Universidade Federal do Rio Grande do Sul

Rhizobium spp. para o controle biológico do fungo fitopatogênico *Sclerotium* (*Athelia*) *rolfsii* no feijoeiro (*Phaseolus vulgaris* L.)

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Porto Alegre

2017

Universidade Federal do Rio Grande do Sul

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Dissertação submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do grau de Mestre em Genética e Biologia Molecular.

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Porto Alegre, novembro de 2017

Agradecimentos

Este trabalho foi realizado com suporte físico e de recursos humanos da Secretaria da Agricultura, Pecuária e Irrigação do Estado do Rio Grande do Sul, contando também com o apoio intelectual e financeiro da Universidade Federal do Rio Grande do Sul. Sou grata a todos os pesquisadores, técnicos e demais funcionários que trabalham nessas instituições.

No âmbito da Secretaria, gostaria de agradecer especialmente ao Dr. Luciano Kayser Vargas pela excelente orientação conjunta com o Dr. Bruno Brito Lisboa. A formulação da pergunta cientifica que originou este trabalho foi elaborada por estes pesquisadores. Devido a eles, posso dizer, sem sombra de dúvida, que trabalhei com pessoas disponíveis, abertas a ideias e muito queridas. Por isso serei eternamente grata. Também tive auxílio de outros profissionais que não podem ser esquecidos pela ajuda e pela convivência. Obrigada aos pesquisadores Drª. Andreia Mara Rotta de Oliveira, Drª. Anelise Beneduzi da Silveira, Dr. Jackson Freitas Brilhante de São José, Dr. Samuel Mazzinghy Alvarenga e às técnicas Marilene Betencourt Silveira, Jamilla Sampaio e Letícia Schonhofen Longoni. Também gostaria de agradecer às estagiárias Debora Jaloto Avila, Carolina Castilho, Simone Lange e Patricia Martins.

No âmbito da Universidade Federal do Rio Grande do Sul, agradeço à Profª. Drª. Luciane Maria Pereira Passaglia por todo auxilio e disponibilidade. Sempre muito querida e atenciosa, assim como é a equipe de pesquisa da Profª. Passaglia, a quem gostaria de agradecer em especial os mais próximos, Drª. Evelise Bach, Drª. Gabriela de Carvalho Fernandes, Fernanda da Silva Moreira, Eliandro Espindula, Priscila Silveira dos Santos, Igor Daniel Ribeiro e Andress Pacheco Pontes. Obrigada também ao Dr. Fernando Hayashi Sant'Anna, que auxiliou muito na questão do *16S rRNA*.

Gostaria de agradecer a minha “primeira família” pelo incentivo e suporte e, em especial, a minha mãe Izolete Gazolla, irmãs Laura e Luana Gazolla e avós Olivia e Avelino Gazolla. Também sou grata a minha “segunda família”, aqui descrita em ordem alfabética e não de importância, visando, é claro, evitar futuras retaliações no Uno e Catan. Então, obrigada Augusto César Cardoso dos Santos, Raissa Pereira Caldeira, Renata de Barros Ruas e Yelena Perevalova. Amo ambas as famílias. Obrigada ao Gabriel Schuch por ajudar a contar sementes de feijão.

Agradeço ao PPGBM-UFRGS. Obrigada em especial ao Elmo Cardoso pelas dezenas de vezes que me auxiliou com burocracias, sendo sempre querido e atencioso. Agradeço também à Profª. Andreia Carina Turchetto Zolet por ter me orientado no estágio didático.

Obrigada ao CNPq pelo auxílio financeiro.

Abreviaturas

Anvisa	Agência Nacional de Vigilância Sanitária;
Conab	Companhia Nacional de Abastecimento;
Emater/RS	Empresa de Assistência Técnica e Extensão Rural do Estado do Rio Grande do Sul;
FAO	Organização das Nações Unidas para Alimentação e Agricultura (do inglês, *Food and Agriculture Organization of the United Nations*);
FBN	Fixação biológica de nitrogênio;
Fe	Átomo de ferro;
IAA	Ácido indol-3-acético (do inglês *indole-3-acetic acid*);
IFA	Associação Internacional de Fertilizantes (do inglês, *International Fertilizer Association*);
MAPA	Ministério da Agricultura, Pecuária e Abastecimento;
N_2	Nitrogênio diatômico;
NH_3	Amônia;
P	Átomo de fósforo;
PGPB	Bactérias promotoras do crescimento vegetal (do inglês, *plant growth-promoting bacteria*);
RELARE	Rede de Laboratórios para a Recomendação de Estirpes de *Rhizobium*.

Resumo

Rizóbios são bactérias fixadoras de nitrogênio utilizadas com sucesso como inoculante microbiano para diminuir a utilização de fertilizantes nitrogenados no cultivo do feijoeiro (*Phaseolus vulgaris* L.) e outras leguminosas. *Sclerotium rolfsii* (sin. *Athelia rolfsii*) é um fungo onipresente que causa perdas severas em culturas importantes, inclusive em espécies de *Phaseolus*. Assim, o objetivo deste estudo foi avaliar a coleção de *Rhizobium* SEMIA para identificar o primeiro agente rizobial para o biocontrole da doença promovida por *S. rolfsii* no feijoeiro. Duplas culturas foram primeiramente realizadas para identificar propriedades de biocontrole entre as estirpes. Entre as 151 estirpes SEMIA testadas, 33 (~22%) mostraram atividade antagonista, sendo 16 delas capazes de inibir ≥84% do crescimento micelial. As estirpes antagonistas produziram de 1,2 a 36,5 $\mu g\ mL^{-1}$ de ácido indol-acético (IAA), um fitohormônio mais conhecido por promover o crescimento de plantas do que por inibir diretamente patógenos. Contudo, obteve-se um r=0,447 (p=0,011) entre a produção de IAA das estirpes antagonistas e a capacidade de inibição do micélio. As estirpes SEMIA 436, 4077, 4088 e 460 foram produtoras de sideróforos, e a atividade antagonista de SEMIA 4088 pode ser, em parte, relacionada a isso. Além de compostos antimicrobianos difusíveis no meio de cultura, SEMIA 460 também inibiu 45% do crescimento micelial através da produção de compostos voláteis. A análise do *16S rRNA* possibilitou a identificação das estirpes SEMIA 456, 4026, 436, 439, 4032, 460, 4085, 4080, 4077 e 4088 como *Rhizobium* spp. Considerando o alto grau de conservação do *16S rRNA* dentro do gênero *Rhizobium*, as linhagens SEMIA 436 e 439 apresentaram similaridades menores que 98,65% com o banco de dados, possivelmente representando um novo táxon. Apesar de terem sido isoladas de nódulos de feijão, as estirpes SEMIA 436, 439, 456, 4026 e 4032 foram alocadas em um ramo filogenético com estirpes de *Rhizobium* tumorigênicas (agrobacteria). Finalmente, para testar a eficiência de biocontrole das estirpes antagonistas selecionadas, plantas de feijão foram individualmente inoculadas e cultivadas em vasos com solo infectado com *S. rolfsii*. Para os parâmetros i) porcentagem de doença e ii) massas secas da parte área os tratamentos com SEMIA 4032, 4077, 4088, 4080, 4085 ou 439 não apresentaram diferenças estatisticamente significativas quando comparadas com o controle (plantas de feijão cultivadas em solo não infectado), demonstrando a grande potencialidade destas estirpes no controle biológico de *S. rolfsii* mediante inoculação de sementes de feijão.

Abstract

Rhizobia are nitrogen-fixing bacteria successfully used as microbial inoculant attempting to diminish synthetic nitrogen fertilizers inputs on the common bean (*Phaseolus vulgaris* L.) and others legume crops. *Sclerotium rolfsii* (syn. *Athelia rolfsii*) is a ubiquitous fungus that causes several losses on important crops, including *Phaseolus* species. In this way, the aim of this study was to evaluate SEMIA *Rhizobium* Culture Collection to identify the first rhizobial biocontrol agent for the *S. rolfsii*-promoted disease on the common bean. Dual cultures were first performed to screening strains for biocontrol proprieties. Among of the 151 SEMIA strains, 33 (~22%) of them showed antagonistic activity on dual cultures, being 16 of them able to inhibit ≥84% of mycelial growth. Antagonistic strains produced 1.2 to 36.5 μg mL^{-1} of indole-acetic acid (IAA), a phytohormone best known to promote plant growth than to direct inhibit plant pathogens. However, a r=0.447 (p=0.011) was obtained between antagonistic strains IAA production and mycelium inhibition ability. Strains SEMIA 436, 4077, 4088 and 460 were siderophore producers, and SEMIA 4088 antagonistic activity can be related to this. Besides antimicrobial diffusible compounds, SEMIA 460 inhibited 45% of mycelial growth through volatiles compounds production. Analysis of *16S rRNA* identified strains SEMIA 456, 4026, 436, 439, 4032, 460, 4085, 4080, 4077 and 4088 as *Rhizobium* spp. Considering the high degree of *16S rRNA* conservation in *Rhizobium* genus. SEMIA 436 and 439 were found to represent new taxa for presenting gene similarities less than 98.65% with the database. Despite being isolated from nodules, SEMIA 436, 439, 456, 4026 and 4032 were placed in a phylogenetic branch with tumorigenic *Rhizobium* (agrobacteria). Finally, to evaluate biocontrol efficiency of the selected antagonists strains, common bean plants were individually inoculated and grown in pots with *S. rolfsii* infected soil. For the parameters i) disease percentage and ii) shoot dry masses, treatments with SEMIA 4032, 4077, 4088, 4080, 4085 and 439 were not found with statistically significant differences from the control (plants grown on uninfected soil), demonstrating the great potentiality of these strains for biological control of *S. rolfsii* through inoculation of common bean seeds.

Sumário

Introdução

A cultura do feijão no Brasil e o uso de fertilizantes nitrogenados

O feijão (*Phaseolus vulgaris* L.) é a mais importante leguminosa produzida mundialmente para uso direto na alimentação humana. De acordo com os últimos dados da Organização das Nações Unidas para Alimentação e Agricultura (FAO), em 2014, o Brasil figurou como maior produtor de feijões secos (*Phaseolus* spp.), seguido pela China, Índia, México, Myanmar e Estados Unidos. Segundo estimativas da Companhia Nacional de Abastecimento (Conab), a produção brasileira relativa à safra total de feijão em 2017/2018 deve flutuar entre 3.304 e 3.345 mil toneladas. A Região Sul do país deve ser responsável pela maior produção, com pouco mais de 950 mil toneladas colhidas. Entretanto, a distribuição desta produção não deve ser homogênea, sendo o Paraná responsável por cerca de 75% da safra.

Com exceção da Argentina, no Brasil e outros países latino-americanos grande parte do cultivo de feijão acontece em pequenas propriedades, em solo com alta declividade, quase sem aporte tecnológico. Essas características de cultivo levam a baixa produtividade em consequência de perdas causadas por fatores climáticos e doenças (Broughton *et al.* 2003). Apesar disto, existe uma tendência à modernização dos sistemas de produção agrícola. A exemplo, aproximadamente 10 mil hectares de feijão já estão sendo cultivados sob modelo de irrigação no Rio Grande do Sul (Bevilaqua *et al.* 2013). Essa é uma quantidade expressiva, uma vez que a área plantada nesse estado em 2017, por exemplo, foi aproximadamente 42 mil hectares, segundo a Empresa de Assistência Técnica e Extensão Rural do Estado do Rio Grande do Sul (Emater/RS).

Conforme ocorre a inserção de tecnologias no âmbito agrícola, espera-se que cresçam os números relativos à produção, concomitantemente com aqueles que revelam o aprisionamento da economia brasileira à importação de insumos. Segundo dados da *International Fertilizer Association* (IFA, em português, Associação Internacional de Fertilizantes), em 2012, aproximadamente 84% dos fertilizantes nitrogenados consumidos no Brasil foram importados. Isso certamente causa um impacto econômico contraproducente. Adicionalmente, também existem implicações ambientais no uso de fertilizantes nitrogenados. Aproximadamente 70% do fertilizante aplicado nos solos se perde através de emissões gasosas, escoamento, erosão e lixiviação, causando

eutrofização de ambientes aquáticos e perda de qualidade do solo (Bhattacharjee *et al.* 2008).

