PAULA ROSE DE ALMEIDA RIBEIRO

TRIPARTITE SYMBIOSIS – SOYBEAN, *BRADYRHIZOBIUM*, ARBUSCULAR MYCORRHIZAL FUNGI: EFFECT OF FORMONONETIN AND DIVERSITY OF *BRADYRHIZOBIUM* IN SOILS FROM DIFFERENT BRAZILIAN REGIONS

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

Orientadora

PhD. Fatima Maria de Souza Moreira

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RESUMO GERAL

Esta tese está dividida em duas partes. A primeira compreende introdução geral e referencial teórico. Na segunda parte são apresentados dois artigos. O primeiro artigo refere-se ao efeito da formononetina associado a adubação fosfatada sobre a colonização micorrizíca e fixação biológica de nitrogênio na produtividade da soja. Este trabalho teve como objetivo avaliar o efeito do isoflavonóide formononetina na soja, em três níveis de adubação fosfatada. Dois experimentos foram instalados em 2010/2011 e 2012/13, constituído de um fatorial 3 x 4 (três níveis de fósforo (0, 60 e 120 kg ha^{-1} de P_2O_5) e quatro níveis de formononetina (0, 25, 50 e 100 g ha^{-1}), e cinco e quatro repetições, respectivamente. Houve efeito positivo da formononetina sobre a colonização micorrízica apenas nos níveis mais baixos de P_2O_5 (0 e 60 kg ha^{-1}). A aplicação de formononetina, associada a 60 kg ha^{-1} P_2O_5 elevou a produtividade de soja para valores equivalentes aqueles observados, quando a dose máxima de P foi aplicada. Esse resultado mostra que sementes tratadas com formononetina, em doses acima de 50 g ha^{-1}, reduzem a necessidade de adubação fosfatada em pelo menos 50% do nível recomendado de fertilizante. O segundo artigo refere-se ao estudo da diversidade genética de estirpes do gênero *Bradyrhizobium*, isoladas de nódulos de soja em solos de diferentes regiões do Brasil. O gênero *Bradyrhizobium* inclui espécies distribuídas em diferentes regiões geográficas. Entretanto, a diversidade observada na análise do gene 16S rRNA é frequentemente baixa. No presente estudo, nós analisamos 46 estirpes de *Bradyrhizobium* oriundas de solos de diferentes regiões brasileiras (Centro-Oeste, Nordeste, Sudeste e Sul), provenientes de áreas de implantação de experimentos similares ao anterior a este trabalho, realizados nas outras instituições participantes do edital 69/2009, por meio da análise do gene 16S rRNA e de cinco genes *housekeeping* (*atpD, gyrB, dnaK, recA*, e *rpoB*) e eficiência simbiótica com plantas de soja, cultivadas em condições axênicas, em vaso Leonard. Em nosso estudo, a análise filogenética dos genes *housekeeping* revelou uma maior diversidade em comparação com a análise do 16S rRNA, indicando possivéis novas espécies, as quais incluem estirpes apresentando alta eficiência simbiótica com plantas de soja. Nossos resultados ressaltam a alta diversidade de estirpes nativas de solos brasileiros, pertencentes ao genêro *Bradyrhizobium*.

Palavras chave: *Glycine max*. Simbioses radiculares. Genes *housekeeping*. Filogenia. Isoflavonóide. Biostimulante.

GENERAL ABSTRACT

This thesis is divided in two parts. The first, contains general introduction and theoretical background. In the second part, two articles are presented. The first article refers to the effect of formononetin associated with phosphorus fertilization on mycorrhizal colonization, nitrogen fixation and soybean productivity. The goal of the present study was to evaluate the effect of the isoflavone formononetin on soybean in combination with three levels of phosphorus (P) fertilization. Two experiments were conducted in 2010/2011 and 2012/13, consisting in a 3 x 4 factorial scheme (three levels of phosphorus: 0, 60 and 120 kg hectare $(ha)^{-1}$ P_2O_5 and four doses of formononetin: 0, 25, 50 and 100 g ha^{-1}) was used with five and four replicates, respectively. Formononetin had a positive effect on mycorrhizal colonization only at lower levels of P addition (0 and 60 kg ha^{-1}). Application of formononetin together with 60 kg ha^{-1} P_2O_5 increased soybean productivity to values equivalent to those observed when the highest level of P was applied. This indicates that seed treatment with formononetin doses higher than 50 g ha^{-1} decreases the need for phosphorus fertilization by up to 50% of the recommended level of fertilizer. The second paper refers to the study of the genetic diversity of *Bradyrhizobium* strains that nodulate soybeans in soils from different Brazilian regions. The *Bradyrhizobium* genus includes species distributed throughout different geographic regions. However, 16S rRNA gene diversity is generally low. In the present study, we analyzed 46 *Bradyrhizobium* strains from soils collected in different regions of Brazil (Midwest, Northeast, Southeast, and South), from areas of the implementation of the previous experiments similar this work, performed in other Institutions participating of the public notice 69/2009, by analyzing the 16S rRNA gene and five housekeeping genes (*atpD, gyrB, dnaK, recA*, and *rpoB*) and their symbiotic efficiency with soybean plants grown under axenic conditions in Leonard jars. In our study, the phylogenetic analysis of housekeeping genes revealed greater diversity compared to the analysis of the 16S rRNA gene, indicating the possibility of novel species, some of which exhibited high symbiotic efficiency with soybean plants. Our results emphasize the great diversity of *Bradyrhizobium* genus strains native to Brazilian soil.

Keywords: *Glycine max*. biostimulant. root symbiosis. Biological nitrogen fixation. housekeeping genes. phylogeny

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PRIMEIRA PARTE

1 INTRODUÇÃO

Na busca por amenizar os impactos ambientais, a ciência vem buscando técnicas que promovam o uso sustentável dos recursos do planeta. Uma das estratégias para a sustentabilidade dos ecossistemas é maximizar o uso dos microrganismos e processos biológicos benéficos ao solo, que promovam a manutenção dos ecossistemas, crescimento, desenvolvimento e produção das plantas. Dentre estes destacam-se os fungos micorrízicos arbusculares - FMAs e as bactérias fixadoras de nitrogênio nodulíferas em leguminosas - BFNNL.

As micorrizas são associações simbiotróficas entre fungos e raízes da maioria das plantas. Os FMAs promovem o crescimento vegetal e a sobrevivência das espécies, por meio de uma relação mutualística, em que fotossintatos são transferidos da planta para os fungos micorrízicos, ao passo que estes transferem água e nutrientes às plantas. Os FMAs atuam como um complemento do sistema radicular da planta hospedeira, capaz de aumentar a absorção de nutrientes, especialmente o fósforo (P), além de proporcionar outros benefícios. Não obstante esta associação ser reconhecidamente importante, a produção de inoculantes de FMAs e seu uso em escala comercial é limitado pelo biotrofismo obrigatório, apresentado pelos FMAs. Em solos tropicais, a baixa disponibilidade de nitrogênio (N) e P é um dos principais fatores limitantes à produção agrícola. Desse modo, FMAs e, também, BFNNL desempenham importante papel na disponibilidade destes nutrientes para as plantas.

As BFNNL, conhecidas como rizóbios, associam-se ao sistema radicular da planta, formando estruturas denominadas “nódulos”, onde ocorre a fixação biológica de nitrogênio – FBN. Estas bactérias transformam o N_2 atmosférico

em amônia, forma assimilável pela planta, sendo capaz de suprir total ou parcialmente a demanda de N da cultura.

No Brasil, o melhor exemplo do uso de bactérias fixadoras de N_2 – BFN, está no cultivo da soja, onde se tem excelentes resultados com o uso de inoculantes comerciais, contendo estirpes do gênero *Bradyrhizobium*. O uso de BFN, por meio de inoculantes comerciais, é uma via alternativa ao uso de fertilizantes nitrogenados, uma vez que os custos com estes são onerosos (DÖBEREINER, 1990; MOREIRA; SIQUEIRA, 2006) e, quando mal manejados, podem causar danos ao meio ambiente e à saúde humana.

Atualmente, o gênero *Bradyrhizobium* possui 19 espécies descritas, oriundas de diferentes regiões geográficas. Estudos realizados por nosso grupo de trabalho (GUIMARÃES et al., 2012; JARAMILLO et al., 2013; LIMA et al., 2009; RUFINI et al., 2014), têm demonstrado alta diversidade de *Bradyrhizobium* nativos, em solos brasileiros, indicando possíveis novas espécies para esse gênero.

As raízes de leguminosas ocorrem, normalmente, micorrizadas na natureza e, quando noduladas, formam uma simbiose tripartite BFN/Leguminosas/FMAs (ANTUNES et al., 2006; CARVALHO; MOREIRA, 2010). Os flavonoides, moléculas emitidas por exsudados radiculares, podem atuar no processo de autorregulação dessa simbiose, para ambos (BFNNL e FMAs).

No Brasil, estudos têm demonstrado a atuação do isoflavonoide formononetina, como estimulante da micorrização em culturas agrícolas como soja e milho (CORDEIRO, 2007; ROMERO, 1999). Entretanto, a maioria dos trabalhos, realizada com soja e milho, além de outras culturas, foi conduzida em condições controladas (NOVAIS; SIQUEIRA, 2009; SILVA-JÚNIOR; SIQUEIRA, 1997, 1998; SIQUEIRA et al., 1999).

Este trabalho faz parte do projeto "Biofertilizante formononetina (isoflavonoide) como estimulante de micorrização em soja e milho para aumento de produtividade associada à eficiência do uso de fertilizantes minerais", coordenado pela Universidade Federal de Lavras, aprovado no Edital MCT/CNPq/CT-AGRO Nº 69/2009: Microrganismos Facilitadores da Nutrição Vegetal. Foi executado em cinco estados brasileiros, Goiás, Minas Gerais, Mato Grosso do Sul, Piauí e Santa Catarina, por diferentes instituições, tendo como objetivo realizar ensaios em campo que comprovem a eficácia de um produto estimulante da micorrização em milho e soja em várias regiões, visando obter as informações necessárias à validação e registro para sua comercialização no Brasil.

O presente trabalho foi realizado com os objetivos de: 1 - Avaliar o efeito do isoflavonoide formononetina associado à adubação fosfatada na colonização micorrízica, nodulação e produtividade de soja em condições de campo; 2 - Caracterizar simbiótica e genotipicamente estirpes bacterianas pertencentes ao gênero *Bradyrhizobium*, isoladas de solos de diferentes regiões do Brasil, de áreas onde foram implantados experimentos com o mesmo objetivo anterior.

2 REFERENCIAL TEÓRICO

2.1 Fixação biológica de nitrogênio em soja

A soja (*Glycine max* (L.) Merrill) é uma importante fonte de proteína na cadeia alimentar humana e animal. Esta cultura apresenta grande importância econômica e social para o Brasil. Atualmente, a soja é a cultura agrícola brasileira que mais cresceu nas últimas três décadas e corresponde a 49% da área plantada em grãos, elevando o Brasil ao maior produtor mundial (BRASIL, 2014). Nas safras de 2010, 2011 e 2012 o Brasil obteve uma produção superior a 65 milhões de toneladas de grãos e em 2013 alcançou 81 milhões de toneladas (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2013).

O N é o nutriente requerido em maior quantidade pelas plantas. Os gastos com fertilizantes nitrogenados são muito onerosos e a produção destes insumos são provenientes de fontes energéticas não renováveis. Deve-se considerar, ainda, que o uso indiscriminado de fertilizantes pode resultar em poluição ambiental, pois a lixiviação, lavagem do perfil do solo por percolação ou escorrimento superficial da água de chuva ou irrigação, pode resultar no acúmulo de formas nitrogenadas, principalmente nitrato (NO^-_3), nas águas de rios, lagos e lençóis subterrâneos, podendo, ainda, atingir níveis tóxicos a animais e humanos (MOREIRA; SIQUEIRA, 2006).

Embora o N_2 se difunda para o espaço poroso do solo, os animais e as plantas não conseguem utilizá-lo como nutriente, em função da tripla ligação existente entre os dois átomos de N. No entanto, bactérias que possuem a enzima nitrogenase são capazes de fixar, biologicamente, o N_2, rompendo a tripla ligação do N_2 atmosférico e reduzindo a amônia (NH_3), a mesma forma obtida no processo industrial. Portanto, as BFNNL se apresentam como uma excelente alternativa ao uso de fertilizantes nitrogenados.

O manejo das BFN, para aumento da produtividade, tem seu exemplo mais significativo na cultura da soja. Por exemplo, no Brasil a exploração da simbiose entre BFNNL do gênero *Bradyrhizobium* e plantas de soja, por meio de inoculantes comerciais, com a utilização de *B. japonicum* (SEMIA 5079 and 5080) e *B. elkanii* (SEMIA 587 and 5019), substitui total ou parcialmente a adubação nitrogenada, economizando algo em torno de 3 bilhões de dólares ao ano, em fertilizantes e tornando esta cultura mais competitiva no mercado em virtude da redução nos custos de produção (MOREIRA; SIQUEIRA, 2006).

2.2 O gênero *Bradyrhizobium*

O gênero *Bradyrhizobium*, dentro de alfaproteobacteria é tradicionalmente associado com plantas da família Leguminosae. Este gênero foi proposto por Jordan (1982), o qual inclui bactérias de crescimento lento que produzem reação alcalina em meio de cultura com manitol como fonte de carbono.

Atualmente, há 19 espécies de *Bradyrhizobium* descritas: *B. japonicum* (JORDAN, 1982), *B. elkanii* (KUYKENDALL et al., 1992), *B. liaoningense* (XU et al., 1995), *B. yuanmingense* (YAO et al., 2002), *B. betae* (RIVAS et al., 2004), *B. canariense* (VINUESA et al., 2005a), *B. denitrificans* (BERKUM et al., 2006), *B. pachyrhizi* e *B. jicamae* (RAMÍREZ-BAHENA et al., 2009), *B. iriomotense* (ISLAM et al., 2008), *B. cytisi* (CHAHBOUNE et al., 2011), *B. lablabi* (CHANG et al., 2011), *B. daqingense* (WANG et al., 2012), *B. huanghuaihaiense* (ZHANG et al., 2012), *B. oligotrophicum* (RAMÍREZ-BAHENA et al., 2012), *B. rifense* (CHAHBOUNE et al., 2012), *B.arachidis* (WANG et al., 2013), *B. retamae* (GUERROUJ et al., 2013) e, mais recentemente, a reclassificação de *B. japonicum*, estirpes do grupo Ia, como *B. diazoefficiens* (DELAMUTA et al., 2013). Na tabela 1 estão listadas as espécies

de leguminosas que se associam simbioticamente com as estirpes tipo de *Bradyrhizobium*. Das 19 espécies descritas. a soja representa a leguminosa com maior número de espécies de *Bradyrhizobium* descritas, as quais incluem (*B. japonicum*, *B. elkanii*, *B. liaoningense*, *B. huanghuaihaiense*, *B. daqingense* e *B. diazoefficiens*).

O gênero *Bradyrhizobium* inclui bactérias simbióticas que induzem a formação de nódulos radiculares em plantas leguminosas, resultando na FBN. Algumas espécies desse gênero (*B. betae* e *B. oligotrophicum*) têm sido encontradas como endofíticas em espécies não leguminosas (RAMÍREZ-BAHENA et al., 2012; RIVAS et al., 2004), porém a capacidade nodulífera e FBN não foi confirmada. O gênero *Bradyrhizobium* inclui espécies eficientes quanto a FBN, algumas dessas estirpes pertencentes às espécies de *B. japonicum* *B. elkanii* foram selecionadas e são usadas como inoculantes comercias para soja, além de outras culturas como caupi, espécies florestais, dentre outras.

O sequenciamento parcial do gene 16S rRNA sozinho não é suficiente para diferenciação de estirpes bacterianas a nível de espécie. Isso ocorre, pois este gene apresenta alto grau de conservação, o que resulta em alta similaridade entre as sequências. Portanto, o gene 16S rRNA é amplamente conhecido por apresentar baixo poder de descriminação entre espécies de *Bradyrhizobium* (VINUESA et al., 2005b; WILLEMS et al., 2001), o que tem sido comprovado por outros autores (DELAMUTA et al., 2012; DELAMUTA et al., 2013; GUIMARÃES, 2013; MENNA et al., 2009; RIVAS et al., 2009). Uma melhor resolução em nível de espécie tem sido obtido pela análise de genes *housekeeping* (DELAMUTA et al., 2012; GUIMARÃES, 2013; MENNA et al., 2009; RIVAS et al., 2009; STEPKOWSKI et al., 2005), os quais têm sido amplamente usados em estudos de classificação taxonômica.

