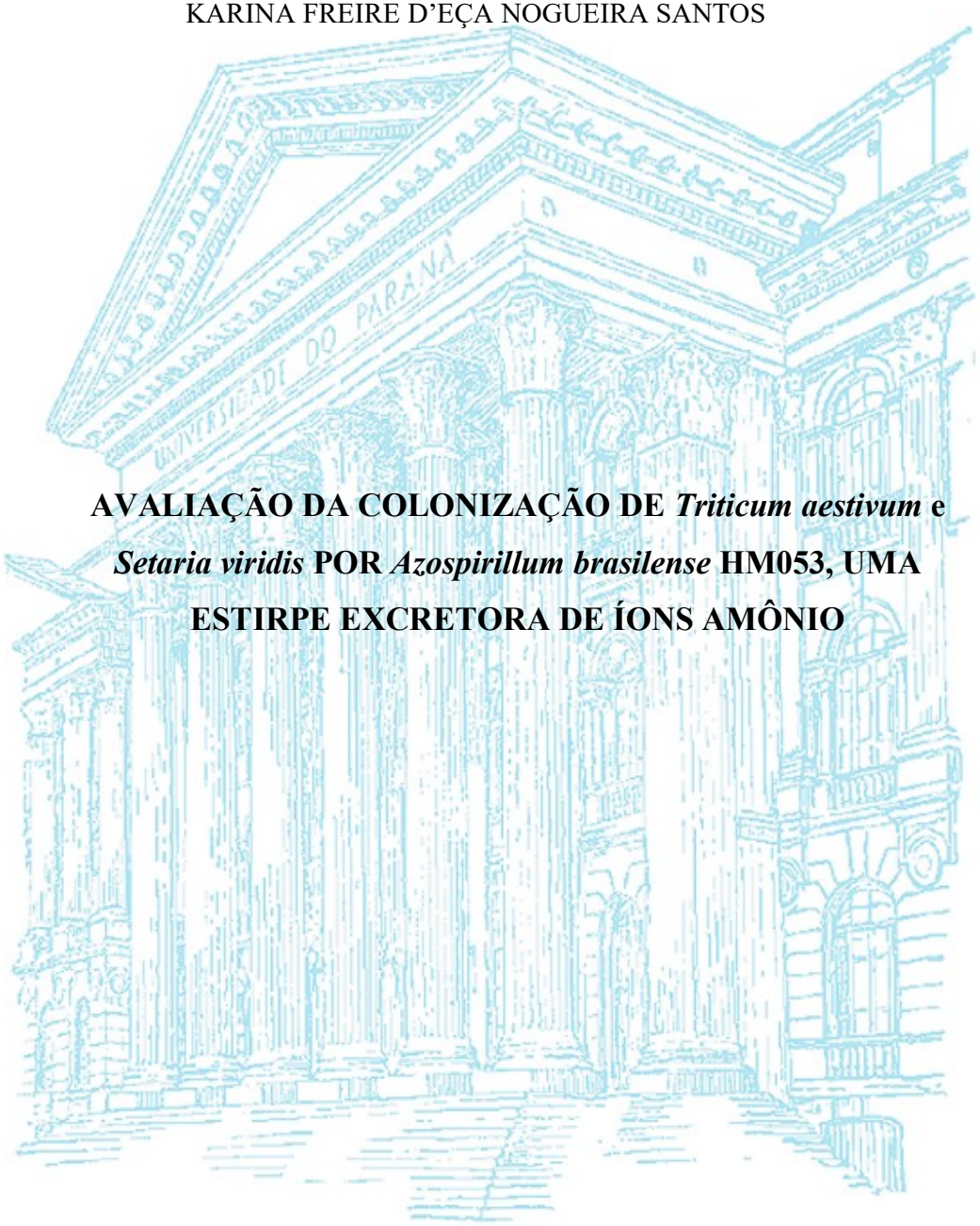
UNIVERSIDADE FEDERAL DO PARANÁ

KARINA FREIRE D'EÇA NOGUEIRA SANTOS



AVALIAÇÃO DA COLONIZAÇÃO DE *Triticum aestivum* e *Setaria viridis* POR *Azospirillum brasilense* HM053, UMA ESTIRPE EXCRETORA DE ÍONS AMÔNIO

CURITIBA

2014

KARINA FREIRE D'EÇA NOGUEIRA SANTOS

AVALIAÇÃO DA COLONIZAÇÃO DE *Triticum aestivum* e *Setaria viridis* POR *Azospirillum brasilense* HM053, UMA ESTIRPE EXCRETORA DE ÍONS AMÔNIO

Tese apresentada como requisito parcial para conclusão do doutorado em Ciências – Bioquímica, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná.

Orientadora: Dra Maria Berenice Reynaud Steffens

Co-orientador: Dr. Emanuel Maltempi de Souza

CURITIBA

2014

Santos, Karina Freire D'eça Nogueira
S237 Avaliação da colonização de *Triticum aestivum* e *Setaria viridis* por *Azospirillum brasilense* HM053, uma estirpe excretora de íons amônio / Karina Freire D'eça Nogueira Santos. - Curitiba, 2014.
140 f.: il., tabs, grafs.

Orientadora: Profª Drª Maria Berenice Reynaud Steffens
Co-orientador: Prof. Dr. Emanuel Maltempi de Souza
Tese (Doutorado) – Universidade Federal do Paraná, Setor de Ciências Biológicas, Curso de Pós-Graduação em Ciências – Bioquímica.

.1.Trigo. 2. *Setaria viridis. 3. Azospirillum brasilense*.
I. Steffens, Maria Berenice Reynaud. II. Souza, Emanuel Maltempi de. III.Título.IV. Universidade Federal do Paraná

CDD 575

TERMO DE APROVAÇÃO

KARINA FREIRE D'EÇA NOGUEIRA SANTOS

Avaliação da colonização de *Triticum aestivum* e *Setaria viridis* por *Azospirillum brasilense* HM053, uma estirpe excretora de íons amônio.

Tese aprovada como requisito parcial para a obtenção do grau de Doutora em Ciências - Bioquímica, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, pela seguinte banca examinadora:

Profa. Dra. Maria Berenice Reynaud Steffens
Departamento de Bioquímica e Biologia Molecular
Universidade Federal do Paraná

Profa Dra Claudia Cristina Garcia Martin-Didonet
Unidade de Ciências Exatas e Tecnológicas
Universidade Estadual de Goiás

Profa Dra Carolina Weigert Galvão
Departamento de Biologia Estrutural, Molecular e Genética
Universidade Estadual de Ponta Grossa

Profa. Dra. Ana Claudia Bonatto
Departamento de Genética
Universidade Federal do Paraná

Prof. Dr. Luciano Fernandes Huergo
Departamento de Bioquímica e Biologia Molecular
Universidade Federal do Paraná

Curitiba, 12 de dezembro de 2014.

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Programa de Pós-Graduação em Ciências – Bioquímica
49 anos

UFPR Biológicas

Ata referente à 225ª defesa de tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências (Bioquímica) da Universidade Federal do Paraná, pela candidata **KARINA FREIRE D'EÇA NOGUEIRA SANTOS**. Aos doze dias do mês de dezembro do ano de dois mil e quatorze, as quatorze horas no anfiteatro do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Paraná, teve início a defesa da tese: **"Avaliação da colonização de *Triticum aestivum* e *Setaria viridis* por *Azospirillum brasilense* HM053, uma estirpe excretora de íons amônio",** perante a Comissão Examinadora constituída pelos professores: Dr.ª Maria Berenice R. Steffens (orientadora) - Departamento de Bioquímica e Biologia Molecular, UFPR, Dr.ª Carolina Weigert Galvão - Departamento de Biologia Molecular, Estrutural e Genética, UEPG, Dr.ª Claudia Cristina Garcia Martin Didonet – Unidade de Ciências Exatas e Tecnológicas, UEG, Dr.ª Ana Claudia Bonatto – Departamento de Genética, UFPR, Dr. Luciano Fernandes Huergo – Departamento de Bioquímica e Biologia Molecular, UFPR. Após a arguição a Comissão Examinadora considerou a candidata aprovada. A Sra. Presidente agradeceu a presença de todos e encerrou os trabalhos, e eu, Thiago Vello, lavrei a presente ata que depois de lida e aprovada será assinada pelos membros da banca.

Parecer da banca (opcional):

Curitiba, 12 de dezembro de 2014.

Prof.ª Dr.ª Maria Berenice R. Steffens

Prof.ª Dr.ª Carolina Weigert Galvão

Prof.ª Dr.ª Claudia Cristina Garcia Martin Didonet

Prof.ª Dr.ª Ana Claudia Bonatto

Prof. Dr. Luciano Fernandes Huergo

AGRADECIMENTOS

Agradeço à Dra Maria Berenice pela boa convivência ao longo do doutorado e pela sua dedicação que foram fundamentais para a conclusão deste trabalho. Ao Dr. Emanuel Maltempi de Souza por sua orientação e estímulo que me ajudaram a decidir pelo doutorado sanduíche. Ao Dr. Gary Stacey pela excelente orientação na Universidade de Missouri, Columbia – EUA. Ao Dr. Richard Ferrieri pela paciência e pelos valiosos ensinamentos no Laboratório Nacional Brookhaven, Long Island NY – EUA. Agradeço também a sua esposa, Marylou pelo carinho durante a minha estadia em Long Island. Ao Dr. Fabio Pedrosa pela oportunidade de usufruir da estrutura do Núcleo de Fixação de Nitrogênio.

Agradeço aos técnicos Valter de Baura, Roseli Prado e Yaya Cui por serem sempre prestativos. A todos os colegas do Núcleo de Fixação pela agradável companhia, discussões e sugestões científicas. Aos meus amigos do Departamento de Bioquímica e Bond Life Science pelos momentos agradáveis que ficarão marcados em minha memória para sempre.

Aos professores do Departamento de Bioquímica e Biologia Molecular que contribuíram imensamente para a minha formação. À coordenação do curso de pós-graduação em bioquímica. Ao CNPq pelo auxílio financeiro no Brasil e Estado Unidos, sem este apoio eu não teria como continuar a minha formação acadêmica. Em especial agradeço pela bolsa sanduíche. Esta foi fundamental para o meu aprimoramento profissional.

Ao meu mestre de Jiu-jitsu Daniel Perez, pelo ensinamento desta arte e por sua amizade que me ajudaram a manter a saúde mental e física.

A toda minha família e amigos. Ao meu pai Cleber (*in memoriam*) que deixou de herança valiosos ensinamentos que levarei por toda a minha vida. À minha mãe Miriam por seu amor incondicional e por apoiar os meus sonhos. A vó Lourdes (*in memoriam*) pelo seu carinho e dedicação. Aos meus queridos irmãos, Luciana e Cristiano, por serem sempre meus companheiros de jornada.

RESUMO

O nitrogênio é um elemento essencial para o crescimento das plantas. Na produção agricultura, fertilizantes nitrogenados têm sido utilizados no campo. No entanto, o seu uso excessivo pode gerar danos ambientais como contaminação dos solos, áreas costeiras e causar eutrofização dos rios. Uma alternativa viável para reduzir esses danos ambientais seria o uso de biofertilizantes. Neste trabalho foi utilizada a bactéria diazotrófica *A. brasilense* que possui a enzima nitrogenase capaz de converter o nitrogênio atmosférico (N_2) em amônio (NH_4^+), forma assimilável pela planta. O primeiro objetivo desta tese foi caracterizar o processo de colonização pelo mutante espontâneo de *A. brasilense* (estirpe HM053) em plantas de trigo. Para isto, o gene repórter *gusA* fusionado ao gene *nifH* foi utilizado nas estirpes selvagem FP2 (denominada FP2-7) e HM053 (denominado HM053-36) que apresentaram a mesma capacidade de fixar nitrogênio *in vitro* e colonizar as raízes de trigo quando comparado às estirpes originais FP2 e HM053. Deste modo, foi possível observar significativa atividade da GusA localizada principalmente nos pontos de emissão das raízes laterais. Para o mutante HM053 esta atividade foi substancialmente mais forte e a expressão do gene *nifH* foi 278 vezes mais alta quando comparado a estirpe selvagem FP2. O segundo objetivo desta tese foi caracterizar o mutante HM053 em plantas de *Setaria viridis*. Com o uso de nitrogênio marcado (^{13}NN) foi possível provar que plantas de *S. viridis* inoculadas com este mutante assimilaram nitrogênio até 16 vezes mais do que plantas inoculadas com a estirpe selvagem FP2. Além disso, plantas de *S. viridis* inoculadas com o mutante HM053 responderam melhor à inoculação quando comparado a FP2 e ao mutante FP10, que é incapaz de fixar nitrogênio. Plantas inoculadas com a estirpe FP10 apresentaram significativo ganho somente no número de raízes laterais, o que pode ser explicado pelo fato de *A. brasilense* produzir fitormônios como a auxina. Já plantas inoculadas com as estirpes HM053 e FP2 apresentaram significativo aumento do comprimento da raiz, número de raízes laterais e massa seca da raiz. Interessantemente, somente plantas inoculadas com a estirpe HM053 apresentaram aumento significativo na produção de grãos (aumento de 34% em relação ao controle). No momento, está em andamento a análise do transcriptoma de raízes de *S. viridis* inoculada com as estirpes FP2, FP10 e HM053 e em breve será possível traçar um perfil molecular entre o processo de interação *S. viridis* - *A. brasilense,* o que contribuirá de maneira geral para o melhor entendimento do processo de interação planta – bactéria. Nesta tese, os efeitos benéficos da inoculação com o mutante espontâneo HM053 de *A. brasilense* tornam esta estirpe um biofertilizante promissor para ser utilizado no campo de acordo com as normas do Ministério da Agricultura.

Palavras-chave: Trigo, *Setaria viridis, Azospirillum brasilense, nifH* e transcriptoma.

ABSTRACT

Nitrogen is an essential nutrient used by plants for growth. In crop production, nitrogen fertilizers have been used in the field. However, excess nitrogen can create environmental problem in soil fields, coastal areas and river eutrophication. A viable alternative way to reduce these environmental problems is with the use of biofertilizers. Here in, it was used the diazotrophic bacterium *A. brasilense* that contain the enzyme nitrogenase, which is capable of converting atmospheric nitrogen (N_2) into ammonia (NH_4^+) as a nitrogen source that can be utilized by plants. The first goal for this thesis was to characterize the colonization process of a spontaneous mutant of *A. brasilense* (strain HM053) in wheat plants. To analyze wheat - *A. brasilense* interaction *nifH::gusA* fusions in the wild type FP2 (denominated FP2-7) and HM053 (denominated HM053-36) were used and showed similar levels of colonization and nitrogen fixation as original strains FP2 and HM053. Although HM053-36 and FP2-7 showed significant GusA activity located mainly at lateral root emergence points, HM053 consistently showed stronger signals and expressed the *nifH* gene 278-fold higher than FP2 *in planta*. The second goal for this thesis was to characterize HM053 in *Setaria viridis* plants. To test this, it was measured the ability of *S. viridis* plants inoculated with HM053 to fix^{13}NN. Plants inoculated with HM053 fixed nitrogen 16-fold higher than plants inoculated with FP2. Furthermore, *S. viridis* plants inoculated with HM053 responded better than plants inoculated with FP2 or an FP10 strain, which is unable to fix nitrogen. Plants inoculated with FP10 only showed a significant increase the number of lateral roots since *A. brasilense* has the ability to produce phytohormones, such as auxin. Inoculation of plants with HM053 and FP2 strains showed an increase of root length, lateral root number and root dry weight. Interestingly, plants inoculated with HM053 showed a 34% increase in the number of plant seeds compared to control plants. At present, transcriptome analysis of *S. viridis* inoculated with FP2, FP10, or HM053 is being processed to help better understand the crosstalk between plants and bacteria at the molecular level. In this thesis, the *A. brasilense* spontaneous mutant HM053 showed a beneficial symbiotic effect, which could be used in the field if it complied with the Brazilian Ministry of Agriculture.

Keywords: Wheat, *Setaria viridis, Azospirillum brasilense*, *nifH* and transcriptome.

SUMÁRIO

1. INTRODUÇÃO

1.1 Fixação biológica de nitrogênio e biofertilizantes

O nitrogênio é um elemento essencial para o desenvolvimento da planta, e portanto sua ausência ou limitação prejudica o crescimento vegetal. A atmosfera é composta de 80% de dinitrogênio gasoso (N_2), porém as plantas não podem absorver diretamente esse gás, sendo as fontes assimiláveis o amônio e o nitrato disponível no solo ou administrados às culturas através de fertilizantes químicos (DOBERMANN, 2007; WESTHOFF, 2009). Em nível mundial, em 2012 foram adicionados 109,9 milhões de toneladas de adubo nitrogenado ao solo. Entre 2011 e 2012 houve um aumento médio de 1,6% ao ano no uso deste fertilizante e a estimativa é que em 2016 serão utilizados cerca de 116 milhões de toneladas (FAO, 2012). A aplicação excessiva deste adubo nitrogenado interfere no ciclo do nitrogênio e causa diversos impactos ambientais, como a eutrofização dos rios, áreas costeiras e a acidificação dos solos (DIXON; KAHN, 2004). Além disso, a adubação com nitrogênio representa um dos principais responsáveis pelo encarecimento da produção agrícola (GOOD et al., 2004).

Alguns procariotos presentes na rizosfera do solo, denominados rizobactérias, são capazes de utilizar o nitrogênio atmosférico através do processo conhecido como fixação biológica de nitrogênio. Estas bactérias são denominadas de diazotróficas e convertem o nitrogênio atmosférico a amônio através da enzima nitrogenase (DIXON; KAHN, 2004). As rizobactérias que estabelecem interação positiva com a raiz da planta são denominadas de bactérias promotoras de crescimento da planta ou PGPB (*Plant Growth Promoting Bacteria*) e são promissoras como biofertilizantes na agricultura sustentável (CELLO DI et al., 1997). Os biofertilizantes são preparados a partir de microrganismos vivos, e quando aplicados na semente ou na superfície da planta podem promover o crescimento desta, seja através do aumento da disponibilidade de nutrientes, produção de fitormônios ou reduzindo os efeitos negativos de patógenos (BLOEMBER; LUGTENBERG, 2001; VESSEY, 2003; SOMERS et al., 2004). Os benefícios da aplicação de biofertilizantes são amplamente reconhecidos na simbiose de leguminosas com bactérias do gênero *Bradyrhizobium.* No Brasil a cultura da soja obtém todo o nitrogênio necessário da atmosfera através da associação com as espécies *Bradyrhizobium japonicum* e *Bradyrhizobium elkanii* (HUNGRIA et al., 1997; VARGAS; HUNGRIA, 1997; MENDES et al., 2000, HUNGRIA et al., 2005, HUNGRIA et al., 2006 a,b).

Outros gêneros de bactérias como *Azospirillum*, *Gluconacetobacter*, *Azoarcus*, *Enterobacter*, *Herbaspirillum* e *Burkholderia,* que colonizam um amplo grupo de gramíneas, também possuem potencial como biofertilizante e por isso têm sido extensivamente estudados (BALANDREAU, 2001; BALDANI et al.,1986; BALLY et al.; 1983; BILAL et al., 1990; DÖBEREINER et al., 1976; ELBELTAGY et al., 2001; GILLIS et al., 1989; REINHOLD-HUREK et al., 1993). Algumas das principais bactérias que podem contribuir com o melhoramento do milho, arroz e o trigo, estão apresentadas na tabela 1.

Entre esses gêneros citados acima, a espécie *Azospirillum brasilense* tem se destacado entre as bactérias diazotróficas devido ao efeito positivo na promoção de crescimento de diferentes espécies de plantas de interesse agronômico (BASHAN et al., 2004). A sua habilidade de promover o crescimento foi atribuída a duas características principais: capacidade de fixar nitrogênio (DÖBEREINER et al., 1976) e produzir fitormônios (TIEN et al., 1979). Como exemplo da promoção de crescimento foi observado o aumento das raízes, o que facilita a captação de água e nutrientes pelas plantas, a produção de fitormônios como giberilina, ácido indolacético e etileno, além de múltiplos outros fatores que ainda não foram completamente estabelecidos entre a interação planta - *Azospirillum* spp (DOBBELAERE et al., 2001; DOBBELAERE et al., 2003; ZEMRANY et al., 2007; BASHAN et al., 2004).

Saubidet e colaboradores (2002) avaliaram em casa de vegetação na Argentina variedades de trigo *(Triticum aestivum L)* inoculadas com *Azospirillum brasilense* (estirpe BNM-10) e observaram aumento significativo das raízes. Isto acarretou em melhor captação de nitrogênio inorgânico, levando ao aumento do conteúdo protéico do grão e melhor produtividade. Em experimentos conduzidos no campo em duas localidades diferentes no Estado do Paraná, Londrina e Ponta Grossa, foram obtidos resultados positivos com plantas de milho (*Zea mays* L) e trigo inoculadas com *A. brasilense* e *A. lipoferum* (HUNGRIA et al., 2010). As plantas de milho e trigo inoculadas com as estirpes de *A. brasilense* Ab-V4, Ab-V5, Ab-V6 e Ab-V7, e com as estirpes Al-V1 e Al-V2 de *A. lipoferum* apresentaram maior produtividade de grãos quando comparadas às plantas não inoculadas, em ambas as cidades. Outro resultado avaliado foi a captação de macro e micronutrientes. Para as plantas de milho inoculadas com Ab-V5, Ab-V6 e Ab-V7 e com a estirpe Al-V1 de *A. lipoferum* foi observado maior acúmulo de P, K e Cu nos grãos e nas folhas. Já o nitrogênio aumentou nas folhas, porém não houve aumento significativo nos grãos. O efeito positivo na produtividade de grãos de milho e trigo foi atribuído a melhor captação de nutrientes e não especificamente a fixação biológica de nitrogênio (HUNGRIA et al., 2010).

Assim, o uso de bactérias diazotróficas como biofertilizantes representa uma alternativa viável para minimizar os efeitos deletérios da utilização de adubos nitrogenados e reduzir o custo da produção agrícola. Essa tese tem como objetivo contribuir com o melhor entendimento entre a interação planta-*Azospirillum brasilense* mutante HM053, estirpe capaz de excretar amônio e fixar nitrogênio constitutivamente, explorando assim a capacidade desse organismo como um candidato promissor a biofertilizante.

TABELA 1 – RESPOSTAS BENÉFICAS OBSERVADAS EM PLANTAS DE INTERESSE AGRONÔMICO INOCULADAS COM BACTÉRIAS DIAZOTRÓFICAS.

Gramínea	Inoculante diazotrófico	Resposta da planta a inoculação	Referência
Arroz	*Azoarcus* spp.	Aumento de 16% da massa seca total da planta	REINHOLD-HUREK; HUREK, 1997*
	Azospirillum brasilense	Aumento da biomassa da parte aérea	SALAMONE et al., 2010**
	Gluconacetobacter diazotrophicus	Aumento de 30% da massa seca total da planta*	MUTHUKUMARASAMY et al., 2005*
	Serratia marcescens	Aumento de 23% da massa seca total da planta*	GYANESHWAR et al., 2001*
	Burkholderia vietnamiensis	Aumento de 13-22% na produtividade**	TRÂN VAN et al., 2000**
Milho	*Klebsiella pneumoniae*	Aumento de 25,8% da produtividade	RIGGS et al., 2001**
	Bacillus spp.	Aumento de 30,5% da produtividade	
	Herbaspirillum seropedicae	Aumento de 7,3% da produtividade	
	Azospirillum brasilense	Aumento de 85% da produtividade	DÍAZ-ZORITA; FERNANDEZ CANIGIA, 2008**
		Aumento de 27% da produtividade e melhor captação de macro e micronutrientes	HUNGRIA et al., 2010**
	Burkholderia spp.	Aumento de 68% da biomassa da parte aérea e 19% da biomassa da semente	BALDANI et al., 2000*
	Azotobacter spp.	Aumento de 19,5% da produtividade**	PANDEY et al., 1998**
	Azospirillum lipoferum	Aumento no número de raízes laterais	EL ZEMRANY et al., 2006
Trigo	*Azospirillum brasilense*	Aumento de 76% na produtividade	DÍAZ-ZORITA;FERNANDEZ CANIGIA, 2008**
		Aumento de 14% da massa fresca da planta e 11% da massa seca da planta	SPAEPEN et al., 2008*
		Aumento de 30% da produtividade e melhor captação de macro e micronutrientes	HUNGRIA et al., 2010**

1.2 Gênero *Azospirillum*

A primeira descrição do gênero *Azospirillum* incluía apenas duas espécies de bactérias fixadoras de nitrogênio: *A. brasilense* e *A. lipoferum* (TARRAND et al., 1978). Porém, ao longo dos anos novos isolados foram descritos e atualmente este gênero contém 18 espécies: *A. brasilense, A. lipoferum* (TARRAND et al., 1978), *A. amazonense* (MAGALHÃES et al., 1983), *A. irakense* (KHAMMAS et al., 1989), *A. halopraeferens* (REINHOLD et al., 1985), *A. largimobile* (DEKHIL et al., 1997), *A. doebereinerae* (ECKERT et al., 2001), *A. oryzae* (XIE; YOKOTA, 2005), *A. melinis* (PENG et al., 2006), *A. canadense* **(**MEHNAZ et al., 2007), *A. fermentarium* (LIN et al., 2013) *A. formosense* (LIN et al., 2012) *, A. humicireducens* (ZHOU et al., 2013)*, A. palatum* (ZHOU et al., 2009), *A. picis* (LIN et al., 2009), *A. rugosum* (YOUNG et al., 2008), *A. thiophilum* (LAVRINENKO et al., 2010)*, A. zeae* (MEHNAZ et al., 2007) e uma provável nova espécie, *A. massiliensis* (FONTE: http://goo.gl/OsB9Vm). Dentre essas espécies *A. brasilense* e *A. lipoferum* possuem vários plasmídeos com tamanho variando de 40 kbp a 550 kbp (PLAZINSKI; DART; ROLFE, 1983; WOOD et al., 1982). As espécies a *A. irakense, A. amazonense* e *A. halopraeferens* também possuem a estrutura do genoma complexo com vários plasmídeos, múltipos replicons, cromossomos circulares e lineares (CABALLERO-MELLADO et al., 1999; MARTIN-DIDONET et al., 2000). Comparado ao genoma da *Escherichia coli* o genoma do *Azospirillum* spp. é 1,8 vezes maior, confirmando o seu maior nível de complexidade genômica (WOOD et al., 1982). A espécie *A. brasilense* é a mais estudada e possui de cinco a sete megareplicons variando de 0,65 a 2,6 Mpb. Diversos megareplicons mostraram sinal de hibridização com o gene rDNA 16S, o que sugere a presença de múltiplos cromossomos para esta espécie, e os genes estruturais relacionados à nitrogenase estão localizados no maior replicon (MARTIN-DIDONET et al., 2000). O genoma das bactérias *A. brasilense* (estirpe Sp245) e *A. lipoferum* (estirpe 4B) foram sequenciados e genes que codificam função crítica para associação com plantas foram encontrados entre os genes de transferência horizontal (WISNIEWSKI-DYÉ et al., 2011). Segundo estes autores cerca de 50% do genoma de *Azospirillum* é composto por genes de transferência horizontal, que incluem genes relacionados a mecanismos de defesa, transporte, metabolismo de aminoácidos, carboidratos, íons inorgânicos e metabólitos secundários. Isto é coerente com o ambiente da rizosfera, que é rica em aminoácidos, carboidratos, íons inorgânicos e metabólitos secundários exsudatos pelas raízes das plantas (DENNIS et al., 2010). Wisniewski-dyé e colaboradores (2011) também concluíram que o

gênero *Azospirillum* é filogeneticamente próximo de bactérias adaptadas ao sistema aquático, o que sugere que o ancestral deste gênero já foi adaptado a este ambiente, e a transferência do ambiente aquático para a rizosfera justifica o elevado nível de genes de transferência horizontal encontrado em seu genoma, uma vez que estes genes possuem um papel importante para a adaptação a mudanças ambientais (HALDESMAN, et al., 2007).

O gênero *Azospirillum* foi isolado a partir de diferentes gramíneas e cereais em regiões de clima tropical e temperado (DÖBEREINER; DAY, 1976; PATRIQUIN; DÖBEREINER; JAIN, 1983). Possui uma ampla versatilidade de metabolizar C e N o que o torna altamente adaptado e competitivo em ambientes como a rizosfera (HARTMANN; ZIMMER, 1994). Em condições desfavoráveis, como limitação de nutrientes, *Azospirillum* pode se converter a forma de cisto e utilizar como fonte de energia grânulos de polihidroxibutirato, podendo assim sobreviver em condições adversas (TAL; OKON, 1985; TAL; SMIRNOFF; OKON, 1990). Além disso, *Azospirillum* é altamente capaz de se locomover. Algumas espécies, como *A. brasilense*, *A. lipoferum* e *A. irakense,* possuem um flagelo polar sintetizado durante o crescimento em meio líquido e flagelos laterais presente durante o crescimento em meio sólido, o que auxilia a bactéria se locomover em superfícies sólidas (MOENS et al., 1995). A capacidade de *Azospirillum* spp. de se locomover no solo em resposta à presença da planta depende da quantidade de água disponível, do tipo de solo e da cultivar. Por exemplo, em solos com uma maior porcentagem de água, *Azospirillum* spp. foi capaz de se locomover melhor comparado a solos mais secos (BASHAN, 1986). Isso indica a importância do flagelo polar na habilidade dessa bactéria se locomover em um ambiente natural como o solo em direção à raiz da planta. Ao utilizar oito cultivares de trigo também foi observado uma capacidade de migração diferenciada, e esta resposta foi atribuída à versatilidade de compostos exsudatos pelas raízes (BASHAN, 1986), uma vez que *Azospirillum* spp. possui quimiotaxia positiva aos ácidos orgânicos, açúcares, aminoácidos e compostos aromáticos (BARAK; OKON, 1983; REINHOLD; HUREK; FENDRIK, 1985; ZHULIN; ARMITAGE, 1993; LOPEZ-DE-VICTORIA; LOVELL, 1993). A quimiotaxia é o primeiro passo para o início da interação *Azospirillum*-planta (BASHAN; LEVANONY, 1990; REINHOLD; HUREK; FENDRIK, 1985). Este assunto será melhor abordado no item 1.7: Interação planta-bactéria.

1.3. Enzima nitrogenase e vias de assimilação de amônio

Como citado anteriormente, a enzima nitrogenase é responsável pela fixação biológica do nitrogênio, e sua função é reduzir o dinitrogênio gasoso (N_2) presente na atmosfera a amônio, em uma reação que envolve gasto energético como esquematizado abaixo:

$$N_2 + 10H^+ + 8e^- + 16\ Mg.ATP \xrightarrow{\textbf{Nitrogenase}} 2\ NH_4^+ + 16Mg.ADP + 16Pi + H_2$$

O complexo da nitrogenase é composto de duas metaloproteínas: proteína-ferro ou NifH; e a proteína ferro-molibdênio ou NifDK. A proteína NifH (produto do gene *nifH*) apresenta um núcleo 4Fe-4S e a sua função é transportar elétrons até a proteína NifDK (produto dos genes *nifDK*) que contém dois grupos prostéticos: 2 centros P e 2 cofatores ferro-molibdênio (BURRIS, 1991). Esta reação de transporte de elétrons depende de ATP. Como este processo envolve gasto energético, o processo de fixação de nitrogênio é altamente regulado a nível transcricional e pós-traducional (POSTGATE, 1982). As enzimas NifH e NifDK, não possuem atividade catalítica isoladamente, sendo necessário a presença simultânea de ambas para que ocorra a redução do N_2, e além disso são sensíveis ao oxigênio. Assim, os microrganismos diazotróficos desenvolveram estratégias ou mecanismos para impedir sua inativação pelo oxigênio, como a aerotaxia que pode guiar o *Azospirillum* spp. para regiões favoráveis para a fixação biológica do nitrogênio na raiz da planta (BARAK et al., 1982; ZHULIN et al., 1996; EADY, 1986; KIM; REES, 1992).

O complexo da nitrogenase responde também às concentrações de amônio. Alguns diazotrofos, como *Azospirillum brasilense*, *Rhodobacter capsulatus* e *Rhodospirillum rubrum,* apresentaram inibição reversível da nitrogenase *in vivo,* um mecanismo de controle pós-traducional da atividade da nitrogenase em presença de amônio (ZUMFT; CASTILLO, 1978; ZHANG et al., 1996). A inativação é denominada desligamento (switch-off) da nitrogenase por íons amônio, e após a exaustão dos íons amônio da cultura a nitrogenase é reativada, e este fenômeno é denominado religamento (switch-on) (ZUMFT; CASTILLO, 1978).

O amônio obtido da fixação biológica ou do meio externo é utilizado na síntese de glutamina e glutamato, que servem como doadores de nitrogênio para reações biossintéticas. Existem três vias que permitem a incorporação de íons amônio em glutamato e glutamina. Estas vias são: glutamina sintetase, glutamato sintase e glutamato desidrogenase (ARCONDÉGUY et al., 2001). Para *A. brasilense* a via predominante é a

via glutamina sintetase-glutamato sintase que independe da fonte de nitrogênio utilizada para crescimento (WESTBY et al., 1987). A glutamina sintetase catalisa a conversão do glutamato e amônio em glutamina. Já a enzima glutamato sintase catalisa a transferência do grupo amina da glutamina para o α-cetoglutarato, resultando em duas moléculas de glutamato (MERRICK; EDWARDS, 1995) conforme a seguinte reação:

1. NH_4^+ + L-glutamato + ATP $\xrightarrow{\textbf{Glutamina sintetase}}$ L-glutamina + ADP + Pi

2. L-glutamina + α-cetoglutarato + NADPH $\xrightarrow{\textbf{Glutamato sintase}}$ 2 L-glutamato + $NADP^+$

1.4. Estirpes mutantes de *Azospirillum brasilense* excretoras de amônio

Os estudos dos mecanismos de regulação da fixação de nitrogênio em *A. brasilense* iniciaram, no Núcleo de Fixação de Nitrogênio - UFPR, com o isolamento de mutantes alterados na atividade da nitrogenase (fenótipo Nif$^-$), que foram caracterizados através de complementação com genes *nif* e *ntr* de *K. pneumoniae* (PEDROSA; YATES, 1984; FUNAYAMA et al., 1985).

Pedrosa e Yates (1984) isolaram a estirpe FP2 (Sp7 ATCC 29145, Nal ^{R}, Sm^R), e a partir desta estirpe Machado (1988) isolou e caracterizou quatro mutantes espontâneos resistentes a etilenodiamina (EDA): HM14, HM26, HM053 e HM210. A EDA é um análogo do amônio e quando incorporado, via glutamina sintetase (GS), produz aminoetil glutamina, um composto aparentemente tóxico a célula (ROWELL et al., 1985). Por essa razão bactérias resistentes a EDA geralmente possuem alguma deficiência na GS e atividade da nitrogenase desreprimida (POLUKHINA et al., 1982). Os quatro mutantes HMs são capazes de excretar amônio e de fixar nitrogênio constitutivamente (fenótipo Nif^c), mesmo na presença de altas concentrações de íons amônio, ou seja, apresentam atividade da nitrogenase insensível ao desligamento (switch-off) por NH_4^+, e a capacidade em excretar amônio produzido pela fixação de nitrogênio está relacionada à deficiência na glutamina sintetase. Além disso, os mutantes da série HM são prototróficos para glutamina, e o amônio excretado é o produto do nitrogênio fixado. Isto foi confirmado, pois, a excreção do NH_4^+ pode ser transitoriamente abolida se houver a substituição do ar atmosférico pelo gás argônio (MACHADO et al., 1991). Os mutantes da série HM

apresentaram atividade da glutamato sintase e glutamato desidrogenase similar à estirpe selvagem FP2. Por outro lado, apresentaram atividade baixa para a glutamina sintetase o que poderia levar a uma deficiência de assimilação de NH_4^+ e explicar sua habilidade em excretar o excesso de amônio derivado do nitrogênio fixado (MACHADO et al., 1991).

1.5 Sistema Ntr

O funcionamento do complexo da nitrogenase em *A. brasilense* requer os produtos de outros genes, além dos genes *nifHDK,* que auxiliam no controle da atividade da nitrogenase em resposta as concentrações de amônio e oxigênio, como os genes do sistema Ntr (Nitrogen regulation system), que regula a assimilação de fontes alternativas de nitrogênio como nitrato (MERRICK; EDWARDS, 1995). Vitorino et al., (2001) caracterizou os genes *nif*, *gln* e *ntr* da série HM e observou que a proteína NifA, responsável por desencadear a fixação biológica de nitrogênio, é expressa e está ativa nos quatros mutantes. Foi observado também que estes mutantes da série HM possuem alterações diferentes na glutamina sintetase (produto do gene *glnA*), porém apresentam os mesmos fenótipos como crescimento deficiente em nitrato, expressão constitutiva dos genes *nif* e falta de regulação do gene *glnB*. Dentre os quatro mutantes destacaremos as alterações na estirpe HM053, que é alvo de estudo nesta tese. A introdução do plasmídeo pAB441, que contém o operon *glnAB* de *A. brasilense,* na estirpe HM053 não restaurou o controle da fixação de nitrogênio na ausência ou na presença de amônio. Porém, este plasmídeo complementou o mutante HM053 para a capacidade de crescimento em meio contendo nitrato ou em meio com baixa concentração de íons amônio como única fonte de nitrogênio. Além disso, o sequenciamento do gene *glnB* não apontou alterações estruturais quando comparado ao gene da estirpe FP2 (VITORINO et al., 2001). Segundo Srivastava e Tripathi (2006) bactérias resistentes a EDA frequentemente possuem mutações em regiões do gene *glnA,* que são os sítios de ligação ao amônio. Em 2012, Hauer sequenciou o gene *glnA* de *A. brasilense* HM053 e identificou a troca do resíduo de prolina na posição 347 por leucina. Esta região da troca está próxima as argininas, as quais são importantes sítios de ligação a ATP e glutamato. Segundo Dhalla e colaboradores (1994), mutantes da glutamina sintetase de *S. typhimurium* em resíduos conservados e próximos, como a arginina 339 e a arginina 359, demonstraram a importância desses sítios para a ligação ao ATP e glutamato. Portanto, essas alterações podem afetar parte da atividade biossintética da glutamina sintetase.

1.6 Genes repórteres e sua aplicação no estudo da interação planta-bactéria

Os genes repórteres são importantes ferramentas da biologia molecular com diferentes aplicações e potencial uso para estudos ecológicos (WILSON et al.,1995; ERRAMPALLI et al.,1999). O gene repórter *gusA*, isolado de *E. coli* e que codifica a β-glucuronidase, foi descrito por Jefferson e colaboradores (1987), que avaliaram a sua atividade em tecidos de plantas transgênicas. Este gene repórter fusionado a genes *nif* tem sido utilizado para mostrar a expressão da nitrogenase *in planta* (RONCATO-MACCARI et al., 2003). Outros genes repórteres utilizados são os da proteína verde fluorescente (*gfp*) isolado da água-viva *Aequorea victoria* (CHALFIE et al.,1994) e o gene *dsred,* que codifica a proteína fluorescente vermelha DsRed, que é homóloga a GFP e foi isolada a partir do gênero de coral *Discosoma* spp. (GROSS et al., 2000). A vantagem das proteínas fluorescentes é a detecção rápida e direta, sem a necessidade de processar o material para análise, enquanto que o gene *gusA* requer processamento com o seu substrato 5-bromo-4-cloro-3-indolil-β-D-glucuronídeo (X-gluc).

O gene repórter *gusA* fusionado ao gene *nifH* foi utilizado para monitorar o processo de colonização em plantas de trigo pela bactéria diazotrófica *A. brasilense* estirpes selvagem FP2 e mutante HM053.

1.7 Interação: planta – bactéria

Diferentes tipos de interação ocorrem entre bactérias diazotróficas e as plantas. Por exemplo, dentre os diazotrofos envolvidos na endossimbiose com leguminosas (família Fabacea) estão os rizóbios que são alfaproteobactérias gram-negativas (SCHULTZE; KONDOROSI, 1998; OLDROYD et al., 2008; DESBROSSES; STOUGAARD, 2011). As plantas não leguminosas (família Cannabaceae) podem se associar com bactérias gram-positivas, como *Frankia* spp*.,* membro da família de Actinobacterias que se associa com um amplo conjunto de plantas (8 diferentes famílias). Estas plantas são denominadas actinorrízicas, ou seja, capazes de formar nódulos radiculares que abrigam fixadores de nitrogênio em simbiose. Já as cianobactérias fixadoras de nitrogênio, como *Nostoc* spp., colonizam diferentes classes de plantas podendo ser encontradas no interior de plantas da família Gunneraceae ou no exterior das plantas da família Cycadaceae (SANTI et al., 2013).

A quantidade de nitrogênio fixado pelas bactérias diazotróficas em associação com plantas da família Poaceae (ou gramíneas) não é tão expressivo quando comparado com a

fixação biológica de nitrogênio em plantas leguminosas ou actinorrízicas. No entanto, resultados positivos entre esta associação têm sido observados no campo (DOBBELAERE et al., 2003; VESSEY, 2003; VERMA et al., 2010; BHATTACHARYYA; JHA, 2012).

A primeira comunicação entre bactérias diazotróficas e a planta se inicia através da quimiotaxia positiva que as bactérias possuem em direção aos exsudatos das raízes das plantas. A composição dos exsudatos da raiz depende do tipo de solo, da disponibilidade de nutriente, do genótipo da planta, do estágio de crescimento e das condições de estresse biótico e abiótico do meio. Segundo Lopez-de-Victoria e Lovell (1993) a quimiotaxia de *A. brasilense* e *A. lipoferum* por compostos aromáticos mostrou-se dependente da capacidade destes microrganismos de catabolizar os compostos. Assim, pode-se concluir que o primeiro indicativo de quimiotaxia se dá através da capacidade do microrganismo de metabolizar o composto aromático. Essas duas espécies tiveram quimiotaxia positiva ao benzoato, catecol, ácido 3,4-dihidroxibenzoico e 4-hidroxibenzoato. Estes compostos são metabolizados por *Azospirillum,* e ainda suportam o seu crescimento e a fixação biológica de nitrogênio (CHEN et al., 1993). Além disso, *Azospirillum* é capaz de detectar baixas concentrações de compostos aromáticos presentes no solo, quando comparado com outras bactérias, o que contribui para a sua melhor adaptação e sobrevivência em solos oligotróficos (CHET; MITCHELL, 1976; ROSZAK; COWELL, 1987).

Uma estirpe mutante de *H. seropedicae,* afetada na proteína AmpG muropeptídeo permease, que permite que os monômeros de açúcares entrem no citoplasma e sejam reciclados e incorporados à peptideoglicanos, apresentou um perfil de lipopolissacarídeos (LPS) diferenciado e reduzida habilidade em colonizar a raiz de milho comparado a estirpe selvagem de *H. seropedicae* SmR1 (TADRA-SFEIR et al., 2011). Balsanelli e colaboradores (2010) realizaram o *knock-out* de dois genes que estão envolvidos na biossíntese de ramnose: *rfbB* (dTDP-D-glucose 3,5-epimerase) e *rfbC* (dTDP-4keto-L-ramnose redutase) em *H. seropedicae*. A habilidade desses mutantes de colonizar a superfície da raiz de milho foi reduzida em 100 vezes, comparada à bactéria do tipo selvagem, e a quantidade de bactérias no interior do tecido da planta também foi 100 vezes menor. Um mutante de *A. brasilense* afetado na biossíntese de dTDP-ramnose teve a produção de LPS afetada reduzindo assim a sua capacidade de colonização da raiz de trigo (JOFRÉ et al., 2004). Além disso, algumas proteínas presentes na membrana externa de *A. brasilense* apresentam um papel importante no processo de adesão e adsorção às raízes das plantas, como a proteína porina *major outer-membrane protein* (MOMP) (BURDMAN et al., 2001). Através de ensaios de Western blotting, estes autores concluíram que a MOMP

apresenta alta capacidade de se aderir a extratos das raízes de trigo, milho e sorgo. O mesmo não foi observado para raízes de tomate, feijão e grão de bico (BURDMAN et al., 2001).

Em *A. brasilense,* além da quimiotaxia, a motilidade também é uma etapa importante para o inicio da colonização da raiz. A perda do flagelo polar por calor, tratamento ácido ou deleção do gene responsável pela sua expressão reduziu a capacidade de colonização da raiz de trigo por *A. brasilense.* Croes e colaboradores (1993) mostraram que o flagelo polar purificado se liga à raiz de trigo, enquanto os flagelos laterais não possuem essa função. A primeira etapa na colonização da raiz por *A. brasilense* é denominada de adsorção, e é caracterizada como sendo uma associação fraca e reversível porque esta bactéria pode se desassociar da raiz facilmente. Nessa etapa inicial a bactéria utiliza o flagelo polar para se ligar a raiz da planta (FIGURA 1). Em seguida, *A. brasilense* se agrega fortemente sendo essa associação irreversível. A segunda etapa é chamada de ancoramento e depende dos exopolissacarídeos (EPS) produzidos pelas bactérias (FIGURA 1) (STEENHOUDT; VANDERLEYDEN, 2000). Deste modo, inicia-se o processo de colonização da raiz pela bactéria *A. brasilense.*

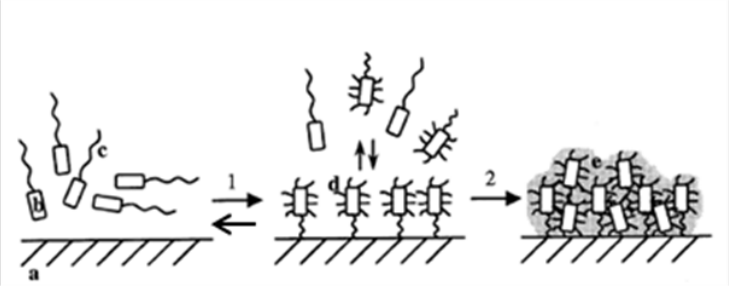


FIGURA 1 - ETAPAS DE COLONIZAÇÃO POR *A. brasilense.* A ETAPA 1 (ADSORÇÃO) REPRESENTA UMA FRACA E REVERSÍVEL ASSOCIAÇÃO CÉLULA POR CÉLULA NA RAIZ DA PLANTA. ESTE PRIMEIRO CONTATO É MEDIADO ATRAVÉS DO FLAGELO POLAR. A ETAPA 2 (ANCORAMENTO) REPRESENTA UMA FIRME E IRREVERSÍVEL ASSOCIAÇÃO, ONDE OS EXOPOLISSACARÍDEOS (EPS) DESEMPENHAM UMA IMPORTANTE FUNÇÃO. AS LETRAS REPRESENTAM: A) SUPERFÍCIE DA RAIZ DA PLANTA; B) *A. brasilense*; C) FLAGELO POLAR; D) FLAGELO LATERAL; E) EPS (ADAPTADO DE STEENHOUDT; VANDERLEYDEN, 2000).

