um complexo etiológico caracterizado por perda de vigor das plântulas (SARTORATO et al., 1994).

De acordo com Casa e colaboradores (2009), as doenças do sistema radicular de feijão-comum são relatadas em praticamente todas as regiões de cultivo no Brasil, com intensidade variando em função do inóculo presente na área, da suscetibilidade da cultivar, das condições climáticas e de práticas de manejo do solo. Somando-se às moléstias fúngicas, bactérias e vírus

totalizam mais de 45 doenças que podem acometer as plantas de feijão, tanto na parte aérea como na rizosfera (SINGH e SCHWARTZ, 2010).

No entanto, perdas significativas na produção são observadas pela podridão da raiz, sendo os fitopatógenos *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina* e *Rhizoctonia solani* considerados os principais patógenos radiculares em feijoeiro comum (CASA et al., 2009). De maneira geral, esses fungos caracterizam-se por apresentar alta habilidade de competição saprofítica e possuir estruturas de resistência, o que dificulta seu controle, pois podem permanecer viáveis por um longo período de tempo, normalmente superior ao tempo da entressafra da cultura (REIS et al., 2005; CASA et al., 2009).

No cultivo do feijão-comum ainda existem outras limitações quanto ao combate a fitopatógenos. Conforme Singh e Schwartz (2010), a baixa quantidade de cultivares resistentes disponíveis aliada à alta capacidade de adaptação dos fitopatógenos a novos genótipos, fazem com que a utilização de agroquímicos seja a principal maneira de controle das doenças. Contudo, a dependência química de agroquímicos e seu uso indiscriminado podem causar vários efeitos prejudiciais ao meio ambiente e à saúde humana (CARNEIRO et al., 2015; KUMAR e SINGH, 2015).

Portanto, a utilização de rizóbios pode ser uma alternativa interessante no controle biológico de doenças causadas por fitopatógenos da podridão radicular do feijão-comum, através da aplicação de inoculantes agrícolas, explorando o potencial de biocontrole dessas bactérias, além da sua bem conhecida capacidade de promover o crescimento de plantas (SINGH e SCHWARTZ, 2010; SCHMIDT et al., 2019).

### 3.5 *Rhizobium* sp. no biocontrole de *Macrophomina phaseolina*

Dentre as podridões radiculares, uma das doenças com maior prevalência é a podridão cinzenta do caule, causada pelo fungo *Macrophomina phaseolina* (Tassi) Goidanisch, o qual infecta principalmente raízes e hastes, interferindo na circulação de seiva, reduzindo o vigor das plantas e podendo levar à redução na produtividade e até mesmo à morte da planta (DALLA PRIA & SILVA, 2010). Nos casos de infestações mais severas, as plantas hospedeiras são destruídas por toxinas fúngicas, como a faseolina, além de provocar a obstrução dos vasos condutores pelo cresimento do micélio (SABATÉ et al., 2019). *M. phaseolina* é um fungo saprófito cosmopolita capaz de infectar mais de 500 espécies de plantas, sendo feijão, caupi, milho, amendoim, girassol, soja, sorgo e crotalária as espécies suscetíveis mais comuns no Brasil (ISLAM et al., 2012; SOBRINHO et al., 2018).

Conforme Islam e colaboradores (2012), a ação desse fitopatógeno é intensificada em altas temperaturas (30 a 35° C) e baixa umidade do solo, sendo o seu controle difícil de ser realizado devido à sua persistência no solo. Essa persistência deve-se a estruturas de resistência, denominadas microescleródios, formadas em condições desfavoráveis ao desenvolvimento fúngico, sendo as práticas de rotação e sucessão de culturas pouco efetivas no controle da doença (REIS et al., 2014).

Na tentativa de controlar a incidência da doença da podridão cinzenta do caule, algumas ações como o manejo de irrigação durante os períodos de seca e a seleção de cultivares de plantas resistentes vêm sendo aplicadas. A utilização de fungicidas nas sementes também é empregada para o controle nos estágios iniciais de crescimento. Porém, o uso de fungicidas pode prejudicar o desenvolvimento de microrganismos benéficos e contribuir para a contaminação da água e do solo (SABATÉ et al., 2017; VOLPIANO et al., 2018).

Nesse sentido, uma alternativa interessante para o biocontrole de fitopatógenos presentes no solo é a utilização de rizóbios em plantas leguminosas. Algumas espécies de rizóbios (*Rhizobium* sp., *R. meliloti* e *Bradirhizobium japonicum*) já foram identificadas como capazes de inibir *M. phaseolina* em culturas como soja, girassol, quiabo, grão-de-bico, fava, tremoço, tomate e amendoim (DAS et al., 2017).

**CAPÍTULO I - Rizóbios no incremento da produção de feijão-comum (*Phaseolus vulgaris* L.)**

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**New indigenous rhizobial isolates for common bean (*Phaseolus vulgaris* L.) inoculation: biotechnological tool aiming a cleaner and more sustainable agriculture**

**Running title:** Biological Nitrogen Fixation in common bean plants

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**New indigenous rhizobial isolates for common bean (*Phaseolus vulgaris* L.) inoculation: biotechnological tool aiming a cleaner and more sustainable agriculture**

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**Abstract**

Inoculation of symbiotic nitrogen fixing rhizobacteria, known as rhizobia, in leguminous plants is an alternative for reducing synthetic N fertilizers inputs on crops. Despite common bean plants are benefited by biological nitrogen fixation performed by rhizobia isolates, the low efficiency observed in this process evidences the need for selection of efficient rhizobial strains. Two new rhizobial strains (SEMIA4107 and SEMIA4108) previously showed a high potential to improve common bean growth in greenhouse experiments. Thus, this work aimed to evaluate common bean plants inoculated with SEMIA4108 and SEMIA4107 in field experiments, and compare the results obtained with a recommended rhizobial strain (*Rhizobium tropici* SEMIA4088). Further characterization of the new strains using *16S rRNA* phylogeny showed SEMIA4108 and SEMIA4107 forming a clade along with *Rhizobium esperanzae*, *Rhizobium pisi*, *Rhizobium fabae* and *Rhizobium phaseoli* strains. Common bean plants inoculated with rhizobia SEMIA4107 presented a slightly higher productivity than the N fertilized. Development of inoculated plants was different from the pattern observed for N+. Nonetheless, no productivity losses were detected. The results demonstrated a high efficiency of rhizobia inoculation in common bean plants. Inoculation of symbiotic N fixing bacteria in leguminous plants is an important practice to achieve a more sustainable agriculture.

**Keywords:** biological nitrogen fixation, synthetic nitrogen fertilizers, environmental contamination, indigenous *Rhizobium*, field experiments.

## Introduction

Until the 1950s, farmers have used organic fertilizers for soil amendments aiming to improve plant growth (Novotny 1999). Thereafter, world population have been continuously increasing, which demands the development and application of new technologies and management intensification to produce more food per cultivated area (Stewart et al. 2005). Thus, the use of synthetic fertilizers in agricultural systems enabled an upgrade of around 50% in food production (Skowrońska and Filipek 2014), supporting the population increase and economic development (Zang et al. 2015).

However, some studies demonstrate that the amount of synthetic fertilizers applied on the soil has been increasing almost exponentially (Skowrońska and Filipek 2014). Besides increasing production costs, it also causes negative environmental effects (Mondal et al. 2017). Such effects are mainly caused by soil application of synthetic nitrogen (N) fertilizers (Vitousek et al. 1997). Eutrophication of water bodies, groundwater contamination, atmospheric pollution and decrease in soil quality are only primary effects of such practices. Therefore, searching for clean and sustainable alternatives is a challenge for sustainable modern agriculture.

A reasonable alternative for the use of synthetic fertilizers, mainly N fertilizers, is the inoculation of Plant Growth Promoting Bacteria (PGPB – Vargas et al. 2009). PGPB promotes plant growth mainly by Biological Nitrogen Fixation (BNF), solubilization of nutrients as Phosphorous (P) and Potassium (K), hormone production, and inhibition of diseases caused by soil-borne pathogens (Rodrigues et al. 2013; Gupta et al. 2015; Volpiano et al. 2018). In leguminous, inoculation of symbiotic N fixing bacteria, known as rhizobia, can supply all N needed for plant development, ensuring an excellent plant growth, yield and productivity (Gourion et al. 2015), besides improving soil fertility for new crops (Bhattacharyya and Jha

2012), and reducing soil application of synthetic N fertilizer. Therefore, selection of new efficient rhizobia isolates for a clean production of leguminous is an important approach to achieve environmental and agricultural sustainability (Sengupta and Gunri 2015).

Common bean (*Phaseolus vulgaris* L.) is one of the most important grains for human consumption. It has economic, nutritional and cultural relevance in several countries (Brougthon et al. 2003; Yanni et al. 2016), superseded only by soybean and peanut (Fageria et al. 2014). In 2016, the estimated world production of dry beans was approximately 27 million tons. Asia accounts for 45.6 % of the total production, the Americas 26.1 %, Africa 24.2 %, Europe 4.0 %, and Oceania 0.1 % (FAO 2017). Common bean plants benefit from BNF, although with a low efficiency. Such low BNF efficiency is result of their short crop cycle and promiscuity on the establishment of symbiosis with a high variety of native rhizobia species, often inefficient in N provision (Dall'Agnol et al. 2016). Currently, some studies demonstrate that inoculation of new rhizobial isolates in common bean plants growing in greenhouse conditions can eliminates the need for N fertilization (Stajkovic et al. 2011; Fageria et al. 2014; de Souza et al. 2016). However, field experiments and recommendation of new rhizobial isolates is a gap in the knowledge transfer from laboratories to producers.

Therefore, the selection of new indigenous rhizobial strains aiming a significant reduction of synthetic N fertilizers in the soil is a biotechnological tool that also has an ecological and health relevance. Thus, this work aimed to evaluate, in field experiments, agronomic parameters of common bean plants inoculated with two new rhizobial isolates previously identified in common bean roots, and compare the results obtained with synthetic N fertilization and a commercial isolate.

## Material and Methods

### *Organisms and 16S rRNA sequence analysis*

Bacteria previously isolated by de Souza et al. (2016) and currently available in the "SEMIA culture collection" (IBP World Catalogue of *Rhizobium* Collections n°443 in the WFCC World Data Center on Microorganisms)" as SEMIA 4108 (=VC28) and SEMIA 4107 (=M3) were employed in this work. In order to further characterize these two new strains, genomic DNA was isolated from bacterial cells using the PureLink™ Genomic DNA Mini Kit (Thermo Scientific). The *16S rRNA* was amplified using BacPaeF (5'AGAGTTTGA TCCTGGCTCAG3') and Bac1542R (5'AGAAAGGAGGTGATCCAGCC3') primers in a final volume of 25 µL, containing 20-50 ng of genomic DNA, 2.5 µL of DreamTaq buffer 10× (Thermo Scientific), 0.5 µL of 10 mM dNTPs mix, 0.5 µL of 100 mM of each primer, 1 µL of DMSO, and 0.1 µL of Dream Taq Polymerase (10 units per µL - Thermo Scientific). The PCR cycling program was: 94ºC for 5 min, followed by 37 cycles of 94ºC for 1 min, 57ºC for 1 min and 10 s, and 72ºC for 1 min; for the final step, reactions were incubated at 72ºC for 5 min. Nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, South Korea) using an ABI3730XL and sequencing primers 785F and 907R. Low-quality sequences were trimmed using Chromas 2.6.4 software. Fragments were assembled into a single sequence using EMBOSS merger tool (http://www.bioinformatics.nl/cgi-bin/emboss/merger). Sequence identity was assessed by comparing the *16S rRNA* sequences of SEMIA 4108 and SEMIA 4107 isolates with the sequences from EzBioCloud *16S rRNA* server database (https://www.ezbiocloud.net/identify). The sequence data reported in this study are publicly deposited in GenBank under accession numbers MN209793 and MN209795.

A *16S rRNA* phylogeny was reconstructed with the 113 type strains of *Rhizobium* species according to the accessions provided on LPSN (available at http://www.bacterio.net). The *16S rRNA* sequences were aligned using SINA 1.2.11 (Pruesse et al. 2012). Bayesian phylogenetic inferences were prepared using BEAST v1.8.4 software. The evolution model used was TN93 (Tamura and Nei 1993). Rate variation among sites was modeled assuming an estimated proportion of invariant sites and a gamma distribution (shape parameter = 4) for both analysis. The Yule process was selected as a tree prior to Bayesian analysis. The Monte Carlo Markov Chain (MCMC) algorithm ran for 10,000,000 generations and sampled every 1,000 generations. Trees were visualized and edited using FigTree 1.4.3 software.