Na tabela 2 são apresentados os testes moleculares usados na identificação das estirpes tipo de *Bradyrhizobium*. Os genes *housekeeping*

(*atpD*, *dnaK*, *glnII*, *gyrB*, *recA* e *rpoB*) estão envolvidos com funções celulares fundamentais e são amplamente utilizados em trabalhos de descrição de espécies de *Bradyrhizobium*. O gene *atp*D codifica a subunidade beta da ATP sintase; o gene *dna*K codifica uma enzima conservada Hsp70 da classe da chaperone; o gene *glnII* codifica a subunidade β da DNA gyrase; o gene *gyr*B codifica a enzima topoisomerase II, responsável pela introdução de supercoil negativo no cromossomo bacteriano, durante a replicação do genoma; o gene *rec*A codifica a recombinase A que está envolvida na recombinação das sequências complementares de DNA e possibilitam a troca de fragmentos de DNA, e o gene *rpo*B codifica a subunidade β da RNA polymerase.

Em estudos prévios do nosso grupo de trabalho, empregando a análise do gene 16S rRNA (GUIMARÃES et al., 2012; JARAMILLO et al., 2013; LIMA et al., 2009; RUFINI et al., 2014) e, mais recentemente com os genes *housekeeping* (GUIMARÃES, 2013), têm indicado uma expressiva diversidade genética de *Bradyrhizobium* nativos em solos brasileiros. Todas as estirpes de *Bradyrhizobium*, analisadas nos trabalhos acima citados, foram provenientes de áreas nunca antes inoculadas. No trabalho de Guimarães (2013), foi verificada uma alta diversidade de *Bradyrhizobium*, no qual se descreve uma nova espécie (*Bradyrhizobium amazonense*), isolada de solos da Amazônia brasileira. Nesse trabalho, também, são apresentados outros grupos com possíveis novas espécies a serem descritas em trabalhos futuros. Estes trabalhos conflitam com relatos de outros autores, os quais indicam que os solos brasileiros são desprovidos de *Bradyrhizobium* nativos e a presença de *Bradyrhizobium*, em nossos solos, foi oriunda dos primeiros inoculantes, vindos dos EUA, para a soja (MARTÍNEZ-ROMERO; CABALLERO-MELLADO, 1996), apesar da predominância destes, em áreas de floresta nativa (MOREIRA et al., 1993).

Tabela 1 Espécies de leguminosas que estabelecem simbiose com as estirpes tipo de *Bradyrhizobium*

Espécies	Teste de nodulação		Origem	Referências
	Positivo	Negativo		
B. japonicum LMG 6138^T	*Glycine max*, *Glycine soja*, *Macroptilium atropurpureum*	nd	China	Jordan (1982)
B. elkanii LMG 6134^T	*Glycine* sp.			Kuykendall et al. (1992)
B. liaoningense LMG 18230^T	*Glycine max*, *Glycine soja*, *Phaseolus aureus*	*Pisum sativum*, *Lotus* sp., *Astragalus sinicus*, *Melilotus* sp.	China	Xu et al. (1995)
B. yuanmingense LMG 21827^T	*Lespedeza* sp., *Vigna unguiculata*, *Glycyrrhiza uralensis*, *Medicago sativa*, *Melilotus albus*	*Glycine max*, *Phaseolus vulgaris*, *Pisum sativum*, *Galega officinalis*, *Trifolium repens*, *Leucaena leucocephala*	China	Yao et al. (2002)
B. betae LMG 21987^T	ns	*Glycine max*, *Pachyrrihizus ahipa*	Espanha	Rivas et al. (2004)
B. canariense LMG 22265^T	*Lupinus* spp., *Adenocarpus* spp., *Chamaecytisus proliferus*, *Spatocytisus supranubius*, *Teline* spp.	*Glycine max*, *Glycine soja*	Ilhas Canárias (Espanha)	Vinuesa et al. (2005a)
B. denitrificans LMG 8443^T	*Aeschynomene indica*	nd	Alemanha	Berkum et al. (2006)
B. iriomotense EK05^T	*Macroptilium atropurpureum*		Japão	Islam et al. (2008)

"Tabela 1, conclusão"

Espécies	Teste de nodulação			Referências
	Positivo	Negativo	Origem	
B. jicamae PAC68^T	*Pachyrhizus erosus*, *Lespedeza* sp.	*Glycine max*	Honduras	Ramírez-Bahena et al. (2009)
B. pachyrhizi PAC48^T	*Pachyrhizus erosus*	*Lespedeza* sp., *Glycine max*	Honduras	Ramírez-Bahena et al. (2009)
B. lablabi CCBAU 23086^T	*Lablab purpureus*, *Arachis hypogaea*, *Vigna unguiculata*	*Glycine max*, *Trifolium repens*, *Lotus corniculatus*, *Vigna radiata*, *Pisum sativum*, *Medicago sativa*	China	Chang et al. (2011)
B. cytisi CTAW11^T	*Cytisus villosus*	*Glycine max*		Chahboune et al. (2011)
B. huanghuaihaiense CCBAU23303^T	*Glycine max*, *Vigna unguiculata*, *Medicago sativa*	*Trifolium repens*, *Lotus corniculatus*, *Phaseolus vulgaris*, *Pisum sativum*	China	Zhang et al. (2012)
B. daqingense CCBAU 15774^T	*Glycine max*, *Vigna unguiculata*, *Medicago sativa*	Trifolium repens, Lotus corniculatus, Phaseolus vulgaris, Pisum sativum	China	Wang et al. (2012)
B. oligotrophicum LMG 10732^T	ns	nd	Japão	Ramírez-Bahena et al. (2012)
B. rifense CTAW71^T	*Cytisus villosus*	*Glycine max*		Chahboune et al. (2012)
B. retamae Ro19^T	*Retama Sphaerocarpa*, *Retama Monosperma*	*Glycine max*	Espanha e Marrocos	Guerrouj et al. (2013)
B. arachidis CCBAU 051107^T	*Arachis hypogaea*, *Lablab purpureus*	nd	China	Wang et al. (2013)
B. diazoefficiens USDA 110^T	*Glycine max*	nd	EUA	Delamuta et al. (2013)

nd = não definido

Tabela 2 Análises moleculares usadas para caracterização e identificação de estirpes tipo de *Bradyrhizobium*: 1 (16S r RNA); 2 (23S rRNA); 3 (ITS 16S-23S rRNA); 4 (IGS 16S-23S rRNA); 5 (*nod*C); 6 (*nod*D); 7 (*nif*H); 8 (*vir*A); 9 (*puf*M); 10 (*atp*D); 11 (*gln*II); 12 (*rec*A); 13 (*dna*K); 14 (*rpo*B); 15 (*gyr*B); 16 (Restriction Fragment Length Polymorfism - RFLP); 17 (Random Amplification of Polymorphic DNA - RAPD); 18 (rep-PCR); 19 (BOX-PCR); 20 (Amplified Fragment Length Polymorfism - AFLP); 21 (Multilocus Enzyme Electrophoresis - MLEE); 22 (DNA-DNA hybridization)

Espécies	**Marcadores Moleculares (1-15)**										**Técnicas moleculares (16-22)**											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
B. japonicum LMG 6138^T																						x
B. elkanii LMG 6134^T	x*															x						x
B. liaoningense LMG 18230^T	x																					x
B. yuanmingense LMG 21827^T	x															x						x
B. betae LMG 21987^T	x		x			x	x	x									x					x
B. canariense LMG 22265^T	x		x		x		x			x	x	x						x			x	x
B. denitrificans LMG 8443^T	x	x	x						x											x		
B. iriomotense EK05^T	x		x		x		x			x	x	x			x							x
B. jicamae PAC68^T	x		x		x		x			x	x						x					x
B. pachyrhizi PAC48^T	x		x		x		x			x	x						x					x
B. lablabi CCBAU 23086^T	x			x	x		x			x	x	x				x			x			x
B. cytisi CTAW11^T	x				x		x			x	x	x					x					x
*B. huanghuaihaiense*CCBAU23303^T	x			x	x		x			x	x	x				x			x			x
B. daqingense CCBAU 15774^T	x			x	x		x			x	x	x							x			x
B. oligotrophicum LMG 10732^T	x									x	x	x										x
B. rifense CTAW71^T	x									x	x	x										x
B. retamae Ro19^T	x				x					x	x	x					x					x
B. arachidis CCBAU 051107^T	x			x	x		x			x	x	x	x	x		x						x
B. diazoefficiens USDA 110^T	x		x							x	x	x	x	x	x							x

* Testes realizados no trabalho original de descrição de cada espécie

2.3 Fungos micorrízicos arbusculares – FMAs

Os FMAs, Filo *Glomeromycota*, Classe *Glomeromycetes* são organismos biotróficos obrigatórios, que se associam com raízes de plantas vasculares terrestres, epífitas, aquáticas e, também, com rizoides e talos de briófitas e outros vegetais basais, formando relação simbiótica mutualista denominada micorriza arbuscular (MA) (STURMER et al., 2010).

Os FMAs são importantes na agricultura e ecossistemas naturais, pois desempenham papel fundamental na ciclagem de nutrientes, especialmente o P. Além desses benefícios destacam se, também: a) favorecimento de microrganismos benéficos, como fixadores de N_2 e solubilizadores de fosfato; b) amenização dos efeitos adversos do pH e excesso de elementos traços na absorção de nutrientes; c) favorecimento na relação água-planta; d) redução dos danos causados por patógenos; e) maior tolerância a estresses ambientais e fatores fitotóxicos; f) melhoria na agregação do solo (MOREIRA; SIQUEIRA, 2006; SILVA-JÚNIOR; SIQUEIRA, 1998; SIQUEIRA et al., 2010).

A melhoria na nutrição fosfatada das plantas é reconhecida como um dos maiores benefícios das micorrizas, que tem sido verificado em vários trabalhos (MOREIRA et al., 2010; NOVAIS; SIQUEIRA, 2009; PAULA et al., 1990; SANTOS et al., 2008; SIQUEIRA et al., 1991). As respostas variam com o nível de P, pH e teor de Al com a espécie de FMA utilizada e com a cultivar ou espécie de planta.

As micorrizas, embora possam ser utilizadas para minerar P, não adicionam P ao solo, mas, sim, alteram sua dinâmica, por meio da produção de ácidos orgânicos específicos, eficazes na solubilização ou alteração da dinâmica do equilíbrio das formas de P no solo, além da produção de quelantes e complexantes capazes de mobilizar principalmente o P. Esses mecanismos são potencializados pelas micorrizas, por estas apresentarem uma capacidade de

maior área de exploração do solo, por meio das hifas fúngicas. As hifas possuem taxa de extensão 823 vezes maior do que a da raiz, o que lhes assegura elevada capacidade de absorção e eficiência (MOREIRA; SIQUEIRA, 2006). Portanto, o potencial das micorrizas está em liberar P que está ou poderia tornar-se fixado.

O biotrofismo obrigatório, apresentado pelos FMAs, ou seja, a dependência do estabelecimento da simbiose com plantas hospedeiras para completar seu ciclo de vida, dificulta o estudo de alguns aspectos biológicos destes organismos, bem como sua aplicação em processos biotecnológicos, a exemplo da produção de inoculante em escala comercial.

2.4 Atuação de isoflavonoides na simbiose tripartite (BFNNL/Leguminosas/FMAs)

Plantas da família Leguminosae formam associação simbiótica com ambos BFN e FMAs, a interação entre os três organismos resulta na simbiose tripartite (ANTUNES et al., 2006). Os processos de infecção radicular por BFNNL e FMAs são mediados por trocas de sinais moleculares entre a planta e os microssimbiontes.

As leguminosas, por exemplo, exsudam diversos metabólitos, principalmente flavonoides, como genisteína, daidzeína, coumestrol, entre outros, que induzem os genes de nodulação (genes Nod) da bactéria. A bactéria, por sua vez, sintetiza fatores de nodulação (fatores nod), também identificados como lipoquitooligossacarídeos, que desencadeiam na planta uma série de respostas, resultando em sua infecção pelos rizóbios e consequente formação de nódulos e FBN (CATFORD et al., 2006; HUNGRIA; STACEY, 1997; KOSSLAK et al., 1987).

As micorrizas, por sua vez, compartilham processos de interação similar ao das BFNNL, em que alguns flavonoides estimulam a germinação de esporos

e o crescimento de hifas, resultando na colonização da raiz por FMAs (ANTUNES et al., 2006; SIQUEIRA et al., 1991).

Flavonoides ou bioflavonoides são metabólitos secundários da classe dos polifenóis, componentes de baixo peso molecular, encontrados em diversas espécies vegetais. Mais de 10.000 flavonoides foram identificados em plantas. Dependendo da estrutura, os flavonoides podem inibir ou estimular a expressão de genes nod em rizóbio, causar quimiotaxia de rizóbios em direção à raiz, inibir patógenos radiculares, estimular a germinação de esporos e ramificação de hifas em FMAs, afetar *quorum sensing*, mediar interações alelopáticas em plantas e quelar nutrientes do solo (HASSAN; MATHESIUS, 2012).

A descoberta do isoflavonoide formononetina como estimulante da colonização micorrízica (NAIR et al., 1991; SIQUEIRA et al., 1991) abriu novas perspectivas para manejo dos FMAs nativos, na agricultura extensiva. Nair et al. (1991) isolaram e identificaram substâncias produzidas por raízes de trevo (*Trifolium repens*) estressadas pela deficiência de P e verificaram que estas substâncias da classe dos isoflavonoides foram ativas sobre propágulos de FMAs, com destaque para a formononetina. Posteriormente, Siqueira et al. (1991) comprovaram que essa substância favoreceu o crescimento de *T. repens* por promover aumento na absorção de nutrientes, especialmente do P, decorrente do estímulo à micorrização.

A principal utilização do isoflavonoide formononetina na agricultura é referente à sua utilização como estimulante a colonização micorrízica. Em países como EUA, Espanha, Holanda, Colômbia e Índia, o produto comercial Myconate®, à base de formononetina, já é comercializado como insumo agrícola biológico. O produto Myconate® foi formulado pela empresa VAMTech L.L.C., na forma de sal de potássio (sal de potássio de 7-hidroxi, 4’-metoxi-isoflavona) com peso molecular 306, também chamado formononetina, solúvel em água (1g em 3 ml de água), apropriados para a aplicação em larga

escala. No site da empresa (http://www.planthealthcare.com/myconate), são mostrados resultados da eficácia do produto no aumento da produção de várias culturas e em vários países.

No Brasil, experimentos com a formononetina, em condições de campo, foram observados aumentos de 14 a 28% na produção da cultura do milho (ROMERO, 1999). Na cultura da soja, Cordeiro (2007) verificou que o número e o peso seco de nódulos foram maiores, quando aplicada formononetina sob condições de baixo nível de P, por outro lado não foram verificadas diferenças para a produção de grãos entre os tratamentos.

Experimentos conduzidos em casa de vegetação, também confirmam a atuação do bioestumulante formononetina nos processos de interação FMAs/planta (NOVAIS; SIQUEIRA, 2009; SILVA-JÚNIOR; SIQUEIRA, 1997, 1998; SIQUEIRA et al., 1991, 1999).

Na soja flavonoides primários, como daidzeína, genisteína e coumestrol induzem a transcrição de genes nod em *B. japonicum* (ANTUNES et al., 2006; BORTOLAN et al., 2009; KOSSLAK et al., 1987). Porém, pouco se sabe sobre a formononetina, na interação rizóbio/leguminosa. Kosslak et al. (1987) verificaram diminuição expressiva na expressão de genes nod em *B. japonicum* na presença deste composto. Antunes et al. (2006), com resultados obtidos com base na avaliação com quatro diferentes cultivares de soja, verificaram que os flavonoides genisteína, daidzeína e coumestrol parecem desempenhar importante função nos sinais iniciais de interação para ambos simbiontes, rizóbio e FMA. Entretanto, flavonoides específicos, como a formononetina, não foram detectados nas cultivares avaliadas. Em estudo com a estirpe CPAC 15 de *B. japonicum*, uma das estirpes aprovada pelo Ministério da Agricultura Pecuária e Abastecimento (MAPA), para a produção de inoculantes comerciais para soja no Brasil, Bortolan et al. (2009) constataram que genes de nodulação são ativados na presença de genisteína ou exsudados de sementes de soja, no estágio inicial

do processo de nodulação. No tratamento com *B. japonicum* e adição de exsudados de sementes de soja a expressão de genes indutores foi superior ao tratamento controle. Contudo, neste trabalho não foi especificado quais compostos estavam presentes nos exsudados de sementes de soja. Estes compreendem uma complexidade de compostos, incluindo flavonoides nos quais podem conter substâncias reconhecidas como indutoras e/ou repressoras da expressão de genes nod.

Em virtude das micorrizas aumentarem a capacidade de absorção de nutrientes, principalmente o P, a nodulação e a FBN é indiretamente beneficiada pela micorrização de leguminosas, principalmente em solos com deficiência de P (BRESSAN et al., 2001; MOREIRA et al., 2010), isso é importante pois, o teor de P no solo é um fator limitante para a FBN e as plantas em solos tropicais. Em contrapartida, o suprimento de N, para a planta, promovido pelas BFNNL, beneficia a micorriza pela maior oferta de fotoassimilados.

