UNIVERSIDADE FEDERAL DO PARANÁ

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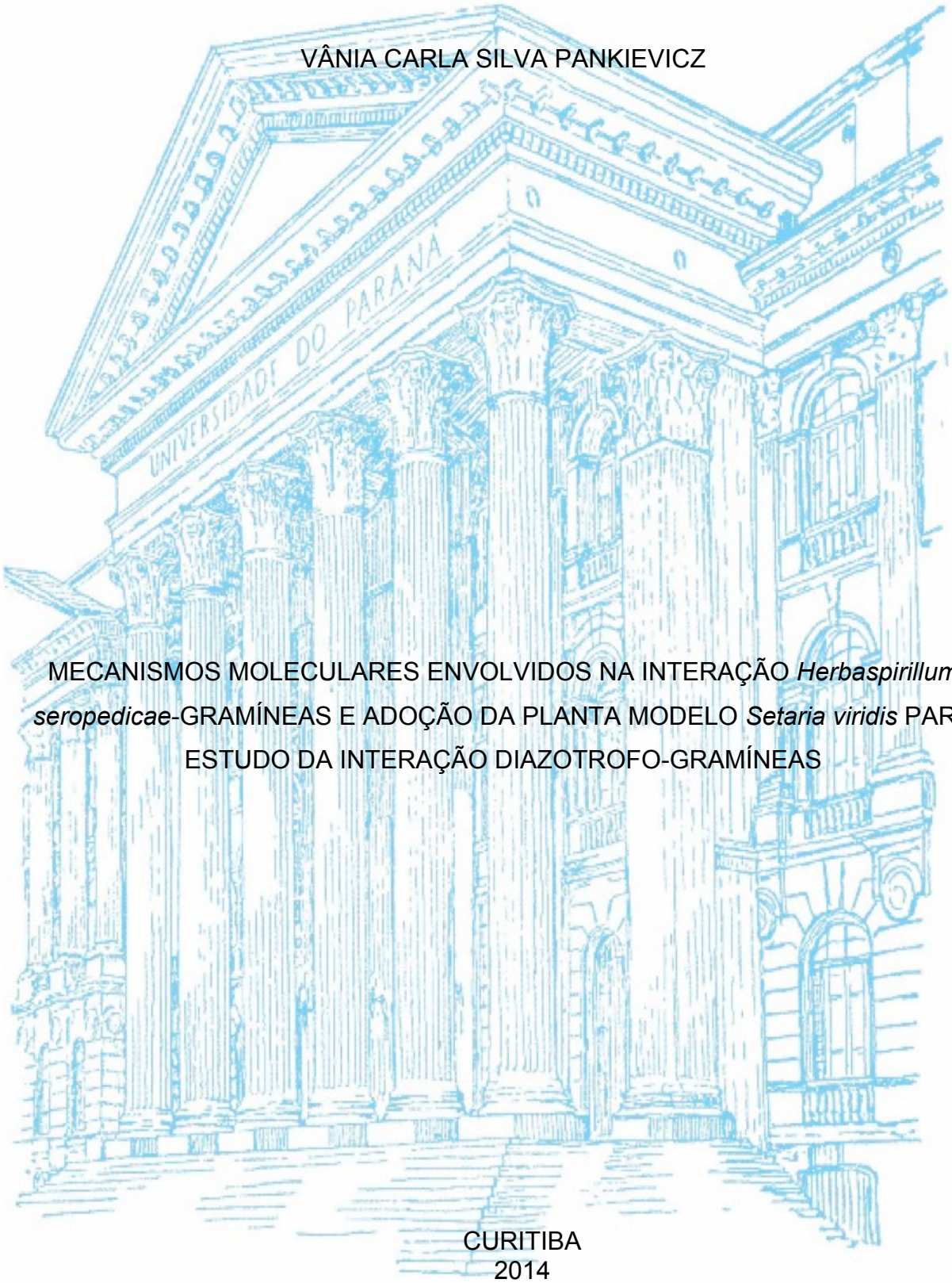

MECANISMOS MOLECULARES ENVOLVIDOS NA INTERAÇÃO *Herbaspirillum seropedicae*-GRAMÍNEAS E ADOÇÃO DA PLANTA MODELO *Setaria viridis* PARA ESTUDO DA INTERAÇÃO DIAZOTROFO-GRAMÍNEAS

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Tese apresentada ao curso de Pós-Graduação em Ciências-Bioquímica, Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências-Bioquímica.

Orientador: Prof. Dr. Emanuel Maltempi de Souza
Co-orientadora: Prof.ª ª. Dr ª. Rose Adele Monteiro
Co-orientador: Prof. Dr. Doumit Camilios Neto

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TERMO DE APROVAÇÃO

VÂNIA CARLA SILVA PANKIEVICZ

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Tese aprovada como requisito parcial para obtenção do grau de Doutor no Curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas da Universidade Federal do Paraná, pela seguinte banca examinadora:

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Curitiba, 18 de setembro de 2014.

DEDICATÓRIA

Ao meu avô, Antônio Pankievicz

AGRADECIMENTOS

Ao meu orientador, Prof. Emanuel Maltempi de Souza, pela orientação e amizade.

Aos meus co-orientadores, Prof. Doumit Camilios Neto e Prof[a]. Rose Adele Monteiro pelo acompanhamento e amizade.

Ao Curso de Pós-Graduação em Ciências-Bioquímica, do Setor de Ciências Biológicas, da Universidade Federal do Paraná, na pessoa do sua coordenadora Prof[a]. Silvia M. S. Cadena, pelo apoio recebido.

Aos Professores do Núcleo de Fixação Biológica de Nitrogênio do departamento de Bioquímica e Biologia Molecular da UFPR, pelas contribuições e sugestões no trabalho.

Ao Prof. Gary Stacey, da Universidade de Missouri - EUA, pela orientação e ensinamentos durante o estágio de doutorado sanduíche.

Ao funcionários Roseli Padro e Valter A. Baura, por estarem sempre prontos a cooperar e ensinar.

A todos os amigos e companheiros de laboratório.

Aos meus pais, Jader e Rosangela e irmãos, Eduardo e Daniel, pelo apoio e entusiasmo.

Ao meu esposo Alex, pelo apoio e dedicação.

RESUMO

As bactérias promotoras do crescimento de planta (BPCP) estimulam o crescimento vegetal por mecanismos que favorecem a assimilação de nutrientes essenciais do solo, produção de fitohormônios e de substâncias que inibem o crescimento de patógenos, além da fixação biológica de nitrogênio (FBN). As bactérias *Herbaspirillum seropedicae* e *Azospirillum brasilense* são fixadores de nitrogênio capazes de colonizar e promover o crescimento de gramíneas. No entanto, os mecanismos moleculares que a bactéria utiliza para gerar os efeitos positivos na planta não são completamente entendidos. O presente trabalho tem como objetivo contribuir para o entendimento da interação entre planta-BPCP. Para isto, tais objetivos incluem: (1) elucidar, através de análise transcritômica, os mecanismos moleculares utilizados por *H. seropedicae* durante a interação com *Triticum aestivum* (trigo); (2) avaliar a promoção do crescimento vegetal pela co-inoculação de *H. seropedicae* e *A. brasilense* em *Setaria viridis* (*green foxtail*), para o desenvolvimento de um novo modelo de estudo da interação planta-bactéria. Na primeira parte deste trabalhado plântulas de *T. aestivum*, cultivadas de forma axênica em meio hidropônico, foram inoculadas com *H. seropedicae*. Duas bibliotecas de RNA-seq foram construídas, uma compreendendo as bactérias aderidas às raízes de trigo e outra pelas bactérias planctônicas crescendo em meio Hoagland's, utilizado para o cultivo da planta. Os dados de transcritoma mostraram que *H. seropedicae* se adere às raízes de trigo provavelmente através de adesinas específicas, ativa o sistema Ntr e os genes *nif*, além de transportadores ABC específicos e expressa genes do metabolismo de polihidroxialcanoatos, produção e secreção de auxina. Na segunda parte deste trabalho, a promoção do crescimento vegetal por bactérias diazotróficas foi avaliada em diferentes acessos da planta *Setaria viridis*. *S. viridis* é uma gramínea modelo para os estudos de fotossíntese do tipo C_4. No presente estudo, 32 acessos desta planta foram co-inoculados com bactérias diazotróficas e cultivadas em casa de vegetação, por 40 dias, em condições de baixo (0,5 mM KNO_3) ou nenhum nitrogênio. Posteriormente, os parâmetros de crescimento foram avaliados e o genótipo A10.1 foi identificado como o mais responsivo à inoculação tendo aumentado o peso seco e fresco da parte aérea, o comprimento da raiz, o número de raízes laterais e o número de sementes. Ainda, foi demonstrado microscopicamente que *H. seropedicae* coloniza os tecidos internos da planta e que *A. brasilense* coloniza a planta epifiticamente e fixa nitrogênio. Em colaboração com o laboratório nacional *Brookhaven*, nos Estados Unidos, experimentos utilizando o isótopo $^{13}N_2$ mostraram evidências da fixação biológica de nitrogênio e incorporação deste nitrogênio em *S. viridis* A10.1. Juntos, os dados deste trabalho trazem novas perspectivas para os estudos da interação *H. seropedicae*-gramínea e revela genes e mecanismos essenciais para a interação. A adoção da planta modelo *S. viridis* deve impulsionar os estudos sobre fixação biológica de nitrogênio com objetivo de consolidar o uso da FBN para a produção de cereais como o milho e o trigo.