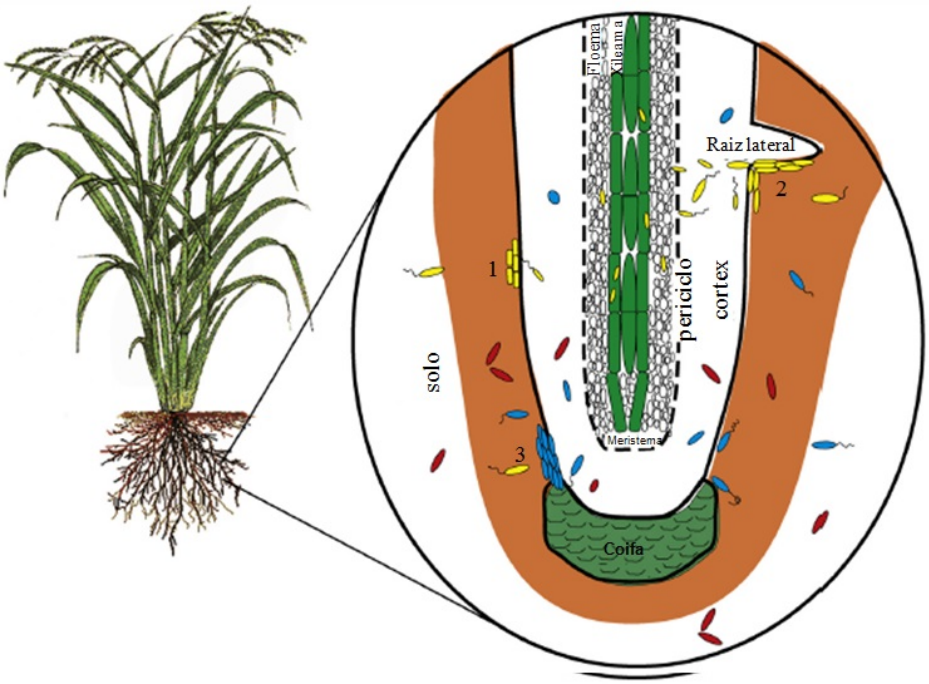
1.8 Colonização da raiz por bactérias diazotróficas

Bactérias diazotróficas das classes Alfa e Betaproteobacteria como *Acetobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Gluconacetobacter* e *Pseudomonas* podem colonizar o interior do tecido vegetal (sendo denominadas de bactérias endofíticas) ou a superfície da raiz (denominadas de bactérias epifíticas) sem causar visível sinal de infecção ou efeito negativo para a planta (BALDANI

et al., 1986; VESSEY, 2003; SCHMID; HARTMANN, 2007; COCKING, 2009; RICHARDSON et al., 2009). Para ambos os tipos de colonização as bactérias não induzem a formação de estruturas diferenciadas nas raízes como os nódulos. Esse tipo de associação não é considerada endossimbiótica onde a bactéria vive intracelularmente nas células das plantas. Entre os gêneros citados acima as espécies *Acetobacter diazotrophicus, Herbaspirillum seropedicae* e *Azoarcus* spp. são efetivamente reconhecidas como endofíticas. Estas primeiramente colonizam a superfície da raiz, e em seguida podem alcançar os tecidos internos (JAMES et al., 1994; JAMES; OLIVARES, 1998; HUREK et al., 1994) que incluem quaisquer regiões internas à epiderme da planta. O espaço intercelular ocupado pelas bactérias compreende uma fração significativa do tecido vegetal. Como exemplo, os espaços entre as células corticais radiculares podem abranger até 30% do volume da raiz, enquanto os espaços entre as células do mesófilo foliar até 70% do volume da folha (GARBEVA et al., 2001). As bactérias endofíticas podem acessar os nutrientes e a água mais facilmente, além de serem menos afetadas pelas flutuações ambientais quando comparado às epifíticas. De maneira geral, as bactérias endofíticas penetram nas plantas através de lesões na epiderme, locais de emissão da radícula, das raízes laterais, através de aberturas naturais como os estômatos, lenticelas, hidatódios ou também através de ferimentos causados por nematódeos patógenos (HALLMAN, 2001; MCCULLY, 2001).

A bactéria *G. diazotrophicus* é capaz de colonizar os vasos do xilema e penetrar os espaços intercelulares da raiz através da coifa e das rachaduras nas junções das raízes laterais (FIGURA 2). O xilema foi sugerido como provável local para a fixação biológica de nitrogênio devido à baixa pressão do oxigênio atmosférico e troca de metabólitos entre a bactéria e o hospedeiro (JAMES et at., 1994). *H. seropedicae* acessa o espaço intercelular também através de rachaduras nos pontos de emissão das raízes laterais e um grande número de bactérias foi encontrada na raiz, caule e folhas de diferentes gramíneas como milho, arroz e sorgo (JAMES; OLIVARES, 1997) (FIGURA 2). Já os sítios primários de colonização de *Azoarcus* spp. são a região da coifa, a zona de elongação e os pontos de emergências das raízes laterais (FIGURA 2). Essa bactéria é capaz de colonizar o espaço intercelular e intracelular na região do córtex e penetrar os vasos do xilema alcançando assim a parte aérea da planta (HUREK et al., 1994). *Azoarcus* spp. expressa dois tipos de enzimas celulolíticas (exo e endoglucanase) que poderia auxiliar na degradação da parede celular, e assim na colonização dos tecidos internos da planta (REINHOLD-HUREK et al., 1993).

Para *A. brasilense* algumas estirpes são classificadas como epifíticas e outras como endofíticas. Após esterilizar a superfície da raiz foi demonstrado que a estirpe Sp 245 de *A. brasilense* é capaz de colonizar o interior da raiz (BALDANI et al., 1986). Esta estirpe Sp 245 foi encontrada no xilema do trigo, enquanto a estirpe Sp 7 somente colonizou a superfície da raiz (SCHLOTER et al., 1994). A pectina é o principal componente da parede celular e lamela média nas plantas (CROMBIE; SCOTT; REID, 2003). As enzimas responsáveis por sua degradação são denominadas de pectinases (VORAGEN et al., 2009). Baixos níveis de pectinases e atividade celulolítica tem sido detectados em culturas de *Azospirillum* spp. A bactéria pode utilizar esta enzima como ferramenta para degradar a parede celular da planta, e assim alcançar os espaços intercelulares do córtex da raiz (UMALI-GARCIA et al., 1980; OKON; KAPULNIK, 1986; TIEN et al., 1981), ou simplesmente penetrar na raiz através de rupturas do tecido como a região de emissão das raízes laterais e pêlos (UMALI-GARCIA et al., 1980). A figura 2 resume os possíveis sítios de entrada das bactérias diazotróficas endofíticas.



Fonte: Adaptado de HARDOIM et al., (2008).

FIGURA 2 - COLONIZAÇÃO ENDOFÍTICA DA RAIZ POR BACTÉRIAS PRESENTES NA RIZOSFERA DO SOLO. AS BACTÉRIAS PODEM FORMAR MICROCOLÔNIAS NA SUPERFÍCIE DA RAIZ (COMO REPRESENTADO EM 1, 2 E 3) ANTES DE ATINGIREM O CORTEX, OU ENTÃO O INÍCIO DA COLONIZAÇÃO PODE PARTIR DE BACTÉRIAS ISOLADAS (REPRESENTADAS EM VERMELHO). EM SEGUIDA, AS BACTÉRIAS PODEM PENETRAR NA RAIZ ATRAVÉS DE FERIMENTOS NATURAIS (BACTÉRIAS AMARELAS REPRESENTADAS EM 1), RACHADURAS QUE SE FORMAM NA COIFA (BACTÉRIAS REPRESENTADAS EM AZUL) E REGIÃO DOS PONTOS DE EMISSÃO DAS RAÍZES LATERAIS (BACTÉRIAS AMARELAS EM 2). ALGUMAS BACTÉRIAS SE RESTRINGEM A COLONIZAÇÃO DO CORTEX (BACTÉRIAS VERMELHAS E AZUIS) E OUTRAS PODEM COLONIZAR O SISTEMA VASCULAR DA RAIZ, O FLOEMA E O XILEMA, COMO REPRESENTADO PELAS BACTÉRIAS AMARELAS.

1.9. Promoção do crescimento vegetal por bactérias diazotróficas

A partir do momento que a bactéria diazotrófica coloniza a raiz, ela pode promover direta ou indiretamente o crescimento vegetal (GLICK et al., 1999) (FIGURA 3). A promoção direta do crescimento inclui a disponibilização de compostos pela bactéria que a planta não pode captar. Por exemplo, a solubilização de minerais contidos no solo, como o fósforo e o ferro, que a planta não pode captar ou, ainda, a fixação do nitrogênio atmosférico. Como já explicado anteriormente, o nitrogênio atmosférico (N_2) não é assimilável pela planta, sendo as bactérias diazotróficas capazes de converter o N_2 a uma forma assimilável. Depois do nitrogênio, o fósforo é o segundo mineral que mais limita o

crescimento da planta. Este mineral é extremamente abundante no solo, porém apenas cerca de 0,1% está disponível para a planta em sua forma solúvel (STEVENSON; COLE, 1999. As bactérias promotoras de crescimento das plantas, ou mais citada em inglês como *plant growth promoting bacteria* (PGPB), são capazes de solubilizar o fósforo do solo, através da produção de ácidos orgânicos, tornando-o assimilável pela planta nas formas de monobase ($H_2PO_4^{-1}$) ou dibase (HPO_4^{-2}) (TAHA et al., 1969; BANIK; DEY, 1982; HALDER et al., 1990; ILLMER et al., 1995; JONES, 1998). Além disso, algumas bactérias solubilizam fosfato orgânico através da enzima fosfatase (GREAVES; WEBLEY, 1965; GLASS, 1989; TARAFDAR; JUNK, 1987; GARCIA et al., 1992). O ferro está presente no solo em sua forma insolúvel, hidróxido férrico. Muitas bactérias produzem um composto orgânico denominado de sideróforo capaz de se ligar ao Fe^{3+} e reduzi-lo para a forma Fe^{2+}, que é assimilável pelas plantas (WEYENS et al., 2009).

Outro exemplo de promoção direta do crescimento pelas PGPB é a produção de fitormônios que podem induzir mudanças morfológicas e fisiológicas das raízes aumentando a área superficial, a taxa de respiração e, desta forma, promovendo o crescimento vegetal (GLICK et al., 1999; PODILE; KISHORE, 2006; BEATTIE, 2006). As bactérias *A. brasilense*, *Aeromonas veronii*, *Agrobacterium* spp., *Alcaligenes piechaudii*, *Bradyrhizobium* spp., *Comamonas acidovorans*, *Enterobacter* spp., *Rhizobium leguminosarum,* dentre outras, são capazes de produzir auxina (ácido indolacético ou simplesmente AIA) e promover o crescimento vegetal através do aumento do crescimento das raízes, proliferação e alongamento de pêlos radiculares, o que amplia a absorção de nutrientes e água pela planta (VESSEY, 2003). As vias biossintéticas de AIA nas bactérias são muito similares àquelas encontradas nas plantas. O triptofano é o principal precursor das rotas biossintéticas de AIA (DOBBELAERE; VANDERLEYDEN; OKON, 2010). Dobbelaere e colaboradores (1999), ao utilizar um mutante capaz de produzir 10% menos de AIA, puderam correlacionar a produção de AIA por *Azospirillum* spp. às alterações na morfologia das raízes de trigo. Plantas de trigo inoculadas com a estirpe selvagem Sp 245 de *Azospirillum brasilense* e dois transconjugantes capazes de produzir maiores concentrações de AIA, pFAJ5005 (produz AIA através da indução pela planta) e pFAJ5002 (produz AIA através da expressão constitutiva do gene *ipdC*), apresentaram encurtamento da raiz e aumento na formação de pêlos radiculares quando comparado as plantas controle sem a bactéria. No entanto, estes efeitos na raiz foram mais pronunciados quando utilizado as bactérias transconjugantes (pFAJ5005 e pFAJ5002) como inoculante do que a estirpe

selvagem Sp 245 (SPAEPE et al., 2008). Isso confirma que a produção de AIA por *A. brasilense* é capaz de alterar significativamente a morfologia da raiz de trigo.

Em *A. brasilense* existem múltiplas vias de produção de AIA, sendo a indol-3-piruvato a via mais importante. A enzima chave desta via é a indol-3-piruvato descarboxilase (codificada pelo gene *ipdC*). Assim, ao colonizar as plantas, *A. brasilense* usa os exsudatos das raízes para sua proliferação. Conforme esses exsudatos se tornam limitados, devido ao crescimento bacteriano, *A. brasilense* aumenta a produção de AIA, levando à formação de raízes laterais e pelos radiculares, que resultam em mais exsudação. Desta forma, cria-se uma regulação que conecta a proliferação bacteriana e o crescimento das raízes (SPAEPE et al., 2009). *Azospirillum* spp. também é capaz de promover o aumento das raízes, através da produção de outros fitormônios como giberilina e citocinina (DOBBELAERE et al., 2001; DOBBELAERE et al., 2003; BASHAN et al., 2004; MARTÍNEZ-MORALES et al., 2003).

A giberilina está envolvida na modificação da morfologia da planta, em particular o caule. A função da giberilina na raiz é menos importante comparado a função do AIA, porém a giberilina é indispensável para o desenvolvimento normal da raiz (TANIMOTO, 2005). Este hormônio foi identificado, através de bioensaio, HPLC ou cromatografia gasosa acoplada à espectrometria de massa, em *Azotobacter* spp. (BAREA; BROWN, 1974; AZCÓN; BAREA, 1975; GONZALEZ-LOPEZ et al., 1986; MARTINEZ-TOLEDO et al., 1988), *Paenibacillus polymyxa* (SATTAR; GAUR, 1987), *Rhizobium leguminosarum* bv. Phaseoli (ATZORN et al., 1988), *A. brasilense* (JANZEN et al., 1992), *A. lipoferum* (BOTTINI et al., 1989, PICCOLI; BOTTINI, 1994; PICCOLI et al., 1996), *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* (BASTIÁN et al., 1998), *Bacillus pumilus* e *Bacillus licheniformis* (GUTIÉRREZ-MAHERO et al., 2001). Ao aplicar 0,1 μg de ácido giberélico em plântulas de milho ou ao utilizar os inoculantes *A. lipoferum* ou *Azospirillum* spp. foi observado efeito similar no aumento dos pêlos da raiz de milho nessas três condições testadas. Foi observado também aumento na altura e peso fresco dessas plantas (FULCHIERI et al., 1993). Esses resultados sugerem que a produção de giberilina por bactérias, como *A. lipoferum* ou *Azospirillum* spp., pode contribuir para melhoramento do desenvolvimento vegetal.

A citocinina é responsável por estimular células em divisão e a expansão de certas partes das plantas (SALISBURY, 1994). Este hormônio pode promover também a abertura estomatal, estimular o crescimento da parte aérea e reduzir o crescimento da raiz. Arkhipova e colaboradores (2007) demonstraram que na presença de estresse moderado à

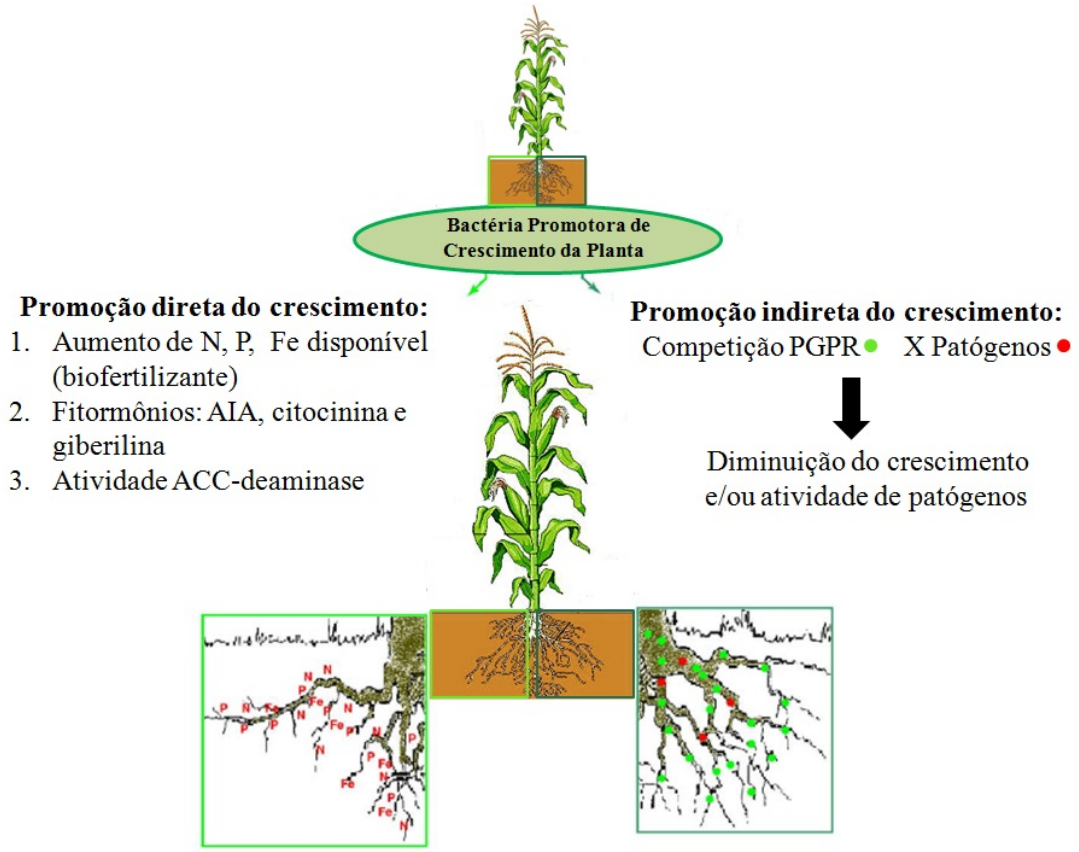
seca, plântulas de alface inoculadas com *Bacillus* respondem melhor quando comparadas com plantas controles sem a bactéria. Algumas evidências de que a citocinina promove o crescimento da planta tem sido investigado. Foi demonstrado que um mutante de *Rhizobium leguminosarum,* auxotrófico para adenosina que é precurssor para a biossíntese de citocinina, foi incapaz de promover o crescimento da raiz de plântulas de canola e alface quando comparado à estirpe selvagem. Isso sugere que a citocinina pode estar envolvida com o desenvolvimento da raiz (NOEL et al., 1996). Ao utilizar bioensaio, radioimunoensaio ou espectrometria HPLC-UV, a produção de citocinina foi observada em *Azotobacter* spp. (BAREA; BROWN, 1974; AZCÓN; BAREA, 1975; GONZALEZ-LOPES et al., 1986; MARTINEZ-TOLEDO et al., 1988; NIETO; FRANKENBERGER, 1989), *Azospirillum* spp. (HOREMANS et al., 1986; CACCIARI et al., 1989), *Rhizobium* spp. (PHILLIPS; TORREY, 1970; UPADHYAYA et al., 1991), e *Paenibacillus polymyxa* (TIMMUSK et al., 1999).

O etileno é um hormônio que ajuda no desenvolvimento da planta quando em condições de estresse biótico e abiótico. Outros efeitos desse hormônio no desenvolvimento da planta são: germinação das sementes, morfogêneses, indução de floração, maturação dos frutos até a senescência, inibição do crescimento da raiz lateral e dos pêlos nas raízes (MAYAK et al., 2004). Assim, a diminuição da produção de etileno, através das bactérias diazotróficas, poderia indiretamente promover o crescimento das raízes laterais. As bactérias podem afetar a produção de etileno através de duas vias, sendo a predominante a 1-aminociclopropano-1-carboxílico-deaminase (ACC deaminase), e deste modo estimular o crescimento vegetal (FIGURA 3) (BELIMOV et al., 2005).

A enzima ACC deaminase produzida pelas PGPB também pode diminuir os efeitos deletérios em condições de estresse ambiental como em presença de metais pesados e estresse hídrico. Plantas de tomate cultivadas em condição de estresse hídrico por excesso de água foram inoculadas com a estirpe mutante de *A. brasilense,* construída a partir da inserção do gene da ACC deaminase (*acdS*) obtido da *Enterobacter cloacae* UW4, e as folhas apresentaram baixos níveis de epinastia quando comparado com plantas inoculadas com a estirpe selvagem (HOLGUIN; GLICK, 2000). Esses resultados sugerem que a superexpressão de ACC deaminase contribui para melhorar a resposta da planta ao estresse hídrico, provavelmente devido à redução da concentração de etileno no tecido da planta. Uma vez que em condição de estresse a planta responde com o aumento da produção de etileno em seus tecidos.

A promoção do crescimento vegetal por PGPB é dita indireta quando ocorre o melhoramento das condições ambientais, como exemplo a competição entre PGPB e bactérias patógenas à planta, ação biopesticida (FIGURA 3), ou ainda através da produção de antibióticos (BLOEMBER; LUGTENBERG, 2001; VESSEY, 2003). Para *A. brasilense* e *A. lipoferum* a produção de bacteriocinas tem sido reportada (OLIVEIRA; DROZDOWICZ, 1987; TAPIA-HERNANDEZ et al., 1990). Somers e colaboradores (2004) identificaram atividade antibactericida contra *Agrobacterium tumefaciens*, *Erwinia carotovora* e *Pseudomonas syringae* pv. *glycinea,* sendo identificado uma atividade moderada contra *E. coli.* Além disso, estes autores mostraram que *A. brasilense* possui atividade antifúngica contra todas as três diferentes espécies de fungos testadas: *Fusarium oxysporum* f. spp., *matthiolae*, *Alternaria brassicicola* e *Neurospora crassa*. As propriedades de biocontrole de *A. brasilense* foram atribuídas à síntese de ácido fenilacético (SOMERS et al., 2004).

Em experimento conduzido em casa de vegetação plantas de tomate inoculadas com *A. brasilense* apresentaram maior resistência a *Pseudomonas syringae (*BASHAN; DE-BASHAN, 2002). Os autores sugeriram que o mecanismo de biocontrole foi a competição entre essas bactérias, sendo que a *A. brasilense* prevaleceu na raiz, devido a melhor adaptação, favorecendo assim a planta. Plantas de morango inoculadas com *A. brasilense* apresentaram maior resistência ao fungo *Colletotrichum acutatum* (TORTORA; DÍAZ-RICCI; PEDRAZA, 2011). Este fungo tem relevância econômica por ser o principal causador da doença antracnose em plantas de morango. Através de cromatografia de camada fina acoplada espectroscopia de fluorescência foi detectada que *A. brasilense* secreta catecol e ácido salicílico (ambos sideróforos) que apresentaram atividade antifúngica contra *Colletotrichum acutatum.* Os resultados obtidos *in vitro* corroboraram os resultados *in planta*, sendo que plantas inoculadas com *A. brasilense* apresentaram 30% a menos de suscetibilidade ao fungo do que as plantas controle (TORTORA; DÍAZ-RICCI; PEDRAZA, 2011).



FONTE: Adaptado de WEYENS et al., 2009.

FIGURA 3. AS BACTÉRIAS PROMOTORAS DE CRESCIMENTO DAS PLANTAS PODEM PROMOVER O CRESCIMENTO DE MANEIRA DIRETA, ATRAVÉS DO AUMENTO DA DISPONIBILIDADE DE NUTRIENTES PARA A PLANTA COMO MAIOR DISPONIBILIDADE DE N, P E Fe, PRODUÇÃO DE FITORMÔNIOS E ATIVIDADE DE ACC-DEAMINASE; OU INDIRETAMENTE ATRAVÉS DO CONTROLE DE ORGANISMOS DELETÉRIOS A PLANTA.

1.10 *Setaria viridis* e *Setaria italica*: plantas modelo

O painço é um grupo de cereais caracterizado por produzirem pequenas sementes, que inclui gramíneas forrageiras como *Setaria italica*, *Pennisetum glaucum, Eleusine coracana, Panicum miliaceum, Echinocloa* spp*., Paspalum scrobiculatum, Eragrotis tef, Digitaria* spp., dentre outras (DWIVEDI et al., 2012). Em 2013, a produção mundial desse grupo alcançou cerca de 30 milhões de toneladas, sendo os quatro principais países produtores: India (10.910.000), Nigéria (5.000.000), Níger (2.995.000) e China (16.205.00) (FAO, 2013).

A *Setaria italica* é uma das espécies mais antigas cultivadas do grupo do painço, e provavelmente o seu cultivo começou na Província de Gansu (noroeste da China) (BARTON et al., 2009). Algumas variedades desta espécie são extremamente adaptadas a clima árido e áreas secas, sendo encontradas na Índia, China e outras partes da Ásia, África do Norte e Américas (LATA; GUPTA; PRASAD, 2012). Enquanto a *S. viridis* provavelmente originou-se na Eurásia, e depois se espalhou por diferentes lugares de clima temperado, tropical e subtropical, sendo encontrada em ecossistemas naturais e agrícolas em todo o mundo (DEKKER, 2003).

S. viridis e *S. italica* pertencem à família Poaceae (subfamília das Panicoideae) que incluem o grupo de gramíneas C_4 de grande importância agronômica como o milho, sorgo e cana-de-açúcar. Além disso, ao comparar a *Arabidopsis thaliana* (eucodiledônea) com a *S. italica e S. viridis* (monocotiledônea), estas últimas são mais próximas de gramíneas de interesse agronômico (monocotiledôneas em geral) tornando-a mais atrativa como planta modelo (BELL et al., 2010; SMITH et al., 2010). Pela análise filogenética de sequências de ribossomo e cloroplasto, por meio da técnica hibridização fluorescente *in situ* do cromossomo, foi possivel concluir que *S. viridis* é um ancestral selvagem da espécie cultivada *S. italica* (WANG et al., 1995; BENABDEMOUNA et al., 2001; DOUST; KELLOG, 2002). Estas duas espécies de plantas são filogeneticamente próximas e o genoma da *S. italica* está disponível no banco de dados Phytozome (http://goo.gl/sn9T0g) (LI; BRUTNELL, 2011).

A *Setaria italica* e *Setaria viridis* possuem muitas características que as tornaram uma espécie modelo para o estudo de processos biológicos básicos. Algumas destas características incluem: genoma pequeno (~ 515 Mega pares de bases), baixa quantidade de DNA repetitivo, endogamia natural, porte pequeno (entre 10 e 15 cm), curto ciclo de vida

(6 a 9 semanas), produção de muitas sementes por planta (cerca de 13.000 sementes) e eficiência de transformação através do método indireto com o uso de *Agrobacterium* (DOUST et al., 2009; BRUTNELL et al., 2010). Além disso, essas espécies possuem uma extensiva coleção de germoplasma, o que possibilita o estudo dos processos de domesticação. Por esses motivos *S. viridis* e *S. italica* são ideais para o estudo da evolução de plantas C_4, genômica comparativa entre gramíneas e modelo para estudo de plantas poliploides como a cana-de-açúcar.

A *S. italica* e *S. viridis* tem sido utilizada também para o estudo da interação com *Azospirillum* spp. (COHEN, et al., 1980; OKON, et al., 1983; KAPULNIK, et al., 1981; PANKIEVICZ et al., 2015). No entanto, ensaios de transcriptoma da *Setaria viridis* inoculada com *Azospirillum brasilense* ainda não foram publicados, sendo apresentados nesta tese os resultados parciais desta interação.

2. OBJETIVOS

2.1 Objetivo geral:

Avaliar a habilidade do mutante espontâneo HM053 de *Azospirillum brasilense*, que é capaz de fixar nitrogênio constitutivamente e excretar amônio, em colonizar as raízes de trigo e compreender os efeitos da inoculação desta estirpe na planta modelo *Setaria viridis.*

2.2 Objetivos específicos:

- Selecionar transconjugantes das estirpes FP2 e HM053 de *A. brasilense* com a marca *nifH-gusA* capazes de fixar nitrogênio;

- Determinar o padrão de colonização das raízes de trigo (*Triticum aestivum* var. CD104) inoculadas com estas estirpes;

- Avaliar, através da microscopica de luz, a expressão do gene *nifH* fusionado ao gene repórter *gusA* dos transconjugantes FP2-7 e HM053-36;

- Quantificar e comparar os níveis de expressão do gene *nifH in planta* por RT-PCR entre o mutante HM053 e selvagem FP2;

- Caracterizar a genética e a bioquímica do mutante HM053 através do sequenciamento do gene *glnA* e análise de *immunoblot* das proteínas NifH e Glutamina Sintetase (GS).

- Utilizar o radioisótopo ^{13}NN para investigar se a planta de *Setaria viridis* é capaz de assimilar o nitrogênio atmosférico fixado pela mistura das bactérias diazotróficas: *A. brasilense* FP2-7 e *Herbaspirillum seropedicae* RAM4 (ambas Nif^{+}) e *A. brasilense* HM053 (Nif^{c});

- Comparar os níveis de nitrogênio assimilado entre plantas de *S. viridis* (var. A10.1) inoculadas com a mistura das estirpes (RAM4 e FP2-7), e plantas inoculadas com o mutante HM053. Utilizar como controle plantas inoculadas com a cultura fervida da bactéria e a estirpe FP10 (Nif^{-}), incapaz de fixar nitrogênio.

- Traçar um perfil molecular, através do transcriptoma da raiz, de genes diferencialmente expressos envolvidos na interação *Setaria viridis – A. brasilense* em quatro condições: 1) *S. viridis* inoculada com a cultura de *A. brasilense* fervida; 2) *S. viridis* inoculada com o mutante HM053 (Nif^{c}), 3) *S. viridis* inoculada com a estirpe selvagem FP2 (Nif^{+}) e 4) *S. viridis* inoculada com a estirpe mutante FP10 (Nif^{-});

- Realizar avaliações fenotípicas do comprimento da raiz e parte aérea, massa seca da raiz e da parte aérea, área folhear, número de sementes e número de raízes laterais de *S. viridis* inoculadas nas quatro diferentes condições como citado acima.

3. REFERÊNCIAS

ARCONDÉGUY, T.; RACHAEL, J.; MERRICK, M. PII signal transduction proteins, pivotal players in microbial nitrogen control. **Microbiol. and Molecular Biology.** v. 65, p. 80-105, 2001.

ATZORN, R.; CROZIER, A.; WHEELER, C. T.; SANDBERG, G. Production of gibberellins and indole-3-acetic acid by *Rhizobium phaseoli* in relation to nodulation of *Phaseolus vulgaris* roots. **Plant Soil,** v. 43,p. 609-619, 1988.

AZCÓN, R.; BAREA, J. M. Synthesis of auxins, gibberellins and cytokinins by *Azotobacter vinelandii* and *Azotobacter beijerinckii* related to effects produced on tomato plants. **Plant Soil**, v. 43, p. 609-619, 1975.

BALANDREAU, J.; VIALLARD, V.; COURNOYER, B.; COENYE, T.; LAEVENS, S.; VANDAMME, P. *Burkholderia cepacia* genomovar III is a common plant-associated bacterium. **Appl Environ Microbiol**, v. 67, p.982-985, 2001.

BALDANI, J. I.; BALDANI, V. L. D.; SELDIN, L.; DÖBEREINER, J. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. **Int J Syst Bacteriol,** v. 36, p. 86-93, 1986.

BALDANI, V. L. D.; BALDANI, J.I.; DÖBEREINER, J. Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. **Biology and Fertility of Soils** v.30, p. 485-491, 2000.

BALDANI, V. L. D.; OLIVEIRA, E.; BALOTA, E.; BALDANI, J. I.; KIRCHHOF, G.; DÖBEREINER, J. *Burkholderia brasilensis* sp. nov., uma nova espécie de bactéria diazotrófica endofítica. **An. Acad. Bras. Ci.,** v. 69, p.116, 1997.

BALLY, I.; THOMAS-BAUZON, D.; HEULIN, T.; BALANDREAU, J.; RICHARD, C.; DE LEY, J. Determination of the most frequent N2-fixing bacteria in a rice rhizosphere. **Can J Microbiol,** v. 29, p. 881-887, 1983.

BAREA, J.M.; BROWN, ME. Effects on plant growth produced by *Azotobacter paspali* related to synthesis of plant growth regulating substances. **J. Appl. Bacteriol**, v. 40, p. 583-593, 1974.

BARAK, R.; NUR, O.; OKON, Y. Detection of chemotaxis in *Azospirillum brasilense.* **Journal of Applied Bacteriology**, v. 54, p. 399-403, 1983.

BAREA, JM.; NAVARRO, E.; MONTOYA, E. Production of plant growth regulators by rhizosphere phosphatesolubilizing bacteria. **J. Appl. Bacteriol**. 40:129-134, 1976.

BARTON, L.; NEWSOME, S. D.; CHEN, F. H.; WANG, H.; GUILDERSON, T.P.; BETTINGER, R. L. Agriculture origins and the isotopic identity of domestication in northern China. **Pro Natl Acad. Sci USA**, v.106, p. 5523-5528, 2009.

BASHAN, Y.; HOLGUIN, G.; DE-BASHAN, L. E. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances. **Can J Microbiol,** v. 50, p 521-577, 2004.

BASHAN, Y. Migration of the Rhizosphere Bacteria *Azospirillum brasilense* and Pseudomonas fluorescens towads wheat roots in the Soil. **Journal of General Microbiology**, v. 132 p. 3407-3414, 1986.

BASHAN, Y.; DE-BASHAN, L. E. Protection of tomato seedlings against infection by *Pseudomonas syringae* pv. *tomato* by using the plant growth-promoting bacterium *Azospirillum brasilense*. **Appl. Environ. Microbiol**. v.68, p. 2637-2643, 2002.

BASHAN, Y.; H. LEVANONY. Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture**. Canadian Journal of Microbiology,** 1990, v. 36, p. 591-608, 1990.

BASTIÁN, F.; COHERN, A.; PICCOLI, P.; LUNA, V.; BARALDI, R.; BOTTINI, R. Production of indole-3–acetic acid and gibberellins A1 and A3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically defined culture media. **Plant Growth Regul,** v. 24, p. 7-11, 1998.

BEATTIE, G.A; Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. In: Gnanamanickam, S. S. **Plant-associated bacteria.** Springer, The Netherlands, v.6 p. 1-56, 2006.

BELIMOV, A. A.; HONTZEAS, N.; SAFRANOVA, V. I.; DEMCHINSKAYA, S. V.; PILUZZA, G.; BULLITTA, S.; GLICK, B. R. Cadmium- tolerant plant growth-ptomoting bacteria associated with the roots of Indian mustard (*Brassica juncea L. Czern*). **Soil Biol. Biochem,** v. 37, p. 241-250, 2005.

BELL, C. D.; SOLTIS, D. E.; SOLTIS, P. S. The age and diversification of the angiosperms re-revisited. **American Journal of Botany,** v. 97, p. 1296–1303, 2010.

BENABDELMOUNA, A.; ABIRACHED, DARMENCY, H. Phylogenetic and genomic relationships in *Setaria italica* and its close relatives based on the molecular diversity and chromosomal organization of 5S and 18S-5.8S-25S rDNA genes. **Theor Appl Genet.** v. 103, p. 668-677, 2001.

BENNETZEN, J. L.; SCHMUTZ, J.; WANG, H.; PERCIFIELD, R.; HAWKINS, J.; PONTAROLI, A. C.; ESTEP, M., et al. Reference genome sequence of the model plant Setaria. **Nature biotechnology**, v. 30, p. 555-561, 2012.

BILAL, R.; RASUL, G.; QURESHI, J.A.; MALIK, K. A. Characterisation of *Azospirillum* and related diazotrophs associated with roots of plants growing in saline soils. **World J Microbiol Biotechnol,** v. 6, p. 46-52, 1990.

BLOEMBERG, G.V.; LUGTENBERG B. J. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. **Curr Opin Plant Biol,** v. 4, p. 343-350, 2001.

BOTTINI, R.; FULCHIERI, M.; PEARCE, D. W.; PHARIS, R.P. Identification of gibberellins, A1, A3 e iso-A3 in cultures of *Azospirillum lipoferum*. **Plant Physiol**. v. 90, p. 45-47, 1989.

BOTTOMLEY, P.J.; NEWTON, W.E. Nitrogen fixation research progress. In: VERMA, D.P.S; BRISSON, N. (Ed.). **Molecular Genetics of Plant-Microbe Interactions,** Canadá: Martinus Nijhoff, 1985**.** p. 427.

BRUTNELL, T. P.; WANG, L.; SWARTWOOD K, GOLDSCHMIDT, A.; JACKSON, D.; ZHU, X. G.; KELLOGG, E.; VAN ECK, J. *Setaria viridis*: a model for C4 photosynthesis. **The Plant Cell,** v. 22, p. 2537-2544, 2010.

BURRIS, R.H. Nitrogenase. **The Journal of Biological Chemistry**. v. 266, p. 9939-9342, 1991.

BURDAMAN, S.D.; OKON, Y.; JURKEVITCH, E. Purification of the major outer membrane protein of *Azospirillum brasilense*, its affinity to plant roots, and its involvement in cell aggregation. **Mol Plant Microbe Interact.** v.14, p. 555-561, 2001.

CABALLERO-MELLADO, J.; LOPEZ-REYES, L.; BUSTILLOS-CRISTALES, R.. Presence of 16S rRNA genes in multiple replicons in *Azospirillum brasilense*. **FEMS Microbiol. Lett**., v. 178, p. 283-288, 1999.

CACCIARI, I.; LIPPI, D., PIETROSANTI, T., AND PIETROSANTI, W. Phytohormone-like substances produced by single and mixed diazotrophic cultures of *Azospirillum* and *Arthrobacter*. **Plant Soil,** v. 115, p. 151-153, 1989.

CASTELLANOS, T.; ASCENCIO, F.; BASHAN, Y. Cell-surface lectins of *Azospirillum* spp. **Curr. Microbiol.** v. 36, p. 241-44, 1998.

CHALFIE, M.; TU, Y.; EUSKIRCHEN, G.; WARD, W.W.; PRASHER, D.C. Green fluorescent protein as a marker for gene expression. **Science**, v. 263, p. 802-805, 1994.

CHEN, Y. P.; LOPEZ-DE-VICTORIA, G.; LOVELL W. D. Utilization of aromatic compounds as carbon and energy sources during growth and N2-fixation by free living nitrogen fixing bacteria. **Arch. Microbiol.** v. 159, p. 207-212, 1993.

CHET, I.; MITCHEL, R. Ecological aspects of microbial chemotactic behavior. Annu. **Rev. Microbiol.** v. 30, p. 221-239, 1976.

COCKING, E. C. The challenge of establishing symbiotic nitrogen fixation in cereals. In: Emerich DW, Krishnan HB. eds. Nitrogen fixation in crop production. **Madison, WI: American Society of Agronomy**, p. 35–63, 2009.

COHEN, E.; OKON, Y.; KIGEL, J.; NUR, I.; HENIS, Y. Increase in dry weight and total nitrogen content in *Zea mays* and *Setaria italica* associated with nitrogen-fixing *Azospirillum* spp. **Plant physiology,** v. 66, p. 746-749, 1980.

CROES, C.; VAN BASTELAERE, E.; DECLERCQ. E.; EYERS, M.; VANDERLEYDEN, J.; MICHIELS, K. Identification and Mapping of loci involved in

motility, adsorption to wheat roots, colony morphology and growth in minimal medium on the *Azospirillum brasilense* Sp7 90-MDa plasmid. **Plasmid,** v. 26, p. 83-93, 1991.

CROMBIE, H.J.; SCOTT, C.; REID, J. S. G. IN: VORAGEN AGJ, SCHOLS HA, VISSER RGF (eds) Advances in pectin and pectinase research. **Kluwer Academic Publishers, Dordrecht**, p. 35-45, 2003.

DALMASTRI, C. Biodiversity of a Burkholderia cepacia population isolated from the maize rhizosphere at different plant growth stages. **Applied and environmental microbiology,** v. 63, p. 4485-4493, 1997.

DHALLA, A.M.; LI, B.; ALIBHAI, M.F.; YOST, K.J.; HEMMINGSEN, J.M.; ATKINS, W.M.; SCHINELLER, J.; VILLAFRANCA, J.J. Regeneration of catalytic activity of glutamine synthetase mutants by chemical activation: Exploration of the role of arginines 339 and 359 in activity. **Protein Science**, v.3, p.476-481, 1994.

DENNIS, P.G.; MILLER, A.J.; HIRSCH, P.R. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? **FEMS Microbiol Ecol.** v.72, p. 313-327, 2010.

DEKHILL, S. B.; CAHILL, M.; STACKBRANDT, E. Transfer of Conglomeromonas largomobilis subs. largomobilis to the genus *Azospirillum* as *Azospirilum largomobile* comb. nov., and elevation of *Conglomeromonas largomobilis* subs. Parooensis to the new type species of *Conglomeromonas, Conglomeromonas parooensis* spp. **Systematic and Applied Microbiology**. v. 20, p. 72-77, 1997.

DEKKER, J. The foxtail (Setaria) species-group. **Weed Science**, v. 51, p. 641-656, 2003.

DESBROSSES, G. J.; STOUGAARD, J. Root nodulation: a paradigm for how plant-microbe symbiosis influences host developmental pathways. **Cell Host and Microbe** v. 10, p. 348-358, 2011.

DÍAZ-ZORITA, M.; FERNANDEZ CANIGIA, M. V. Análisis de la producción de cereales inoculados con *Azospirillum brasilense* en la República Argentina. In: Cassán FD, Garcia de Salamone I (eds) *Azospirillum* spp.: cell physiology, plant interactions and agronomic research in Argentina. **Asociación Argentina de Microbiologia**, p. 153-164, 2008.

DIXON, R.; KAHN, D. Genetic regulation of biological nitrogen fixation. **Nature reviews Microbiology**, v. 2, p. 621-31, 2004.

DOBBELAERE, S.; VANDERLEYDEN, J.; OKON, Y. Plant growth-promoting effects of diazotrophs in the rhizosphere. **Critical Review in Plant Sciences** v. 22, p.107–149, 2003.

DOBBELAERE, S.; CROONENBORGHS, A.; AMBER, T.; PTACEK, D.; VANDERLEYDEN, J.; DUTTO, P.; LABANDERA-CONZALEZ, C.; CABALLERO-MELLADO, J.; AGUIRRE, J. F. Responses of agronomically important crops to inoculation with Azospirillum. **Aust J Plant Physiol** v. 28, p. 871-879, 2001.

DOBBELAERE, S.; VANDERLEYDEN, J.; OKON, Y. Plant growth-promoting effects of diazotrophs in the rhizosphere. **Crit Rev Plant Sci,** v. 22, 107-149, 2003.

DÖBEREINER, J.; DAY, J. M. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. In: Newton WE, Nyman CJ (eds) Proceedings of the 1st International Symposium on Nitrogen Fixation. **Washington State University Press,** WA, p. 518-538, 1976.

DÖBEREINER, J. The genera *Azospirillum* and *Herbaspirillum*. In BALLOWS, A., TRUPPER, H.G., DWORKING, M., HARDER, W. **The Prokaryotes.** v. 3, p. 2236-3353, Springer-Verlag, 1991.

DOBERMANN, A. Nutrient use efficiency-measurement and management in a time of new challenges. In: **Proceedings of the IFA International Workshop on Fertilizer Best.** Fertilizer Best Management Practices, 7–9 March 2007, Brussels, Belgium. International Fertilizer Industry Association, 1–28. Dobritsa SV, Potter D, Gookin TE, Berry AM. 2001, 2007

DOUST, A. N.; KELLONG, E. A.; Inflorescence diversification in the panicoid "bristle grass" clade (Paniceae, Poaceae): evidence from molecular phylogenies and developmental morphology. **American Jornal of Botany,** v. 89, p.1203-1222, 2002.

DWIVEDI, S.; UPADHYAYA, H.; SENTHILVEL, S. HASH, C.; FUKUNAGA, K.; DIAO, X.; SANDRA, D.; BALTENSPERGER, D.; PRASAD, M. Millets: Genetics and Genomic Resourcer. In. Janick J (ed) **Plant Breed Rev**, v. 35, USA: John Wiley & Sons, Inc., p. 247-375, 2012.

ECKERT, B.; WEBER, O.B.; KIRCHHOF, G.; HALBRITTER, A.; STOFFELS, M.; HARTMANN, A. *Azospirillum doebereinerae* sp. nov., a nitrogen-fixing bacterium associated with the C4-grass Int. J. **Syst. Evol. Microbiol.,** v. 51, p. 17-26, 2001.

EL ZEMRANY, H.; CORTET, J, LUTZ M.P.; CHABERT, A.; BAUDOUIN, E.; HAURAT, J.; MAUGHAN, N.; FELIX, D.; DE´FAGO, G.; BALLY, R. Field survival of the phytostimulatory *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation. **Soil Biology & Biochemistry**, v. 38, p. 1712–1727, 2006

ENGELHARD, M.; HUREK T, REINHOLD-HUREK B. Preferential occurrence of diazotrophic endophytes, *Azoarcus* sp., in wild rice species and land races of *Oryza sativa* in comparison with modern races. **Environmental Microbiology,** v. 2, p 131-141, 2000.

ERRAMPALLI, D., LEUNG, K.; CASSIDY, M. B.; KOSTRZYNSKA, M.; BLEARS, M.; LEE, H.; TREVORS, J. T. Applications of green fluorescent protein as a molecular marker in environ- mental microorganisms. **J. Microbiol. Methods,** v. 35, 187-199, 1999.

FAO. Current world fertilizer trends and outlook to 2016. FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS Rome, 2012

FAO. FAOSTAT. Food and Agriculture Organisation of the United Nations. Disponível em: < http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor>.

Acesso em: 20 Jan. de 2013.
FULCHIERI, M.; LUCANGELI, C.; BOTTINI, R. Inoculation with *Azospirillum lipoferum* affects growth and gibberelin status of corn seedling roots. **Plant Cell Physiol**. v. 34, p. 1305-1309, 1993.

FUNAYAMA, S.; RIGO, L. U.; PEDROSA, F. O. Nif A$^-$ mutants of *Azospirillum brasilense* strain Sp 245. In: EVANS, H. J.; BOTTOMLEY, P.J.; NEWTON, W.E. eds, 1985.

FRINK, C. et al. Nitrogen fertilizer: retrospect and prospect. **Proc. Natl. Acad. Sc**, v. 96, p. 1175-1180, 1999.

GARBEVA, P.; VAN OVERBEEK, L. S; VAN VUURDE, J. W. L.; VAN ELSAS, J. D. Analysis of endophytic bacterial communities communities of potato by planting and denaturing gradient gel electrophoresis (DGGE) of 16s rDNA based PCR fragments. **Microb Ecol,** v. 41, p. 369-383, 2001.

GARCÍA DE SALAMONE, I. E; DI SALVO L. P.; ORTEGA, J. S. E.; BOA SORTE, P. M. F.; URQUIAGA, S.; TEIXEIRA, K. R. S. Field response of rice paddy crop to Azospirillum inoculation: physiology of rhizosphere bacterial communities and the genetic diversity of endophytic bacteria in different parts of the plants. **Plant Soil,** v. 336, p. 351-362, 2010

GARCIA, C.; FERNANDEZ, T.; COSTA, F.; CERRANTO, B.; MASCIANDARO, G. Kinetics of phosphatase activity in organic wastes. **Soil biol. Biochem.** v. 25, p. 361-365, 1992.

GILLIS, M.; KERSTERS, K.; HOSTE, B.; JANSSENS, D.; KROPENSTEDT, R. M.; STEPHEN, M.P.; TEIXEIRA, K. R. S.; DÖBEREINER, J.; DE LEY, J. *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane. **Int J Syst Bacteriol,** v. 39, p. 361-364, 1989.

GLICK, B. R.; PATTEN, C. L.; HOLGUIN, G.; PENROSE , D. M. Biochemical and genetic mechanisms used by plant growth promoting bacteria. London: **Imperial College Press**, 1999.

GONZALEZ-LOPEZ, J.; SALMERON, V.; MARTINEZ-TOLEDO, M. V.; BALLESTEROS, F.; RAMOS-CORMENZANA, A. Production of auxins, gibberellins and cytokinins by *Azotobacter vinelandii* ATCC 12837 in chemically defined media and dialysed soil media. **Soil Biol. Biochem.** v. 18, p. 119-120, 1989.

GOOD, A. G.; SHRAWAT, A. K.; MUENCH, D. G. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? **Trends in plant science,** v. 9, p. 597-605, 2004.

GORDONJ, K.; BRILL, W. J. Mutants that produce nitrogenase in the presence of ammonia. **Proceedings of the National Academy of Sciences of the United States of America,** v. 69, p. 3501-3503, 1972.

GROSS L. A., BAIRD G. S., HOFFMAN, R. C., BALDRIDGE, K. K.; TSIEN ROGER Y. The structure of the chromophore within DsRed, a red fluorescent protein from coral. **PNAS**. v. 97, p.11990-11995, 2000.

GUTIERREZ-MANERO, F. J.; RAMOS, S. B.; PROBANZA, A.; MEHOUACHI, J.; TADEO, F. R.; TALON, M. 2001. The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. **Physiol. Plant.** v. 111, p. 206-211, 2001.