Rizóbios para a promoção de crescimento no feijoeiro

Diante do cenário até aqui descrito, é sensato considerar que a modernização de culturas alimentares básicas como o feijão, de forma sustentável e independente do mercado importador de insumos, é questão estratégica para a economia brasileira. Para atender essa demanda é possível explorar tecnologias de inoculação em plantas com micro-organismos benéficos.

O feijoeiro é capaz de estabelecer associação com bactérias conhecidas como rizóbios (Djordjevic *et al.* 1987). Esta associação muitas vezes resulta no fornecimento de nitrogênio para a planta, que se dá através do processo de fixação biológica de nitrogênio (FBN) que ocorre em nódulos na raiz. A FBN ocorre quando moléculas de nitrogênio presentes na atmosfera (N_2) sofrem a quebra da ligação tripla diatômica ($N{\equiv}N$) e são convertidas em amônia (NH_3), sendo estas moléculas, então, capazes de serem bioassimiladas em aminoácidos ou outros compostos nitrogenados. A disponibilidade de nitrogênio é considerada o fator mais limitante para o desenvolvimento das plantas. No Brasil, a dose média de nitrogênio aplicado é insuficiente de forma crítica para o feijão e outras culturas alimentares básicas, fazendo então com que estas não atinjam seu potencial de produtividade (Lopes *et al.* 2007). Todavia, a simbiose, que resulta no processo de FBN, é capaz de suprir se não toda, parte da necessidade da aplicação destes fertilizantes.

Os rizóbios estão dentro do grupo de bactérias conhecidas como rizobactérias promotoras do crescimento vegetal (PGPB, do inglês, *plant growth-promoting bacteria*). Além do fornecimento de nitrogênio, as PGPB podem apresentar uma série de outros mecanismos que promovem o crescimento de plantas (Ahemad e Kibret 2014). É comum a divisão destes mecanismos em diretos e indiretos. A ação direta se daria pelo fornecimento de nutrientes, como o nitrogênio, ferro, fósforo e da modulação dos níveis de hormônios vegetais. A ação indireta estaria relacionada com a habilidade de controle de patógenos de plantas. Ambos tipos de ação serão brevemente descritos abaixo.

O ferro (Fe) é um nutriente essencial que se encontra no solo de forma relativamente insolúvel. Sideróforos são moléculas orgânicas de baixo peso molecular (400 a 1000 Da) capazes de quelar e transportar átomos de Fe para dentro das células

bacterianas (Neilands 1995). A FBN acontece mediada por um complexo enzimático dependentes de Fe, a nitrogenase. Devido a isso, a produção de sideróforos pode ser uma estratégia para obtenção de ferro importante para as PGPB capazes de realizar FBN (Wichard *et al.* 2009). Além disso, outro possível efeito positivo dos sideróforos é a complexação de alumínio, diminuindo assim a toxicidade deste para as plantas (Roy e Chakrabartty 2000). A produção de sideróforos também pode estar relacionada à ação indireta, através da indisponibilização de Fe para o crescimento de fitopatógenos (Solano *et al.* 2008).

O fósforo (P) é outro nutriente que constitui um importante fator limitante do crescimento de plantas. Sendo o P altamente reativo com elementos como alumínio, Fe e cálcio, menos que 5% do total deste contido no solo está disponível para assimilação pelos vegetais (Dobbelaere *et al.* 2003). Algumas PGPB promovem um melhor aproveitamento do P existente no solo por ter habilidade de externar ácidos orgânicos, fosfatases e fitases que facilitam processos de mineralização e conversão das formas insolúveis em solúveis (Chen *et al.* 2006; López-López *et al.* 2010).

A produção de auxinas, uma classe de hormônios estimuladores de crescimento em plantas, é outro mecanismo que parece ser consideravelmente comum em PGPB, principalmente rizóbios (Boiero *et al.* 2007; Vargas *et al.* 2009). A principal auxina encontrada nas plantas é o ácido indol-3-acético (IAA, do inglês *indole-3-acetic acid*). A exposição ao IAA produzido por rizóbios pode afetar a planta de diversas maneiras (Spaepen *et al.* 2007). Um cenário positivo é a promoção de mudanças morfológicas no sistema radicular, como o aumento da densidade e comprimento dos pêlos radiculares, gerando maior captação de nutrientes do solo.

Rizóbios para o biocontrole *Sclerotium rolfsii* no feijoeiro

Os rizóbios, além da ação direta na promoção de crescimento vegetal, também apresentam evidências de exercerem controle de efeitos inibitórios de patógenos de plantas. A utilização de organismos vivos para a supressão de patógenos é conhecida como “controle biológico” ou “biocontrole”, exercida então por “agentes de controle biológico” ou “biocontroladores”. Para os rizóbios, já foi reportado o controle de podridões radiculares causadas por fungos, como *Fusarium solani* (Dar *et al.* 1997), *F. oxysporum*, *Rhizoctonia solani* (de Jensen *et al.* 2002), *Phytium* sp. (Huang e Erickson 2007) e *Verticillium* sp. (Vargas *et al.* 2009). Este processo de supressão é muitas vezes

resultado da expressão de diferentes mecanismos de ação, como a síntese de compostos antimicrobianos, como antibióticos (Robleto *et al.* 1998) e ácido cianídrico (Chandra *et al.* 2007); a competição por nutrientes, mediada pela produção de sideróforos; e a produção de enzimas extracelulares, como β-1,3-glucanases, proteases (Compant *et al.* 2005) e quitinases (Kacem *et al.* 2009) atuantes sobre a parede celular do patógeno. Também podem atuar na detoxificação de moléculas responsáveis pela virulência do patógeno, como o ácido oxálico (Nagarajkumar *et al.*, 2005) e enzimas que provocam a degradação de tecidos.

Evidentemente, o biocontrole é relevante para diminuição do uso de agrotóxicos. Isso tem importância no panorama brasileiro, uma vez que, desde 2008, o país ocupa o lugar de maior consumidor de agrotóxicos do mundo. Enquanto nos últimos dez anos, o mercado mundial de agrotóxicos cresceu 93%, no Brasil esse crescimento foi de 190%, sendo os fungicidas correspondentes a 14%, segundo dados da Anvisa (Agência Nacional de Vigilância Sanitária) e Observatório da Indústria dos Agrotóxicos da Universidade Federal do Paraná disponibilizados no dossiê da Associação Brasileira de Saúde Coletiva (Carneiro *et al.* 2015).

O biocontrole de fungos se torna muito interessante para a cultura do feijão, uma vez que o feijoeiro é suscetível a várias moléstias causadas por estes. Tratando-se de doenças causadas por fungos, no feijão, podem ocorrer perdas de até 50% de produção na lavoura, sendo a podridão-do-colo uma das principais doenças que ocorrem na Região Sul do Brasil, segundo informações da Comissão Técnica Sul-Brasileira de Feijão (2012). Seu agente causal é o fungo necrotrófico *Sclerotium rolfsii* (sinônimo de *Athelia rolfsii*). Este fungo ocorre principalmente em regiões tropicais e subtropicais, principalmente em solos arenosos e pouco ácidos. O fungo fixa-se à superfície da planta em contato com o solo, e produz ácido oxálico fitotóxico e enzimas pectolíticas que colaboram para matar células (Punja *et al.* 1985). *S. rolfsii* penetra na epiderme das raízes, invadindo inter e intracelularmente o parênquima radicular causando extensiva degradação dos tecidos rapidamente produzindo uma zona apodrecida que geralmente circunda a haste ou raíz. Se a parte mais baixa da haste ou superior da raíz principal é circundada, o hospedeiro murcha repentinamente e morre (Bianchini e Maringoni 1997).

Este fungo é de difícil controle, entre outras razões, devido à sua disseminação por insumos e equipamentos agrícolas; a alta habilidade de sobrevivência no solo e o fato de infectar mais de 500 espécies de plantas (Iquebal *et al.* 2017; Punja e Grogan 1981).

Existem poucos relatos na literatura a respeito do emprego de biocontrole para a podridão-do-colo do feijoeiro. Os relatos encontrados até a presente data estão dispostos na Tabela 1. Não foram encontrados relatos sobre o uso eficiente de rizóbios para o tratamento desta doença no feijoeiro até o momento. Entretanto, existem publicações sobre o emprego de tratamento com rizóbios em outras culturas, conforme listado na Tabela 2.

Tabela 1. Agentes microbianos para o biocontrole da podridão-do-colo do feijoeiro causada por *S. rolfsii*.

Biocontrolador(es) testado(s)	**Metodologia(s)**	**Maior(es) eficiência(s) obtida(s)**	**Referência**
Bradyrhizobium japonicum	Teste em casa de vegetação utilizando solo artificialmente contaminado	Não houve redução na severidade da doença mediante a inoculação.	Pereira Neto e Bassay Blum (2010)
Diferentes espécies dentro do gênero *Trichoderma*	Teste em câmara de crescimento com solo artificialmente contaminado	Inoculações com *T. harzianum, T. lacteal* e *T. pseudokoningii* reduziram o índice da doença em 66,8%, 65,8% e 63%, respectivamente	Barakat *et al.* (2006)
Trichoderma harzianum	Teste em casa de vegetação utilizando solo artificialmente contaminado	A inoculação de *T. harzianum* proporcionou 8% de plantas atacadas. Nas testemunhas apenas com o patógeno esse número foi de 47%	Elad *et al.* (1980)
	Teste a campo em solo naturalmente contaminado	A inoculação de *T. harzianum* proporcionou cerca de 10% de plantas atacadas. Nas testemunhas apenas com o patógeno esse número foi cerca de 30%	
Talaromyces flavus	Teste em casa de vegetação com solo artificialmente contaminado com escleródios tratados com *T. flavus*	A doença foi reduzida em 64% mediante infecção com escleródios tratados.	Madi *et al.* (1997)
Diferentes espécies dentro do gênero *Bacillus e Streptomyces*	Teste em casa de vegetação utilizando solo artificialmente contaminado	Inoculações com *B. subtilis* subsp. *spizizenii*, *Bacillus subtilis* subsp. *subtilis*, *B. atrophaeus, B. tequilensis* e *S. cyaneofuscatus* reduziram de 50% até 58,5% a severidade da doença	Gholami *et al.* (2014)
B. subtilis, Pseudomonas fluorescens, Saccharomyces cerevisiae e *Trichoderma viride*	Teste em casa de vegetação com solo artificialmente contaminado	Inoculações com *B. subtilis, P. fluorescens* e *T. viride* e reduziram a doença em 82%, 74% e 75%, respectivamente	Eid (2014)
	Teste à campo em solo naturalmente contaminado	Inoculações com *T. viride, S. cerevisiae* e *B. subtilis* reduziram a doença em 63%, 50% e 47%, respectivamente	

Tabela 2. Emprego de biocontrole utilizando rizóbios para o tratamento de doenças causadas por *S. rolfsii* em diversas culturas.