***Field experiments***

Field experiments were carried out at the Viamão Research Center (30°2'10.64" S and 51°1'17.65" W) of the Department of Agricultural Research and Diagnosis of Rio Grande do Sul state, located in Viamão city, Brazil. The soil of this region is classified as Ultisol (Soil Survey Staff, 1999), and the regional climate is subtropical with warm and wet summer (Cfa), according to Köppen classification. Weather records were available on-site from a local weather station, providing information on minimum and maximum air temperature, relative humidity, and rainfall level during the execution of the experiments. To evaluate soil fertility, soil nutrient analyses were performed before soil amendments. Organic matter (OM), clay, macro and micronutrients, and pH were determined using standard methods (Sparks 1996). Field experiment was performed according to the Official Protocol for Evaluation of Agronomic Efficiency of Inoculants related to BNF process in leguminous plants (Brazilian Ministry of Agriculture, Livestock, and Supply - MAPA - BRASIL 2011) during the growing periods of 2016-2017 and 2018-2019 (October to January).

Inoculants were prepared with SEMIA 4108 and SEMIA 4107, and additionally with SEMIA 4088 (*Rhizobium tropici*), a rhizobial strain approved by MAPA as an inoculant for common bean cultivation. Log-phase rhizobial cells were grown in Yeast Mannitol broth (Vincent 1970). Thereafter, cells were inoculated in sterile peat at a 3:2 (w:v) ratio and stored at room temperature for ten days. Inoculant quality was monitored by counting the colony-forming units (CFU) with a minimum number of viable cells of $10^9$ CFU per gram of peat. To each kg of common bean seeds (*Phaseolus vulgaris* L. - Triunfo cultivar), 54 g of the respective inoculant was added.

The experimental design was randomized blocks with five treatments and four replicates. The area selected for the experiment has never been cropped with rhizobia inoculation. The five treatments used were: N-, no mineral N and no seed inoculation; N+, 80 kg of mineral N (177 kg of urea) per hectare (45 kg at sowing plus 132 kg after 20 days of plant emergence) and no seed inoculation; SEMIA 4088, no mineral N and SEMIA 4088 inoculation; SEMIA 4108, no mineral N and SEMIA 4108 inoculation; SEMIA 4107, no mineral N and SEMIA 4107 inoculation. All treatments were fertilized with P (61 $kg.ha^{-1}$ of triple superphosphate in 2016-17, and 73 $kg.ha^{-1}$ in 2018-19), K (52 $kg.ha^{-1}$ of potassium chloride in 2016-17, and 69 $kg.ha^{-1}$ in 2018-19), and limestone (2.7 $tons.ha^{-1}$, in 2016-17).

Experimental units had 5 m length x 2 m width (10 $m^2$), with rows spaced 0.5 m apart, totalizing four rows per experimental unity (Supplementary Figure 1). After seed inoculation, sowing was performed at a density of 14 seeds per meter, which represents 70 seeds per row and 280 seeds per sample unit.

When plants achieved full flowering (R6 stage of growth cycle - around 30 days after plant emergence), 10 plants of each experimental unit were harvested, and root and shoots were separated. Roots were used to determine nodule number and nodule dry matter. Shoots were

dried at 65°C for three days and used to determine dry matter and N, P and K content (Tedesco et al. 1995). At harvest (R9 stage of growth cycle - around 90 days after plant emergence), seeds from 10 plants of each experimental unit were harvested and used to determine seed yield and N, P and K contents (Tedesco et al. 1995).

Obtained results were subjected to One-Way ANOVA followed by Tukey test ($p < 0.05$) using InfoStat 2018 software. Correlation analyses between plant agronomic parameters observed in both sampling periods (R6 and R9 stages of growth cycle), and both growing periods 2016-17 and 2018-19, were performed by Principal Component Analysis (PCA).

## Results

### *16S rRNA phylogeny of indigenous rhizobia*

According to similarity-based searches against databases of *16S rRNA* sequences, SEMIA 4108 and SEMIA 4107 presented high gene similarities with species belonging to the *Rhizobium* genus. A 16S rRNA phylogenetic tree was generated with SEMIA 4108 and SEMIA 4107 sequences and the *Rhizobium*-type strains (Figure 1 and Supplementary Figure 2). Both SEMIA 4108 and SEMIA 4107 formed a clade along with *Rhizobium esperanzae*, *R. pisi*, *R. fabae* and *R. phaseoli* type strains.

### *Field experiments*

Weather data in the experimental field showed temperature varying from 15.8° C to 31.3° C in 2016-17, and from 9.0° C to 39.7° C in 2018-19. The average of monthly rainfall was around 140 mm in 2016-17, and 100 mm in 2018-19 (Table 1). The soil is acidic with medium clay content, and presented low percentage of OM in both growing periods (Table 2). The

effective cation exchange capacity (CEC) presented intermediary levels, with high content of P and K, and low content of Ca and Mg. When soil nutrient was analyzed in both growing periods 2018-19 presented higher CEC and Ca, and lower Al, P and K (Table 2). Micronutrients (Cu, Zn, and Mn) were available in high amounts in both periods.

The plant growth-promoting traits of these new indigenous rhizobial isolates (SEMIA 4108 and SEMIA 4107) were previously evaluated, and SEMIA 4108 was characterized as strong siderophore and low indolic compounds producer (33.35 $\mu g.mL^{-1}$); and SEMIA 4107 was characterized as strong siderophore and high indolic compounds producer (109.61 $\mu g.mL^{-1}$). In this work, the common bean seeds were inoculated and cropped (without irrigation) in field experiment, and agronomic parameters were evaluated at the R6 stage (Table 3). At this stage, plants cropped in 2016-17 growing period showed that rhizobial inoculated treatments presented higher nodule number and nodule dry matter when compared to non-inoculated treatments. The control treatment, which received synthetic N fertilizer (N+), presented the highest shoot dry matter, followed by inoculated treatments SEMIA 4108, SEMIA 4088 and SEMIA 4107. As expected, negative control without inoculation nor N fertilizer (N-) presented the lowest shoot dry matter. Macronutrients (N, P and K) on shoots followed the same pattern: high amounts in N+, an intermediary group composed by inoculated treatments, and low amount in N-. In the 2018-19 growing period, common bean plants inoculated with SEMIA 4088 presented highest values of nodule number and nodule dry matter. Shoot dry matter, N and P contents of inoculated treatments were similar to N fertilized treatment (N+).

At harvest (R9 growth stage), the crop showed remarkable results, once the inoculated treatment SEMIA 4107 presented a slightly higher productivity than N+ (around 2.5 $tons.ha^{-1}$ in SEMIA 4107, and 2.4 in N+ in 2016-17; and around 3.1 $tons.ha^{-1}$ in SEMIA 4107, and 2.0 in N+ in 2018-19; Figure 2A and D). The percentage of nutrients accumulated on the grains is

an intrinsic characteristic of plant variety. Therefore, N, P and K percentage on the grains were the same for all evaluated treatments: 2.8 % of N, 0.4 % of P, and 1.4 % of K in 2016-17; and 2.6 % of N, 0.5 % of P, and 1.6 % of K in 2018-19. However, if we consider the amount of nutrients accumulated on the grains per hectare, N accumulation increases from 50 $kg.ha^{-1}$ in N- to more than 63 $kg.ha^{-1}$ in inoculated treatments and N+ in 2016-17. Total P accumulation increases from 7.7 $kg.ha^{-1}$ in N- to 8.6 $kg.ha^{-1}$ in the other four treatments, and K accumulation increases from 26.2 $kg.ha^{-1}$ in N- to more than 30 $kg.ha^{-1}$ in the other four treatments (Figure 2C, E and G). In 2018-19, N accumulation increased from 31 $kg.ha^{-1}$ in N- to around 75 $kg.ha^{-1}$ in inoculated treatment SEMIA 4108. Total P accumulation increases from 6.7 $kg.ha^{-1}$ in N- to around 15 $kg.ha^{-1}$ in SEMIA 4107 and SEMIA 4108, and K accumulation increases from 21 $kg.ha^{-1}$ in N- to around 52 $kg.ha^{-1}$ in SEMIA 4107 (Figure 2D, F and H).

Correlation analysis among agronomic parameters obtained by the five treatments during R6 and R9 stages in both growing evaluated periods helped to elucidate our results (Figure 3). PCA analysis explained 80.2% of total variability, and considering all agronomic parameters evaluated at the first sampling period (R6 stage), the results obtained for inoculated treatments (SEMIA 4088, SEMIA 4108 and SEMIA 4107) were related (forming a group). Control treatments (N+ and N-) were arranged out of this group, at opposite sides of the PC1 axis (Figure 3A). Inoculated treatments were related with high amounts of all evaluated agronomic parameters (nodule number and dry matter, shoot dry matter and N, P and K contents), while N+ was related with high shoot dry matter and shoot nutrient contents. At harvest period (R9 stage) PCA analysis explained 94.2% of total variability. Inoculated treatments SEMIA 4088 and SEMIA 4108 showed related results to N+ control (forming a group), while SEMIA 4107 was arranged out of this group, presenting the most promising results. Finally, N- control treatment was allocated opposite to inoculated treatments and N+.

These analyses confirm the high efficiency obtained by the inoculation of common bean plants with the new selected rhizobial strains. If correctly applied, it can provide economic and environmental advantages for producers. These data showed that the development of inoculated plants does not follow the same pattern as observed for N fertilized plants (N+). However, no yield and productivity losses were detected at the end of the growing process, with agronomic parameters similar or higher than N+ control treatment.

## Discussion

Rhizobia inoculation in leguminous can supply all the N needed for plant development (Peoples et al. 2009). Selection of new rhizobia strains for inoculation in different leguminous aiming to replace synthetic N fertilization is a potential biotechnological tool (Simon 2006; Ali et al. 2012; Koskey et al. 2017). The consolidated knowledge regarding rhizobia inoculation allowed the recommendation of several new rhizobial strains for leguminous inoculation, and inoculants are available for all producers in specialized stores (Herrmann 2015). The most successful case is the experience of bradyrhizobial inoculation in soybean plants. Many strains of *Bradyrhizobium japonicum*, *B. diazoefficiens* and *B. elkanii* are commercialized as specific inoculants for soybean plants around the world. Such inoculation improves soybean growth and productivity without synthetic N fertilization (Suzuki et al. 2014; Chibeba et al. 2017). Some studies relate that such bradyrhizobial strains also solubilize nutrients (Fernández et al. 2007), prevent diseases caused by soil-borne pathogens (Al-Ani et al. 2012; Volpiano et al. 2018), and produce hormones that improve the nutrient uptake area through root enlargement (Molla et al. 2001).

The knowledge regarding the symbiosis of *Bradyrhizobium* spp. with soybean plants is much deeper than the symbiosis of *Rhizobium* spp. with common bean plants. Promiscuity of common bean plants and fast-growing cycle may reduce BNF efficiency (Martínez-Romero 2003; Kaschuk et al. 2006). However, some greenhouse studies showed that new rhizobial isolates from common bean plants present high potential of BNF. Korir et al. (2017) demonstrate that inoculation with rhizobial strains CIAT899, IITA-PAU987 and IITA-PAU983. Samavat et al. (2012) studied five rhizobial isolates (RH3 to RH7) which improved the growth of common bean plants and nutrient absorption. Moreover, the growth promotion effect was much higher when the treatments were co-inoculated with *Pseudomonas fluorescens* UTPF68 or UTPF109.

However, the knowledge regarding efficiency of rhizobia-common bean symbiosis also need to be proved in field experiments. As seen in our study, selection of new efficient rhizobial isolates for inoculation in greenhouse experiments (de Souza et al. 2016) also present successful potential to be used in field experiments. Analyzing field practices for common bean cultivation, plant inoculation is a neglected biotechnological tool that shows similar (or even better) results when compared with crops fertilized with synthetic N. Traditional common bean crops are usually found in small lands, such as family farming, and we have observed a resistance by farmers regarding the use of this biotechnological approach, mainly due to the lack of knowledge regarding their possibilities. Therefore, the classical agricultural management that uses synthetic fertilizers to ensure a good harvest (with no concerns regarding human health and food security), needs to be rethought.