Bressan et al. (2001) constataram que a inoculação com FMAs proporcionou aumentos na matéria seca dos nódulos, porém este aumento variou de acordo com as espécies de FMAs utilizados e os níveis de P no solo. Moreira et al. (2010) verificaram que o P foi o nutriente mais limitante, seguido por N, para o desenvolvimento das espécies de leguminosas avaliadas *Albizia lebbeck*, *Enterolobium contortisiliquum*, *Leucaena leucocephala* e *Sesbania virgata*. A dupla inoculação com BFN e FMAs aumentou o crescimento de todas as plantas quando comparado ao tratamento com uma única inoculação de BFN ou FMAs, exceto para *A. lebbeck* que não respondeu à inoculação com BFN e/ou FMA.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 **Effect of formononetin associated with phosphorus fertilization on mycorrhizal colonization, nitrogen fixation and soybean productivity**

Artigo preparado de acordo com as normas para submissão do periódico Biology and Fertility of soils

Effect of formononetin associated with phosphorus fertilization on mycorrhizal colonization, nitrogen fixation and soybean productivity

Running title: Formononetin and phosphorus on soybean symbiosis

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Abstract The goal of the present study was to evaluate the effect of the biostimulant isoflavone formononetin on soybean and its symbiosis with beneficial microorganisms in combination with three levels of phosphorus (P) fertilization. Two experiments were conducted during the 2010/2011 and 2012/13 agricultural years at the experimental farm of Lavras Federal University in Ijaci municipality, Minas Gerais. A 3 x 4 factorial scheme (three levels of P: 0, 60 and 120 kg hectare $(ha)^{-1}$ P_2O_5 and four doses of formononetin: 0, 25, 50 and 100 g ha^{-1}) was used with five replicates. The three levels of P tested were 0, 50 and 100% of the recommended levels of fertilizer, with soil analysis as reference. Plant height, shoot dry weight (SDW), nodule number, nodule dry weight, mycorrhizal colonization (MC) and shoot nitrogen (N) and P concentrations at full bloom and productivity at the end of the crop cycle were quantified. Application of formononetin had no effect on shoot dry weight, nodule number and nodule dry weight. Formononetin had a positive effect on MC only at lower levels of P (0 and 60 kg ha^{-1}), with colonization increasing from 56 to 64%. Application of formononetin together with 60 kg ha^{-1} P_2O_5 increased soybean productivity to values equivalent to those observed when the highest level of P was applied. Seed treatment with formononetin doses higher than 50 g ha^{-1} decreases the need for P fertilization by up to 50% of the recommended levels of fertilizer.

Keywords *Glycine max* · isoflavonoid · biostimulant · root symbiosis

Introduction

Soybean (*Glycine max* (L.) Merrill) is an important source of protein in the human and animal food chains. This crop has high economic and social importance in Brazil, which is its greatest producer worldwide. Soybean production levels in Brazil from the 2010, 2011 and 2012 harvests were greater than 65 million tons of grains, reaching 81 million tons in 2013 (CONAB 2013).

Nitrogen (N) and phosphorus (P) are important nutrients for plant growth. However, their availability is generally low in tropical soils, making them the main limiting factors of the growth and productivity of important crops. In tropical soils, as

found in Brazil, clays are mainly of the 1:1 type, which increases the immobilization of P due to the formation of phosphates, iron and aluminum. Because of this, the annual addition of high volumes of P fertilizers to the soil is necessary.

However, P-based fertilizers are agricultural inputs obtained from non-renewable sources, and N fertilizers are obtained from atmospheric N_2 using industrial processes that consume energy from oil derivatives. In both cases, the processes involved in their production make these inputs onerous. In addition, when badly managed, the use of fertilizers may cause damage to the environment and human health.

In the case of leguminous plants such as soybean, demand for N may be completely suppressed when supplied by symbiosis with N-fixing bacteria (NFB) belonging to the genus *Bradyrhizobium*. On the other hand, arbuscular mycorrhizal fungi (AMF) have been shown to improve P uptake by their host plants due to the establishment of a hyphal network and consequent increase in nutrient uptake from the soil. Despite the known beneficial effects of these fungi, their use in agriculture is limited because they are obligate biotrophs and their inoculum mass production is economically unviable.

However, the discovery of the effects of the isoflavone formononetin stimulant on mycorrhizal colonization (MC) (Nair et al. 1991; Siqueira et al. 1991a) has opened new possibilities for the management of native AMF in extensive agriculture. Nair et al. (1991) isolated and identified substances produced by the roots of P-deficient clover (*Trifolium repens*) and observed that the identified isoflavones affected AMF propagules, especially formononetin. Siqueira et al. (1991a) confirmed that the formononetin stimulated the growth of clover (*T. repens*), resulting in increased plant nutrient uptake, especially P, due to mycorrhization stimulation.

Soybean plants form symbiotic associations with both NFB and AMF, with the interaction among the three organisms resulting in a tripartite symbiosis (Antunes et al. 2006). Synergetic effects of this tripartite symbiosis in leguminous plants have been observed, mainly in conditions of low levels of P and N (Moreira et al. 2010; Wang et al. 2011).

Formononetin affects in vitro sporulation of AMF (Romero and Siqueira 1996) and colonization and sporulation of AMF in *Brachiaria* (Novais and Siqueira 2009) and

stimulates colonization in maize and soybean (Silva-Júnior and Siqueira 1997; Silva-Júnior and Siqueira 1998). However, these studies were all performed under controlled conditions that do not represent the complexity of the soil environment. Field experiments are therefore essential to confirm the effect of formononetin on AMF/rhizobium/Leguminosae interactions and on parameters of phytotechnic interest, such as productivity. This is especially important because an increase in P uptake efficiency, associated with satisfactory production yields, can considerably decrease the need for P fertilization. Beneficial effects of formononetin on MC were reported for crops, such as alfalfa, potato and watermelon (Davies et al. 2005a, 2005b; Catford et al. 2006; Westphal et al. 2008). However, most of these experiments were conducted under controlled conditions.

The goal of the present study was to evaluate the effect of the isoflavone formononetin associated with P fertilization on mycorrhizal colonization, nodulation and productivity of soybean under field conditions over two years of cultivation.

Materials and methods

Site characterization

Two experiments were conducted at the Experimental Farm at Federal University of Lavras in Ijaci municipality, Minas Gerais (MG) (experiment 1: 21º12', 17''S, 44º 58'49''W and 957 m altitude; experiment 2: 21º12' 10''S, 44º 58'50''W and 957 m altitude) during the agricultural years of 2010/11 and 2012/13. The region's climate is mesothermal, with dry winters (Cwb), a 19.3ºC annual average temperature, and a 1.411 mm annual average rainfall. Average temperatures and accumulated rainfall during the experimental period are presented in Figure 1.

The experimental areas were fallow land until 2009 and were then cultivated with maize until April 2010. The areas were again left to lie fallow with growth of spontaneous vegetation, predominantly *Brachiaria*, until prepared for the experiments.

The soil at the experimental sites is Yellow Red Latosol (Oxisol), with the following chemical and physical characteristics: experiment 1 - water pH = 6.5, H + Al

= 2.3 $cmol_c$ dm^{-3}, Al = 0.01 $cmol_c$ dm^{-3}, Ca = 3.8 $cmol_c$ dm^{-3}, Mg = 1.5 $cmol_c$ dm^{-3}, cation exchange capacity (CEC) = 7.9 $cmol_c$ dm^{-3}, K= 101.0 mg dm^{-3}, P = 10.3 mg dm^{-3}, clay = 570 g kg^{-1}, silt = 80 g kg^{-1}, and sand = 350 g kg^{-1}; experiment 2 - water pH= 5.9, H + Al = 2.9 $cmol_c$ dm^{-3}, Al = 0.0 $cmol_c$ dm^{-3}, Ca = 4.7 $cmol_c$ dm^{-3}, Mg = 1.3 $cmol_c$ dm^{-3}, CEC = 9.2 $cmol_c$ dm^{-3}, K = 118.0 mg dm^{-3}, P = 7.2 mg dm^{-3}, clay = 570 g kg^{-1}, silt = 80 g kg^{-1}, and sand = 350 g kg^{-1}.

Density of native nodulating nitrogen-fixing bacteria in Leguminosae (NNFBL) and AMF spores

An experiment was conducted to estimate the most probable number (MPN) of cells of NNFBL native to the cultivated area. Prior to preparation for experiment 1 (2010/11), three composite samples obtained from five subsamples were collected from the 0-20 cm soil layer along the experimental area. The samples were placed in sterile plastic bags and stored at 4ºC until analysis.

The experiment was conducted in a greenhouse at the Biology, Microbiology and Soil Biology Processes sector of Lavras Federal University. Longneck glass bottles (500 mL), recyclable and sterilized, containing Hoagland and Arnon (1950) nutrient solution with 5.25 mg L^{-1} N as NH_4NO_3 and KNO_3 were used. Filter paper was used as support for root development of the soybean plants (Florentino and Moreira, 2009). The same soybean cultivar used in the field experiment was used as a trap plant. Soybean seeds were previously surface sterilized with 70% ethanol for 30 seconds and 2-3% sodium hypochlorite for 2 minutes and then washed six times with sterile distilled water. The seeds were pre-germinated in Petri dishes containing filter paper and moistened sterile cotton at 28ºC for 2 days.

Serial dilutions of each of the three soil samples were performed (10^{-1} to 10^{-7}). The pre-germinated seeds were inoculated with 1 mL of each of the serial dilutions, with three replicates per dilution. A positive control inoculated with *Bradyrhizobium elkanii* Br 29 (SEMIA 5019), which is approved as an inoculant for soybean by the Brazilian Ministry of Agriculture, Livestock and Food Supply, and two uninoculated negative

controls, one with the addition of 52.5 mg L^{-1} mineral N and another with 5.25 mg L^{-1} mineral N, a low N supply, were included for the control of possible contamination.

Nodulation was quantified 30 days following inoculation. Determination of the MPN was performed by classifying the presence of nodules as positive and the absence of nodules as negative for each dilution and using the "Most Probable Number Estimate" software (Woomer et al., 1988).

Native AMF spore density was quantified according to Gerdemann and Nicolson (1963) using 50 g of soil from the same soil samples used for the quantification of native NNFBL. These quantifications were performed only during the first year of cultivation.

Experimental design

A randomized block experimental design was used with a 3 x 4 factorial scheme, with the factors being three levels of P addition: 0, 60 and 120 kg ha^{-1} P_2O_5, corresponding to 0, 50 and 100% of the recommended levels (Novais et al. 1999), and four addition doses of the biostimulant formononetin (0, 25, 50 and 100 g ha^{-1}), as the commercial product PHC-506, according to the manufacturers' recommendations (Plant Health Care). The soybean cultivar FAVORITA RR, which is appropriate for the region, was used in both experiments. Six rows, with 6 m long each were set at the experimental plots, with a 0.45 m space between them. Five replicates were used for experiment 1 (2010/11), and 4 replicates were used for experiment 2 (2012/13). The four central row were used for data collection.

Soil preparation consisted of one plowing and one harrowing, followed by the opening of plantation furrows. Fertilization was performed manually prior to sowing. The different levels of P_2O_5 for each treatment were applied as a single superphosphate together with 40 kg K_2O ha^{-1}, as KCl, to the plantation furrows. Soybean seeds were previously inoculated with a commercial peat-based inoculant. Experiment 1: Biomax premium peat (100 g inoculant per 50 kg seeds) containing *Bradyrhizobium japonicum* (SEMIA 5079 and 5080). Experiment 2: Adhere 60^R (60 g inoculant per 50 kg seeds) containing *Bradyrhizobium elkanii* (SEMIA 587 and 5019). The seeds from experiment 2 also received different doses of formononetin. Sowing was performed in December

2010 and 2012, and harvests occurred in April 2011 and 2013. Crop handling (manual weeding) was performed during the course of crop development.

Parameters quantified at flowering (R2 stage) in the field

Mycorrhizal colonization

Approximately 1 g of fine roots was collected from four plants per experimental plot. The roots were washed, cleared with 10% KOH, washed in running water and placed in 1% HCl for 5 minutes. The roots were then stained with Trypan Blue (Koske and Gemma 1989). Stained roots were placed in gridded Petri dishes, and percentage colonization was quantified according to Giovannetti and Mosse (1980).

Number of nodules and nodule dry weight

Plant nodulation was quantified by removing the nodules of the root system of five plants per plot. Roots were placed on a sieve, and the nodules were separated, counted, and dried in an oven at 60ºC to obtain the nodules' dry weight.

Plant height, shoot dry weight, concentrations and total contents of nutrients

Five plants per plot were collected at the R2 stage for the quantification of plant height, shoot dry weight (SDW), concentrations and total contents of nutrients. The collected plants were placed in paper bags and maintained in a forced air circulation oven at 60°C until constant weight was reached, and the SDW was measured using a digital balance. The dry shoots were homogenized and used for the determination of shoot nutrient concentrations. P concentrations were measured following nitric-perchloric digestion of the samples. Ca, Mg, Zn, Cu and Fe concentrations were determined by atomic absorption spectroscopy, P concentrations were determined using colorimetry, and N concentrations were determined using the micro-Kjeldahl method (Malavolta et al. 1997). Shoot total nutrient contents were calculated by multiplying the SDWs by the

nutrient concentrations. For the second cultivation year, only the N and P concentrations were quantified, according to the methodology described above.

Productivity

Productivity was quantified at the end of the crop cycle. Plants of the two central row of each plot were collected, excluding 0.5 m from the transect extremities, with a total 4.5 m^2 useful area. Productivity was corrected for 13% humidity.

Statistical analysis

An analysis of variance followed by a Scott-Knot test and regression analysis were performed using the Sisvar software, version 4.0 (Ferreira 2011).

Results and discussion

Density of NNFBL and AMF

The average density of native NNFBL measured in soybean trap plants was 540 CFU g^{-1} soil. AMF spore density was 3.5 spores per g^{-1} soil. The number of soil spores varies with AMF species, climate, soil characteristics and seasonality (Smith and Read 1997). Siqueira et al. (1991a) observed that the stimulating effect of formononetin on the growth of clover plants mediated by AMF colonization depended on its concentration, the growth period and spore density. In their work, the highest response was found with the application of 2 to 4 spores per g^{-1} soil. It should be noted that in addition to the spores, other structures, such as hyphae fragments, mycelium and colonized roots, can be used for propagation. Because spontaneous vegetation grew in the area prior to soybean planting, part of these structures may have remained active in the soil even following its preparation. This is relevant because the stimulation of MC by formononetin varies with soil infectivity (Siqueira 1991a; Silva-Júnior and Siqueira 1997).

Mycorrhizal colonization

MC varied with the addition of formononetin from approximately 56% up to 66% with different levels of P addition during the two years of study. In 2010/11, with no P addition and 60 kg ha^{-1} P_2O_5, increasing doses of formononetin increased MC from approximately 57% to 64% (Figure 2a). A similar effect was observed in 2012/12 with 60 kg ha^{-1} P_2O_5, with MC increasing from approximately 55% to 61% (Figure 2b). However, with no P_2O_5 addition, formononetin had no effect on MC, which was already high (~63%). In 2010/11, when 100% of the recommended level of P fertilizer (120 kg ha^{-1} P_2O_5) was added to the soil, formononetin had no effect on MC. In fact, the addition of high levels of P tends to inhibit or limit MC, and in conditions of low P availability, mycorrhization tends to be higher compared with plants receiving the maximum supply of P (Moreira et al. 2010).

Several studies, especially those performed under controlled conditions, have shown the benefits of the use of formononetin on mycorrhization (Siqueira et al. 1991a,b; Romero and Siqueira 1996; Silva-Júnior and Siqueira 1997; Novais and Siqueira 2009). For example, Silva-Júnior and Siqueira (1997) observed that in addition to increasing the percentage of colonization, the densities of arbuscules and vesicles were also increased in maize and soybean treated with formononetin. Siqueira et al. (1991a) observed that the effect of formononetin on white clover growth was mediated by higher MC and that the effects depended on the formononetin concentration and spore density in the soil. An 89% increase in sporulation and a 60% increase in MC were reported for *Brachiaria* (Novais and Siqueira 2009). Formononetin was also observed to favor in vitro *Gigaspora gigantea* mycelial growth (Romero and Siqueira 1996).

Ozan et al. (1997), found that, in nonsterile soil about 40% and 95% of the added formononetin could not be recovered after one and three weeks, respectively. This indicates that the soil microbial community is able to metabolize this isoflavone. In fact, the added formononetin was able to stimulate the growth of some soil microorganisms (e.g. actinobacteria and bacteria). In contrast, the concentration of formononetin in the sterile soil did not vary over a period of 15 days after its application. Therefore, the

ability of the soil microrganisms to degradate formononetin is possibly one of the factors that control the outcome of formononetin application on plant growth.