Planta(s) testada(s)	**Rizóbio(s) testado(s)**	**Metodologia(s)**	**Maior(es) eficiência(s) obtida(s)**	**Referência**
Grão-de-bico *Cicer arietinum*	*Rhizobium* spp. cepas BARI Rca 201, BARI Rca 220, BARI Rca 202, BARI *chickpea* Rajshahi, BARI Rca IC 59 e BARI *chickpea pulse* 1.	Teste a campo em solo naturalmente contaminado	Tratamentos com BARI chickpea Rajshahi reduziram a incidência somada das doenças causadas por *S. rolfsii* e *Fusarium oxysporum* em até 67,5%.	Khalequzzaman (2015)
	Rhizobium spp. cepa RL091	Teste em casa de vegetação utilizando solo artificialmente contaminado	O rizóbio proporcionou mortalidade de cerca de 60% das plantas, enquanto os controles com apenas *S. rolfsii* apresentaram cerca de 80%.	Singh *et al.* (2013)
Feijão-da-China *Vigna mungo*	Biofertilizantes compostos de *Rhizobium* spp. (cepa/espécie não descrita)	Teste a campo em solo naturalmente contaminado	Tratamentos com os fertilizantes reduziram a incidência somada das doenças causadas por *S. rolfsii* e *Fusarium oxysporum* em até 73%.	Mohammad e Hossain (2003)
Chícharo *Lathyrus sativus*	*Rhizobium* spp. (cepa/espécie não descrita)	Teste em casa de vegetação utilizando solo artificialmente contaminado	Os testes foram realizados duas vezes. Os tratamentos com o rizóbio não tiveram efeitos em um teste. No segundo teste, pioraram a porcentagem de infecção com *S. rolfsii*.	Rahman *et al.* (2017)
Amendoim *Arachis hypogaea*	*Bradyrhizobium* spp. isolados de nódulos de amendoim	Teste em casa de vegetação utilizando solo artificialmente contaminado	Um índice indicador da doença foi reduzido de aproximadamente 3 para cerca de 0.6 até 1.6 mediante tratamentos com os rizóbios.	Ghasemi *et al.* (2017)

Reexplorando a coleção SEMIA de isolados do feijoeiro

Atualmente não existe legislação definindo as características de inoculantes microbianos nos Estados Unidos ou nos países da União Europeia (Malusá and Vassilev 2014). No Brasil, entretanto, desde 1975 existem regulamentações para o uso de inoculantes compostos exclusivamente de estirpes de bactérias que são recomendadas por instituições de pesquisa pública brasileiras. Para reforçar as recomendações, em 1985, foi criada a RELARE (Rede de Laboratórios para a Recomendação de Estirpes de *Rhizobium*). Essa rede tinha o objetivo de identificar as estirpes de rizóbios mais efetivas para a fixação de nitrogênio para cada espécie de legume. Após, estabeleceu-se a coleção SEMIA ("*Rhizobium Culture Collection*" da Seção de Microbiologia Agrícola; nº. 443 na base de dados *World Data Center on Microorganisms*). Essa coleção visa a manutenção e distribuição para a indústria de inoculantes das estirpes reconhecidas como mais eficientes, mas também contém centenas de outras estirpes que não obtiveram *status* de recomendação por falta de eficiência na fixação de nitrogênio. A Coleção SEMIA ficou a cargo do Laboratório de Microbiologia Agrícola pertencente à extinta Fundação Estadual de Pesquisa Agropecuária do Rio Grande do Sul. Recentemente a coleção foi designada para ser mantida pela a Secretaria da Agricultura, Pecuária e Irrigação do Estado do Rio Grande do Sul.

Atualmente, as estirpes de *Rhizobium* spp. SEMIA 4077, SEMIA 4080 e SEMIA 4088 são as únicas aprovadas (Ministério da Agricultura, Pecuária e Abastecimento, instrução normativa n. 13 de 24/03/2011) para a inoculação do feijoeiro, considerando exclusivamente a característica de disponibilização de nitrogênio. A respeito da eficácia destas bactérias, Pelegrin *et al.* (2009) e Soares *et al.* (2006) avaliaram a resposta da inoculação da estirpe SEMIA 4077 e obtiveram rendimentos equivalentes à aplicação de cerca de 80 kg ha^{-1} e 70 kg ha^{-1} de nitrogênio, respectivamente, o que propiciaria uma redução em cerca da metade da necessidade de utilização de fertilizantes nitrogenados.

Evidentemente, as estirpes SEMIA 4077, SEMIA 4080 e SEMIA 4088 estão armazenadas na coleção SEMIA. Todavia, a coleção também possui outras mais de 150 estirpes que foram previamente isoladas de nódulos do feijoeiro. Este trabalho se propõe re-explorar essas bactérias, desta vez visando a formulação de inoculantes capazes de exercer o biocontrole eficiente de *S. rolfsii* no feijoeiro.

Objetivo geral

Avaliar estirpes de bactérias isoladas de nódulos de feijoeiro pertencentes à coleção SEMIA quanto à sua capacidade de inibir o desenvolvimento de *Sclerotium rolfsii in vitro.* Além disso, determinar possíveis mecanismos de ação envolvidos no processo de antagonismo. Por fim, testar a inoculação de estirpes selecionadas visando o biocontrole da murcha-do-colo do feijoeiro.

Objetivos específicos

- Dentre 151 estirpes que compõe a coleção SEMIA de isolados do feijoeiro, avaliar a presença de característica de inibição do crescimento micelial de *S. rolfsii in vitro* empregando duplas-culturas;

- Dentre as estirpes antagonistas detectadas, avaliar a produção de compostos indólicos (IAA), produção de sideróforos e enzimas extracelulares (proteases e celulases);

- Selecionar estirpes conforme os dados obtidos nos itens anteriores para i) determinar a taxonomia, através do sequenciamento do *16S rRNA*, ii) avaliar a produção de compostos voláteis com capacidade de inibição do micélio de *S. rolfsii*, e iii) inocular sementes de feijão visando aferir a capacidade de proteção da doença causada por *S. rolfsii* em condições de vaso.

Capítulo I

***Rhizobium* strains as plant growth-promoting rhizobacteria and biological control agents against *Sclerotium* (*Athelia*) *rolfsii* on the common bean.**

Artigo a ser enviado para submissão na revista *Applied Microbiology and Biotechnology*.

Rhizobium strains as plant growth-promoting rhizobacteria and biological control agents against _Sclerotium_ (_Athelia_) _rolfsii_ on the common bean.

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Abstract

Rhizobium strains often establish nitrogen-fixing root-nodules stimulating leguminous plant growth, while *Sclerotium rolfsii* is a fungus recognized to serious damage crops. To describe the first *Rhizobium* for biocontrolling *S. rolfsii*-promoted disease on the common bean, *SEMIA* culture collection was screened. Among 151 strains, 33 (~22%) showed antagonistic activity on dual cultures, being 16 of them able to inhibit ≥84% of mycelial growth. Antagonistic strains produced 1.2 to 36.5 μg mL^{-1} of indole-acetic acid (IAA), a phytohormone best known to promote plant growth than to direct inhibit plant pathogens. However, an r= 0.447 (p=0.011) was obtained between antagonistic strains IAA production and mycelium inhibition ability. Strains SEMIA 436, 4077, 4088 and 460 were siderophore producers, and SEMIA 4088 antagonistic activity can be related to this. Besides diffusible compounds, SEMIA 460 inhibited 45% of mycelial growth through volatiles. Analysis of *16S rRNA* classified strains SEMIA 456, 4026, 436, 439, 4032, 460, 4085, 4080, 4077 and 4088 as *Rhizobium* spp. SEMIA 436 and 439 were found to represent new taxa for presenting gene similarities less than 98.65% with the database. SEMIA 436, 439, 456, 4026 and 4032

were placed in a phylogenetic branch with tumorigenic *Rhizobium* despite being isolated from nodules. To evaluate biocontrol efficiency, common bean seeds were inoculated and grown in infected soil, where SEMIA 4032, 4077, 4088, 4080, 4085 and 439 treatments presented disease percentage and shoot dry masses statistically the same of the uninfected control, demonstrating the potentiality of these strains for *S. rolfsii* biological control on common bean.
Keywords: common bean; biocontrol; rhizobium; pathogenic fungi; agrobacteria.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is considered one of most important grain legume produced worldwide. Brazil, China, India, Mexico, Myanmar and the USA are the most important *Phaseolus* spp. producing countries (FAO, 2014). As others crops, common bean cultivated genotypes demand high nutritional requirements and proper phytosanitary conditions for expressing their genetic potential. In this way, microbial inoculation technologies have been attempting to diminish production and environmental costs due to agrochemicals inputs on crops.

Rhizobia are collectively known as bacteria able to induce root nodules on leguminous plants and convert atmospheric nitrogen into available ammonia (Lindström and Martinez-Romero 2005), thus being a widely used microbial inoculant. Pelegrin *et al.* (2009) and Soares *et al.* (2006) evaluated SEMIA 4077 and obtained common bean grain yields equivalent to the application of 70-80 kg ha^{-1} of nitrogen. Rhizobia can also stimulate both leguminous and non-leguminous growth through the secretion of molecules analogous to plant hormones such as auxins (Ghosh *et al.* 2015); solubilization of inorganic insoluble phosphates (Kumar and Ram 2014); mineralization of organic phosphates (López-López *et al.* 2010); improving iron acquisition and/or alleviating aluminum toxicity mediated by chelating molecules knows as siderophores (Datta and Chakrabartty 2014; Roy and Chakrabartty 2000); ACC deaminase activity decreasing ethylene inhibitory action (Glick 2005); leading to abiotic stress tolerance by secreting exopolysaccharides and formatting biofilm structures (Qurashi and Sabri 2012).

There are currently no specific legal provisions defining microbial inoculants characteristics in the European Union and in the USA (Malusá and Vassilev 2014). Since

1975 in Brazil, Ministry of Agriculture, Livestock, and Supply (*Ministério da Agricultura, Pecuária e Abastecimento*, abbreviated as MAPA) regulated the commercialization of inoculants. Moreover, SEMIA, an official *Rhizobium* Culture Collection was created to maintain recommended strains and provide their distribution to the inoculant industry. In example, SEMIA 4077, SEMIA 4080 and SEMIA 4088 (also known as CIAT 899, PRF 81 and H 12) are the recommended strains for common bean inoculation. SEMIA also contains hundreds of other strains, which did not obtain recommendation status due to lack of efficiency in nitrogen fixation. Presently, SEMIA is maintained by our laboratory located at Secretaria da Agricultura, Pecuária e Irrigação of the Brazilian State of Rio Grande do Sul.

Besides nitrogen-fixing characteristics rhizobia could also perform as biological control agents, especially of phytopathogenic fungi such as *Fusarium solani* (Dar *et al.* 1997), *F. oxysporum*, *Rhizoctonia solani* (de Jensen *et al.* 2002), *Phytium* sp. (Huang and Erickson 2007) and *Verticillium* sp. (Vargas *et al.* 2009). Biocontrol is often a result of different mechanisms such as antibiotics (Robleto *et al.*, 1998) and HCN (Chandra *et al.*, 2007) action; nutrients competition (siderophores could be often involved); action of extracellular cell wall-degrading enzymes, i.e. β-1,3-glucanases, proteases (Compant *et al.*, 2005) and chitinases (Kacem *et al.*, 2009); parasitization of hyphal tips and inhibition of reproductive structures, like sclerotia (a compact mass of hardened fungal mycelium) or zoospores; detoxificating pathogen's virulence molecules as oxalic acid (Nagarajkumar *et al.*, 2005) and hydrolytic enzymes. In addition, rhizobia could exert "indirect antagonism" by eliciting reactions of plant defense (Elbadry *et al.* 2006).

Sclerotium rolfsii (also known as *Athelia rolfsii*) is a necrotrophic ubiquitous soil-borne phytopathogenic fungus responsible for severe losses particularly on crops from tropical and subtropical regions. On the common bean, this fungus is known to promote collar rot disease leading to complete wilt of plants (Bianchini and Maringoni 1997). *S. rolfsii* chemical controlling is difficult for many reasons, such as the dissemination by contaminated equipment and machinery, the fungal ability to form sclerotia (Punja and Grogan 1981), and the extensive fungal host range of at least 500 plant species (Iquebal *et al.* 2017). Besides, fungicides use lead to the killing of non-target beneficial microorganisms and contribute to water and soil pollution.

The pronounced information available on rhizobial application and the possibility to screening biocontrol traits among the great number of strains/isolates stored in different

culture collection centers could promptly reduce agricultural pesticides use. Therefore, the aim of this study was revaluate *SEMIA* Culture Collection focusing on identify the first effective *Rhizobium* biocontrol agent for the collar rot of the common bean.