Herrige et al. (2008) estimate that BNF contribution to crop legumes reaches around 20-22 million tons of N per year. Due to high losses observed during the application of synthetic N fertilizers (urea, 45% of N), it would be necessary to apply approximately 100 million tons

of this fertilizer in leguminous to achieve the same N amount provided by BNF (Peoples et al. 2009). Considering only common bean crops, the recommended N dose for each hectare is approximately 80 kg (177 kg of urea.ha$^{-1}$- BRASIL 2011). More than 25 million hectares are cultivated with common beans around the world (FAO 2018), therefore, it is estimated that BNF has the potential to reduce about 4.5 million tons of synthetic N fertilizers (as urea) applied on soil only in this crop. Urea costs are estimated around US$ 305 per ton (Fertilizer price trends 2017), while one inoculant dose costs around US$ 4 per hectare. Using this data as reference, inoculation technique of new rhizobial isolates aiming to improve BNF may represent an economy of more than US$ 1.4 billion per year, only in common bean cultivation.

Besides economic gains, BNF is an environmentally friendly technique. Synthetic N fertilizers are produced by the Haber-Bosch process, which is an energy-intensive conversion of highly inert $N_2$ to highly reactive $NH_3$ (Mulvaney et al. 2009) under high pressure and temperature, which is achieved by burning fossil fuels (Galloway et al. 2008). The high dose of synthetic N fertilizer applied is a result of the low persistence time of this fertilizer on the soil. It is estimated that about 50% of this reactive N (present in synthetic fertilizers) are lost to the environment (Galloway et al. 2003). Therefore, this environmental contamination is, somehow, planned and largely accepted as usual.

Such N losses to the environment lead to the production of tropospheric ozone and aerosols, which induce respiratory illness, cancer and cardiac disease in humans; eutrophication, hypoxia, loss of biodiversity and habitat degradation in coastal ecosystems (by effects of NOx and $NH_3$); global climate changes (by the uptake of greenhouse gases) and stratospheric ozone depletion, which affects human and ecosystem health (Erisman et al. 2007; Galloway et al. 2008; Mulvaney et al. 2009; Sutton et al. 2011). To each hectare cropped with urea (177 kg.ha$^{-1}$, this compound present 45% of N), the greenhouse gas emission of $N_2O$ is approximately 2.1 kg

(GHG 2010). The release of 1kg of $N_2O$ into the atmosphere is equivalent to 288.45 kg.$CO_{2eq}$. So, rhizobial inoculation practices could reduce the release of about 15 million tons of $CO_{2eq}$ into the atmosphere per year. Considering our results with common bean plants, around 2.2 million tons of synthetic N fertilizers would not return to the environment in toxic forms.

There is a massive difficulty to transfer the results obtained in laboratory and greenhouse conditions to the field and, consequently, to the development of new inoculant formulations. In our previous work (de Souza et al. 2016), we performed an initial screening, evaluation of plant growth-promoting traits and the first experiment of common bean inoculation in greenhouse. A similar screening was performed by Figueiredo et al. (2008), Stajkovic´ et al. (2011), Oliveira-Longatti et al. (2013), Ribeiro et al. (2013) and others. Like us, all of them found new rhizobial isolates with potential to be recommended and commercialized. However, there are no reports on the literature regarding the continuity of their studies. We believe that we are finally close to recommend a new indigenous rhizobial isolate for common bean inoculation in Brazil.

We recognize the importance of synthetic fertilizers for food production, which supported population growth in the last century. However, the synthetic N fertilization in leguminous plants needs to be rethought and replaced by rhizobial inoculation, improving the BNF process and reducing environmental and health costs. We believe that associations between research institutions and food industries could facilitate this replacement aiming a cleaner and more sustainable food production.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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**Table 1:** Weather data in both common bean growing periods (2016-17 and 2018-19).

| Growing period | Max Temp | Min Temp | Relative humidty | Total rainfall |
|---|---|---|---|---|
| **2016-17** | **(°C)** | **(°C)** | **(%)** | **(mm)** |
| October | 24.5 | 15.8 | 76.1 | 176.4 |
| November | 27.1 | 16.5 | 69.3 | 92.0 |
| December | 30.6 | 20.2 | 70.2 | 120.5 |
| January | 31.3 | 21.7 | 74.3 | 167.5 |
| **2018-19** | | | | |
| October | 33.2 | 9.0 | 81.5 | 110.8 |
| November | 34.6 | 12.3 | 79.4 | 108.6 |
| December | 38.9 | 11.0 | 77.7 | 24.8 |
| January | 39.7 | 19.5 | 81.8 | 151.6 |

**Table 2: Soil nutrient analysis before soil amendments.**

| Basic Analysis | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Growing period** | **P** | **K** | **Clay** | **OM** | **pH** | **Al** | **Ca** | **Mg** | **CEC** | |
| | | | | | | | | | **pH 7** | **Effective** |
| | **mg . (dm$^3$) $^{-1}$** | | **… % …** | | | **… cmol$_c$ . (dm$^3$)$^{-1}$ …** | | | | |
| **2016-17** | 18.4 | 95.0 | 26.0 | 1.6 | 5.1 | 0.4 | 1.0 | 0.6 | 5.7 | 2.3 |
| **2018-19** | 12.1 | 77.0 | 26.0 | 1.3 | 5.6 | 0.1 | 2.5 | 1.7 | 6.9 | 4.5 |

| Micronutrients | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Growing period** | **Zn** | **Cu** | **Mn** | **Na** | **Fe** | **Sat CEC effect** | | **Relation** | | |
| | | | | | | **Na** | **Al** | **Ca/Mg** | **Ca/K** | **Mg/K** |
| | **… mg . (dm$^3$) $^{-1}$ …** | | | | **%** | **… % …** | | | | |
| **2016-17** | 4.2 | 1.2 | 11.8 | 4.0 | 0.2 | 0.8 | 17.7 | 1.7 | 4.1 | 2.5 |
| **2018-19** | 14.1 | 0.5 | 12.0 | 1.0 | 0.2 | 0.1 | 2.2 | 1.5 | 12.7 | 8.6 |

nd = not determined; OM = organic Matter; CEC = cation exchange capacity

**Table 3:** Agronomic parameters of common bean plants evaluated after 30 days of cultivation (R6 stage).

| **Growing period 2016-17** | **Nodule** | | **Shoot dry matter** | **N** | **P** | **K** |
|---|---|---|---|---|---|---|
| **Treatment** | **number . plant$^{-1}$** | **mg . plant$^{-1}$** | **g . plant$^{-1}$** | **mg . shoot$^{-1}$** | | |
| **N -** | 6.3 ± 2.4 b | 7.9 ± 5.7 ab | 2.7 ± 0.2 c | 47.9 ± 9.7 b | 8.9 ± 7.6 b | 81.0 ± 15.4 b |
| **N +** | 3.7 ± 2.7 b | 3.2 ± 2.6 b | 5.7 ± 1.0 a | 109.7 ± 25.9 a | 17.1 ± 3.8 a | 167.4 ± 19.5 a |
| **SEMIA 4088** | 15.3 ± 2.2 a | 20.1 ± 4.1 a | 3.7 ± 0.4 bc | 72.0 ± 16.3 ab | 10.6 ± 1.7 b | 103.7 ± 22.0 b |
| **SEMIA 4108** | 15.3 ± 5.5 a | 13.6 ± 5.6 ab | 4.0 ± 0.5 b | 76.7 ± 11.3 ab | 12.2 ± 1.7 ab | 114.4 ± 36.6 ab |
| **SEMIA 4107** | 12.0 ± 0.8 ab | 23.1 ± 12.0 a | 3.5 ± 0.7 bc | 69.9 ± 23.1 b | 11.1 ± 2.5 b | 106.6 ± 28.1 b |
| **2018-19** | | | | | | |
| **N -** | 7.9 ± 2.3 ab | 15.6 ± 8.1 ab | 2.9 ± 0.6 b | 40.5 ± 7.5 b | 19.9 ± 4.0 b | 52 ± 30.4 a |
| **N +** | 5.2 ± 1.5 b | 7.4 ± 3.8 b | 8.0 ± 2.5 a | 108.2 ± 33.7 a | 54.4 ± 15.1 a | 95.0 ± 37 a |
| **SEMIA 4088** | 24.9 ± 12.0 a | 52.4 ± 36.9 a | 7.2 ± 1.6 a | 118.8 ± 33.7a | 50.3 ± 8.5 a | 74.7 ± 39.0 a |
| **SEMIA 4108** | 23.6 ± 12.7 a | 26.7 ± 5.8 ab | 6.8 ± 1.5 a | 92.1 ± 32.5ab | 46.4 ± 9.7 a | 100.2 ± 48.2 a |
| **SEMIA 4107** | 16.7 ± 4.0 ab | 25.5 ± 11.3 ab | 7.0 ± 2.0 a | 102.0 ± 35.1 ab | 48.4 ± 11.8 a | 94.0 ± 63.2 a |

Values followed by the same letter in the same column were not significantly different, as determined by Tukey test ($p < 0.05$).

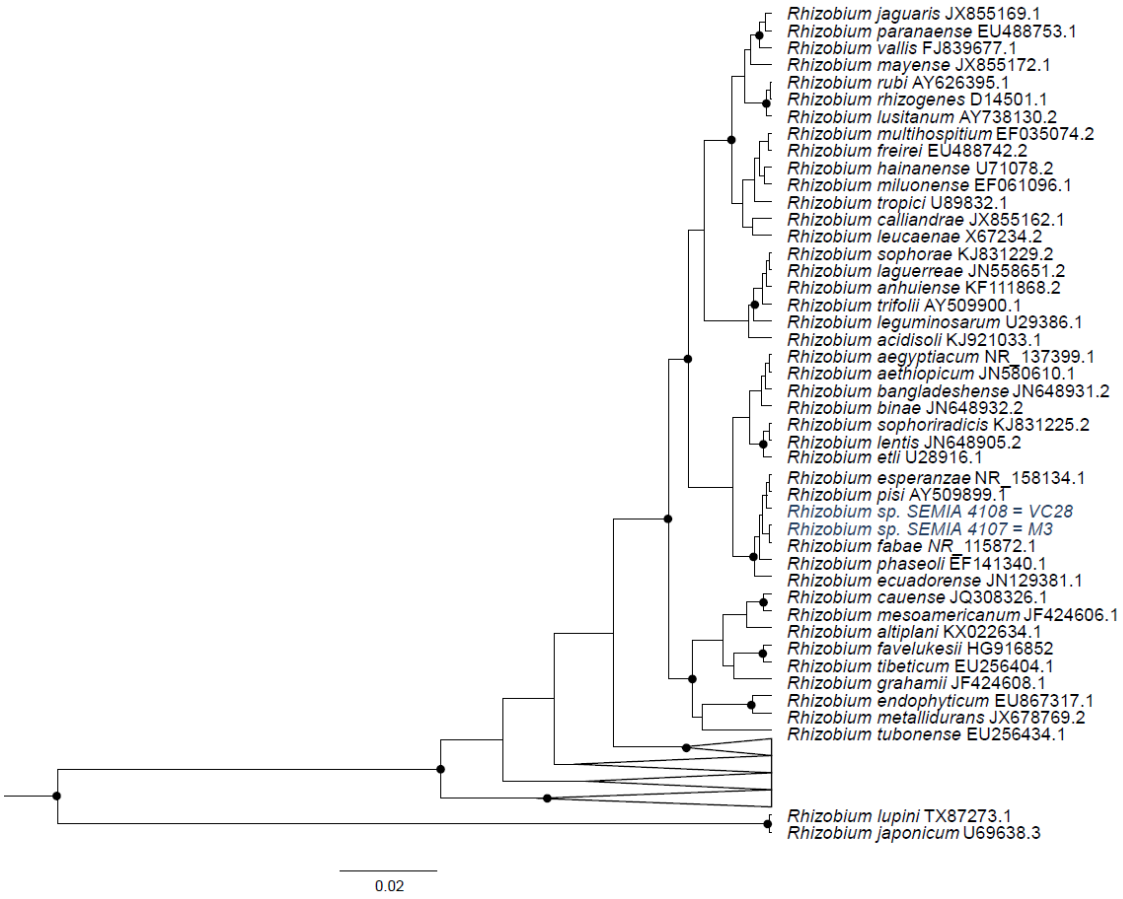



**Figure 1:** Phylogenetic tree of 16S rRNA sequences from *Rhizobium* sp. SEMIA 4108 (=VC28), *Rhizobium* sp. SEMIA 4107 (=M3) and *Rhizobium* species, as inferred by Bayesian analysis. The black circles at the branching points indicate posterior probability >95%. Genbank IDs are shown after the bacterial species names. The same tree with posterior probability values and nodes in expanded state is available in the Supplementary Figure 2.