GYANESHWAR, P.; JAMES, E. K.; MATHAN, N.; REDDY, P. M.; REINHOLD-HUREK, B.; LADHA, J. K. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. **Journal of Bacteriology,** v. 183, p. 2634-2645, 2001.

ZEMRANY, H. EL.; CZARNES, S.; HALLETT, P.D.; ALAMERCERY, S.; BALLY, R.; MONROZIER, L. J. Early changes in root characteristics of maize (Zea mays) following seed inoculation with the PGPR *Azospirillum lipoferum* CRT1, **Plant Soil,** v. 291, 109-118, 2007.

HALLMANN, J. Plant interactions with endophytic bacteria. In: Jeger, M.J., Spence, N.J. (Eds.), **Biotic Interactions in Plante Pathogen Associations**. CABI Publishing, Wallingford, United Kingdom, p. 87-119, 2001.

HANDELSMAN, J.; TIEDJE, J.; ALVAREZ-COHEN, ASHBURNER, M.; CANN, I.K.O. The New Science of Metagenomics: Revealing the Secrets of Our Microbial Planet. National Academies Press, Washington, DC. p.158, 2007.

HARTMANN, A.; ZIMMER, W. Physiology of Azospirillum. In: ***Azospirillum*/Plant Associations**. Okon, Y., ed., CRC Press, Boca Raton, F. L., p. 15-39, 1994.

HAUER, V. **Sequenciamento do gene glna das estirpes mutantes HM14, HM26, HM053 e HM210 de *Azospirillum brasilense.*** 49 f.Trabalho de conclusão de curso (Bacharelado em Ciências Biologia) - Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, 2012.

HOLGUIN, G.; GLICK, B.R. Inoculation of tomato plants with *Azospirillum brasilense* transformed with ACC deaminase gene from Enterobacter cloacae UW4. **Microbial Ecology**, v. 3, p. 281-288, 2000.

HOREMANS, S., D.E.; KONINCK, K.; NEURAY, J.; HERMANS, R.; VLASSAK, K. Production of plant growth sub- stances by *Azospirillum* sp. and other rhizosphere bacteria. **Symbiosis,** v. 2, p. 341-346, 1986.

HUNGRIA, M.; VARGAS, M. A. T.; CAMPO, R.; GALERANI, P. R. Adubação nitrogenada na soja? Londrina: Embrapa Sojaa (Comunicado Tecnico), 1997.

HUNGRIA, M. FRANCHINI, J.C, CAMPO, R.J, GRAHA, P.H. The importance of nitrogen fixation to soybean cropping in South America. In: Newton WE (ed) Nitrogen fixation: origins, applications and research progress, v.4, Werner, W, Newton, WE (Ed). Nitrogen fixation in agriculture, forestry, ecology and the environment. Springer, Dordrecht, Amsterdam, p. 25-42, 2005.

HUNGRIA, M.; CAMPO, R.J.; MENDES, I.C GRAHAM, P.H. Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: the success of soybean (*Glycine max L.*Merr) in South America. In: SINGH, R.P.; SHANKAR, N.; JAIWA, P.K. Nitrogen nutrition and sustainable plant productivity. **Stadium Press**, Houston, p. 43 – 93, 2006a.

HUNGRIA, M.; FRANCHINI, J.C.; CAMPO, R.J.; CRISPINO, C.C.; MORAES, J.Z.; SIBALDELLI, R.N.R.; MENDES, I.C.; ARIHARA, J. Nitrogen nutrion of soybean in Brazil: contributions of biological N2 fixation and of N fertilizer to grain yield. **Can J Plant Science**, v. 86, p. 927 – 939, 2006b.

HUREK, T.; REINHOLD-HUREK, B.; VAN MONTAGU, M.; KELLENBERGER, E. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. **J. Bacteriol.** v. 176, p. 1913-1923, 1994.

ILMER, P.; BARBATO, A.; SCHINNER, F. Solubilization of hardly soluble AIPO4 with P-solubilizing microorganisms. **Soil biochem**. v. 27, p. 265-270, 1995.

JAMES, E. K.; OLIVARES, F. L. Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. **Crit. Rev. Plant Sci.** 17:77-119, 1998.

JAMES, E. K.; REIS, V. M.; OLIVARES, F. L.; BALDANDI, J. I.; DÖBEREINER, J. Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. **J. Exp. Bot.** 45:757-766, 1994.

JAZEN, R.; ROOD, S., DORMAR, J.; MCGILL, W. *Azospirillum brasilense* produces gibberellins in pure culture and chemically medium and in co-culture on straw. **Soil Biol. Biochem**. v. 24, p. 1061-1064, 1992.

JEFFERSON, R.A. Assaying chimeric genes in plants: the GUS gene fusion system. **Plant Mol Biol Rep,** v. 5, p. 387-405, 1987.

JOFRÉ E, LAGARES A, MORI G. Disruption of dTDP-rhamnose biosyn- thesis modifies lipopolysaccharide core, exopolysaccharide production and root colonization in *Azospirillum brasilense*. **FEMS Microbiology Letters**, v. 231, p. 267-275, 2004.

JONES, DL. Organic acids in the rhizosphere – a critical review. **Plant soil,** v. 205, p. 25-44, 1998

KAPULNIK, Y.; OKON, Y.; KIGEL, J.; NUR, I.; HENIS, Y. Effects of temperature, nitrogen fertilization, and Plant age on nitrogen fixation by *Setaria italica* inoculated with *Azospirillum brasilense* (strain cd). **Plant Physiol.** v. 68, p. 340-343, 1981.

KHAMMAS, K. M.; AGERON, E.; GRIMOT, P. A.; KAISER, P. *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soil. **Res. Microbiol.,** Paris, v.140, p. 679-694, 1989.

KIM, J.; REES, D.C. Strutural models for the metal centers in the nitrogenase molybdenum-iron protein. **Science**, Washington D.C, v. 275, p. 1677-1682, 1992.

LAVRINENKO, K.; CHERNOUSOVA, E.; GRIDNEVA, E.; DUBININA, G.; AKIMOV, V.; KUEVER, J.; LYSENKO, A.; GRABOVICH, M. *Azospirillum thiophilum* sp. nov., a diazotrophic bacterium isolated from a sulfide spring. **Int. J. Syst. Evol. Microbiol.** v. 60, p. 2832-2837, 2010.

LATA, C.; GUPTA, S.; PRASAD, MANOJ. Foxtail millet: a model crop for genetic and genomic studies in bioenergy grasses. **Informa Healthcare,** v. 33, p. 328-343, 2012.

LI, P.; BRUTNELL, T. *Setaria viridis* and *Setaria italica*, model genetic systems for the Panicoid grasses. **Experimental Botany**. V. 62, n° 9, 3031-3037, 2011.

LIN, S. Y.; LIU, Y. C.; HAMEED, A.; HSU, Y. H.; LAI, W.A., SHEN, F. T., YOUNG, C.C. *Azospirillum fermentarium* sp. nov., a novel nitrogen-fixing species isolated from a fermenter in Taiwan. **Int. J. Syst**. **Evol. Microbiol**., 2013.

LIN, S. Y.; SHEN, F. T.; YOUNG, L. S.; ZHU, Z. L.; CHEN, W. M.; YOUNG, C. C. *Azospirillum formosense* sp. nov., a diazotroph from agricultural soil. **Int. J. Syst. Evol. Microbiol.** v. 62, p.1185-1190, 2012.

LIN, S.Y.; YOUNG, C.C.; HUPFER, H.; SIERING, C.; ARUN, A.B.; CHEN, W.M.; LAI, W.A.; SHEN, F.T.; REKHA, P.D.;YASSIN, A.F. *Azospirillum picis* sp. nov., isolated from discarded tar. **Int. J. Syst. Evol. Microbiol**. v. 59, p. 761-765, 2009.

LOPEZ-DE-VICTORIA, G.; LOVELL, C.R. Chemotaxis of *Azospirillum* species to aromatic compounds. **Appl. Environ. Microbiol**.59, 2951-2955, 1993.

MACHADO HB, FUNAYAMA S, RIGO LU, PEDROSA FO. Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine. **Canadian Journal of Microbiology.** v.29, p.549-553, 1991.

MACHADO, H. B **Organização estrutural e regulação do operon ORF1-ntrB-ntrC em *Azospirillum brasilense***. Tese de Doutorado. Departamento de Bioquímica da Universidade Federal do Paraná, 1995.

MAGALHÃES, F. M.; BALDANI. J. I.; SOUTO, S. M.; KUYKENDALL, J. R.; DOBEREINER, J. A new acid tolerant Azospirillum species. **An. Acad. Bras. Cienc., Rio de Janeiro,** v. 6, p. 417-430, 1983.

MARINEZ-TOLEDO, M. V.; DELA RUBIA, T.; MORENO, J.; GONZALEZ-LOPEZ, J. Root exudate of *Zea mays* and production of auxins, gibberellins and cytokinins by *Azotobacter chroococcum*. **Plant soil**, v. 110, p. 149-152, 1988.

MARTIN-DIDONET, C. C.; CHUBATSU, L S; SOUZA, E M; et al. Genome structure of the genus *Azospirillum*. **Journal of bacteriology**, v. 182, n. 14, p. 4113-4116, 2000.

MARTINEZ-TOLEDO, M. V.; DE LA RUBIA, T.; MORENO, J.; GONZALEZ-LOPEZ, J. Root exudates of Zea mays and production of auxins, gibberellins and cytokinins by *Azotobacter chroococcum*. **Plant Soil**, v. 110, p. 149-152, 1998.

MAYAK, S.; TIROSH, T.; GLICK, B. R. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. **Plant Physiol Biochem,** v. 42, p.565-572, 2004.

MEHNAZ, S.,; WESELOWSKI, B.; LAZAROVITS, G. *Azospirillum zeae* sp. nov., a diazotrophic bacterium isolated from rhizosphere soil of Zea mays. **Int. J. Syst. Evol. Microbiol.** v.57, p. 2805-2809, 2007.

MEHNAZ, S.; WESELOWSKI, B.; LAZAROVITS, G. *Azospirillum canadense* sp. nov., a nitrogen-fixing bacterium isolated from corn rhizosphere. **Int. J. Syst**. **Evol. Microbiol**. v. 57, p.620-624, 2007.

MENDES, I. C.; VARGAS, M. A. T.; HUNGRIA, M. Resposta da soja adubação nitrogenada na semeadura, em sistemas de plantio direto e convencional na região do Cerrado. Planaltina: Embrapa Cerrados. Embrapa Cerrados. Boletim de Pesquisa, 12, 15p, 2000.

MCCULLY, M. E., 2001. Niches for bacterial endophytes in crop plants: a plant biologist's view. **Australian Journal of Plant Physiology,** v. 28, p. 983-990, 2001.

MERRICK, M. J.; EDWARDS, R. A. Nitrogen control in bacteria. **Microbiol. Rev**. v. 59, p. 604-622, 1995.

MUTHUKUMARASAMY, R. CLEENWERCK, I, REVATHI G. et . Natural association of gluconacetobacter diazotrophicus and diazotrophic acetobacter peroxydans with wetland rice. **Systematic and applied microbiology,** v. 28, p. 227-286, 2005.

NACIRI, Y.; DARMENCY, H.; BELLIARD, J.; DESSAINT, F.; PERNÈS, J. Breeding strategy in foxtail millet, *Setaria italica* (L.P. Beauv.), following interspecific hybridization. **Euphytica**, v. 60, p. 97-103, 1992.

NIETO, K. F.; FRANKENBERGER, W. T., JR. Biosynthesis of cytokinins by Azotobacter chroococcum. **Soil Biol. Biochem.** v. 21, p. 967-972, 1989.

NOEL, T. C.; SHENG, C.; YOST, C. K.; PHARIS, R. P.; HYNES, M. F. *Rhizobium leguminosarum* as a plant growth-promoting rhizobacterium: direct growth promotion of canola and lettuce. **Can. J. Microbiol**. v. 42, p. 279-283, 1996.

OKON, Y.; CAKMAKCI, L.; NUR, I.; CHET, I. Aerotaxis and chemotaxis of A. brasilense. **Microbial Ecology**, v.6, p. 277-280, 1980.

OKON, Y.; HEYTLE, P.G.; HARDY, R.W Fixation by *Azospirillum brasilense* and Its incorporation into host *Setaria italica*. **Appl Environ Microbiol**, v. 46, p. 694-697, 1983.

OKON, Y.; KAPULNIK, Y. Development and function of Azospirillum-inoculated roots. **Plant soil,** v. 90, p. 3-16, 1986.

OLDROYD, G. E.; MURRAY, J. D.; POOLE, P. S.; DOWNIE, J. A. The rules of engagement in the legume-rhizobial symbiosis. **Annual Review of Genetics,** v. 45, p. 119-144, 2010.

OLIVARES, F. L.; BALDANI, V. L. D.; REIS, V.; BALDANI, J. I.; DOBEREINER, J. Occurrence of the endophytic diazotrophs Herbaspirillum spp. in roots, stems, and leaves, predominantly of Gramineae. **Biol Fertil Soils,** v. 21, p. 197-200, 1996.

OLIVEIRA, R. G. B.; DROZDOWICZ, A. Inhibition of bacteriocin producing strains of *Azospirillum lipoferum* by their own bacteriocin. **Zentbl. Mikrobiol**, v. 142, p. 387-391-, 1987.

PANDEY, A.; SHARMA, E.; PALNI, L. M. S. Influence of bacterial inoculation on maize in upland farming systems of the Sikkim Himalaya. **Soil Biology and Biochemistry,** v. 30, p. 379-384, 1998.

PATRIQUIN, D.G.; DÖBEREINER, J.; JAIN, D. K. Sites and processes of association between diazotrophs and grasses. **Can J Microbiol,** v. 29, p. 900-915, 1983.

PEDROSA, F. O.; YATES, M. G. Regulation of nitrogen fixation (nif) genes of *Azospirillum brasilense* by nifA and ntrC (glnG) type genes. **FEMS Microbiol. Lett**. v. 55, p. 95-101, 1984.

PENG, G.; WANG, H.; ZHANG, G.; HOU, W.; LIU, Y.; WANG, E. T.; TAN, Z. *Azospirillum melinis* sp. nov., a group of diazotrophs isolated from tropical molasses grass. **Int J Syst Evol Microbiol,** v. 56, p. 1263-1271, 2006.

PHILLIPS, D. A.; TORREY, J. G. Cytokinin production by *Rhizobium japonicum*. **Physiol. Plant**, v. 23, p. 1057-1063, 1970.

PICCOLI, P.; BOTTINI, R. Effect of C/N ration, N content, pH, and incubation time on growth and gibberellins production by *Azospirillum lipoferum*. **Symbiosis** v. **17**, p. 229-236, 1994.

PICCOLI, P.; MASCIARELLI, O.; BOTTINI, R. Metabolism of 17,17–[2H2]-gibberellins A4, A9 and A20 by *Azospirillum lipoferum* in chemically-defined culture medium. **Symbiosis**, v. 21, p. 263-274, 1996.

PLAZINSKI, J.; DART, P. J.; ROLFE, B. G. Plasmid visualization and nif gene location in nitrogen-fixing Azospirillum strains. **J Bacteriol,** v. 155, 1429-1433, 1983.

POLUKHINA, L.E.; SAKHURIEVA, G.N.; SHESTAKOV, S.V. Ethylenediamine-resistant Anabaena variabilis mutants with derepressed nitrogen-fixing system. **Microbiology**, v. 51, p.80-85, 1982.

POSTGATE, J. R. The fundamentals of nitrogen fixation. Cambridge, Cambridge **Uni. Press**. v. 252, 1982.

REINHOLD, B.; HUREK, T.; FENDRIK, I. *Azospirillum halopraeferans* sp. nov., a diazotroph associated with roots of *Leptochloa fusca* (Linn.) Kunth. In: EVANS, H.J.;

REINHOLD-HUREK, B.; HUREK, T. *Azoarcus* spp. and their interactions with grass roots. **Plant and Soil,** v. 194, p. 57-64, 1997.

REINHOLD-HUREK, B. R.; HUREK, T.; GILLIS, M.; HOSTE, B.; VANCANNEYT, M.; KERSTERS, K. DE LEY. *Azoarcus* gen. nov. Nitrogen-fixing proteobacteria associated with roots of kallar grass (Leptochoa fusca (L.) Kunth) and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. **Int J Syst Bacteriol**, v. 43, p. 574-584, 1993.

RICHARDSON, A. E.; BAREA, J. M.; MCNEILL, A. M.; PRIGENT-COMBARET, C. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. **Plant and Soil**, v. 321, p.111-117, 2009.

RIGGS, P.J.; CHELIUS, M. K.; INIGUEZ, A.L.; KAEPPLER, S.M.; TRIPLETT, E W. Enhanced maize productivity by inoculation with diazotrophic bacteria. **Australian Journal of Plant Physiology,** v. 28, p. 829-836, 2001.

RODRIGUEZ, H.; FRAGA, R. Phosphate solubilizing bacteria and their role in plant growth promotion. **Biotechnol.** v. 17, p. 319-339, 1999.

RONCATO-MACCARI, L. D. B.; RAMOS, H. J. O.; F. O, P., ALQUINI, YEDO, CHUBATSU, L. S., YATES, M G, RIGO, L U, STEFFENS, M. B. R., SOUZA, E. M. Endophytic *Herbaspirillum seropedicae* expresses nif gene in gramineous plants. **FEMS Microbiology Ecology**, v. 45, p. 39-47, 2003.

ROSZAK, D. B.; COLWELL, R. R. Survival strategies of bacteria in natural environments. **Microbiol. Rev**. v. 51, p. 365-379, 1987.

ROWELL, P.; KERBY, N.W.; DARLING, A.J.; STEWART, W.D.P. Physiological and biochemical aspects of N_2-fixing cyanobacteria. In Nitrogen fixation research progress. Edited by H.J. Evans, P.J. Bottomley and W.E Newton. Martinus Nijhoff Publishers, Dordrecht, Netherlands, p. 387-393, 1985.

SALISBURY, F. B. The role of plant hormones. In Plant-Environment Interactions. Ed. R. E Wilkinson. p. 39-81. Marcel Dekker, New York, USA, 1994.

SANTI, C.; BOGUSZ, D.; FRANCHE, C. Biological nitrogen fixation in non-legume plants. **Annals of Botany**, v. 111, n. 5, p. 743–67, 2013.

SATTAR, M. A; GAUR, A. C. Production of auxins and gibberellins by phosphate-dissolving microorganisms. **Zentralbl. Mikrobiol.,** v. 142, p. 393-395, 1987.

SAUBIDET, M. I.; FATTA, N.; BARNEIX, A. J. The effect of inoculation with *Azospirillum brasilense* on growth and nitrogen utilization by wheat plants. **Plant and Soil,** v. 245, p.215-222, 2002.

SCHLOTER, M.; KIRCHHOF, G.; HEINZMANN, J.; DÖBEREINER, J.; HARTMANN, A. Immunulogical studies of the wheat-root-colonization by *Azospirillum brasilense* strains Sp7 and Sp245 using strain-specific monoclonal antibodies. In: Hegazi, N.A., Fayes, M., Monib, M. (Eds.), **Nitrogen Fixation with Non-leguminous.** American University in Cairo Press, Cairo, p. 291-297, 1994.

SCHMID, M.; HARTMANN, A. Molecular phylogeny and ecology of root associated diazotrophic alpha and beta-proteobacteria. In: Elmerich C, Newton WE. eds. Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. New York, NY: **Springer Verlag**, 21-40, 2007.
SCHULTZE, M.; KONDOROSI, A. Regulation of symbiotic root nodule development. **Annu. Rev. Gen**. v.32, p. 33-57, 1998.

SCOTT, B. A.; VANGESSEL, M. J.; WHITE-HANSEN, S. Herbicide-resistant weeds in the United States and their impact on extension. **Weed Technol.** v. 23, p. 599-603, 2009.

SHANMUGAMK, T.; CHAN, I.; MORANDI, C. Regulation of nitrogen fixation. Nitrogenase- derepressed mutants of *Klebsiella pneumoniae*. **Biochim. Biophys. Acta**, v. 408, p. 101-111, 1975.

SMITH, S. A.; BEAULIEU, J. M.; DONOGHUE, M. J. An uncorrelated relaxed-clock analysis suggests an earlier origin for flowering plants. **Proceedings of the National Academy of Sciences,** USA 107, 5897-5902, 2010.

SOMERS, E.; VANDERLEYDEN, J.; SRINIVASAN, M. Rhizosphere bacterial signalling: a love parade beneath our feet. **Crit Rev Microbiol**. v. 30, p. 205-240, 2004.

SPAEPEN, S.; DOBBELAERE, S.; CROONENBORGHS, A.; VANDERLEYDEN J. Effects of *Azospirillum brasilense* indole-3-acetic acid production on inoculated wheat plants. **Plant and Soil,** v. 312, p.15-23, 2008.

SPAEPEN, S.; DAS, F.; LUYTEN, E.; MICHIELS, J.; VANDERLEYDEN, J. Indole-3-acetic acid-regulated genes in Rhizobium etli CNPAF512. **FEMS Microbiol Lett,** v. 291, 195-200, 2009.

SRIVASTAVA, A.; TRIPATHI, A.K. Adenosine Diphosphate Ribosylation of Dinitrogenase Reductase and Adenylylation of Glutamine Synthetase Control Ammonia Excretion in Ethylenediamine-Resistent Mutants of *Azospirillum brasilense* Sp7. **Current Microbiology,** v. 53, p. 317-323, 2006.

STEENHOUDT, O.; VANDERLEYDEN, J. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. **FEMS Microbiol. Rev.,** v. 24, p. 487-506, 2000.

STEVENSON, F. J.; COLE, M. A. **Cycles of Soil: Carbon, Nitrogen, Phosphorus, Sulfur, Micronutrients**, 2 Ed. Wiley, New York, USA. 1999.

TADRA-SFEIR, M. Z.; SOUZA, E M; FAORO, H. et al. Naringenin regulates expression of genes involved in cell wall synthesis in *Herbaspirillum seropedicae*. **Applied and environmental microbiology**, v. 77, n. 6, p. 2180–3, 2011.

TAHA, S. M.; MAHMOUD, S. A. Z, HALIM EL-DAMATY, A.; ABD EL-HAFEZ, A. M. Activity of phosphate dissolving bacteria in Egyptian soils. **Plant Soil.** v. 31, p.149-160, 1969.

TAL, S.; Y. OKON. *Production of the material polyhydroxybu tyrate and its function in Azospirillum brasilense* Cd. **Can. J. Microbiol**. 31: 608–613, 1985.

TAL, S.; SMIRNOFF, P.; OKON, Y. The regulation of poly-β-hydroxybutyrate metabolism in *Azospirillum brasilense* during balanced growth and starvation. **J. Gen. Microbiol**, 136, 1191-1196, 1990.

TAPIA-HERNANDEZ, A.; MASCARUA-ESPARZA, M. A.; CABALLERO-MELLADO, J. Production of bacteriocins and siderophore-like activity in *Azospirillum brasilense*. **Microbio,** v. 64, p. 73-83, 1990.

TARRAND, J. J.; KRIEG, N. R.; DÖBEREINER, J. A. taxonomic study of the *Spirillum lipoferum* group with description of a nem genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. **Can. J. Microbiol**, v. 24, p. 967-80, 1978.

TIMMUSK, S.; WAGNER, E. G. H. The plant-growth- promoting rhizobacterium *Paenibacillus polymyxa* induces changes in Arabidopsis thaliana gene expres- sion: a possible connection between biotic and abiotic stress responses. **Mol. Plant-Microbe Interact,** v. 12, p. 951- 959, 1999.

TORTORA, K. L.; DÍAZ-RICCI, J. C.; PEDRAZA, R. O. *Azospirillum brasilense* siderophores with antifungal activity against *Colletotrichum acutatum.* **Arch Microbiol** 4:275-86, 2011.

TRÂN VAN, V.; BERGE, O.; KÊ, S.N.; BALANDREAU, J.; HEULIN, T. Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. **Plant and Soil,** v. 218; p. 273-284, 2000.

UMALI-GARCI, M.; HUBBEL, D. H.; GASKINS, M. H; DAZZO, F. B. Association of Azospirillum with grass roots. **Appl. Environ. Microbiol,** v. 39, p. 219-226, 1980.

UPADHYAYA, N. M.; LETHAM, D. S.; PARKER, C. W.; HOCART, C. H.; DART, P. J. Do rhizobia produce cytokinins? **Biochem. Int.** 24: 123-130, 1991.

VERMA, J. P.; YADAV, J.; TIWARI, K. N.; LAVAKUSH, S.V. Impact of plant growth promoting rhizobacteria on crop production. **International Journal of Agricultural Research, v.** 5, p. 954-983, 2010.

VARGAS, M. A. T.; HUNGRIA, M. Fixacao biologica do N_2 na cultura da soja. In:. In:VARGAS, M.A.T.; HUNGRIA, M (Ed.). Biologia dos solos de cerrados. Planaltina Planaltina, **Biologia dos solos de cerrados,** 1997.

VESSEY, J. K. Plant growth promoting rhizobacteria as biofertilizers. **Plant Soil**, v. 255, p. 571-586, 2003.

VITORINO, J. C.; STEFFENS, M. B. R.; MACHADO, H. B.; YATES, M. G.; SOUZA, E. M.; PEDROSA, F. O. Potential roles for the glnB and ntrYX genes in *Azospirillum brasilense*. **FEMS Microbiol. Lett.** v. 201, p. 199-204, 2001

VORAGEN, A.; COENEN, G-J.; VERHOEF, R.; SCHOLS, H. Pectin a versatile polysaccharide present in plant cell walls. **J. Struct. Chem**, v. 20, p. 263-275, 2009.
WALL, J. D.; GEST, H. Derepression of nitrogenase activity in glutamine auxotrophs of Rhodopseudomonas capsulate. **J. Bacteriol**. v. 137, p.1459-1463, 1979.

WANG, R. L.; WENDEL, J. F.; DEKKER, J. H. Weedy adaptation in Setaria spp. I. Isozyme analysis of genetic diversity and population genetic structure in *Setaria viridis.* **Am J Bot** v. 82, p. 308-317, 1995.

WESTBY, C. A.; ENDERLIN, C. S.; STEINBERG, N. A.; JOSEPH, C. M.; MEEKS, J. C. Assimilation of 13$NH4^+$ by *Azospirillum brasilense* grown under nitrogen limitation and excess. **J. Bacteriol**. v. 169, p. 4211-4214, 1987.

WESTHOFF, P. **The economics of biological nitrogen fixation in the global economy**. In: Emerich DW, Krishnan HB. eds. Nitrogen fixation in crop production. Agronomy. Monogr. 52, ASA, CSSA, SSSA, Madison, WI, p. 309-328, 2009.

WILSON, R. N.; SOMERVILLE, C. R. Phenotypic suppression of giberellin-insensitive mutant (gai) of Arabidopsis. **Plant Physiology**, v.. 108, p. 495-502, 1995.

WISNIEWSKI-DYE, F.; BORZIAK, K.; KHALSA-MOYERS, G.; ALEXANDRE, G.; LEONID, O.S.; WUICHET, K.; HURST, G.B.; MCDONALD, W.H.; ROBERTSON, J.S.; BARBE, V.; CALTEAU, A.; ROUY, Z.; MANGENOT, S.; Prigent-combaret, C.; NORMAND, P.; BOYER, M.; SIGUIER, P.; DESSAUX, Y.; ELMERICH, C.; CONDEMINE, G.; KRISHNEN, G.; KENNEDY, I.; PATERSON, A.H.; GONZÁLEZ, V.; MAVINGUI, P.; ZHULIN, I. B. *Azospirillum* genomes reveal transition of bacteria from aquatic to terrestrial environments. **Plos genetics,** v. 7, p. 1-13, 2011.

WOOD, A. G.; MENEZES, E. M.; DYKSTRA, C.; DUGGAN, D. E. **Methods to demonstrate the megaplasmids (or minichromosomes) in *Azospirillum*.** In: KLINGMULLER, W. Azospirillum genetics, phisiology, ecology, Birthauser, Basel, p. 18-34, 1982.

XIE, C. H.; YOKOTA, A. *Azospirillum oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the roots of the rice plant *Oryza sativa*. **Int. J. Syst. Evol. Microbiol**. v. 55, p. 1435-1438, 2005.

YOUNG, J. P. W. **Phylogenetic classification of nitrogen-fixigin organisms**. In STALEY, G.; BURRIS, R. M.; EVANS, M. S. ed. Biological Nitrogen Fixation. New York. p. 943, 1992.

YOUNG, C.C.; HUPFER, H.; SIERING, C.; HO, M.J.; ARUN, A.B.; LAI, W.A.; REKHA, P.D.; SHEN, F.T.; HUNG, M.H.; CHEN, W.M.; YASSIN, A.F. *Azospirillum rugosum* sp. nov., isolated from oil-contaminated soil. **Int. J. Syst. Evol. Microbiol**. v.58, p.959-963, 2008.

ZHANG, Y.; BURRIS, R. H.; LUDDEN, P. W.; ROBERTS, G. P. Presence of a second mechanism for the posttranslation regulation of nitrogenase activity in *Azospirillum brasilense* in response to ammonium. **J. Bacteriol**, v. 178, p.2948-2953, 1996.

ZHOU, S.; HAN, L.; WANG, Y.; YANG, G.; ZHUANG, L.; HU, P. *Azospirillum humicireducens* sp. nov., a nitrogen-fixing bacterium isolated from a microbial fuel cell. **Int.J. Syst. Evol. Microbiol**.v.63, p. 2618-2624, 2013

ZHOU, Y.; WEI, W.; WANG, X.; XU, L.; LAI, R. *Azospirillum palatum* sp. nov., isolated from forest soil in Zhejiang province, China. **Journal of general and applied microbiology**. v.55, 2009.

ZHULIN, I. B.; ARMITAGE, J. P. Motility, chemokinesis, and methylation independent chemotaxis in *Azospirillum brasilense*. **J Bacteriol,** v. 175, p. 952-958, 1993.

ZHULIN, I. B.; BESPALOV, V. A.; JOHNSON, M. S.; TAYLOR, B. L. Oxygen taxis and proton motive force in *Azospirillum brasilense*. **J. Bacteriol**. 178, 5199- 5204, 1996.

ZUMFT, W. G.; CASTILLO, F. Regulatory properties of the nitrogenase from *Rhodopseudomonas palustris*. **Arch. Microbiol.** v. 117, p. 53-60, 1978.

4. ARTIGO 1: Wheat colonization by an *Azospirillum brasilense* ammonium-excreting strain reveals up-regulation of nitrogenase and superior plant growth promotion

K. F. D. N. Santos[a] • V. R. Moure[a] • V. Hauer[b] • A. R. S, Santos[a] • L. Donatti[c] • C. W. Galvão[d]
F. O. Pedrosa[a] • E. M. Souza[a] • R. Wassem[b] • M. B. R. Steffens [a]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Rua Coronel Francisco Heráclito dos Santos, C. Postal 19046, 81531-980, Curitiba, PR, Brasil
[b] Departamento de Genética, Universidade Federal do Paraná
[c] Departamento de Biologia Celular, Universidade Federal do Paraná,
[d] Departamento de Biologia Estrutural, Molecular e Genética, Universidade Estadual de Ponta Grossa, Avenida General Carlos Cavalcanti, C. Postal 4748, 84030-900, Ponta Grossa, PR, Brasil

---

Abstract

Aims: In this work an ammonium-excreting strain (HM053) of *A. brasilense* was further characterized genetically and biochemically, and its capacity of colonizing and promoting wheat growth was determined.

Methods: Immunoblot, reverse transcription-qPCR and DNA sequencing were used for HM053 characterization. To analyze wheat - *A. brasilense* interaction *nifH::gusA* fusions in the wild-type FP2 (FP2-7) and HM053 (HM053-36) backgrounds were employed.

Results: HM053 glutamine synthetase (GS) was not adenylylated in response to an ammonium shock or under any condition tested. Sequencing of the *glnA* gene revealed a substitution of a proline residue by a leucine at position 347 of the GS. HM053 was capable of colonizing the surface of wheat roots and increased by 30% and 49% the shoot and root dry weight, respectively, when compared with uninoculated plants, and by 30% and 31% when compared with the parent strain FP2. Although HM053-36 and FP2-7 showed GUS activity located mainly at lateral root emergence points, HM053-36 consistently showed stronger signals and expressed the *nifH* gene at a level 278 fold higher than strain FP2 *in planta*, according to qPCR data.

Conclusions: HM053, a spontaneous mutant in GS, enhanced plant development several fold higher when compared to the wild-type FP2. HM053 ability to excrete ammonium and fix nitrogen constitutively, even in the presence of high NH_4^+ concentration, could explain why this mutant performed better than FP2, and suggests HM053 as a better potential nitrogen biofertilizer.

Keywords: *Azospirillum brasilense*, ammonium-excreting mutant, biofertilizer, glutamine synthetase, *glnA* gene

Abbreviations:
PGPR: Plant-Growth-Promoting Rhizobacterium (PGPR)
BNF: Biological Nitrogen Fixation
GS: Glutamine Synthetase
GOGAT: Glutamate Synthase
GUS: β-glucuronidase
CFU: Colony Forming Units
D.a.i: Days after inoculation

4.1 INTRODUCTION

Plant-Growth-Promoting Rhizobacteria (PGPR) constitutes a heterogeneous group of bacteria that has the potential to improve plant growth and productivity. These microorganisms can promote the development of roots increasing the uptake of water and mineral nutrients by plants, and can be used as biofertilizer (Vessey 2002). The ability of these bacteria to promote plant growth has been attributed to a set of features, including but not limited to, biological nitrogen fixation, synthesis of phytohormones such as auxin (Martínez-Morales et al. 2003), gibberellins (Bottini et al. 1989), cytokinins (Pennisetum et al. 1979) and abscisic acid (Cohen et al. 2007), production of siderophores, phosphate solubilization and also indirectly by suppression of plant pathogens such as fungi, bacteria, viruses, nematodes and insects (Ryu et al. 2004). In most cases only a few of such capabilities are present in a given PGPR.

In *A. brasilense*, a well known PGPR, nitrogen fixation genes (*nif*) are subjected to transcriptional regulation while nitrogenase activity is under post-translational control. Transcriptional regulation involves the control of NifA synthesis and activity by NH_4^+ and O_2 (Arsene et al. 1996; Araújo et al. 2004; Fadel-Picheth et al. 1999). Post-translational regulation of nitrogenase activity involves reversible ADP-ribosylation of dinitrogenase reductase, the NifH protein, catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DraT), leading to nitrogenase inactivation (switch-off) under conditions of high ammonium levels or energy depletion. Under ammonium ions depletion or limitation or the energy levels are restored, dinitrogenase reductase-activating glycohydrolase (DraG) removes the ADP-ribosyl group, promoting nitrogenase reactivation (Moure et al. 2013; Huergo et al. 2012). Intracellular ammonium assimilation into glutamine and glutamate, involves two major pathways. Under low NH_4^+ concentrations glutamate is converted to glutamine by glutamine synthetase (GS) in an ATP-dependent manner. Glutamine then donates its amide nitrogen to 2-oxoglutarate yielding 3 moles of glutamate in a reaction catalysed by glutamate synthase (GOGAT) (Meers and Tempest 1970; Elmerich and Aubert 1971). Under high NH_4^+ concentrations, ammonium is mainly used for reductive amination of 2-oxoglutarate, a reaction catalysed by glutamate dehydrogenase. Under the latter condition, GS is inactivated by successive adenylylation of each of its 12 subunits in a reaction catalysed by the bifunctional enzyme adenylyltransferase (GlnE) (Arcondéguy et al. 2001).

Nitrogen availability is one of the major limiting factors for crop growth and productivity. Symbiotic diazotrophs have been widely used as inoculants for leguminous plants with which they can form efficient symbiotic associations. The most successful association is between soybean and *Bradyrhizobium* spp, where the bacteria can provide all the nitrogen required by the plant (Sij, Turner and Craigmiles 1979; Koutroubras, Papakosta and Gagianas 1998; Mendes, Hungria and Vargas 2003). Although cereals are not capable of such symbiosis, their beneficial association with N_2-fixing PGPR has reduced the use of synthetic N fertilizer (Curatti and Rubio 2014). The genus *Azospirillum* has emerged as one of the most promising PGPR (Okon and Vanderleyden 1997; Döbereiner, 1992; Fibach-Paldi et al. 2012). *Azospirillum brasilense* has been used as biofertilizer for production improvement of economically important cereal crops such as rice, wheat and maize (Hungria et al. 2010; García de Salamone et al. 2010; Spaepen et al. 2008; Fallik and Okon 1996). The mechanism by which *Azospirillum*-based inoculant promotes plant growth is not fully understood. Phytohormones production and biological nitrogen fixation have been attributed as the main factors (Steenhoudt and Vanderleyden 2000). However, the contribution of each of these factors remains controversial. Several studies showed transfer of N from bacteria to plant (Kapulnik et al. 1985; Döbereiner 1992; Dobbelaere and Okon 2007) while others failed (Hurek et al. 1994; Egener et al. 1998; Gerk et al. 2000).

Machado and coworkers (1991) characterized a spontaneous mutant, HM053, resistant to ethylenediamine (EDA^R) derived from *A. brasilense* FP2 (Sp7 ATCC 29145, Sm^R, Nal^R). This mutant is able of excreting ammonium and fixing nitrogen in the presence of high concentrations of NH_4^+, hence a candidate for use as biofertilizer to supply nitrogen to gramineaceous plants. Machado et al. (1991) suggested that HM053 ability to excrete ammonium is related to low GS activity, resulting in deficiency of NH_4^+ assimilation and explaining the excretion of excess ammonium produced during nitrogen fixation. Recently Pankievicz et al. (2015) showed that this strain can promote growth of *Setaria viridis* and was capable of providing 100% of the plant N needs. Here, this strain was further characterized genetically and biochemically, and its wheat root colonization capacity assessed. A *nifH-gusA* chromosomal fusion and qPCR analysis confirmed *nif* expression *in planta* during wheat root colonization.

4.2 MATERIALS AND METHODS

4.2.1 Bacteria and growth conditions

The spontaneous ethylenediamine resistant mutant HM053 (Nif^C, EDA^R, Nal^R, Sm^R) was isolated by Machado et al. (1991) from *A. brasilense* FP2 strain (Sp7 ATCC 29145, Sm^R, Nal^R) (Pedrosa and Yates 1984). To monitor the colonization efficiency, strains containing chromosomal *nifH-gusA* fusions, FP2-7 (Nif^+, Nal^R, Sm^R, Tc^R, Km^R) and HM053-36 (Nif^C, EDA^R, Nal^R, Sm^R, Tc^R, Km^R), were generated by single-recombination (Santos, A. R. S., personal communication). Cells were grown at 30°C in NFbHP-lactate liquid medium under shaking at 120 rpm or in NFbHP-lactate semi-solid medium (0.175% agar w/v) with appropriate antibiotics. The nitrogen source was added as required (2 mM or 20 mM NH_4Cl or 5 mM sodium glutamate).

4.2.2 Analytical assays

Nitrogenase activity was determined by acetylene reduction method (Pedrosa and Yates 1984), where the strains FP2, HM053 and their derivate strains were grown in semi-solid medium supplemented with different nitrogen sources: 5 mM glutamate or NH_4Cl in different concentrations (5 mM to 20 mM). The quantification of the excreted ammonium was performed at 24, 48, 72 and 96 hours after bacteria inoculation in semi-solid medium. Ammonium in the supernatant solution was determined by the indophenol procedure (Chaney and Marbach 1961). The total protein concentration was determined as described (Bradford 1976), after cell lysis with 0.2 M NaOH. Western blot assays and electrophoresis was performed as described (Huergo et al. 2006).

4.2.3 Plant assays

The seeds of *Triticum aestivum* var. CD104 were kindly provided by Coodetec (Cooperativa Central de Pesquisa Agrícola – Brazil). For optical microscopic analysis the seeds were surface-sterilized, germinated, inoculated and grown as described by Camilios-Neto et al. (2014). GUS activity was detected in bright field transmitted light images of the roots that were incubated for 1 hour in 50 mM sodium cacodylate buffer pH 7.5 containing 0.5 mg. ml^{-1} X-gluc at 45°C (Hurek et al. 1994). Wheat root images were analyzed in a Carl Zeiss Jenaval microscope equipped with an AxioCan MRC camera. For scanning

electron microscopy inoculated wheat roots were fixed using amodified Karnovsky fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at 4°C) (Karnovsky 1965). The samples were dehydrated through a gradient series of ethanol, where the critical point to dry the samples was obtained in a Bal-Tec CPD - 030 with carbon dioxide. The dried samples were coated with ionized gold film (SCD030, Balzers Union, Fl, USA) and examined in a JEOL-JSM 6360 LV scanning electron microscope.

For plant growth promotion assay, axenic seedlings were incubated with 1 ml of 4.5 x 10^6 cells of HM053 and 3 x 10^6 cells of FP2 per plantlet for 30 minutes. As control, axenic plantlets were incubated for 30 minutes in sterile Hoagland's nutrition solution (Hoagland and Arnon 1950) without inoculum. The seedlings were planted in pots containing a mixture of sterilized quartz sand and vermiculite in a proportion of 3:1, respectively. The plantlets were watered twice a week with Hoagland's nutrient solution containing low nitrogen (0.5 mM KNO_3). The study was conducted in a growth chamber at 28°C, 16 hour photoperiod with nominal irradiance of 220 µmol m^{-2} s^{-1} and 60% relative humidity for 32 days. Then, the plants were harvested and growth parameters were measured: plant height, shoot and root dry weight. Statistical analyses were assessed by Test-t (parametric) or Wilcox test (non-parametric), according to the distribution of the samples. It was used fifteen plants per condition (N =15).

4.2.4 Quantification of mRNA levels by RT-qPCR

The roots inoculated with FP2 and HM053 strains were harvested 6 d.a.i, when there were about 10^7 bacterial cells per gram of roots for both strains. Roots from six plants were collected and used for RNA extraction. This experiment was performed in independent duplicates. The *nifH* primer specific for *A. brasilense* (Camilios-Neto et al. 2014) was used to evaluate mRNA levels.

Total RNA was isolated from 200 mg wheat roots using the RNAqueous kit (Ambion, Austin, TX). The cDNA was produced from 1 µg DNase-treated total RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA reaction was diluted 5 times before using Power SYBR-Green PCR Master Mix on a StepOne Plus Real Time-PCR System (both from Applied Biosystems, Foster City, CA). The housekeeping gene *rpoC* was used as internal control (Camilios-Neto et al. 2014). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

4.3 RESULTS

4.3.1 *A. brasilense* HM053 glutamine synthetase is not adenylylated in response to ammonium addition

A. brasilense HM053 fixes nitrogen constitutively in the presence of high NH_4^+ concentration. This mutant was reported to have low levels of constitutively adenylylated glutamine synthetase (GS) (Machado et al. 1991). However, the post-translational modification profiles of nitrogenase and glutamine synthetase were not determined.

To assess the effect of post-translational modification of both NifH and GS in response to ammonium in the wild-type FP2 and HM053 strains, cells were grown under nitrogen-fixing conditions using 5 mM glutamate or 2 mM NH_4Cl (condition –N), and then shocked by the addition of 2 mM NH_4Cl for 5 min (condition +N) (Fig.1). The cells were collected and submitted to Western Blot analysis using antibodies against *A. brasilense* NifH and *Escherichia coli* GS. Only one subunit of NifH dimer is ADP-ribosylated, hence a double band is observed in the immunoblot, since the modified subunit has a lower migration rate than the unmodified subunit on SDS-PAGE (Kanemoto and Ludden 1984). For GS, the adenylylation status can also be detected as a double band pattern under similar electrophoresis conditions (Huergo et al. 2006).

In the wild-type strain, both NifH and GS modifications were synchronized upon ammonium shocking in cells grown with low NH_4Cl or glutamate as nitrogen source (Fig.1). When HM053 cells were grown in glutamate as nitrogen source, nitrogenase was inactivated by ADP-ribosylation, but not when grown in low NH_4^+. This result suggests that not only *nif* genes are derepressed in this mutant, but nitrogenase ADP-ribosylation is impaired. HM053 GS was not subject to post-translational regulation upon ammonium shock in both nitrogen sources, although it was similarly expressed. These results suggest that the GS post-translational control in the spontaneous mutant is also impaired. Also, since HM053 fixes nitrogen when grown in high ammonium (10 or 20 mM) the mutation in this strain overrides ADP-ribosylation signaling under high ammonium.

4.3.2 Identification of *glnA* mutation in HM053

In *A. brasilense* the signal transducing PII proteins, GlnB and GlnZ, are involved in the control of DraT and DraG activities, respectively (Huergo et al. 2012; Moure et al.

2013). GlnB is also involved in the activation of the *nif* specific transcriptional factor, the NifA protein (Araújo et al. 2004). In contrast to *E. coli*, *A. brasilense* GS post-translational modification apparently is not regulated by PII proteins, being regulated by the glutamine level (De Zamaroczy 1998). The PII protein genes *glnB* and *glnZ* of mutant HM053 were sequenced and no modification was found (Vitorino et al. 2001). Also, plasmids pJC1 (which contains the *glnB* gene of *A. brasilense*) failed to complement the HM053 mutant (Vitorino et al. 2001). Nitrosoguanidine mutant strains 7028 and 7029 of *A. brasilense*, which are glutamine auxotrohophs and excrete ammonium when grown in NO_3^-, have point mutations in the *glnA* gene (Van-Dommelen et al. 2003). Since GS activity is impaired in HM053 (Machado et al. 1991) and it seems unable to be adenylylated by GlnE, its *glnA* and *glnE* gene were PCR-amplified and sequenced. No modification in the sequence of the *glnE* gene was detected. On the other hand, in *glnA* gene a change of cytosine to thymine in codon 1040 leading to the replacement of a proline residue at position 347 to a leucine residue was found. It has been shown that point mutations in GS active site alters profoundly its activity (Witmer et al. 1994; Abell et al. 1995; Dhalla et al. 1994; Liaw and Eisenberg 1994). Although Pro-347 is conserved in GS sequences and is located close to the active site, it does not seem to be involved in metal or nucleotide binding (Fig.1S). However, proline is a helix destabilizing residue. Hence its replacement by leucine, which is a α-helix stabilizing residue, may cause considerable conformational changes in GS folding which may explain the phenotype of HM053.

4.3.3 Colonization of wheat by the mutant HM053

The interaction between wheat roots and *A. brasilense* HM053 was analyzed and compared with the wild-type strain FP2. The strains HM053-36 and FP2-7, which contain the *gusA* reporter gene, were used to monitor the colonization of wheat roots.

A. brasilense FP2-7 and HM053-36 strains were first tested for the capacity to excrete ammonium ions and fix nitrogen in semi-solid medium. These strains contain a *nifH::gusA* fusion inserted in the chromosome as a result of single cross-over, thus they contain a functional *nifHDK* operon and a *nifH-gusA* fusion. Nitrogenase activity in -N condition for FP2-7 (14.9 nmol of ethylene mg protein^{-1} min^{-1}) and HM053-36 (28.5 nmol of ethylene mg protein^{-1} min^{-1}) was similar to that of the parental FP2 (18.9 nmol of ethylene mg protein^{-1} min^{-1}) and HM053 strains (30 nmol of ethylene mg protein^{-1} min^{-1}) (2S). As expected, in presence of nitrogen (NH_4^+), strains FP2 and FP2-7 failed to fix

nitrogen, while HM053 and HM053-36 were able to fix (2S). The total concentration of ammonium excreted by mutant HM053 (3.3 mM) and its derivative strain HM053-36 (2.2 mM) after 96 hours of the inoculation were not statistically different (Table 1S). This result indicates that the insertion of the cassette did not affect the ammonium excretion capacity and fix nitrogen of these strains. As expected, strains FP2 and FP2-7 were not able to excrete ammonium (Table 1S) (Machado et al. 1991). These results validated the use of these derivative strains in the foregoing experiments.