Palavras-chave: Fixação biológica de nitrogênio, *H. seropedicae*, *A. brasilense*, RNA-seq, bactérias diazotróficas, bactérias promotoras de crescimento de planta, *S. viridis*.

ABSTRACT

The plant growth-promoting bacteria (PGPB) stimulate plant growth by mechanisms that increase assimilation of essential nutrients from the soil, phytohormones release and production of substances that inhibit phytopathogens and the biological nitrogen fixation (BNF). *Herbaspirillum seropedicae* and *Azospirillum brasilense* are proteobacteria that fix nitrogen and are able to establish an efficient colonization with plants and its benefit in promoting plant growth has already been demonstrated. However, the molecular mechanisms utilized by the bacteria to cause the positive effects to the plant are not completely understood. In the present work we intend to study the mechanisms that contributes to the beneficial plant-bacteria interaction. The main goals include: (1) Transcriptomic analysis of *H. seropedicae* colonizing *T. aestivum* roots to identify genes related to bacterial ability of colonizing plant; and (2) evaluate the plant growth promotion of *Setaria viridis* upon co-inoculation with *H. seropedicae* and *A. brasilense*. In the first part of this work *T. aestivum* seedlings growing hydroponically in Hoagland's cultivation medium were inoculated with *H. seropedicae*. Two RNA-seq libraries were constructed; one from wheat-roots-attached bacteria (WRA) and another from planktonic bacteria (PLANK) growing in the Hoagland's medium. The transcriptome data showed that *H. seropedicae* attached to the wheat roots probably through specific adhesins. The bacterial metabolism was adapted to low oxygen concentrations and the *nif* genes were activated. ABC transporters genes were also expressed, providing insights into specific adaptations. Genes of polyhydroxyalkanoate synthesis were activated and genes related to the ability of promote plant growth, such as phytohormones production were up-regulated. In the second part of this study, the plant growth promotion by diazotrophic bacteria was evaluated in different accessions of *Setaria viridis*. *S. viridis* is a grass model for C_4-type photosynthesis, and has been extensively employed in studies of plant genetics. Here, 32 accessions were co-inoculated with *H. seropedicae* and *A. brasilense* in greenhouses during 40 days, under conditions of low (0.5 mM KNO_3) or no nitrogen addition. Finally, the *S. viridis* genotype A10.1 was identified as the most responsive to inoculation with increased fresh and dry shoot weight, total root length, number of lateral roots and number of seeds. Indeed, it was shown that *H. seropedicae* colonizes the internal tissues of the plant and that *A. brasilense* epiphytically colonizes the plant and fix nitrogen. In collaboration with the Brookhaven National Laboratory-USA, the utilization of the $^{13}N_2$ isotope showed evidence of BNF and incorporation of fixed nitrogen in *S. viridis* A10.1. Together, the results provide new perspectives for the studies of *H. seropedicae*-grass interaction and reveals genes and essential mechanisms for this interaction. The adoption of the model plant *S. viridis* should enhance the studies on biological nitrogen fixation with the objective of establishing the use of BNF to increase the production of cereals and uses less chemical nitrogen fertilizers.

Key Words: Biological Nitrogen Fixation, *H. seropedicae*, *A. brasilense*, RNA-seq, diazotrophic bacteria, plant growth promoting rhizobacteria, *S. viridis*.

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LISTA DE ABREVIATURAS

PHA	-	polihidroxialcanoato
EPS	-	exopolissacarídeo
LPS	-	lipopolissacarídeo
CPS	-	polissacarídeos capsulares
AIA	-	ácido indol acético
SSTIII/SST3	-	sistema de secreção do tipo três
SSTVI/SST6	-	sistema de secreção do tipo seis
cDNA	-	DNA complementar
mRNA	-	RNA mensageiro
rRNA	-	RNA ribossomal
SEC	-	sec protein-translocation pathway
TAT	-	twin-arginine translocation system
PGPR	-	plant growth promoting rhizobacteria
BNF	-	biological nitrogen fixation
NGS	-	next generation sequencing
RNA-seq	-	RNA sequencing
WRA	-	wheat root adhered library
PLANK	-	planktonic library
RPKM	-	reads per kilobase per million mapped reads
IAA	-	indole acetic acid
RT-qPCR	-	real time quantitative polymerase chain reaction
CFU	-	colonies forming units
d.a.i.	-	days after inoculation
h.p.i	-	hours post inoculation
EST	-	expressed sequence tags

SUMÁRIO

1 INTRODUÇÃO

1.1 PROMOÇÃO DO CRESCIMENTO VEGETAL POR BACTÉRIAS

As raízes vegetais sofrem intensa influência do ambiente que as envolve. A zona do solo que sofre influência das raízes chama-se rizosfera e a superfície limítrofe entre a raiz e o solo é o rizoplano, que inclui a epiderme vegetal e a mucilagem. A rizosfera sustenta o desenvolvimento e a atividade de comunidades microbianas, incluindo os microrganismos capazes de promover o crescimento vegetal (BLOOM, JACKSON e SMART, 1993).

Kloepper e Schroth (1978) descreveram pela primeira vez o termo *plant-growth promoting rhizobacteria* - PGPR (em português BPCP - Bactérias Promotoras do Crescimento de Planta) para descrever as bactérias do solo que colonizam a rizosfera, crescendo internamente ou ao redor dos tecidos vegetais, sendo capazes de estimular o crescimento da planta através de diversos mecanismos. Desde então, pesquisadores visam compreender como estas bactérias realizam seus efeitos positivos, de modo a utilizar estes microrganismos como forma de diminuir o impacto ambiental negativo decorrente da intensa utilização de fertilizantes, herbicidas e pesticidas.

Os benefícios provenientes destas interações incluem: aumento na taxa de germinação de sementes, crescimento de raiz, aumento da área foliar, teor de clorofila, absorção de nutrientes, teor de proteínas, atividade hidráulica, tolerância ao estresse abiótico, comprimento e peso de raiz e folhas, biocontrole e adiamento da senescência vegetal (Figura 1) (PÉREZ-MONTAÑO *et al.*, 2014). Desta forma, bactérias ou rizobactérias que promovem o crescimento da planta, através de mecanismos que aprimoram a absorção de nutrientes pela planta são chamadas biofertilizantes.