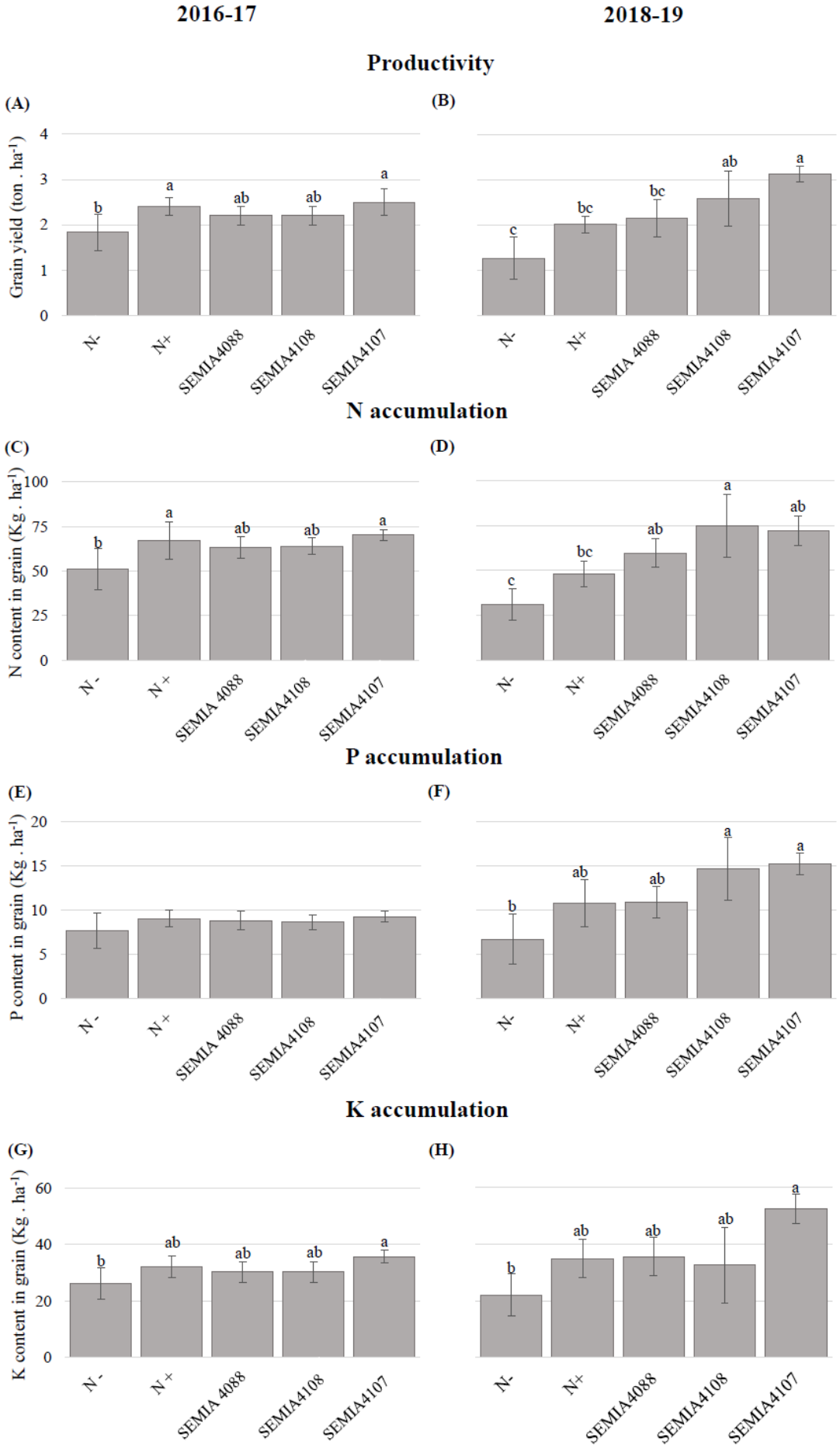

2016-17
2018-19
Productivity
(A)
(B)
Grain yield (ton . ha-1)
N-
N+
SEMIA4088
SEMIA4108
SEMIA4107
N accumulation
(C)
(D)
N content in grain (Kg . ha-1)
P accumulation
(E)
(F)
P content in grain (Kg . ha-1)
K accumulation
(G)
(H)
K content in grain (Kg . ha-1)

**Figure 2:** Agronomic parameters evaluated at harvest (R9 growth stage). (A and B) Grain yield (in each hectare), (C and D) nitrogen, (E and F) phosphorous, and (G and H) potassium content in grains per hectare. The results were compared by One-Way ANOVA test and the means by Tukey test ($p < 0.05$). * means did not present significant differences.

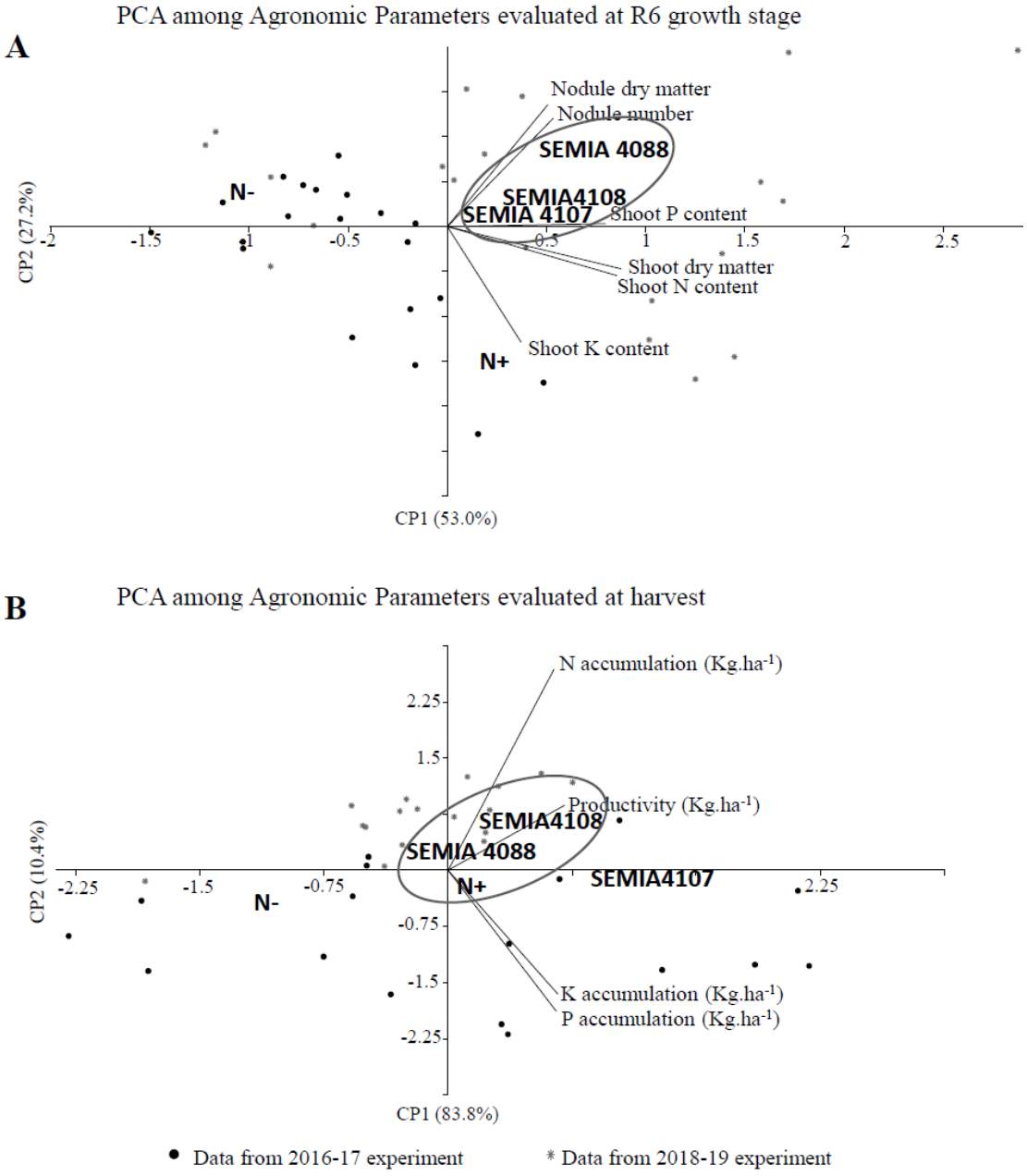
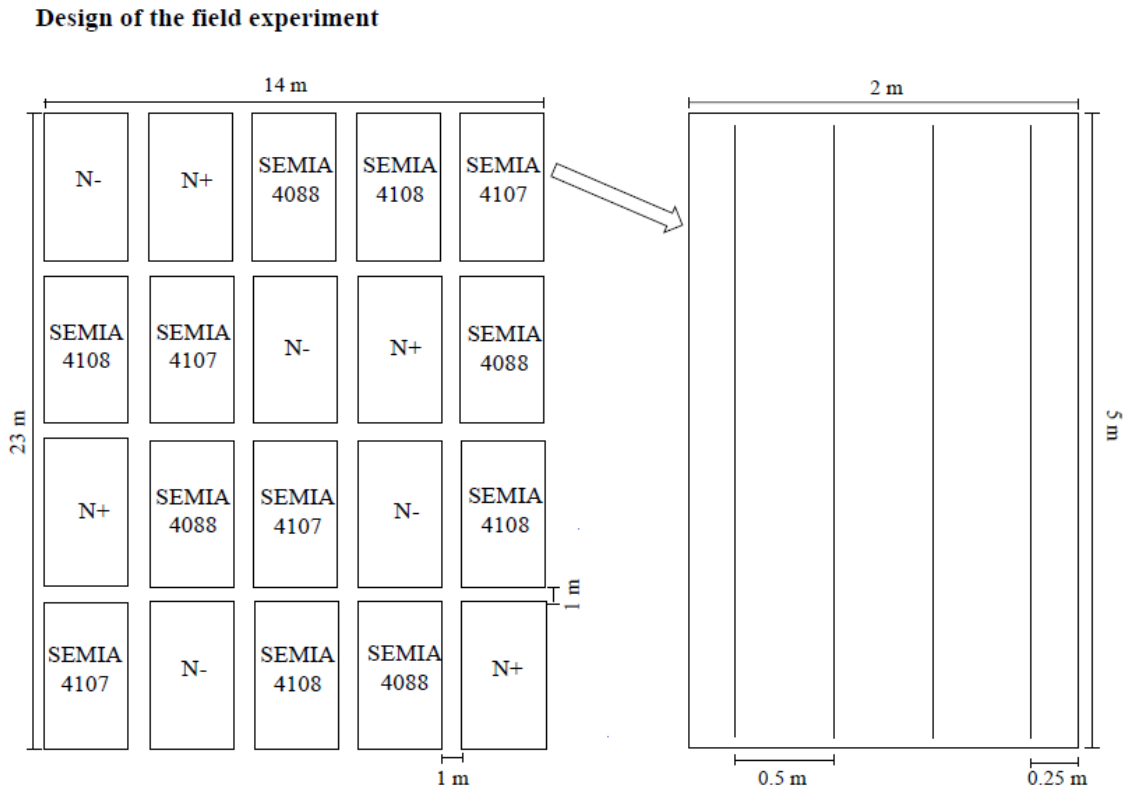
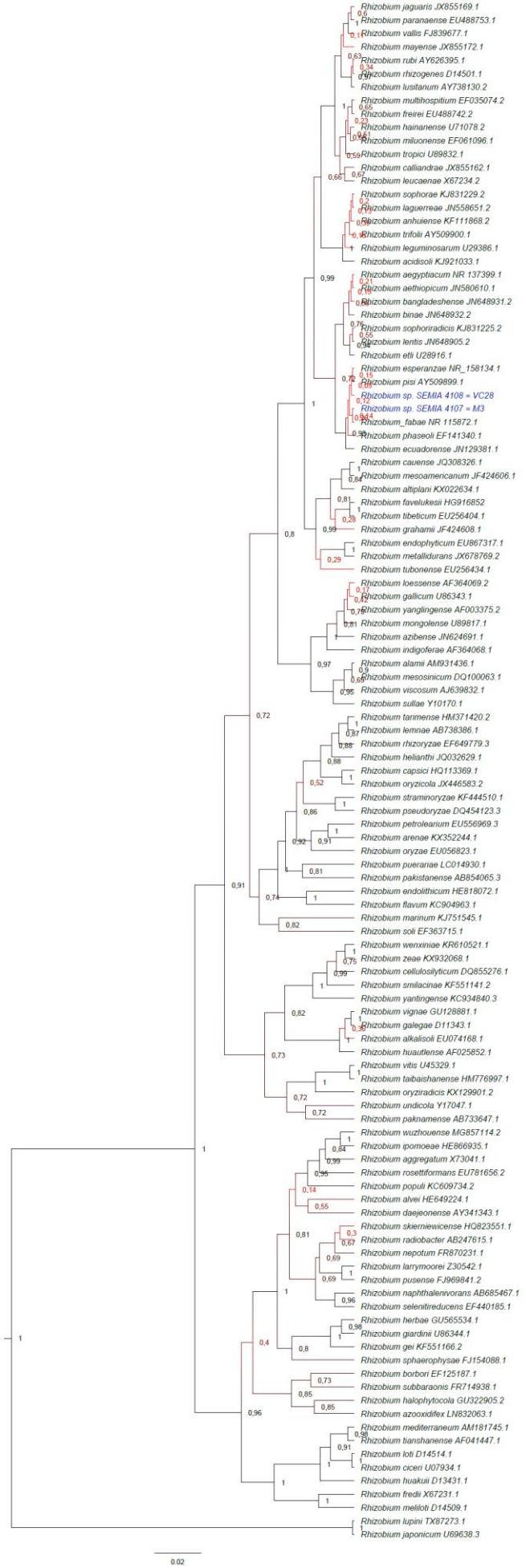



**Figure 3:** Principal component analysis (PCA) of agronomic characteristics evaluated (A) at flowering stage (R6), and (B) at harvest (R9) in both growing periods (2016-17 and

2018-19). CP 1 and CP 2 accounts for approximately 80.2% of total variability in (A) and 94.2 % in (B).