2. Materials and methods

2.1 Organisms, culturing media, and conditions

Bacteria previously isolated from common bean nodules stored at *SEMIA Rhizobium Culture Collection* (World Data Center on Microorganisms no. 443) were used in this study. Lyophilized bacteria were rehydrated and grown on YM medium plates (Somasegaran and Hoben 2012) at 28ºC. Whenever a log-phase liquid culture was required, bacteria were multiplied in 15 mL tubes containing 5 mL of YM broth, in a rotary shaker (120 rpm) for 72h.

The fungal pathogen was previously isolated from common bean plants and identified as *S. rolfsii* based on etiology and morphological characteristics (Mordue and Holliday 1974). Cultures of *S. rolfsii* were obtained by cultivation on PDA medium (Beever and Bollard 1970) for five days at 23°C and 12 hours of photoperiod. Fungal identity was further confirmed by PCR amplification of internally transcribed spacer region using ITS1/ITS4 universal primer (Kawasaki *et al.* 1990). Amplified PCR product was sequenced and BLASTN 2.7.0+ (Altschul *et al.* 1997) search a query length of 597 revealing 99% homology to *Sclerotium* (*Athelia*) *rolfsii* (GenBank MF425542.1). The sequence of the isolate used in this work was deposited at GenBank under accession number MF817710.1.

2.2 Dual culture assay

High-throughput screening of 151 SEMIA strains for *S. rolfsii in vitro* antagonism was performed based on a dual culture method on TY medium plates (Somasegaran and Hoben 2012). A 0.7 cm *S. rolfsii* agar plug was disposed at the center of 9 cm plates and the bacterium was streaked in a square form. Antagonistic activity of bacterium plates was measured through comparison to solely cultivated fungal plates after incubation of both for five days at 23°C and 12 hours of photoperiod. Inhibition is described in percentages according to the equation inhibition (%) = [(C – 0.7) – (T – 0.7) / (C – 0.7)] × 100, where C

is fungal colony diameter (cm) of the control, and T is fungal colony diameter (cm) in the dual culture. Each SEMIA strain was tested on three different plates.

2.3 Indole acetic acid, siderophore, proteases and cellulases detection

Antagonistic SEMIA strains detected in dual cultures were tested in triplicates for indole acetic acid (IAA), siderophore, proteases and cellulases production. IAA was evaluated based on Asghar *et al.* (2002). Bacterial log-phase liquid culture in broth supplemented with 0.5 g L^{-1} tryptophan was centrifuged for 5 min at 10.000 rpm and 500 µL of the supernatants generated were placed in microtubes to react with 500 µL of Salkowski reagent (2 mL 0.5 mol L^{-1} $FeCl_3$ + 98 mL 35 % $HClO_4$). The mixture was left in the dark for 15 min at room temperature, following spectrophotometer mensuration at 520 nm. IAA concentration was inferred from a standard curve. Proteolytic and cellulolytic activity of bacterial strains were inferred by detection of hydrolyzing zones on agar plates containing skim milk (Montanhini *et al.* 2013) or carboxymethyl cellulose (Kasana *et al.* 2008) after inoculation with 5 µL drops of bacterial log-phase liquid cultures.

For siderophores detection, the bacterium strain was multiplied in 100% and 1:2 water diluted iron-deficient liquid King's B medium according to conditions previously described (Schwyn and Neilands 1987). Subsequently, cultures were centrifuged for 5 min at 10.000 rpm and an aliquot of 50 µL was collected and pipetted into a microplate along with 50 µL of chrome azurol-S (CAS) reagent following incubation for 15 min. Bacterial strains that changed the color of the reaction mixture from blue to orange were considered positive for siderophore production. Antagonistic activity related to siderophores was determined through comparison of iron-deficient King's B with ones supplemented with 100 µM of $FeCl_3$ (Bevivino *et al.* 1998) on dual culture s assays as described before.

2.4 Volatiles detection through double plate assay

SEMIA strains with different characteristics were selected for further testing. A 0.7 cm *S. rolfsii* agar plug was disposed at the center of PDA medium plates. Subsequently, 30 µL of bacterial log-phase liquid culture were spread on Congo Red YM medium plates. Petri dishes containing fungal plug were then placed inverted over the plate with the bacterium, and control treatments were prepared without it. The plates were incubated for five days at 23°C and 12 hours of photoperiod. Each SEMIA strain was tested on three different plates.

Inhibition through volatiles is presented in percentages according to inhibition equation previously described for dual-cultures.

2.5 16S rRNA sequence analysis

Genomic DNA of each selected SEMIA strain was extracted and purified according to Joseph and David (2001). The *16S rRNA* was amplified using primers BacPaeF (5'AGA GTT TGA TCC TGG CTC AG3') and Bac1542R (5'AGA AAG GAG GTG ATC CAG CC3') according to Stackebrandt and Liesack (1993) and Edwards *et al.* (1989) in a final volume of 25 µL containing 20-50 ng of genomic DNA, 1 µL of 100 pmol of each primer, 1 µL of 0.25 mmol L^{-1} dNTP mix, 1 µL of 50 mmol L^{-1} $MgCl_2$, 1 µL of DMSO, 2.5 µL of Taq DNA Polymerase PCR Buffer (10×) and 0.2 µL of Taq DNA Polymerase (Thermo Scientific). The cycling program used was: 94ºC for 5 min, followed by 37 cycles of 94ºC for 1 min, 50ºC for 1 min and 10 s, and 72ºC for 1 min; finally, the reaction was 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. Low-quality sequences were automatically trimmed using Chromas 2.6.4 (Goodstadt and Ponting 2001). Sequences from *16S rRNA* gene of selected SEMIA strains were deposited at GenBank under accession number MG209100 to MG209109.

Sequence identity was assessed by comparing the *16S rRNA* sequences of SEMIA strains with the sequences from EzBioCloud (https://www.ezbiocloud.net/identify), a quality-controlled *16S rRNA* server database. In addition, each SEMIA *16S rRNA* sequence was aligned with MUSCLE to a counterpart sequences containing all type strains of *Rhizobium* available in LPSN (available at http://www.bacterio.net), and due to the wide heterogeneity in length of the *16S rRNA* sequences, tail gaps from partial *16S rRNA* sequences were manually removed with MEGA7 software. Sequences were aligned again using SINA (https://www.arb-silva.de/aligner/), an alignment software for ribosomal sequences. Finally, sequence identity matrixes were generated with BioEdit 7.2.6.

Molecular phylogenetic analysis of SEMIA and *Rhizobium* type strains sequences were generated with Bayesian analyses using BEAST v1.8.4 software. The mode of nucleotide evolution used in all analyses HKI. The Yule process was selected as a tree prior

to Bayesian analysis, and 10,000,000 generations were performed for the16S rRNA analysis. The trees were visualized and edited using FigTree 1.4.3 software.

2.6 Biocontrol pot experiment

S. rolfsii inoculum was made according to Falcão *et al.* (2005), with slight modifications. Erlenmeyer flasks containing moistened (20% m/v) maize (*Zea mays* L.) grains were autoclaved at 120 °C for 20 min. After, two 0.7 cm *S. rolfsii* agar plugs were disposed in the flask and then incubated for 14 days at 23°C and 12 h of photoperiod. Pre-sprouting common bean cv. BRS Triunfo (Brazilian National Register of Cultivars no. 31.376) seeds were obtained after disposing seeds in filter paper moistened following incubation in germination chambers for 2 days (16 h of light at 30°C and 8 h of dark at 20°C). Pre-sprouting seeds were immersed in SEMIA strain log-phase liquid cultures for 5 min. Subsequently, 180 mL pots were filled with a blend of clay and sandy non-autoclaved soil, resulting in the following characteristics: pH = 5.40, organic matter = 0.4%, CEC pH 7 = 3.5 and effective CEC (ECEC) = 1.8, P = 42.9 mg/dm^{-3}, K = 41 mg/dm^{-3}, Al = 0.1 $cmol_c/dm^{-3}$, Ca = 1.1 $cmol_c/dm^{-3}$, Mg = 0.5 $cmol_c/dm^{-3}$, H+Al = 1.7 $cmol_c/dm^{-3}$, B = 0.4 mg/dm^{-3}, Zn = 3.2 mg/dm^{-3}, Cu = 0.3 mg/dm^{-3}, Mn = 29 mg/dm^{-3}, Na = 4 mg/dm^{-3}, Arg = 7% and Fe = 0.04%. Soil infestation was made with two *S. rolfsii*-infected maize grains disposed along the pots containing one rhizobial-inoculated pre-sprouting common bean seed. Two control treatments were arranged as follows: “treatment -” composed of common beans not inoculated nor infected, and “treatment +” composed of common bean in *S. rolfsii*-infected soil as previously described. Three replicates were used per treatment. Disease percentage was recorded as a day-to-day score based on points from 0 to 8 considering the date of onset of the wilt. One point was subtracted from 8 every 4 days without disease symptoms. Shoot dry masses were recorded through separation of aerial part of each plant following dry at 60°C to constant mass.

2.7 Statistical analysis

The results obtained from dual cultures, double plates and biocontrol tests were submitted to one-way analysis of variance and means compared by Scott-Knott (SK) test at 5% error probability using Sisvar 5.6 platform (Ferreira 2011). Spearman's correlation between IAA production and fungal mycelium inhibition on dual cultures

was determined. Data from antagonistic activity related to siderophores were prior submitted to F test then means were compared by Student's t-test at 5% of significance.

3. Results

3.1 SEMIA strains exhibit high ability to antagonize S. rolfsii

Dual cultures were performed to screening a SEMIA, a *Rhizobium* spp. culture collection for biocontrol proprieties against pathogenic fungus *S. rolfsii* (Table 1). Among of the 151 screened-strains, 33 (~22%) of them showed significant antagonistic activity, and 16 were able to decrease over 84% of the fungal radial mycelium growth. SEMIA 456, 4026 and 436 presented inhibition of over 98% of fungal growth. MAPA-recommended strains SEMIA 4080, 4077 and 4088 were able to decrease in 86%, 86% and 60% the mycelium diameter, respectively.

3.2 Bacterial IAA production correlates with S. rolfsii inhibition

IAA *in vitro* production among 1.2 to 36.5 μg mL^{-1} was detected among antagonistic SEMIA strains. MAPA-recommended strains 4077, 4080 and 4088 produced 2.5, 6.1, 4.7 μg mL^{-1} of IAA, respectively. The prominent IAA producers, SEMIA 456 (34.7 μg mL^{-1}) and 439 (36.5 μg mL^{-1}), were grouped along with major *S. rolfsii* antagonists in dual cultures. A significant correlation ($r = 0.447$, $p = 0.011$) was obtained between bacterial IAA production with *S. rolfsii* mycelial growth inhibition on dual cultures (Figure 1).

3.3 SEMIA strains produce lytic enzymes and siderophores

Among all the 33 antagonistic strains detected before, siderophore production was detected only in SEMIA 436, 460, 4077 and 4088. A 100-μM $FeCl_3$ concentration on growth medium has been found to inhibit siderophores compounds synthesis (Visca *et al.* 1992). In this way, dual cultures on both iron-deficient (to stimulate siderophore synthesis) and $FeCl_3$ supplemented King's B (to repress siderophore synthesis) medium were performed to confirm siderophore influence on *S. rolfsii* mycelium growth (Figure 2). SEMIA 4088 demonstrated antagonistic activity related to siderophore production once plates had 13% mycelium widespread in $FeCl_3$ supplemented medium. No more statistical differences on mycelium growth between different media were found. Lytic enzymes were also tested, and

SEMIA 4026, 4031 and 4079 were the only ones detected producing proteases. Cellulase production was not detected (*data not shown*).

In order to perform further testing, we selected the antagonistic strains i) SEMIA 456, 4026, 436, 439 and 4032 due to their strongly fungal inhibition (≥93%) and IAA production features; ii) SEMIA 460 as a median inhibitor (51%), and for the presence of a mucoid colony phenotype (often an indication of enhanced biofilm formation with possible biocontrol implication (Chen *et al.* 2013)); iii) SEMIA 4085 as poor inhibitor (10%); and iv) SEMIA 4080, 4077 and 4088, MAPA-recommended strains.