The expression of the *nifH::gusA* fusion was assessed in wheat roots inoculated with FP2-7 and HM053-36 strains 3 to 12 d.a.i (Fig. 2). Both strains were found preferentially adhered at lateral root emerging points (Fig. 2). Few cells were found in the root cap (Fig. 3S). These data were complemented by scanning electron microscopy showing cell aggregates and single cells at the surface on the wheat roots (3 to 15 d.a.i) (Fig. 4S). Furthermore, the ability of *A. brasilense* strains HM053 and FP2 to colonize the internal tissues was assessed after surface-sterilization, as described by Camilios-Neto et al. (2014). No bacterial colony was recovered from surface sterilized roots, suggesting these strains are epiphytic colonizers, supporting the previous characterization of *A. brasilense* strain FP2 (Camilios-Neto et 2014) and Sp7 (Schloter et al. 1994) as epiphytic. It is noteworthy that stronger β-glucuronidase activity signals were visualized with HM053-36 inoculated roots in comparison to FP2-7, suggesting that *nifH* is more expressed in the ammonium-excreting mutant. To further investigate this finding, *nifH* mRNA levels of wheat inoculated were estimated by RT-qPCR. The *nifH* gene was 278 fold (±13.06) more expressed in HM053 than in FP2 inoculated plants. These results clearly suggest that HM053 has higher nitrogen-fixing ability when compared with FP2.

4.3.4 Plant growth promoting activity of *A. brasilense* HM053

In order to verify if *A. brasilense* HM053 could stimulate plant growth, dry weight and length were measured in inoculated wheat plants and compared with uninoculated controls. Plants inoculated with HM053 performed better than the uninoculated or inoculated with FP2. The difference in root and shoot length was small, but statistically significant (p-value ≤ 0.05). Furthermore, the dry weight of plant inoculated with HM053 was substantially higher; the root dry weight of wheat inoculated with HM053 was 49% and 31% higher than in wheat uninoculated and inoculated with FP2, respectively (Fig. 3).

For the shoots the dry weight of HM053 inoculated wheat plants was 30% higher compared to uninoculated or FP2 inoculated plants (Fig. 3).

4.4 DISCUSSION

A. brasilense HM053 is an ethylenediamine (EDA) spontaneous resistant mutant and has the ability to fix nitrogen constitutively and excrete ammonium (Machado et al. 1991). This mutant showed a low level of constitutively adenylylated GS, suggesting that it is defective in both activity and adenylylation of GS. However, Vitorino et al. (2001) showed that the *glnA* gene is normally expressed and no mutation was found in *glnB* or *glnZ* genes. Here, Western blot of cells grown under low fixed nitrogen condition and shocked with 2 mM NH_4Cl was used to further characterize this strain. Interestingly the GS of HM053 was not adenylylated, indicating a possible mutation in the adenylylation system or GS itself. Analysis of the *glnE* gene sequence from HM053 showed no mutations in this gene. However, sequencing of the *glnA* gene revealed a single mutation leading to a change of the conserved proline-347 for a leucine residue. This mutation seems to have two effects: drastic reduction of GS activity and inhibition of GS adenylylation by GlnE. Since this residue is located close the active site and its substitution is predicted to lead to substantial secondary structure change it is feasible that this single mutation is responsible for the GS phenotype of mutant HM053. The low GS activity restricts HM053 capacity to assimilate $NH_4^+$and produce glutamine causing two effects: a) under high NH_4^+, glutamine is produced at low rate and its low concentration signals to the cell low NH_4^+ condition. Under this condition GlnD protein will catalyze the uridylylation of the GlnB protein that will, in turn, activate NifA. Active NifA will activate the transcription of the *nif* genes constitutively (biological nitrogen fixation); b) under nitrogen fixation condition, the low GS activity is unable to assimilate NH_4^+ produced by nitrogenase, which in turn is excreted to the culture medium. HM053 cells grown in low NH_4^+ were unable to ADP-ribosylate NifH, whereas glutamate grown cells did. It is likely that under low NH_4^+ the levels of glutamate are also depleted since it is synthesized via the combined action of GS and GOGAT. In this case, a sudden increase in NH_4^+ would not translate in quick increase of glutamine, the signal for GlnB protein deuridylylation by GlnD. Deuridylytated GlnB is required in *A. brasilense* for DraT activation (Moure et al. 2013), thus under this condition HM053 does not ADP ribosylate NifH. However when grown in glutamate, the levels of

intracellular glutamate are higher allowing enough glutamine synthesis to trigger GlnB deuridylylation and NifH ADP ribosylation.

The higher capacity of nitrogen fixation and ammonium excretion of HM053 seems to be beneficial for inoculated plants. To test if *A. brasilense* HM053 could fix nitrogen *in planta* and promote growth, this strain was inoculated in wheat seedlings (10^6 cells per plant). Colonization, *nifH* expression and plant dry weight were followed. Not only *nifH* expression in plants inoculated with HM053 was almost 300-fold higher than when inoculated with the parental strain FP2, but shoot and root weights were over 30% higher in plants inoculated with HM053 compared with those uninoculated or inoculated with FP2.

Superior plant growth promotion activity has been observed by mutants with the ability to excrete ammonium. Rice seedlings inoculated with ammonium-excreting strain SA1 from cyanobacterium *Anabaena variabilis* ATCC29413 weighted 5-times more than those inoculated with wild-type strain (Latorre et al. 1986). Similarly, wheat inoculated with SA1 also improved weight (Spiller and Gunasekaran 1990). A winter wheat variety inoculated with the *A. brasilense* ammonium-excreting mutant (strain 7029) showed up to 50% higher whole plant dry weight than those inoculated with the wild-type Sp7 strain (Van Dommelen et al. 2009). More recently a study assessing artificial symbiosis between the ammonium-excreting *Azotobacter vinelandii* mutant and a microalgae showed increase in biomass (Ortiz-Marquez et al. 2012). Together with the results reported here, these studies strongly suggest the associated plant is benefited by ammonium-excreting PGPR. Indeed, it was shown recently that the inoculation of HM053 mutant in the graminaceous plant model *Setaria viridis* led to an incorporation of 16-fold more nitrogen than the mix of wild-type strains of *A. brasilense* and *Herbaspirillum seropedicae* (Pankievicz et al. 2015). Furthermore, fixed nitrogen derived from labeled $^{13}N_2$ excreted by strain HM053 was shown to be incorporated into plant rubisco.

In summary the evidence presented in this paper shows that a spontaneous single mutation in GS of *A. brasilense* HM053 lead to a constitutive nitrogen fixation and an ammonium-excreting phenotype. Although this mutant has not been tested under field conditions to evaluate not only its effectiveness as ammonium-excreting bacteria, but also its capacity to compete with indigenous microflora, the results suggest that discrete site directed changes in the genome of plant growth promoting rhizobacteria may allow construction of new and more effective *Azospirillum brasilense* strains.

Acknowledgments

This work was supported by National Institute of Science and Technology on Biological Nitrogen Fixation (INCT/CNPq), We thank Roseli Prado, Valter A. Baura and Marilza Doroty Lamour for technical assistance.

4.5 REFERENCES

Abell LM, Schineller J, Keck PJ, Villafranca JJ (1995) Effect of metal-ligand mutations on phosphoryl transfer reactions catalyzed by *Escherichia coli* glutamine synthetase. Biochemistry 34:16695–16702.

Araújo LM, Monteiro RA, Souza EM, et al (2004) GlnB is specifically required for *Azospirillum brasilense* NifA activity in *Escherichia coli*. Research in microbiology 155:491–495. doi: 10.1016/j.resmic.2004.03.002

Arcondéguy T, Jack R, Merrick M (2001) PII Signal Transduction Proteins, Pivotal Players in Microbial Nitrogen Control. Microbiology and Molecular Biology Reviews 65:80–105. doi: 10.1128/MMBR.65.1.80-105.2001

Arsene F, Kaminski PA, Elmerich C (1996) Modulation of NifA activity by PII in *Azospirillum brasilense*: evidence for a regulatory role of the NifA N-terminal domain. Journal of Bacteriology 178:4830–4838.

Bottini R, Fulchieri M, Pearce D, Pharis RP (1989) Identification of Gibberellins A1, A3, and Iso-A3 in cultures of *Azospirillum lipoferum*. Plant Physiol 90:45–47.

Bradford M (1976) A rapid and sensive method for the quantification of microgram quantities of protein utilization: the principle of protein-dye binding. Anal Biochem 72:248–254.

Camilios-Neto D, Bonato P, Wassem R, et al (2014) Dual RNA-seq transcriptional analysis of wheat roots colonized by *Azospirillum brasilense* reveals up-regulation of nutrient acquisition and cell cycle genes. BMC genomics 15:378. doi: 10.1186/1471-2164-15-378

Chaney L, Marbach P (1961) Modified Reagents of Urea and for Determination Ammonia. Clinical Chemistry 8:130–132.

Cohen AC, Bottini R, Piccoli PN (2007) *Azospirillum brasilense* Sp 245 produces ABA in chemically-defined culture medium and increases ABA content in arabidopsis plants. Plant Growth Regulation 54:97–103. doi: 10.1007/s10725-007-9232-9

Curatti L, Rubio LM (2014) Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. Plant Science 225:130–137. doi: 10.1016/j.plantsci.2014.06.003

De Zamaroczy M (1998) Structural homologues PII and Pz of *Azospirillum brasilense* provide intracellular signalling for selective regulation of various nitrogen-dependent

functions. Molecular Microbiology 29:449–463. doi: 10.1046/j.1365-2958.1998.00938.x

Dhalla AM, Li BIN, Alibhai ME, et al (1994) Regeneration of catalytic activity of glutamine synthetase mutants by chemical activation : Exploration of the role of arginines 339 and 359 in activity. Protein Science 3:476–481.

Dobbelaere S, Okon Y (2007) The plant growth-promoting effect. In: Elmerich, Claudine, Newton WE (ed) Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations. pp 145–170

Döbereiner J (1992) History and perspectives of diazotrophs in association with non leguminous plants. Symbiosis 13:1–13.

Dommelen A Van, Keijers V, Wollebrants A, Vanderleyden J (2003) Phenotypic Changes Resulting from Distinct Point Mutations in the *Azospirillum brasilense* glnA Gene , Encoding Glutamine Synthetase. Applied and Environmental Microbiology 7028:5699–5701. doi: 10.1128/AEM.69.9.5699

Egener T, Hurek T, Reinhold-Hurek B (1998) Use of green fluorescent protein to detect expression of nif genes of *Azoarcus* sp. BH72, a grass-associated diazotroph, on rice roots. Molecular plant-microbe interactions : MPMI 11:71–5. doi: 10.1094/MPMI.1998.11.1.71

Elmerich C, Aubert J-P (1971) Synthesis of glutamate by a glutamine : 2-oxo-glutarate amidotransferase (NADP oxidoreductase) in *Bacillus megaterium*. Biochemical and biophysical research communications 42:371–376.

Fadel-picheth CMT, Souza EM, Rigo LU, Funayama S (1999) Regulation of *Azospirillum brasilense* nifA gene expression by ammonium and oxygen. 179:281–288.

Fallik E, Okon Y (1996) The response of maize (Zea mays) to *Azospirillum* inoculation in various types of soils in the field. World Journal of Microbiology and Biotechnology 12:511–515.

Fibach-Paldi S, Burdman S, Okon Y (2012) Key physiological properties contributing to rhizosphere adaptation and plant growth promotion abilities of *Azospirillum brasilense*. FEMS microbiology letters 326:99–108. doi: 10.1111/j.1574-6968.2011.02407.x

García de Salamone IE, Salvo LP, Escobar Ortega JS, et al (2010) Field response of rice paddy crop to *Azospirillum* inoculation: physiology of rhizosphere bacterial communities and the genetic diversity of endophytic bacteria in different parts of the plants. Plant and Soil 336:351–362. doi: 10.1007/s11104-010-0487-y

Gerk LP, Gilchrist K, Kennedy IR (2000) Mutants with Enhanced Nitrogenase Activity in Hydroponic *Azospirillum brasilense* -Wheat Associations. Applied and Environmental Microbiology 66:2175–2184. doi: 10.1128/AEM.66.5.2175-2184.2000.Updated

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Circular California Agriculture Experimental Station 347:1-32.

Huergo L, Souza E, Araujo M, et al (2006) ADP-ribosylation of dinitrogenase reductase in *Azospirillum brasilense* is regulated by AmtB-dependent membrane sequestration of DraG. Molecular microbiology 59:326–337.

Huergo LF, Pedrosa FO, Muller-Santos M, et al (2012) PII Signal Transduction Proteins: Pivotal Players in Post-Translational Control of Nitrogenase Activity. Microbiology 158:176–190. doi: 10.1099/mic.0.049783-0

Hungria M, Campo RJ, Souza EM, Pedrosa FO (2010) Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. Plant and Soil 331:413–425. doi: 10.1007/s11104-009-0262-0

Hurek T, Reinhold-hurek B, Montagu MVAN, Kellenberger E (1994) Root Colonization and Systemic Spreading of *Azoarcus* Strain BH72 in Grasses. Journal of bacteriology 176:1913–1923.

Kanemoto RH, Ludden PW (1984) Effect of Ammonia, Darkness, and Phenazine Methosulfate on Whole-Cell Nitrogenase Activity and Fe Protein Modification in *Rhodospirillum Rubrum*. Journal of Bacteriology 158:713–720.

Kapulnik Y, Okon Y, Henis Y (1985) Yield response of spring wheat cultivars (*Triticum aestivum* and *T . turgidum*) to inoculation with *Azospirillum brasilense* under field conditions. Biology and Fertility of Soils 27–35.

Karnovsky M (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. Journal of Cell Biology 27:137A–138A.

Koutroubras SD, Papakosta D, Gagianas A (1998) The importance of early dry matter and nitrogen accumulation in soybean yield. Europ JAgron 9:1–10.

Latorre C, Lee JH, Spiller H, Shanmugam KT (1986) Ammonium ion-excreting cyanobacterial mutant as a source of nitrogen for growth of rice: A feasibility study. Biotechnology Letters 8:507–512. doi: 10.1007/BF01025211

Liaw SH, Eisenberg D (1994) Structural model for the reaction mechanism of glutamine synthetase, based on five crystal structures of enzyme-substrate complexes. Biochemistry 33:675–681.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif) 25:402–8. doi: 10.1006/meth.2001.1262

Machado, Funayama S, Rigo LU, Pedrosa F (1991) Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine. Canadian Journal of Microbiology 37:549–553.

Martínez-Morales L, Soto-Urzúa L, Baca B, Sánchez-Ahédo J (2003) Indole-3-butyric acid (IBA) production in culture medium by wild strain *Azospirillum brasilense*. Media 228:167–173. doi: 10.1016/S0378-1097(03)00694-3

Meers J, Tempest D (1970) "Glutamine(amide): 2-Oxoglutarate Amino Transferase Oxido-reductase (NADP)", an Enzyme Involved in the Synthesis of Glutamate by Some Bacteria. Journal of General Microbiology 64:187–194.

Mendes IC, Hungria M, Vargas VM (2003) Soybean response to starter nitrogen and *Bradyrhizobium* inoculation on Cerrado Oxisol under no-tillage and conventional tillage systems. Revista Brasileira de Ciência do Solo Journal 27:81–87.

Moure VR, Danyal K, Yang Z-Y, et al (2013) The nitrogenase regulatory enzyme dinitrogenase reductase ADP-ribosyltransferase (DraT) is activated by direct interaction with the signal transduction protein GlnB. Journal of bacteriology 195:279–86. doi: 10.1128/JB.01517-12

Okon Y, Vanderleyden J (1997) Root associatied *Azospirillum* species can stimulate plants. ASM News 63:364–370.

Ortiz-Marquez JCF, Nascimento M Do, Dublan M de los A, Curatti L (2012) Association with an Ammonium-Excreting Bacterium Allows Diazotrophic Culture of Oil-Rich Eukaryotic Microalgae. Applied and Environmental Microbiology 78:2345–2352. doi: 10.1128/AEM.06260-11

Pankievicz VCS, Do Amaral FP, Santos KFDN, et al (2015) Robust biological nitrogen fixation in a model grass-bacterial association. The Plant journal : for cell and molecular biology 81:907–19. doi: 10.1111/tpj.12777

Pedrosa FO, Yates MG (1984) Regulation of nitrogen fixation (nif) genes of A*zospirillum brasilense* by nifA and ntrC (glnG) type genes. FEMS Microbiol. Lett. 55:95–101.

Pennisetum L, Tien T M, Gaskins MH (1979) Plant Growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet. 37:1016–1024.

Ryu C, Farag MA, Hu C, et al (2004) Bacterial Volatiles Induce Systemic Resistance in. 134:1017–1026. doi: 10.1104/pp.103.026583.with

Sij JW, Turner FT, Craigmiles J (1979) Starter nitrogen fertilization in soybean culture. Comm Soil Sci Plant Anal 10:1451–1457.

Spaepen S, Dobbelaere S, Croonenborghs A, Vanderleyden J (2008) Effects of *Azospirillum brasilense* indole-3-acetic acid production on inoculated wheat plants. Plant and Soil 312:15–23. doi: 10.1007/s11104-008-9560-1

Spiller H, Gunasekaran M (1990) Ammonia-excreting mutant strain of the cyanobacterium *Anabaena variabilis* supports growth of wheat. Applied Microbiology and Biotechnology 33:477–480. doi: 10.1007/BF00176670

Steenhoudt O, Vanderleyden J (2000) Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS microbiology reviews 24:487–506.

Van Dommelen A, Croonenborghs A, Spaepen S, Vanderleyden J (2009) Wheat growth promotion through inoculation with an ammonium-excreting mutant of *Azospirillum brasilense.* Biology and Fertility of Soils 45:549–553. doi: 10.1007/s00374-009-0357-z

Vessey JK (2002) Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255:571–586.

Vitorino JC, Steffens MB, Machado HB, et al (2001) Potential roles for the glnB and ntrYX genes in *Azospirillum brasilense*. FEMS microbiology letters 201:199–204.

Witmer MR, Palmieri-Young D, Villafranca JJ (1994) Probing the catalytic roles of n2-site glutamate residues in *Escherichia coli* glutamine synthetase by mutagenesis. Protein Science : A Publication of the Protein Society 3:1746–1759.

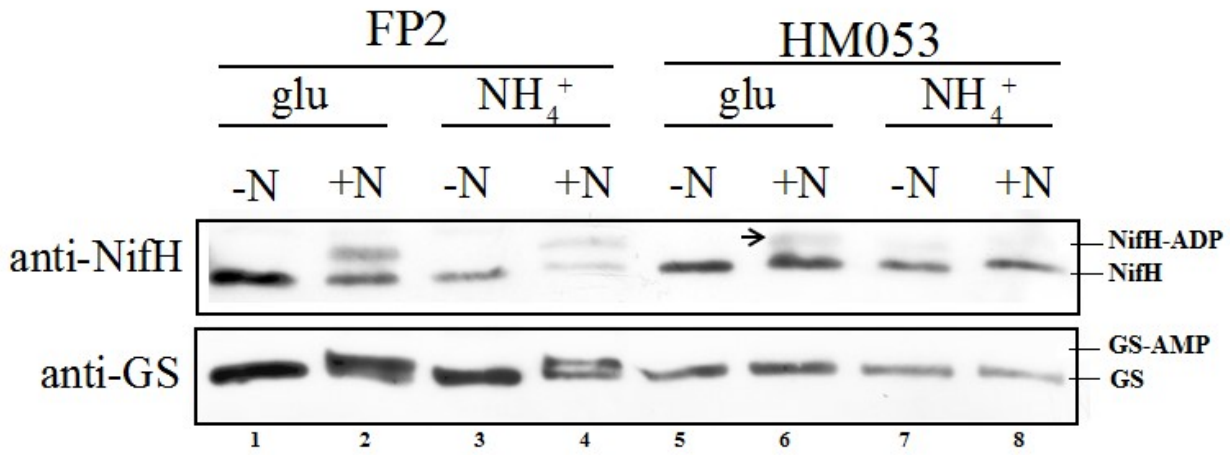


Figure 1.Western blotting analysis. *A. brasilense* wild-type FP2 and mutant HM053 strains were grown in 5 mM glutamate (glu) or 2 mM NH_4Cl (NH_4^+) as nitrogen source. Nitrogen-fixing cells (-N) were subjected to an ammonium shock using 2 mM NH_4Cl during 5 min (+N). Whole cell extracts were collected, analyzed by SDS-PAGE and Western blotting with anti-NifH and anti-GS antibodies. The arrow indicates partial modification of the NifH protein.

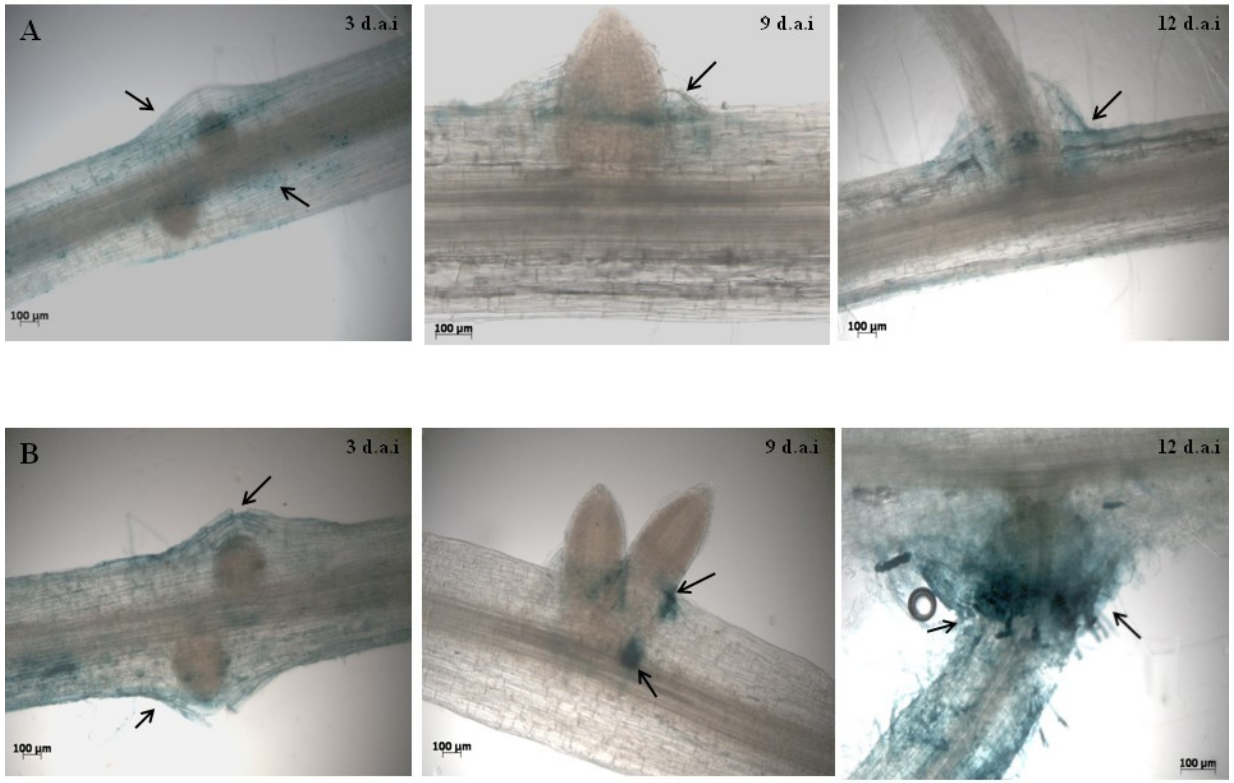


Figure 2. Expression of *nifH-gusA* by *Azospirillum brasilense* on the lateral root emergence of wheat (*Triticum aestivum* var. CD104). A) Wheat root inoculated with derivative FP2-7 strain (from wild-type FP2): 3, 9 and 12 days after inoculation (d.a.i) B) Wheat root inoculated with derivative HM053-36 strain (from mutant HM053): 3, 9 and 12 d.a.i. Seedlings were inoculated with *Azospirillum brasilense* (10^7cells per plant) and the GUS activity staining was analyzed by light microscopy.

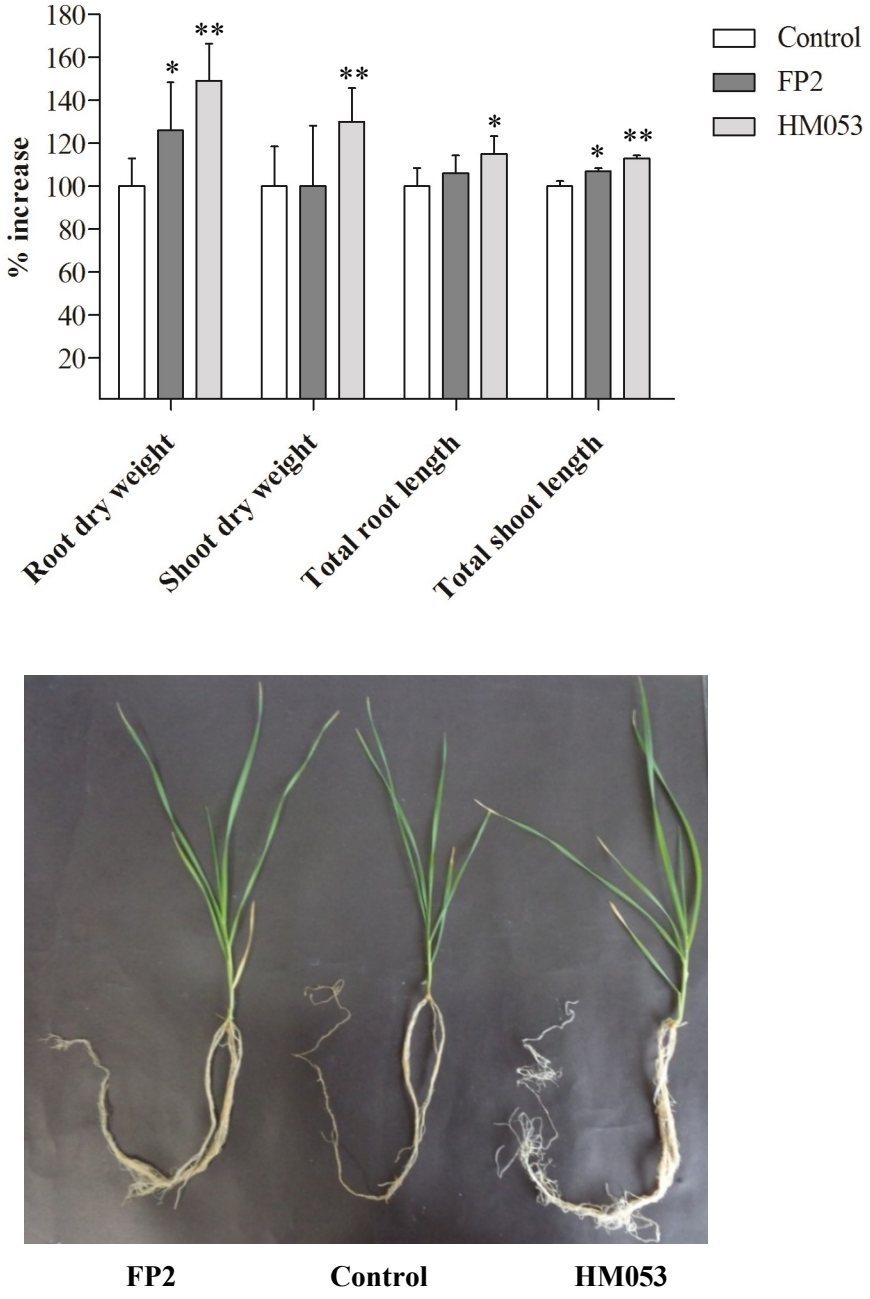
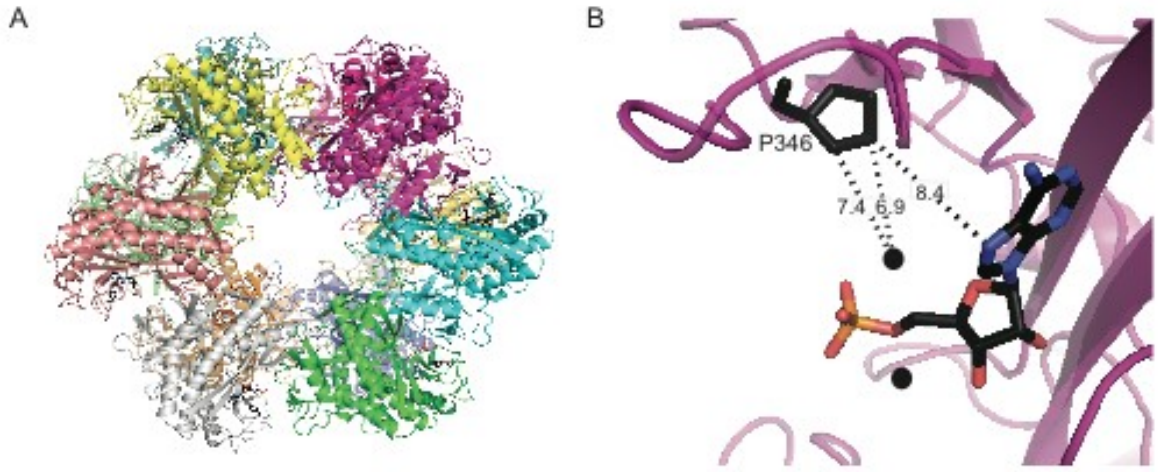


Figure 3. Wheat (*Triticum aestivum* var. CD104) responses to inoculation with *Azospirillum brasilense* wild-type FP2 and mutant HM053. The plants were incubated with 1 ml (total 10^6 cells) per plant for 30 minutes, watered with Hoagland's solution containing 0.5 mM KNO_3 and harvested 32 days after inoculation. The number of colony forming units (CFU) per gram of fresh root after 96 hours of inoculation was evaluated, and there were 1.5 x 10^7 CFU of HM053 and 1 x 10^7 CFU of FP2 per gram of fresh root. The root and shoot were measured by dry weight (mg) and length (cm). Each asterisk denotes a statistically significant difference with *p-value ≤ 0.05 or **p-value ≤ 0.01 using the appropriate test (T-test or Wilcox test).



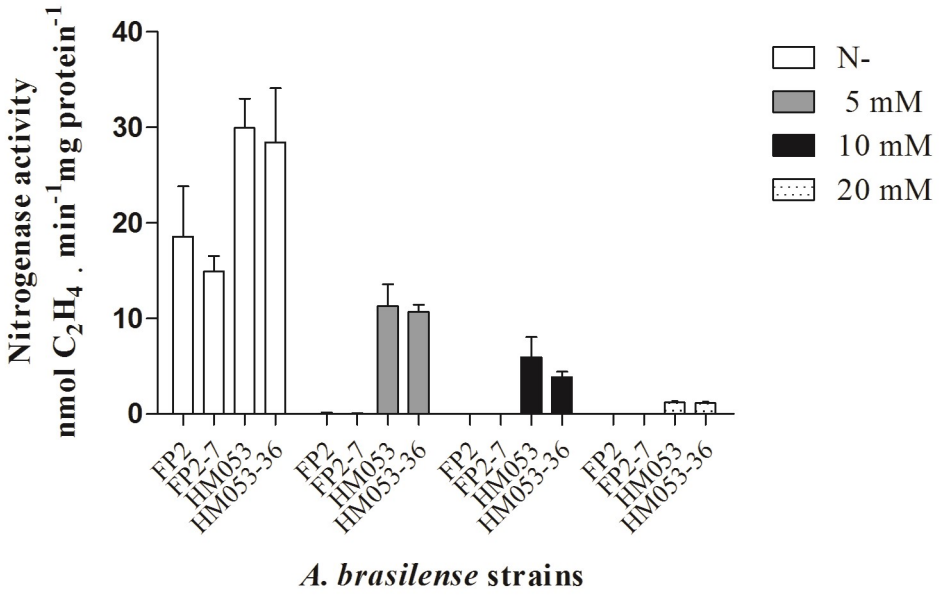
1S. Structural model of glutamine synthetase. A) Overall structure of *Salmonella enteric* serovar Typhimurium glutamine synthetase with bound AMP and Mn^{2+} ions (PDB 1LGR). B) Detailed interactions between glutamine synthetase proline residue at position 346 and AMP (black sticks) and Mn^{2+} (black balls). Polar contacts are shown as black dashed lines and the respectives distances are indicated in Å. The structure was generated using Pymol.

Table 1S. Ammonium excretion by *A. brasilense* mutant HM053 and HM053-36 strains (derivate strain from HM053 that contain *nifH-gusA* fusion) grown in nitrogen-free semisolid NFbHP medium at 30°C for 96 hours. NH_4^+ excreted into the medium was determined by the indophenols procedureas in Chaney and Marbach (1961). The concentration of protein was determined by the Bradford (1976) procedure.

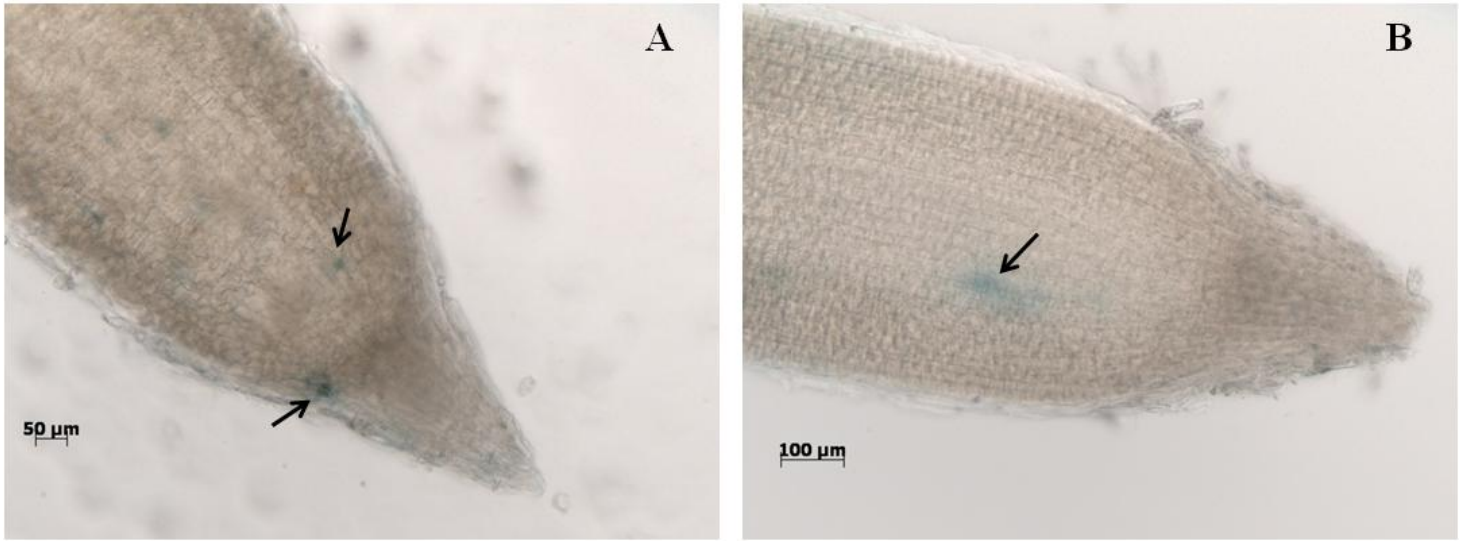
Strain	24 hours		48 hours		72 hours		96 hours	
	NH_4^+ (mM)	**Protein (mg.mL^{-1})**	**NH_4^+ (mM)**	**Protein (mg.mL^{-1})**	**NH_4^+ (mM)**	**Protein (mg.mL^{-1})**	**NH_4^+ (mM)**	**Protein (mg.mL^{-1})**
FP2	ND	0.01	ND	0.03	ND	0.06	ND	0.10
FP2-7	ND	0.01	ND	0.04	ND	0.08	ND	0.13
HM053	0.26 ± 0.08	0.01	0.68 ± 0.03	0.02	2.01 ± 0.41	0.03	3.27 ± 0.68	0.06
HM053-36	0.28 ± 0.01	0.01	0.66 ± 0.19	0.01	1.25 ± 0.27	0.02	2.18 ± 0.76	0.04

ND = not detected

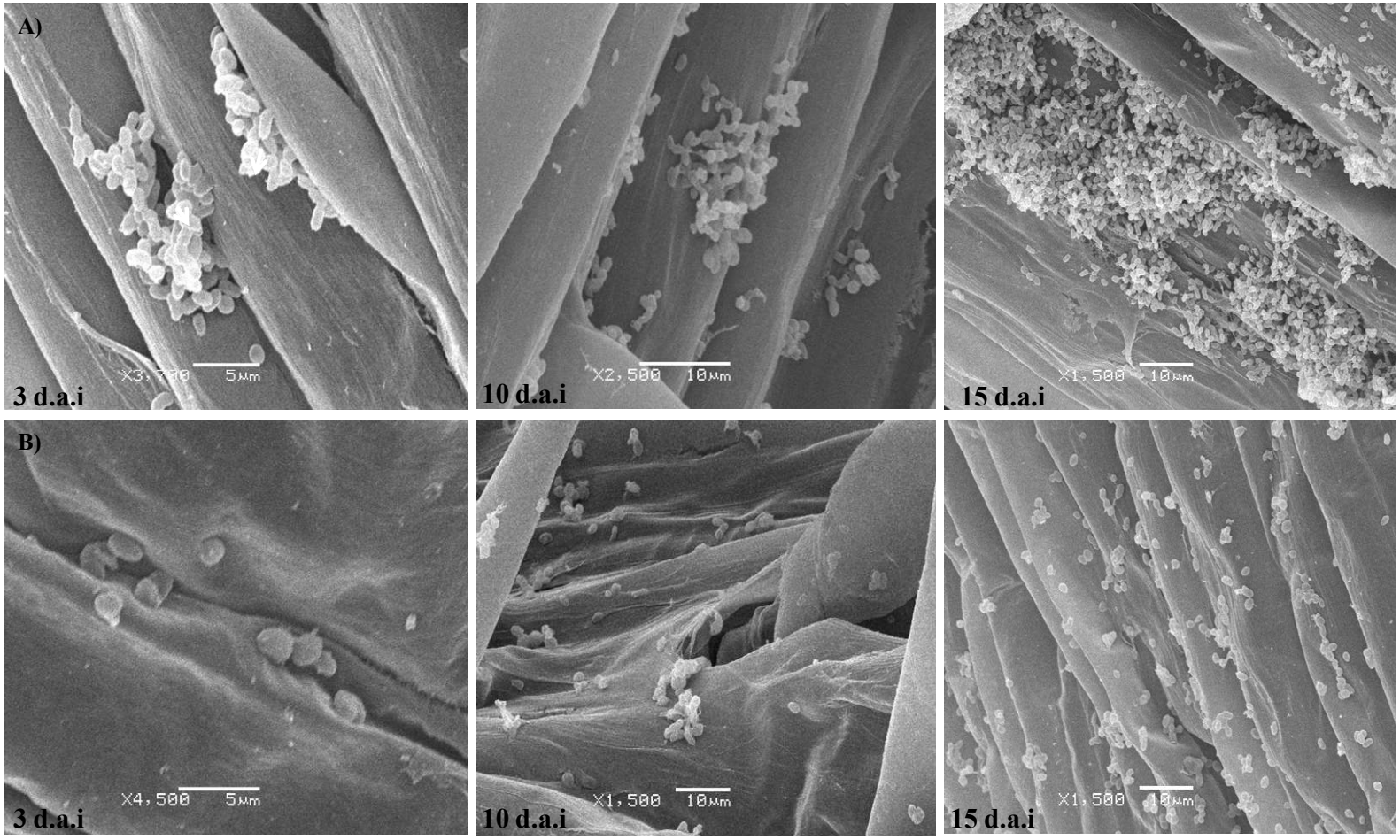
Test-t or Wilcox test $p \leq 0.05$ was used to compare statistical difference between HM053 and HM053-36



2S. Nitrogenase activity of *A. brasilense* strains: wild-type FP2, mutant HM053, derivate strains FP2-7 and HM053-36 that contain *nifH-gusA* fusion. Cells were grown at 30°C for 20h in semisolid NFbHP medium containing NH_4Cl at three indicated concentrations (5 mM to 20 mM) or for around 24h in semisolid NFbHP medium containing 5 mM glutamate (N-). The data shows the average of at least three independent determinations.



3S. Light microscopy in wheat root cap 9 day after inoculation. A) Wheat inoculated with FP2-7 strain (from wild-type FP2); B) Wheat inoculated with HM053-36 strain (from mutant HM053). The arrows indicate cells of *A. brasilense* expressing *nifH* gene from a cromossomal *nifH-gusA* fusion. Seedlings were inoculated with *Azospirillum brasilense* (10^7cells per plant) and the GUS activity staining was analyzed by light microscopy.



4S. Scanning electron microscopy of *A. brasilense* on wheat root (*Triticum aestivum* var. CD 104) 3, 10 and 15 days after inoculation (d.a.i). Seedlings were inoculated with *Azospirillum brasilense* (10^7cells per plant). A) Epiphytic colonization of the wheat root by FP2 strain. B) Epiphytic colonization of the wheat root by HM053 strain.

The low level of root colonization by HM053 as seen in these pictures is not mentioned in this paper .

O segundo artigo da tese "*Robust biological nitrogen fixation in a model grass-bacterial association"* envolveu o projeto de tese das doutorandas Vânia Pankievicz (UFPR) e Fernanda Amaral (UFSC). A minha contribuição ao projeto destas alunas foi a realização do experimento com ^{13}NN, como descrito abaixo no item 4.3.8 até 4.3.13 de material e métodos.

5. ARTIGO 2: Robust biological nitrogen fixation in a model grass-bacterial association

Authors: Vânia C.S. Pankievicz[1], Fernanda P. Amaral[2], Karina F. D. N. Santos[1], Beverly Agtuca[3], Youwen Xu[5], Michael J. Schueller[5], Ana Carolina M. Arisi[2], Maria. B.R. Steffens[1], Emanuel M. de Souza[1], Fábio O. Pedrosa[1], Gary Stacey[4], Richard A. Ferrieri[5]

[1]Department of Biochemistry and Molecular Biology, Federal University of Paraná, 81531-980, Curitiba, Brazil.
[2]Department of Food Science and Technology, Federal University of Santa Catarina, 88034-001, Florianópolis, Brazil.
[3]Department of Environmental Biology, State University of New York College of Environmental Science and Forestry, Syracuse, NY 13210, USA;
[4]Divisions of Plant Science and Biochemistry, C.S. Bond Life Science Center, Universityof Missouri, Columbia, MO, 65211.
[5]Biological, Environmental & Climate Sciences Department, Brookhaven National Laboratory, Building 901, Upton, NY, 11973, USA.

Abstract

Nitrogen-fixing rhizobacteria can promote plant growth; however, it is controversial whether biological nitrogen fixation (BNF) contributes to growth promotion. The roots of *Setaria viridis,* a model C_4 grass, were effectively colonized by bacterial inoculants resulting in a significant enhancement of growth. ^{13}NN tracer studies provided direct evidence for tracer uptake by the host plant and incorporation into protein. Indeed, plants showed robust growth under nitrogen limiting conditions when inoculated with an ammonium excreting strain of *Azospirillum brasilense*. ^{11}C-labeling experiments showed that patterns in central carbon metabolism and resource allocation exhibited by nitrogen starved plants were largely reversed by bacterial inoculation, such that they resembled plants grown under nitrogen sufficient conditions. Adoption of *S. viridis* as a model should promote research into the mechanisms of associative nitrogen fixation with the ultimate goal of greater adoption of BNF for sustainable crop production.

One Sentence Summary: *Setaria* is an attractive model for associative nitrogen fixation.

5.1 MAIN TEXT

Nitrogen (N) is the major limiting nutrient that promotes growth of most plants. Acquisition and assimilation of N is second in importance only to photosynthesis for plant growth and development (*1*). For this reason common agricultural practices make use of chemical inputs including fertilizers to maintain high crop yields. Excessive use of fertilizers,

however, can have the adverse effects on the environment through extensive chemical runoff into the waterways (*2*). Legumes provide the advantage that, in symbiosis with soil rhizobial bacteria, they can obtain N through biological nitrogen fixation (BNF). However, most agricultural plants, especially grasses, lack this ability and, hence, there has been sustained interest in transferring BNF ability into grass crops, such as corn (*3*).

Plant growth promoting bacteria (PGPB) colonize roots and engage in associative symbiosis with various host plants, including bioenergy grass species (*4*). In most cases, the mechanism of plant growth promotion is unknown. In selected cases, plant growth promotion is attributed to antagonism toward phytopathogens (*5*) and/or the induction of plant resistance (6). Other PGPB may act mostly by phytostimulation (e.g., release of phytohormones (*7*)). Several nitrogen-fixing PGPB have been identified as endophytes of grass species, including *Azoarcus* spp. in Kallar grass and rice (*8*, *9*), *Herbaspirillum seropedicae* in sugarcane (*10*) and sorghum (*11*), and *Gluconacetobacter diazotrophicus* in sugarcane (*12*). Unlike rhizobia that form an intimate intracellular symbiosis with their legume hosts, PGPB do not induce the formation of observable plant structures. These associations are strictly defined by the lack of any evidence of intracellular infection (*10*, *13*).

Many PGPB are capable of BNF. However, the role of BNF in plant growth promotion has not been well documented. For example, most publications simply report the presence of nitrogen fixing PGPB or perhaps the *in planta* expression of bacterial nitrogenase protein or genes; for example, *Azoarcus* sp. in rice (*14*, *15*) and *Herbaspirillum* spp. in sugarcane and rice (*16*, *17*). Only a few rare studies have provided convincing data for fixation *in planta* and even fewer for incorporation of fixed nitrogen by the plant host. Notable positive examples include the interaction between *G. diazotrophicus* and sugarcane (*18*), *Azoarcus* sp. strain BH72 and Kallar grass (*19*), *Klebsiella* sp. and wheat (*20*) or *H. seropedicae* Z67 and rice (*21*,*22*) and *Azospirillum brasilense* in *Setaria italica* (*23*).

However, the levels of nitrogen fixation reported would provide little or no contribution to the overall nitrogen demand. In contrast, some field studies with wild grass species suggest that BNF can provide 30% or more of the plant nitrogen demand (*24*, *25*), attesting to the promise of this approach. What is clearly needed is a tractable experimental system that exhibits appreciable levels of associative nitrogen fixation in which more detailed, mechanistic studies can be conducted.

We screened over 30 genotypes of *S. viridis* for their response to inoculation with *H. seropedicae* and *A. brasilense*. Both of these diazotrophic bacteria have been utilized as commercial inoculants, mainly in Latin America. *H. seropedicae* is a diazotrophic and

endophytic bacterium, which belongs to the *Betaproteobacteria* class. In contrast, *A. brasilense* is a member of the *Alphaproteobacteria* and its colonization is confined to the plant root surface (*26-28*). We identified three genotypes (EstepME034, EstepE017 and A10.1) that responded strongly to inoculation with increased growth, biomass, seed production and root growth. A total of nine parameters of plant growth were measured: total root length, lateral root number, root fresh and dry weight, plant height, inflorescence length, seed number, shoot fresh weight, and dry weight (Table S1). Dual inoculation of *S. viridis* seedlings was used to reduce the total number of plants needed for screening, which was necessary since relatively few seeds were available for some of the genotypes. A detailed description of the responses of each genotype under conditions of no-nitrogen addition (i.e., no nitrogen addition to the nutrient solution) or when grown under low nitrogen (0.5 mM KNO_3) is provided in supplemental Fig. S1-S6.