Plant height, shoot dry weight, number of nodules and nodule dry weight

The application of formononetin associated with the addition of P_2O_5 at different levels had no effect on plant height, SDW, number of nodules or nodule dry weight (Table 1). This indicates that formononetin did not have a negative effect on soybean nodulation, which is in accordance with previous reports (Silva-Júnior and Siqueira 1997; Silva-Júnior and Siqueira 1998). In a pot experiment using soil, formononetin stimulated mycorrhizal development but had a reduced effect on soybean dry weight (Silva-Júnior and Siqueira 1997). In white clover, formononetin was observed to increase nodulation in the presence of AMF, which was thought to result from higher MC and plant growth (Siqueira 1991a).

Very little is known about the relationship of formononetin and the nodulation of soybean. The flavones genistein, daidzein and coumestrol appear to have an important role in the early signaling events of the establishment of symbioses with both rhizobium and AMF. However, some flavones, such as glycitein and formononetin, do not appear to act as signaling molecules in the establishment of the tripartite symbiosis in soybean (Antunes et al. 2006). Formononetin was not detected in root and seed of soybean in the study by Antunes et al. (2006). However, the formononetin significantly decreased expression of the *nodABC-lacZ* genes in *B. japonicum* (Kosslak et al. 1987).

A significant effect of P on plant height and SDW was only observed in 2012-13. The highest values were obtained when 120 kg ha^{-1} P_2O_5 was used.

Concentrations and total contents of N and P

Shoot N and P concentrations were not influenced by the application of formononetin or fertilization with P_2O_5 during the two years of cultivation (Table 2). The average N and P concentrations were 21.23 and 1.17 g/kg in 2010/11 and 23.38 and 1.20 g/kg in 2012/13, respectively. These values were below the levels considered adequate for

soybean (Malavolta 2006), which are 55-58 g/kg for N and 4-6 g/kg for P. Leaf concentrations of Ca, Mg, Zn, Cu, Fe and Mn, which were quantified only in 2010-11, were not influenced by the application of formononetin and P_2O_5 (results not shown).

On the other hand, the use of different doses of formononetin and different levels of P_2O_5 had a significant effect on total content of N and P shoot contents only in 2012-13 (Table 3). Increasing doses of formononetin together with the addition of 60 kg P_2O_5 increased the uptake of N and P. In 2010-11, only the Ca and S total contents varied significantly ($P < 0.05$) with the levels of P_2O_5 addition, with no effect of formononetin (results not shown).

Productivity

Average soybean productivity varied from approximately 2010-2674 kg ha^{-1} in 2010/11 and from 1969-2604 kg ha^{-1} in 2012/13 (Figure 3a and 3b). With no addition of P_2O_5, mycorrhizal colonization did not result in increased soybean productivity, regardless of stimulation by formononetin (Figure 2 and 3). The absence of significant effects of formononetin on soybean productivity with no addition of P_2O_5 indicates that application of this isoflavone alone is not sufficient to stimulate mycorrhization to the point of meeting soybean P demand. However, mycorrhization stimulated by formononetin resulted in increased soybean productivity when this crop received 60 kg ha^{-1} P_2O_5. This effect was significant only in 2010/11, when the addition of formononetin together with the addition of 60 kg ha^{-1} P_2O_5 increased productivity from 2100 to 2500 kg ha^{-1}, levels similar to those obtained following the application of the maximum level of P_2O_5 (Figure 3a).

The use of this technology can result in financial gains by the farmers. These gains may be direct, through an increase in productivity, or indirect, through a decrease in P fertilization and decreased costs of transportation and handling. Because formononetin can be applied directly to the seed, labor costs may be reduced. Additionally, a decrease in the application of P_2O_5 by up to 50% of the recommended level from the beginning of the plantation would result in a 50% saving in P fertilization costs.

There are also environmental gains to be considered because P fertilizers used in agriculture originate from non-renewable natural sources. There is an increasing demand for the use of these resources, with a consequent increase in the costs of the agriculture production chain. A rational use of natural resources is therefore urgent. The use of formononetin may be a sustainable alternative for reducing the use of P fertilizers.

It should be pointed out that the effect of formonetin can be very context-dependent, even within the farm scale. For that reason, future studies are necessary to elucidate the factors (e.g. native microbial communities, soil P level, density of AMF propagules, soil class, climate, etc) controlling the effect of formononetin on mycorrhizal colonization, plant growth and yield, especially for crops extensively cultivated, like soybean, which are responsible for an overall large consuption of P fertilizers. These studies may contribute to make the effect of formononetin more predictable allowing for its recommendation and large-scale usage in agriculture.

Conclusions

Formononetin stimulates mycorrhization in the absence of P fertilization but does not increase soybean productivity. However, when the application of formononetin is combined with the application of 60 kg ha^{-1} P_2O_5,productivity increases to levels equivalent to those obtained with the use of the maximum recommended level of P_2O_5 (120 kg ha^{-1}) for soybean cultivation.

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Conflict of interest

The authors declare that have no conflict of interest.

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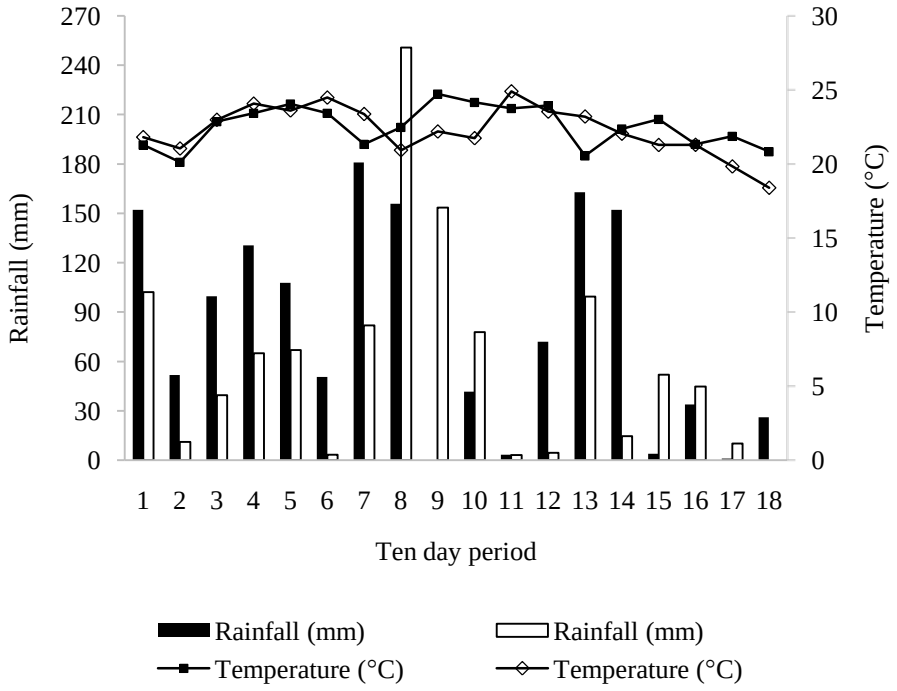

Fig. 1 Average temperature (lines) and accumulated rainfall (bars) per ten day period from 01/11/2010 to 29/04/2011 and from 01/11/2012 to 30/04/2013

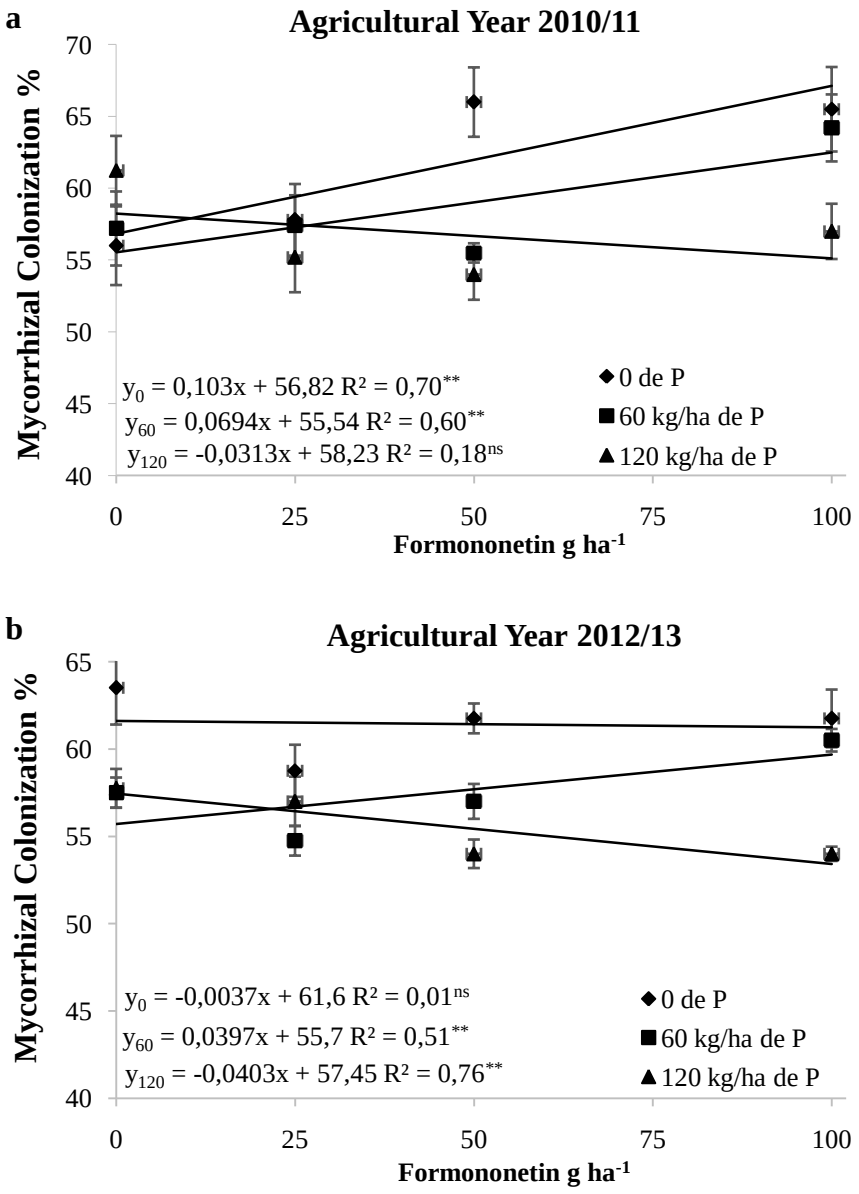

Fig. 2 Mycorrhizal colonization of soybean after treatment with different doses of formononetin and different levels of P for the agricultural years (**a**) 2010/11 and (**b**) 2012/13. **Significant at $P < 0.05$ and ns not significant according to the F test

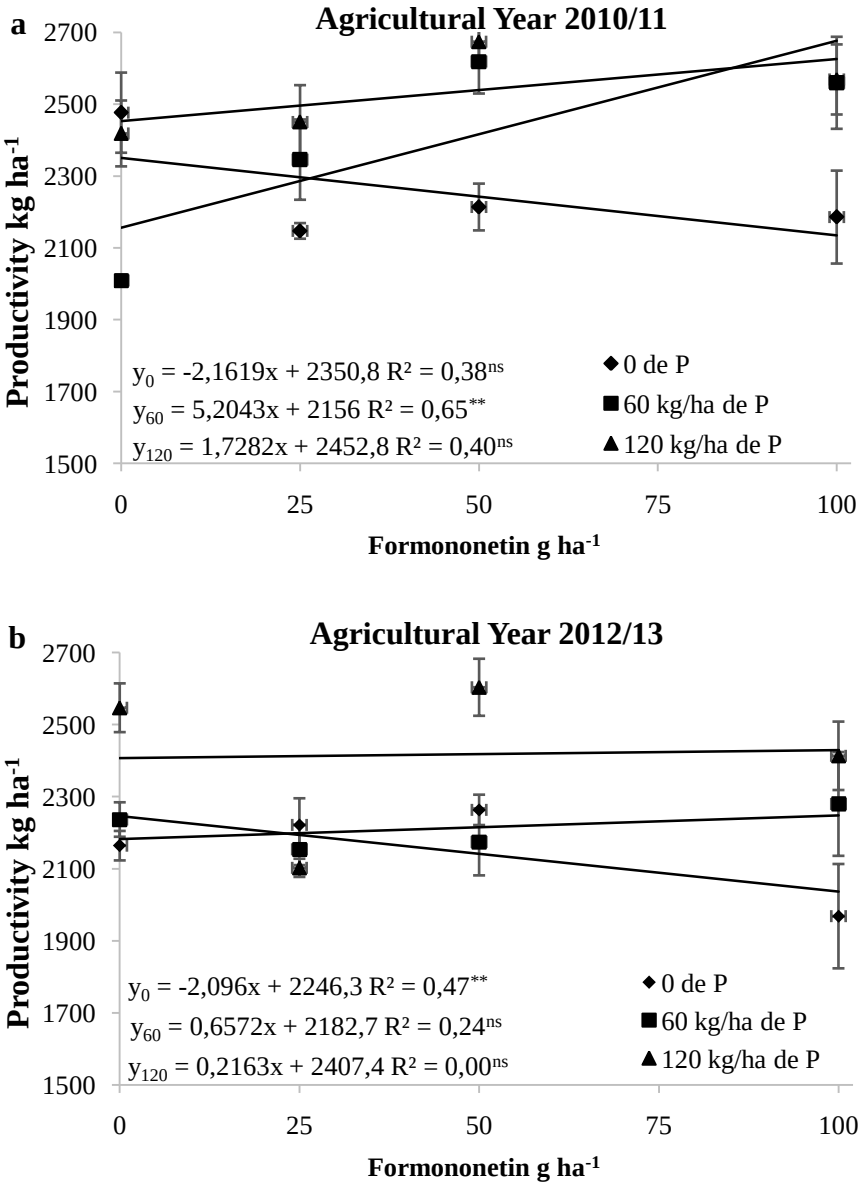

Fig. 3 Productivity of soybean after treatment with different doses of formononetin and different levels P for the agricultural years (**a**) 2010/11 and (**b**) 2012/13. ** Significant at $P < 0.05$ and ns not significant according to the F test

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Table 1 Average values of plant height, shoot dry weight, nodules number and dry weight of soybean cv. Favorita RR at flowering in two crop seasons with different phosphorus levels and formononetin doses.

Phosphorus Levels	Formononetin doses g ha^{-1}							
	2010-11				2012-13			
(kg ha^{-1})	0	25	50	100	0	25	50	100
Plant height (cm)								
0	39.22 aA	46.28 aA	43.18 aA	46.52 aA	37.68 cB	37.80 cB	39.65 cA	41.58 cA
60	45.96 aA	43.36 aA	43.56 aA	48.58 aA	42.83 bB	41.85 bB	43.63 bB	46.60 bA
120	47.72 aA	46.16 aA	42.50 aA	46.82 aA	46.88 aB	48.75 aB	51.05 aA	51.23 aA
Shoot dry weight (g)								
0	9.09 aA	11.42 aA	8.82 aA	8.97 aA	9.46 bB	9.27 cB	10.53 bA	11.14 bA
60	10.69 aA	9.81 aA	12.15 aA	11.61 aA	10.57 bB	10.92 bB	11.64 aA	12.36 aA
120	10.57 aA	11.66 aA	10.78 aA	11.62 aA	13.32 aA	14.96 aA	12.53 aA	13.14 aA
Number of nodules								
0	102 aA	88 aA	88 bA	94 aA	97 aA	108 aA	106 aA	86 bA
60	121 aA	92 aA	95 bA	105 aA	119 aA	85 aA	95 aA	94 bB
120	91 aA	113 aA	124 aA	120 aA	101 aA	100 aA	96 aA	126 aA
Nodule dry weight (g)								
0	1.53 aA	1.62 bA	1.39 bA	1.40 aA	1.82 aA	1.56 bA	1.67 aA	1.54 bA
60	2.13 aA	2.31 aA	1.68 aA	1.63 aA	1.98 aA	1.57 bA	1.81 aA	1.91 aA
120	1.83 aA	1.83 bA	1.90 aA	2.00 aA	1.83 aA	1.94 aA	1.99 aA	2.11 aA

Averages followed by the same letter were not significantly different according to the Scott-Knott test at $P < 0.05$. Upper case letters compare averages among different formononetin application doses, and lower case letters indicate comparisons among different P_2O_5 addition levels within the same agricultural year

Table 2 Average values of N and P concentrations in the shoots of soybean cv. Favorita RR at flowering in two crop seasons with different phosphorus levels and formononetin doses.