**Supplementary Figure 1:** Experimental design (randomized blocks) of the field experiment. The experiment occupies 322 $m^2$ and had four replicates of five treatments (three rhizobia inoculation: SEMIA 4088, SEMIA 4108 and SEMIA 4107; and two control treatments: with and without synthetic N fertilization, N+ and N-, respectively), totaling 20 experimental sample unities.


Rhizobium jaguaris JX855169.1
Rhizobium paranaense EU488753.1
Rhizobium vallis FJ839677.1
Rhizobium mayense JX855172.1
Rhizobium rubi AY626395.1
Rhizobium rhizogenes D14501.1
Rhizobium lusitanum AY738130.2
Rhizobium multihospitium EF035074.2
Rhizobium freirei EU488742.2
Rhizobium hainanense U71078.2
Rhizobium miluonense EF061096.1
Rhizobium tropici U89832.1
Rhizobium calliandrae JX855162.1
Rhizobium leucaenae X67234.2
Rhizobium sophorae KJ831229.2
Rhizobium laguerreae JN558651.2
Rhizobium anhuiense KF111868.2
Rhizobium trifolii AY509900.1
Rhizobium leguminosarum U29386.1
Rhizobium acidisoli KJ921033.1
Rhizobium aegyptiacum NR 137399.1
Rhizobium aethiopicum JN580610.1
Rhizobium bangladeshense JN648931.2
Rhizobium binae JN648932.2
Rhizobium sophoriradicis KJ831225.2
Rhizobium lentis JN648905.2
Rhizobium etli U28916.1
Rhizobium esperanzae NR_158134.1
Rhizobium pisi AY509899.1
Rhizobium sp. SEMIA 4108 = VC28
Rhizobium sp. SEMIA 4107 = M3
Rhizobium_fabae NR 115872.1
Rhizobium phaseoli EF141340.1
Rhizobium ecuadorense JN129381.1
Rhizobium cauense JQ308326.1
Rhizobium mesoamericanum JF424606.1
Rhizobium altiplani KX022634.1
Rhizobium favelukesii HG916852
Rhizobium tibeticum EU256404.1
Rhizobium grahamii JF424608.1
Rhizobium endophyticum EU867317.1
Rhizobium metallidurans JX678769.2
Rhizobium tubonense EU256434.1
Rhizobium loessense AF364069.2
Rhizobium gallicum U86343.1
Rhizobium yanglingense AF003375.2
Rhizobium mongolense U89817.1
Rhizobium azibense JN624691.1
Rhizobium indigoferae AF364068.1
Rhizobium alamii AM931436.1
Rhizobium mesosinicum DQ100063.1
Rhizobium viscosum AJ639832.1
Rhizobium sullae Y10170.1
Rhizobium tarimense HM371420.2
Rhizobium lemnae AB738386.1
Rhizobium rhizoryzae EF649779.3
Rhizobium helianthi JQ032629.1
Rhizobium capsici HQ113369.1
Rhizobium oryzicola JX446583.2
Rhizobium straminoryzae KF444510.1
Rhizobium pseudoryzae DQ454123.3
Rhizobium petrolearium EU556969.3
Rhizobium arenae KX352244.1
Rhizobium oryzae EU056823.1
Rhizobium puerariae LC014930.1
Rhizobium pakistanense AB854065.3
Rhizobium endolithicum HE818072.1
Rhizobium flavum KC904963.1
Rhizobium marinum KJ751545.1
Rhizobium soli EF363715.1
Rhizobium wenxiniae KR610521.1
Rhizobium zeae KX932068.1
Rhizobium cellulosilyticum DQ855276.1
Rhizobium smilacinae KF551141.2
Rhizobium yantingense KC934840.3
Rhizobium vignae GU128881.1
Rhizobium galegae D11343.1
Rhizobium alkalisoli EU074168.1
Rhizobium huautlense AF025852.1
Rhizobium vitis U45329.1
Rhizobium taibaishanense HM776997.1
Rhizobium oryziradicis KX129901.2
Rhizobium undicola Y17047.1
Rhizobium paknamense AB733647.1
Rhizobium wuzhouense MG857114.2
Rhizobium ipomoeae HE866935.1
Rhizobium aggregatum X73041.1
Rhizobium rosettiformans EU781656.2
Rhizobium populi KC609734.2
Rhizobium alvei HE649224.1
Rhizobium daejeonense AY341343.1
Rhizobium skierniewicense HQ823551.1
Rhizobium radiobacter AB247615.1
Rhizobium nepotum FR870231.1
Rhizobium larrymoorei Z30542.1
Rhizobium pusense FJ969841.2
Rhizobium naphthalenivorans AB685467.1
Rhizobium selenitireducens EF440185.1
Rhizobium herbae GU565534.1
Rhizobium giardinii U86344.1
Rhizobium gei KF551166.2
Rhizobium sphaerophysae FJ154088.1
Rhizobium borbori EF125187.1
Rhizobium subbaraonis FR714938.1
Rhizobium halophytocola GU322905.2
Rhizobium azooxidifex LN832063.1
Rhizobium mediterraneum AM181745.1
Rhizobium tianshanense AF041447.1
Rhizobium loti D14514.1
Rhizobium ciceri U07934.1
Rhizobium huakuii D13431.1
Rhizobium fredii X67231.1
Rhizobium meliloti D14509.1
Rhizobium lupini TX87273.1
Rhizobium japonicum U69638.3
0.02

**Supplementary Figure 2:** Phylogenetic tree of 16S rRNA sequences from *Rhizobium* sp. SEMIA 4108 (=VC28), *Rhizobium* sp. SEMIA 4107 (=M3), and 113 type strains of *Rhizobium* species, as inferred by Bayesian analysis. Genbank IDs are shown after the bacterial species names. The significance of each branch is indicated at the branching points by posterior probability.

**CAPÍTULO II - Rizóbios no biocontrole de fungos fitopatogênicos do feijão-comum (*Phaseolus vulgaris* L.)**

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# Effect of rhizobia inoculation on the development of soil-borne pathogens infecting common bean plants

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**Abstract** Common bean (*Phaseolus vulgaris*) plants are one of the most important legumes for human consumption due to its nutritional value and the fact it is rich in protein, iron, carbohydrates, and bioactive compounds. Diseases in common bean plants caused by soil-borne pathogens cause important economic losses in the world. Rhizobia is a well-known bacterial group that directly and indirectly promotes plant growth. The steps for biological nitrogen fixation performed in leguminous plants are well-known, as it is also known that the symbiosis between rhizobia and leguminous presents inhibitory effects against fungal diseases in plants. Thus, this work gathers information about the indirect effect in plant growth caused by rhizobia inoculation in common bean plants against the fungal pathogens *Rhizoctonia solani, Fusarium oxysporum*, *Fusarium solani* and *Macrophomina phaseolina*. Literature shows that the inoculation of common bean plants with different rhizobia isolates reduces or blocks the symptoms of disease caused by these phytopathogenic fungi. It is already known that rhizobia produce extracellular enzymes that hamper fungal development and induce physiological and molecular changes in plants. However, the specific mechanisms that govern these interactions and inhibitions still need to be clarified.

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## Introduction

Plant Growth Promoting Rhizobacteria (PGPR) is a group of non-pathogenic bacteria that includes both free living (e.g. *Azospirillum brasilense*) and symbiotic (e.g. *Rhizobium* spp. and *Bradyrhizobium* spp.) nitrogen fixing microorganisms (Das et al. 2017). This bacterial group promotes plant growth directly usually by facilitating nutrient uptake (mainly nitrogen [N], phosphorous [P] and potassium [K]) and hormone production, and indirectly by decreasing inhibitory effects of fungal diseases on plant development, acting as biocontrol agents (Glick 2012).

Rhizobia group is a recognized PGPR that establishes symbiotic relationships with leguminous species and creates specialized structures called nodules on the plant root (Mortier et al. 2012). Metabolic activity of these bacteria inside the nodules is able to supply the whole N needed for the leguminous plants development through a process known as Biological Nitrogen Fixation (BNF). Inoculation of rhizobia in leguminous plants shows potential for eliminating all costs related to N fertilization in crops (Fageria et al. 2014). Because of their economic importance, the symbiosis (Mortier et al. 2012) and BNF steps have already been studied and well-described, aiming to improve the efficiency of these processes (Wagner 2012).

Screening of rhizobia as an inoculant for leguminous plants mainly considers the BNF efficiency and the growth-promoting effect caused by inoculation (Olivares et al. 2013). Therefore, the knowledge about the mechanisms that involve the direct effect in plant growth caused by bacterial production of indole-acetic acid and ACC deaminase (Bhattacharjee et al. 2012), gibberellins (Hayashi et al. 2014), nutrient solubilization (Granada et al. 2013), and BNF (Fageria et al. 2014) is consolidated. However, it is also known that rhizobia-leguminous symbiosis has inhibitory effects against the development of fungal diseases in plants (Das et al. 2017). Its inhibitory effect against soil-borne fungal pathogens could be explained through direct effect caused by mycoparasitism, production of antibiotics, hydrogen cyanide and siderophores, or indirect effect caused by the induction of plant defense mechanisms, which reduces their susceptibility (Gopalakrishnan et al. 2015; Das et al. 2017).

Recently, the general population and environmental protection agencies are criticizing the excessive use of pesticides. Environmental agencies particularly recommend the use of microbial inoculants (mainly *Rhizobium* spp. isolates) as a biological disease-controlling agent (Bashan et al. 2014). Therefore, this work gathers information about the indirect plant growth effect caused by rhizobia inoculation in common bean (*Phaseolus vulgaris*) plants infected with the phytopathogenic fungi *Rhizoctonia solani*, *Fusarium oxysporum, Fusarium solani* and *Macrophomina phaseolina*, the main causes of root rot.

## Common bean (*Phaseolus vulgaris*) plants

Common beans (CB) are leguminous plants with economic, nutritional, and cultural importance around the world (Broughton et al. 2003). Due to its nutritional value and high levels of protein, iron, carbohydrates, and bioactive compounds, CB are one of the most important legumes for human consumption (Broughton et al. 2003; Vital et al. 2014). More than 23 million ha are cultivated around the world, mainly by family agriculture in small lands (Broughton et al. 2003), which generates income and employment for these families (CONAB 2014). The estimated total production ranges from 11 to 13 million tons (Beebe et al. 2013), and Brazil, India, China, USA, and Mexico account for 61% of global production (FAO 2014). Consumption of this grain in Latin American countries ranges from 12 to 18 kg per year per person, reaching 36 and 60 kg in Nicaragua and some African countries, respectively (Beebe et al. 2013).

Concerned about yield and quality losses, United Nations (UN) has expanded the discussion about legume cropping and legume-protein-based food consumption (IYOP – International Year of Pulses – Vollmann 2016). The goals include searching for drought-resistant cultivars and understanding the challenges faced by producers of the CB crops (Beebe et al. 2013), since most of the farms do not use modern technologies (Recchia et al. 2013), and climatic variations as well as incorrect soil tillage have facilitated the spreading of fungal pathogens (Broughton et al. 2003).