The genotype that responded most strongly to inoculation was *S. viridis* A10.1 (Fig. 1A), the genotype used for genome sequencing by the U.S. Department of Energy Joint Genome Institute (http://www.jgi.doe.gov), as well as for the further development of genomic and genetic resources (*29*, *30*). Some parameters analyzed for the *S. viridis* A10.1, plant height, inflorescence length, root fresh and dry weight, did not show a significant response to inoculation when plants were grown in the absence of added nitrogen. However, the shoot fresh weight for inoculated plants increased 24.5%, shoot dry weight increased 82.2%, root length increased 28.6% and lateral root numbers increased 39.6% (Fig. 1B). Under such severe nitrogen limitation, the plants produced very few seeds. However, the inoculated plants showed a remarkably higher yield of seed production in comparison to the uninoculated control plants. To check if the growth promotion effect of bacterial inoculation was occurring in the early developmental stages, the total root length and number of lateral roots were measured in plants of 11 and 17 d.a.i. (days after inoculation). Interestingly, the total root length and the lateral root number showed a significant increase in the inoculated plants compared to uninoculated plants at 17 d.a.i., whereas no increase was observed at 11 d.a.i. (Fig. 1C).

Bacterial colonization of the plant internal tissue is well described in many plant species. For example, nitrogen fixing *Burkholderia*, *Acetobacter* and *Pseudomonas* were found to colonize internal plant tissues (*31*). *H. seropedicae* was also shown to be an endophyte of a variety of plant species (*32-35*). Inoculation of *S. viridis* plants with *H. seropedicae* strains expressing fluorescent proteins (i.e., RAM4 expressing DsRed and RAM10 expressing GFP) revealed colonization of the exterior and interior of the root,

including the vascular tissue (Fig. 2 & Fig. S7-S10). Likewise, inoculation with *A. brasilense* strain FP2-7, expressing a *nifH::gusA* gene, demonstrated the expression of nitrogenase but only on the root surface at the base and tip of lateral roots, and along the root elongation zone (Fig. S11).

Bacterial plating was used to follow the time course of colonization by the two bacteria, taking advantage of the ability to select for each strain using their unique antibiotic resistance patterns (Fig. S12). The *H. seropedicae* population reached 10^8 CFU/g of root at 1 d.a.i. with numbers consistently dropping to 10^6 CFU/g by 17 d.a.i. In contrast, colonization by *A. brasilense* was more stable remaining at ~10^6 CFU/g until 17 d.a.i. The mechanism of colonization of *H. seropedicae* is well described and initiates by crack-entry, usually at the site of lateral root emergence (*10*, *36*). At 3 and 4 d.a.i., *H. seropedicae* DsRed expressing cells were attached to the root hair cells (Fig. S7& S10) but by 7 and 8 d.a.i. bacteria were visible in the intercellular spaces of internal root tissues (Fig. S8, S9B & S10). We used the imaging software Axiovision to integrate the serial, confocal images to clearly document the presence of *H. seropedicae* cells in the interior of roots (Fig. 2). Measurements derived from these images showed endophytic colonization of *S. viridis* roots 9, 10 and 18μm below the root surface.

Expression activity of the *nifH::GUS* fusion in *A. brasilense* was examined in two parts of the roots, the upper zone, at 3 cm down to the hypocotyl zone, and the lower zone, at 10 cm down to the hypocotyl, at the lateral root emergence points and the maturation zone. The *nifH::GUS* staining was visible mostly in the lower part of the root system, including lateral root cracks (Fig. S11A), root tips (Fig. S11B) and in the elongation zone (Fig. S11C). A roughly similar localization of *A. brasilense* on wheat roots was reported (*37*). These authors suggested that the lower part of the root may provide a microaerobic environment conducive to *A. brasilense* colonization.

Demonstration of nitrogenase gene expression is not sufficient to conclude that BNF is occurring or, more importantly, that fixed nitrogen is being used by the plant. Hence, to address these issues, we measured the fixation and incorporation of ^{13}NN gas after either mock inoculation or inoculation with *A. brasilense* (FP2-7) and *H. seropedicae* (RAM4) (Fig. S13-S14). Radiographic images of inoculated plants revealed a measurable amount of radioactivity in the roots but it was not possible to quantify the levels using this approach (Fig. S14). We also noted a significant amount of radioactivity fixed in photosynthetically active tissues that were unavoidably captured beneath the stem flange (Fig. S15). Follow-up decay analysis (Fig. 3A-D) on those targeted tissues verified that the radioactivity isolated in

the aerial portions of the plant immediately after the tracer pulse only had a carbon-11 signature (Fig. 3A) attributable to fixation of the small amount of $^{11}CO_2$ in the pulse. A trace of carbon-11 is expected due to the method used to generate the ^{13}NN gas (*38*). Roots from the same inoculated plants, however, revealed a mixture of nitrogen-13 and carbon-11 isotopes when subjected to decay analysis immediately following the tracer pulse (Fig. 3B). Isotopic identification by decay analysis was based on a strong correlation between the experimentally derived half-lives and the published values. To verify that the nitrogen-13 signature was the result of bacterial N_2 fixation, roots from the uninoculated control plants were also analyzed, which revealed only a carbon-11 signature (Fig. 3D). Finally, to distinguish our work from prior studies that measured nitrogenase enzyme activity using the acetylene reduction assay, we applied decay analysis to the aerial portions of the plant 15 minutes after the pulse, during which the plant was subjected to high illumination (1500 μmol m^{-2} sec^{-1}) as a means to promote a strong water transpiration stream. By monitoring the radioactivity profile in the lower stem area, it was obvious that a measurable amount of radioactivity was transported from roots-to-shoots over this 15 minute time course (Fig.S15). Decay analyses performed on aerial tissues after 15 minutes (Fig. 3C) revealed a strong nitrogen-13 signature along with carbon-11 suggesting that some portion of the fixed ^{13}NN is transported upward within the host plant. Furthermore, protein extraction from source leaves followed by radio thin layer chromatography revealed that a portion of the transported nitrogen-13 tracer was metabolized into ribulose-1,5-bisphosphate carboxylase (Fig.S16). This provides further evidence that the plant is indeed incorporating N provided by BNF.

Similar experiments with the commensurate decay analyses (data not shown) were conducted using *S. viridis* (A10.1) plants inoculated with a mixture of the nitrogen-fixing defective mutant strains of *A. brasilense* (FP10) (*39*) and *H. seropedicae* (SmR54) (*40*), as well as after inoculation with *A. brasilense* strain HM053 (*41*). This latter strain fixes *in vitro* approximately 2 times more N than its parent strain *A. brasilense* FP2 and excretes ammonium into the medium(*41*, *42*). Results from these studies, as well as those using the reporting strains RAM4 and FP2-7, were tabulated together in Table 1 and presented as the fractional fixation of ^{13}NN and $^{11}CO_2$ tracers delivered to the soil column. These data were normalized for plant variability in dried tissue mass, as well as for the variability in the daily delivered tracer dose. No evidence was seen of ^{13}N incorporation into tissue of either uninoculated plants or plants inoculated with the nitrogen fixation defective mutant strains (FP10 and SmR54). In contrast, plants inoculated with the reporting wild-type FP2-7 and RAM4 strains fixed 747 ± 141 ppt N_2 on a dry root mass basis, which equates to a cumulative

daily fixation rate of 125 ± 36 nmoles N_2 and meets ~7% of the plant's daily nitrogen demands (*43*, *44*). However, 16-fold higher levels of ^{13}NN fixation (i.e., 12,231 ± 5922 ppt on a root dry mass) were seen in plants inoculated with the ammonium-excreting, *A. brasilense* mutant strain HM053. This level is sufficient to provide the plant's daily N demand, which was reflected by the healthy growth phenotype depicted in Fig. 1A. Taken together, these studies constitute the first hard evidence demonstrating incorporation of significant levels of fixed nitrogen into the host plant with biological transport of ^{13}N to aerial portions of the plant.

Carbon and nitrogen metabolism in the plant are tightly linked and, hence, we used $^{11}CO_2$ fixation and incorporation as a means to monitor the effects of nitrogen sufficiency or insufficiency. As shown in Table 1, the levels of ^{11}C incorporation in roots strongly correlated with the BNF levels. For example, plants inoculated with the ammonium-excreting strain, HM053, assimilated 14-times more $^{11}CO_2$ than plants inoculated with the wild-type strains, which, in turn, fixed 3-times more $^{11}CO_2$ than the mock inoculated plants or plants inoculated with BNF defective strains. This positive correlation between N_2 fixation and root CO_2 assimilation is similar to reports on pea root nodules using $^{14}CO_2$ (*45*), which was explained by enhanced activity of phosphoenolpyruvate carboxylase. This enhanced carbon fixation could also contribute to the growth promotion seen by PGPB inoculation.

Two additional studies were conducted in order to better understand the source of the root carbon-11 radioactivity. In one study, we subjected plants to darkness and then detached the aboveground tissues (sealing the base of the stem with lanolin) just prior to administering a pulse of tracer to the soil column and remaining roots. Decay analysis of the root system immediately after the pulse revealed strong ^{13}N and ^{11}C signatures (Table 1). We also compared the effect of the light and dark cycles on the isotopic composition fixed within the root systems of plants inoculated with *H. seropedicae* (RAM4) and *A. brasilense* (FP2-7) and grown with low nitrate, as well as within roots of uninoculated plants grown with high nitrate. Both $^{13}N_2$ and $^{11}CO_2$ fixation was apparent in detached roots. Furthermore, ^{13}NN fixation was independent of diurnal effects, which is similar to what is observed in legumes (*46*). On the other hand, $^{11}CO_2$ fixation was positively correlated with the diurnal cycle being higher during the light period. So while $^{11}CO_2$ fixation is clearly regulated by the N_2-fixing capacity of the bacteria, it appears that the host can also exert some fine control of this process.

Though $^{11}CO_2$ fixation was reduced for uninoculated plants grown under nitrate limitation, leaf export of ^{11}C-photoassimilates (Fig. 4B) were significantly higher in these plants (P=0.006), relative to control plants grown under normal nitrate conditions. Inoculated

plants, however, showed a significant reduction in ^{11}C-photoassimilate export (P=0.034), relative to uninoculated controls grown under the same nitrate level, indicating a shift in resource transport dynamics back to a normal unstressed state.

Radiographic images (Fig. S17A), reflecting the bio-distribution of those ^{11}C-photoassimilates exported from the load leaf, showed a trend of increased radiotracer distribution belowground for control plants grown under nitrate limitation.This trend was quantitatively verified using "cut-and-count" techniques to measure the amount of radioactivity in the different tissues (Fig. S17B), and showed that plants grown under nitrate limitation exhibited a significant increase in belowground allocation (P=0.054) relative to normal control plants. This trend was again reversed (P=0.041) when plants were inoculated with bacteria, demonstrating once again that the presence of the bacteria re-instated "normal" plant physiological responses. Furthermore, we observed a significant reduction in ^{11}C-photoassimilate exudation (Fig. S17C) from control roots under nitrogen limitation, as well as from bacteria association relative to normal nitrogen roots (P=0.014). Although not significant *(*P=0.279), there was a systematic increase observed in total root mass (gfw) of uninoculated control plants grown under nitrate limitation (Fig. S17D), as compared with control plants grown under normal nitrate conditions. The slight increase in control plant root mass under nitrate limitation was observed to be due to increased primary root length and not due to increased branch root patterning. However, this trend of increased root mass was reversed significantly in inoculated plants (P=0.041). Although total root mass was lower under these conditions, a slight increase in branch root patterning was observed.

Metabolic fluxes of new carbon, reflected as ^{11}C, were traced through and into the various pools of soluble and insoluble (i.e., storage) substrates (Fig. 5) of load leaf tissue 1 hr after exposure to $^{11}CO_2$. Results showed no significant change in the insoluble ^{11}C-fractions measured across the three growth conditions (control, 5 mM nitrate; control, 0.5 mM nitrate; bacteria inoculated, 0.5mM nitrate). However, nitrate limitation of uninoculated plants resulted in a decrease (from 40.5 ± 5.0% total ^{11}C-activity to 30.0 ± 1.9%) in the soluble ^{11}C-sugar pool relative to normal controls that was significant (P=0.052). This response was compensated for by an increase in the ^{11}C-amino acid pool (from 7.2 ± 1.9% to 17.3 ± 4.3%) that was marginally significant (P=0.066). Results further demonstrated that bacteria inoculated plants grown under nitrate limitation exhibited similar metabolic behavior as control plants grown under normal nitrate conditions. Specifically, an increase in the ^{11}C-soluble sugar pool (from 30.0 ± 1.9% to 37.1 ± 3.8%) was observed relative to nitrate limited control plants, though this change was only marginally significant (P=0.082). This trend was

also compensated for by a reduction in the ^{11}C-amino acid pool (from 17.3 ± 4.3% to 11.4 ± 1.5%), though the change was not considered significant (P=0.148).Taken together, these observed changes in new carbon partitioning into total soluble ^{11}C-sugar and ^{11}C-amino acid pools was seen as a re-instatement of "normal" plant metabolic behavior under nitrate limitation that was invoked by bacterial inoculation.These data present perhaps the strongest argument that nitrogen fixed by the bacteria is incorporated by the plant and directly affect overall plant metabolism.

There was, however, one subtle difference in that the remaining ^{11}C-soluble fraction (i.e., the fraction of soluble radioactivity that was not accounted for as sugars and amino acids) was reduced to zero in the presence of bacteria. This fraction may likely contain some lipid compounds that were solubilized during the methanol:water extraction process, however, further testing is needed.

The data presented above presents a detailed analysis of *S. viridis* plants following both the levels of colonization, nitrogen fixation, plant nitrogen incorporation and direct effects on overall plant metabolism. These results clearly indicate that, under suitable conditions, *S. viridis* can obtain sufficient nitrogen via BNF to promote robust plant growth. Although the study of diverse biological systems can sometimes be very informative, greater and more in depth discovery occurs when research is focused on a suitable model system. For example, the adoption of the model legumes *Medicago truncatula* and *Lotus japonicus* resulted in rapid advances in our understanding of the rhizobium-legume nitrogen fixing symbiosis (e.g., (*47-50*)). In a similar manner, we propose that the adoption of a suitable plant model to investigate the mechanisms of associative nitrogen fixation and its effects on plant growth promotion would accelerate our understanding of this complex system. The ultimate goal would be to transfer the knowledge gained to improve the productivity of valuable crop species (e.g., corn). This is an important goal given the challenges faced by agricultural due to changing climatic conditions and a rapidly growing human population. The demonstration of robust BNF activity in *S. viridis* under controllable laboratory conditions shows that this experimentally tractable, grass species holds significant promise as a model for the study of associative nitrogen fixation and plant growth promotion.

Table 1 - Root isotope analysis

Conditions	Cycle	Root ^{13}NN Fractional Fixation (per mg dry wt.)	Root $^{11}CO_2$ Fractional Fixation (per mg dry wt.)
Inoculated[a.] (RAM4 + FP2-7)	Light	580.8 ppt	0.0166
Inoculated[a.] (RAM4 + FP2-7)	Light	838.4 ppt	0.0326
Inoculated[a.] (RAM4 + FP2-7)	Light	818.2 ppt	0.0309
Mean ± SD	**Light**	**747.5 ± 141.4 ppt[b.]**	**0.0267 ± 0.0088[c.]**
Inoculated[a.] (RAM4 + FP2-7)	Dark	747.5 ppt	0.0127
Inoculated[a.] (RAM4 + FP2-7)	Dark	621.2 ppt	0.0204
Inoculated[a.] (RAM4 + FP2-7)	Dark	676.8 ppt	0.0171
Mean ± SD	**Dark**	**681.8 ± 65.7 ppt[b.]**	**0.0167 ± 0.0039[c.]**
Control (uninoculated)	Light	0.0 ppt	0.0072
Control (uninoculated)	Light	0.0 ppt	0.0116
Control (uninoculated)	Light	0.0 ppt	0.0101
Mean ± SD	**Light**	**0.0 ppt**	**0.009.7± 0.0022[d.]**
Control (uninoculated)	Dark	0.0 ppt	0.0068
Control (uninoculated)	Dark	0.0 ppt	0.0054
Mean ± SD	**Dark**	**0.0 ppt**	**0.0061± 0.0010[d.]**
Inoculated (RAM4 + FP2-7)	Detached Root[e.]	419.2 ppt	0.0038
Inoculated (HM053)[f.]	Light	7,470.9 ppt	0.2666
Inoculated (HM053)[f.]	Light	10,524.7 ppt	0.1284
Inoculated (HM053)[f.]	Light	20,883.5 ppt	0.5524
Inoculated (HM053)[f.]	Light	10,043.5 ppt	0.5689
Mean ± SD	**Light**	**12,230.7 ± 5922.3 ppt**	**0.3791 ± 0.2172**
Inoculated[g.] (SmR54 + FP10)	Light	0.0 ppt	0.0010
Inoculated[g.] (SmR54 + FP10)	Light	0.0 ppt	0.0038

Table 1 - Root isotope analysis

Conditions	Cycle	Root ^{13}NN Fractional Fixation (per mg dry wt.)	Root $^{11}CO_2$ Fractional Fixation (per mg dry wt.)
Inoculated[g.] (SmR54 + FP10)	Light	0.0 ppt	0.0023
Inoculated[g.] (SmR54 + FP10)	Light	0.0 ppt	0.0043
Mean ± SD	**Light**	**0.0 ppt**	**0.0026 ± 0.0019**

p.p.t., parts per trillion

a. Plants were inoculated with reporting wild-type strains of *H. seropedicae* (RAM4) and *A. brasilense* (FP2-7).
b. No difference was seen in the light/dark N_2 fixation for both bacterial strains. Based on the ^{13}N data we calculate a cumulative N_2 fixation rate of 125 nmoles ± 36 N_2 fixed/day which equates to 7% of the plants daily nitrogen demands.
c. $^{11}CO_2$ fixation in the roots of plants inoculated with reporting wild-type strains of *H. seropedicae* (RAM4) and *A. brasilense* (FP2-7) showed significantly higher (P = 0.0073) fixation amounts during the light cycle than during the dark cycle. Plants inoculated with reporting wild-type strains of *H. seropedicae* (RAM4) and *A. brasilense* (FP2-7) showed 2.8-times higher $^{11}CO_2$fixation levels than uninoculated control plants.
d. Similar levels of $^{11}CO_2$ fixation were seen in the uninoculated plants as that in plants inoculated with strains of *H. seropedicae* (RAM4) and *A. brasilense* (FP2-7).
e. The plant was maintained in darkness in the time prior to removing the shoot for this detached root study.
f. Plants were inoculated with the super N_2-fixing mutant strain of *A. brasilense* (HM053). On average this strain was fixing ^{13}NN at 16-times higher than the reporting wild-type strain. Fractional $^{11}CO_2$ fixation was also 14-times higher than the reporting wild-type strains.
g. Plants were inoculated with *nifA* minus strains of *H. seropedicae* (SmR54) and *A. brasilense* (FP10).

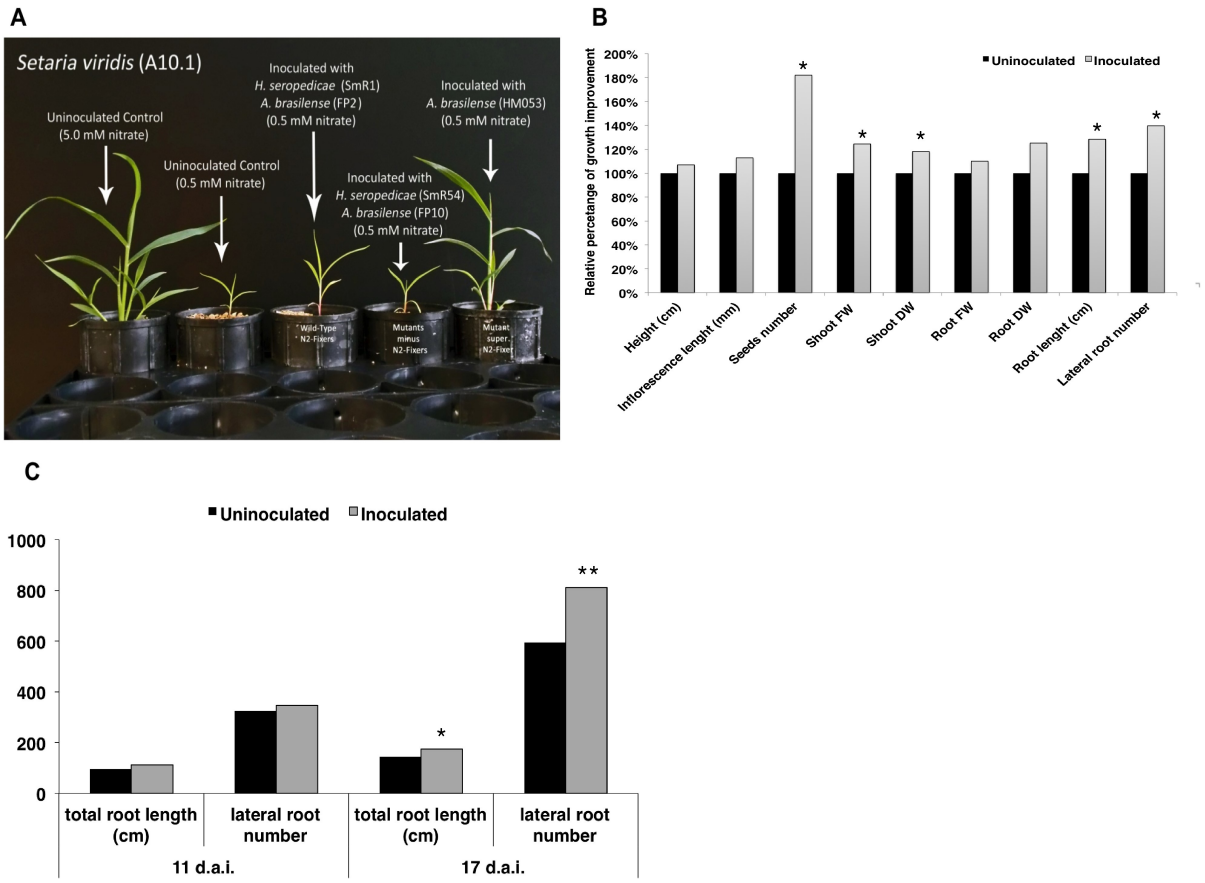


Figure 1 - Representative pictures of *S. viridis* grown under different nitrogen regimes with or without bacterial inoculation. SmR1 and FP2 are wild-type strains of *H. seropedicae* and *A. brasilense*, respectively. SmR54 and FP10 are mutants of the same bacteria that are unable to fix nitrogen. Strain HM053 is an ammonium-excreting mutant strain of *A. brasilense*. We measured a 14-fold higher level of fixation on a dry root mass basis (determined by $^{13}N_2$ incorporation) with this strain providing up to 100% of the nitrogen needs of the plant. B**:** Relative percentage of growth improvement by bacterial inoculation for the *S. viridis* A10.1 genotype grown without added nitrogen. In comparison to uninoculated plants, plants inoculated with a mixture of *A. brasilense* and *H. seropedicae* showed significant growth promotion in seed number, shoot fresh weight (shoot FW), shoot dry weight (shoot DW), total root length, and total lateral root number at 40 days after inoculation. Each asterisk denotes a statistically significant difference with p-value ≤ 0.05. C: Growth promotion effects by bacterial inoculation on early stages of root growth of the *S. viridis* A10.1 genotype. Plants were inoculated with a mixture containing equal CFUs of *A. brasilense* and *H. seropedicae* (total CFU 1 x 10^8 per plant). Inoculated plants showed a total root length increased and higher lateral root numbers at 17 days after inoculation, but not at 11 days after inoculation. * p-value ≤ 0.05, ** p-value ≤ 0.01.

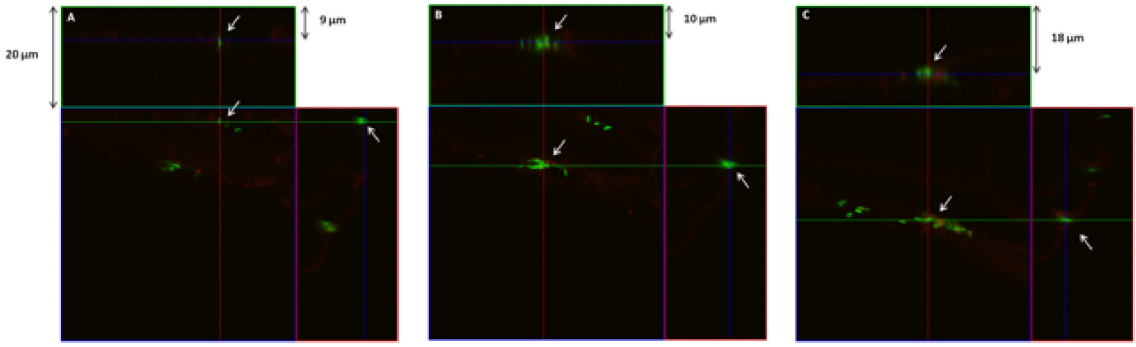


Figure 2 - Orthogonal optical sections from cortical cells of *S. viridis* colonized by *H. seropedicae.* The *H. seropedicae* cells expressing GFP (green) in the red background of the root stained with propidium iodide. The central views framed in blue show x–y focal plans from the z-stacks. The red and green lines represent vertical optical slices through the z-stacks, which produce the side images outlined in red and green, respectively. In these side views, the blue lines mark the position where the central view images are located within the z-stacks. The bacteria are located at different depths in the root tissue indicating the endophytic colonization.

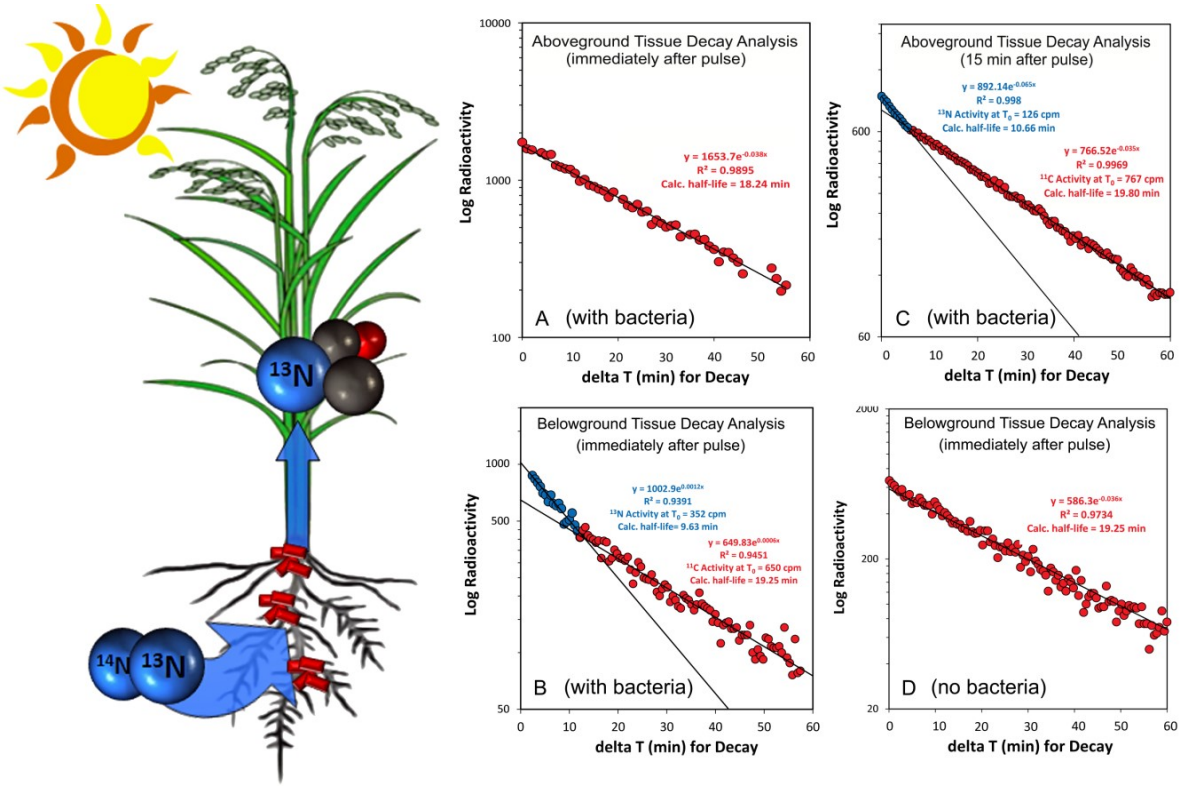


Figure 3 - ^{13}NN fixation and uptake by plants was determined using radioactive decay analysis on different tissues. Comparative decay analyses of aboveground (a, c) and belowground tissues (b, d) immediately after the ^{13}N-nitrogen pulse and 15 min after the pulse during high illumination. In this treatment, a logarithmic plot of the radioactivity level against time will yield a straight line for single isotope decay, and two straight lines for a mixture of two isotopes with differing decay rates. In the upper plots, the ^{13}N signature is depicted by the blue data points and the ^{11}C signature by the red data points. Inoculated plants always had a measureable amount of ^{13}N in the roots immediately after the pulse, but the aerial portions only showed an ^{11}C signature. After 15 min and high illumination, we were able to demonstrate that a portion of the root ^{13}N signature is transported to the aerial portions of the plant.

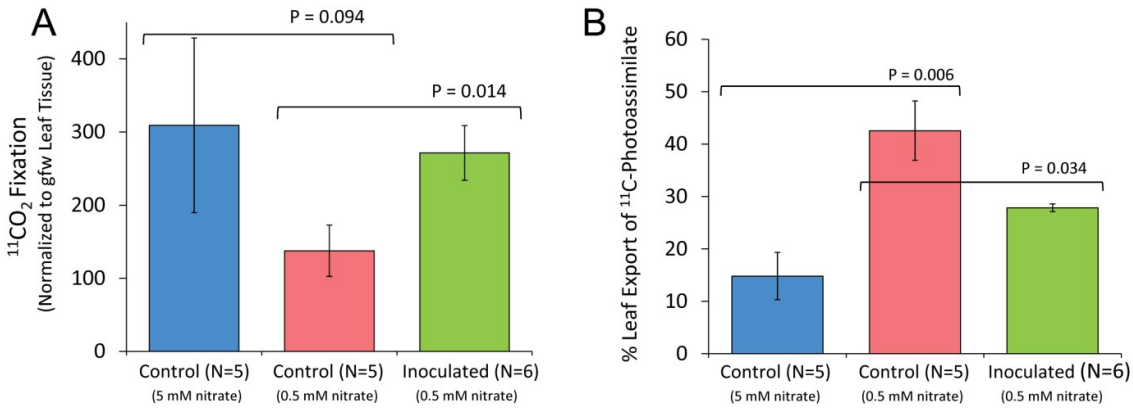


Figure 4 - Whole-plant physiology parameters measured as a function of three growth regimes including: uninoculated control plants (5.0 mM nitrate), uninoculated control plants (0.5 mM nitrate) and inoculated plants (0.5 mM nitrate). (a) $^{11}CO_2$ fixation normalized to gram fresh weight (gfw) of leaf tissue exposed to tracer during administration. (b) Percentage of fixed ^{11}C exported as photoassimilates by the load leaf over a 1-h period. All bars are mean values ± SE. Statistical significance across the growth regimes is shown by the P-values.

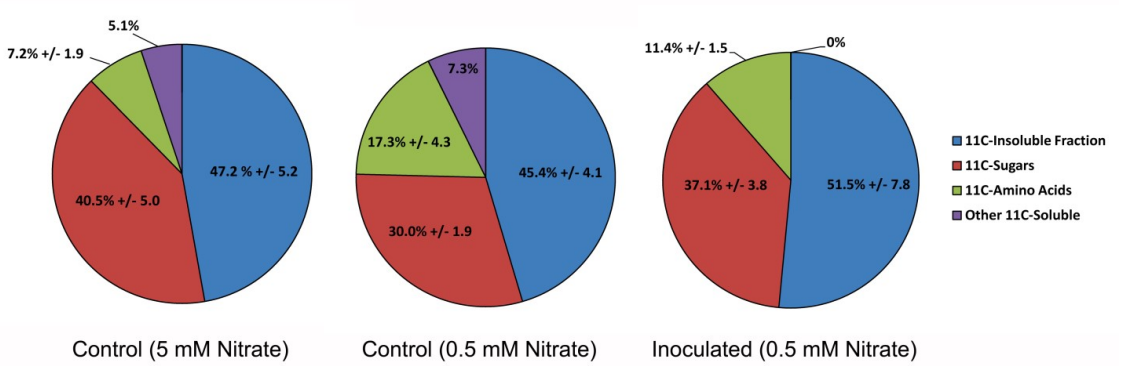


Figure 5 - Metabolic partitioning of plant new carbon (as ^{11}C) into soluble and insoluble fractions of load leaf extracts 1 h after tracer administration. The soluble component was further subdivided into sugars, amino acids and an undefined fraction. Data are presented as a function of three growth regimes including: un- inoculated control plants (5.0 mM nitrate), uninoculated control plants (0.5 mM nitrate) and inoculated plants (0.5 mM nitrate) and reflects mean values ± SE.

5.2 MATERIALS AND METHODS:

5.2.1 Bacteria growth conditions

H. seropedicae strain RAM4 (Nif+, Sm^R, Km^R, *dsRed*) expressing the DsRed fluorescent protein (*51*) and *H. seropedicae* RAM10 (Nif+, Sm^R, Km^R, *gfp*; Monteiro, R.A., unpublished) expressing GFP were grown in liquid NFbHP-malate medium (*52*) and *A.*

brasilense strain FP2-7 (Nif+, Nal^R, Sm^R, Tc^R, Km^R) carrying a *nifH::gusA* was grown in liquid NFbHP-lactate medium (*53*). NH_4Cl (20 mM) was added to both media, which are here after called NFbHPN-malate or NFbHPN-lactate. These strains have nitrogenase activity and colonization pattern identical to the wild type strains (*51*). *A. brasilense* HM053 (Nif^C, Nal^R, Sm^R) was grown in liquid NFbHP-lactate medium with added NH_4Cl (20 mM). This strain is a spontaneous mutant of *A. brasilense* and fixes nitrogen constitutively in the presence of high ammonium concentrations and excretes NH_4^+derived from nitrogen fixation (*53*). *H. seropedicae* SmR54 (Nif^- *nifA*::Tn5-B21, Sm^R, Km^R) and *A. brasilense* FP10 (Nif^-, *nifA*$^-$, Sm^R) are Nif minus strains. The bacterial cultures were grown at 30°C with shaking at 130 rotations per min (rpm). Streptomycin at a final concentration of 80 μg/mL was added to cultures of both bacterial strains. Kanamycin at a final concentration of 200 μg/mL was added to *H. seropedicae* cultures. Tetracycline and kanamycin were added to *A. brasilense* FP2-7 cultures at a final concentration of 10 μg/mL and 50 μg/mL, respectively.

5.2.2 *S. viridis* seed sterilization and germination

Seeds of various accessions of *S. viridis* were obtained through the generosity of Drs. Thomas Brutnell (Donald Danforth Plant Science Center, St. Louis, MO), KatrynDevos (University of Georgia, Athens, GA), and Elizabeth Kellogg (University of Missouri-St. Louis, St. Louis, MO). The seeds were first sterilized with 6% (v/v) bleach plus 0.1% (v/v) Tween 20 for 3 min and rinsed five times with sterile distillated water. The sterile seeds were plated with the embryos facing upwards onto medium containing modified 1/10 strength Hoagland's nutrient solution [54] and phytagel 1% (w/v) in square plates. For germination, the plates were placed in the dark for two to three days and one day in the light at 30°C.

For carbon–11 experiments the sterilized seeds were planted in 100 mL glass test tubes (Fisher Scientific, Inc., Pittsburg, PA, USA) filled with Hoagland's fortified agar gels adjusted to pH 5.9. For the uninoculated control studies, the nitrate level was adjusted to either 5.0 mM potassium nitrate (here called normal nitrate level) or 0.5mM potassium nitrate (limiting nitrate level). For the bacterial inoculated studies, the nitrate level in the gel was adjusted to 0.5mM potassium nitrate.

The Hoagland's solutions were prepared from the individual salts in order to control the nitrate concentrations listed above. Gel solutions were prepared using 2 L of distilled water, 1 mL of 1 M potassium phosphate monobasic, 1 mL of 1 M potassium dibasic, 1 mL of

1 M micronutrient solution, 1 mL of 0.028 M ferric ethylenediaminetetraacetic acid, 2 mL of 1 M calcium chloride, 2 mL of 1 M magnesium sulfate, potassium nitrate (adjusted to obtain either a 5.0mM or 0.5mM nitrate concentration), 1.1 g MES hydrate, and 5.6 g Gelzan CM. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO USA). Solutions were autoclaved (Harvey SterileMax, Thermo Fisher Scientific, Inc., Pittsburg, PA, USA) for 15 min at 121°C and mixed at high speed to enable aeration of the viscous solution before it had a chance to set as a gel. All growth tubes were placed and cultivated in a commercial growth chamber (Percival, Inc) at 23°C using 120 µmol m^{-2} s^{-1} of light intensity on a 12 h photoperiod. These three-week-old plants had a height of about 5 cm and were used for experiments.

5.2.3 *S. viridis* seedling inoculation and growth

The *S. viridis* seedlings were inoculated with both *H. seropedicae* RAM4 and *A. brasilense* FP2-7. The cultures were grown as described above. When the bacterial cultures reached $O.D._{600nm}$ = 1.0 (10^8 cells/mL), the bacteria were washed 3 times with Hoagland's nutrient solution. Equal amounts of *H. seropedicae* and *A. brasilense* cultures were mixed and used as inoculum at a final $O.D._{600nm}$ = 1.0. The seedlings were inoculated with 1 mL of inoculum per plantlet for 30 min. This bacterial suspension was counted through serial dilutions to confirm the bacterial numbers inoculated. After inoculation, the seedlings were transferred to pots containing a mixture of sterilized turface (Turface MVP®) and vermiculite in a proportion of 3:1, respectively. This mixture was arrived at through trial and error since it allowed robust plant growth but also provided for easy access to the roots without a lot of adhering soil. The plants were growth in the green house at 30°C with a 16-h light/8-h dark cycle. The greenhouse plants were watered twice a week with one-tenth strength Hoagland's solution. The plants were supplemented with potassium nitrate depending upon the treatment.

5.2.4 Plant growth promotion assay

To assess the phytostimulatory effect of *H. seropedicae* and *A. brasilense* in different *S. viridis* genotypes, the seedlings were inoculated as described above and the control plants were inoculated with boiled cultures (uninoculated), which contained the same inoculum boiled for 15 min at 100°C. This control strategy was used to further control for any possible

nitrogen carryover from the inoculant. The plants were harvested after 40 days of inoculation. Several growth parameters were measured: plant height, inflorescence length, number of seeds, shoot fresh and dry weight and root fresh and dry weight. The root length and number of lateral roots were measured using the WinRHIZO pro software (Régent Instrument Inc., Québec City, Canada).

5.2.5 Bacterial quantification

To evaluate the ability of the bacteria to colonize the selected *S. viridis* genotypes, the rootsand leaves were sampled in selected days after inoculation (d.a.i.). To count the total number of *H. seropedicae* and *A. brasilense* colonizing the rhizoplane, the roots were washed once and macerated with 1 mL of 0.9% (w/v) NaCl saline solution. In order to gauge the level of endophytic colonization by *H. seropedicae* counting, the roots were surface-sterilized with 70% (v/v) ethanol for 40 sec, followed by 1% (w/v) chloramine-T for 40 sec and washed four times in sterile distilled water, before maceration in 0.9% (w/v) NaCl saline solution. Total root homogenates were serially diluted and plated onto the appropriate selection medium for each bacterium. For *H. seropedicae* selection, the NFbHPN-malate medium containing 80 µg/mL streptomycin and 200 µg/mL kanamycin was used. *A. brasilense* selection used the NFbHPN-lactate medium supplemented with 80 µg/mL streptomycin, 100 µg/mL kanamycin and 10 µg/mL tetracycline. Colony forming units (CFU) were counted after three days of incubation at 30°C and converted to CFUs per gram of fresh tissue. To ensure that the colonies were from the inoculated bacteria, the colonies were observed under fluorescent stereoscope for *H. seropedicae* or were visualized by staining with 30 mg/mL of X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) for *A. brasilense* (55).

5.2.6 Analyses of bacterial root colonization by light, fluorescence and laser scanning microscopy

H. seropedicae strain RAM4, which constitutively expresses the DsRed protein that codes for a red fluorescent protein (*51*), and strain RAM10, which constitutively expresses the green fluorescent protein (GFP), were used to investigate bacterial colonization of the roots of the various *S. viridis* genotypes. Roots from two to four plants were sampled and analyzed through two different root parts: upper part (zone of differentiation) and lower part (zone of elongation). Dissected root fragments from control and inoculated plants were placed on a

slide in a drop of water, and then covered with a glass coverslip to observe under a fluorescence microscope (Olympus IX70 inverted microscope) using the ET-DSRed 590-650 nm bandpass filter. For confocal microscopy, the roots were prepared as described above and observed using a Zeiss LSM 510 META laser scanning confocal microscope equipped with 488 nm argon and 543 nm He–Ne lasers to detect green fluorescence emitted by a GFP-tagged *H. seropedicae* (excitation at 488 nm and detection at 500-550 nm) and red fluorescence emitted by *dsRed*-tagged RAM4 strain (excitation at 543 nm and detection at 488-633 nm). For better localization of the bacteria in the internal root tissue, the plant roots were incubated with 2 μM of propidium iodide (PI) for 5-7 min and washed five times with distilled water. PI staining was visualized with the argon laser (excitation at 488 nm, detection at 565–615 nm). When PI staining was performed, images of red PI fluorescence were overlaid with the images of the GFP-tagged bacteria and with brightfield transmitted light images of the root parts. The red fluorescence images from DsRed-tagged bacteria were overlaid with the transmitted images (brightfield mode) of the root parts. All composite images were produced using the LSM Image Browser 4.0 software (Carl Zeiss Microimaging). Additional images were performed in a Nikon Eclipse Ti inverted laser scanning confocal microscope equipped with ion laser to detect green fluorescence emitted by a GFP-tagged strain, RAM10 (excitation 488 nm and detection at 525-550 nm) and blue auto-fluorescence emitted by the plant root (excitation 405,8 and detection at 450-550 nm). The orthogonal section images and the 3D view images were obtained using the NIS-element v.4.1 software.

5.2.8 ^{13}NN production

^{13}NN was produced *via* the ^{14}N (p,pn)^{13}N reaction using 18 MeV protons from the BNL Ebco TR-19 Cyclotron (Ebco Industries Ltd, Richmond, BC, Canada). These protons were focused onto the front metal foil of a 50 mL volume gas target leaving 15.5 MeV protons to interact with the gas inside. This target was typically pressurized to 400 psi with 99.9% N_2 + 0.1% O_2. Nitrogen-13 manifests as a by-product during the proton irradiation of this target system which is used at BNL generate $^{11}CO_2$ from the ^{14}N(p, α)^{11}C reaction (*56*). Typically, a 125 μA·min irradiation (25 μA on target for 5 min) would produce 12.95 GBq of $^{11}CO_2$, 4.14 GBq of [^{14}O/^{15}O]O_2 and 1.37 GBq of ^{13}NN at the end-of-bombardment. This radioactive gas was immediately processed through a stripping trap (*57*); (Fig. S13) which was comprised of molecular sieve 4 Å (100 mesh; Alltech, Inc., Deerfield, IL, USA). At room

temperature this trap removed the majority of the $^{11}CO_2$ component and enabled the remaining components to equilibrate at STP in a 2 L volume tube that was located downstream and inside of a CRC-12 Dose Calibrator (Capintec, Inc, Ramsey, NJ, USA) for direct radiation measurement. At this point, the $^{11}CO_2$ component was reduced substantially to only 148 Bq. Composition of trapped gas was tested using radio gas chromatography analysis (Hewlett Packard 5890 Series; Agilent Technologies, Santa Clara, CA, USA) where components were separated using a spherocarb (100 mesh) packed column (10 ft. x 0.125 in. o.d.; Alltech Associates, Inc., Deerfield, IL, USA).

5.2.9 $^{11}CO_2$ production and administration

$^{11}CO_2$ was produced *via* the $^{14}N(p, \alpha)^{11}C$ nuclear transformation (*58*) from a 20 mL target filled with high-purity nitrogen gas (400 mL @ STP) using 18MeV protons from the TR-19 (Ebco Industries Ltd, Richmond, BC, Canada) cyclotron at BNL, and captured on a molecular sieve (4Å). The $^{11}CO_2$ that was trapped on the molecular sieve was desorbed and quickly released into an air stream at 200 mL/min as a discrete pulse for labeling a leaf affixed within a 5 x 10 cm lighted (320 μmol m^{-2} s^{-1}) leaf cell at 21°C to ensure a steady level of fixation. The load leaf affixed within the cell was pulse-fed $^{11}CO_2$ for 1 minute, then chased with normal air for the duration of exposure. A PIN diode radiation detector (PIN refers to an intrinsic semiconductor layer sandwiched between p-type and n-type semiconductor layers: Carroll Ramsey Associates, Inc, Berkeley, CA, USA) affixed to the bottom of the leaf cell enabled continuous measurement of radioactivity levels within the cell during the initial pulse and in the minutes right after the pulse giving information on $^{11}CO_2$ fixation.

5.2.10 Plant growth for ^{13}NN studies

The A10.1 genotype of *S. viridis* was selected for further testing of nitrogen uptake using our radiotracer technology because it exhibited the strongest phenotype of the thirty genotypes tested with *Herbaspirillum seropedicae* and *Azospirillum brasilense* growth promoting bacteria. Seeds were surface sterilized and either inoculated with the bacteria mix, as previous described, or left uninoculated. Seeds were germinated in Petri dishes filled with Hoagland's fortified agar gel. Agar gels were prepared out of 3 L of de-ionized water, 4.9 g

Hoagland modified basal salt mixture (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) and 1.66 g MES hydrate. The pH of the solution was adjusted to 5.9 by adding 1N potassium hydroxide solution. While stirring, 8.4 g Gelzan CM (Sigma-Aldrich Corp. St. Louis, MO USA) were added. The solution was autoclaved (Harvey SterileMax, Thermo Fisher Scientific, Inc., Pittsburg, PA, USA) for 15 min at 121°C and mixed at high speed to enable aeration of the viscous solution before it had a chance to set as a gel. After germination, seedlings were transplanted to pots (10 in. x 2.5 in. i.d. tapered cylindrical pot; Stuewe& Son, Inc., Tangent, OR, USA) filled with a 3:1 mix of Turface:Vermiculite (Turface was purchased from Profile Products LLC, Buffalo Grove, IL, USA; Vermiculite-A4 course grain was purchased from Whittemore Company, Inc., Lawrence, MA, USA). Plants were grown in Conviron growth chambers (Conviron, Inc., Winnipeg, Manitoba, Canada) set to a 12 h photoperiod at 600 μmol m^{-2} s^{-1} and 22°C. Plants were watered with Hoagland's nutrient solution adjusted for nitrate levels at either 5mM (for uninoculated control plants) or 0.5mM (for the uninoculated control plants and the bacteria inoculated plants).

5.2.11 ^{13}NN tracer administration

Prior to a ^{13}NN experiment a planting pot containing a single study plant would be sealed into the tracer receiving chamber (Fig. S13). This chamber couples to the plastic pot making a gas-tight seal via an imbedded o-ring. A split Plexiglas™ flange is installed around the stem area of the plant and sealed to the pot using tape as a means to isolate the aerial portions of the plant from the belowground portions when tracer was introduced. A small diaphragm pump is affixed to the exhaust line located on this flange and is adjusted to maintain a slight vacuum (~5 Torr below atmosphere). This action ensures that contents of the tracer pulse do not contact the aerial portions of the plant. During pulsing, the contents of the ^{13}NN collection tube are displaced with an air flow of 200 mL min^{-1}. This flow of gas enters the pot through the bottom holes allowing tracer to flow from bottom up through the soil column and exiting through the flange port. A small PIN diode radiation detector (Carroll Ramsey Associates, Inc., Berkeley, CA, USA) positioned on the stem area provides real time feedback of radiation levels during, and after pulsing.