Phosphorus Levels	Formononetin doses g ha^{-1}							
	2010-11				2012-13			
(kg ha^{-1})	0	25	50	100	0	25	50	100
N g/kg								
0	22.2 aA	22.0 aA	21.8 aA	20.8 aA	23.7 aA	23.7 aA	19.7 aA	24.0 aA
60	21.2 aA	23.2 aA	21.8 aA	21.6 aA	20.7 aA	27.7 aA	23.0 aA	26.2 aA
120	20.8 aA	18.2 aA	23.2 aA	20.0 aA	21.5 aA	24.0 aA	25.7 aA	20.2 aA
P g/kg								
0	1.00 aA	1.00 aA	1.20 aA	1.00 aA	1.10 aA	1.10 aA	1.20 aA	1.20 aA
60	1.00 aA	1.00 aA	1.20 aA	1.00 aA	1.10 aA	1.30 aA	1.30 aA	1.20 aA
120	1.00 aA	1.20 aA	1.20 aA	1.00 aA	1.20 aA	1.20 aA	1.20 aA	1.30 aA

Averages followed by the same letter were not significantly different according to the Scott-Knott test at $P < 0.05$. Upper case letters compare averages among different formononetin application doses, and lower case letters indicate comparisons among different P_2O_5 addition levels within the same agricultural year

Table 3 Average values of N and P contents in the shoots of soybean cv. Favorita RR at flowering in two crop seasons with different phosphorus levels and formononetin doses.

Phosphorus Levels (kg ha^{-1})	Formononetin doses g ha^{-1}							
	2010-11				2012-13			
	0	25	50	100	0	25	50	100
N mg/plant								
0	178.47 aA	254.01 aA	192.46 aA	183.60 aA	225.72 aA	224.35 bA	205.84 cA	266.44 bA
60	225.26 aA	229.39 aA	264.12 aA	260.51 aA	218.88 aB	303.19 aA	268.29 bB	323.23 aA
120	216.45 aA	209.83 aA	253.25 aA	229.13 aA	266.01 aA	308.18 aA	321.73 aA	267.89 bA
P mg/plant								
0	9.87 aA	12.00 aA	10.72 aA	10.62 aA	10.54 bB	10.03 bB	12.19 bA	13.93 bA
60	11.27 aA	12.11 aA	14.78 aA	14.35 aA	11.59 bB	14.38 aA	15.55 aA	14.52 bA
120	11.70 aA	15.46 aA	14.41 aA	12.50 aA	15.09 aA	15.59 aA	14.72 aA	16.62 aA

Averages followed by the same letter were not significantly different according to the Scott-Knott test at $P < 0.05$. Upper case letters compare averages among different formononetin application doses, and lower case letters indicate comparisons among different P_2O_5 addition levels within the same agricultural

ARTIGO 2 **Genetic diversity of *Bradyrhizobium* strains that nodulate soybeans from soils at different brazilian regions**

Artigo preparado de acordo com as normas para submissão do periodico

Soil Biology and Biochemistry

Genetic diversity of *Bradyrhizobium* strains that nodulate soybeans from soils at different Brazilian regions

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Abstract

The symbiotic N_2-fixing genus *Bradyrhizobium* includes 19 species distributed throughout different geographic regions. Only one species has recently been described based on isolates from tropical soils, although tropical region is considered to be the origin of legume rhizobia symbiosis. Besides, some authors suggested that *Bradyrhizobium* was introduced in Brazil with first soybeans inoculants from USA. In this work, 46 *Bradyrhizobium* strains were isolated from soils collected in different regions of Brazil (Midwest, Northeast, Southeast, and South), using soybean as a trap plant. These strains were characterized genetically by analyzing the 16S rRNA gene and five housekeeping genes (*atpD, gyrB, dnaK, recA,* and *rpoB*). They were also characterized in terms of their symbiotic efficiency with soybean plants grown under axenic conditions in Leonard jars. The phylogenetic analysis of housekeeping genes revealed the possible presence of novel species in the Northeast and Southeast soils, some of which exhibited high symbiotic efficiency with soybean plants. These results emphasize the great diversity among native strains belonging to *Bradyrhizobium* genus in Brazilian soils as well as potential ones to be used as inoculants.

***Keywords*:** Biological nitrogen fixation; *Glycine max*; housekeeping genes; phylogeny; taxonomy

1. Introduction

The genus *Bradyrhizobium* encompasses N_2-fixing bacteria that can live symbiotically with legumes or associated with non-legumes. In Brazil, efficient symbiotic N_2-fixing strains were already selected and are available to be used as inoculants for soybean and several other forest, forage, and green manure legume species (Moreira and Siqueira, 2006). Soybean is an important commoditie in Brazil, USA and Argentina, which are the largest world producers. Inoculation with N_2-fixing *Bradyrhizobium* strains is a common practice that contributes largely to decreasing the production costs of this crop by replacing mineral N- fertilizers. This success arose from the succesful breeding of this crop with symbiotic N_2-fixation. However, some authors attributed the origin of selected inoculant strains to the first inoculants introduced in Brazil from USA, which lead some authors to consider the absence of *Bradyrhizobium* strains in Brazilian soils (Lopes et al., 1976; Martínez-Romero and Caballero-Mellado, 1996; Ferreira and Hungria 2002; Torres et al., 2012).

The genus *Bradyrhizobium* includes slow growing bacteria that alkalinize culture medium containing mannitol as a carbon source (Jordan, 1982). Currently, there are 19 described *Bradyrhizobium* species: *B. japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall et al., 1992), *B. liaoningense* (Xu et al., 1995), *B. yuanmingense* (Yao et al., 2002), *B. betae* (Rivas et al., 2004), *B. canariense* (Vinuesa et al., 2005a), *B. denitrificans* (van Berkum et al., 2006), *B. pachyrhizi* and *B. jicamae* (Ramírez-Bahena et al., 2009), *B. iriomotense* (Islam et al., 2008), *B. cytisi* (Chahboune et al., 2011), *B. lablabi* (Chang et al., 2011), *B. daqingense* (Wang et al., 2012), *B. huanghuaihaiense* (Zhang et al., 2012), *B. oligotrophicum* (Ramírez-Bahena et al., 2012), *B. rifense* (Chahboune et al., 2012), *B. arachidis* (Wang et al., 2013), *B. retamae* (Guerrouj et al., 2013), and

B. diazoefficiens (Delamuta et al., 2013). The number of described novel species has increased in the past two years along with the use of appropriate molecular techniques, such as housekeeping gene analysis (Willems et al., 2001; Martens et al., 2008; Rivas et al., 2009), as well as the study of new host plants and unexplored geographic regions.

However, these species were described mainly based on strains from temperate regions, although diverse *Bradyrhizobium* strains have been isolated from various species in tropical ecosystems (Moreira et al., 1993, 1998; Lima et al., 2005; Lima et al., 2009; Doignon-Bourcier et al., 2000; Guimarães et al., 2012; Jaramillo et al, 2013). These authors applied phenotypic (SDS-PAGE of total proteins) and genotypic methods (AFLP, IGS PCR-RFLP and Rep-PCR) that show interspecific variability.

The high degree of conservation of the 16S rRNA gene sequence indicates low diversity within the genus *Bradyrhizobium*. A better resolution at the species level has been achieved by the analysis of housekeeping genes (Stepkowski et al., 2005; Rivas et al., 2009; Menna et al., 2009; Delamuta et al., 2012; Guimarães, 2012), which have been widely employed in bacterial taxonomic classification studies. Considering the importance of the genus *Bradyrhizobium* as a source of genetically stable strains to be used as inoculants for soybeans and many other species, bioprospection studies are carried out to map its occurrence and to evaluate its diversity in Brazilian soils.

The present study aimed to analyze the symbiotic and genotypic diversity of 46 *Bradyrhizobium* bacterial strains, which were isolated from soils of different Brazilian regions, by performing nodulation and symbiotic efficiency tests in soybean plants (*Glycine max*), as well as a phylogenetic analysis of 16S rRNA and housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA*, and *rpoB*).

2. Materials and methods

2.1. Origin of soil samples for analysis

Soil samples were collected from different regions of Brazil: Midwest (Dourados, Mato Grosso do Sul state-MS), Northeast (Bom Jesus, Piauí state-PI), Southeast (Ijaci, Minas Gerais state-MG), and South (Campos Novos, Santa Catarina state-SC) (Fig. S1).

Soil from Bom Jesus-PI municipality was collected at the Novo Horizonte Farm, which is located in the Serra do Quilombo (mountain ridge), Bom Jesus municipality. The geographic coordinates of the area are 9º19'21''S and 44º48'55''W, and it is at an altitude of 660 m. Soil in this area is classified as Dystrophic Yellow Latosol (Oxisol).

Soil from Dourados-MS municipality was collected at the Experimental Farm of the Federal University of Góias. The geographic coordinates of the area are 22º13'56"S and 54º59'25"W, and it is at an altitude of 408 m. Soil in this area is classified as Distroferric Red Latosol (Oxisol).

Soil from Ijaci-MG municipality was collected at the Experimental Farm of the Federal University of Lavras. The geographic coordinates of the area are 21º12'17''S, 44º58'49''W, and it is at an altitude of 956 m. Soil in this area is classified as Yellow Red Latosol (Oxisol) with a clay-like texture.

Soil from Campos Novos-SC municipality was collected at the experimental area of the Agricultural Research and Rural Extension of Santa Catarina. The geographic coordinates of the area are 27°29'13"S and 51º 24'58"W, and it is at na altitude of 900 m. Soil in this area is classified as Distroferric Red Latossol (Oxisol).

All sampled areas have a history of inoculated soybean cropping.

The soil used in every experiment came from three composite samples, which consisted of five sub-samples each, collected from within the 0-20 cm depth layer. The samples were stored in sterilized plastic bags after collection and kept refrigerated (4ºC) until use. The experiments with soil samples collected from the different states were carried out in 2011, in the months of March (Ijaci-MG), May (Bom Jesus-PI and Dourados-MS), and October (Campos Novos-SC).

2.2. Isolation of bacterial strains using soybean as the trap plant

Bacterial strains were captured over four experiments conducted in a greenhouse at the Soil Biology, Microbiology and Biological Processes Laboratory of the Department of Soil Science, Federal University of Lavras.

The experiments were carried out in recyclable, sterilized long-neck bottles (500 mL) using filter paper as a support for plant root development (Florentino et al., 2009). The bottles were covered in aluminum foil and filled with a 4-fold dilution of Hoagland and Arnon (1950) solution. Hoagland and Arnon (1950) solution with a low nitrogen concentration (5.25 mg L^{-1}, considered as a start-up dose for nitrogen fixation) was used in the inoculated treatments and the uninoculated control. In the uninoculated control with high nitrogen concentration, i.e., Hoagland and Arnon (1950) complete solution containing 52.5 mg L^{-1} of nitrogen was used.

Soybean was used as a trap plant. The cultivars used were Monsoy 7980 (Bom Jesus-PI), CD 235 RR (Dourados-MS), Favorita RR (Ijaci-MG, and Campos Novos-SC). The soybean seed surfaces were disinfected with 70% alcohol for 30 seconds, immersed in 2-3% sodium hypochlorite solution for 2 minutes, and rinsed six times with sterilized distilled water. The seeds were then

germinated in a Petri dish containing filter paper and moistened sterile cotton and incubated at 28ºC for 2 days.

The soil samples were then subjected to a series of serial dilutions. The procedure was carried out by suspending 10 g of soil in 90 mL of sterile saline solution (0.85% NaCl), stirring for 30 minutes at 125 rpm (10^{-1} dilution), and conducting six successive steps of adding a 1.0 mL aliquot to a tube containing 9.0 mL of saline solution (dilutions from 10^{-2} to 10^{-7}).

The seedlings were transferred to the bottles containing the nutrient solution and inoculated with 1 mL of each serial dilution (three replicates per dilution). *B. elkanii* strain Br 29 (SEMIA 5019) approved as a soybean inoculant by the Ministry of Agriculture, Livestock and Supply (Brazil) was used as a positive control; two uninoculated negative controls were also used, one with high and one with low mineral N content (52.5 mg L^{-1} and 5.25 mg L^{-1}, respectively). The latter condition was used to test for possible contamination, while the former was used to determine whether the conditions were adequate for plant growth.

After 35 days of growth, the presence or absence of nodules was evaluated to estimate the most probable number (MPN) of noduliferous nitrogen fixing bacteria in legumes (NNFBL), which were then isolated.

2.3. Isolation of bacteria

Four nodules were randomly selected from each soybean plant, detached, and used for rhizobia isolation. They were hydrated in sterile distilled water and immersed in 95% ethanol. They were then immersed in hydrogen peroxide (H_2O_2) for 3 minutes and rinsed six times with sterile distilled water for surface disinfection. The nodules were subsequently macerated in plates containing 79 culture medium (Fred and Waksman, 1928), also known as YMA

(Vincent, 1970), the medium contained bromothymol blue and was at a pH of 6.8. The material was spread in streaks to obtain single colonies, and the bacteria were left to grow at 28ºC. Pure colonies were harvested and characterized morphologically.

2.4. Cultural characteristics

The bacterial culture characterization was performed using the same conditions and culture medium described for purifying isolates. The characteristics under study included the growth rate measured by the time to the appearance of isolated colonies (1-3 days - fast growth; 4 days - intermediate growth; 6 days or more days - slow growth), changes in culture medium pH (acidification, neutralization, or alkalinization), shape (circular or irregular), colony elevation, border appearance, color (yellow, orange, white, beige, or salmon), and the consistency of the exopolysaccharides produced (gummy, aqueous, or dry).

Purification of the bacteria resulted in a total of 699 isolates from all the four different regions. Only representatives of cultural groups with slow to intermediate growth and alkalinizing culture medium were selected for partial analysis of 16S rRNA genes. They comprised 21 strains isolated from Northeast soil, 12 strains isolated from the Southeast soil, 8 strains isolated from the Midwest soil and 5 strains isolated from the South soil.

2.5. Strains selected for DNA extraction and 16S rRNA sequencing

A total of 46 *Bradyrhizobium* strains were selected. The strains were grown at 28ºC on solid 79 medium (Fred and Waksman, 1928) and were

checked for purity by streaking them onto solid 79 medium and microscopically examining them.

The strains used in the present work originated from the Northeast Region (Bom Jesus-PI) (UFLA 06-13, UFLA 06-14, UFLA 06-15, UFLA 06-16, UFLA 06-17, UFLA 06-18, UFLA 06-19, UFLA 06-20, UFLA 06-21, UFLA 06-22, UFLA 06-23, UFLA 06-24, UFLA 06-25, UFLA 06-26, UFLA 06-27, UFLA 06-28, UFLA 06-29, UFLA 06-30, UFLA 06-31, UFLA 06-32, and UFLA 06-33); Midwest Region (Dourados-MS) (UFLA 06-34, UFLA 06-35, UFLA 06-36, UFLA 06-37, UFLA 06-38, UFLA 06-39, UFLA 06-40, and UFLA 06-41); Southeast Region (Ijaci-MG) (UFLA 06-01, UFLA 06-02, UFLA 06-03, UFLA 06-04, UFLA 06-05, UFLA 06-06, UFLA 06-07, UFLA 06-08, UFLA 06-09, UFLA 06-10, UFLA 06-11, and UFLA 06-12); and South Region (Campos Novos-SC) (UFLA 06-42, UFLA 06-43, UFLA 06-45, UFLA 06-46, and UFLA 06-47).

Bacterial DNA was extracted using the "ZR Fungal/Bacterial DNA MiniPrep" extraction kit (Zymo Research Corporation, Irvine, CA, USA) after growth in liquid 79 medium at 28°C. A partial amplification of the 16S rRNA gene was performed using 5 µL DNA, 5 µL dNTP (2 mmol L^{-1}), 5 µL 10X buffer, 4 µL $MgCl_2$ (2.5 mmol L^{-1}), 1 µL of each primer (10 mmol L^{-1}), 0.4 µL Taq DNA polymerase (5U μL^{-1}), and sterile Milli-Q water to a final reaction volume of 50 µL. The primers, temperatures, and cycling conditions are listed in Table S1. The amplification was carried out using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). The presence of PCR products and their concentrations were verified by electrophoresis of 5 µl of product on a 1% agarose gel and staining with SYBR Green (Invitrogen). A molecular mass marker (Smartladder-Eurogentec) was included to estimate the lengths of the amplification products. The PCR products were sent to Macrogen Inc. (Macrogen Inc., Seoul, Korea) for sequencing. The sequence quality was

analyzed using BioNumerics 7.1 software (Applied Maths, Austin, TX, USA). The sequences were then compared with those deposited in GenBank.

2.6. DNA extraction and housekeeping gene (atpD, gyrB, dnaK, recA, and rpoB) sequencing

The bacterial DNA was prepared using the alkaline lysis method as described by Niemann, et al., (1997) from cells grown for 5 days. Internal fragments of housekeeping genes (*atpD, gyrB, dnaK, recA*, and *rpoB*) were PCR-amplified and sequenced.