Fungal diseases were pointed as the main cause of low productivity in CB crops around the world (Broughton et al. 2003), causing losses of 80 to 100% in yield and quality (Singh and Schwartz 2010; Beebe et al. 2013). More than 45 bacterial, fungal, and viral diseases are described in aerial and underground parts of the CB. However, significant losses in production observed by root rots stimulate studies with their causal agents *Rhizoctonia solani*, *Fusarium oxysporum*, *Fusarium solani* and *Macrophomina phaseolina* (Singh and Schwartz 2010). Inoculation with rhizobia isolates plays an important role in nutrient acquisition (Beebe et al. 2013), besides being an interesting alternative to control the dissemination of such plant diseases (Deshwal et al. 2003; Das et al. 2017). However, the specific mechanisms behind this kind of control are largely unknown. Table 1 summarizes the available information regarding biocontrol of soil-borne pathogens by inoculation of rhizobia isolates in CB plants.

## Biocontrol of *Rhizoctonia solani* infecting common bean plants by rhizobia inoculation

*Rhizoctonia* spp. infect at least 200 plant species and are some of the most common soil-borne pathogens in crop plants (Lehtonen et al. 2008). The most common *Rhizoctonia* species infecting CB plants is *Rhizoctonia solani* (Godoy-Lutz et al. 2003; Nerey et al. 2010). Different symptoms caused by *Rhizoctonia solani* infection have already been observed, and pre- and post-emergence damping-off, root and hypocotyl rot, and foliar blight are the most reported (Rusuku et al. 1997; Nerey et al. 2010). As far as we know, there is no

**Table 1** Biocontrol of soil-borne pathogens *Rhizoctonia solani*, *Fusarium oxysporum* and *Fusarium solani* by inoculation of rhizobia isolates in common bean plants

| Fungal pathogen | Biocontrolling rhizobia | Mode of Action | Biocontrol effect | Reference |
|---|---|---|---|---|
| *Rhizoctonia solani* | *Rhizobium* spp. | Production of antimicrobials, siderophores, and hydrogen cyanide | Improved (more than 100%) shoot fresh weight | Samavat et al. (2011) |
| | Native rhizobia | NI | Reduced disease symptoms | Naseri (2013) |
| | *Rhizobium leguminosarum* | NI | Reduced incidence of disease in field experiments | Mora-Umaña and Cavallini (1996) |
| *Fusarium oxysporum* | *Rhizobium leguminosarum* | NI | Reduced pathogen attack | Barquero et al. (2016) |
| *Fusarium solani* | *Rhizobium sp.* | NI | Improved plant biomass | Buonassisi et al. (1986) |
| | *Rhizobium tropici* | NI | Reduced (~ 50%) disease severity | de Jensen et al. (2002) |
| | *Rhizobium leguminosarum* | NI | Improved (~ 25%) plant biomass | Dar et al. (1997) |
| | *Rhizobium tropici* | ISR | Induced ISR (through Nod factors) | Lange et al. (1999) |

*ISR* Induction of Systemic Resistance, *NI* not identified

available variety of CB resistant to this pathogen. To prevent the disease establishment, fungicides such as pencycuron, fludioxonil, tolclofos-methyl, flutolanil, azoxystrobin, mancozeb, iprodione, imazalil, and propiconazole are recommended (Tsror 2010). This pathogen is considered a species complex classified into 14 hyphal anastomosis groups (AG - Nerey et al. 2010). Some AG were linked to web blight of the aboveground structures of the plant (AG 1-IA, AG 1-IB, AG 1-IE, AG 1-IF, AG 2–2, and AG 4), and others AG have been linked to root rot (AG 4 and AG 2–2 - Valentin Torres et al. 2016). Meinhardt et al. (2002) studied 18 *Rhizoctonia solani* isolated from CB plants, and all were correlated with the AG4 anastomosis group and their genetic subgroups. Infection of CB plants by this fungal species can cause losses ranging from 5 to 40% of the yield (Valentin Torres et al. 2016), and the knowledge about genetic composition of populations infecting CB plants can influence management practices that will be implemented (Godoy-Lutz et al. 2003).

In the soil, *Rhizoctonia solani* presents a facultative sporophyte, able to infect at a wide range of soil temperatures, although cool (11–18 °C) and wet soil conditions are ideal for infection (Seethapathy et al. 2017). Its multinucleate hyphae grows on the root plant surface and attaches to it after a short period (12 h maximum). Penetration of the fungus into root tissues occurs in cracks or by excretion of extracellular enzymes that degrade cell walls (Borras-Hidalgo et al. 2012). Then, additional inter- or intra-cellular growth continues from roots to shoots causing tissue collapse, which forms brown lesions, ultimately leading to plant death (Guerrero-González et al. 2011). This pathogen is very persistent in the soil, causing continuous disease in different crops after plants die, which generates a cycle that is very difficult to control (Al-Askar and Rashad 2010).

The first studies regarding the inhibition of *Rhizoctonia solani* by rhizobia isolates were only performed in vitro. Drapeau et al. (1973) tested the antagonistic effect of three new *Rhizobium* spp. strains and none was able to inhibit this pathogen. Later, Kelemu et al. (1995) showed that new bradyrhizobial isolates reduced *Rhizoctonia solani* mycelial weight, sclerotial production and sclerotial "germination", probably by the production/secretion of antimicrobial metabolites. Until now, most of the studies reporting a decrease on the development of *Rhizoctonia solani* caused by rhizobia isolates have only been performed in vitro (Kumar et al. 2016). In the 90's, in vivo experiments directly conducted in plants evaluating the biocontrol of fungi diseases by rhizobia isolates started to be performed. Such studies showed reductions of up to four times in disease symptoms caused by *Rhizoctonia solani* in CB plants cultivated in greenhouse conditions and inoculated with rhizobia cultural filtrates (Samavat et al. 2011). The same effect was observed in CB plants inoculated with *Rhizobium leguminosarum* biovar *phaseoli* in field experiments (Mora-Umaña and Cavallini 1996). Such protective effect was also found in non-CB plants. Mazen et al. (2008) performed field experiments with Faba bean plants and showed that soaking the seeds in rhizobial cultural filtrates, individually or in combination with arbuscular mycorrhiza fungi, acts as a bioprotective agent against root rot caused by *Rhizoctonia solani*. Recently, Choudhary and Sindhu (2015) showed that co-inoculation of *Bacillus* and

*Pseudomonas* with *Rhizobium*/*Bradyrhizobium* isolates avoid root rot and damping off symptoms in cluster bean plants infected by *Rhizoctonia solani*. These works show high efficiency of rhizobial inoculation to control infection by this fungal species and highlight the importance of more in-depth studies to reveal the exact mechanisms of inhibition.

## Biocontrol of *Fusarium* spp. infecting common bean plants by rhizobia inoculation

The genus *Fusarium* comprises a high number of filamentous fungal species that can be plant-pathogenic and harmful for humans and animals (Srivastava et al. 2018; Moretti 2009). Some species have been considered major pathogens of cereals (Placinta et al. 1999). These phytopatogenic isolates inhabit the upper layer of the soil (0 to 25 cm), present a cosmopolitan saprophyte lifestyle, and can act as biotrophic parasites (Kikot et al. 2009; Basallote-Ureba et al. 2016). Their chlamydospores or mycelium secrete hydrolytic enzymes which degrade cell wall polymers, invade the plant tissue to the xylem vessels, searching for nutrients, and consequently cause cortical deterioration, root rot, leaf yellowing and wilting, and ultimately plant death (Coleman 2016; Srivastava et al. 2018). However, production of secondary metabolites by some *Fusarium* spp. isolates can act as plant growth promoters, and have a protective effect against other phytopathogens (Gao et al. 2010).

Fusarium wilt (Fw) is caused by fungal species belonging to *Fusarium oxysporum* sensu *lato* complex. The disease causes one of the major losses in different leguminous crops (Dean et al. 2012). Such species spread themselves through infected soil particles in seeds, wind and irrigation water (Xue et al. 2017). In CB plants, the fungus responsible for Fw (*Fusarium oxysporum* f. sp. *phaseoli* (Fop)) causes chlorosis, leaf loss, vascular tissues necrosis, and finally plant death (Alves-Santos et al. 2002; de Borba et al. 2017). Chlamydospores of Fop remain viable on the soil for a long time, which reduces the suppressive potential of crop rotation. Fw causes around 10% loss in grain yield, and for this reason, the use of seed treatment with fungicides is highly recommended (Cross et al. 2014).

The selection of CB genotypes resistant to Fop is being focused on plants that slow down pathogen progression to the xylem (de Borba et al. 2017). Meanwhile, rhizobia inoculation is proven as an efficient technique in Fop biocontrol (Mazen et al. 2008). In vitro experiments have demonstrated that *Rhizobium phaseoli* isolated from CB plants can inhibit the growth of four different Fop isolates (Kucuk 2013). Recently, such inhibition was attributed to *Rhizobium* spp. production of chitinases, β-1,3- and β-1,4-glucanases (Kumar et al. 2016), which are responsible for the lysis of fungal cell walls (Das et al. 2017). Production of bacteriocins by *Rhizobium leguminosarum* also presents biocontrol potential against Fop (Das et al. 2017). However, the biocontrol effect promoted by rhizobia seems to be connected with the synthesis of elicitors or liposaccharides that induce plant systemic resistance (Reitz et al. 2000).

*Fusarium solani* is another phytopathogen that causes yield loss in important crops, including olive trees, pea, sweet potato, cucurbits, soybean and common bean (Filion et al. 2003; Gao et al. 2004; Zhang et al. 2006; Amira et al. 2017). Root rot disease in CB plants is caused by *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burkholder) W.C. Snyder & N.H. Hans (Fsp) (Filion et al. 2003). This disease has been studied since the 70's, and is stimulated by low temperature, soil compaction and high soil moisture, which is common in fields cropped with CB plants (Buttery et al. 1998). As with Fop, this pathogen is also persistent in the soil. The initial symptoms of plant disease are longitudinal streaks (reddish) on the roots of young plants. Subsequently, irregular reddish lesions turn brown, with no defined margins. Destruction of the roots reduces water and nutrient absorption, hindering the normal plant development (Sasan and Bidochka 2013).

One of the first studies regarding biocontrol of Fsp infecting CB plants by rhizobia was performed in vitro by Buonassisi et al. (1986). These authors evaluated the antagonistic effect of 42 *Rhizobium* spp. nodulating CB plants against Fsp, and observed that 41 presented inhibition potential. Then, 15 isolates were selected for in vivo experiments with CB plants, and only two caused a significant reduction in *Fsp* root rot symptoms. Inoculation of *Rhizobium leguminosarum* in CB plants showed a biocontrol effect against Fsp, even though such effect was evaluated only by agronomic parameters such as plant biomass and height, leaf area, and nitrogen/phosphorus accumulation (Dar et al. 1997). de Jensen et al. (2002) showed that co-inoculation of *Rhizobium tropici* and *Bacillus subtilis* inhibits Fsp development, as well as improving shoot dry weight and yield of CB plants.

### Biocontrol of *Macrophomina phaseolina* infecting common bean plants by rhizobia inoculation

The fungal genus *Macrophomina* belongs to the Ascomycota phylum and the Botryosphaeriaceae family. *Macrophomina phaseolina* Tassi (Goid) is a plant pathogen able to infect over 400 plant species, causing charcoal rot disease (Mihail and Taylor 1995). This fungal genus is distributed around the world, but the economic impacts caused by its infection are higher in tropical and subtropical regions, with arid and semi-arid climates, especially in warm and dry environments (Kaur et al. 2012). Symptoms of charcoal rot disease are most significant on the first stages of CB development, causing wilting and leaf discoloration. Roots may also turn gray, and black spots appear under the stem epidermis and on the roots, giving the plants an appearance of charcoal-sprinkles (Mayek-Pérez et al. 2002).

Control of *M. phaseolina* dissemination is difficult due to its low plant specificity. This fungus presents resistant structures called sclerotia, which can survive up to 10 months in dry conditions (Khan 2007). Severity of plant disease is directly related to the number of viable fungal cells on the soil (Kendig et al. 2000). The physiological and histopathological changes caused by *M. phaseolina* in CB plants are poorly understood. It has been suggested that CB cultivars resistant to charcoal rot could also resist drought stress (Mayek-Pérez et al. 2002). The infection process in CB plants has not yet been described; however, it is already known that it is able to infect the cotyledons, roots or stems in either pre- or post-emergence stages of soybean plants. The hyphae develop inter- and intracellularly through enzymatic action. Then, *M. phaseolina* colonizes the vascular system, blocking xylem vessels and causing disease symptoms (Ilyas and Sinclair 1974).