A associação benéfica entre planta-bactéria mais bem conhecida é a interação simbiótica entre plantas leguminosas e bactérias do gênero *Rhizobium*, onde as estruturas formadas são nódulos colonizados por bacteróides capazes de fixar nitrogênio atmosférico e fornecê-lo para a planta, em forma de amônia ou outro

composto nitrogenado. As bactérias promotoras do crescimento de planta que não formam nódulos nas raízes se associam de outras duas maneiras principais: colonização epifítica, onde os microrganismos ficam na superfície da planta, ou endofítica quando entram nos tecidos internos das plantas sem causar danos. Assim, uma vez em interação com o vegetal as BPCP passam a expressar características que têm como consequência uma melhora no estado nutricional da planta por meio de mecanismos como a fixação biológica de nitrogênio, aumento da disponibilidade de nutrientes na rizosfera e aumento da área de superfície da raiz (PÉREZ-MONTAÑO *et al.*, 2014). Normalmente, a combinação desses vários elementos que fazem da bactéria uma boa promotora de crescimento, e a visualização dos benefícios causados pela interação planta-bactéria, constituem a primeira etapa no estudo dos mecanismos bacterianos de promoção do crescimento vegetal.

O nitrogênio é um dos nutrientes vegetais mais importantes. As plantas podem assimilar o nitrogênio do solo sob a forma de nitrito, nitrato ou amônia. Estas formas de nitrogênio não são disponíveis em quantidades necessárias na maioria dos solos e devem ser fornecidos para as culturas agrícolas. Entretanto, grande parte do adubo nitrogenado, empregado na agricultura é perdido por lixiviação ou pelas chuvas. Assim a inoculação de bactérias fixadoras de nitrogênio de vida livre pode ter importante papel na manutenção da fertilização de plantas de interesse comercial. Exemplos bem estabelecidos da contribuição da fixação biológica de nitrogênio para a fertilização de plantas são aqueles onde experimentos utilizando a técnica de diluição do isótopo $^{15}N_2$ mostraram a alocação do nitrogênio fixado na planta. Entre estes estão a inoculação de *Azospirillum* em trigo e em cana-de-açúcar (BODDEY e VICTORIA, 1986; RAMOS *et al.*, 2001), *Azoarcus* em sorgo (STEIN, HAYEN-SCHNEG e FENDRIK, 1997) e *Azospirillum* em milho (GARCIA DE SALAMONE *et al.*, 1996).

Levando em conta que os efeitos das bactérias promotoras de crescimento vegetal representam importante contribuição no rendimento das culturas, não é surpreendente que inoculantes comerciais tenham sido desenvolvidos para uma variedade de culturas. Por exemplo, várias cepas de *Azospirillum brasilense* e *Azospirillum lipoferum* têm sido aplicadas com sucesso em culturas agrícolas

(OKON e LABANDERA-GONZALEZ, 1994; DOBBELAERE, S. *et al.*, 2001; PEDRAZA *et al.*, 2009; HUNGRIA *et al.*, 2010). Uma crescente aceitação do uso desses tratamentos biológicos na agricultura e as alterações na indústria de produção de sementes (por exemplo, como uma maior adoção da tecnologia de tratamento de sementes) estão contribuindo para o aumento do mercado de inoculantes.

O fósforo também é um nutriente essencial para a planta e, apesar da sua alta disponibilidade nos solos, este elemento encontra-se na forma de compostos insolúveis não absorvíveis pela planta, portanto, limitando seu crescimento. Alguns BPCP são capazes de transformar compostos insolúveis de fosfato do solo em uma forma assimilável pela planta, através de acidificação ou atividade enzimática (HAMEEDA *et al.*, 2008; RICHARDSON *et al.*, 2009). Bactérias dos gêneros *Azospirillum*, *Bacillus*, *Burkholderia*, *Erwinia*, *Pseudomonas*, *Rhizobium, Serratia* e *Herbaspirillum* são capazes de solubilizar fosfato (SUDHAKAR *et al.*, 2000; MEHNAZ e LAZAROVITS, 2006).

A assimilação de outros nutrientes também é favorecida quando plantas são inoculadas com bactérias promotoras de crescimento. Nutrientes como Ca, K, Fe, Cu, Mn e Zn podem ser absorvidos durante a acidificação da rizosfera através da produção de ácidos orgânicos ou estimulação das bombas de próton, pois um ambiente com pH reduzido aumenta a solubilização destes nutrientes (MANTELIN e TOURAINE, 2004).

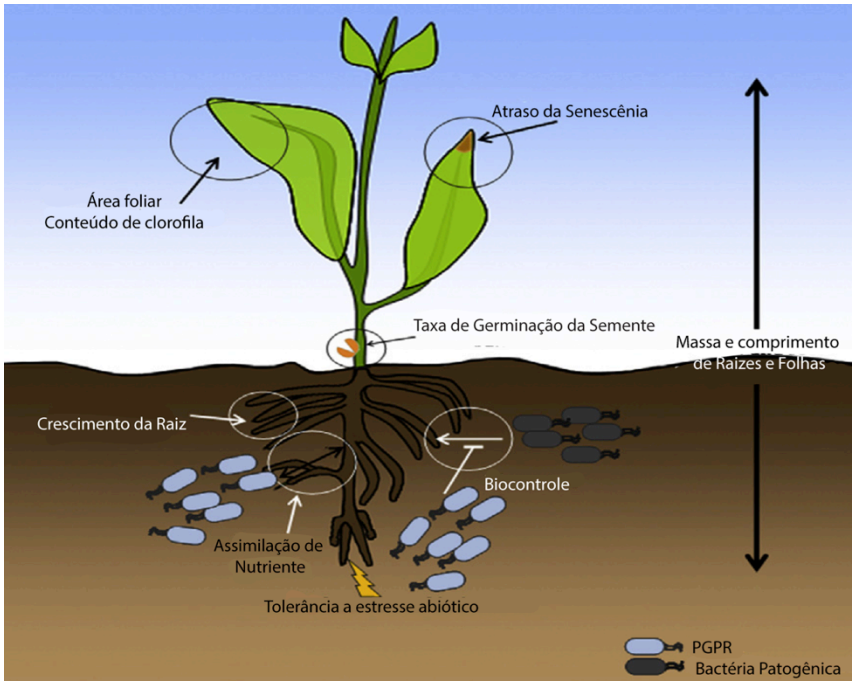


FIGURA 1 - Resposta fisiológica da planta à inoculação com bactérias promotoras do crescimento de planta. FONTE: adaptado de PÉREZ-MONTAÑO *et al.*, 2014.

Tendo em vista os benefícios que a inoculação com bactérias promotoras do crescimento de planta proporciona aos seus hospedeiros é importante entender quais são os mecanismos tanto fisiológicos como moleculares que estes microrganismos utilizam nessa interação a fim de permitir a definição de que espécie de BPCP seria melhor empregada como inoculante em cada cultura agrícola. Entender como as plantas selecionam os microrganismos do solo que formam a comunidade microbiana na rizosfera é um tema que tem ganhado importância, em vista do uso crescente das rizobactérias para a promoção do crescimento vegetal (DROGUE *et al.*, 2012).

Em trabalhos recentes, dois grupos usaram técnicas de metagenômica para descrever de forma muito detalhada a composição microbiana da rizosfera e da microbiota endofítica da planta modelo *Arabidopsis thaliana*. Ambos os estudos demonstraram que o tipo de solo define o microbioma da rizosfera, o qual era diferente das espécies identificadas colonizando a planta endofiticamente. A comunidade endofítica foi dominada por *Actinobacteria*, seguida por *Proteobacteria*, *Firmicutes*, *Bacteroidetes* e *Cyanobacteria* (BULGARELLI *et al.*, 2012). Trabalho semelhante conduzido por Lundberg e colaboradores (2012) utilizou 600 genótipos diferentes de *A. thaliana* e demostraram que além da composição do solo, há uma

contribuição da planta hospedeira na formação do microbioma rizosférico e endofítico. No entanto, não está claro se os microrganismos presentes no microbioma da raiz são recrutados aleatoriamente da comunidade local do solo ou ativamente por meio de seleção por exsudatos das plantas. Da mesma forma, embora alguns endófitos sejam benéficos, a identidade e as funções da maioria das espécies microbianas que habitam os espaços intercelulares da raiz são desconhecidas (HIRSCH e MAUCHLINE, 2012).