PCR amplification of the housekeeping genes was performed with a thermocycler (BIO RAD) in 25 µl reaction mixtures containing 1-2.5 µl DNA, 3.125 µl dNTP (2 mM), 2.5 µl PCR buffer (10X) (Qiagen), 5 µl Q-solution (Qiagen), 0.5 µl of each primer (50 µM), 0.2 Taq (5U/µl) (Qiagen), and Mili-Q water sterile. The primers, temperatures, and cycling conditions are listed in Table S1. The presence of PCR products and their concentrations were verified by electrophoresis of 5 µl of product on a 1% agarose gel and staining with ethidium bromide. A molecular mass marker (Smartladder-Eurogentec) was included to estimate the lengths of the amplification products.

The PCR product was cleaned using a NucleoFast^{R} 96 PCR plate clean up kit under vacuum (GENESIS workstation 200 - TECAN). The PCR products were washed and recovered in Mili-Q water. The purified DNA was sequenced using the dideoxynucleotide chain-termination method with fluorescent ddNTPs (BigDye Direct Cycle sequencing kit, Applied Biosystems) on an ABI Prism 3100xl capillary sequencer, according to the manufacturer's instructions (Applied Biosystems). Prior to sequencing, the amplified products were purified using 10 µL Bigdye Xterminator and 45 µL SAM in a MicroAmp^{TM} Optical 96-well Reaction Plate (Applied Biosystems).

Individual sequence files were compiled into consensus sequences using BioNumerics 5.10 software (Applied Maths).

2.7. Phylogenetic analysis

The 46 strains identified as belonging to the *Bradyrhizobium* genus were used to build phylogenetic trees using the partial sequences of the 16S rRNA genes. The sequences were aligned using the ClustalW Multiple Alignment algorithm in BioEdit. The phylogenetic tree was built using the neighbor-joining method using the Kimura 2 Parameter model (Kimura, 1980) for the 16S rRNA gene, and the maximum likelihood method was used for the *atpD*, *gyrB*, *dnaK*, *recA* and *rpo*B genes, available in the Molecular Evolutionary Genetic Analysis software package (MEGA, version 5) (Tamura et al., 2011). For comparison, the alignment included the sequences for type strains of *Bradyrhizobium* species, as well as those of the four strains used as soybean inoculants in Brazil (*B. japonicum* - SEMIA 5079 and 5080 and *B. elkanii* - SEMIA 587 and 5019). The sequences are available in GenBank (National Center for Biotechnology Information, NCBI) and are detailed in Table S2.

The four strains used since 1979 as soybean inoculants and approved by Ministry of Agriculture, Livestock and Supply (Brazil), were included only in the trees built separately for the 16S rRNA, *dnaK*, and *recA* genes. For the *atpD*, *gyrB* and *rpoB* genes the sequences are not available in GenBank, thus it was not possible to include them.

For the gene analyses alignments were trimmed so all strains had the same length for a particular gene, resulting in the following lengths: (sizes in parentheses) 16S rRNA (913 bp), *gyrB* (554 bp), *dnaK* (222 bp), *atpD* (426 bp), *recA* (381 bp), and *rpoB* (446 bp). Sequences for reference type strains, and the four strains used as soybean inoculants in Brazil are available in GenBank.

2.8. Strain efficiency test with soybean plants in Leonard jars

The 46 strains identified as *Bradyrhizobium* spp. were also analyzed in terms of their symbiotic ability and efficiency with soybean plants (*Glycine max*). The experiments were conducted using the soybean cultivar Favorita RR in Leonard jars (Vincent, 1970) for a period of 55 days.

To minimize the risk of contamination, the experiment was divided into two stages. Experiment 1 analyzed 24 strains, experiment 2 analyzed 22 strains. Experiment 1 was set up on August 16th, 2013, and experiment 2 was set up on August 30th, 2013. Both experiments were carried out under similar conditions. During the experimental period, the temperature varied between 5ºC and 32ºC, and the relative air humidity varied between 48% and 96%. Both experiments included a positive control inoculated with the *B. elkanii* Br 29 (SEMIA 5019), approved soybean inoculant by Ministry of Agriculture, Livestock and Supply (Brazil) and two negative, uninoculated controls, one with a high (52.5 mg L^{-1}) and one with a low (5.25 mg L^{-1}) mineral N concentration. The study was completely randomized and performed in triplicate.

The steps described earlier for the preparation of the Hoagland and Arnon (1950) nutrient solution, superficial disinfection, and seed germination for the capture of isolates in long neck bottles were also applied in this stage.

A 1:2 mixture of sand (150 cm^3) and vermiculite (300 cm^3) was added to the upper part of the jar, a 4-fold diluted nutrient solution was added to the lower part. The jars were subsequently autoclaved (121°C for 1 h).

Four seedlings were added to each jar. In the preparation for bacterial inoculation, the strains were grown in solid 79 medium (Fred and Waksman, 1928) and later inoculated in liquid 79 medium with the aid of a sterile platinum loop. The strains were incubated for five days at 28ºC with constant stirring. At

the planting stage, each seed was inoculated with 1 mL of bacterial culture containing approximately 10^9 bacterial cells.

After sowing and inoculation, the jars were covered with about 1 cm thick layer consisting of a mixture of 10 kg of sand, 1 L of chloroform, and 10 g of paraffin to avoid possible contaminations. Thinning was conducted five days after sprouting, with two plants kept in each jar.

During the experiment, the jars were periodically refilled with a freshly prepared and autoclaved nutrient solution according to the plant absorption rate. At the start of the flowering stage (55 days after sowing), the plants were collected for the determination of the nodule number (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), and efficiency compared to the controls with mineral N (ECCN).

For the NN determination, the nodules were detached from the roots and counted. NDM determination involved placing the nodules in glass tubes and drying them in a forced-air oven at 60ºC to a constant weight. For the SDM and RDM determinations, shoots or roots were placed separately in paper bags and dried in a forced-air oven at 60ºC to a constant weight. The relative efficiency of each treatment was calculated using the formula ECCN = (inoculated SDM/SDM with N) x 100.

The data were tested for normality using the Shapiro-Wilk test (Shapiro et al., 1968) and for homoscedasticity using the Bartlett test (Bartlett, 1937). The experimental data were subjected to analysis of variance using the statistical analysis software program SISVAR v. 5.3 (Ferreira, 2011). The treatment effects were compared using the Scott-Knott test, with 5% significance.

3. Results

3.1. Phylogenetic analysis of 16S rRNA and housekeeping genes (gyrB, dnaK, atpD, recA and rpoB)

The phylogenetic tree built from 16S rRNA gene sequences identified two large groups of strains (G-I and G-II) (Fig. S2). The former (G-I) includes 32 strains, which grouped with *B. elkanii* LMG 6134^T, *B. jicamae* PAC68^T, *B. elkanii* SEMIA 587 and 5019, *B. pachyrhizi* PAC48^T, and *B. lablabi* CCBAU 23086^T. The latter (G-II) includes 7 strains, which all grouped with *B. japonicum* LMG 6138^T and SEMIA 5079. The UFLA 06-24 strain was not grouped with any other strain analyzed, nor with any other *Bradyrhizobium* type strain or the soybean inoculant strains; it exhibited a closer relationship with the type strains *B. cytisi* CTAW11^T and *B. rifense* CTAW71^T.

Strains UFLA 06-03, UFLA 06-05, UFLA 06-08, UFLA 06-35, UFLA 06-41, and UFLA 06-43 were not included in the 16S rRNA gene phylogenetic tree due to the poor quality of the reverse sequences, which resulted in a reduced sequence length.

The housekeeping gene trees were built using the Maximum Likelihood (ML) method. However, similar results were obtained when the data were analyzed by the Neighbor Joining (NJ) method (data not shown).

Phylogenetic trees were built for each housekeeping gene (Figs. S3-S7). Trees built for genes *gyrB* (Fig. S3), *dnaK* (Fig. S4), *atpD* (Fig. S5), *recA* (Fig. S6), and *rpoB* (Fig. S7) resulted in the formation of two main groups (G-I and G-II). Subgroups within the major groups were also distinguishable for each gene. As in the 16S rRNA genetree, strain UFLA 06-24 was not grouped with any of the other strains in this study, nor with *Bradyrhizobium*-type strains or soybean inoculating strains, for any of the genes (Figs. S3-S7).

The *dnaK* gene showed the least power of discrimination between groups because two groups which were clearly separated when comparing the remaining housekeeping genes, were grouped in G-I.I. These two groups include one group with 12 strains isolated from Northeast soils (UFLA 06-13, UFLA 06-14, UFLA 06-15, UFLA 06-16, UFLA 06-17 UFLA 06-19, UFLA 06-20, UFLA 06-21, UFLA 06-22, UFLA 06-26, UFLA 06-27, and UFLA 06-33) and another group with three strains isolated from the Southeast soil (UFLA 06-05, UFLA 06-06, and UFLA 06-10). Strain UFLA 06-10 was not included in the gyrB tree due to low sequence quality.

3.2. Concatenated analysis of gyrB, dnaK, atpD, recA, and rpoB

All five sequences of the housekeeping genes were concatenated in order to gain a better understanding of the relatedness of the strains. A concatenated sequence with 2.128 bp was obtained.

The analysis of the five concatenated housekeeping genes showed a high degree of differentiation between groups for the analyzed strains (Fig. 1), with two groups clearly separated and a single strain (UFLA 06-24), these results suggest the possible presence of novel species among these Brazilian bradyrhizobia. These results were similar with the analysis of individual housekeeping genes, specially for *gyrB*, *atpD*, *recA*, and *rpoB*.

3.3. Most probable number of native NNFBL

The most probable number of NNFBL in all areas was determided by using soybean plants as traps inoculated with decimal serially diluted soil samples to determine the following Most Probale numbers: Bom Jesus-PI =

80.000, Dourados-SC = 14.000, Ijaci-MG = 540, Campos Novos-SC = 68.000 cell g^{-1} soil.

Isolates were obtained from nodules formed in plants of all positive dillutions, i.e, dillutions where nodules appeared. In all analyzed areas some atypical fast-growing soybean microsymbionts that acidified the culture medium, were isolated, however in smaller percentage (19.5%) compared to the slow growing bacteria such as bradyrhizobia that alkalinize culture medium (80.5%).

3.4. Soybean efficiency test

The negative controls (no inoculation, low and high mineral N content) did not exhibit nodulation, indicating no contamination in both experiments. The positive control inoculated with *B. elkanii* Br 29 SEMIA 5019, one of the strains approved by Ministry of Agriculture, Livestock and Supply (Brazil) as a soybean culture inoculant in Brazil, nodulated efficiently in both experiments, demonstrating that the culture conditions were favorable for nodulation and BNF. All 46 strains analyzed in the present study were able to nodulate soybeans (Table 1 and 2).

For experiment 1 (Table 1), approximately 71% of the analyzed strains exhibited NN values similar to that of the control strain, Br 29 (SEMIA 5019) ($p < 0.05$), while the remaining 29% exhibited higher NN values ($p < 0.05$), especially UFLA 06-10, which exhibited the highest NN values compared to the control strain ($p < 0.05$).

The NDM varied between 0.17 and 0.67 g $plant^{-1}$. The lowest value was observed for strain UFLA 06-40, and the highest value was observed for strains UFLA 06-10, UFLA 06-13, and UFLA 06-22, which exhibited NDM values

higher than all the other strains in the study, as well as the strain recommended as an inoculant ($p < 0.05$).

SDM production varied between 0.93 and 3.28 g plant^{-1}, for the negative uninoculated controls with low and high mineral N content, respectively (Table 1).

Strain UFLA 06-24 exhibited SDM, TDM, and ECCN values larger than those of all the other strains, including Br 29 (SEMIA 5019) ($p < 0.05$). Strains UFLA 06-19 and UFLA 06-20 exhibited values equal to those of Br 29 (SEMIA 5019) ($p < 0.05$) in all analyzed parameters. Strains UFLA 06-13, UFLA 06-15, and UFLA 06-22 exhibited values equal to or larger than those of Br 29 (SEMIA 5019) in all analyzed parameters ($p < 0.05$).

For experiment 2 (Table 2), most strains (59%) exhibited NN values equal to that of the control strain Br 29 (SEMIA 5019) ($p < 0.05$). Similar results were observed for NDM.

For SDM, RDM, TDM, and ECCN values, the largest values were observed for the control with high mineral N ($p < 0.05$). Strains UFLA 06-03, UFLA 06-12, UFLA 06-28, and UFLA 06-29 exhibited larger SDM and EFCN values than Br 29 (SEMIA 5019) and all other strains ($p < 0.05$); the remaining parameters remained equal to those of Br 29 (SEMIA 5019) ($p < 0.05$).

Strains UFLA 06-01, UFLA 06-23, UFLA 06-31, UFLA 06-32, and UFLA 06-43 showed similar values to those of Br 29 (SEMIA 5019) in all parameters analyzed ($p < 0.05$).

4. Discussion

4.1. Phylogenetic analysis of 16S rRNA and housekeeping genes (gyrB, dnaK, atpD, recA, and rpoB)

The present study corroborates previous reports showing that sequencing of the 16S rRNA gene alone is not sufficient for differentiating *Bradyrhizobium* species (Willems et al., 2001; Vinuesa et al., 2005a,b; Vinuesa et al., 2008; Rivas et al., 2009; Delamuta et al., 2012; Delamuta et al., 2013). The phylogenetic tree for this gene showed low differentiation between strains and grouped different *Bradyrhizobium* species in the same group. This result stems from the high degree of gene conservation, resulting in high sequence similarity.

The phylogenetic analysis of the housekeeping genes showed, except for *dnaK*, a high degree of differentiation between groups for the analyzed strains. Strains isolated from the Northeast soil are of particular interest because they exhibited good symbiotic efficiency and may possibly represent novel species. Strains UFLA 06-15 and UFLA 06-27 in particular show values equal to or greater than those of Br 29 (SEMIA 5019) for all analyzed efficiency parameters ($p < 0.05$). Strains UFLA 06-19 and UFLA 06-20 exhibited high efficiency parameters, with values similar to those of Br 29 (SEMIA 5019) ($p < 0.05$). Strain UFLA 06-24, which was not grouped with any other strain studied in the present work, nor with any previously described *Bradyrhizobium* spp. type strains or soybean inoculant strains, exhibited SDM, TDM, and ECCN values larger than those of all strains under analysis, including Br 29 (SEMIA 5019) ($p < 0.05$). These results contradict previous reports in the literature, which emphasizes the lack of native *Bradyrhizobium* strains in Brazilian soils that are able to establish an efficient symbiotic association with soybean plants (Lopes et

al., 1976; Martínez-Romero and Caballero-Mellado, 1996; Ferreira and Hungria 2002; Torres et al., 2012).

4.2. Soybean efficiency test

Soybean is the species most commonly used in nodulation tests in studies describing novel *Bradyrhizobium* species (Jordan, 1982; Kuykendall et al., 1992; Xu et al., 1995; Yao et al., 2002; Rivas et al., 2004; Vinuesa et al., 2005a; van Berkum et al., 2006; Ramírez-Bahena et al., 2009; Chahboune, et al., 2011; Chang et al., 2011; Chahboune et al., 2012; Wang et al., 2012; Zhang et al., 2012; Ramírez-Bahena et al., 2012; Wang et al., 2013; Guerrouj et al., 2013; Delamuta et al., 2013). The use of soybean in nodulation tests to describe novel species is related to its economic importance.

Strain UFLA 06-24 exhibited higher values than strain Br 29 (SEMIA 5019) (approved as inoculant for soybean cultures) for all parameters ($p < 0.05$). To complete the evaluation of their agronomic potential, the strains should be studied in terms of agronomic efficiency and competitive ability under field conditions because these aspects were only evaluated under axenic conditions in the absence of interactions with other soil microorganisms and components.

5. Conclusions

Our results confirm the importance of housekeeping genes as a tool to reveal the diversity of the still poorly known *Bradyrhizobium* diversity, especially in soils from different Brazilian regions.

In our study, the use of five housekeeping genes revealed greater diversity when compared to the 16S rRNA analysis alone, clearly indicating that strain UFLA 06-24, which remained alone in all analyzed genes, may represent a

novel species, as well as a group of 12 strains, including strains isolated from the Northeast soil, and one other group with three strains isolated from the Southeast soil. The strains will be further characterized to allow species description. All these strains exhibited high symbiotic efficiency.