3.4 SEMIA strains are able to antagonize S. rolfsii through volatiles

Selected antagonistic SEMIA strains were tested employing double plate technique for production of volatile substances inhibiting fungal growth (Table 2). SEMIA 460, 4077 and 4088 decreased mycelium diameter in 45%, 28% and 28% through volatiles, respectively.

3.5 16S rRNA sequence analysis and phylogenetics of SEMIA strains

The genera of SEMIA strains was confirmed through comparisons with EzBioCloud, a curated *16S rRNA* database, and also with a *Rhizobium* type strains local database (Figure 3 and Supplementary Table 1), were all SEMIA strains presented gene similarities higher than 98.4% with species from *Rhizobium* genus. Interestingly, SEMIA 436 and 439 presented maximum similarity values of 98.4% (EzBioCloud) and 97.6% (both databases), respectively. These values are inferior to the recommended species circumscription threshold of 98.65% (Kim *et al.* 2014).

Sequence analysis from *16S rRNA* set of selected SEMIA revealed SEMIA 4077 and 4080 presenting similarity of 99%. Similarity analysis exclusively of *Rhizobium* type strains local database revealed that ~80.4% of sequences shared high similarities with others (Figure 4).

A phylogenetic tree was generated with SEMIA sequences and the *Rhizobium* type strains (Figure 5). Considering the last node with posterior value over 98%, SEMIA 4077, 4080, 4088 where grouped along with *R. hainanense*, *R. miluonense*, *R. multihospitium*, *R. freire* and *R. tropici*; SEMIA 4085 with *R. gallicum*, *R. mongolense*, *R. yanglingense* and *R. loessense*; and SEMIA 436, 439, 456, 4026, 4032 with *R. pusense*. SEMIA 460 was placed

along *R. leguminosarum, R. sophorae, R. ecuadorense, R. laguerreae, R. anhuiense, R. trifolii* and *R. acidisoli* with less (64% posterior) support.

3.6 SEMIA strains exhibit high biocontrol efficacy on collar rot of the common bean

Biocontrol efficacy of selected SEMIA strains was measured employing pot tests (Figure 6). Common bean cultivated in *S. rolfsii*-infected soil and inoculated with 4026, 436, 460, 456 did not statistically differ from plants with no inoculation (positive control) for disease percentage and shoot dry masses. In the other hand, inoculation with SEMIA 4032, 4077, 4088, 4080, 4085 or 439 were grouped with same disease percentage and shoot dry masses with the negative control (no soil infection).

4. Discussion

Dual cultures screens have been demonstrating an effective approach to prospecting biocontrol agents. In an attempt to promptly obtain a biocontrol agent against *S. rolfsii*-promoted disease in the common bean, *SEMIA*, a previously stabilized *Rhizobium* culture collection was evaluated. Other authors reported bacteria and fungi antagonistic toward *S. rolfsii* mycelium growth on dual culture experiments. Rhizobial isolates from groundnut (*Arachis hypogaea* L.) were able to inhibit up to 62.5% of mycelium growth diameter (Ganesan *et al.* 2007). Shaban and El-Bramawy (2011) reported 85.5% of inhibition evaluating a *Rhizobium leguminosarum* isolate. *Bacillus tequilensis* (Gholami *et al.* 2014), *Trichoderma harzianum* (Sabet *et al.* 1998) and *Trichoderma viride* (Manjula *et al.* 2004) were found inhibiting 73.6%, 45% and 58% of mycelium growth, respectively. Here, prominent antagonistic SEMIA strains demonstrated up to 99% of *S. rolfsii* mycelium inhibition on dual cultures.

Antagonistic SEMIA strains were found producing IAA concentrations compatible to a previously reported *Rhizobium* spp. with beneficial effects on plants (Bhattacharjee *et al.* 2012). IAA synthesis is considered a common feature in soil beneficial bacteria, being a part of their plant-colonization strategy. This molecule is often considered one of the most effective plant-growth inducer (Vargas *et al.* 2017). IAA produced by rhizobia is involved in nodulation process (Boiero *et al.* 2007) and root architecture modification (Yanni *et al.* 2001). However, high IAA concentrations have shown an unresponsive effect and/or adverse impact on plant growth. As an example, Schlindwein *et al.* (2008) reported lettuce seeds

with abnormal germination when treated with an IAA-overproducing (171.1 μg.ml^{-1}) *R. trifolii* strain.

In a biocontrol context, phytostimulation action of IAA produced by beneficial bacteria could be helpful; however, this action relies on the plant. Exogenous IAA was already found to exert stimulatory and inhibitory effects on fungi (Fu *et al.* 2015). Kulkarni *et al.* (2013) reported that 0.5, 5, and 50μM concentrations of exogenous IAA can induce *Fusarium delphinoides* growth. However, at higher concentrations (500 and 5000 μM), IAA has considerably decreased the growth of this fungus. Interesting, SEMIA 439, the most preeminent IAA producer detected here, also grouped along the greater fungal inhibitors, produce 208 μM of IAA. IAA was also reported triggering protection against external adverse conditions by coordinately enhancing different cellular defense systems in *Escherichia coli* (Bianco *et al.* 2006). Here, we hypothesized also a direct relationship between *in vitro* bacterial IAA production with *S. rolfsii* mycelial growth inhibition and found a significant ($p=0.011$), but weak correlation ($r=0.447$) according to Hinkle *et al.* (2003). Importantly, this result points out a function in soil competitiveness for beneficial bacterial IAA than merely the improvement of plant-bacteria interaction fitness. However, remains to be elucidated whether IAA has a direct impact on fungal mycelium growth or/and a secondary role stimulating responses in the prokaryotic cell.

Besides possible IAA action, biological control agents can negatively affect the growth of plant pathogens by others several mechanisms. For example, Rodriguez-Kabana *et al.* (1978) reported that proteolytic activity of *Trichoderma viride* was crucial for *S. rolfsii* enzymatic activity disruption. Thus, we tested proteases export to the culture medium and found this characteristic in only three (~9%) of 33 antagonistic SEMIA strains. Siderophore-producing antagonistic rhizobia also have been reported (Granada *et al.* 2014; Vargas *et al.* 2009). However, siderophore production is not always related to antagonistic activity, as was already reported to *Burkholderia cepacia* (Bach *et al.* 2016). Genome sequencing revealed that SEMIA 4077 possesses a siderophore-biosynthesis gene cluster while SEMIA 4080 does not (Ormeño-Orrillo *et al.* 2012). In agreement, SEMIA 436, 460 and the MAPA-recommended strains SEMIA 4077 and 4088 were identified as siderophore producers. However, SEMIA 4088 was the only which demonstrated a slightly antagonistic activity related to siderophore production.

In addition to diffusible antifungal molecules as lytic enzymes and siderophores (Kümmerli *et al.* 2014), some bacteria also synthetize volatile compounds which influence fungi growth (Bhagat *et al.* 2014; Wheatley 2002). As an example, soil bacterial volatiles were reported to completely cease laccase activity from *Phanerochaete magnoliae* (Mackie and Wheatley 1999). Laccase represents an important virulence factor for various phytopathogenic fungi protecting them from plant defense molecules such as tannins and phytoalexins (Pezet *et al.* 1992). Ganesan *et al.* (2007) reported inhibition of up to 11% of *S. rolfsii* growth by volatiles produced by *Rhizobium* spp. Here, SEMIA 460 was the major volatile inhibitor (45%), being this probably a major antagonism mechanism for its, considering the 51% of inhibition detected in dual cultures.

Bacterial strains from SEMIA culture collection were previously identified as *Rhizobium* spp. based on the prospecting from common bean root nodules and cultural characteristics. To confirm bacterial identification, *16S rRNA* sequence analysis as a standard method was employed. To provide reliable identification, once it may be compromised by the quality of sequences deposited in public databases (i.e. NCBI), the curated *16S rRNA* database EzBioCloud (Yoon *et al.* 2017) was chosen as an alternative for conventional comparisons. Once *Rhizobium* spp. identity of SEMIA 456, 4026, 436, 439, 4032, 460, 4085, 4080, 4077 and 4088 strains was confirmed through this generalist database, results were validated through specific comparison with sequences of 102 *Rhizobium* type strains with validly published prokaryotic names.

Similarity analysis of the *16S rRNA* sequences constituting the *Rhizobium* type strains database has shown ~80.4% of sequences sharing high similarity (≥98.65%) with others. As expected, although *16S rRNA* analysis allows identification of *Rhizobium* genus, this high degree of gene conservation seriously limited the separation at the species level.

SEMIA 4077 and 4080 sequences obtained presented a high (99%) 16S rRNA gene similarity. Often considered one of the most successful symbionts of common bean, SEMIA 4077 is the type strain of *Rhizobium tropici* (Martínez-Romero *et al.* 1991). SEMIA 4080, which in the past was considered a *Rhizobium tropici* strain, shares only 52% of its genome sequences with SEMIA 4077 (Ormeño-Orrillo *et al.* 2012). Recently, SEMIA 4080 was recognized as the first *Rhizobium freirei* strain described (Dall'Agnol *et al.* 2013).

SEMIA 456 was the only strain theoretical identified in species level with high pairwise similarity exclusively with *Rhizobium radiobacter*. In the other hand, SEMIA 436

and 439 presented gene similarities lower than the recently recommended threshold to separate two prokaryotic species (Kim *et al.* 2014), being phylogenetically related to *Rhizobium radiobacter*. Further molecular analysis and phenotypic characterization will be explored in the future to describe SEMIA 436 and 439, isolated in 1960's, from Argentina and former Czechoslovakia, respectively, as possible new taxa.

In the phylogenetic tree with *Rhizobium* type strains sequences, SEMIA 436, 439, 456, 4026 and 4032 were placed in a branch with *R. pusense*, closely with *Agrobacterium larrymoorei* (former *Agrobacterium larrymoorei*), *R. skierniewicense, R nepotum* and *R. radiobacter* (former *Agrobacterium tumefaciens*). These bacteria, which in the past were/would have been called *Agrobacterium* (Young *et al.* 2001), are usually known for being tumorigenic, therefore they do not form nitrogen-fixing nodules on roots but induce tumorous cell growth often mediated by tumor-inducing (Ti) plasmid expression. However, Ribeiro *et al.* (2013) reported strains related to *R. radiobacter, R. nepotum* and *R. pusense* according to *16S rRNA* analysis, which were isolated from common bean and are able to form white nodules on roots. At this point, we need to endorse/clarify that "agrobacteria" and "rhizobia" are terms to be exclusively used to describe a bacterial phenotypic characteristic. 'Agrobacteria' is a tumorigenic *Rhizobium* while 'rhizobia' is a root-nodulating bacterium, which could currently belong to 13 different genera (Willems 2006) with, but no matter how efficient, nitrogen-fixation ability.

Nodulation (*nod*) and nitrogen-fixation genes (*nif* and *fix*) are often clustered on large plasmids (Sym plasmid), or within genomic symbiosis islands (SI), often found within insertion sequence elements, transposases, and related genes (MacLean *et al.* 2007). We speculate that the SEMIA strains placed in the branch with tumorigenic *Rhizobium* could represent a case where "agrobacteria" turned into a "rhizobia" via horizontal gene transfer. Root-nodulating bacteria which according to ribosomal gene analysis were related to "non-rhizobial" species/genus have been found to naturally harboring the nod genes essential for establishing rhizobial symbiosis (Moulin *et al.* 2001; Trujillo *et al.* 2006). We found up to date no reports of agrobacteria naturally harboring these genes; however, Martínez *et al.* (1987) engineered agrobacteria to harbor Sym plasmid and obtain a mutant able to form nitrogen-fixing nodules (Martínez *et al.* 1987). In addition, agrobacteria could lose tumorigenic characteristic i.e. high temperature (>28°C) culturing leads to "loosening" of the Ti plasmid in the bacterial population (Schilperoort *et al.* 1980). Tumor-inducing,

nodulation and nitrogen-fixation genes presence/composition/location in SEMIA 436, 439, 456, 4026 and 4032 will be evaluated in the future.