5.2.12 Root imaging

After exposure to tracer, roots were separated from the Turface:Vermiculite plant mix and washed in a PBS solution (x1 strength adjusted to pH 7.4) containing 0.1% Tween 20. No attempt was made to disentangle the root mass into individual root structures for imaging (Fig. S14). Roots were imaged using autoradiography (Typhoon 7000: GE Healthcare, Piscataway, NJ, USA). Images were post-processed using ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

5.2.13 Radioisotope decay analysis

All radioisotopes undergo decay with characteristic properties of radiation release. The radioisotopes that were noted above (^{11}C, $t_{½}$ 20.4 min; ^{13}N, $t_{½}$ 9.97 min; ^{15}O, $t_{½}$ 2.0 min; ^{14}O, $t_{½}$ 77 sec) as being present in the pulse mix all decay by positron emission. However, after annihilation these positrons, regardless of their initial energy, all give rise to the same energy (511 keV) gamma radiation making them indistinguishable by gamma spectroscopy, with the exception of ^{14}O which also emits a 2314 keV gamma ray. The one distinguishing feature of these radioisotopes is they each possess a unique temporal signature for decay. All radioisotopes decay by first-order kinetics according to the following equation where A_0 is defined as the activity at time point zero, A_t as the observed activity at time point t, λ as the decay constant (equal to Log 2/radioactive half-life) and t as the elapsed time:

$$A_0 = A_t e^{-\lambda t}$$

By virtue of this equation, a plot of the Log A_t versus time will yield a straight line whose slope is the half-life and y-intercept is A_0. For a mixture of radioisotopes multiple lines will manifest from this data treatment enabling the user to extract precise information on isotopic purity.

Tissues targeted for decay analysis were sealed into 10 mL glass vials and placed in a well-type gamma counter where levels of radioactivity were measured every 0.5 min for the duration of at least 1 hr. Decay analysis plots were constructed as described above (Fig. 4A-D) and linear regression analysis was performed on the data and the fractional radioisotope values calculated. Isotope identification was made on the basis of the calculated half-life from these plots.

5.2.14 Rubisco extraction and analysis for ^{13}N-incorportion

Following 15 minutes of high illumination at 1500 μmol m^{-2} s^{-1}, 500 mg of source leaf tissue from ^{13}N-exposed plants was harvested and ground fresh in 500 μL of acetone using a mortar and pestel. The liquid extract was then mixed with 1.5 mL of hexane solvent, vortexed for 2 minutes and then centrifuged at 2000 rpm for 2 minutes to effect phase separation. We found that the initial acetone extraction removed all the leaf pigments. However, upon mixing with the hexane solvent, acetone became sufficiently miscible helping to drive the bulk of these extracts into that fraction. After phase separation, we were left with 50 μL or less of a water fraction (derived from the leaf tissue) that was subjected to radio thin layer chromatography (TLC) analysis for labeled proteins. Samples were spiked using authentic Rubisco enzyme (Sigma-Aldrich, Inc., St. Louis, MO, USA) and TLC was performed on glass-backed cellulose plates (100 μm thick, Sigma-Aldrich, Inc., St. Louis, MO, USA) using a solvent of methanol: ammonia: water (6:3:1 by vol(*59*)). After development, nitrogen-13 was measured using autoradiography (Typhoon 7000: GE Healthcare, Piscataway, NJ, USA) with typical exposures of 30 minutes. Following imaging, plates were treated with ninhydrin spray reagent (Sigma-Aldrich, Inc., St. Louis, MO, USA) and then heat treated at 200^{o}C for 5 minutes for protein visualization under long wavelength UV light.

5.2.15 Plant physiological measurements

Four physiological components were measured using the carbon-11 radiotracer included: (i) leaf $^{11}CO_2$ fixation; (ii) leaf export of ^{11}C-photoassimilates, (iii) allocation of ^{11}C-photoassimilates belowground; and (iv) root exudation of ^{11}C-photoassimilates.
Positron autoradiography (Typhoon 7000: GE Healthcare, Piscataway, NJ, USA) was used to obtain two-dimensional whole-plant images of the plant shoots and roots. The source leaf section where $^{11}CO_2$ was administered was removed prior to imaging. This data was later used to calculate distributions of radioactivity within targeted tissues using ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Afterwards, plant tissues were harvested, weighed and counted for radioactivity levels using a Capintec Radioisotope Dose Calibrator CRC-15W (Capintec, Inc, Ramsey, NJ, USA). Radioactivity was corrected for decay using the end-of-bombardment time as time zero. The fresh tissue masses were used to normalize radioactivity amounts enabling us to accurately project ^{11}C-photoassimilate

allocation patterns independent of sink mass. Aliquots of the growth medium were also sampled and counted for radioactivity levels using a gamma scintillation counter enabling quantification of root exudation.

5.2.16 Tissue extraction

Source leaf tissues exposed to the $^{11}CO_2$ were harvested, weighed and counted in the Capintec Radioisotope Calibrator for radioactivity levels. Tissues were placed into 1.5 mL Eppendorf™ tubes into which 4x w/v of methanol was added. Tissues were fresh ground using a Retsch Miximill MM400 ball and mill grinder (Retsch GmbH, Germany) after which they were briefly vortexed (VWR analog vortex mixer; Sigma-Aldrich Corp. St. Louis, MO, USA) and then sonicated (Branson Bransonic 32; Sigma-Aldrich Corp. St. Louis, MO, USA) in an iced water bath for 10 min with intermittent vortexing to ensure complete mixing. Tubes were centrifuged (Eppendorf Centrifuge 5424) for 2 min at 15,000 rpm and the supernatant removed by pipette. Pellets contained all insoluble components, which were comprised mostly of cell-wall polymers and starch. Filtrates contained small, soluble compounds, including soluble sugars and amino acids. Aliquots of the soluble fraction and the total pellet were measured for ^{11}C-radioactivity using a gamma scintillation counter.

5.2.17 Carbon-11 label sugar analysis

Soluble ^{11}C-sugars were separated and analyzed by thin layer chromatography (TLC) (*60*). Glass backed NH_2-silica HPTLC-plates (200µm, w/UV254) were used for the sugar separation (Sorbent Technologies, Atlanta, GA, USA). Plates were pre-spotted with sugar standards of glucose, sucrose and fructose for registration of ^{11}C-sugar R_f signatures against those of authentic compounds and then with 1 and 2 µL aliquots of radioactive leaf extract using a semi-automatic Linomat 5 sample applicator (Camag Scientific Inc., Wilmington, NC, USA) for high precision of spot size and sample volume. The larger volume of extract was sometimes needed to visualize the ^{11}C-hexose sugars, which were usually lower in concentration than sucrose. TLC plates were developed using a mobile phase consisting of 75:25 acetonitrile:water (v/v). Developed plates were imaged using autoradiography (Typhoon FLA 7000) to determine the fraction of each radiolabeled sugar. ImageQuant TL software 7.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to analyze both

the radiographic images to determine the relative amount of ^{11}C within the individual sugars. These values were summed across the soluble sugar pool, and presented as the total^{11}C-sugars corrected to reflect the fraction of the total ^{11}C-activity within the targeted tissue using ^{11}C-soluble and ^{11}C-insoluble fractions.

5.2.18 Carbon-11 labeled amino acid analysis

A 50 µL volume of the soluble extract was delivered into a 0.5 mL Eppendorf™ tube (Fisher Scientific, Inc., Pittsburgh, PA, USA) and mixed with an equal volume of o-phthalaldehyde amino acid derivatizing reagent (OPA: Sigma-Aldrich Inc., St. Louis, MO, USA) containing 0.1% (v/v) mercaptoethanol and 0.1% (v/v) sodium hypochlorite. The mixture was vortexed and then allowed to react at ambient temperature for 3 min. Primary amino acids are readily converted into iso-indole derivatives by OPA enabling their separation with reversed-phase high-performance liquid chromatography and quantification by fluorescence detection (*61*). A 20 µL volume of the derivatized mixture was injected onto a reversed-phased analytical HPLC column (Phenomenex, Torrance, CA, USA: Ultramex™ C18, 10 µm particle size, 250 × 4.6 mm i.d.) using a pre-column gradient mixer (Isco, Lincoln, NE, USA) and a mobile phase comprised of A (DI water), B (0.01 M potassium phosphate (monobasic) solution buffered at pH 6.5), and C (methanol). At injection, the mobile phase (1.8 mL min^{-1}) was sustained at 75% A: 25% B for 5 min and then programmed to attain 20% B: 80% C by 30 min using a Knauer solvent gradient mixer (Sonntek Inc., Upper Saddle River, NJ, USA). Elution profiles were calibrated against standards using a fluorescence detector (Hitachi FL Detector L-2485). The outlet of the fluorescent detector was connected in series to a NaI gamma radiation detector (OrtecInc, Oak Ridge TN, USA) that enabled direct measurement of the amount of radioactivity associated with each substrate eluting the column. Analog outputs from detectors were fed to a chromatography data acquisition station (SRI Instruments, Torrance, CA, USA). The radiation detector was also cross-calibrated against the gamma detector that was used to measure the soluble and insoluble extract fractions so that ^{11}C-amino acids could be correlated to total ^{11}C-activity levels within the plant (*62*).Individual ^{11}C-amino acids were summed as one pool of labeled metabolites and presented as the fraction of the total ^{11}C-activity within the targeted tissue using ^{11}C-soluble and ^{11}C-insoluble fractions.

5.3 REFERENCES AND NOTES:

1. S.Sulieman, Does GABA increase the efficiency of symbiotic N2 fixation in legumes? *Plant Signal Behav*. 32-36 (2011).
2. V.Walker *et al*., Variation of secondary metabolite levels in maize seedling roots induced by inoculation with *Azospirillum*, *Pseudomonas* and *Glomus* consortium under field conditions. *Plant Soil.* **356,** 151-163 (2012).
3. M.Charpentier, G. Oldroyd, How close are we to nitrogen-fixing cereals? *Curr.Opin. Plant Biol*. **13** (5), 556-564 (2010).
4. C.Santi, D.Bogusz, C. Franche, Biological nitrogen fixation in non-legume plants. *Annals of Botany*. 743-767 (2013).
5. J.Raaijmakers*et al*., The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil*. **321** (1), 341-361 (2009).
6. B.W.Verhagen *et al*., The transcriptome of rhizobacteria-induced systemic resistance in arabidopsis. *Molecular Plant-Microbe Interactions*. **17** (8), 895-908 (2004).
7. A.Richardson *et al*., Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*. **321** (1), 305-339 (2009).
8. T.Hurek *et al*., Root colonization and systemic spreading of *Azoarcus sp.* strain BH72 in grasses. *J.Bacteriol*. **176,** 1913 (1994).
9. B.Reinhold*et al*., Close Association ofAzospirillum and Diazotrophic Rods with Different Root Zones of Kallar Grass. *Appl. Environ. Microbiol.* **52** (3), 520-526 (1986).
10. E.K. James, F.B.L. Olivares, Infection and Colonization of Sugar Cane and Other Graminaceous Plants by Endophytic Diazotrophs.*Critical Reviews in Plant Sciences*. **17** (1), 77-119 (1998).
11. E.K. James*et al*., *Herbaspirillum,* an endophytic diazotroph colonizing vascular tissue in leaves of *Sorghum bicolor* L. Moench.*J. Exp. Bot.* **48**, 785-797 (1997).
12. E.K.James*et al*., Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus. J. Exp. Bot*. **45,** 757-766 (1994).
13. B.Reinhold-Hurek, T. Hurek, Life in grasses: diazotrophic endophytes.*Trends Microbiol*. **6** (4), 139-144 (1998).
14. T. Hurek *et al*., Augmented rates of respiration and efficient nitrogen fixation at nanomolar concentrations of dissolved O_2 in hyperinduced *Azoarcus* sp. strain BH72.*J.Bacteriol*. **176**, 4726 (1994).
15. T. Egener, T. Hurek, B. Reinhold-Hurek, Endophytic Expression of nif Genes of *Azoarcus* sp. Strain BH72 in Rice Roots. *Molecular Plant-Microbe Interactions*. **12** (9), 813-819 (1999).
16. F.L.Olivares *et al*., Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirilium*. *New Phytologist*. **135** (4), 723-737 (1997).
17. E.K.James*et al*., Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. *Molecular Plant-Microbe Interactions*. **15** (9), 894-906 (2002).
18. M.Sevilla*et al*., Comparisonof Benefit to Sugarcane Plant Growth and $^{15}N_2$ Incorporation Following Inoculation of Sterile Plants with *Acetobacter diazotrophicus* Wild-Type and NifA Mutant Strains.*Molecular Plant-Microbe Interactions.* **14** (3), 358-366 (2001).
19. T.Hurek *et al*., Azoarcus Grass Endophytes Contribute Fixed Nitrogen to the Plant in an Unculturable State. *Molecular Plant-Microbe Interactions*. **15** (3), 233-242 (2002).

20. A.L.Iniguez, Y. Dong, E.W. Triplett, Nitrogen Fixation in Wheat Provided by *Klebsiella pneumoniae* 342. *Molecular Plant-Microbe Interactions.***17** (10), 1078-1085 (2004).
21. P.Gyaneshwar *et al*., Herbaspirillumcolonization increases growth and nitrogen accumulation in aluminium-tolerant rice varieties. *New Phytologist*. **154** (1), 131-145 (2002).
22. R.M. Boddey*et al*., Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. *Plant Soil* **174**, 195–209 (1995).
23. Y.Okon, P.G. Heytler, R.W.F. Hardy, N_2-fixation by *Azospirillum brasilense* and its incorporation into host *Setaria italica. Applied and Environmental Microbiology*. **46** (3), 694-697 (1983).
24. R.M.Boddey, R.L. Victoria, Estimation of biological nitrogen fixation associated with Brachiaria and Paspalum grasses using^{15}N labelled organic matter and fertilizer. *Plant and Soil*. **90** (1-3), 265-292 (1986).
25. R. Morais*et al*., Contribution of biological nitrogen fixation to Elephant grass *(Pennisetum purpureum Schum.). Plant and Soil*. **356** (1-2), 23-34 (2012).
26. S. Dobbelaere*et al*., Responses of agronomically important crops to inoculation with *Azospirillum. Functional Plant Biology*. **28** (9), 871-879 (2001).
27. J.J. Tarrand, N.R.Krieg, J. Dobereine, A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, Azospirillum gen. nov. and two species, *Azospirillum lipoferum (Beijerinck)* comb. nov. and *Azospirillum brasilense* sp. nov. *Can J Microbiol*. **24** (8), 967-980 (1978).
28. Y.Bashan, G. Holguin, L.E. de-Bashan, *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). *Canadian Journal of Microbiology*. **50** (8), 521-577 (2004).
29. T.P.Brutnell *et al*., *Setaria viridis:* a model for C4 photosynthesis. *The Plant cell*. **22** (8), 2537-44 (2010).
30. P.Li, T.P. Brutnell, *Setaria viridis* and *Setaria italica,* model genetic systems for the Panicoid grasses. *J Exp Bot*. **62** (9), 3031-7 (2011).
31. C.Lodewyckx*et al*., Endophytic bacteria and their potential applications. *Crit Rev Plant Sci*.**6**: p.583-606 (2002).
32. J.I. Baldani*et al*., Characterization of *Herbaspirillum seropedicae,* a root-associated nitrogen fixing bacterium. *Intl. J. Sys. Bact.* **36**, 86-93 (1986).
33. L. Magalhães Cruz*et al*., 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* sp.) and pineapple (*Ananas comosus* (L.)Merril). *Appl Environ Microbiol*. **67** (5), 2375-9 (2001).
34. M.A.Schmidt *et al*., Evidence for the endophytic colonization of *Phaseolus vulgaris* (common bean) roots by the diazotroph *Herbaspirillum seropedicae. Braz J Med Biol Res*. **44** (3), 182-5 (2011).
35. L.D.B.Roncato-Maccari*et al*., Endophytic *Herbaspirillum seropedicae* expresses *nif* genes in gramineous plants. *Fems Microbiology Ecology*. **45** (1), 39-47 (2003).
36. E.K. James,Nitrogen fixation in endophytic and associative symbiosis. *Field Crops Research*. **65**(2-3), 197-209 (2000).
37. A.Vande Broek*et al*., Spatial-temporal colonization patterns of *Azospirillum brasilense* on the wheat root surface and expression ofthe bacterial *nifH* gene during association. *Molecular Plant Microbe Interaction*. **6**(5), 592-600 (1993).
38. M.C.K.Kasel, M.J. Schueller, R.A. Ferrieri, Optimizing [^{13}N]N_2 radiochemistry for imaging nitrogen-fixation in root nodules of legumes. *J. Label Cmpds & Radiopharm*. **53**, 592-597 (2010).

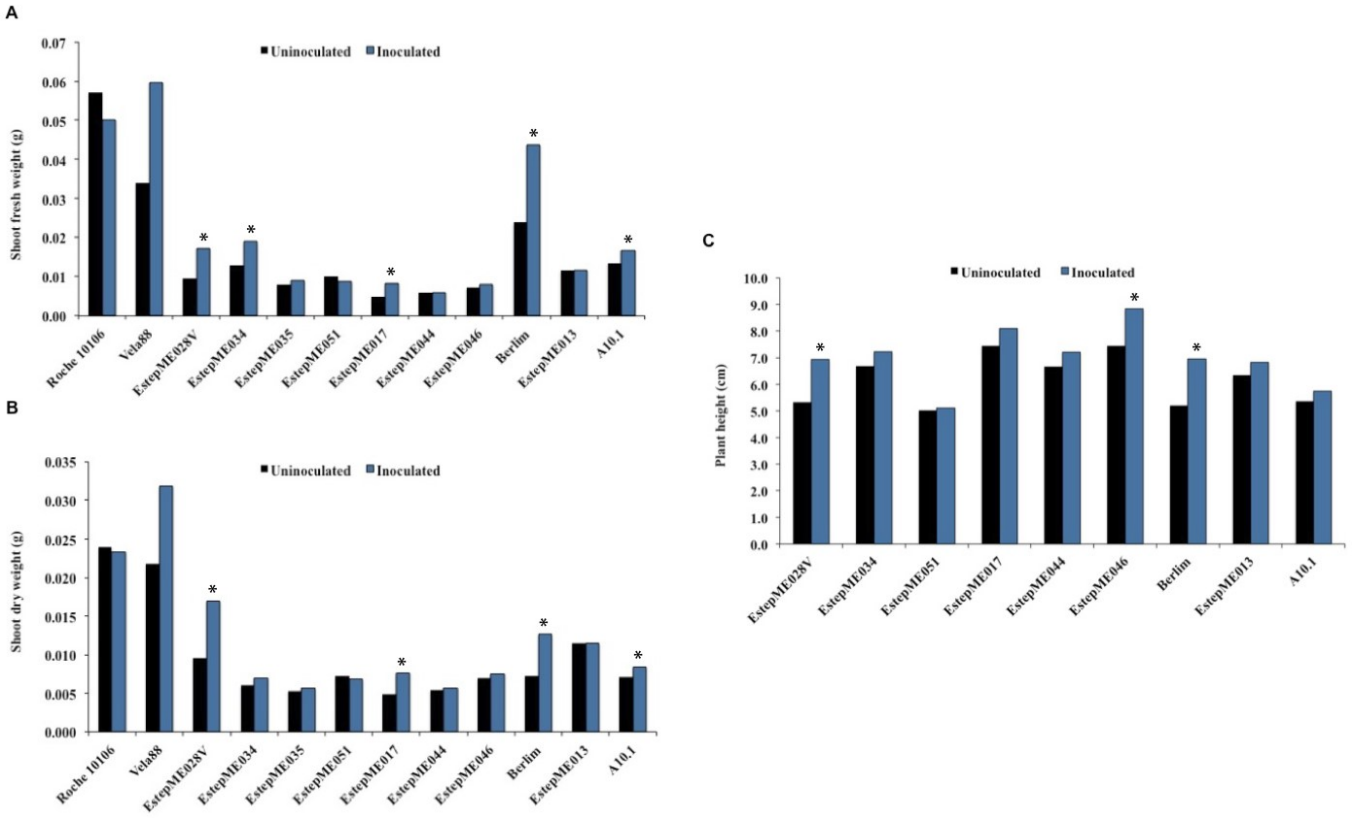
39. F.O. Pedrosa, M.G. Yates, Regulation of nitrogen fixation *nif* (genes) of *Azospirillum brasilense* by *nifA* and *ntrC (glnB)* type gene products. *FEMS Microbiol Lett*. **23**, 95-101 (1984).
40. E.M.Souza *et al*., Expression of the *nifA* gene of *Herbaspirillum seropedicae:* role of the NtrC and NifA binding sites and of the -24/-12 promoter element. *Microbiology*. 2000. **164**: p. 1407-1418 (2000).
41. H.B. Machado*et al*., Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine. *Can J Microbiol*. **37**(7), 549-553 (1991).
42. J.C.Vitorinoet al., Potential roles for the *glnB* and *ntrYX* genes in *Azospirillum brasilense. FEMS Microbiol Lett*. **201**(2), 199-204 (2001).
43. A.J.Bloom. Nitrogen as a limiting factor: crop acquisition of ammonium and nitrate, in Ecology in Agriculture. *Academic Press: San* Diego. 145-172 (1997).
44. A.J.Bloom*et al.,* Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. *Science*. **328** (5980), 899-903 (2010).
45. L.Rosendahl, C.P. Vance, W.B. Pedersen. Products of dark CO_2 fixation in pea root nodules support bacteroid metabolism. *Plant Physiology*. **93**, 12 (1990).
46. P.R.Weisz, T.R. Sinclair, Soybean nodule gas permeability, nitrogen fixation and diurnal cycles in soil temperature. *Plant and Soil* **109**(2), 227-234 (1988).
47. G.E.D. Oldroyd, J.A. Downie. Coordinating Nodule Morphogenesis with Rhizobial Infection in Legumes. *Annual Review of Plant Biology.* **59**(1), 519-546 (2008).
48. S.B.Cannon *et al*. Legume genome evolution viewed through the *Medicago truncatula* and *Lotus japonicus* genomes. *Proceedings of the National Academy of Sciences*. **103** (40),14959-14964 (2006).
49. Q.Y. Jiang, P.M. Gresshoff. Classical and molecular genetics of the model legume *Lotus japonicus. Mol.Plant Microbe. Interaction*. **10**, 59 (1997).
50. N.Sandal*et al*. Genetics of symbiosis in *Lotus japonicus:* Recombinant inbred lines, comparative genetic maps, and map position of 35 symbiotic loci. *Molecular Plant-Microbe Interactions*. **19** (1), 80-91 (2006).
51. R.A. Monteiro *et al*. Early colonization pattern of maize(*Zea mays L.* Poales, Poaceae) roots by *Herbaspirillum seropedicae* (*Burkholderiales, Oxalobacteraceae*). *Genetics and Molecular Biology.* **31** (4), 932-937 (2008).
52. G.Klassen *et al*., Effect of nitrogen compounds on nitrogenase activity in *Herbaspirillum seropedicae* strain SmR1. *Can. J. Microbiol*. **43**, 841-846 (1997).
53. H.B. Machado *et al*.Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine., 1991: *Can. J. Microbiology*. (1991).
54. D.R.Hoagland, D.I. Arnon, The water-culture method for growing plants without soil. *Journal Circular California Agricultural Experiment Station*. **347**, 32 (1950).
55. L.D.B. Roncato-Maccari *et al*. Root colonization, systemic spreading and contribution of *Herbaspirillum seropedicae* to growth of rice seedlings. *Symbiosis*. **35**, 1-10 (2003).
56. L.Wang *et al*. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics*. **26**(1), 136-8 (2010).
57. R.A.Ferrieri *et al.* Use of carbon-11 inPopulus shows that exogenous jasmonic acid increases biosynthesis of isoprene from recently fixed carbon. *Plant Cell and Environment*. **28**, 591-602 (2005).
58. R.A. Ferrieri, A.P. Wolf. The chemistry of positron emitting nucleogenic (hot) atoms with regard to preparation of labeled compounds of practical utility. *Radiochimica Acta*. **34**, 69-83 (1983).
59. S.Chakrabarti,S. Bhattacharya, S.K. Bhattacharya. A nonradioactive assay method for determination of enzymatic activity of D-ribulose-1,5-bisphosphate

carboxylase/oxygenase (Rubisco). *Journal of Biochemical and Biophysical Methods*. **52,** 179-187 (2002).

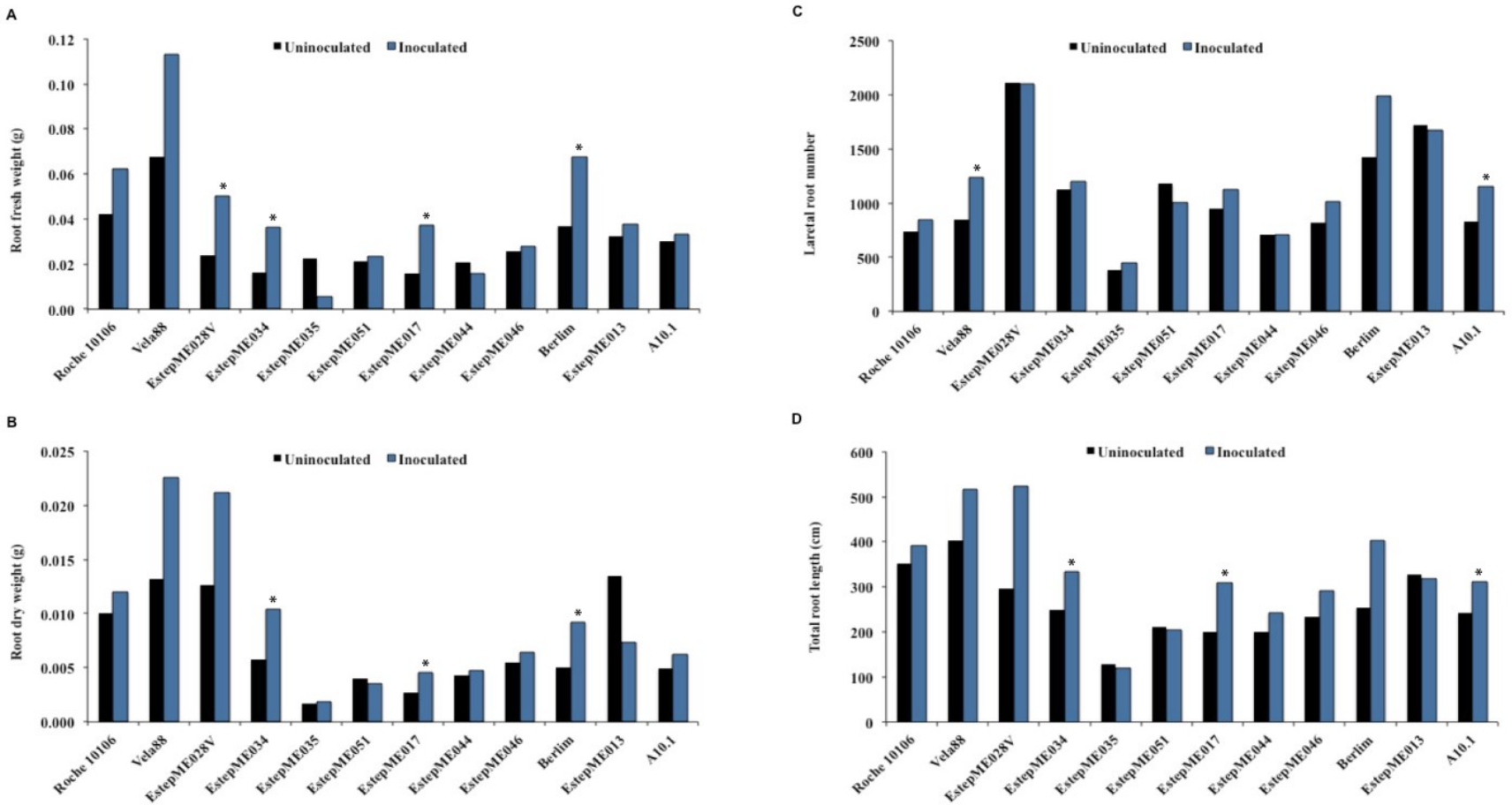
60. B.A.Babst, A.A. Karve, T. Judt. Radio-Metabolite Analysis of Carbon-11 Biochemical Partitioning to Non-Structural Carbohydrates for Integrated Metabolism and Transport Studies. *Plant and Cell Physiology*. **54,** 1016-1025 (2013).
61. J.Chow, J. Orenberq, K. Nugent. Comparison of automated pre-column and post-column analysis of amino acid oligomers. *J Chromatogr*. 386:243 (1987).
62. N. Haniket *al*. Partitioning of New Carbon as C-11 in *Nicotiana tabacum* Reveals Insight into Methyl Jasmonate Induced Changes in Metabolism. *Journal of Chemical Ecology*. **36**, 1058-1067 (2010).

Acknowledgments: This article has been authored by Brookhaven Science Associates, LLC under contract number DE-AC02-98CH10886 with the U.S. Department of Energy (DOE) which supported R.A. Ferrieri and the BNL team in this effort. Furthermore, this was financially supported by the National Institute of Science and Technology-Biological Nitrogen Fixation, INCT-FBN, through the Brazilian Research Council – CNPq/MCT and the CiênciaSemFronteiras Program, Brazil (fellowships supporting V.C.S. Pankievicz, F. P. Amaral and K.F Santos). Support was also provided by grant DE-FOA-0000223 (to G. Stacey) from the Department of Energy, Office of Biological and Environmental Research. Additional support was provided by SUNY School of Environmental Science and Forestry Honors Internship Program (supporting B. Agtuca). We gratefully acknowledge the technical support of Dr. AleksandrJurkevic from the University of Missouri molecular cytology core and Israel H. Bini from the Federal University of Paraná for assisting with the microscopy, and Dr. Carolina Galvão from UEPG, Brazil for the *A. brasilense* strain FP2-7.

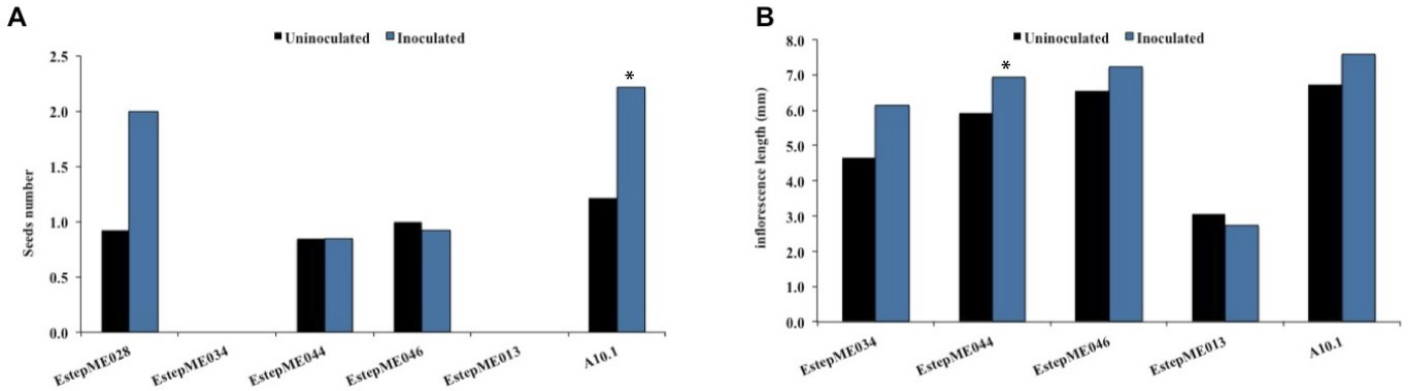
5.4 SUPPLEMENTAL FIGURES



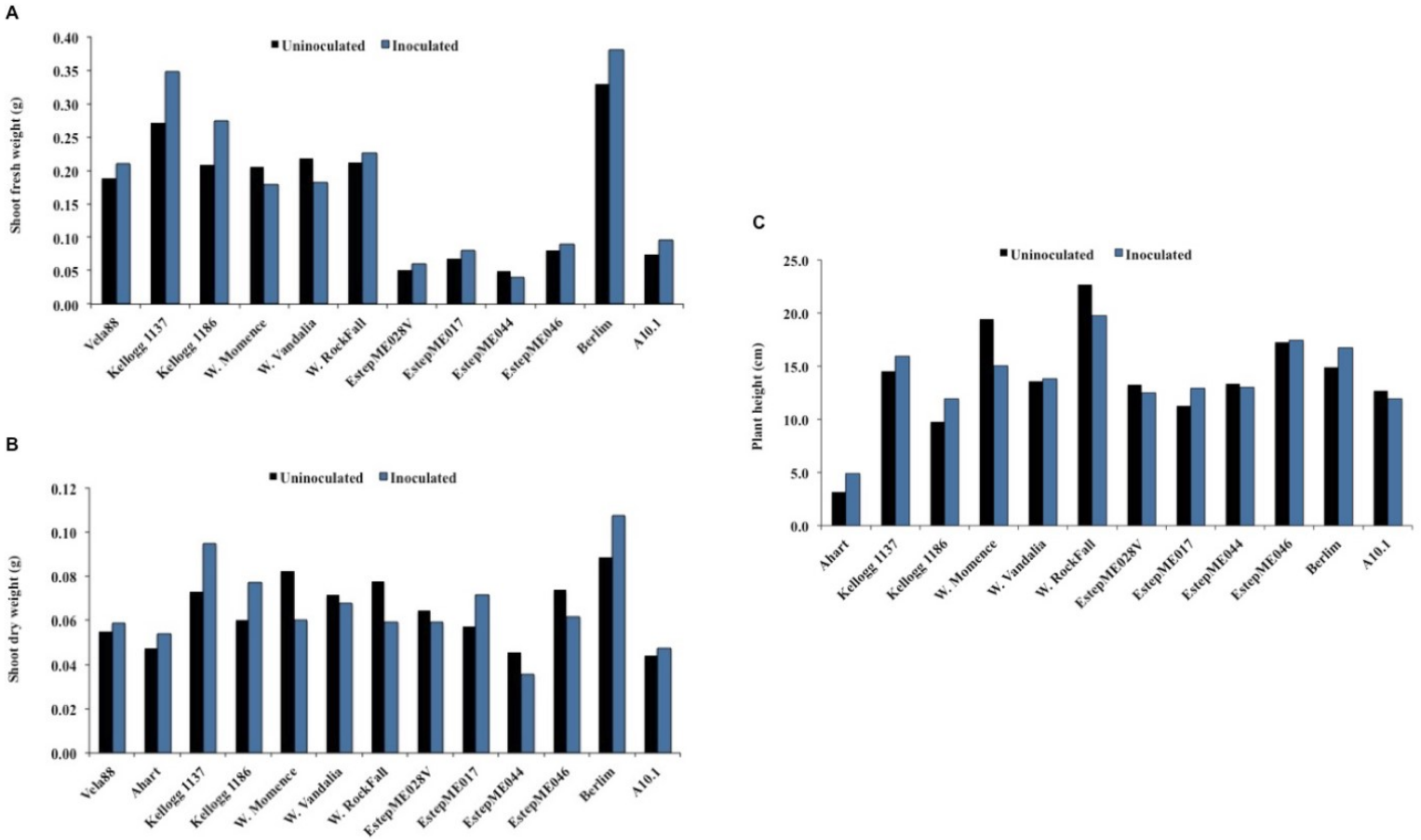
S1 - Shoot growth promotion parameters of *S. viridis* genotypes grown without added nitrogen. (A) Shoot fresh weight of the *S. viridis* genotypes, EstepME028V, EstepME034, EstepME017, Berlim, and A10.1 showed a significant increase when the plants were inoculated with *A. brasilense* and *H. seropedicae*. (B) Shoot dry weight for the genotypes EstepME028V, EstepME017, Berlim and A10.1 showed a significant increase upon inoculation. (C) Genotypes,EstepME028V, EstepME046 and Berlim showed a significant increase in the plant height when plants were inoculated. Each asterisk indicates statistically significant difference with p-value ≤ 0.05.



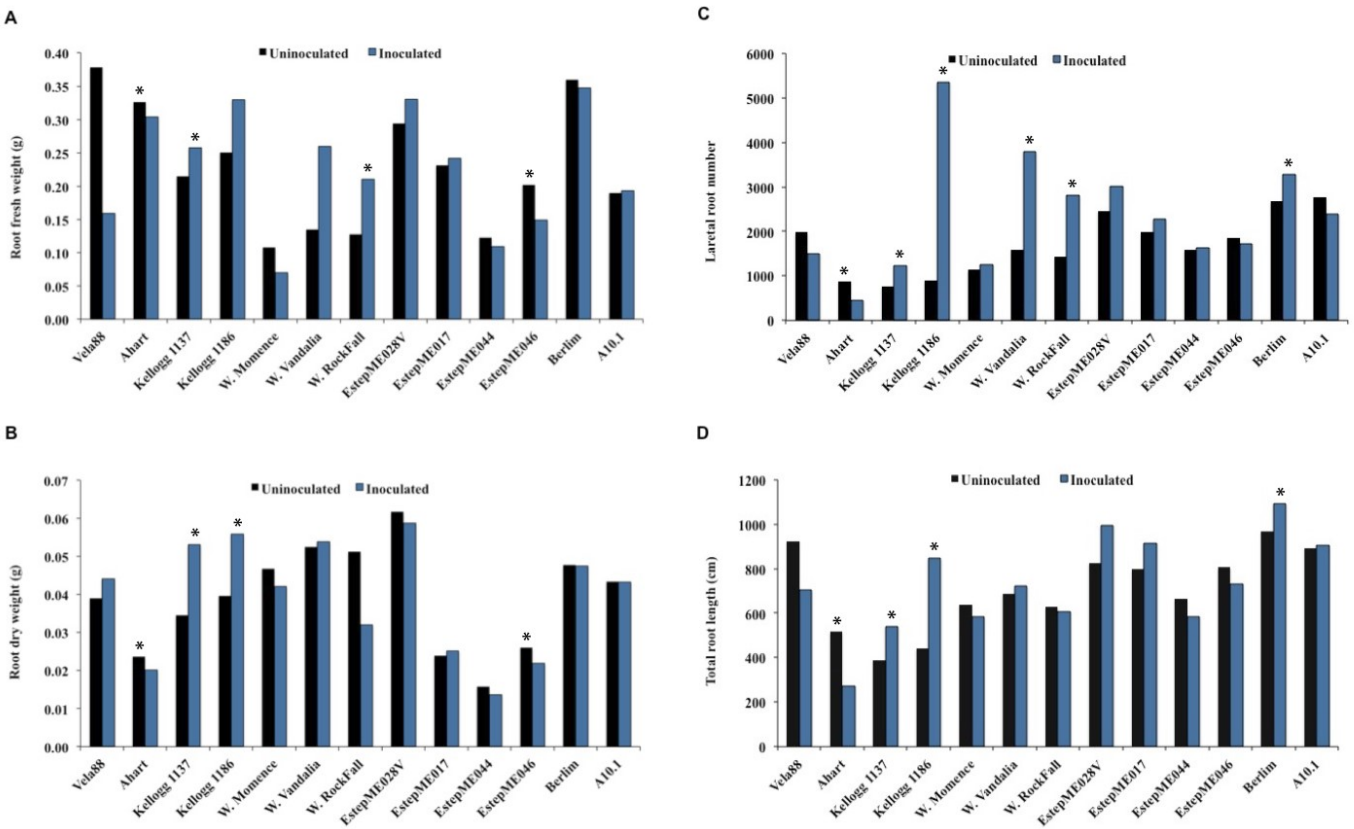
S2 - Root growth promotion parameters of *S. viridis* genotypes grown without added nitrogen. (A) Genotypes EstepME028V, EstepME034, EstepME017 and Berlim showed a significant increase in the root fresh weight when plants were inoculated with *A. brasilense* and *H. seropedicae.* (B) Genotypes, EstepME034, EstepME017 and Berlim showed a significant increase in the root dry weight when plants were inoculated. (C) The lateral root number increased for genotypes Vela88 and A10.1, upon inoculation. (D) Total root length increased for EstepME034, EstepE017 and A10.1 when plants were inoculated. Asterisk indicates statistically significant difference with p-value ≤ 0.05 between uninoculated plants and inoculated plants.



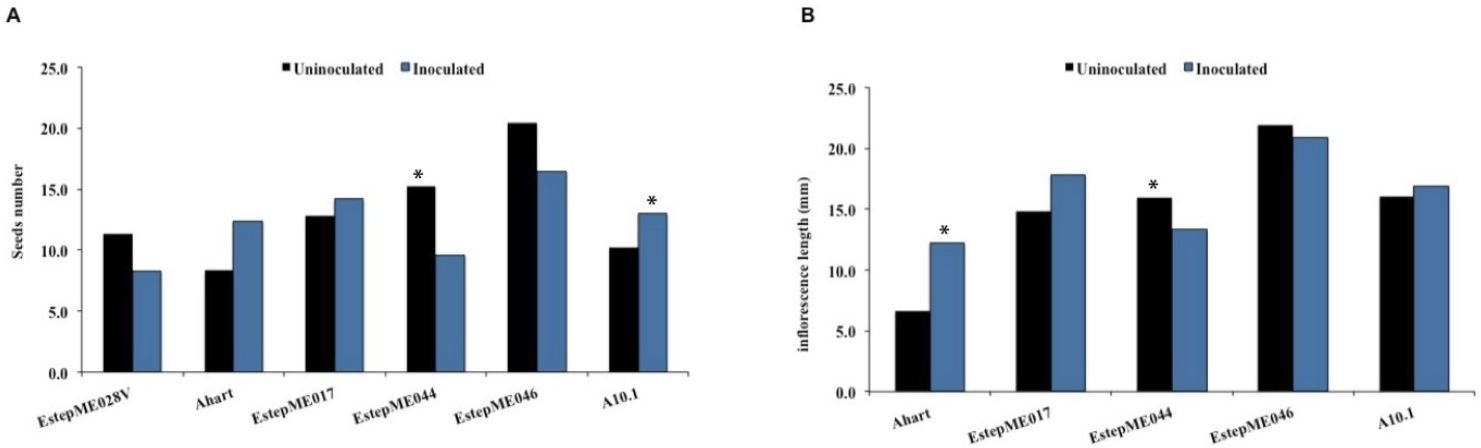
S3 - Number of seeds produced and inflorescence length for the *S. viridis* genotypes grown without added nitrogen (A) Only genotype A10.1 showed a significant increase in seed number when plants were inoculated with *A. brasilense* and *H. seropedicae,* while (B) the EstepME044 and EstepME034 was the genotypes that showed a significant increase in the inflorescence length when plants were inoculated. Asterisk indicates a statistically significant difference with p-value ≤ 0.05 between uninoculated plants and inoculated plants.



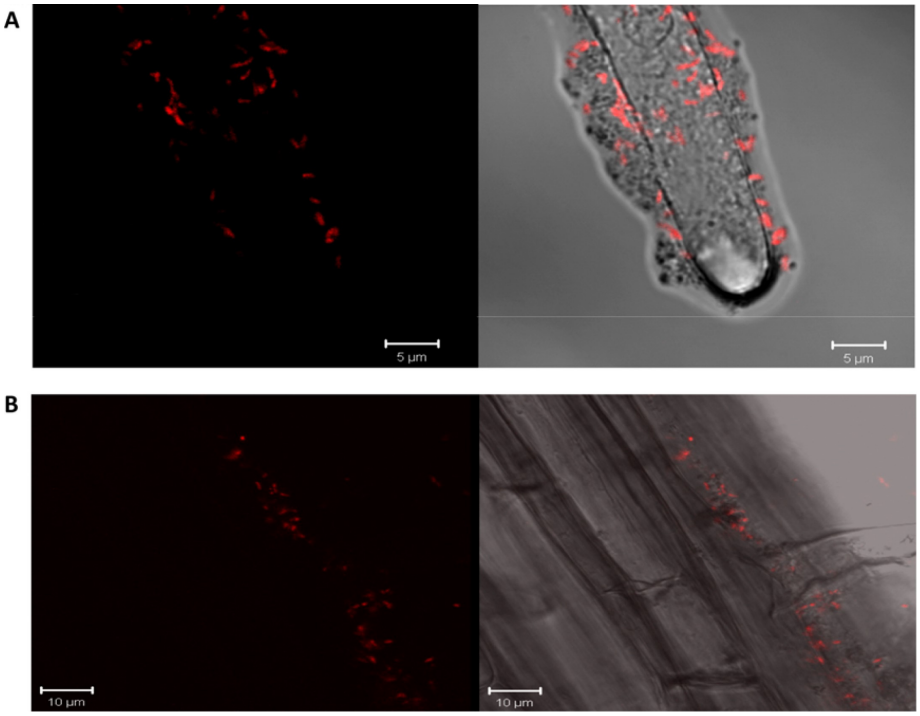
S4 - Shoot growth promotion parameters of *S. viridis* genotypes grown with low-nitrogen addition (i.e., 0.5 mM nitrate). None of the genotypes showed a significant increase in shoot fresh or dry weight and plant height when plants were inoculated with *A. brasilense* and *H. seropedicae*. No statistical significant differences were observed.



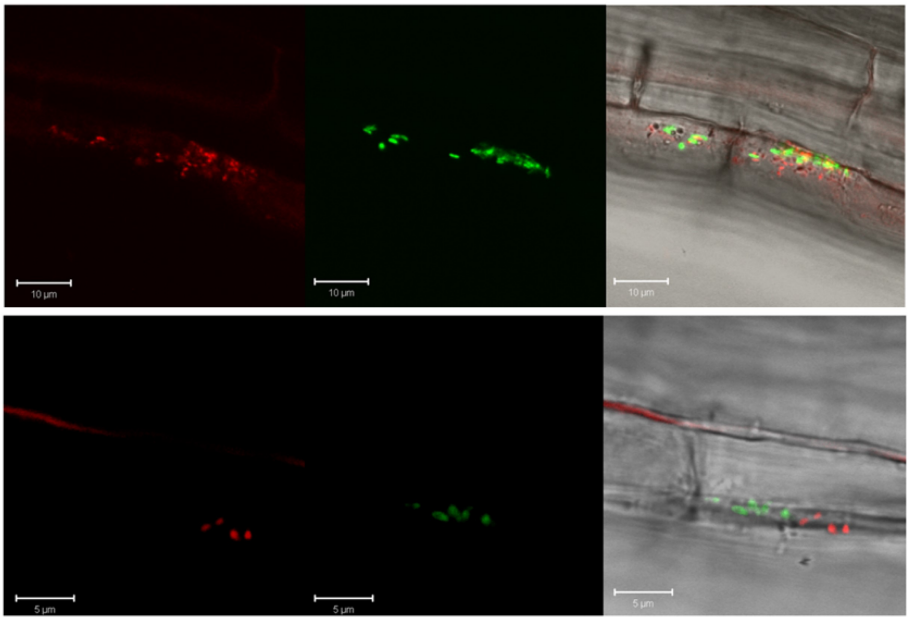
S5 - Root growth promotion parameters of *S. viridis* genotypes grown with low-nitrogen addition (i.e., 0.5mM nitrate). (A) Genotypes Kellogg1137 and W. Rockfall showed a significant increase in the root fresh weight when plants were inoculated with *A. brasilense* and *H. seropedicae*, while the genotypes, Ahart and EstepME046 showed a decreased in the root fresh weight upon inoculation. (B) The Genotypes, Kellogg1137 and Kellogg1186 showed a significant increase in the root dry weight when plants were inoculated with bacteria, while the accession Ahart, and EstepME046 showed decreased root dry weight upon inoculation. (C) The lateral root number increased for genotypes, Kellogg1137, Kellogg1186, W. Vandalia, W. Rockfall and Berlim, while the genotypeAhart had a decreased lateral root number upon the inoculation. (D)Genotypes, Kellogg1137, Kellogg1186 and Berlim showed a significant increase in the root length when plants were inoculated, while the genotypeAhart showed a decreased total root length upon the inoculation with bacteria.Asterisk indicates a statistically significant difference with p-value ≤ 0.05 between uninoculated plants and inoculated plants



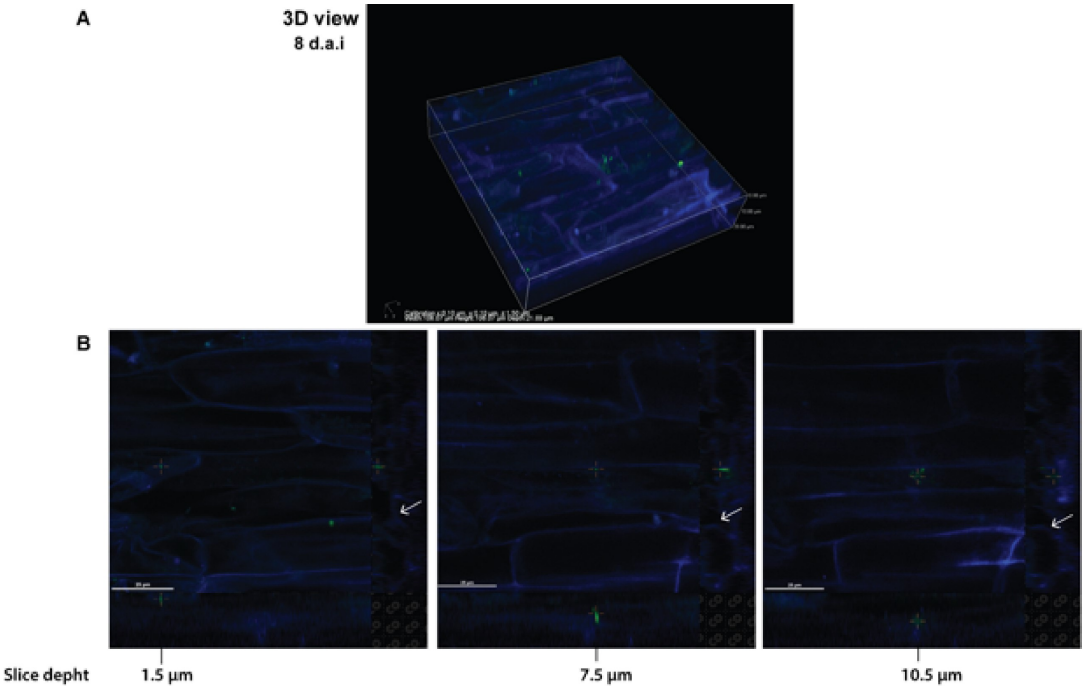
S6 - Number of seeds produced and inflorescence length for the genotypes of *S. viridis* grown with low-nitrogen addition (i.e., 0.5mM nitrate). The genotype A10.1 showed a significant increase in seed number (A) when plants were inoculated with *A. brasilense* and *H. seropedicae*, while the accession EstepME044 showed a decreased number of seeds and inflorescence length. (B) The genotype Ahart showed increased inflorescence length. Asterisk indicates a statistically significant difference with p-value ≤ 0.05 between uninoculated plants and inoculated plants.