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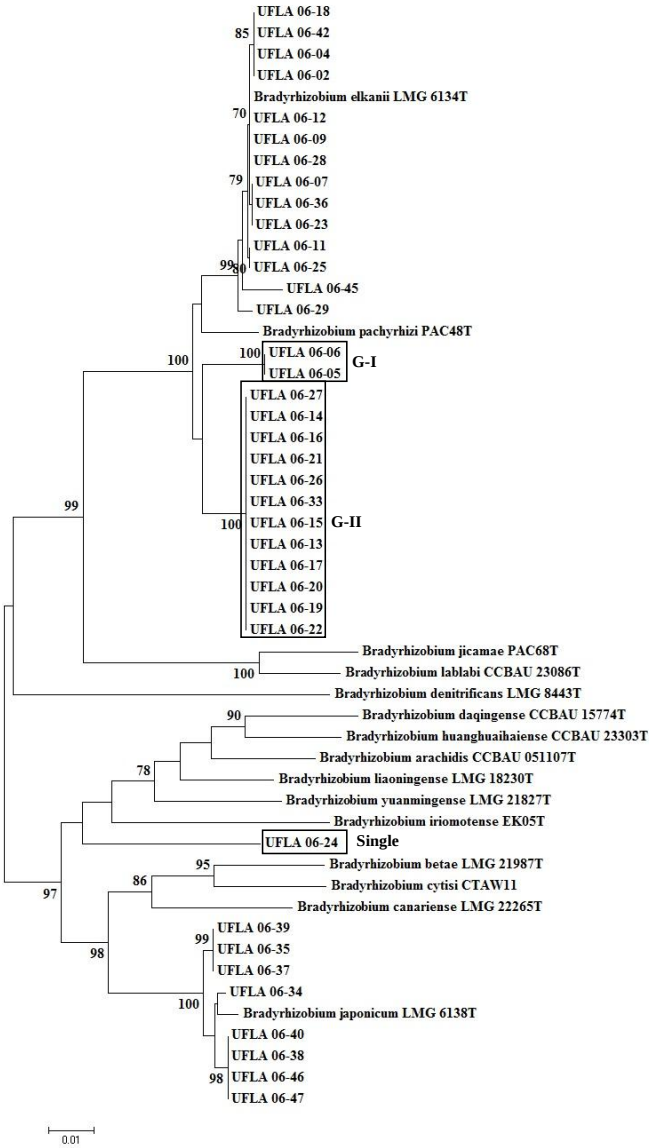

Fig. 1. Maximum likelihood phylogenetic tree based on the concatenated sequence data for the genes (*gyrB*, *dnaK*, *atpD*, *recA*, and *rpoB*) of 37 *Bradyrhizobium* strains and the type strains of the remaining species of *Bradyrhizobium*. Bootstrap values were based on 1000 trials. All positions containing gaps and missing data were eliminated from the dataset. Bootstrap values >70% are indicated at nodes. Phylogenetic analyses were conducted in Mega5

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Table 1. Results of the first series of nodulation tests of soybean plants. Mean values for the nodule number (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), and efficiency compared to controls with mineral N (ECCN). Treatments were inoculations with strains isolated from soils of different Brazilian regions (Midwest-MW, Northeast-NE, Southeast-SE, and South-S), two controls uninoculated, a with mineral N (+N) and another with low mineral N (-N) and control inoculated with *B. elkanii* Br 29 (SEMIA 5019).

Treatments	Origin	NN	NDM	SDM	RDM	TDM	ECCN
	State/Region	-	---------- g plant^{-1} ----------				%
UFLA 06-05	MG/SE	128 b	0.44 c	1.39 e	0.43 c	1.81 e	42 e
UFLA 06-06	MG/SE	136 b	0.37 d	1.32 e	0.42 c	1.74 e	39 e
UFLA 06-10	MG/SE	159 a	0.67 a	1.86 d	0.61 b	2.48 d	58 d
UFLA 06-46	SC/S	102 c	0.41 c	1.72 d	0.51 c	2.23 d	50 d
UFLA 06-47	SC/S	98 d	0.51 b	1.78 d	0.67 b	2.45 d	56 d
UFLA 06-34	MS/MW	79 d	0.37 d	1.77 d	0.49 c	2.26 d	56 d
UFLA 06-35	MS/MW	86 d	0.57 b	2.17 c	0.53 c	2.70 c	67 c
UFLA 06-37	MS/MW	71 d	0.40 c	1.42 e	0.45 c	1.88 e	42 e
UFLA 06-38	MS/MW	88 d	0.43 c	2.24 c	0.60 b	2.84 c	69 c
UFLA 06-39	MS/MW	68 d	0.43 c	1.80 d	0.56 b	2.36 d	55 d
UFLA 06-40	MS/MW	67 d	0.17 e	1.18 f	0.44 c	1.63 f	38 e
UFLA 06-13	PI/NE	121 c	0.60 a	2.37 c	0.61 b	2.98 c	69 c
UFLA 06-14	PI/NE	69 d	0.45 c	1.79 d	0.62 b	2.41 d	55 d
UFLA 06-15	PI/NE	105 c	0.52 b	2.12 c	0.65 b	2.77 c	67 c
UFLA 06-16	PI/NE	110 c	0.49 b	1.80 d	0.50 c	2.30 d	57 d
UFLA 06-17	PI/NE	74 d	0.30 d	1.79 d	0.51 c	2.30 d	56 d
UFLA 06-19	PI/NE	77 d	0.51 b	2.21 c	0.56 b	2.77 c	69 c
UFLA 06-20	PI/NE	89 d	0.54 b	2.02 c	0.56 b	2.59 c	62 c
UFLA 06-21	PI/NE	78 d	0.55 b	2.28 c	0.54 c	2.82 c	72 c
UFLA 06-22	PI/NE	95 d	0.60 a	2.33 c	0.72 b	3.05 c	68 c
UFLA 06-24	PI/NE	74 d	0.35 d	2.80 b	0.67 b	3.46 b	85 b
UFLA 06-26	PI/NE	73 d	0.45 c	1.50 e	0.50 c	2.01 e	47 e
UFLA 06-27	PI/NE	112 c	0.54 b	2.11 c	0.61 b	2.71 c	62 c
UFLA 06-33	PI/NE	80 d	0.36 d	1.45 e	0.41 c	1.85 e	44 e
SEMIA 5019		89 d	0.53 b	2.39 c	0.57 b	2.95 c	70 c
-N		0 e	0.00 f	0.93 g	0.43 c	1.36 f	28 f
+N		0 e	0.00 f	3.28 a	1.22 a	4.50 a	100 a
CV (%)		16.47	13.77	7.60	12.04	6.88	7.84

Averages followed by the same letter within each column are not significantly different according to the Scott-Knott test at ($p < 0.05$) significance.

Table 2. Results of the second series of nodulation tests of soybean plants. Mean values for the nodule number (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), and efficiency compared to controls with mineral N (ECCN). Treatments were inoculations with strains isolated from soils of different Brazilian regions (Midwest-MW, Northeast-NE, Southeast-SE, and South-S), two controls uninoculated, a with mineral N (+N) and another with low mineral N (-N) and control inoculated with *B. elkanii* Br 29 (SEMIA 5019).

Treatments	Origin	NN	NDM	SDM	RDM	TDM	ECCN
	State/Region	-	---------- g plant^{-1} ----------				--- % ---
UFLA 06-01	MG/SE	97 a	0.30 a	2.26 c	0.60 b	2.86 b	66 c
UFLA 06-02	MG/SE	71 b	0.15 b	1.31 e	0.37 c	1.69 c	38 e
UFLA 06-03	MG/SE	89 a	0.29 a	2.74 b	0.65 b	3.39 b	80 b
UFLA 06-04	MG/SE	68 b	0.25 a	2.36 c	0.61 b	2.97 b	68 c
UFLA 06-07	MG/SE	73 b	0.27 a	2.58 c	0.52 c	3.10 b	75 c
UFLA 06-08	MG/SE	83 a	0.19 b	1.65 d	0.40 c	2.05 c	47 d
UFLA 06-09	MG/SE	61 b	0.19 b	1.63 d	0.36 c	1.99 c	47 d
UFLA 06-11	MG/SE	26 c	0.04 c	0.59 f	0.18 c	0.76 d	17 f
UFLA 06-12	MG/SE	93 a	0.31 a	2.80 b	0.73 b	3.53 b	82 b
UFLA 06-18	PI/NE	49 c	0.19 b	2.27 c	0.45 c	2.72 b	67 c
UFLA 06-23	PI/NE	91 a	0.25 a	2.39 c	0.59 b	2.98 b	69 c
UFLA 06-25	PI/NE	86 a	0.21 b	1.82 d	0.38 c	2.20 c	52 d
UFLA 06-28	PI/NE	97 a	0.32 a	2.77 b	0.67 b	3.45 b	80 b
UFLA 06-29	PI/NE	107 a	0.28 a	2.73 b	0.65 b	3.38 b	80 b
UFLA 06-30	PI/NE	97 a	0.20 b	2.00 c	0.58 b	2.58 b	57 c
UFLA 06-31	PI/NE	103 a	0.25 a	2.19 c	0.65 b	2.84 b	65 c
UFLA 06-32	PI/NE	95 a	0.31 a	2.42 c	0.60 b	3.01 b	70 c
UFLA 06-42	SC/S	68 b	0.20 b	2.55 c	0.57 b	3.12 b	73 c
UFLA 06-43	SC/S	107 a	0.25 a	2.49 c	0.57 b	3.07 b	72 c
UFLA 06-45	SC/S	98 a	0.26 a	2.24 c	0.51 c	2.75 b	65 c
UFLA 06-36	MS/MW	69 b	0.22 b	1.87 d	0.43 c	2.29 c	54 d
UFLA 06-41	MS/MW	38 c	0.08 c	0.76 f	0.28 c	1.04 d	22 f
SEMIA 5019		104 a	0.30 a	2.47 c	0.65 b	3.12 b	71 c
-N		0 d	0.00 c	1.20 e	0.43 c	1.63 c	35 e
+N		0 d	0.00 c	3.49 a	1.20 a	4.70 a	100 a
CV (%)		27.48	25.80	12.63	21.46	13.61	13.54

Averages followed by the same letter within each column are not significantly different according to the Scott-Knott test at ($p < 0.05$) significance.

Supplementary material

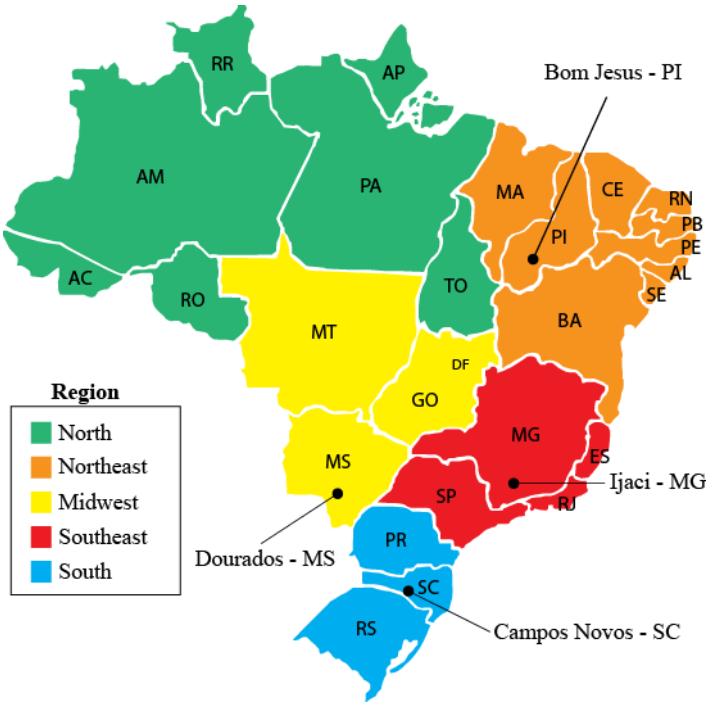

Fig. S1. Map showing the sites where the soil samples used in this study were collected: Midwest (Dourados-MS), Northeast (Bom Jesus-PI), Southeast (Ijaci-MG), and South (Campos Novos-SC).

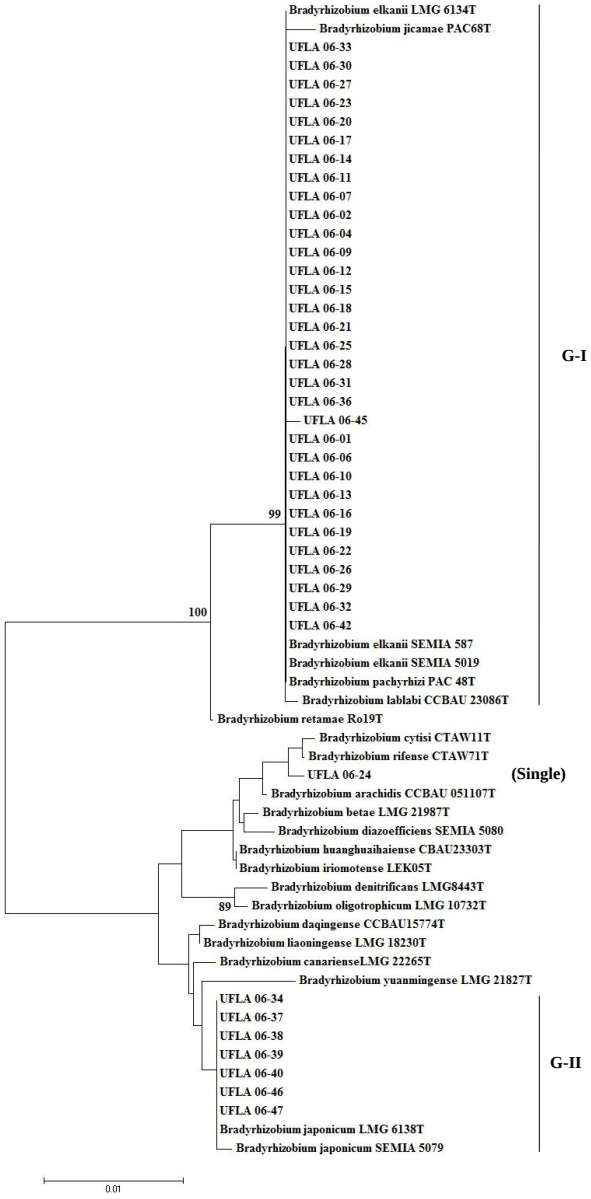

Fig. S2. Neighbor Joining phylogenetic tree based on partial sequences of the 16S rRNA genes of 40 *Bradyrhizobium* strains and the type strains of the remaining species of *Bradyrhizobium*. Bootstrap values were based on 1000 trials. All positions containing gaps and missing data were eliminated from the dataset. Bootstrap values > 70% are indicated at the nodes. Phylogenetic analyses were conducted in Mega5.

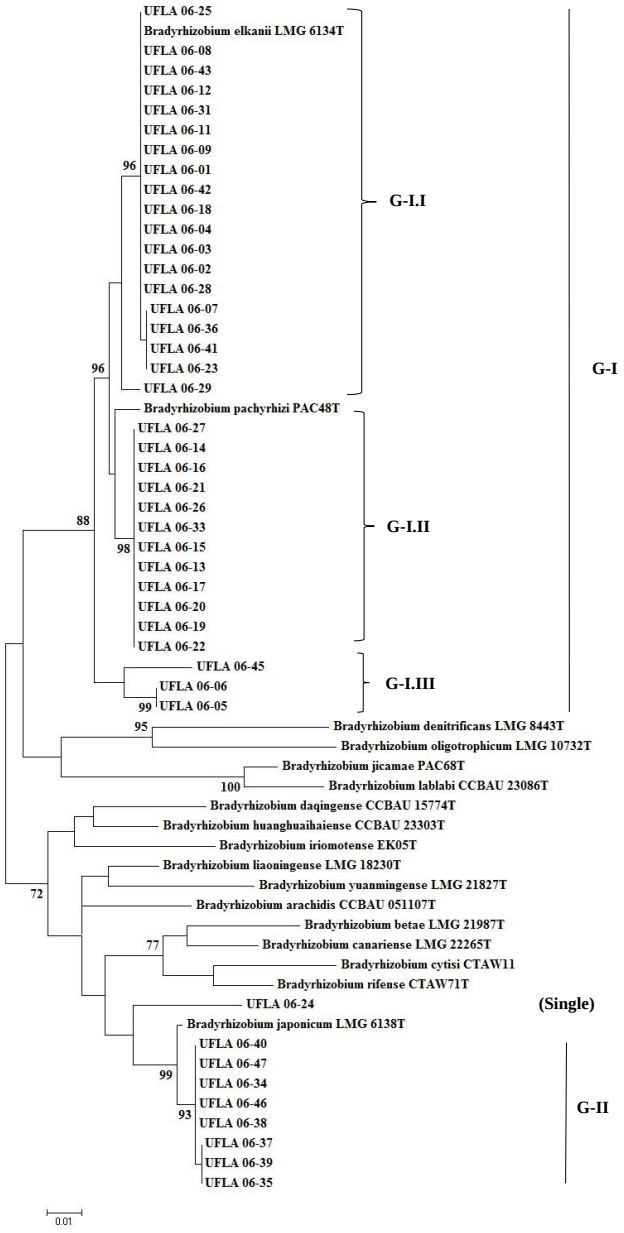

Fig. S3. Maximum likelihood phylogenetic tree based on partial sequences of the *gyrB* genes of 43 *Bradyrhizobium* strains and the type strains of the remaining species of *Bradyrhizobium*. Bootstrap values were based on 1000 trials. All positions containing gaps and missing data were eliminated from the dataset. Bootstrap values > 70% are indicated at the nodes. Phylogenetic analyses were conducted in Mega5.