In this work, dual cultures screens were assessed in order to prospect an efficient *Rhizobium* biocontrol agent of *S. rolfsii*-promoted disease on the common bean. Dual cultures are widely used for this purpose and results strongly correlates with microbial activities *in planta* (Shehata *et al.* 2016). However, SEMIA 4026, 436 and 456 were strong *in vitro* fungal antagonists and did not succeed in controlling the disease *in planta*. A dual culture-screened isolate may not succeed *in planta* for many reasons (i.e not proper colonize the plant and/or compete with native microbiota). In the other hand, SEMIA 4085 succeeded in controlling collar rot disease, despite being selected as a poor *in vitro* inhibitor. Dual culture screens could not detect microorganisms that are effective *in planta* major/only through "indirect antagonism", such as inducing host resistance or competing for ecological plant niches (Knudsen *et al.* 1997; Pang *et al.* 2009). In fact, rhizobia were previously reported to elicit systemic resistance on plants (Elbadry *et al.* 2006).

Previous research has shown the efficacy of different biological agents in controlling *S. rolfsii* disease in the common bean. Barakat *et al.* (2006) reported that *Trichoderma harzianum*, *T. lacteal* and *T. pseudokoningii* reduced the disease index by 66.8%, 65.8% and 63%, respectively. Madi *et al.* (1997) obtained 64% of disease reduction in soil infected with sclerotia treated with *Talaromyces flavus*. Inoculations with *B. subtilis* subsp. *spizizenii*, *B. subtilis* subsp. *subtilis*, *B. atrophaeus*, *B. tequilensis* and *S. cyaneofuscatus* reduced disease severity from 50% to 58.5% (Gholami *et al.* 2014). Eid (2014) reported *B. subtilis*, *Pseudomonas fluorescens*, and *T. viride* as able to reduce disease by 82%, 74% and 75%, respectively. Here, inoculation with SEMIA 4032, 4077, 4088, 4080, 4085 and 439 onto common bean cultivated in *S. rolfsii*-infected soil have had same disease percentage as the negative control (uninfected soil), thus these rhizobial strains exhibited 100% of disease reduction. Besides action of antifungal molecules, this suppression of collar rot may be due to plant growth promotion, especially considering MAPA-recommended strains which are well known to significantly increase plant shoot and root mass (Fageria *et al.* 2014; Kellman *et al.* 2005; Korir *et al.* 2017).

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6. Tables

Table 1. Antagonism against *S. rolfsii* and IAA production of SEMIA strains.

SEMIA strain	Fungal inhibition %	IAA µg mL^{-1}
456	99	34.7
4026	99	3.3
436	98	18.0
439	95	36.5
457	94	20.6
4032	93	22.3
464	93	16.6
461	92	15.5
4033	92	10.9
462	90	4.6
4031	89	16.8
480	87	13.5
4080	86	6.1
472	86	12.8
4077	86	2.5
465	84	3.6
4079	81	1.2
4022	78	5.0
4084	78	1.8
455	76	17.3
4016	76	4.1
4036	76	5.0
4070	73	2.0
463	70	6.2
481	65	7.3
4039	65	1.2
4088	60	4.7
485	58	4.8
460	51	9.2
4029	50	21.7
449	49	7.5
4085	10	1.8
422	5	10.7

Data in brackets do not differ by SK test at 5% error probability.

Table 2. Antagonistic activity on *S. rolfsii* of volatile compounds produced by SEMIA strains.

SEMIA strain	Volatile inhibition (%)
460	45
4077	28
4088	28
456	17
436	15
4026	13
4080	13
439	11
4032	9
4085	9
422	0

Data in brackets do not differ by SK test at 5% error probability.

7. Figures

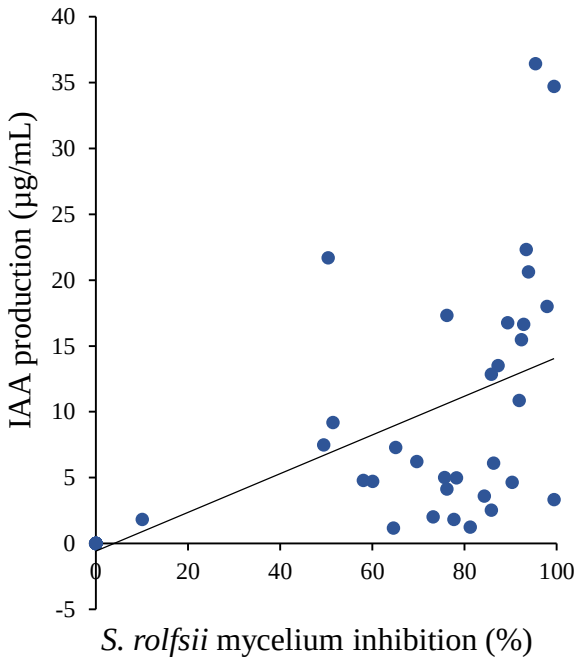


Figure 1. Scatter plot between rhizobial IAA production and ability to inhibit *S. rolfsii* mycelium growth. Spearman's r = 0.447, p-value = 0.011.

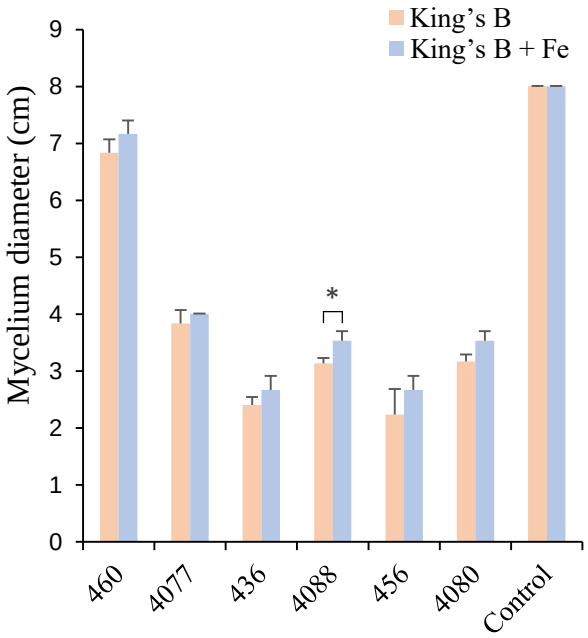


Figure 2. Siderophore influence on *S. rolfsii* mycelium growth. Data with an asterisk were found significant different (Student's t-test at 5%) between iron-deficient and $FeCl_3$ supplemented King's B medium.

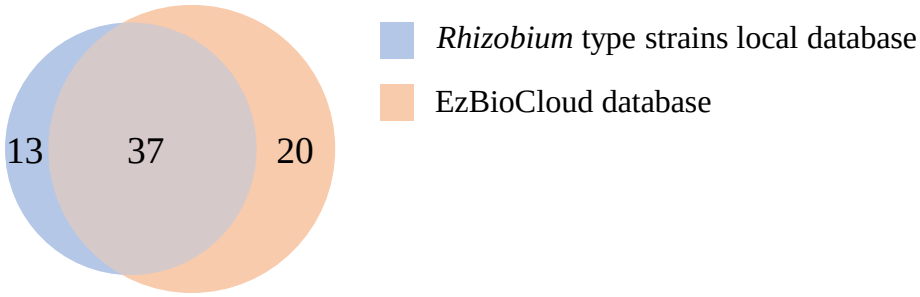


Figure 3. Hits considering the similarity threshold of 98.65% from *16S rRNA* of SEMIA strains with *Rhizobium* spp. sequences from different databases.

a)

	436	439	456	460	4026	4032	4077	4080	4085
439	64.7								
456	95	64.7							
460	89.4	59.8	90.5						
4026	95.8	65	95.8	89.7					
4032	95.2	66.2	95	89	96.8				
4077	87.1	62.5	87.2	90.4	88.1	90.3			
4080	86.9	62.5	87.2	90.2	88.1	90.1	99		
4085	87.1	62.9	87.5	90	88.6	90.6	95.8	95.9	
4088	69.8	75.2	70.1	72.6	70.7	72.4	80.3	80.4	77
SEMIA									

Sequence similarity ≤ 98.7%

Sequence similarity ≥ 98.7%

b)

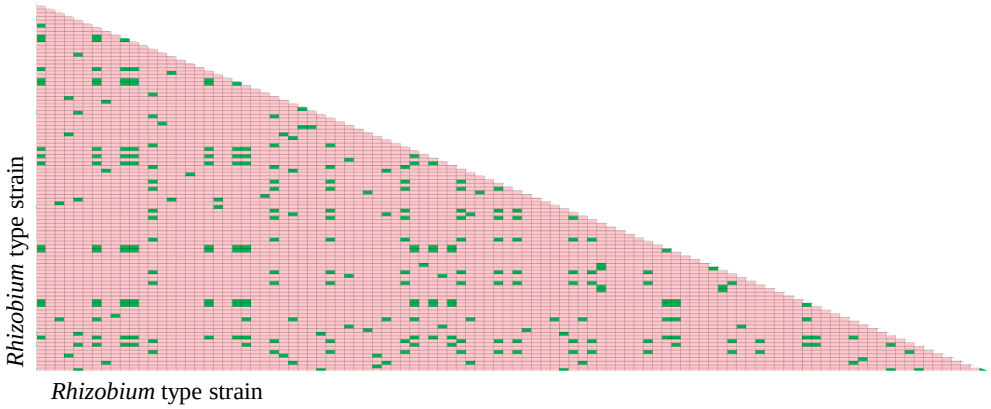
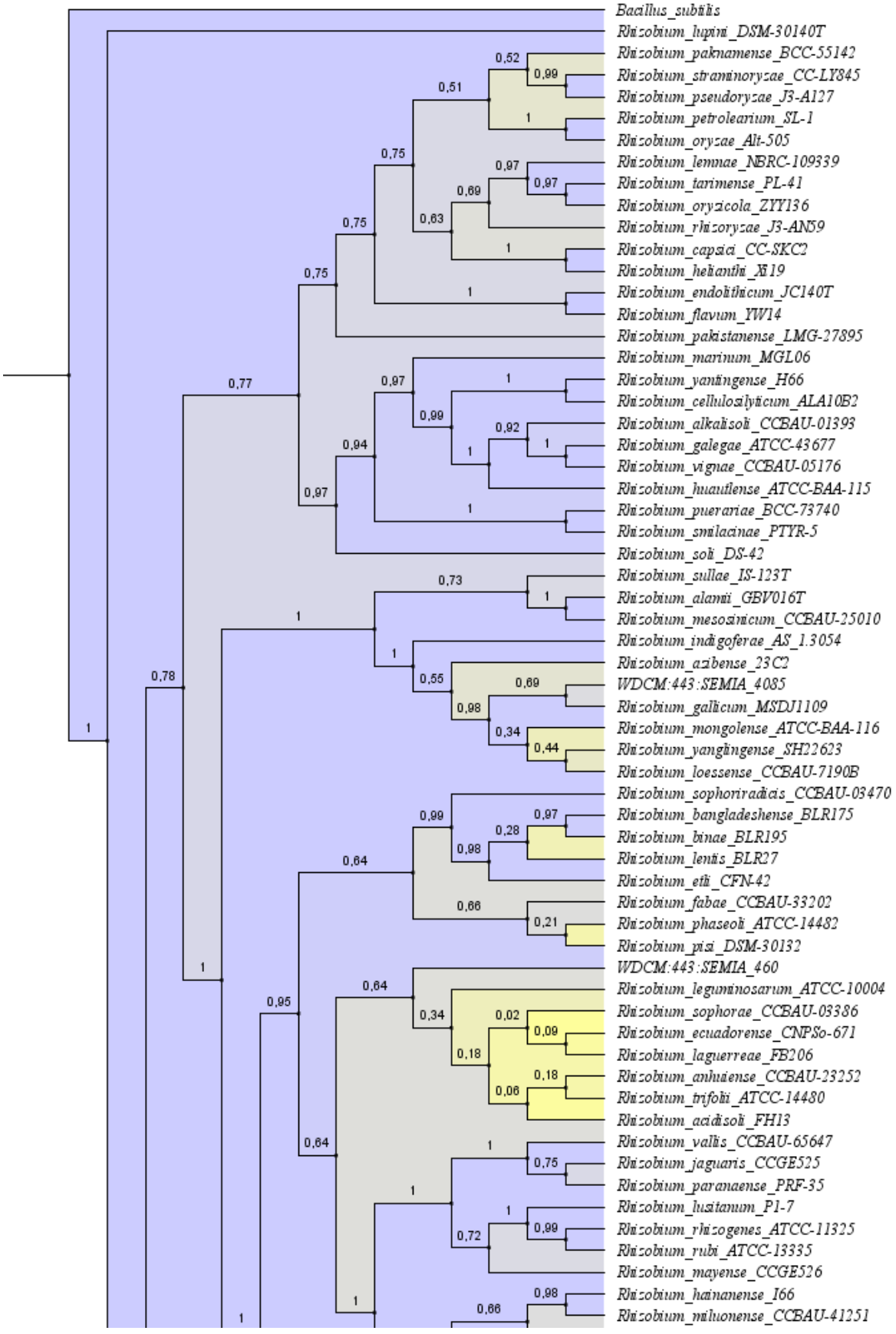


Figure 4. Heatmap of *16S rRNA* similarity matrix between sequences sets of a) SEMIA selected antagonistic strains and b) 102 *Rhizobium* type strains.