Apesar da grande diversidade encontrada nos microrganismos da rizosfera e endofíticos, o conhecimento científico acerca deste tema aumenta cada vez mais. Sabe-se que o primeiro mecanismo que contribui para a especificidade planta-bactéria, e consequentemente para os efeitos fitoestimulatórios, é a atração dos microrganismos em direção a um composto específico, como ácidos orgânicos exsudados da planta hospedeira. Este mecanismo chamado de quimiotaxia está relacionado com a adaptação nutricional da bactéria, uma vez que estes atraentes servem como fontes de carbono para as células bacterianas (BAIS *et al.*, 2006; DROGUE *et al.*, 2012; SHIDORE *et al.*, 2012).

Após a atração, a competência para uma colonização eficiente às raízes também determina o sucesso da interação. Além disso, recentemente alguns estudos mostraram que as BPCP produzem uma gama de metabólitos diferenciados durante a interação e que estas substâncias têm papel importante nos mecanismos de defesa e competição, mas também podem estar envolvidas com interações específicas e em comunicação com a planta hospedeira. Mais ainda, há alguns exemplos onde os endófitos podem modular a síntese de metabólitos da planta (BRADER *et al.*, 2014). Por exemplo, foram observadas mudanças específicas na composição de açúcares, aminoácidos e ácidos orgânicos exsudados pelas raízes de plantas de algodão, após a inoculação com *Azotobacter* (KUMAR *et al.*, 2007). Da mesma forma, a inoculação com *Azospirillum* em raízes de milho também alterou os metabólitos secundários produzidos tanto nos tecidos radiculares como nas folhas de diferentes cultivares de milho, sugerindo uma adaptação específica da planta, como um todo, em relação a estirpe bacteriana utilizada (WALKER *et al.*, 2011).

1.2 BACTÉRIAS PROMOTORAS DO CRESCIMENTO DE PLANTA: *Herbaspirillum seropedicae* E *Azospirillum brasilense.*

1.2.1 *Herbaspirillum seropedicae*

O gênero *Herbaspirillum* pertence à classe Betaproteobactéria, que agrupa muitas bactérias que se associam a plantas, como aquelas dos gêneros *Azoarcus*, *Burkholderia* e *Ralstonia* (BALDANI *et al.*, 1986; BALDANI *et al.*, 1992). *Herbaspirillum seropedicae* é uma bactéria gram-negativa vibrióide, apresenta 0,6 a 0,7 µm de diâmetro, 1,5 a 5 µm de comprimento e possui três flagelos. É um microrganismo capaz de fixar nitrogênio sob condições microaeróbicas e em uma ampla faixa de pH (5,3 a 8,0) (BALDANI *et al.*, 1986). Este microrganismo foi primeiramente isolado no município de Seropédica, Rio de Janeiro (Brasil), de tecidos internos de raízes de milho, sorgo e arroz cultivados em dois solos diferentes (BALDANI *et al.*, 1986). Cruz e colaboradores (2001) também identificaram esta bactéria, através do sequenciamento do gene 16SrRNA, em colmo, folhas, raízes e frutos de abacaxi (*Ananas comosus*) e banana (*Musa* sp.) cultivados nos estados da Bahia e Rio de Janeiro. Esta bactéria é capaz de colonizar raízes, caules e folhas de seus hospedeiros sem causar doença e apresenta baixa sobrevivência no solo (OLIVARES *et al.*, 1996; JAMES e OLIVARES, 1998).

H. seropedicae é uma bactéria endofítica, mas precisa ser recrutada do solo e provavelmente possui uma etapa de colonização do rizoplano, onde a bactéria necessita expressar características conhecidas com “competência de rizosfera” (ou *rhizosphere competence*) (REINHOLD-HUREK e HUREK, 2011). Assim, a associação de *H. seropedicae* com plantas começa através de quimiotaxia, seguida pela adesão de bactérias nas superfícies das raízes. A entrada da bactéria ocorre nos pontos de emergência de raízes secundárias e ferimentos; e a disseminação ocorre através dos espaços intercelulares com posterior ocupação do xilema (BALDANI, JAMES*, et al.*, 1992; JAMES *et al.*, 2002). Em arroz, as bactérias entram pelas fissuras das raízes laterais e ocupam os espaços intercelulares, colonizando o aerênquima e o xilema das raízes, posteriormente chegam aos caules e folhas. A ocupação dos tecidos internos ocorre muito rapidamente, após um dia de

inoculação, em laboratório. Em trigo e sorgo *H. seropedicae* apresenta densa colonização na superfície da raiz e após 12 e 15 dias foi encontrado no aerênquima radicular e no parênquima foliar, respectivamente (RONCATO-MACCARI *et al.*, 2003). Em milho, a adesão por *H. seropedicae* às raízes foi observada 30 min após a inoculação; a colonização epifítica da raiz continua por todo o ciclo da planta (BALSANELLI *et al.*, 2010). Da Silva e colaboradores (2003) sugeriram que alterações no envelope celular são necessárias para a adesão, pois foram observadas bactérias em estreito contato com a parede celular de raízes de cana-de-açúcar e no ponto de adesão foi possível observar saliências no envelope celular em direção às células vegetais.

1.2.2 *Azospirillum brasilense*

O gênero *Azospirillum* pertence à classe Alfaproteobactéria e é formado por bactérias de vida livre, fixadoras de nitrogênio, de formato vibrióide, possuindo flagelo polar ou peritricoso (BALDANI e BALDANI, 2005). As fontes de carbono preferencialmente utilizadas por *Azospirillum* spp. são ácidos orgânicos como malato, lactato, succinato e piruvato. Carboidratos como D-frutose e D-glucose podem ser utilizados por algumas espécies (DÖBEREINER e PEDROSA, 1987). O metabolismo de nitrogênio é versátil, podendo ser utilizados como fonte de nitrogênio: nitrato, nitrito, amônio, aminoácidos e nitrogênio atmosférico (DÖBEREINER e PEDROSA, 1987; STEENHOUDT e VANDERLEYDEN, 2000).

As espécies de *Azospirillum* estudadas em mais detalhes são *Azospirillum brasilense* e *Azospirillum lipoferum* (BALDANI e BALDANI, 2005). Os microrganismos pertencentes a este gênero são na maioria rizosféricos ou epifíticos, mas algumas estirpes podem colonizar também o interior de raízes (STEENHOUDT e VANDERLEYDEN, 2000). Acredita-se que a etapa inicial de adesão de *A. brasilense* ocorra por quimiotaxia mediada pelo flagelo, em seguida ocorre a etapa irreversível de adesão mediada por polissacarídeos da própria bactéria (ALEXANDRE, GREER e ZHULIN, 2000; ALEXANDRE, 2010). Alguns trabalhos mostram a variabilidade dos polissacarídeos de parede e sua influência na agregação celular, adsorção na raiz e sobrevivência da célula bacteriana

(BURDMAN *et al.*, 2001; LERNER *et al.*, 2009).

Estas bactérias são capazes de fixar o nitrogênio atmosférico e se associar a raízes de diversas plantas economicamente importantes como milho, trigo, sorgo entre outras; apresentando efeitos positivos de promoção do crescimento (OKON, 1985 ; STEENHOUDT e VANDERLEYDEN, 2000; ECKERT *et al.*, 2001; SPAEPEN, VANDERLEYDEN e OKON, 2009 ; HELMAN, BURDMAN e OKON, 2011). Inoculantes comerciais têm sido testados e utilizados em centenas de milhares de hectares, principalmente na América Latina (FUENTES-RAMIREZ e CABALLERO-MELLADO, 2005 ; HUNGRIA *et al.*, 2010; HELMAN, BURDMAN e OKON, 2011). No Brasil as estirpes AbV5 e AbV6 tem sido largamente utilizadas. Na safra 2012-2013 foram inoculadas aproximadamente 2,5 milhões de hectares de milho e trigo (fonte: ANPII).