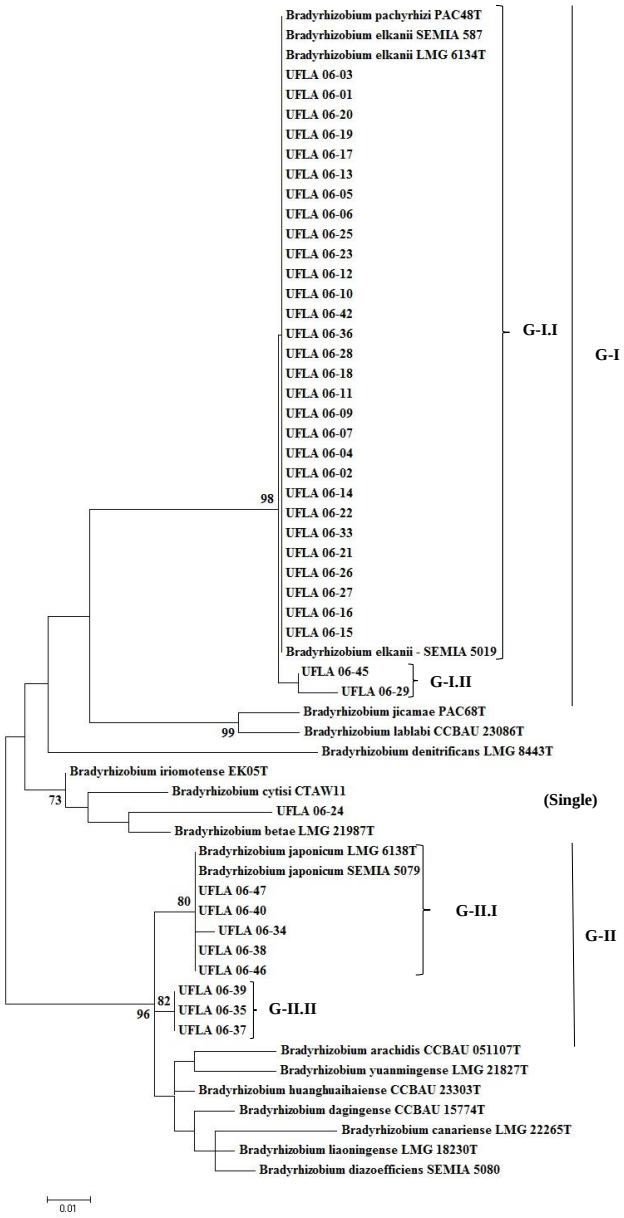

Fig. S4. Maximum likelihood phylogenetic tree based on partial sequences of the *dnaK* genes of 40 *Bradyrhizobium* strains and the type strains of the remaining species of *Bradyrhizobium*. Bootstrap values were based on 1000 trials. All positions containing gaps and missing data were eliminated from the dataset. Bootstrap values > 70% are indicated at the nodes. Phylogenetic analyses were conducted in Mega5.

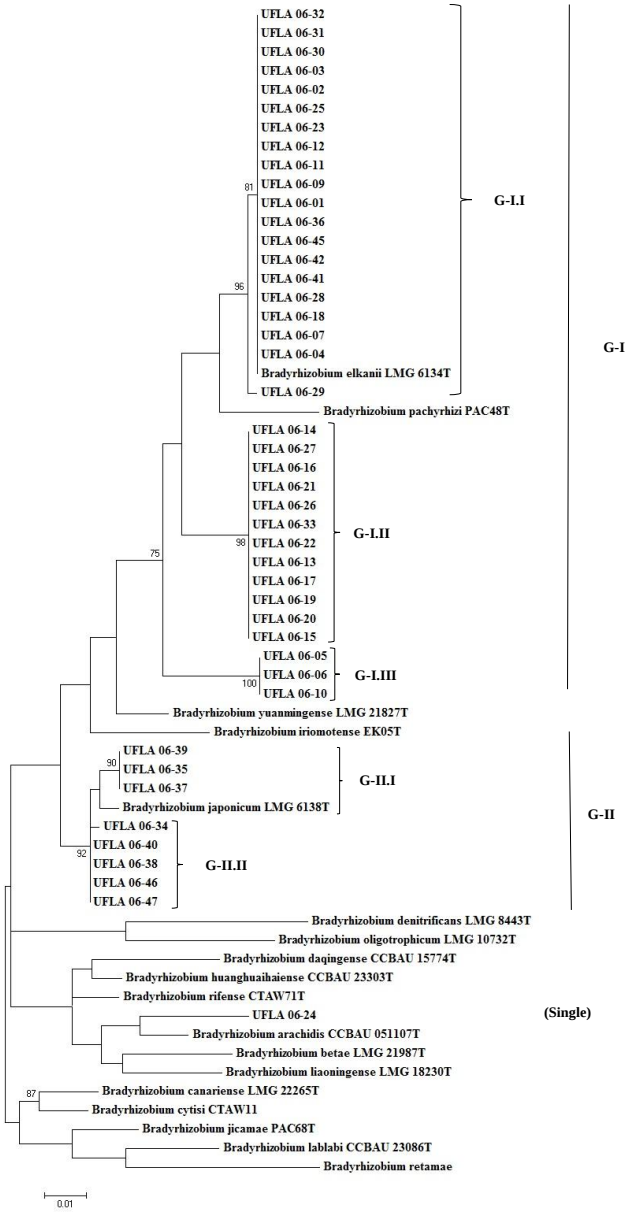

Fig. S5. Maximum likelihood phylogenetic tree based on partial sequences of the *atpD* genes of 44 *Bradyrhizobium* strains and the type strains of the remaining species of Bradyrhizobium. Bootstrap values were based on 1000 trials. All positions containing gaps and missing data were eliminated from the dataset. Bootstrap values > 70% are indicated at the nodes. Phylogenetic analyses were conducted in Mega5.

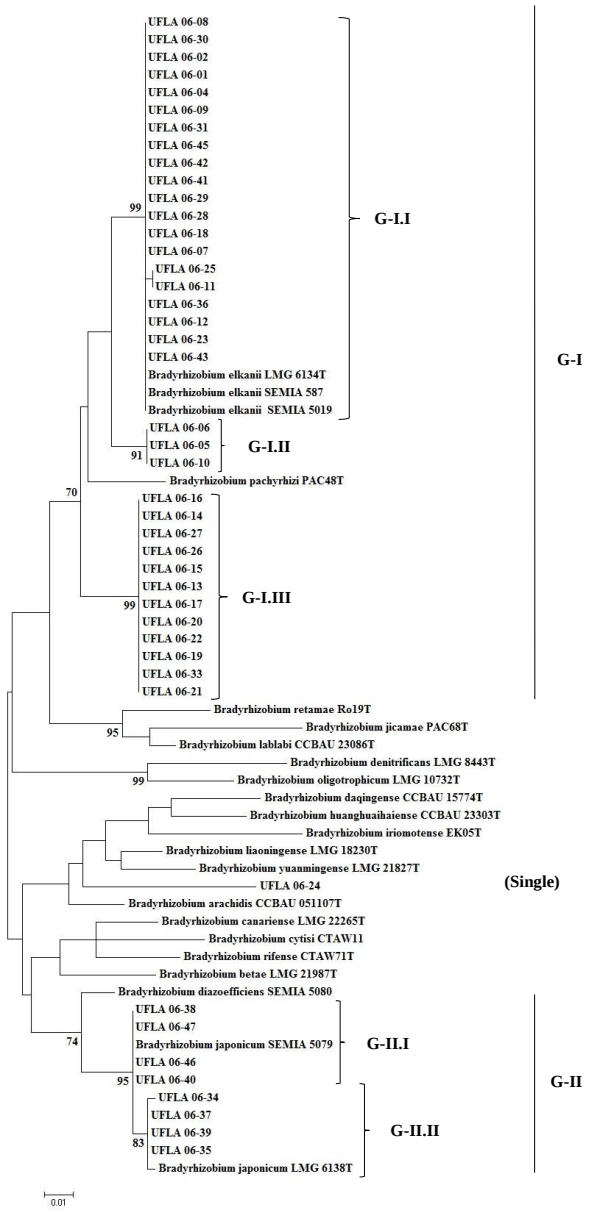

Fig. S6. Maximum likelihood phylogenetic tree based on partial sequences of the *recA* genes of 44 *Bradyrhizobium* strains and the type strains of the remaining species of *Bradyrhizobium*. Bootstrap values were based on 1000 trials. All positions containing gaps and missing data were eliminated from the dataset. Bootstrap values > 70% are indicated at the nodes. Phylogenetic analyses were conducted in Mega5.

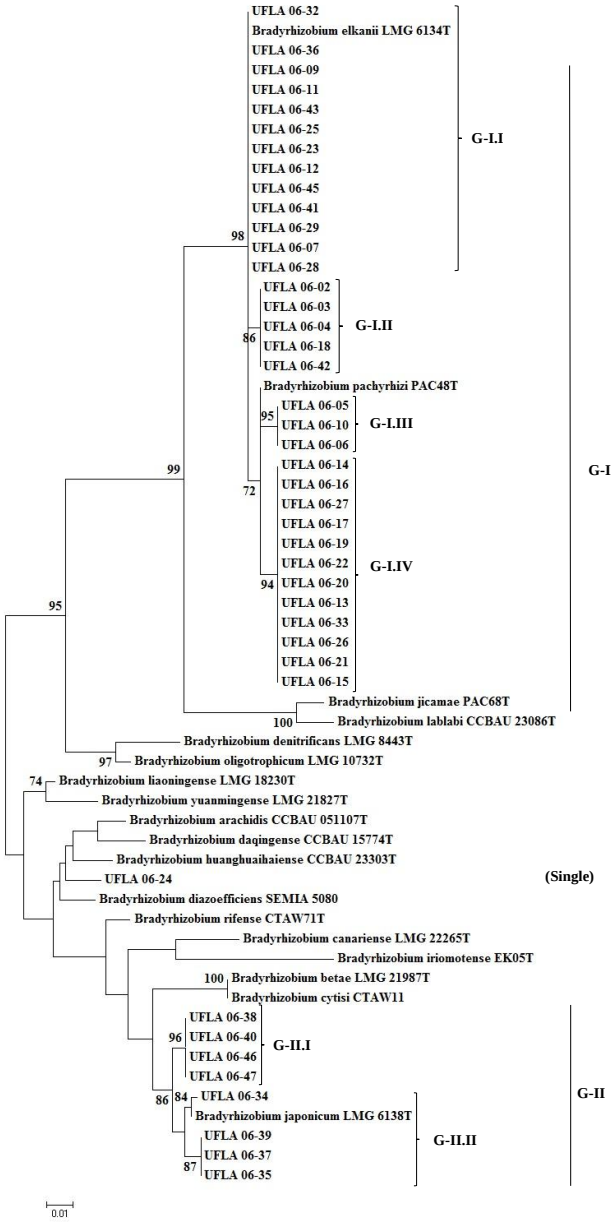

Fig. S7. Maximum likelihood phylogenetic tree based on partial sequences of the *rpoB* genes of 42 *Bradyrhizobium* strains and the type strains of the remaining species of *Bradyrhizobium*. Bootstrap values were based on 1000 trials. All positions containing gaps and missing data were eliminated from the dataset. Bootstrap values > 70% are indicated at the nodes. Phylogenetic analyses were conducted in Mega5.

Table S1. Oligonucleotide primers used and PCR cycling conditions.

Primer[a]	Sequence 5'-3'	Position[b]	PCR cycling	Reference
16S rRNA 27F	AGAGTTTGATCCTGGCTCAG	8-27	5'94ºC, 35x(40''94ºC,	Lane, 1991
16S rRNA 1492R	GGTTACCTTGTTACGACTT	1507-1492	40''55ºC, 1'30''72ºC), 7'72ºC	
TSrecAf	CAACTGCMYTGCGTATCGTCGAAGG	8-32	5'95ºC, 32x (45''94ºC,1'60º,	Stepkowski et al.,
TSrecAr	CGGATCTGGTTGATGAAGATCACCATG	620-594	1'30''74ºC), 5'72ºC	2005
gyrB343F	TTCGACCAGAAYTCCTAYAAGG	343-364	5'95ºC, 5x (2'94ºC, 2'57ºC,	Martens et al., 2007
gyrB1043R	AGCTTGTCCTTSGTCTGCG	1061-1043	1'30''72ºC), 28x (30''94ºC, 1'57ºC,1'30''72ºC), 5'72ºC	
dnaK1466F	AAGGARCANCAGATCCGCATCCA	1466-1488	5'94ºC, 35x (1'94ºC, 1'62ºC,	Stepkowski et al.,
dnaK1777R	TASATSGCCTSRCCRAGCTTCAT	1799-1777	40''72ºC), 5'72ºC	2003
atpD352F	GGCCGCATCATSAACGTSATC	352-372	5' 95ºC, 2x (2'94ºC, 1'64.5ºC,	Stepkowski et al.,
atpD871R	AGMGCCGACACTTCMGARCC	890-871	1'72ºC), 30x (30''94ºC, 1'64.5ºC, 1'72ºC), 5'72ºC	2005
rpoB83F	CCTSATCGAGGTTCACAGAAGGC	83-103	5'95ºC, 3x (2'94ºC, 2'58.2ºC,	Martens et al., 2007
rpoB1061R	AGCGTGTTGCGGATATAGGCG	1081-1061	1'72ºC), 30x (30''94ºC, 1'58.2ºC, 172ºC), 5'72ºC	
rpoB1061R	AGCGTGTTGCGGATATAGGCG	1081-1061		
rpoB456F	ATCGTYTCGCAGATGCACCG	456-475		
rpoB458R	GAACGGTGCATCTGCGARACG	478-458		

[a]Primers given in bold were used for initial amplification; [b]Position relative to the gene sequences.

Table S2. GenBank accession numbers of *Bradyrhizobium* reference sequences used in alignment and sequence comparison

Strain	16S rRNA	*gyrB*	*dnaK*	*atpD*	*recA*	*rpoB*
B. canariense LMG 22265^T	AY577427	FM253220	FM253306	FM253135	FM253177	FM253263
B. japonicum LMG 6138^T	X66024	AM418801	AM182120	AM418753	AM182158	AM295349
B. elkanii LMG 6134^T	AF362942	AM418800	AM168363	AM418752	AY591568	AM295348
B. jicamae PAC68^T	AY624134	HQ873309	JF308945	FJ428211	HM047133	HQ587647
B. lablabi CCBAU 23086^T	GU433448	JX437670	JX437663	GU433473	GU433522	JX437677
B. pachyrhizi PAC48^T	AY624135	HQ873310	JF308946	FJ428208	HM047130	HQ587648
B. daqingense CCBAU 15774^T	HQ231274	JX437669	JX437662	HQ231289	HQ231270	JX437676
B. huanghuaihaiense CBAU23303^T	HQ231463	JX437672	JX437665	HQ231682	HQ231595	JX437679
B. liaoningense LMG 18230^T	AF208513	FM253223	FM253309	AY386752	FM253180	FM253267
B. yuanmingense LMG 21827^T	AF193818	FM253226	FM253312	FM253140	FM253183	FM253269
B. iriomotense L EK05^T	AB300992	AB300997	JF308944	AB300994	AB300996	HQ587646
B. betae LMG 21987^T	AY372184	AB353735	FM253303	FM253129	AB353734	FM253260
B. cytisi CTAW11^T	EU561065	JN186292	JN186290	GU001613	GU001575	JN186288
B. oligotrophicum LMG 10732^T	JQ619230	KC569467	-	JQ619232	JQ619231	KC569469
B. rifense CTAW71^T	EU561074	KC569466	-	GU001617	GU001585	KC569468
B. retamae Ro19^T	KC247085	-	-	KC247101	KC247094	-
B. arachidis CCBAU 051107^T	HM107167	JX437675	JX437668	HM107217	HM107233	JX437682
B. denitrificans LMG 8443^T	X66025	AB070583	FJ347273	FM253153	EU665419	FM253282
B. diazoefficiens SEMIA 5080	AF234889	-	FJ390997		FJ391157	JX867243
B. japonicum SEMIA 5079	AF234888	-	FJ390996		FJ391156	-
B. elkanii SEMIA 5019	AF237422	-	FJ390990		FJ391150	-
B. elkanii SEMIA 587	AF234890	-	FJ390985		FJ391145	-