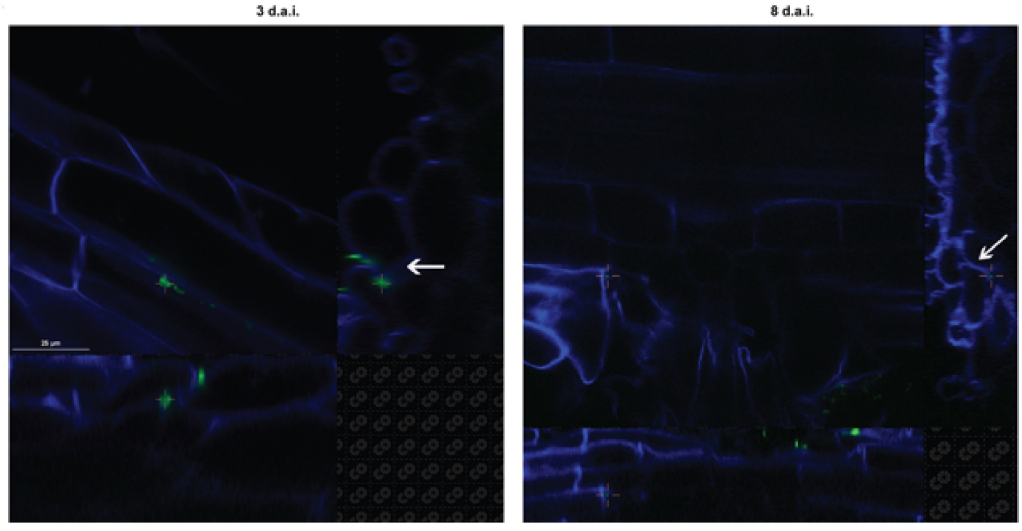
S7 - Confocal laser scanning microscope images of *H. seropedicae* on roots of *S. viridis* A10.1 after four days of inoculation. A root hair (A) and the base of a root hair (B) colonized by *H. seropedicae* RAM4 expressing DsRed shown in the left panels and gray backgrounds shown in the right panels correspond to the root image formed by the transmitted light. Superposed images are laid on the brightfield image shown in the right panels.



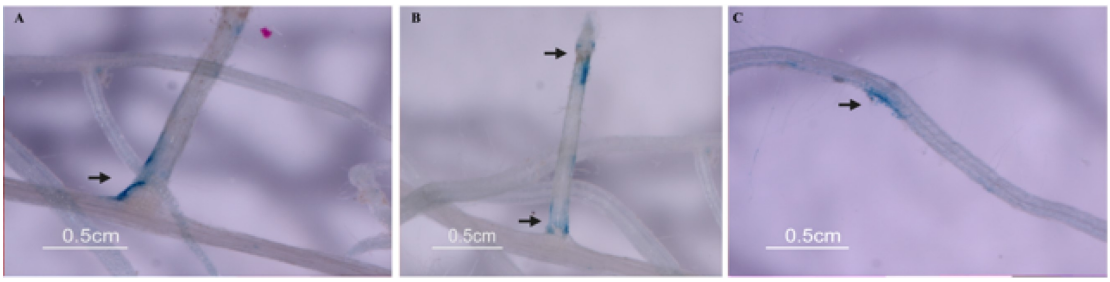
S8 - Confocal laser scanning microscope images of *H. seropedicae* on roots of *S. viridis* A10.1 genotype after seven days of inoculation.Bacterial cells colonize the intercellular spaces, delimited by the membranes of the plant cells. *H. seropedicae* RAM10 expressing GFP protein are shown in the middle panel; the plants cells stained with propidium iodide are shown in the left panels. Superposed images are laid on the bright field image shown in the right panels.



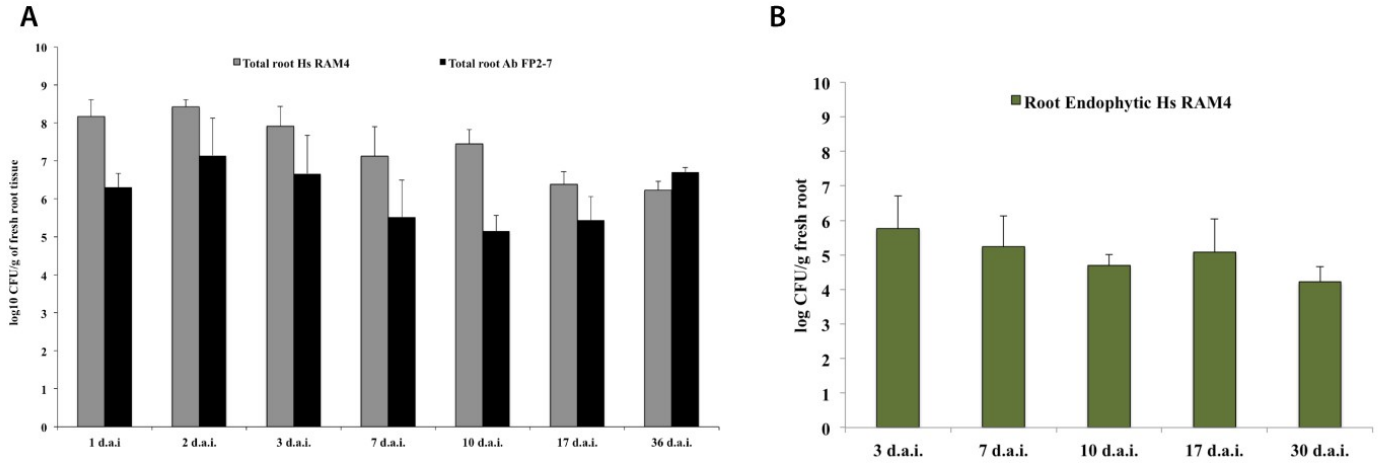
S9 - 3D view and orthogonal optical sections from cortical cells of *S. viridis* colonized by *H. seropedicae.* The *H. seropedicae* cells expressing GFP (green) in the blue background of the root auto-fluorescence. (A) The 3D view of a colonized *S. viridis* root 8 days after inoculation (d.a.i.) was used to make the orthogonal optical sections from the cortical cells of the plant. The central views show the focal plans from the z-stacks. The side views represent vertical and horizontal optical slices through the z-stacks. The red cross marks the position where the central view images are located within the z-stacks. The bacteria were localized on the root surface attached to a root hair (B, left panel) and inside the root cortex 7.5 µm deep (B, middle panel) in the root and 10.5 µm (B, right panel) deep in the root cortex.



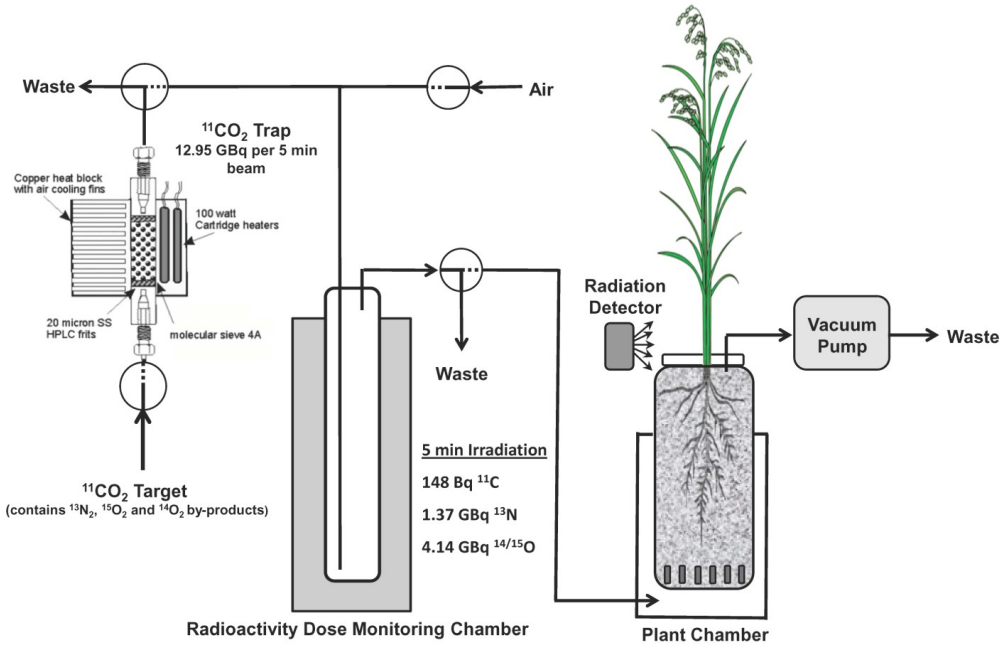
S10 - Orthogonal optical slices from confocal laser scanning images of *H. seropedicae* colonizing *S. viridis* roots after 3 and 8 days of inoculation. The *H. seropedicae* cells expressing GFP (green) in the blue background of the root auto-fluorescence. The bacteria are located above the epidermal plant cells 3 d.a.i. (left panel) and is colonizing the cortex cells 8 d.a.i. (right panel).



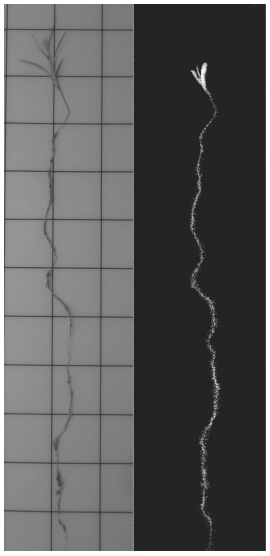
S11 - Epiphytic colonization and *nifH:gusA* expression by *A. brasilense* FP2-7 on the root surface of *S. viridis* A10.1 genotype. The plants were inoculated with *A. brasilense* FP2-7 expressing the *nifH:gusA* gene for 24 days, and then stained for GUS expression. Black arrows indicate regions of colonization at the lateral root cracks (Panel A), lateral roots tips (Panel B), and elongation zones of parent roots (Panel C). The images were obtained using a Leica MZFLIII stereomicroscope with color digital camera.



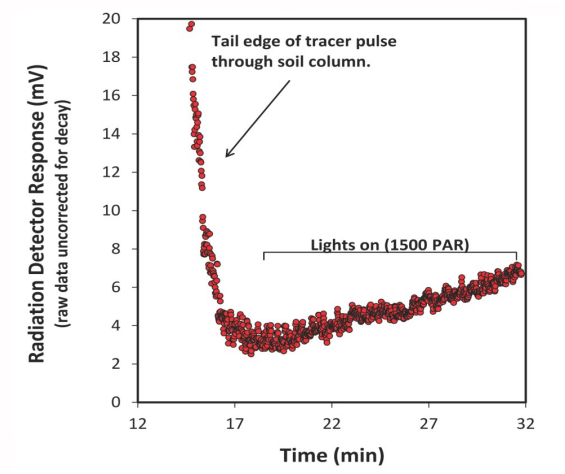
S12 - Pattern of bacterial colonization of the roots of *S. viridis* A10.1 genotype. (A) Data are expressed in colony forming units (CFU) per gram of fresh tissue by the following sampling: *H. seropedicae* RAM4 recovered from total root tissue (Total root Hs RAM4) and *A. brasilense* FP2-7 recovered from total root tissue (Total root Ab FP2-7). (B) Internal *H. seropedicae* were recovered from surface sterilized root (Root Endophytic Hs RAM4). No uninoculated plants presented natural contamination with *H. seropedicae* or *A. brasilense*, although some natural colonizer were recovered from total roots and leafs of control plants Values are mean ± SE of at least five replicates. (d.a.i.= days after inoculation).



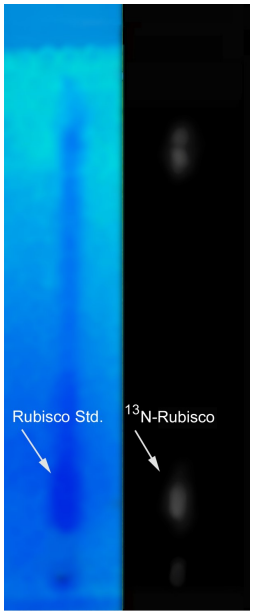
S13 - Schematic drawing of the BNL ^{13}NN plant pulsing station. This schematic depicts the setup by which doses of ^{13}NN were produced on the BNL cyclotron. ^{13}NN was generated from a gaseous target system that is designed to produce high levels of $^{11}CO_2$. ^{13}NN is a by-product of the irradiation process. Most, but not all the $^{11}CO_2$ tracer is removed during gas handling using a molecular sieve trap leaving a tiny but fixed amount of $^{11}CO_2$ in the ^{13}NN pulse. The gas stream also contains substantial amounts of ^{14}O and ^{15}O as labeled O_2 which decay rapidly ($t_{½}$ 77 sec and $t_{½}$ 2min, respectively) and are not measureable during decay analysis of the plant tissues. The waste stream from this sieve trap accumulates in a 4 L flow-through bulb, and once a maximum level of activity is reached the contents are pulsed through a soil column containing a study plant. A vacuum pump affixed to the air exhaust flange located at the base of the stem prevents tracer from exhausting through the stem penetration hole. A small amount of photosynthetically active tissue below this flange is unavoidable. That tissue was not included in any root tissue decay analysis for isotopic composition measurement.



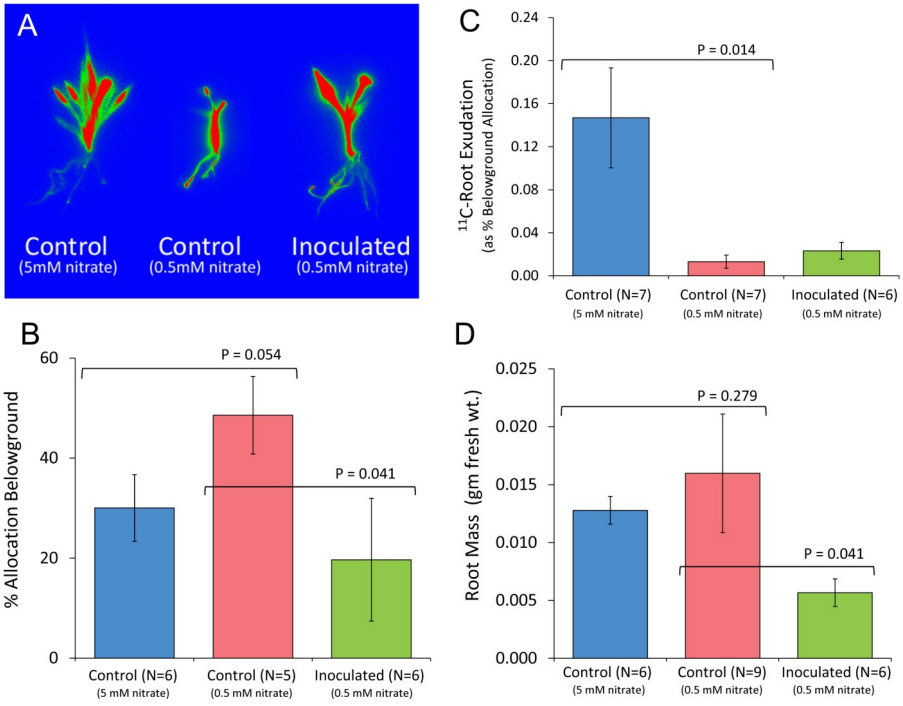
S14 - Photograph and autoradiography image. The photograph was taken after the roots were removed from the Turface soil column. Roots were washed using a phosphate buffered saline solution then laid out for radiographic imaging. It was not possible to disentangle the fine roots for this image. Images were acquired using GE Medical Systems Typhoon 7000 imager.



S15 - Time-activity trace showing transport of root activity aboveground. Data presented in this graph is the uncorrected millivolt response of the CsI (diode) radiation detector affixed to the upper aerial portions of the plant. The initial data shows the tail edge of the pulse. Shoot lights were turned on after the initial pulse had passed through the soil column. Illumination was set to 1500 µmol m^{-2} sec^{-1} force water transpiration and drive tracer upward. The rise in the raw counts clearly demonstrates that biological transport is occurring. Shoots were collected after 15 minutes and subjected to decay analysis in order to tease apart the isotopic composition. A strong ^{13}N signature was seen in those tissues after this transport.



S16 - Radio thin layer chromatography analysis of source leaf protein reveals a ^{13}N-labeled ribulose-1,5-bisphosphate carboxylase component suggestive of plant metabolism.



S17 - Whole-plant physiology parameters and root growth measured as a function of three nitrogen growth regimes. Panel A: radiographic images depicting allocation patterns of ^{11}C-photoassimilates across the entire plant 1 hr after tracer administration. Color sealing reflects red-to-blue for high-to-low levels of radioactivity. Panel B: allocation of ^{11}C-photoassimilates belowground 1 hr after tracer administration and based on percentage of fixed ^{11}C. Panel C: root exudation of ^{11}C-photoassimilates 1 hr after tracer administration and based on percentage of belowground allocation. Panel D: root biomass presented in grams of fresh weight. All bars are mean values +/- SE. Statistical significance across the growth regimes is shown by the P-values.

Table S1 - Growth promotion parameters for the various *S. viridis* genotypes screened under the no-nitrogen (no-N) and low-nitrogen (Low-N) condition (i.e., 0.5mM KNO_3) for uninoculated (Uninoc) and inoculated plants (Inoc).

		Root length (cm)		Lateral root number		Root fresh Weight (g)		Root dry weight (g)		Plant height (cm)		Seeds number		Inflorescence length (mm)		Shoot fresh weight (g)		Shoot dry weight (g)	
		Uninoc	**Inoc**	**Uninoc**	**Inoc**	**Uninoc**	**Inoc**	**Uninoc**	**Inoc**	**Uninoc**	**Inoc**	**Uninoc**	**Inoc**	**Uninoc**	**Inoc**	**Uninoc**	**Inoc**	**Uninoc**	**Inoc**
A10.1	**No-N**	241.9	311.1	826.3	1154.0	0.030	0.033	0.005	0.006	5.4	5.7	1.2	2.2	6.7	7.6	0.014	0.017	0.007	0.008
	Low-N	893.1	902.8	2770.0	2393.9	0.190	0.194	0.043	0.043	12.7	11.9	10.2	13.0	16.1	16.9	0.074	0.096	0.044	0.047
Ahart	**No-N**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Low-N	515.4	271.0	877.3	456.2	0.326	0.304	0.024	0.020	3.2	4.9	8.3	12.4	6.7	12.2	-	-	0.047	0.054
Berlim	**No-N**	254.4	402.9	1426.4	1989.3	0.037	0.068	0.005	0.009	5.2	7.0	NP	NP	NP	NP	0.024	0.044	0.007	0.013
	Low-N	967.5	1093.1	2670.1	3288.7	0.360	0.347	0.048	0.048	14.9	16.7	NP	NP	NP	NP	0.330	0.381	0.088	0.108
EstepME013	**No-N**	327.4	317.6	1722.5	1669.6	0.032	0.038	0.013	0.007	6.3	6.8	NP	NP	3.1	2.7	0.012	0.012	0.012	0.011
	Low-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EstepME017	**No-N**	198.9	310.2	948.5	1127.7	0.016	0.037	0.003	0.005	7.5	8.1	NP	NP	NP	NP	0.005	0.008	0.005	0.008
	Low-N	795.7	914.9	1978.8	2269.8	0.232	0.242	0.024	0.025	11.3	12.9	12.8	14.2	14.8	17.8	0.068	0.080	0.057	0.071
EstepME028V	**No-N**	296.3	523.1	2105.4	2104.6	0.024	0.050	0.013	0.021	5.3	6.9	0.9	2.0	-	-	0.010	0.017	0.010	0.017
	Low-N	825.5	995.2	2463.0	3010.6	0.294	0.331	0.062	0.059	13.3	12.5	11.3	8.3	-	-	0.051	0.061	0.065	0.059
EstepME034	**No-N**	249.5	334.4	1127.0	1203.9	0.016	0.037	0.006	0.010	6.7	7.2	NP	NP	4.7	6.1	0.013	0.019	0.006	0.007
	Low-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EstepME035	**No-N**	129.4	119.7	384.0	445.5	0.023	0.006	0.002	0.002	-	-	NP	NP	NP	NP	0.008	0.009	0.005	0.006
	Low-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EstepME044	**No-N**	200.2	242.7	704.0	708.8	0.021	0.016	0.004	0.005	6.7	7.2	0.8	0.8	5.9	6.9	0.006	0.006	0.005	0.006
	Low-N	662.5	586.1	1589.8	1635.4	0.123	0.110	0.016	0.014	13.3	13.0	15.3	9.5	15.9	13.3	0.049	0.039	0.045	0.035
EstepME046	**No-N**	232.7	292.5	818.0	1015.0	0.026	0.028	0.006	0.006	7.4	8.8	1.0	0.9	6.5	7.2	0.007	0.008	0.007	0.007
	Low-N	806.0	729.5	1847.6	1726.2	0.202	0.150	0.026	0.022	17.2	17.4	20.4	16.4	21.9	20.9	0.081	0.090	0.074	0.062
EstepME051	**No-N**	211.0	203.5	1176.8	1000.6	0.021	0.024	0.004	0.004	5.0	5.1	NP	NP	NP	NP	0.010	0.009	0.007	0.007
	Low-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kellogg 1137	**No-N**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Low-N	390.3	541.3	767.0	1236.0	0.215	0.258	0.034	0.053	14.5	15.9	NP	NP	NP	NP	0.271	0.348	0.073	0.095
Kellogg 1186	**No-N**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Low-N	442.6	845.5	897.4	5359.2	0.251	0.330	0.040	0.056	9.8	12.0	NP	NP	NP	NP	0.208	0.275	0.060	0.077
Roche 10106	**No-N**	351.8	392.7	738.5	846.0	0.042	0.062	0.010	0.012	-	-	NP	NP	NP	NP	0.057	0.050	0.024	0.023
	Low-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vela88	**No-N**	401.9	515.8	847.8	1240.4	0.068	0.113	0.013	0.023	-	-	NP	NP	NP	NP	0.034	0.060	0.022	0.032
	Low-N	922.3	703.5	1978.8	1505.0	0.378	0.159	0.039	0.044	-	-	NP	NP	NP	NP	0.188	0.210	0.055	0.059

Table S1 - Growth promotion parameters for the various *S. viridis* genotypes screened under the no-nitrogen (no-N) and low-nitrogen (Low-N) condition (i.e., 0.5mM KNO_3) for uninoculated (Uninoc) and inoculated plants (Inoc).

		Root length (cm)		Lateral root number		Root fresh Weight (g)		Root dry weight (g)		Plant height (cm)		Seeds number		Inflorescence length (mm)		Shoot fresh weight (g)		Shoot dry weight (g)	
		Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc
Waselkov Momence	**No-N**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Low-N	637.5	582.3	1136.5	1261.5	0.108	0.071	0.047	0.042	19.4	15.1	NP	NP	NP	NP	0.205	0.179	0.082	0.060
WaselkovRockFall	**No-N**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Low-N	627.9	607.6	1423.5	2802.4	0.128	0.211	0.051	0.032	22.7	19.8	NP	NP	NP	NP	0.213	0.226	0.078	0.059
Waselkov Vandalia	**No-N**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Low-N	688.8	721.7	1579.8	3796.7	0.135	0.260	0.052	0.054	13.6	13.8	NP	NP	NP	NP	0.219	0.182	0.071	0.068
Vela 86	**not enought to statistics**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
EsteME015	**not enought to statistics**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
EstepME025V	**not enought to statistics**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
EstepME026	**not enought to statistics**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
EstepME032V	**not enought to statistics**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
1253-1	**plants died**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
Thompson	**plants died**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
EstepME043	**plants died**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
EstepME019	**plants died**	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na

NP= no production of seeds
na=not applicab

6. RESULTADOS PARCIAIS: Transcriptoma da planta modelo *Setaria viridis* inoculada com *Azospirillum brasilense* estirpe HM053.

Para complementar o estudo da interação *Setaria viridis* - *A. brasilense*, foi realizado o transcriptoma (RNA-seq) de raízes de *Setaria viridis* (var. A10.1), coletadas 5 dias após a inoculação com as estirpes FP2 (Nif^+), FP10 (Nif^-), HM053 (Nif^e) e plantas controle, inoculadas com a cultura fervida de *A. brasilense* por 15 minutos a 100 °C. As sementes de *S. viridis* foram desinfectadas com solução de hipoclorito 6% e tween 20, germinadas (dois dias no escuro e um dia no claro a 30 °C), inoculadas com 10^7 células de *A. brasilense* pré-lavadas por centrifugação a 5000 rpm e transferidas para potes contendo o meio turface e vermiculita (3:1). Como foram utilizadas plantas jovens para o RNA-seq, estas foram regadas apenas com água. Para as avaliações fenotípicas foram utilizadas as mesmas condições como descrito acima, exceto que as plantas foram coletadas 30 dias após a inoculação e foram regadas com 50 ml de solução Hoagland's enriquecido com 0,5 mM KNO_3 duas vezes na semana (FIGURA 1).

O RNA da raiz foi extraído segundo o protocolo de Zhang e colaboradores (2014) (dados não publicados). A cauda poli-A do RNAm foi purificada a partir do RNA total e foram feitas as construções das bibliotecas de cDNA, no DNA Core Facility (University of Missouri). Nesta etapa foi utilizado NEBNext Ultra Directional RNA sample preparation kit. Em seguida, as bibliotecas foram sequenciadas no Illumina HiSeq 2000, a corrida foi "*single end*" e foram lidos 100 base por comprimento (FIGURA 2). Assim, foram geradas doze bibliotecas (quatro diferentes condições, como descrito acima, em triplicatas biológicas) com uma média total de 26 milhões de sequencias mapeadas apenas uma vez no genoma de *S. italica* (Phytozome version 10.0). Em seguida, as sequencias foram pré-processadas através do programa Trimmomatic v. 0.32 (BOLGER et al., 2014), sendo utilizadas sequências com no mínimo 50 pares de base. As sequências foram alinhadas através do programa TopHat v.2.0.12 (TRAPNELL et al., 2009). Para a análise dos genes expressos foi utilizado o programa Cufflinks v.2.2.1 (TRAPNELL et al., 2010) e os fragmentos por kilobase por milhão de sequências mapeadas (FPKM) foram contados utilizando o programa htseq-count v.0.5.4p5 (ANDERS; PYL, HUBER et al., 2014) (FIGURA 2). O trabalho de bioinformática foi realizado em colaboração com o estudante de doutorado da Universidade de Missouri – EUA, Ning Zhang.

Os dados brutos do sequenciamento estão apresentados na tabela 1 e através da análise de correlação de Pearson foi verificado um alto coeficiente de correlação entre os FPKM das

triplicatas biológicas, o que indica que as repetições apresentaram boa reprodutibilidade ($R^2 \geq$ 0.98), exceto a biblioteca de plantas inoculadas com a estirpe FP10 ($R^2 \geq 0,95$) (TABELA 2). Na figura 3 foram selecionadas replicatas biológicas para ilustrar algumas correlações descritas na tabela 2.

Foram expressos 35.470 genes, sendo os diferencialmente expressos obtidos a partir da correlação de dois softwares EdgeR (3.6.8) e Cuffdiff (v.2.2.1), considerando um valor-p < 0,05 (FIGURA 4). A partir destes resultados foi possível comparar as condições e selecionar os genes com variação de expressão (ou *fold change*) maior ou igual a 1 (considerados genes induzidos), enquanto os genes com variação de expressão menor ou igual a -1, reprimidos. Os valores de *fold change* foram calculados a partir da função de $\log_2$, e os valores já transformados estão apresentados na tabela 3.

O maior número de genes diferencialmente expressos (664 genes) foi obtido a partir da comparação entre plantas inoculadas com o mutante HM053 e o controle (acrônimo HM053 x C), sendo 598 genes induzidos e 66 reprimidos (TABELA 3). Entre plantas controle e plantas inoculadas com a estirpe selvagem FP2 (FP2 x C), foram encontrados 186 genes diferencialmente expressos contendo 89 genes induzidos contra 97 genes reprimidos (TABELA 3). O número de genes diferencialmente expressos na biblioteca de plantas inoculadas com a estirpe FP10 (Nif$^-$) foi de apenas 13 genes (5 induzidos e 8 reprimidos) e foi considerado insuficiente. Nova análise estatística será realizada.

Nesta tese, serão focadas as anotações de algumas proteínas obtidas através dos genes diferencialmente expressos entre as plantas controle e plantas inoculadas com as estirpes FP2 e HM053. Posteriormente para a publicação serão feitas as comparações entre todas as condições.

A partir da classificação dos genes pelo Gene Ontology (GO), um banco de dados que padroniza a representação dos genes e a anotação funcional desses considerando a homologia entre as diferentes espécies, foi realizada a análise de enriquecimento separando assim os genes em três diferentes grupos: Processos Biológicos, Componentes Celulares e Função Molecular (FIGURA 5). Em plantas inoculadas com o mutante HM053 foram identificados 72.46% de genes relacionados com processos biológicos e 27.5% relacionados com função molecular. Não foram encontrados genes diferencialmente expressos relacionados com componentes celulares como apoplasto, membrana e tilacóide, entre plantas controles e plantas inoculadas com o mutante HM053 (FIGURA 5). Já para plantas inoculadas com a estirpe selvagem FP2 foram encontrados 15.69% dos genes diferencialmente expressos relacionados com componentes celulares, 13.30% relacionados com função molecular e

71.01% de genes foram relacionados com processos biológicos (FIGURA 5). De alguma maneira ainda não esclarecida, as plantas inoculadas com a estirpe selvagem FP2 foram capazes de modificar significativamente os componentes celulares de *S. viridis* enquanto para o mutante HM053 a resposta à inoculação não foi capaz de modificar significativamente a expressão de genes desta categoria ($p < 0.05$). Alguns genes relacionados com processos biológicos e função molecular estão apresentados na figura 6. Em geral, o número de genes diferencialmente expressos na biblioteca de *S. viridis* inoculadas com o mutante HM053 foi superior ao número de genes expressos na biblioteca de *S. viridis* inoculadas com a estirpe selvagem FP2. Isso sugere que o mutante HM053 teve uma maior capacidade de afetar a expressão de genes de processos biológicos e funções moleculares de *S. viridis* quando comparado à estirpe selvagem FP2 (FIGURA 6).

O programa Mapman é capaz de categorizar os genes e gerar figuras, como as figuras 7 e 8, que representam os genes relacionados ao estresse biótico e abiótico. Para esta análise foi utilizado como arquivo de entrada o conjunto de genes diferencialmente expressos com variação da expressão maior ou igual a 1 ou menor ou igual a -1 (valores de $\log_2$ transformados). Nesta categoria de classificação, para a biblioteca de *S. viridis* inoculada com o mutante HM053, foram encontrados 45 genes regulados (42 induzidos e somente 3 reprimidos) (FIGURA 7). Já na biblioteca de *S. viridis* inoculada com a estirpe selvagem FP2, foram encontrados 29 genes regulados, sendo 24 induzidos e 5 reprimidos (FIGURA 8). Estes genes estão relacionados com a sinalização por:

1) Hormônio (foram encontrados 7 genes induzidos na biblioteca de *S. viridis* inoculada com HM053 e apenas 1 gene induzido na biblioteca de *S. viridis* inoculada com a FP2); **2) Parede celular** (3 genes induzidos na condição inoculada com HM053); **3) Proteólise** (5 genes induzidos nas plantas inoculadas com HM053 e 1 reprimido para FP2); **4) Sinalização** (6 genes induzidos para HM053 e 3 genes induzidos para FP2); **5) Fatores de transcrição** (6 induzidos na condição inoculada com HM053 e 1 reprimido para condição inoculada com FP2); **6) Estado redox** (2 genes induzidos para ambas as bibliotecas); **7) Metabolismo secundário** (3 genes induzidos e 2 reprimidos na biblioteca HM053, 1 gene induzido e 2 reprimidos na biblioteca FP2) (FIGURAS 7 e 8).

O fator de transcrição WRKY foi induzido tanto na biblioteca *S. viridis* inoculada com o mutante HM053 (2 genes induzidos) quanto na biblioteca *S. viridis* inoculada com a estirpe selvagem FP2 (1 gene induzido) (FIGURAS 7 e 8). Este fator se caracteriza pela presença de motivos de aminoácidos altamente conservados na região N-terminal (WRKYGQK) e um dedo de zinco na região C-terminal (MANGELSEN et al., 2008) e desempenha um papel

importante no mecanismo de processos de resistência a doenças, sinalização hormonal, resposta a stresse biótico e abiótico, processos de desenvolvimento e senescência (KIM et al., 2008; LAI et al., 2008; NAOUMKINA et al., 2008; ZHANG et al., 2008) (FIGURAS 7 e 8).

Entre os genes relacionados a estresse abiótico foram encontrados 8 genes induzidos para a biblioteca de *S. viridis* inoculada com a FP2 e 6 para a biblioteca de *S. viridis* inoculada com o HM053, sendo 1 destes genes reprimidos (FIGURA 7 e 8). Entre os genes induzidos, para ambas as bibliotecas foram encontradas as proteínas de choque térmico Hsp20 (6 genes na biblioteca de plantas inoculadas com a estirpe selvagem FP2 e 2 genes na biblioteca inoculada com o mutante HM053), Hsp70 (1 gene na biblioteca FP2 e 3 genes na biblioteca HM053) e a Hsp90 (1 gene identificado apenas na biblioteca de plantas inoculadas com a FP2). As proteínas de choque térmico da família Hsp são divididas em proteínas de choque térmico de alta massa molecular (200-800kDa), como a Hsp100, Hsp90, Hsp70/DnaK e Hsp60/GroE, e em proteínas de choque térmico de baixa massa molecular (16-42kDa), como a Hsp20 e sHsps. Essas proteínas estão relacionadas à resistência a fatores de estresse ambiental, como estresse ao calor, e possuem a função molecular de chaperona auxiliando no dobramento de outras proteínas em suas formas nativas tanto em condições de estresse como em condições normais. Nesta última condição as proteínas Hsps têm uma função importante em prevenir a agregação irreversível e a desnaturação da protéica (CASHIKAR et al., 2005; HASLBECK et al., 2005; LEE; VIERLING, 2000). Assim, essas proteínas são importantes para o desenvolvimento normal da planta, uma vez que as altas temperaturas influenciam negativamente o seu crescimento e produtividade (GROVER et al., 1991). O único gene reprimido dentro dessa via, encontrado na biblioteca de plantas inoculadas com o HM053, codifica a proteína *germin-like protein* (GLP) que está presente em diferentes tecidos da planta como raiz, folhas e flores (LI et al., 2010; DUNWELL et al., 2008; BHATTACHARJEE, 2005). A GLP tem um papel importante na resposta a defesa. Em condições de estresse, como ataque de microrganismos e insetos patógenos, estresse a seca, estresse a toxinas e pressões salinas, ocorre o aumento da expressão do gene que codifica esta proteína. Outra função da GLP é de oxalato oxidase e superóxido dismutase, enzimas que podem gerar espécies reativas de oxigênio, como H_2O_2, influenciando na defesa da planta. Outras atividades da GLP relacionada a defesa das plantas incluem a ADP glicose pirofosfatase/fosfodiesterase (RODRIGUEZ-LOPES et al., 2001) e inibidores de serina protease (SEGARRA et al., 2003). Assim, pode-se concluir que *A. brasilense,* por não ser uma bactéria patógena, não induziu este gene específico de defesa em plantas de *S. viridis*, o que pode indicar que existe um sistema de reconhecimento planta-patógeno.

A espécie *A. brasilense* não é capaz de induzir a formação de nódulos nas raízes de *S. viridis.* No entanto, a proteína *nodulin* MtN21, que está relacionada com os estágios iniciais de formação dos nódulos, foi induzida em ambas as bibliotecas (TABELA 4). A proteína *nodulin* está relacionada também com a resposta morfogênica e organogênica da planta ao processo de colonização pelas bactérias (PERLICK; PÜHLER, 1993). As respostas morfogênicas e organogênicas são induzidas na presença de auxina e citocinina, hormônios estes produzidos por *A. brasilense* (APPLEWHITE et al., 1994). Esta proteína foi anotada em *M. truncatula* e tem similaridade com 5NG4, membro da família MtN21, que está relacionada com a formação das raízes adventícias (BUSOV et al., 2004). Isto sugere que *A. brasilense* pode estimular a formação de raízes laterais em *S. viridis.* Resultado este observado nas avaliações fenotípicas, realizadas nas mesmas condições, e que serão discutidas posteriormente.

Esses foram alguns exemplos de proteínas encontradas na tabela de dados gerada nos ensaios de RNA-seq e no programa Mapman. Uma análise mais detalhada e aprofundada dos resultados do transcriptoma de *S. viridis* inoculada com *A. brasilense* está em andamento e será apresentada no terceiro artigo vinculado a esta tese de doutorado.

Para os experimentos de promoção de crescimento foram analisados: massa seca da parte aérea e da raiz, número de raízes laterais, comprimento da raiz e parte aérea, área foliar e número de sementes. A partir desses resultados foi possível concluir que as três estirpes de *A. brasilense* foram capazes de promover o crescimento vegetal de *S. viridis* e que essa habilidade não é restrita apenas a aquelas capazes de fixar nitrogênio (TABELA 5). A inoculação com a estirpe FP10 também modificou a arquitetura da planta, sendo observado maior número de raízes laterais quando comparado as plantas controle. Este resultado é esperado uma vez que *A. brasilense* é capaz de produzir fitormônios (como AIA, ácido indolbutírico, citocinina e giberilina) tanto em cultura quanto em associação com a planta estimulando o seu crescimento (CROZIER et al., 1988, MARTÍNEZ-MORALES et al., 2003; BOTTINI et al., 1989, TIMMUSK et al., 1999; CASSÁN et al., 2001 a, b; JAZEN et al., 1992; BOTTINI et al., 2004). Além disso, foi demonstrado que a inoculação com *Azospirillum* spp. ou aplicação de hormônios induziu a proliferação de raízes laterais e pêlos (FULCHIERI et al., 1993, GLICK 1995, EL-KHAWAS, ADACHI, 1999). O mutante HM053 e a estirpe selvagem FP2, diferentemente da estirpe FP10, foram capazes de promover o aumento de 33,2% do comprimento da raiz e 23,9%, respectivamente. Aumento significativo na massa seca da raiz também foi observado para essas duas estirpes (TABELA 5). O número de sementes produzidas em solo turface com 0,5 mM de nitrogênio foi extremamente baixo

(média de 45 sementes por planta) (TABELA 5) quando comparado com plantas de *S. viridis* crescidas em solo rico em nitrogênio (média de aproximadamente 13.000 sementes por planta) (BRUTNELL et al., 2010). Isso indica que a limitação de nitrogênio afetou muito a produtividade de grãos, mesmo assim plantas inoculadas com o mutante excretor de amônio HM053 apresentaram 33,9% de aumento na produção de sementes (TABELA 5). Outros autores também encontraram resultados positivos da inoculação de *A. brasilense* em plantas de *Setaria italica,* espécie filogeneticamente próxima da *S. viridis*, corroborando os nossos dados (COHEN et al., 1980; OKON, et al., 1983; KAPULNIK, et al., 1981).

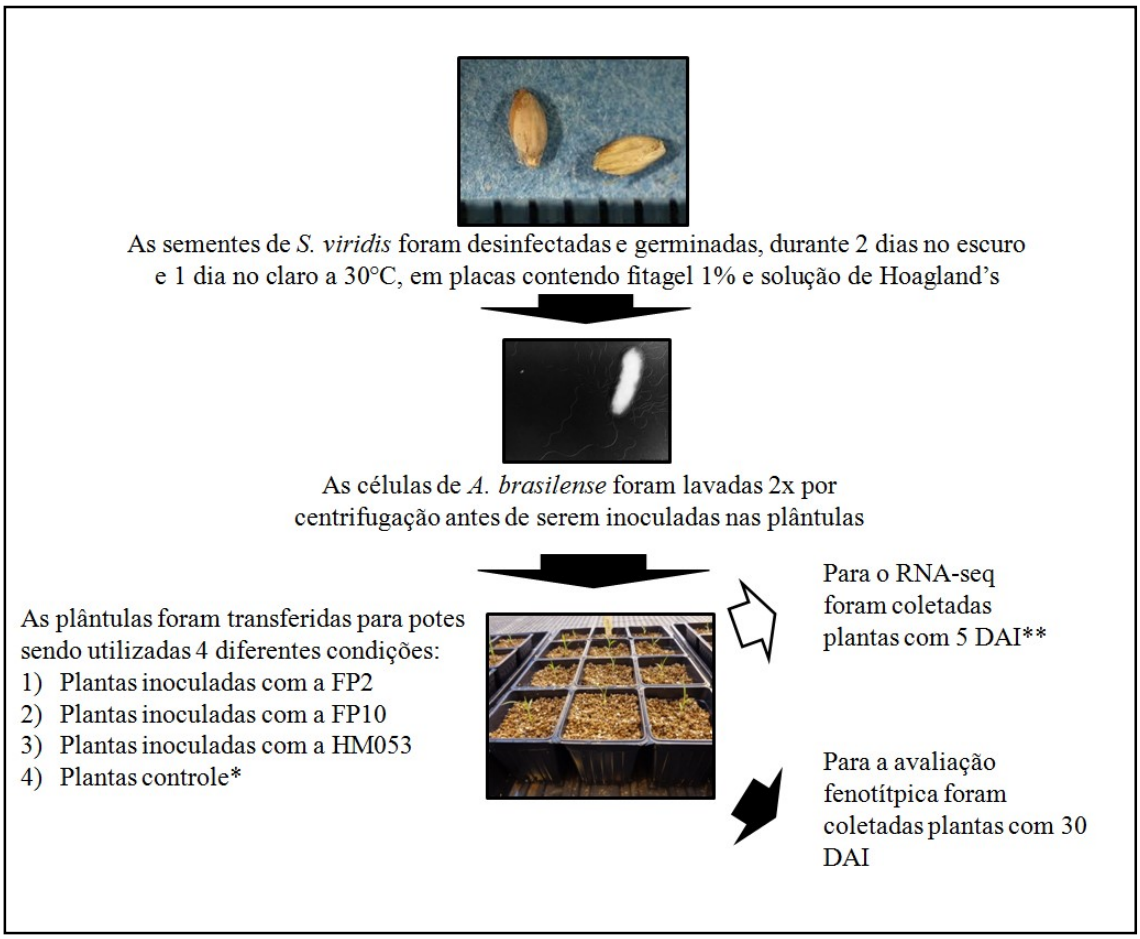


FIGURA 1. FLUXOGRAMA DOS EXPERIMENTOS REALIZADOS PARA O RNA-SEQ E AVALIAÇÃO FENOTÍPICA.
*AS PLANTAS CONTROLE FORAM INOCULADAS COM A CULTURA DE *A. brasilense* FERVIDA POR 15 MINUTOS A 100°C.
** DAI: DIAS APÓS A INOCULAÇÃO

As raízes foram maceradas em nitrogênio líquido e o RNA foi extraído segundo o protocolo de Quan Zhang (2014) (Dados não publicados)

As bibliotecas de cDNA foram construídas a partir do mRNA purificado (sem a cauda poli-A) segundo o protocolo do fabricante (NEBNext Ultra Directional RNA samples preparation Kit)

As amostras foram seqüenciadas segundo o protocolo do Illumina Hiseq 2000

Foram geradas 12 bibliotecas
(4 diferentes condições em triplicatas biológicas)

As sequências foram pré-processadas (Trimmomatic v 0.32)
sendo utilizadas sequências com no mínimo 50 pb

As sequencias foram alinhadas através do TopHat v 2.0.12 .
O genoma de referência utilizado foi o da *S. italica* (a partir do Phytozome v 10.0)

Para análise dos genes expressos foi utilizado o programa Cufflinks (v.2.2.1) e o FPKM foi determinado utilizando o programa htseq-count (0.5.4p5)

Os genes diferencialmente expressos foram analisados utilizando 2 programas:
Cuffdiff (v2.2.1) e o EdgeR (3.6.8)

FIGURA 2. ESTRATÉGIA UTILIZADA PARA A OBTENÇÃO DA BIBLIOTECA DE cDNA E AS ANÁLISES DE BIOINFORMÁTICAS UTILIZADAS A PARTIR DO DADOS DE RNA-SEQ.

TABELA 1. QUANTIDADE DE SEQUÊNCIAS BRUTAS E MAPEADAS NO GENOMA DE *S. viridis* EM QUATRO DIFERENTES CONDIÇÕES: PLANTAS CONTROLE, PLANTAS INOCULADAS COM A ESTIRPE FP10 (NIF^-), HM053 (NIF^C) E FP2 (NIF^+) DE *A. brasilense.*

	Controle*	**FP10***	**HM053***	**FP2***	**Média****
Número de sequências (bruto)	53,380,621.0	51,205,725.7	57,748,123.3	56,815,112.0	54,787,395.5
Número de sequências mapeados no genoma	44,236,225.7	43,742,065.0	47,978,109.7	46,416,846.0	45,593,311.6
Porcetagem de sequências mapeadas no genoma	95,5%	96,1%	96,1%	95,9%	95,9%
Número de sequências únicas mapeadas no genoma	26,246,967.3	24,176,518.3	28,043,900.3	27,102,793.0	26,392,544.7
Número de múltiplas sequências	17,989,258.3	19,565,546.7	19,934,209.3	19,314,053.0	19,200,766.8
Porcetagem de única sequências	59,3%	55,6%	58,6%	58,4%	58,0%

* Os valores representam a média das triplicatas biológicas.