Apesar do grande conhecimento e estudo acerca da fixação biológica de nitrogênio em *Azospirillum*, somente Okon (1985) mostrou dados convincentes da contribuição da FBN para o crescimento vegetal. Porém, o sucesso desta bactéria como BPCP se deve ao fato dela expressar eficientemente diversas outras características que promovem o crescimento da planta como: (1) produção e secreção de fitohormônios como auxinas, citocininas e giberelinas (SPAEPEN *et al.*, 2007); (2) produção de óxido nítrico, que age como um sinalizador celular (MOLINA-FAVERO *et al.*, 2008; BASHAN e DE-BASHAN, 2010); (3) síntese e utilização de polihidroxialcanoatos e carotenoides (KADOURI, JURKEVITCH e OKON, 2003; FIBACH-PALDI, BURDMAN e OKON, 2012).

1.3 MECANISMOS MOLECULARES ENVOLVIDOS NA COLONIZAÇÃO E NA PROMOÇÃO DO CRESCIMENTO VEGETAL POR BPCP.

1.3.1 Fixação Biológica de Nitrogênio

Os fatores genéticos que regulam a formação de comunidades endofíticas ou rizosféricas no solo são pouco compreendidos, bem como as adaptações moleculares que regulam tais interações. Dentre os mecanismos de promoção de

crescimento vegetal e estabelecimento da interação com o hospedeiro, a fixação biológica de nitrogênio é o mais antigo e mais estudado.

O nitrogênio é um elemento essencial para a sobrevivência de todos os organismos, sendo necessário para a síntese de proteínas, ácidos nucléicos e outras biomoléculas. Ele é abundante no ar atmosférico (aproximadamente 80%) na forma de nitrogênio molecular (N_2), mas somente certas bactérias e arqueas são capazes de transformar o N_2 atmosférico em NH_3 (POSTGATE e CANNON, 1981).

A fixação biológica de nitrogênio é uma parte importante do ciclo do nitrogênio porque repõe o conteúdo global de nitrogênio da biosfera e compensa as perdas devido à desnitrificação. Bactérias fixadoras de nitrogênio, também chamadas de diazotróficas são microrganismos capazes de realizar o processo de fixação de nitrogênio. Através do complexo enzimático da nitrogenase estas bactérias convertem o nitrogênio atmosférico em amônia, em uma forma assimilável pela planta (TJEPKEMA e BURRIS, 1976).

Estes microrganismos podem ser classificados em dois grupos de acordo com sua interação com parceiros vegetais: 1) diazotrofos de vida livre, que são capazes de fixar N_2 independente de um hospedeiro; e 2) diazotrofos simbióticos que dependem do estabelecimento de uma interação com o hospedeiro vegetal. Dentre estas bactérias promotoras de crescimento vegetal se encontram fixadores de nitrogênio dos gêneros *Azospirillum*, *Gluconacetobacter, Azoarcus* e *Herbaspirillum* (HUREK *et al.*, 1993; JAMES e OLIVARES, 1998; REINHOLD-HUREK, e HUREK, 1998).

Os genes relacionados a fixação de nitrogênio tanto em bactérias de vida livre como em bactérias simbióticas são chamados genes *nif*. Eles incluem os genes estruturais da maquinaria da nitrogenase, genes necessários para a biossíntese dessa enzima e os genes regulatórios. O complexo enzimático da nitrogenase é formado por metaloenzimas com estruturas conservadas e mecanismos característicos (REES e HOWARD, 2000) e catalisa a reação:

$$N_2 + 8\ e^- + 8\ H^+ + 16\ MgATP \longrightarrow 2\ NH_4^+ + 16\ MgADP + 16\ Pi + H_2$$

A fixação biológica de nitrogênio é altamente regulada a nível transcricional e

pós-traducional, por mecanismos regulatórios que variam dependendo da bactéria fixadora (DIXON e KAHN, 2004). A proteína NifA é o principal regulador deste processo em Proteobacteria. A modulação de sua atividade ocorre em resposta a concentrações de oxigênio e nitrogênio fixado. Em espécies do filo Proteobacteria, os genes *nif* estão sujeitos a ativação transcricional por NifA (proteína da família EBP - *enhancer-binding protein*) juntamente com o fator σ^{54} da RNA polimerase. O sistema regulatório de dois componentes NtrB-NtrC por sua vez, controla a expressão de NifA e é responsável pelo sensoriamento global e controle em resposta a concentrações de nitrogênio. Proteínas da família PII de transdução de sinal também são importantes para comunicar o status de nitrogênio a vários reguladores responsáveis pela transcrição dos genes *nif* em resposta à disponibilidade de nitrogênio. Em *H. seropedicae*, a proteína transdutora de sinal GlnK é um regulador chave da fixação de nitrogênio em ambos os níveis transcricional e pós-traducional, pois GlnK interage com NifA, provavelmente modulando sua atividade. Além disso, juntamente com a proteína transportadora de amônio AmtB, está envolvida na regulação pós-traducional da atividade da enzima nitrogenase (revisado por CHUBATSU *et al.*, 2011).

1.3.2 Quimiotaxia

Quimiotaxia bacteriana é um fenômeno pelo qual as bactérias percebem o ambiente químico onde estão inseridas e controlam seu movimento em direção a substâncias atrativas ou se afastam das repelentes (BERG, 1975). A quimiotaxia auxilia as bactérias na adaptação a ambientes adversos e na amplificação dos sinais. Este sistema serve como modelo para o estudo de vias de sinalização de dois componentes que são únicos aos grupos bacterianos e vegetais. Em *E. coli,* os principais fatores envolvidos na via de transdução de sinal de quimiotaxia é a proteína de membrana aceptora de metil (MCP – *methyl-accepting protein*) e 6 proteínas citosólicas: CheA, CheB, CheR, CheW, CheY e CheZ. A quimiotaxia é facilitada pela metilação ou desmetilação dos quimioreceptores, catalisada por CheR e CheB (revisado por HAZELBAUER *et al.*, 2008).

Na interação planta-bactéria a importância da quimiotaxia é devido ao fato de

que as plantas exsudam grandes quantidades de nutrientes, os quais podem agir como quimioatraentes para as bactérias (BRENCIC e WINANS, 2005). Nessas interações, a bactéria visa obter nutrientes do exsudato ou mesmo se instalar no interior da planta (DROGUE *et al.*, 2012). O flagelo é a primeira estrutura da bactéria que entra em contato com a célula vegetal, sendo responsável pelo movimento em resposta à quimiotaxia do microrganismo em direção à planta. A quimiotaxia em direção a ácidos orgânicos, aminoácidos e compostos aromáticos é bem documentada no caso da bactéria *A. brasilense* e *A. lipoferum* (REINHOLD, HUREK e FENDRIK, 1985; LOPEZ-DE-VICTORIA e LOVELL, 1993). Ácidos orgânicos como malato e succinato, aminoácidos como aspartato e glutamato e os compostos aromáticos benzoato e protocatecoato, mostraram ter um efeito quimiotático em diferentes estirpes desta bactérias (VRANOVA *et al.*, 2010). Em *Pseudomonas*, a expressão de *cheY*, mostrou-se dependente da variedade de beterraba inoculada (MARK *et al.*, 2005). As bases moleculares das vias de sinalização de quimiotaxia podem envolver uma regulação gênica na bactéria que é dependente do cultivar da planta hospedeira e da espécie/estirpe, e investigações acerca deste tema têm sido frequentes e foram bem revisadas por Drogue e colaboradores, (2012).