Bacillus_subtilis
Rhizobium_lupini_DSM-30140T
Rhizobium_paknamense_BCC-55142
Rhizobium_straminoryzae_CC-LY845
Rhizobium_pseudoryzae_J3-A127
Rhizobium_petrolearium_SL-1
Rhizobium_oryzae_Alt-505
Rhizobium_lemnae_NBRC-109339
Rhizobium_tarimense_PL-41
Rhizobium_oryzicola_ZYY136
Rhizobium_rhizoryzae_J3-AN59
Rhizobium_capsici_CC-SKC2
Rhizobium_helianthi_Xi19
Rhizobium_endolithicum_JC140T
Rhizobium_flavum_YW14
Rhizobium_pakistanense_LMG-27895
Rhizobium_marinum_MGL06
Rhizobium_yantingense_H66
Rhizobium_cellulosilyticum_ALA10B2
Rhizobium_alkalisoli_CCBAU-01393
Rhizobium_galegae_ATCC-43677
Rhizobium_vignae_CCBAU-05176
Rhizobium_huautlense_ATCC-BAA-115
Rhizobium_puerariae_BCC-73740
Rhizobium_smilacinae_PTYR-5
Rhizobium_soli_DS-42
Rhizobium_sullae_IS-123T
Rhizobium_alamii_GBV016T
Rhizobium_mesosinicum_CCBAU-25010
Rhizobium_indigoferae_AS_1.3054
Rhizobium_azibense_23C2
WDCM:443:SEMIA_4085
Rhizobium_gallicum_MSDJ1109
Rhizobium_mongolense_ATCC-BAA-116
Rhizobium_yanglingense_SH22623
Rhizobium_loessense_CCBAU-7190B
Rhizobium_sophoriradicis_CCBAU-03470
Rhizobium_bangladeshense_BLR175
Rhizobium_binae_BLR195
Rhizobium_lentis_BLR27
Rhizobium_etli_CFN-42
Rhizobium_fabae_CCBAU-33202
Rhizobium_phaseoli_ATCC-14482
Rhizobium_pisi_DSM-30132
WDCM:443:SEMIA_460
Rhizobium_leguminosarum_ATCC-10004
Rhizobium_sophorae_CCBAU-03386
Rhizobium_ecuadorense_CNPSo-671
Rhizobium_laguerreae_FB206
Rhizobium_anhuiense_CCBAU-23252
Rhizobium_trifolii_ATCC-14480
Rhizobium_acidisoli_FH13
Rhizobium_vallis_CCBAU-65647
Rhizobium_jaguaris_CCGE525
Rhizobium_paranaense_PRF-35
Rhizobium_lusitanum_P1-7
Rhizobium_rhizogenes_ATCC-11325
Rhizobium_rubi_ATCC-13335
Rhizobium_mayense_CCGE526
Rhizobium_hainanense_I66
Rhizobium_miluonense_CCBAU-41251

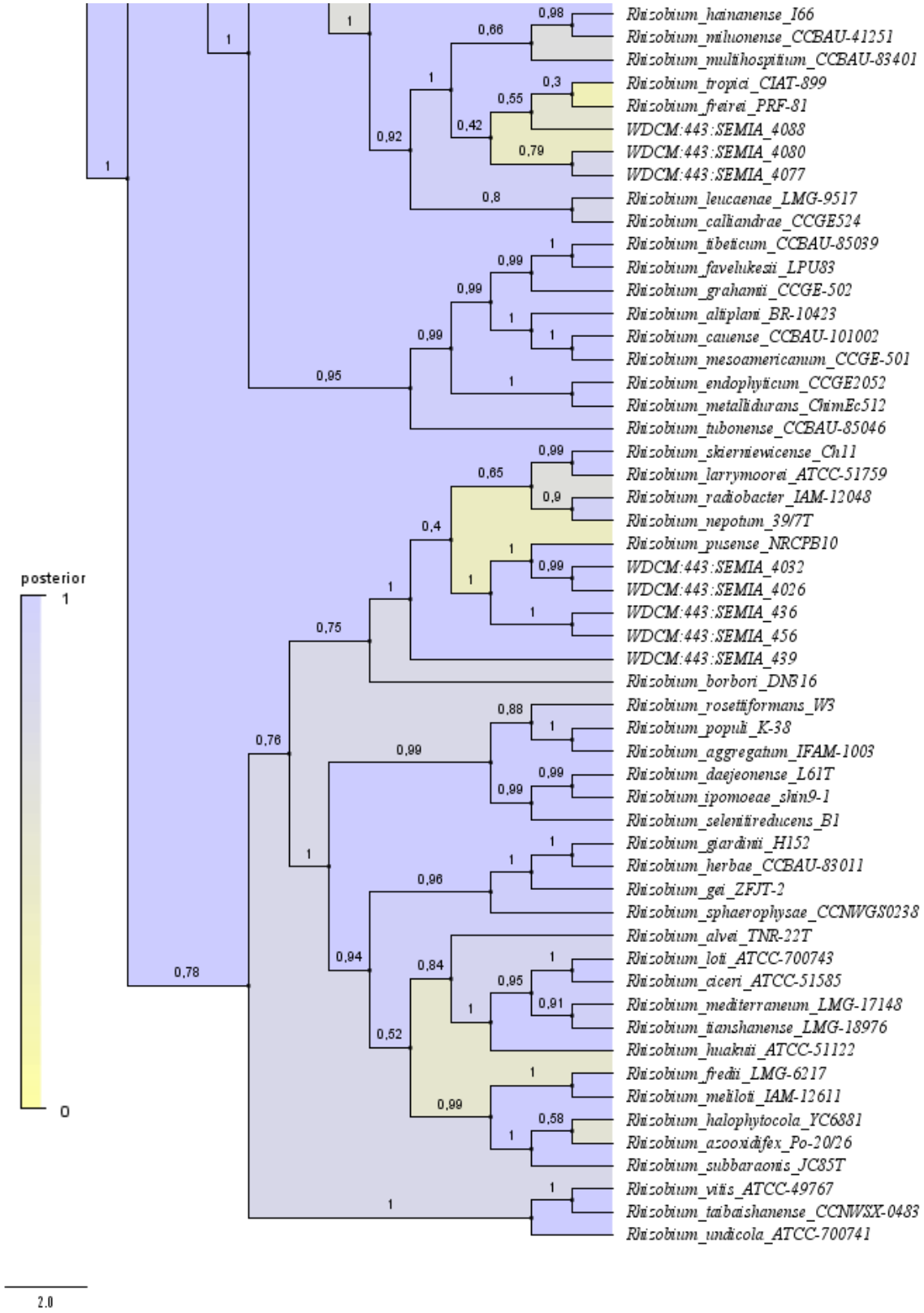

Rhizobium_hainanense_I66
Rhizobium_miluonense_CCBAU-41251
Rhizobium_multihospitium_CCBAU-83401
Rhizobium_tropici_CIAT-899
Rhizobium_freirei_PRF-81
WDCM:443:SEMIA_4088
WDCM:443:SEMIA_4080
WDCM:443:SEMIA_4077
Rhizobium_leucaenae_LMG-9517
Rhizobium_calliandrae_CCGE524
Rhizobium_tibeticum_CCBAU-85039
Rhizobium_favelukesii_LPU83
Rhizobium_grahamii_CCGE-502
Rhizobium_altiplani_BR-10423
Rhizobium_cauense_CCBAU-101002
Rhizobium_mesoamericanum_CCGE-501
Rhizobium_endophyticum_CCGE2052
Rhizobium_metallidurans_ChimEc512
Rhizobium_tubonense_CCBAU-85046
Rhizobium_skierniewicense_Ch11
Rhizobium_larrymoorei_ATCC-51759
Rhizobium_radiobacter_IAM-12048
Rhizobium_nepotum_39/7T
Rhizobium_pusense_NRCPB10
WDCM:443:SEMIA_4032
WDCM:443:SEMIA_4026
WDCM:443:SEMIA_436
WDCM:443:SEMIA_456
WDCM:443:SEMIA_439
Rhizobium_borbori_DN316
Rhizobium_rosettiformans_W3
Rhizobium_populi_K-38
Rhizobium_aggregatum_IFAM-1003
Rhizobium_daejeonense_L61T
Rhizobium_ipomoeae_shin9-1
Rhizobium_selenitireducens_B1
Rhizobium_giardinii_H152
Rhizobium_herbae_CCBAU-83011
Rhizobium_gei_ZFJT-2
Rhizobium_sphaerophysae_CCNWGS0238
Rhizobium_alvei_TNR-22T
Rhizobium_loti_ATCC-700743
Rhizobium_ciceri_ATCC-51585
Rhizobium_mediterraneum_LMG-17148
Rhizobium_tianshanense_LMG-18976
Rhizobium_huakuii_ATCC-51122
Rhizobium_fredii_LMG-6217
Rhizobium_meliloti_IAM-12611
Rhizobium_halophytocola_YC6881
Rhizobium_azooxidifex_Po-20/26
Rhizobium_subbaraonis_JC85T
Rhizobium_vitis_ATCC-49767
Rhizobium_taibaishanense_CCNWSX-0483
Rhizobium_undicola_ATCC-700741
posterior
1
0
2.0

Figure 5. Phylogenetic tree of the SEMIA selected antagonistic strains and 102 *Rhizobium* type strains inferred by Bayesian analysis using a 16S rRNA gene. The significance of each branch is indicated at the branching points by posterior probability.