** Média entre as quatro condições.

TABELA 2. VALORES DA CORRELAÇÃO DE PEARSON DOS FRAGMENTOS POR KILOBASE, POR MILHÃO DE SEQUÊNCIAS MAPEADAS (FPKM) ENTRE AS TRIPLICATAS BIOLÓGICAS DOS GENES EXPRESSOS A PARTIR DAS QUATRO BIBLIOTECAS DE RNA-SEQ: **1)** *S. viridis* INOCULADAS COM A CULTURA DE *A. brasilense* FERVIDA (CONDIÇÃO CONTROLE); **2)** BIBLIOTECA FP2; **3)** BIBLIOTECA HM053 4**)** BIBILIOTECA FP10. A ANÁLISE DOS GENES EXPRESSOS FOI REALIZADA ATRAVÉS DO PROGRAMA CUFFLINKS E OS FPKM CONTADOS ATRAVÉS DO HTSEQ-COUNT.

FPKM 1)	**Controle_B**	**Controle_C**
Controle_A	0.99	0.99
Controle_B		0.99

FPKM 2)	**FP2_B**	**FP2_C**
FP2_A	0.99	0.99
FP2_B		0.99

FPKM 3)	**HM053_B**	**HM053_C**
HM053_A	0.99	0.98
HM053_B		0.99

FPKM 4)	**FP10_B**	**FP10_C**
FP10_A	0.96	0.95
FP10_B		0.99

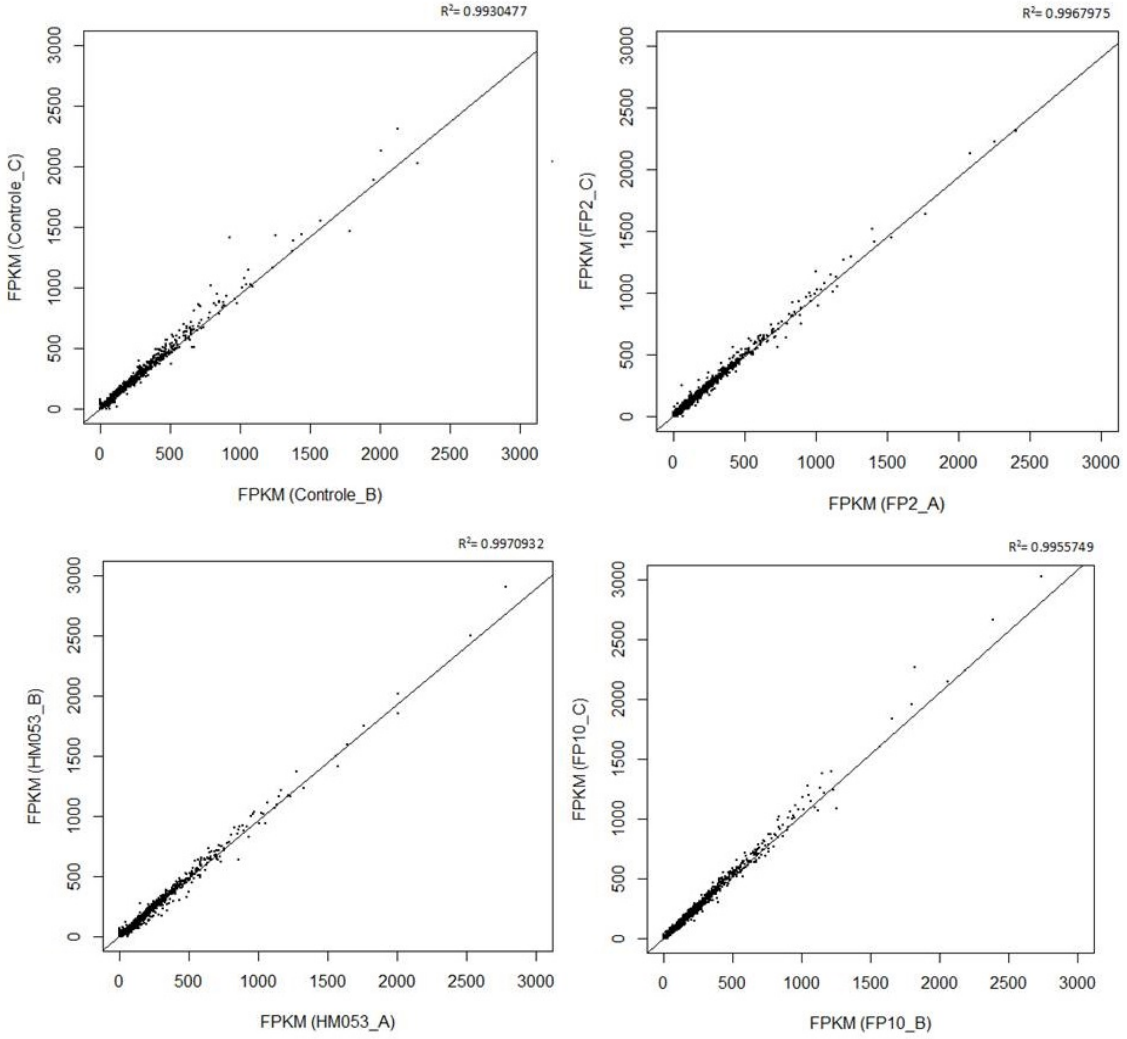


FIGURA 3. CORRELAÇÃO DE PEARSON DOS FPKM* DAS BIBLIOTECAS DE *S. viridis* INOCULADAS COM *A. brasilense* estirpes FP2, HM053 e FP10. FORAM ESCOLHIDOS 2 VALORES REPRESENTATIVOS ENTRE AS TRIPLICATAS BIOLÓGICAS PARA GERAR OS GRÁFICOS ACIMA.
*FPKM: FRAGMENTOS POR KILOBASE, POR MILHÃO DE SEQUÊNCAIS MAPEADAS

TABELA 3. NÚMERO DOS GENES DIFERENCIALMENTE EXPRESSOS ENTRE AS BIBLIOTECAS INOCULADAS COM AS TRÊS ESTIRPES DE *A. brasilense* EM RELAÇÃO AO CONTROLE: FP10 X C; HM053 X C E FP2 X C. DENTRE OS GENES DIFERENCIALMENTE EXPRESSOS ($p < 0.05$) FORAM SELECIONADOS OS GENES REPRIMIDOS (FC* < -1) E INDUZIDOS (FC > 1).

Condições	Número total de genes diferencialmente expressos	Induzidos	Reprimidos
FP10 x Controle	13	5	8
HM053 x Controle	664	598	66
FP2 x Controle	186	89	97

FC: Fold change ou valor da expressão foi calculado a partir da função logarítma $\log_2$.

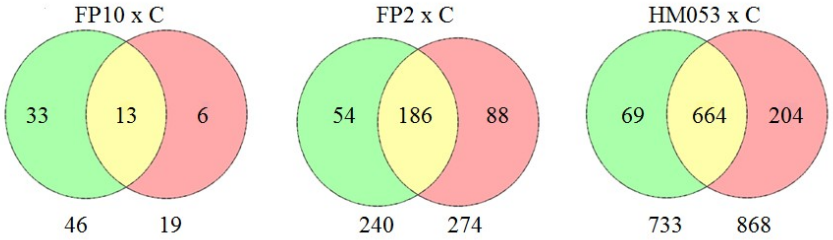


FIGURA 4. NÚMERO TOTAL DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE PLANTAS CONTROLE E PLANTAS INOCULADAS COM *A. brasilense.* EM AMARELO ESTÃO REPRESENTADAS AS INTERSEÇÕES ENTRE OS GENES DIFERENCIALMENTE EXPRESSOS OBTIDOS A PARTIR DA ANÁLISE DE DOIS SOFTWARES EDGER (V.3.6.8) EM VERMELHO E CUFFDIFF (V.2.2.1) EM VERDE. FOI UTILIZADO COMO PONTO DE CORTE $p < 0.05$ E FOLD CHANGE (FC) < -1 OU (FC) > 1.
FP10 x C: REPRESENTA A BIBLIOTECA DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE PLANTAS INOCULADAS COM A ESTIRPE FP10 (Nif^-) E PLANTAS CONTROLE.
FP2 x C: REPRESENTA A BIBLIOTECA DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE PLANTAS INOCULADAS COM A ESTIRPE SELVAGEM FP2 (Nif^+) E PLANTAS CONTROLE.
HM053 x C: REPRESENTA A BIBLIOTECA DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE PLANTAS INOCULADAS COM A ESTIRPE MUTANTE HM053 (Nif^C) E PLANTAS CONTROLE.

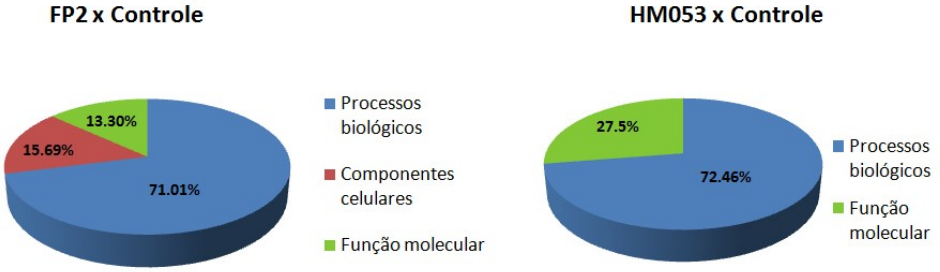


FIGURA 5. ANÁLISE DE ENRIQUECIMENTO DOS GENES DIFERENCIALMENTE EXPRESSOS ENTRE PLANTAS INOCULADAS COM AS ESTIRPES FP2 E HM053 DE *A. brasilense* EM RELAÇÃO AO CONTROLE. OS GENES FORAM CLASSIFICADOS EM PROCESSOS BIOLÓGICOS, COMPONESTES CELULARES E FUNÇÃO MOLECULAR PELO GENE ONTOLOGY (GO).

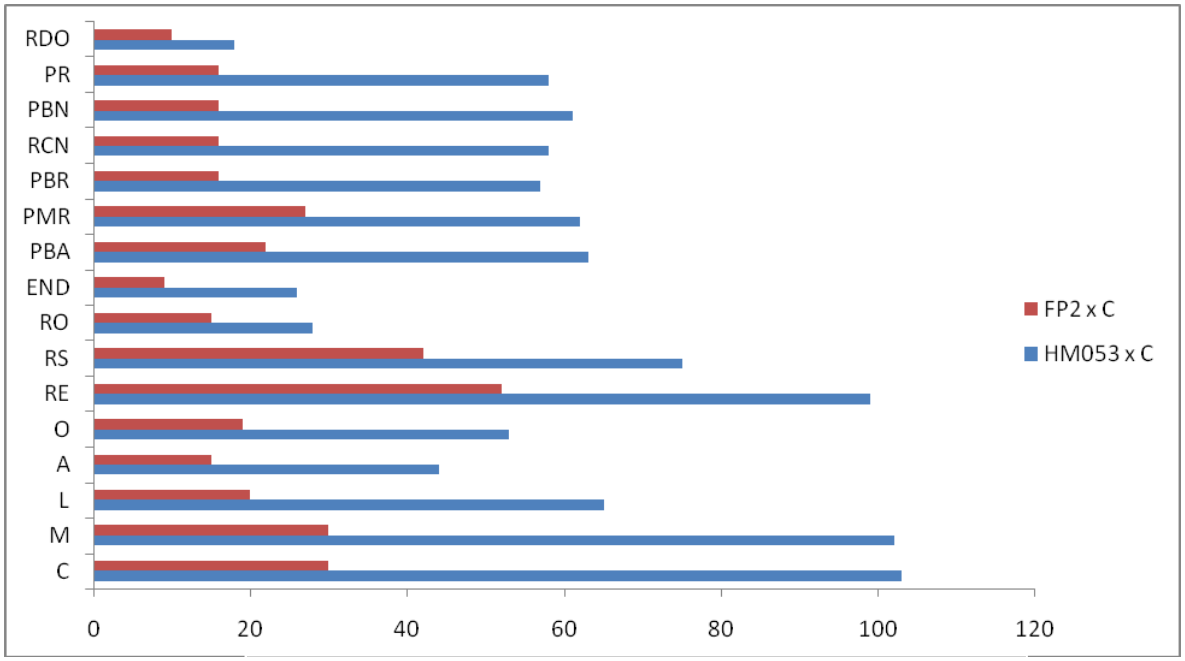


FIGURA 6. CLASSIFICAÇÃO E NÚMEROS DOS GENES DIFERENCIALMENTE EXPRESSOS A PARTIR DA ANÁLISE DE ENRIQUECIMENTO DO GENE ONTOLOGY (GO). **RDO:** RESPOSTA DE DEFESA A OUTROS ORGANISMOS; **PR:** PROCESSOS DE REGULAÇÃO DE METABOLISMOS DE RNA; **PBN:** PROCESSO BIOSSINTÉTICO DE COMPONENTES DE NITROGÊNIO; **RCN:** REGULAÇÃO DE COMPONENTES METABÓLICOS DE NITROGÊNIO; **PBR:** PROCESSOS BIOSINTÉTICO DE REGULAÇAO; **PMR:** PROCESSOS METABÓLICOS DE REGULAÇÃO; **PBA:** PROCESSOS BIOSSINTÉTICO DE COMPOSTOS AROMÁTICOS; **END:** RESPOSTA A ESTÍMULO ENDÓGENO; **RO:** RESPOSTA A SUBSTÂNCIA ORGÂNICA; **RS:** RESPOSTA A ESTRESSE; **RE:** RESPOSTA A ESTÍMULOS; **O:** ATIVIDADE DE OXIREDUTASE; **A:** ATIVIDADE DE TRANSCRIÇÃO E LIGAÇÃO A ÁCIDOS NUCLÉICOS; **L:** LIGAÇÃO AO DNA; **M:** LIGAÇÃO A ÍONS METAL; **C:** LIGAÇÃO A ÍONS CÁTIONS.
FP2 x C: REPRESENTA A BIBLIOTECA DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE PLANTAS INOCULADAS COM A ESTIRPE SELVAGEM FP2 E PLANTAS CONTROLE.
HM053 x C: REPRESENTA A BIBLIOTECA DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE PLANTAS INOCULADAS COM A ESTIRPE MUTANTE HM053 E PLANTAS CONTROLE.

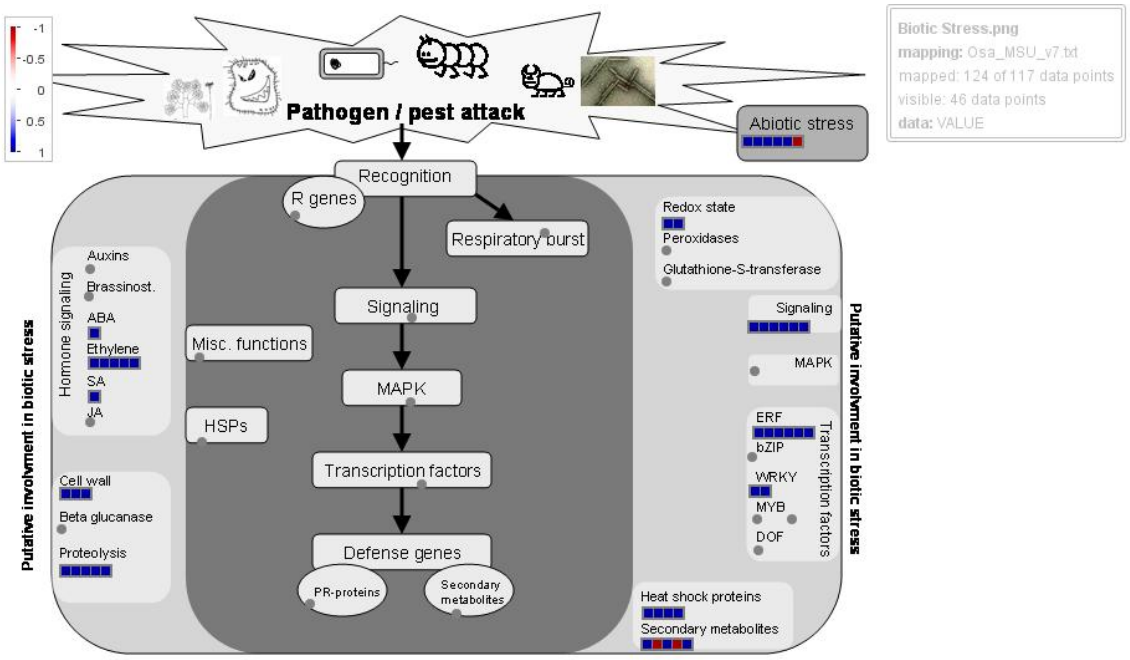


FIGURA 7. GENES DIFERENCIALMENTE EXPRESSOS REGULADOS NA BIBLIOTECA DE *S. viridis* INOCULADA COM O MUTANTE DE *A. brasilense* HM053. OS GENES ENVOLVIDOS COM ESTRESSE BIÓTICO E ABIÓTICO ESTÃO REPRESENTADOS NO CAMPO CINZA ESCURO, SENDO OS GENES ENCONTRADOS NO CONJUNTO DE DADOS DESSA BIBLIOTECA ASSINALADOS EM AZUL (GENES INDUZIDOS) OU EM VERMELHO (GENES REPRIMIDOS). ENQUANTO NO CAMPO CINZA CLARO ESTÃO REPRESENTADAS AS POSSÍVEIS FUNÇÕES GÊNICAS. OS GENES TRANSCRITOS COM FOLD CHANGE (FC) < - 1 OU (FC) > 1 FORAM CATEGORIZADOS ATRAVÉS DO SOFTWARE MAPMAN.

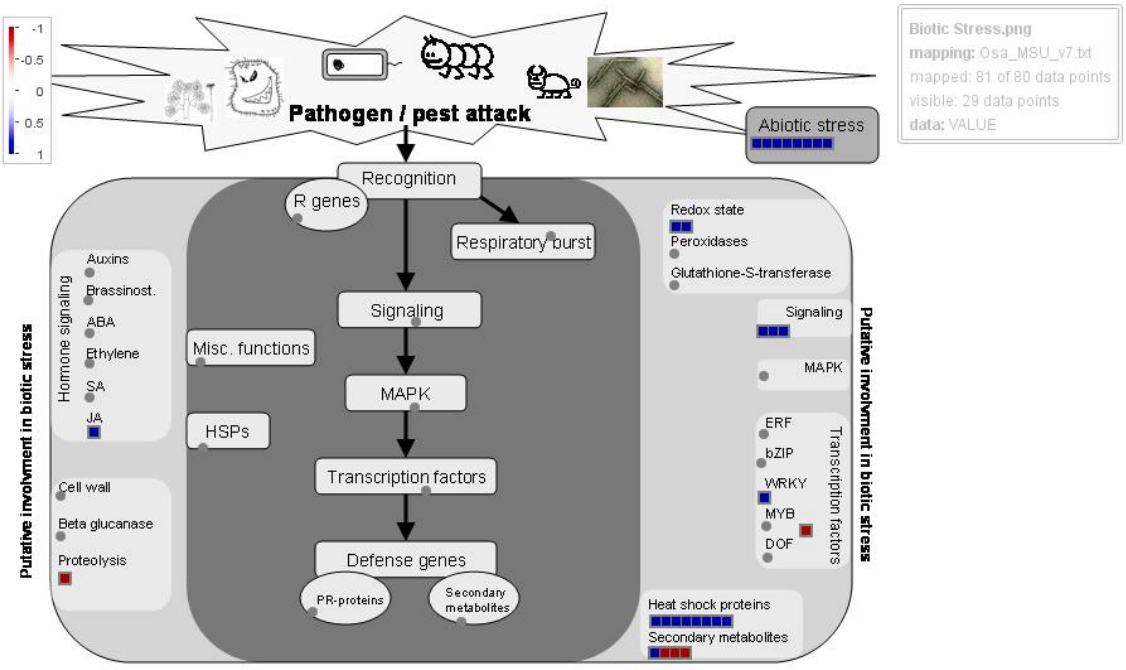


FIGURA 8. GENES DIFERENCIALMENTE EXPRESSOS REGULADOS NA BIBLIOTECA DE *S. viridis* INOCULADA COM *A. brasilense*, ESTIRPE SELVAGEM FP2. OS GENES ENVOLVIDOS COM ESTRESSE BIÓTICO E ABIÓTICO ESTÃO REPRESENTADOS NO CAMPO CINZA ESCURO, SENDO OS GENES ENCONTRADOS NO CONJUNTO DE DADOS DESSA BIBLIOTECA ASSINALADOS EM AZUL (GENES INDUZIDOS) OU EM VERMELHO (GENES REPRIMIDOS). ENQUANTO NO CAMPO CINZA CLARO ESTÃO REPRESENTADAS AS POSSÍVEIS FUNÇÕES GÊNICAS. OS GENES TRANSCRITOS COM FOLD CHANGE (FC) < - 1 OU (FC) > 1 FORAM CATEGORIZADOS ATRAVÉS DO SOFTWARE MAPMAN.

TABELA 4. ANOTAÇÃO DAS PROTEÍNAS DIFERENCIALMENTE EXPRESSAS ENTRE PLANTAS INOCULADAS COM AS ESTIRPES FP2 (NIF+) E HM053 (NIF^C) DE *A. brasilense* COMPARADAS ÀS PLANTAS CONTROLE. NA TABELA ESTÃO APRESENTADOS OS VALORES ABSOLUTOS DA VARIAÇÃO DE EXPRESSÃO.

(Continua)

Gene ID	Produto do gene	Variação de expressão	Valor p
	HM053 x Controle		
Si022469m	NAD(P)-binding Rossmann-fold superfamily protein	3.6	1.07E-12
Si027962m	Zinc finger (C3HC4-type RING finger) family protein	3.5	1.76E-15
Si039986m	CAP160 protein	3.4	1.87E-12
Si024999m	PAR1 protein	3.4	9.32E-15
Si039585m	AWPM-19-like family protein	3.2	1.03E-12
Si015178m	tetraspanin9	3.1	0.00815
Si039983m	nicotianamine synthase 2	3.1	7.72E-09
Si039071m	embryonic cell protein 63	3	1.64E-07
Si028044m	wall-associated kinase 2	3	1.15E-90
Si003245m	HSP20-like chaperones superfamily protein	3	1.40E-08
Si022691m	NAD(P)-linked oxidoreductase superfamily protein	3	2.32E-11
Si040145m	Calcium-binding EF-hand family protein	2.8	2.95E-09
Si038941m	Nodulin MtN3 family protein	2.8	3.69E-30
Si030563m	Family of unknown function (DUF716)	2.8	2.16E-36
Si011005m	dehydration response element B1A	2.7	6.54E-13
Si034589m	slufate transporter 2;1	2.7	5.89E-42
Si004892m	Transmembrane amino acid transporter family protein	2.7	3.02E-33
Si037154m	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	2.7	9.68E-09
Si001772m	glutamate-cysteine ligase	2.7	3.38E-06
Si034355m	heat-shock protein 70T-2	2.7	5.20E-09
Si026639m	O-methyltransferase 1	2.6	9.15E-12
Si030856m	redox responsive transcription factor 1	2.6	6.15E-11

TABELA 4. ANOTAÇÃO DAS PROTEÍNAS DIFERENCIALMENTE EXPRESSAS ENTRE PLANTAS INOCULADAS COM AS ESTIRPES FP2 (NIF+) E HM053 (NIF^C) DE *A. brasilense* COMPARADAS ÀS PLANTAS CONTROLE. NA TABELA ESTÃO APRESENTADOS OS VALORES ABSOLUTOS DA VARIAÇÃO DE EXPRESSÃO.

(Continuação)

Gene ID	Produto do gene	Variação de expressão	Valor p
	HM053 x Controle		
Si034355m	heat-shock protein 70T-2	2.6	5.20E-09
Si012688m	Integrase-type DNA-binding superfamily protein	2.6	1.01E-10
Si018237m	homeobox 7	2.6	3.08E-18
Si030869m	C-repeat-binding factor 4	2.5	2.07E-14
Si027645m	cytochrome P450, family 71, subfamily A, polypeptide 24	2.5	6.56E-08
Si026926m	Dehydrin family protein	2.5	2.91E-14
Si027645m	cytochrome P450, family 71, subfamily A, polypeptide 24	2.5	6.56E-08
Si026583m	Dehydrin family protein	2.5	1.60E-05
Si024975m	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	2.4	1.36E-17
Si029316m	nine-cis-epoxycarotenoid dioxygenase 9	2.4	1.79E-12
Si007199m	C-repeat/DRE binding factor 2	2.4	4.39E-14
Si004341m	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	2.4	5.04E-12
Si021980m	mitogen-activated protein kinase kinase kinase 15	2.3	1.74E-17
Si003242m	HSP20-like chaperones superfamily protein	2.3	4.58E-13
Si029036m	Protein of unknown function (DUF3133)	2.3	1.80E-17
Si019730m	tRNAse Z1	2.3	1.40E-05
Si035445m	Glycosyl hydrolase superfamily protein	2.3	7.87E-47
Si019551m	RmlC-like cupins superfamily protein	2.3	2.14E-36
Si019636m	Integrase-type DNA-binding superfamily protein	2.2	5.00E-05
Si022693m	Peroxidase superfamily protein	2.2	5.36E-09
Si033567m	dehydration-responsive element-binding protein, putative, expressed	2.2	3.24E-09
Si014327m	RING/U-box superfamily protein	2.2	1.23E-16
Si001317m	mitogen-activated protein kinase kinase kinase 16	2.2	5.19E-20

TABELA 4. ANOTAÇÃO DAS PROTEÍNAS DIFERENCIALMENTE EXPRESSAS ENTRE PLANTAS INOCULADAS COM AS ESTIRPES FP2 (NIF+) E HM053 (NIF^C) DE *A. brasilense* COMPARADAS ÀS PLANTAS CONTROLE. NA TABELA ESTÃO APRESENTADOS OS VALORES ABSOLUTOS DA VARIAÇÃO DE EXPRESSÃO.

(Continuação)

Gene ID	Produto do gene	Variação de expressão	Valor p
	HM053 x Controle		
Si022667m	Peroxidase superfamily protein	2.2	6.49E-13
Si039737m	Eukaryotic aspartyl protease family protein	2.2	2.45E-12
Si037251m	C2H2-type zinc finger family protein	2.2	1.47E-13
Si018295m	HSP20-like chaperones superfamily protein	2.1	5.00E-05
Si024895m	EF hand calcium-binding protein family	2.1	7.31E-24
Si032711m	salt tolerance zinc finger	2.1	7.00E-10
Si036171m	Calcium-binding EF-hand family protein	2.1	1.53E-07
Si026670m	WRKY family transcription factor	2.1	5.81E-08
Si016692m	terpene synthase 21	2.1	3.12E-94
Si034533m	Heat shock protein 70 (Hsp 70) family protein	2.1	6.32E-08
Si026830m	Ribosomal protein L16p/L10e family protein	2.1	1.85E-19
Si037714m	Integrase-type DNA-binding superfamily protein	2.1	1.79E-11
Si034600m	heat shock cognate protein 70-1	2.1	3.45E-06
Si000808m	NADP-malic enzyme 3	2	1.76E-08
Si038745m	jasmonate-zim-domain protein 11	2	3.35E-22
Si013654m	Protein kinase superfamily protein	2	2.91E-14
Si002121m	WRKY family transcription factor	2	1.89E-10
Si032749m	hydroxyproline-rich glycoprotein family protein	2	2.20E-25
Si014237m	redox responsive transcription factor 1	2	6.44E-19
Si036287m	late embryogenesis abundant domain-containing protein / LEA domain-containing protein	2	3.70E-09

TABELA 4. . ANOTAÇÃO DAS PROTEÍNAS DIFERENCIALMENTE EXPRESSAS ENTRE PLANTAS INOCULADAS COM AS ESTIRPES FP2 (NIF+) E HM053 (NIF^C) DE *A. brasilense* COMPARADAS ÀS PLANTAS CONTROLE. NA TABELA ESTÃO APRESENTADOS OS VALORES ABSOLUTOS DA VARIAÇÃO DE EXPRESSÃO.

(Continuação)

Gene ID	Produto do gene	Variação de expressão	Valor p
	HM053 x Controle		
Si012456m	RING/U-box superfamily protein	1.9	6.96E-07
Si002446m	bifunctional nuclease in basal defense response 1	1.9	1.79E-15
Si001608m	Transmembrane amino acid transporter family protein	1.9	5.55E-24
Si022284m	nodulin MtN21 /EamA-like transporter family protein	1.9	0.0047
Si037536m	jasmonate-zim-domain protein 11	1.8	1.55E-14
Si023330m	ethylene responsive element binding factor 4	1.7	1.69E-31
Si002401m	bifunctional nuclease in basal defense response 1	1.7	2.08E-14
Si026260m	cytochrome P450, family 94, subfamily C, polypeptide 1	1.7	2.95E-14
Si002401m	WRKY DNA-binding protein 46	1.7	2.08E-14
Si015433m	WRKY family transcription factor	1.6	9.27E-13
Si029773m	cytochrome p450 78a9	1.6	5.76E-13
Si024816m	WRKY DNA-binding protein 70	1.6	1.11E-07
Si021144m	heat shock protein 101	1.5	1.27E-15
Si010228m	ethylene responsive element binding factor 3	1.5	1.79E-21
Si017443m	WRKY DNA-binding protein 40	1.5	1.43E-18
Si023279m	WRKY DNA-binding protein 50	1.5	2.67E-21
Si022676m	WRKY family transcription factor	1.4	9.97E-12
Si017671m	gibberellin 2-oxidase 8	1.4	4.43E-07
Si037496m	hemoglobin 1	-1.3	3.47E-06
Si024989m	HOPZ-ACTIVATED RESISTANCE 1	-2.1	9.58E-16
Si014533m	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein	-2.2	1.04E-07
Si013704m	alpha-amylase-like	-2.6	2.30E-10
Si011531m	inhibitor I family protein, putative, expressed	-2.6	4.06E-32

TABELA 4. ANOTAÇÃO DAS PROTEÍNAS DIFERENCIALMENTE EXPRESSAS ENTRE PLANTAS INOCULADAS COM AS ESTIRPES FP2 (NIF+) E HM053 (NIF^C) DE *A. brasilense* COMPARADAS ÀS PLANTAS CONTROLE. NA TABELA ESTÃO APRESENTADOS OS VALORES ABSOLUTOS DA VARIAÇÃO DE EXPRESSÃO.

(Continuação)

Gene ID	Produto do gene	Variação de expressão	Valor p
	FP2 x Controle		
Si026764m	HSP20-like chaperones superfamily protein	6.57	9.96E-05
Si037076m	PsbP-like protein 2	3.84	5.00E-05
Si035707m	glyceraldehyde-3-phosphate dehydrogenase B subunit	3.66	0.0005
Si003245m	HSP20-like chaperones superfamily protein	3.66	0.00005
Si010261m	glyceraldehyde 3-phosphate dehydrogenase A subunit 2	3.13	5.00E-05
Si001775m	sedoheptulose-bisphosphatase	3.04	6.99E-08
Si009497m	heat shock protein 90.1	2.92	0.00087
Si009661m	rotamase FKBP 1	2.85	0.00001
Si005789m	phosphoenolpyruvate carboxylase 2	2.75	0.00005
Si003151m	HSP20-like chaperones superfamily protein	2.72	0.00074
Si034355m	heat-shock protein 70T-2	2.71	0.00059
Si011036m	HSP20-like chaperones superfamily protein	2.67	0.00004
Si011175m	thioredoxin M-type 4	2.65	0.0016
Si003242m	HSP20-like chaperones superfamily protein	2.6	0.00003
Si034600m	heat shock cognate protein 70-1	2.54	0.00093
Si026536m	O-methyltransferase 1	2.43	0.0008
Si003202m	HSP20-like chaperones superfamily protein	2.42	5.00E-05
Si007560m	early nodulin-related	1.89	5.00E-05
Si003893m	Auxin-responsive GH3 family protein	1.5	0.00125
Si019474m	Leucine-rich repeat protein kinase family protein	1.4	5.00E-05
Si030429m	WRKY DNA-binding protein 18	1.38	1.53E-09
Si033102m	nodulin MtN21 /EamA-like transporter family protein	1.33	0.0002

TABELA 4. ANOTAÇÃO DAS PROTEÍNAS DIFERENCIALMENTE EXPRESSAS ENTRE PLANTAS INOCULADAS COM AS ESTIRPES FP2 (NIF+) E HM053 (NIF^C) DE *A. brasilense* COMPARADAS ÀS PLANTAS CONTROLE. NA TABELA ESTÃO APRESENTADOS OS VALORES ABSOLUTOS DA VARIAÇÃO DE EXPRESSÃO.

(Conclusão)

Gene ID	Produto do gene	Variação de expressão	Valor p
	FP2 x Controle		
Si003298m	dehydrin xero 1	-2.1	1.78E-15
Si003179m	Thioesterase superfamily protein	-2.11	0.00033
Si037627m	K-box region and MADS-box transcription factor family protein	-2.4	0.00005
Si013704m	alpha-amylase-like	-2.43	1.02E-09
Si010630m	Phosphoenolpyruvate carboxylase family protein	-3.32	4.96E-07

TABELA 5. RESPOSTA DE *S. viridis* (VAR. A10.1) A INOCULAÇÃO COM QUATRO DIFERENTES ESTIRPES DE *A. brasilense*.

	Comprimento da raiz (cm)/planta	**Número de raízes laterais/planta**	**Comprimento da parte aérea (cm)/planta**	**Área foliar (cm^2)/planta**	**Massa seca da raiz (g)/planta**	**Massa seca da parte aérea (g)/planta**	**Número de sementes/planta**
Controle	1274.9 ± 83.0	1483.6 ± 541.1	19.15 ± 2.9	5.13 ± 1.00	0.028 ± 0.01	0.060 ± 0.02	37.73 ± 10.7
FP2	1579.8 ± 189.4**	2719.7 ± 534.4**	21.50 ± 3.9	5.64 ± 1.10	0.042 ± 0.02**	0.065 ± 0.02	48.23 ± 15.8
FP10	1511.9 ± 294.4	2442.2 ± 933.1**	18.57 ± 3.4	5.57 ± 1.30	0.034 ± 0.03	0.065 ± 0.02	45.2 ± 5.6
HM053	1698.1 ± 237.8**	2757.9 ± 1023.5**	23.38 ± 4.5	5.83 ± 1.00	0.053 ± 0.02**	0.078 ± 0.02	50.53 ± 10.7**

**Significante para $p < 0.01$. Foi utilizado o teste estatístico Wilcox.

7. REFERÊNCIAS

ANDERS, S.; PYL, P. T.; HUBER, W. HTSeq - A Python framework to work with high-throughput sequencing data. **Bioinformatics.** v.31, p.166-169, 2014.

APPLEWHITE, P.B,. K-SAWHNEY, R.; GALSTON A.W. Isatin as an auxin source favoring floral and vegetative shoot regeneration from calli produced by thin layer explants of tomato pedicel. **Plant growth regul.** v.15, p. 17-21, 1994.

BHATTACHARJEE, S. Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plants. **Cur. science bangalore**-,v.89, p. 1113, 2005.

BOLGER, A. M. et al. Trimmomatic: a flexible trimmer for Illumina sequence data. **Bioinformatics.** v.30, n.15, p.2114-2120, 2014

BOTTINI, R.; CASSA, ´N. F.; PICCOLI, P. Gibberellin production Candau R, Avalos J, Cerda Cassa by bacteria and its involvement in plant growth promotion and yield increase. **Appl Microbiol Biotech**. v. 65, p.497-503, 2004.

BOTTINI, R.; FULCHIERI, M.; PEARCE, D. PHARIS, R.P. Identification of gibberellins A1,A3 and iso-A3 in cultures of *Azospirillum lipoferum*. **Plant Physiol** v.90, p. 45-47, 1989.

BRUTNELL, T. P.; WANG, L.; SWARTWOOD K, GOLDSCHMIDT, A.; JACKSON, D.; ZHU, X. G.; KELLOGG, E.; VAN ECK, J. *Setaria viridis*: a model for C4 photosynthesis. **The Plant Cell,** v. 22, p. 2537-2544, 2010.

BUSOV, V.B.; JOHANNES, E.; WHETTEN, R.W.; SEDEROFF, R.R.; SPIKER, S.L.; LANZ-GARCIA, C.; GOLDFARB, B. An auxin-inducible gene from loblolly pine (Pinus taeda L.) is differentially expressed in mature and juvenile-phase shoots and encodes a putative transmembrane protein. **Planta,** v. 218, p.916–927, 2004.

CASHIKAR, A.G.; DUENNWALD, M.; LINDQUIST, S.L A chaperone pathway in protein disaggregation. Hsp26 alters the nature of protein aggregates to facilitate reactivation by Hsp104. **J Biol Chem**, v. 280, p. 23869-23875, 2005.

CASSÁN F, LUCANGELI C, BOTTINI R, PICCOLI P. *Azospirillum sp.* Metabolize [17,17-2H2] Gibberellin A20 to [17,17-2H2] Gibberellin A1 in vivo in dy rice mutant seedlings. **Plant Cell Physiol**, v.42, p. 763-767, 2001b.

CASSÁN, F.; BOTTINI, R.; SCHNEIDER, G.; PICCOLI, P. *Azospirillum brasilense* and *Azospirillum lipoferum* hydrolyze conjugates of GA20 and metabolize the resultant aglycone to GA1 in seedlings of rice dwarf mutants. **Plant Physiol.** v.125, p. 2053–2058, 2001a.

COHEN, E.; OKON, Y.; KIGEL, J.; NUR, I.; HENIS, Y. Increase in dry weight and total nitrogen content in Zea mays and *Setaria italica* associated with nitrogen-fixing *Azospirillum* sp. **Plant physiology,** v. 66, p. 746-749, 1980.

CROZIER, A.P.; ARRUDA, P.; JASMIM, J.M.; MONTEIRO, A.M.; Sandberg G. Analysis of indole-3-acetic acid and related indoles in culture medium from *Azospirillum lipoferum* and *Azospirillum brasilense*. **Appl Environ Microbiol, v.** 54, p. 2833-2837, 1988.

DUNWELL, J.M.; GIBBINGS, J.G.; MAHMOOD, T.; NAQVI, S.S. Germin and germinlike proteins: evolution, structure, and function. **Plant Sciences**, v. 27, p. 342-375, 2008.

EL-KHAWASH, H.; ADACHI, K. Identification and quantifi- cation of auxins in culture media of *Azospirillum* and *Klebsiella* and their effect on rice roots. **Biol Fert Soils.** v. 28, p.377-381, 1999.

FULCHIERI, M.; LUCANGELI, C.; BOTTINI, R. Inoculation with *Azospirillum lipoferum* affects growth and gibberellins status of corn seedling roots. **Plant Cell Physiol.** v. 34, p.1305-1309, 1993.

GAMAS, P.; NIEBEL, F.C.; LESCURE, N.; CULLIMORE, J.V. Use of a subtractive hybridization approach to identify new *Medicado truncatula* Genes induced during root nodule development. **Mol. Plant-Microbe interactions,** v. 9, p.233-242, 1996**.**

GLICK, B.R.The enhancement of plant growth by free living bacteria. **Can J Microbiol.** v. 41, p.109-117, 1995.

GROVER, A.; AGGARWAL, P.K.; KAPOOR, A.; KATIYAR-AGARWAL, S.; AGARWAL M.; CHANDRAMOULI, A: Addressing abiotic stresses in agriculture through transgenic technology. **Curr Sci,** v. 84, p. 355-367, 2003.

HASLBECK, M.; MIESS, A.; STROMER, T.; WALTER, S.; BUCHNER, J. Disassembling protein aggregates in the yeast cytosol. The cooperation of Hsp26 with Ssa1 and Hsp104. **J Biol Chem,** v. 280, p**.** 23861-23868, 2005.

JANZEN, R.; ROOD, S.; DORMAR, J.; MCGILL, W. *Azospirillum brasilense* produces gibberellins in pure culture and chemically-medium and in co-culture on straw. **Soil Biol Biochem**. v. 24, p. 1061–1064, 1992.

KAPULNIK, Y.; OKON, Y.; KIGEL, J.; NUR, I.; HENIS, Y. Effects of temperature, nitrogen fertilization, and Plant age on nitrogen fixation by *Setaria italica* inoculated with *Azospirillum brasilense* (strain cd). **Plant Physiol.** v. 68, p. 340-343, 1981.

KIM, K.C., LAI, Z.B., FAN, B.F., CHEN, Z.X. *Arabidopsis* WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defence. **Plant Cell** v.20, p. 2357–2371, 2008.

LAI, Z.B., VINOD, K., ZHENG, Z.Y., FAN, B.F., CHEN, Z.X. Roles of Arabidopsis WRKY3 and WRKY4 transcription factors in plant responses to pathogens. **BMC Plant Biol.** v.8, 68, 2008.

LEE, G.J.; VIERLING, E. A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. **Plant Physiol**. v. 122, p.189-198, 2000.

LI, H.Y.; JIANG, J.; WANG, S.; LIU, F.F. Expression analysis of ThGLP, a new germin-like protein gene, in Tamarix hispida. **Journal of Forestry Research**, v. 21p. 323–330, 2010

MANGELSEN, E., KILIAN, J., BERENDZEN, K.W., KOLUKISAOGLU, U.H., HARTER, K., JANSSON, C.,WANKE,D., Phylogenetic and comparative gene expression analysis of barley (Hordeum vulgare) WRKY transcription factor family reveals putatively retained functions between monocots and dicots. **BMC Genomics.** v. 9, 194, 2008.

MARTÍNEZ-MORALES, L.J.; SOTO-URZÚA, L.; BACA, B.E.; SÁNCHEZ-AHEDO, J.A. Indole-3-butyric acid (IBA) production in culture medium by wild strain *Azospirillum brasilense*. **FEMS Microbiol Lett,** v.228, p. 167–173, 2003.

NAOUMKINA, M.A.; HE, X., DIXON, R.A. Elicitor-induced transcription factors formetabolic reprogramming of secondary metabolism in Medicago truncatula. **BMC Plant Biol.** v.8, 132, 2008.

NARUSAKA, Y.; NAKASHIMA, K.; SHINWARI, Z.K.; SAKUMA, Y.; FURIHATA, T.; ABE, H.; NARUSAKA, M.; SHINOZAKI, K.;YAMAGUCHI-SHINOZAKI, K. Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. **Plant Journal**, v. 34, p.137-148, 2003.

OKON, Y.; HEYTLER, P.G.; HARDY, R.W.F. N_2 fixation by *Azospirillum brasilense* and Its incorporation into Host *Setaria italica.* **Applied and Environm. Microb**. v.46, p-694-697, 1983.

PERLICK, A.; PÜHLER, A. A survey of transcripts expressed specifically in root nodules of broadbean (Vicia faba L.). **Plant Mol. Biol,** v. 22, p. 957-970, 1993.

RODRIGUEZ-LOPEZ, M.; BAROJA-FERNANDEZ, E.; ZANDUETA-CRIADO, A.; MORENO-BRUNA, B.; MUNOZ, F. Two isoforms of a nucleotide-sugar pyrophosphatase/phosphodiesterase from barley leaves (Hordeum vulgare L.) are distinct oligomers of HvGLP1, a germin-like protein. **FEBS Lett,** v. 490, p. 44-48, 2001.

SEGARRA CI, CASALONGUE CA, PINEDO ML, RONCHI VP, CONDE RD. A germin-like protein of wheat leaf apoplast inhibits serine proteases. **J Exp Bot**, 2003.

TIMMUSK, S.; NICANDER, B.; GRANHALL, U.; TILLBERG, E. Cytokinin production by *Paenibacillus polymyxa.* **Soil Biol Biochem**. v. 31, p. 1847–1852, 1999.

TRAPNELL, C. et al. TopHat: discovering splice junctions with RNA-Seq. **Bioinformatics**. v. 25, n. 9, p. 1105-1111, 2009.

TRAPNELL, C.; WILLIAMS, B.A.; PERTEA, G.; MORTAZAVI, A.; KWAN, G.; VAN BAREN M.J.; SALZBERG, S.L.; WOLD, B.J.; PACHTER, L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. **Nature biotechnology**. v. 28 p. 511–515, 2010.

ZHANG, J., PENG, Y.L., GUO, Z.J. Constitutive expression of pathogen-inducible OsWRKY31 enhances disease resistance and affects root growth and auxin response in transgenic rice plants. **Cell Res.** v. 18, p.508–521, 2008.

8. CONCLUSÕES GERAIS

1. O mutante espontâneo HM053 é capaz de fixar nitrogênio constitutivamente em presença de NH_4^+. Este fenótipo pode ser explicado devido a ausência de ADP ribosilação da proteína NifH e a baixa atividade da glutamina sintetase (GS), levando a baixos níveis de glutamina intracelular. Estes eventos sinalizam para célula condição de fixação de nitrogênio. Logo, GlnB permanecerá uridililada e regulará o gene *nifA* (ativador transcricional dos genes *nif*) catalisando a redução do dinitrogênio a amônio pela nitrogenase, promovendo a fixação biológica do nitrogênio;

2. O sequenciamento do gene *glnA*, que codifica a GS, revelou a substituição do resíduo de prolina por leucina na posição 347, esta alteração pode explicar a ausência de adenililação na GS em presença de amônio e a sua baixa atividade, uma vez que a mutação pode levar a substancial alteração em sua estrutura secundária. A baixa atividade da GS também explica a incapacidade de assimilar o NH_4^+ produzido pela nitrogenase, levando assim a sua excreção no meio;

3. Plantas de trigo (var. CD104) inoculadas com *A. brasilense* estirpe mutante HM053 responderam melhor a inoculação do que plantas inoculadas com a estirpe selvagem FP2;

4. Os transconjugantes FP2-7 e HM053-36, contendo a fusão *nifH-gusA,* apresentaram a mesma capacidade de fixar nitrogênio e excretar amônio (habilidade restrita a estirpe mutante HM053) quando comparado a seus parentais FP2 e HM053;

5. Através da fusão *nifH-gusA* foi sugerido que ambas as estirpes expressam o gene *nifH* em associação com raiz de trigo;

6. Por RT-qPCR a expressão do gene *nifH in planta* do mutante HM053 foi 278 vezes maior comparado a estirpe selvagem FP2;

7. Com o uso de radioisótopos (^{13}NN) foi possível concluir que o nitrogênio fixado pelas espécies diazotróficas *A. brasilense* e *H. seropedicae*, é incorporado pela planta. Uma vez que plantas controle (inoculadas com a cultura da bactéria fervida) e plantas inoculadas com as estirpes Nif$^-$ não apresentaram a fixação do radioisótopo ^{13}NN nos tecidos;

8. Plantas de *S. viridis* inoculadas com o mutante HM053 assimilaram até 16 vezes mais nitrogênio comparado à mistura das espécies *A. brasilense* (FP2-7) e *H. seropedicae* (RAM4). *S. viridis* inoculada com este mutante HM053 apresentou também 14 vezes mais habilidade de incorporar carbono (^{11}C);

9. Parte do nitrogênio fixado pela bactéria é transportado para a parte aérea da planta e metabolizado. Isso foi comprovado através de traços de radioisótopo ^{13}NN detectados na enzima ribulose-1,5-biphosphate carboxylase;

10. *S. viridis* var. A10.1 respondeu positivamente a inoculação com *A. brasilense*, uma vez que houve aumento significativo no número de raízes laterais, comprimento da raiz e aumento de produtividade. Esta última característica foi observada apenas para plantas inoculadas com o mutante HM053;

11. Esses resultados indicam o mutante HM053 como um forte candidato a biofertilizante para uso no campo, uma vez que é um mutante espontâneo e que poderá ser utilizado em acordo com o Ministério da Agricultura Brasileira. Como perspectiva, os resultados do ensaio de RNA-seq complementarão os resultados apresentados nesta tese e contribuirão com o entendimento da interação entre *S. viridis – A. brasilense*.