1.3.3 Adesão

As bactérias aderem-se às raízes normalmente por dois mecanismos aparentemente independentes um do outro: adsorção, caracterizada pela fraca adesão mediada principalmente por proteínas ou moléculas de ancoragem, e aderência propriamente dita quando a bactéria se adere através de polissacarídeos (RODRÍGUEZ-NAVARRO, DARDANELLI E RUÍZ-SAÍNZ, 2007; HORI e MATSUMOTO, 2010). Em ambos os mecanismos, a interação física entre os dois organismos parece requerer especificidade entre os componentes da superfície da bactéria e os receptores na superfície da raiz vegetal (DROGUE *et al.*, 2012).

Proteínas especializadas expostas à superfície da bactéria são normalmente essenciais para a adsorção das células aos componentes da superfície da raiz vegetal e geralmente funcionam como adesinas. As flagelinas Fla1 e Fla2 de *A. brasilense* Sp7 aparentemente são necessárias para a adsorção da bactéria à raiz,

uma vez que estas flagelinas purificadas são capazes de aderir à raiz de trigo *in vitro* (CROES *et al.*, 1993). No endófito *Azoarcus* sp. BH72 o sistema pili do tipo IV têm papel essencial para o estabelecimento da bactéria na superfície radicular de plântulas de arroz. Mais ainda, mutantes nos genes *pilA* e *pilT* são incapazes de aderir e colonizar internamente as raízes de arroz (DÖRR, HUREK e REINHOLD-HUREK, 1998; BÖHM, HUREK E REINHOLD-HUREK, 2007). O pilus do tipo IV também é responsável pela motilidade do tipo *twitching.* Esse tipo de motilidade é independente do flagelo, sendo uma forma da bactéria se movimentar em superfícies úmidas, como a superfície radicular. Este movimento é mediado pela extensão seguida de retração do pilus. A motilidade tipo *twitching* é importante para a colonização por diversas bactérias de hospedeiros tanto vegetais como animais (MATTICK, 2002).

O modo como diferentes espécies se aderem às raízes é variado. Por exemplo, a análise genômica de *Enterobacter* sp. estirpe 638, um endófito de árvores do gênero *Populus*, mostrou diversos genes codificando para proteínas envolvidas na adesão às raízes, incluindo hemaglutininas, adesinas auto-transportadoras (YadA), pili tipo I e IV, biossíntese de celulose e polissacarídeos (TAGHAVI *et al.*, 2010). Por outro lado, o genoma de *A. brasilense* Sp245 não possui genes *pil*, mas possui genes TAD (*tight adhesion*) pili. Este genes estão localizados na ilha genômica WCI (*widespread colonization island*) e codificam uma maquinaria necessária para a montagem da adesina Flp (*fimbrial low-molecular-weight protein*), uma proteína fibrilar de baixo peso molecular.

A adesão dependente de produtos dos genes TAD é inexistente em bactérias do gênero *Herbaspirillum*. Mas, uma vez na superfície radicular, a ancoragem de bactérias deste gênero pode ser mediada por lipopolissacarídeos (LPS), exopolissacarídeos (EPS) e polissacarídeos capsulares (CPS) em contato com a superfície da raiz vegetal. Resultados recentes sugerem que é através da ligação do antígeno-O da cadeia de LPS à lecitinas específicas que *H. seropedicae* adere às raízes de milho (BALSANELLI *et al.*, 2010). Como exposto, existem diversos mecanismos bacterianos utilizados para a aderência eficiente às raízes vegetais, este deve ser um dos fatores que controlam a especificidade entre bactéria e hospedeiro vegetal.

1.3.4 Produção de fitohormônios e nutrientes

As bactérias promotoras de crescimento vegetal normalmente produzem mais de um tipo de hormônio vegetal, incluindo: auxina (Ácido Indol Acético - AIA), ácido giberélico, citocininas, ácido abscísico e etileno (SPAEPEN, VANDERLEYDEN e REMANS, 2007; AHMAD, AHMAD e KHAN, 2008). A produção de fitohormônios por estas bactérias depende da composição do exsudato da raiz e tem como consequência principalmente o aumento do tamanho do sistema radicular, permitindo um melhor uso do solo e consequentemente maior aporte de minerais e água melhorando a nutrição da planta (SPAEPEN, VANDERLEYDEN e REMANS, 2007). A auxina é o hormônio mais estudado, sua biossíntese ocorre principalmente pela via do triptofano, o qual atua como um precursor biosintético do ácido indol acético (SPAEPEN, VANDERLEYDEN e REMANS, 2007).

O etileno é um fitohormônio produzido pelas plantas em resposta a estresses bióticos ou abióticos. O etileno inibe o crescimento da planta, estimula a senescência, a abscisão de folhas e frutos e a morte celular programada perto de pontos de infecções bacterianas (BASHAN e DE-BASHAN, 2005). Algumas bactérias produzem uma enzima denominada 1-aminociclopropano-1-carboxilato (ACC) deaminase, que promove a clivagem do precursor do etileno, ACC, em amônia e α-cetobutirato (GLICK *et al*., 2007). Esta enzima é responsável por estimular o crescimento vegetal pelo decréscimo dos níveis de etileno na planta (SUN, CHENG e GLICK, 2009). Apesar de não haver nenhuma via descrita para esta enzima no metabolismo das bactérias, seu efeito no crescimento vegetal foi demonstrado em cepas expressando a ACC deaminase em *A. brasilense* Cd e Sp245 (HOLGUIM *et al.*, 2001). Em *P. putida*, a estirpe selvagem UW4 estimulou o crescimento de plantas de canola em condições de estresse salino, o mesmo não foi observado para a estirpe mutante no gene que codifica a enzima ACC deaminase (CHENG *et al.*, 2007). O gene *acdS*, que codifica para a ACC deaminase, foi identificado no genoma de *H. seropedicae* SmR1. A produção conjunta de auxina e ACC deaminase por *H. seropedicae* pode ser um mecanismo provável para estímulo do crescimento vegetal por este microrganismo (PEDROSA *et al.*, 2011).

1.3.5 Solubilização de fosfato

Os microrganismos solubilizadores de fosfato são caracterizados pela sua capacidade de solubilizar formas precipitadas de P em meios de cultura de laboratório. Incluem uma diversidade de microrganismos tanto simbióticos como de vida livre. Algumas bactérias dos gêneros *Bacillus*, *Enterobacter* ou *Pseudomonas* promovem o crescimento da planta através da produção de ácidos orgânicos e mineralização de fosfato orgânico via fosfatases ácidas e fitases, solubilizando o fosfato do solo (RODRÍGUEZ e FRAGA, 1999; RICHARDSON *et al.*, 2009). A quantidade de fósforo solúvel liberado depende do tipo do ácido orgânico produzido pela BPCP. A habilidade da bactéria promotora de crescimento de produzir ácidos orgânicos e solubilizar o P mineral é dependente da disponibilidade de carbono na rizosfera, e pode ser regulada por repressão catabólica por ácidos orgânicos como malato e succinato no caso de bactérias do gênero *Pseudomonas* (RODRÍGUEZ e FRAGA, 1999; RICHARDSON *et al.*, 2009; PATEL *et al.*, 2011).