Biocontrol of charcoal rot by rhizobia has been scarcely reported. In vitro experiments showed up to 90% inhibition of *M. phaseolina* growth by rhizobia isolates (Sagolshemcha et al. 2017). Rhizobitoxins produced by *Bradyrhizobium japonicum* are able to inhibit the development of this fungus and charcoal rot in soybean plants (Chakraboraty and Purkayastha 1984). Arora et al. (2001) showed that the growth inhibition of *M. phaseolina* by *Rhizobium meliloti* in groundnut plants was positively related to bacterial siderophore production. Also, rhizobia isolated from root nodules of CB plants inhibited in vitro growth of this fungus through the production of β-1,3- and β-1,4-glucanases (Kumar et al. 2016). In field experiments using CB plants, these authors showed that a bacterial consortium composed of *Rhizobium leguminosarum*, *Bacillus* sp. and *Pseudomonas* sp. improved the biocontrol efficiency of soil-borne pathogens. To date, there are no studies reporting in vivo biocontrol potential of *M. phaseolina* infecting CB plants by rhizobia isolates.

## Conclusion and future directions

Evidence from the literature shows that inoculation of CB plants with different rhizobia isolates reduces or blocks the root rot symptoms caused by the phytopathogenic fungi *Rhizoctonia solani*, *Fusarium oxyporum*, *Fusarium solani* and *Macrophomina phaseolina*. These studies also report that co-inoculation studies present a challenge for the future. However, elucidation of the molecular, biochemical and physiological mechanisms involved with inhibition of fungal development and plant infection are neglected and still largely unknown. The studies focused in this subject are limited, only suggesting compounds and/or enzymes/pathways that could be involved in the biocontrol process, such as bacterial production of siderophores, extracellular enzymes, antimicrobial molecules and systemic resistance induction in plants.

Knowledge about the plant, bacteria and phytopathogen interaction, and biomolecules involved in this process, needs to be uncovered through new multidisciplinary approaches. Advances in chromatography, spectroscopy and nuclear magnetic resonance techniques could be helpful to identify potential biomolecules, and structural bioinformatics techniques could be used to predict their targets. However, in vitro studies are only the first step in gaining knowledge, and in vivo approaches with plant studies are needed to understand the inhibition mechanisms. For this purpose, molecular techniques evaluating differential gene expression data (transcriptomics, proteomics, metabolomics) in plants infected by phytopatogens and inoculated with rhizobia can be an excellent tool to elucidate such complex interaction. Thus, aiming to facilitate these studies, the use of well-known plant species and rhizobia interactions, such as CB-rhizobia or soybean-*Bradyrhizobium* spp., should be the first option.

The BNF and indirect plant growth promoting effect caused by rhizobial inoculation in CB plants still need to be elucidated and communicated for producers. The

classical agricultural management that uses chemical fertilizers and fungicides to ensure good harvests with no human health and food security concerns (Margni et al. 2002) is the most common practice in CB crops. Also, we can observe a clear resistance by farmers to the use of biotechnological approaches, mainly due to them being unaware of their potential. Rhizobial inoculation and crop rotation, if correctly applied, ensures the sustainability of the cropped areas, with high nutrient availability and low levels of fungal infections (Beatty and Good 2011), and is environmentally safe and economically advantageous to the farmers (Ndakidemi et al. 2006).

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that the present work was developed without any potential conflict of interest, with no human or animal participants. All authors read and approved the final version of this manuscript.

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***In vitro* evaluation of *Macrophomina phaseolina* inhibition by rhizobia isolates**

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**Abstract**

Bacteria of the genus *Rhizobium* induce nodule formation and biological nitrogen fixation (BNF) in legumes. These bacteria promote plant growth through atmospheric $N_2$ fixation, nutrient supply, phytohormone synthesis, and mineral solubilization. In addition to the role in soil fertility, these microorganisms can reduce incidence of diseases caused by phytopathogens in various crops. Thus, the aim of this study was to isolate and evaluate *Rhizobium* sp. strains from root nodules of common bean plants (*Phaseolus vulgaris* L.) and evaluate their inhibitory effect on the phytopathogenic fungus *Macrophomina phaseolina* MP53. Among the 40 strains of isolated rhizobia, only isolates R26, L5 and VC28 presented inhibitory *in vitro* effect against *M. phaseolina* MP53, with a reduction of approximately 50% in the mycelial mass. GC-MS analysis showed that the presence of compounds that may be associated with microbial antagonism. Development of inoculants using *Rhizobium* sp. could be considered a biological control agent, and may contribute to increased crop growth and productivity. The data obtained in our *in vitro* study show that *Rhizobium* isolates has the potential for inhibition the development of *M. phaseolina* MP53 and the obtained volatile compound 1-butanol, 9,10-Dihydro-12'-

hydroxy-ergotoman-3',6',18-trione and Hexahydro-pyrrolo [1,2-a] pyrazine-1,4-dione may be responsible for the antagonism activity.

**Key words:** antagonism, rhizobial isolates, metabolites, *Macrophomina phaseolina*.

## Introduction

Rhizobia is a diverse group of soil bacteria that induce the formation of nitrogen-fixing nodules on the roots of legumes (Deakin and Broughton, 2009). These bacteria promote the growth of leguminous plants directly through $N_2$ fixation, phytohormone synthesis and mineral solubilization (Deshwal et al., 2003). Besides playing a major role in biological nitrogen fixation, which improves soil fertility (Gurion, 2015), rhizobia can also promote plant growth reducing the level of disease incidence in several leguminous crops, such as soybean, bean, faba bean, ground nut, chickpea, pea, pigeon pea, tomato and fenugreek (Das et al., 2017).

Some mechanisms are involved on the reduction of pathogenic fungi development by rhizobia inoculation, such as mycoparasitism, production of antibiotics and antifungal secondary metabolites, including hydrogen cyanide (HCN), siderophore production, competition for nutrients, and induction of plant defense mechanisms, which reduce susceptibility to pathogenic attack (Arora et al., 2001; Das et al., 2017). The understanding of physiological mechanisms controlling fungal biocontrol by rhizobia, and the use of rhizobia inoculants instead of chemical pesticides, became an eco-friendly alternative for agriculture (Glick, 2012).

Some rhizobia strains can inhibit the growth of various soil borne pathogens as *Fusarium* sp., *Rhizoctonia solani*, and *Macrophomina phaseolina* in both leguminous and non-leguminous plants (Mazur et al., 2004; Das et al., 2017). *M. phaseolina* Tassi (Goid) is an imperfect fungus of the phylum Ascomycota, which causes dry root rot, stem

canker, stalk rot and charcoal rot in over 500 plant species (Sexton et al. 2016; Martínez Villarreal et al., 2016). Charcoal root rot severely damages susceptible cultivars, and epidemics are prevalent in drought areas with high temperatures (Mihail and Taylor, 1995). To control charcoal rot, bean germplasm and cultivars have been identified (Sabaté et al., 2017). Seed treatment with fungicides is used to control charcoal rot in the early growth stages (Sabaté et al., 2017) however, the chemical dependence of pesticides and their indiscriminate use can cause several detrimental effects on the environment (Kumar and Singh, 2015). Alternatively, the use of microbial inoculants, mainly *Rhizobium* spp. isolates, can be considered a biological disease-controlling agent (Schmidt et al., 2019).

The development of soil management techniques to control pathogens, targeting a more sustainable agriculture, is increasing on the last few years (Ranjbar et al., 2017). Thus, the aim of this study was to screening rhizobia isolates from common bean (*Phaseolus vulgaris* L.) root nodules based on their efficiency to inhibit the development of the fungi *M. phaseolina*. In addition, we have performed the identification of metabolites produced in response to rhizobia-fungi interaction.

## Material and Methods

### Rhizobia isolation

Rhizobia were isolated by the plant-bait method as described by Melloni et al. (2006). First, soil samples were collected (July 2017) in the city of Santa Cruz do Sul, Brazil (29°42"21' S and 52°22"55' W), and packed in 500 mL pots. Common bean seeds were disinfested with one rinse in 70% ethanol for one min, followed by 1% sodium hypochlorite for three min, and finally in sterile distilled water (Andrade and Hakawa, 1994). Disinfested seeds were cultivated in the soil and kept in a greenhouse for 15 days. Afterwards, plants were harvested, the root nodules were extracted, and surface

disinfested by the same methodology described for seed disinfestation. These nodules were pulverized in 0.1 mL of sterile distilled water, and the suspension obtained was inoculated in Yeast Mannitol Agar (YMA) at 28°C until the development of isolated colonies, which were purified and preserved in 40% glycerol at -20°C (Somasegaran and Hoben, 1994). Rhizobia VC28 and L5, used in this work, were previously characterized as *Rhizobium fabae* (de Souza et al., 2016). The Brazilian Agricultural Research Corporation (Embrapa) kindly provided the fungal strain.

**Rhizobia antagonism against *Macrophomina phaseolina***

The screening of rhizobial isolates able to inhibit the development of *M. phaseolina* MP53 was evaluated by the methodology of Cavaglieri et al. (2005) with minor modifications. An halo of 6 mm diameter of *M. phaseolina* MP53 was inoculated in the center of petri dishes with TY Agar (Beringer, 1974), and rhizobia was inoculated around the fungus, forming a barrier. The petri dishes were incubated at 28°C for 15 days, and fungal inhibition was visually checked. The whole experiment was carried out in triplicates.

An aliquot of each rhizobia previously selected on preliminary screening was inoculated in TY Broth for two days (until reach approximately $10^8$ CFU.mL$^{-1}$), at 28°C under stirring. After this, a 6 mm disc of the fungus was inoculated on the TY broth which rhizobia were grown. Control condition comprises only the fungal isolate, without adding any rhizobia. The suspensions were incubated for seven days at 28°C under stirring. Subsequently, the broth was filtered, and the mycelia suspension was dried at 65°C for three days and weighed. The resulting liquid was used to determine the metabolites produced by the microorganisms (CHAO, 1990). The whole experiment was carried out in triplicates.

Statistical analyses were performed using One-Way Anova, and the means were compared by the Tukey test ($p < 0.05$), using InfoStat software.

**Extraction of metabolic compounds**

Metabolites of the filtrate were extracted with the same volume of ethyl acetate and n-butanol. The organic fractions were dried using anhydrous magnesium sulfate ($MgSO_4$), and concentrated in a vacuum rotary evaporator (Veloso et al. 2013).

**Identification of microbial metabolites produced**

Identification of metabolites was performed by gas chromatography coupled with mass spectrometry (GC-MS) using the methodology described by Telke et al. (2010) with minor modifications. Concentrated samples were dissolved in methanol and analyzed on Shimadzu's QP2010 Ultra GC-MS mass spectrometer employing a Resteck RTx-5MS capillary column (30 m x 0.25 mm di x 0.25 mm film thickness), with initial column heating temperature of 40°C for four min, which was linearly increased at 10°C per minute to 270°C, and held for four min. The injector temperature was 275°C and GC-MS interface was maintained at 300°C with the temperature of 260°C ion source and employing an ionization voltage of 70 eV. The carrier gas used was helium at a flow rate of 1 mL per min, and the run time of the analysis was 144 min. Compounds were identified based on mass spectra compared to the NIST library spectra of GC-MS-solution software (NIST / EPA / NIH Mass Spectral Library, NIST11). These analyses were performed on the Instrumental Analytical Chemistry Laboratories of Tecnovates (The Science and Technology Park of Taquari Valley).

## Results and discussion

Isolation of rhizobia resulted in 38 isolates (named R1 to R38). Previously isolated rhizobia isolates L5 and VC 28 (de Souza et al., 2016) were added to the study. The screening of 40 rhizobia isolates showed that only three (R26, L5, and VC28) present inhibitory effect against *M. phaseolina* MP53, being able to inhibit 50% of *M. phaseolina* MP53 growth in TY broth. The preliminary screening method used for selection of rhizobia able to inhibit the development of *M. phaseolina* MP53 was shown in Figure 1.

As seen in Figure 2, the mycelial mass weight from inoculated treatments is lower than control condition (no rhizobia added), corroborating the inhibition pattern seen in agar TY. Kumar et al. (2016) obtained similar results, with *Rhizobium leguminosarum* being able to inhibit around 50-70% the *M. phaseolina* growth. Chakraborty and Purkayastha (1984) also observed a reduction in biomass dry weight of *M. phaseolina* (both in broth and agar media) after inoculation of *Bradyrhizobium* sp. In addition, the authors verified that the antagonistic effect was not associated to direct competition for nutrients, but rather by the action of ribotoxins, also detected in the roots of soybean plants inoculated with the bacteria alone or in association with the fungus.

## Identification of metabolites generated in the antagonism

The compounds extracted with ethyl acetate are listed in Table 1. We were able to detect alkaloids esters and other solvents.