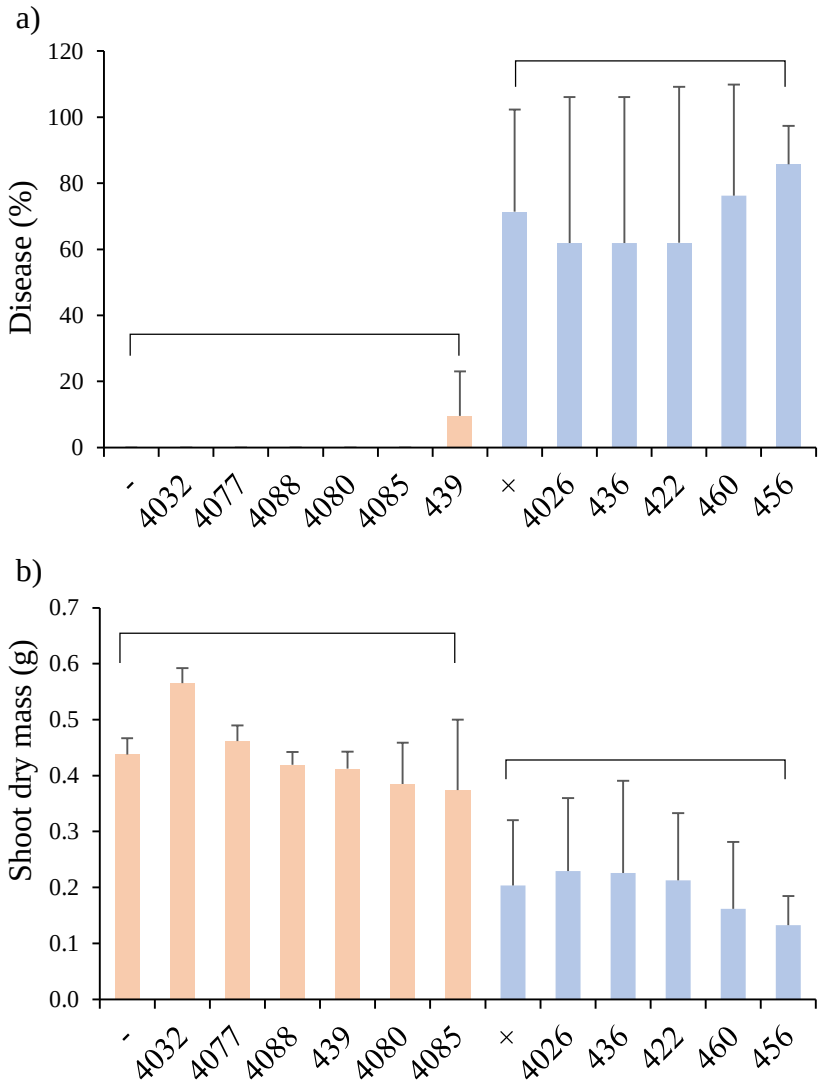


Figure 6. Biocontrol of *S. rolfsii*-promoted disease on the common bean via inoculation with SEMIA strains. a) Disease percentage according to a day-to-day score. b) Shoot dry masses. Treatment "-" composed of common beans not inoculated nor infected, and "treatment +" composed of common bean in *S. rolfsii*-infected soil. Data in brackets do not differ by SK test at 5% error probability.

8. Supplementary material

Supplementary Table 1. Molecular identification based on 16S ribosomal DNA sequencing.

SEMIA strain	*Rhizobium* type strains local database				EzBioCloud database		
	Query length	Pairwise similarity (%)	Accession	Top-hit taxon	Accession	Pairwise similarity (%)	Query length
436	1360	98.8	AB247615	*Rhizobium radiobacter*	AJ389904	98.4*	1428
439	939	97.6*	AB247615	*Rhizobium radiobacter*	AJ389904	97.6*	945
456	1345	99.7	AB247615	*Rhizobium radiobacter*	AJ389904	98.8	1422
460	1142	100	KJ921033	*Rhizobium acidisoli*	KJ921033	99.9	1425
		100	KF111868	*Rhizobium anhuiense*	KF111868	99.0	
		100	FJ392873	*Rhizobium fabae*	DQ835306	98.9	
		100	JN558651	*Rhizobium laguerreae*	JN558651	99.0	
		99.5	U29386	*Rhizobium leguminosarum*	U29386	98.8	
		100	KJ831229	*Rhizobium sophorae*	KJ831229	99.9	
		99.3	JN648931	*Rhizobium bangladeshense* *Rhizobium gallicum*	ARDC01000036	99.0	
		99.3	JN648932	*Rhizobium binae*			
		99.3	JN129381	*Rhizobium ecuadorense*			
		99.3	U28916	*Rhizobium etli*			
		99.2	JN648905	*Rhizobium lentis*			
		98.8	FR870231	*Rhizobium nepotum*			
		99.9	NR044112	*Rhizobium phaseoli*			
		100	AY509899	*Rhizobium pisi*			
		99.9	AY509900	*Rhizobium trifolii*			
4026	1347	99.4	AB247615	*Rhizobium radiobacter*	AJ389904	99.1	1405
		99	FJ969841	*Rhizobium pusense*	jgi.1102370	98.8	
				Rhizobium massiliae	AF531767	98.8	
4032	1346	99.3	AB247615	*Rhizobium radiobacter*	AJ389904	99.3	1374
		98.8	FJ969841	*Rhizobium pusense*	jgi.1102370	99.1	
		97.8	HQ823551	*Rhizobium skierniewicense* *Rhizobium massiliae*	AF531767	99.1	

		97.3	NR115518	*Rhizobium rubi*	*Agrobacterium* sp.	CP002249	98.7	
4077	1321	98.7	U89832	*Rhizobium tropici*		CP004015	99.5	1321
		99.5	EF035074	*Rhizobium multihospitium*		jgi.1052913	99.5	
		99.6	EU488742	*Rhizobium freirei*		AQHN01000056	99.5	
		99.3	U71078	*Rhizobium hainanense*		FMAC01000030	99.4	
		99.3	EF061096	*Rhizobium miluonense*		jgi.1052910	99.2	
		99.1	JX855162	*Rhizobium calliandrae*		JX855162	99.1	
		98.8	D14501	*Rhizobium rhizogenes*		BAYX01000035	98.9	
		99	X67234	*Rhizobium leucaenae*		AUFB01000074	98.9	
		98.9	JX855172	*Rhizobium mayense*		JX855172	98.9	
		98.8	AY738130	*Rhizobium lusitanum*		jgi.1052907	98.9	
		98.7	X855169	*Rhizobium jaguaris*		JX855169	98.7	
		98.7	EU488753	*Rhizobium paranaense*		EU488753	98.7	
4080	1319	99.1	EF035074	*Rhizobium multihospitium*		jgi.1052913	99.5	1319
		99	EU488742	*Rhizobium freirei*		AQHN01000056	99.5	
		98.9	U71078	*Rhizobium hainanense*		FMAC01000030	99.4	
		98.9	EF061096	*Rhizobium miluonense*		jgi.1052910	99.2	
		98.7	JX855162	*Rhizobium calliandrae*		JX855162	99.1	
				Rhizobium tropici		CP004015	99.4	
				Rhizobium leucaenae		AUFB01000074	98.9	
				Rhizobium mayense		JX855172	98.9	
				Rhizobium lusitanum		jgi.1052907	98.9	
				Rhizobium jaguaris		BAYX01000035	98.8	
				Rhizobium jaguaris		JX855169	98.7	
				Rhizobium paranaense		EU488753	98.7	
4085	1269	99.9	AF003375	*Rhizobium yanglingense*		AF003375	99.5	1318
		99.8	AF364069	*Rhizobium loessense*		jgi.1041465	99.5	
		99.3	U89817	*Rhizobium mongolense*		ATTQ01000080	99.5	
		98.8	Y10170	*Rhizobium sullae*		Y10170	98.7	

		99.6	U86343	*Rhizobium gallicum*			
		98.8	AF364068	*Rhizobium indigoferae*			
		98.8	EU488742	*Rhizobium freirei*	AQHN01000056	99.4	
		98.7	U71078	*Rhizobium hainanense*	FMAC01000030	99.3	
		98.7	EF035074	*Rhizobium multihospitium*	jgi.1052913	99.4	
				Rhizobium tropici	CP004015	99.5	
				Rhizobium miluonense	jgi.1052910	99.2	
4088	1077			*Rhizobium calliandrae*	JX855162	99.0	1080
				Rhizobium rhizogenes	BAYX01000035	98.8	
				Rhizobium leucaenae	AUFB01000074	98.8	
				Rhizobium mayense	JX855172	98.7	
				Rhizobium lusitanum	jgi.1052907	98.7	

Data with asterisk stands for gene similarities lower than the threshold to separate two prokaryotic species

Considerações finais

Retomando os objetivos iniciais deste trabalho, primeiramente se buscava encontrar capacidade de inibição do micélio de *S. rolfsii* explorando as 151 estirpes bacterianas que compõe a coleção SEMIA de isolados do feijoeiro. Os resultados de dupla-cultura demonstraram que, de fato, existiam estirpes altamente eficientes para o antagonismo dentro da coleção. Especificamente, cerca de 22% das estirpes SEMIA testadas foram capazes de inibir o micélio fúngico, sendo 16 capazes de exercer mais de 84% de inibição.

Também se tinha como objetivo avaliar a produção de IAA, sideróforos, proteases e celulases para as possíveis estirpes antagonistas detectadas. Os resultados revelaram que a produção de IAA está presente em concentrações variáveis entre as estirpes. Além disso, também foi observado que a produção de IAA está correlacionada ($r=0.447$, $p=0.011$) com a habilidade de antagonismo. A produção de proteases foi detectada em apenas três estirpes (SEMIA 4026, 4031, 4079), enquanto a de sideróforos foi detectada em quarto estirpes (SEMIA 436, 4077, 4088 e 460). Ainda mais, foi demonstrado que para SEMIA 4088 a produção de sideróforos está em parte relacionada com a capacidade inibidora do micélio fúngico. A produção de celulases não foi detectada.

Subsequentemente, se buscava selecionar algumas estirpes dentre as possíveis antagonistas obtidas para se determinar a taxonomia e capacidade de produção de compostos voláteis inibidores. O sequenciamento do *16S rRNA* demonstrou que todas as estirpes avaliadas pertenciam ao gênero *Rhizobium.* A análise do *16S rRNA* das estirpes selecionadas também trouxe informações além das esperadas pelo objetivo pré-estabelecido, sendo essas: i) a existência de possíveis novas espécies ainda não descritas dentro da coleção SEMIA, e ii) um grupo de estirpes encontradas no mesmo clado de *Rhizobium* tumorigênicos, levantando questionamentos interessantes sobre transferência horizontal de genes relativos a simbiose. A liberação de compostos voláteis foi também comprovada como um mecanismo de inibição, mais especificamente para SEMIA 4077, 4088 e 460.

O objetivo final do trabalho era demonstrar a capacidade de proteção da doença promovida por *S. rolfsii* em plantas de feijão inoculadas com as possíveis estirpes antagonistas detectadas, considerando condições de vaso em solo infectado. Os testes de biocontrole em plantas foram realizados e mostraram as estirpes antagonistas de *Rhizobium* spp. SEMIA 4032, 4077, 4088, 4080, 4085 e 439 como agentes eficazes para o controle da doença. Mais especificamente, para os parâmetros de i) porcentagem de doença e ii) massas

secas da parte área, os tratamentos com as estirpes citadas, em solo infectado com o patógeno, não apresentaram diferenças estatisticamente significativas de plantas de feijão cultivadas em solo livre de infecção.

Perspectivas

Questões levantadas diante dos resultados encontrados nesse trabalho ainda serão investigadas em profundidade. Em resumo, no momento, está instalado teste a campo na Unidade de Viamão da Secretaria da Agricultura, Pecuária e Irrigação. Ainda, visando explorar *clusters* de biossíntese de compostos secundários, os genomas das estirpes SEMIA 4032, 4088, 4085 e 439 estão sendo sequenciados utilizando as facilidades do Departamento de Bioquímica da Universidade Federal do Paraná. Importantemente, em parceria com a Secretaria de Desenvolvimento Tecnológico da UFRGS, um relatório de invenção está sendo redigido, a fim de dar início à avaliação da pertinência do pedido de depósito de uma patente de invenção denominada "Inoculante composto de estirpes de *Rhizobium* spp. selecionadas para controle de doenças em plantas".

Além das questões acima, outras também serão exploradas. Com mais destaque, testes estão sendo delimitados para avaliar a influência da presença das estirpes biocontroladoras encontradas na germinação de sementes de feijão e avaliar a elicitação de resposta imune em feijoeiro. Também será a avaliada a capacidade de degradação de ácido oxálico das estirpes com capacidade de biocontrole.

Também serão delimitadas análises diante da possibilidade de descrição de SEMIA 436 e 439 como novas espécies. Além da possibilidade de explorar a presença de genes relacionados à nodulação e fixação biológica de nitrogênio. No momento, testes confirmatórios de nodulação estão instalados para estas e as outras estirpes que estavam relacionadas com estirpes de *Rhizobium* tumorigênicos.

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