1.3.6 Secreção de Proteínas

A secreção de proteínas tem um papel central nas interações entre bactéria e planta. Seis tipos de sistema de secreção foram descritos para bactérias gram-negativas (TSENG, TYLER e SETUBAL, 2009). As proteínas podem ser translocadas através da membrana interna e externa em um único passo, utilizando os sistemas de secreção do tipo I, tipo III, tipo IV e tipo VI, ou então transportadas até o espaço periplasmático pela via geral de transportadores dependentes de SEC ou TAT e em seguida são exportadas para o espaço extracelular pelo sistema de secreção do tipo II e tipo V. Os sistemas do tipo III, IV e VI utilizam uma unidade de translocação, semelhante a uma agulha, a qual permite a injeção direta da proteína para o citoplasma da célula do hospedeiro (Revisado por TSENG, 2009). A importância destes 3 sistemas tanto para as interações patogênicas como benéficas tem sido descritas (TSENG, TYLER e SETUBAL, 2009). Muitas proteínas (toxinas ou efetoras) secretadas pelos microrganismos têm a capacidade de causar uma resposta no sistema de defesa da planta de forma a contribuir para a fisiologia da bactéria ajudando no sucesso da colonização, nutrição e na proliferação da bactéria

na rizosfera ou nos tecidos vegetais internos (TORTO-ALALIBO *et al.*, 2009). O genoma de *H. seropedicae* contém genes que codificam para os sistema de secreção do tipo I, tipo II, tipo III, tipo V e tipo VI. Dentre estes, os sistemas de secreção dos tipos III e VI são os mais prováveis de estarem envolvidos com a interação planta-bactéria. No entanto, para o sistema de secreção do tipo III (SSTIII) ainda não foi observada nenhuma evidência de sua funcionalidade (dissertação PANKIEVICZ, 2010 e este trabalho). Já para a bactéria *H. rubrisubalbicans*, causadora de doença da estria vermelha em cana-de-açúcar vr. B4362, mutantes no gene *hrpE* do SSTIII não foram capazes de produzir sintomas, mostrando o envolvimento deste sistema com o estabelecimento da doença (SCHMIDT *et al.*, 2012).

O sistema de secreção do tipo VI (SSTVI) é o mais comum entre as bactérias endofíticas e foi encontrado em análises metagenômicas de endófitos de raízes de arroz, fortalecendo a ideia de que este sistema é relevante para a interação planta-bactéria (REINHOLD-HUREK e HUREK, 2011). O SSTVI é comum em Proteobactérias (BOYER *et al.*, 2009). Bactérias patogênicas do gênero *Burkholderia*, *Yersinia* e *Pseudomonas* parecem ter vários genes que codificam para este sistema (PUKATZKI, MCAULEY e MIYATA, 2009). Em rizóbios, o sistema Imp, como também é chamado o SSTVI, afeta a capacidade de infecção da bactéria em diferentes hospedeiros (BLADERGROEN, BADELT e SPAINK, 2003). No fitopatógeno *Agrobacterium tumefaciens* a proteína Hcp do SSTVI é secretada, sugerindo que este sistema está relacionado com a formação de tumor por esta bactéria (WU *et al.*, 2008).

Devido ao aumento de publicações relatando o envolvimento do SSTVI com a interação planta-bactéria, parece que este sistema está envolvido com o estilo de vida das bactérias promotoras de crescimento de planta. Descobrir qual o papel deste sistema e quais proteínas são secretadas são objetivos a serem alcançados futuramente.

1.3.7 Mecanismos de Defesa das bactérias

As BPCP sintetizam algumas enzimas que podem modular o crescimento e

desenvolvimento da planta hospedeira. Estas enzimas normalmente estão relacionadas com a defesa da bactéria ou podem hidrolisar a parede vegetal de modo a invadir a célula. A produção de enzimas como quitinases, celulases, beta-1-3-glucanases, proteases e lipases podem lisar as células de fungos ou suprimir a presença de rizobactérias deletérias (MULETA, ASSEFA e GRANHALL, 2007). Outra forma de defesa é a produção de antibióticos que contribuem para o sucesso da colonização e previnem doenças que podem ser causadas por outras bactérias nas raízes das plantas. Algumas bactérias do gênero *Bacillus* produzem antibióticos e promovem o crescimento das plantas (CHOUDHARY e JOHRI, 2009). Além disso, a exposição das plantas à estresses bióticos e abióticos leva a uma grande produção de óxido nítrico e de espécies reativas de oxigênio. A produção de espécies reativas de oxigênio é a mediadora da resposta imune da planta contra um possível patógeno e assim é usada como sinalização molecular (BOLWELL e WOJTASZEK, 1997). Muitas BPCP possuem mecanismos para a destoxificação destas espécies reativas tais como, superóxido dismutases, peroxidases, hidroperóxido redutases e glutationa-S-transferases (MITTER *et al.*, 2013).

1.3.8 Metabolismo de Polihidroxialcanoatos

Dentre todas as capacidades bacterianas expostas anteriormente, a capacidade da bactéria de ajustar seu metabolismo diante à situações adversas ou durante a interação, influencia no sucesso do estabelecimento da colonização e promoção do crescimento. A capacidade de sintetizar e armazenar compostos de reserva como os polihidroxialcanoatos (PHA) é uma destas que podem ser utilizados como fonte energética e reserva de compostos orgânicos (MADISON e HUISMAN, 1999). Em *A. brasilense*, as reservas de PHA são determinantes para a bactéria superar períodos de deficiência de carbono, além disso, a sobrevivência de *A. brasilense* Sp7 em condições de baixo nutriente é maior quando comparada com um mutante deficiente na produção de PHA (KADOURI *et al.*, 2002; KADOURI, JURKEVITCH e OKON, 2003; KADOURI *et al.*, 2005). Experimentos de campo mostraram que houve uma promoção do crescimento de milho e trigo utilizando inoculação com bactérias que produziam quantidades aumentadas de PHA

(DOBBELAERE, S. *et al.*, 2001; HELMAN, BURDMAN e OKON, 2011). *H. seropedicae* sintetiza PHA em condições de fixação de nitrogênio em meio de cultura (CATALAN *et al.*, 2007) e o seu genoma contém 13 genes envolvidos com sua síntese e degradação (PEDROSA *et al.*, 2011).

A via de síntese e degradação de PHA é bem caracterizada para algumas bactérias. Elas acumulam o polímero em condições onde há disponibilidade de ácidos graxos, mas limitação de nutrientes vitais como N, P, S e O_2. O polímero acumulado, por sua vez, pode ser degradado quando a limitação de nutrientes é diminuída, mas há menor disponibilidade de carbono. Em alguns casos síntese e degradação acorrem ao mesmo tempo (WITHOLT e KESSLER, 1999). Alguns autores, incluindo Ren e colaboradores (2009) mostraram que a polimerização e degradação de PHA ocorre simultaneamente. Em *Pseudomonas putida* o ácido 3-hidroxicarboxílico é liberado pela depolimerase PhaZ e excretado para o meio, enquanto que, simultaneamente 3-hidroxiacil-CoA é incorporado ao polímero. Isso resulta em um ciclo metabólico onde o balanço energético é a hidrólise de um ATP a AMP e pirofosfato. A vantagem metabólica deste mecanismo não é clara, já que energia na forma de ATP é gasta no que parece ser um ciclo fútil. No entanto, pode ser que esse ciclo sirva como mecanismo regulatório para o controle do fluxo de ácido graxo através da oxidação de ácidos graxos, uma vez que as células apresentando altos níveis energéticos possuem uma alta razão [Acetil-CoA]/[CoA] e [NADH/NAD] e a oxidação de ácidos graxos é inibida, havendo acúmulo de 3-hidroxiacil-CoA. Por sua vez, a alta razão [3-hidroxiacil-CoA]/[CoA] direciona os ácidos 3-hidroxicarboxílicos para o polímero de PHA. Assim, as flutuações ambientais das concentrações de carbono e outros nutrientes vitais serão compensadas pela absorção ou liberação de carbono dos polímeros (REN *et al.*, 2009).

1.4 RNA-SEQ COMO FERRAMENTA PARA ESTUDO DOS MECANISMOS MOLECULARES DA INTERAÇÃO PLANTA-BACTÉRIA

O transcritoma é o conjunto completo de transcritos de uma célula em um estágio específico de desenvolvimento ou condição fisiológica. Compreender o transcritoma é essencial para interpretar os elementos funcionais de um genoma e revelar os componentes moleculares de células e tecidos (WANG *et al.*, 2008). RNA-seq (do inglês *RNA sequencing*) é essencialmente o sequenciamento paralelo massivo de cDNA derivado de mRNA celular. Este método é baseado nas plataformas de sequenciamento de nova geração (*NGS-next generation sequencing*). Nesta técnica, todas as moléculas de RNA são reversamente transcritas a cDNA e, dependendo da plataforma onde são sequenciadas, as moléculas de cDNA devem ser amplificadas e sequenciadas. Depois da reação de sequenciamento, as leituras (sequências obtidas) são mapeadas contra um genoma de referência, de onde se pode deduzir a estrutura ou expressão de um dado transcrito da amostra sequenciada (WESTERMANN, GORSKI e VOGEL, 2012).