The compound 9,10-dihydro-12'-hydroxy-ergotoman-3',6',18-trione was the most abundant alkaloid found, representing 28.4% of the compounds found in R26, 42.1% in L5, and 44.2% in VC28. This alkaloid was not identified on the control treatment. According to Metzger et al. (2009), ergot alkaloids are toxins, nitrogen-containing natural products belonging to the group of indole alkaloids. The main producers are fungi of the

phylum Ascomycota, as *Claviceps*, *Epichloë*, *Penicillium* and *Aspergillus* species (Gerhards et al., 2014). Our results suggest that *M. phaseolina* produces this alkaloid only in the presence of the evaluated bacteria. Beyond competition for nutrients, a possible explanation for the production of such alkaloid may be related to the ergoline biosynthesis on *M. phaseolina* induced by *Rhizobium* sp. (Siridev and Mallaiha, 2007; Gerhards et al., 2014). As far as we know, this is the first report that shows ergoline biosynthesis in *M phaseolina*. It was also observed the presence of hexahydro-pyrrolo [1,2-a] pyrazine-1,4-dione in all treatments inoculated with rhizobia. This same compound was also obtained in the ethyl acetate extract from *Streptomyces* sp., showing an antifungal activity against *Pyricularia oryzae* (Awla et al., 2016). In addition, an antibacterial activity against *Escherichia coli*, *Pseudomonas aeroginosas* and *Enterococcus faecalis* was reported by Melo et al. (2014), which isolated such compound from the fungus *Mortierell alpina.*

In addition, Baskaran et al. (2015) attributed the inhibitory effect of eleven bacteria and six fungi to the compounds ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy e pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3, highlighting that ethyl acetate was the most suitable solvent for the extraction of antimicrobial agents. These compounds are similar to those obtained in this work. Further, compounds with antimicrobial activity such as ergotamine could be used in future agricultural applications (Melo et al., 2014; Awla et al., 2016).

The n-butanol extract showed that the main produced compound was 1-butanol (Table 2). Lim et al. (2017) attributed the antifungal action to volatile compounds, including 1-butanol, against different phytopathogenic fungi. Some studies show that alcohols such as 1-hexanol and allyl alcohol also have antifungal activity, which can be used to prevent diseases (Archibold et al., 1997; Huang et al., 1997). The other

compounds have no bacterial activity and can be related to residues of solvents or extraction residues (Secundo et al., 1992; Campos et al., 2017).

Production of metabolites is a potent and broad-spectrum trait for biocontrol (Mishra and Arora, 2018). However, the mechanisms involved in inhibition of fungal development are still largely unknown, and the biocontrol of charcoal rot by rhizobia has been scarcely reported (Schmidt et al., 2019). Recently, Sabaté et al. (2017) evaluated the *in vitro* inhibition of *M. phaseolina* and verified that the phytopathogen is susceptible to lipopeptides action, such as surfactins, iturins, and fengycins. Volpiano et al. (2018) reported the ability to biocontrol *Sclerotium rolfsii* by *Rhizobium* sp. (SEMIA 460). These authors reported that such strain inhibited 45% of mycelial growth through production of volatile compounds. Thus, more research is needed in this area to identify more effective disease-suppressive strains of rhizobia (Das et al., 2017).

The use of beneficial microorganisms is considered one of the most promising methods for safe crop management practices (Ongena and Jacques, 2008). In this way, in order to reduce the use of chemical products, alternative methods such as biological control are being suggested for the control of charcoal root rot caused by *M. phaseolina*. Biocontrol of phytopathogens through inoculation of beneficial organisms is an effective method to treat plant diseases. The direct advantage would be a reduction in the use of pesticides and limiting root-attacking diseases, plus protection of transplants in the field by virtue of its ability to colonize roots.

Development of *Rhizobium* inoculants with dual attributes of nitrogen fixation and antagonism against phytopathogens can contribute to increased plant growth and productivity (Das et al., 2017). Thus, the data obtained in this work support that the studied bacterial isolates possess the potential of controlling the development of *M. phaseolina* MP53.

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**Table 1.** Metabolites produced by rhizobia isolates and *M. phaseolina* MP53 in the ethyl acetate extract.

| Compound | Area % | | | |
|---|---|---|---|---|
| | **Control** | **R26** | **L5** | **VC28** |
| 2,2-dimethoxybutane | 28.8 | 27.5 | 2.1 | 3.1 |
| octadecyl ester | - | - | 11.9 | 11.4 |
| tetradecyl ester | - | - | 3.1 | 2.3 |
| pentadecyl ester | - | - | 3.1 | 2.3 |
| 9,10-dihydro-12'-hydroxy-ergotoman-3',6',18-trione | - | 28.4 | 42.1 | 44.2 |
| hexahydro-pyrrolo [1,2-a] pyrazine-1,4-dione | 20.1 | 14.1 | 16.1 | 14.3 |
| undecyl ester | - | 14.0 | 14.4 | 13.6 |
| heptadecyl ester | 20.1 | 10.7 | 2.3 | 3.0 |

**Table 2.** Metabolites produced by rhizobia isolates and *M. phaseolina* MP53 in the butanol extract.

| Compound | Area % | | |
|---|---|---|---|
| | **R26** | **L5** | **VC28** |
| 3-pentanol | - | 2.6 | - |
| 1,3-dioxolane-4-methanol, 2-ethyl | - | 0.3 | - |
| 1-butanol | 1.9 | 60.5 | 69.6 |
| hydrazinecarbothioamide | 1.7 | 1.2 | 0.7 |
| carbonic acid, dipropyl ester | - | - | 20.3 |

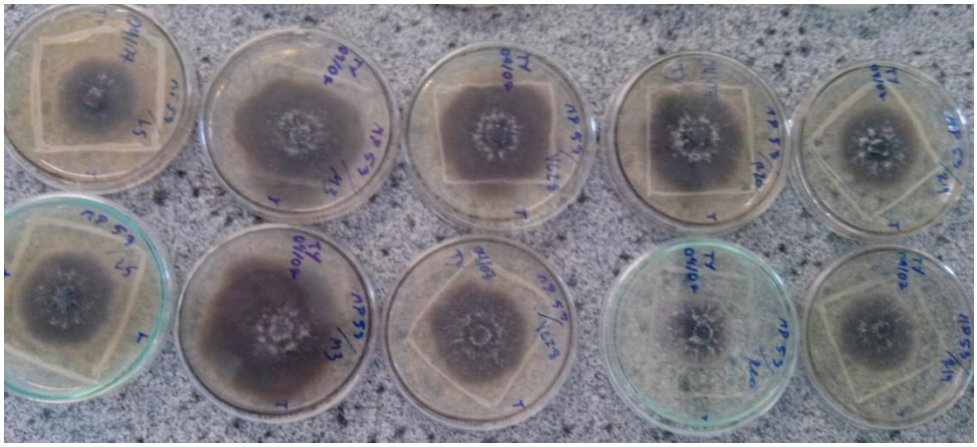

**Figure 1**. Preliminary screening method used for selection of rhizobia able to inhibit the development of *M. phaseolina* MP53.

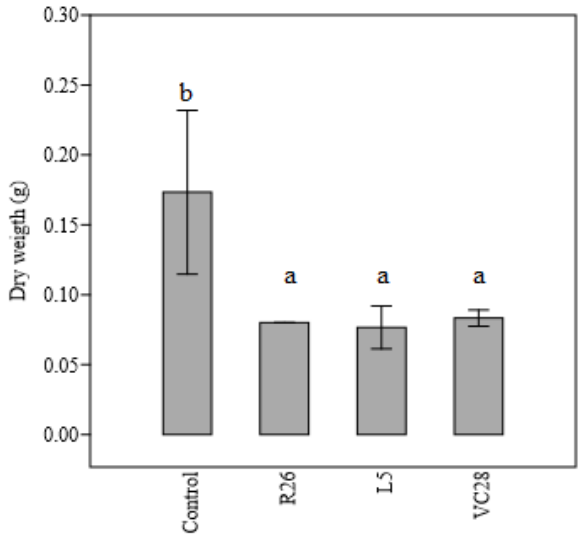


**Figure 2.** Dry weight of fungal mycelia with no addition of rhizobia (Control), and inoculated with R26, L5 and VC28 rhizobia isolates. Means with different letters are statistically different ($p < 0.05$).

# 4 DISCUSSÃO GERAL

A Fixação Biológica de Nitrogênio (FBN) em leguminosas, através da inoculação de microrganismos do grupo de rizóbios, é um processo bastante elucidado, capaz de reduzir os custos relativos à utilização de N nessas culturas, inclusive para a cultura do feijão-comum. Entretanto, além de possuir um período curto de desenvolvimento, o feijão-comum é considerado uma planta promíscua, capaz de estabelecer relações simbióticas com diversas espécies de rizóbios nativos que podem realizar a FBN de maneira pouco eficiente. Assim, a avaliação a campo de rizóbios pré-selecionados quanto à sua eficiência simbiótica precisa ser realizada, pois demonstra o potencial de utilização desses microrganismos na agricultura moderna.

Nesse sentido, os resultados apresentados no presente trabalho mostram que as estirpes SEMIA 4107 (=M3) e SEMIA 4108 (=VC28) podem ser usadas alternativamente à estirpe de referência SEMIA 4088 (*R. tropici*), bem como substituir a prática de adubação nitrogenada do feijão-comum, obtendo uma produtividade semelhante. Sob o ponto de vista econômico, e considerando que o nitrogênio é o elemento que mais onera a produção do feijão, por ser o insumo utilizado em maior quantidade e o fator limitante para o crescimento da planta, o uso de inoculantes com alta eficiência na simbiose e na FBN é recomendado (PELEGRIN et al., 2009).

Dessa forma, conclui-se que estudos objetivando a seleção de novas linhagens de rizóbios nativos, bem como sua aplicação no cultivo do feijoeiro, através de testes a campo, são essenciais para a indicação e/ou recomendação de bactérias que possuam alta eficiência na FBN para essa cultura. Também, na busca por processos que vão ao encontro da sustentabilidade agrícola, a utilização de inoculantes agrícolas com esses rizóbios é uma ferramenta biotecnológica com potencial para reduzir o uso de adubos nitrogenados, evitando diversos impactos ambientais associados a essa prática.

Referente ao biocontrole, o uso de diferentes espécies de rizóbios podem reduzir, ou até mesmo evitar a manifestação de doenças no feijão-comum associadas aos fitopatógenos *Rhizoctonia*, *Fusarium* e *Macrophomina*, principais causadores da podridão radicular nessa leguminosa. Entretanto, os mecanismos de inibição desses fitopatógenos por *Rhizobium* sp. ainda não estão bem elucidados, sendo a compreensão das interações entre o agente de biocontrole, o fitopatógeno e a planta, essencial para o sucesso dessa abordagem (WHIPPS, 2001).

Nesse sentido, a intensificação na busca por novas espécies de rizóbios ou sua obtenção diretamente a partir de isolados armazenados nos diferentes centros de cultura (VOLPIANO et al., 2018), bem como a avaliação do seu potencial com foco no biocontrole, pode viabilizar a utilização desses microrganismos objetivando a redução no uso de agroquímicos. Além disso, estudos quanto à produção de metabólitos secundários por rizóbios precisam ser explorados objetivando maior benefício para a agricultura.

O uso de rizóbios como agentes de biocontrole é uma alternativa promissora aos agroquímicos, pois podem contribuir para a redução da contaminação do solo e águas subterrâneas, e ainda incrementar a produção com a redução de perdas nas lavouras. Esta perspectiva vem sendo observada pelo mercado global de biopesticidas, cuja taxa anual de crescimento no uso de bactérias promotoras do crescimento de plantas (BPCP), desde 2013, é de 16% , demonstrando o potencial econômico dessa prática (SINGH et al., 2019).

O biocontrole da doença da podridão cinzenta do caule, causada pelo fitopatógeno *Macrophomina phaseolina*, através do uso de rizóbios no cultivo do feijão-comum, ainda é pouco relatada na literatura. Dessa forma, este trabalho traz informações quanto à inibição *in vitro* de *M. phaseolina* através de isolados de rizóbios obtidos a partir de plantas de feijão-comum, e considera a possibilidade de inibição desse fitopatógeno pela produção de determinados compostos orgânicos voláteis.

# 5 CONTINUIDADE DO TRABALHO / EXPERIMENTOS FUTUROS

- Avaliação a campo (conforme MAPA) para recomendação da SEMIA 4107;
- Identificação molecular da estirpe R26;
- Avaliação *in silico* de genes relacionados à produção de ergotamina em *Macrophomina phaseolina*;
- Avaliação da inibição de *M. phaseolina* pelas estirpes R26, L5 e VC28 em casa de vegetação.

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