A eficiência da interação planta-bactéria depende de um conjunto de mecanismos adaptativos por parte do microrganismo (propriedade quimiotáticas, adaptações metabólicas, capacidade de colonização da raiz e regulação de fatores genéticos envolvidos na promoção do crescimento vegetal) e da planta hospedeira (padrão de compostos exsudados, propriedade da superfície radicular e sensibilidade a hormônios bacterianos). Deste modo, o estudo de transcritoma durante a interação planta-bactéria pode fornecer informações ou revelar determinantes genéticos únicos envolvidos com estas associações. Mais ainda, o estudo de genomas e transcritomas de bactérias que colonizam plantas podem revelar pistas importante da dinâmica de evolução destas associações e adaptações espécie-específica (DROGUE *et al.*, 2012).

Apesar da quantidade de aspectos positivos que o sequenciamento de nova geração trouxe aos estudos de biologia molecular, estudar um transcritoma de procarioto traz diversos desafios, pois a abundância relativa de transcritos individuais pode variar em muitas ordens de magnitude. Por isso, para gerar perfis transcritômicos abrangentes utilizando RNA-seq deve-se obter um número grande o suficiente de leituras para detectar as transcrições biologicamente relevantes que compõem uma percentagem relativamente pequena da biblioteca de cDNA.

A detecção e quantificação de transcritos de baixa abundância podem ser

aprimoradas de duas maneiras principais. Primeiro, o número total de leituras por biblioteca pode ser aumentado. Em segundo lugar, a proporção de leituras que representam transcritos raros pode ser aumentada através da remoção do RNA ribossômico que compreende de 80-95% dos transcritos bacterianos (GIANNOUKOS *et al.*, 2012). Em experimentos focados em comparar expressão gênica entre estirpes ou diferentes condições de crescimento, a inclusão de mais cepas, replicatas biológicas ou amostras coletadas em diferentes tempos podem compensar a baixa profundidade do sequenciamento e permitir descobertas biológicas e confiabilidade estatística, o que pode ser mais valioso do que detectar transcritos de baixa abundância (HAAS *et al.*, 2012). Estudos recentes sugerem que a quantificação de 95% de transcritos em células de mamíferos requer 700 milhões de leituras (BLENCOWE, AHMAD e LEE, 2009); no entanto, nenhuma estimativa do número de leituras necessárias para a saturação de um transcritoma bacteriano foi realizada ou relatada (HAAS *et al.*, 2012).

1.5 ADOÇÃO DE *Setaria Viridis* COMO MODELO VEGETAL PARA O ESTUDO DA PROMOÇÃO DO CRESCIMENTO VEGETAL POR BPCP

Pertencente ao grupo Paniceae e subfamília Panicoideae, cerca de 125 espécies de *Setaria* estão distribuídas em todo mundo, incluindo a planta daninha *green foxtail* ou *Setaria viridis*, a forragem *Setaria sphacelata*, o cereal semi-domesticado *yellow foxtail* ou *Setaria glauca* e talvez a mais antiga cultura agrícola domesticada, *foxtail millet ou Setaria italica*. A planta *green foxtail* é conhecida pela sua capacidade invasora agressiva e ampla distribuição nas regiões tropicais e temperadas, e tem sido considerada com o ancestral selvagem da cultura *foxtail millet*. O gênero *Setaria* está evolutivamente perto de cereais como o milho (*Zea mays*), cana-de-açúcar (*Saccharum officinarum*), sorgo (*Sorghum bicolor*) milheto (*Pennisetum miliaceum*), e de culturas utilizadas na produção de biocombustíveis como *switchgrass* (*Panicum virgatum*) e capim-elefante (*Pennisetum purpureum*).

Setaria italica é cultivada nas regiões áridas e semiáridas do mundo por

milhares de anos, sendo um importante grão na dieta alimentar chinesa e indiana. É conhecida pela sua excepcional tolerância a seca e ao estresse salino. Apesar do progresso feito com melhoramento clássico de novos cultivares na China a produtividade de grãos e o rendimento desta planta ainda são baixos se comparado com milho, sorgo e outros cereais. O estudo molecular de *Setaria* irá impulsionar o modo de melhoramento, considerando o contexto genômico de novas cultivares.

As plantas do gênero *Setaria* são plantas C_4. A fotossíntese do tipo C_4 está associada com a produção eficiente de biomassa e melhor aproveitamento de água e nitrogênio. Acredita-se ser um componente responsável pela alta produtividade de muitas culturas destinadas a alimentação como milho e sorgo, e de culturas destinadas a produção de biocombustíveis como cana-de-açúcar e "*switchgrass*". Há um grande interesse em transferir a maquinaria responsável pela fotossíntese do tipo C_4 para outras espécies, devido aos desafios encontrados em trabalhar com plantas de grande porte como milho, cana-de-açúcar e outras culturas C_4. Com intuito de transferir o mecanismo de fotossíntese C_4 para uma planta C_3 foi criado o projeto Arroz C_4. Este projeto é financiado pela Fundação Gates e planeja usar a *Setaria* como doador genético do metabolismo C_4 para criação de um arroz com fotossíntese do tipo C_4, o que pode aumentar a produtividade do arroz significativamente (VON CAEMMERER, QUICK e FURBANK, 2012). *Setaria* foi escolhida para este projeto por possuir uma sequência genômica de referência e mapas de haplótipos disponíveis, pequeno porte físico e rápido ciclo de vida (BRUTNELL *et al.*, 2010).

Assim, *Setaria* está bem posicionada para ser o modelo mais adequado para estudos de genômica funcional de cereais e produtores de biocombustível, uma vez que muitos são difíceis de estudar diretamente devido aos genomas poliplóides, extensos tempos de geração e grande porte da planta. *S. viridis* possui um genoma pequeno e diplóide (~ 490 Mb) , curto ciclo de vida (50-80 dias) e baixa estatura (30-50 cm) (FIGURA 2) (BENNETZEN *et al.*, 2011; JIANG, BARBIER e BRUTNELL, 2013).

Em março de 2014 aconteceu a primeira conferência internacional sobre genética de *Setaria*, reunindo cientistas do mundo todo que estudam diferentes aspectos da biologia desta planta. Nesta conferência *Setaria viridis* foi estabelecida

como um organismo modelo para os geneticistas de planta e foi criada a comunidade internacional de *Setaria* (*Internacional Setaria Community*).

Como exposto anteriormente, a literatura estabelece firmemente que as bactérias promotoras de crescimento vegetal podem afetar o crescimento das plantas e aumentar a produtividade das culturas. No entanto, pouco se sabe sobre os mecanismos moleculares por onde estas bactérias induzem seus efeitos benéficos. Um exemplo disso é o diazotrófico *A. brasilense*, que é considerada uma das BPCP mais bem estudadas e um colonizador eficiente de raízes de plantas (FIBACH-PALDI, BURDMAN e OKON, 2012). No entanto, até o momento, nenhum mecanismo exclusivo foi estabelecido para explicar a capacidade destas bactérias promoverem o crescimento vegetal. Embora o estudo de sistemas biológicos diversos possa ser muito informativo, os progressos são mais rápidos quando a investigação se concentra em um sistema modelo adequado permitindo um aprofundamento das descobertas. Por exemplo, a adoção da leguminosa modelo *M. truncatula* e *L. japonicus* resultou em avanços rápidos na compreensão da fixação de nitrogênio na simbiose entre *Rhizobium*-leguminosa (JIANG e GRESSHOFF, 1997; CANNON *et al.*, 2006).

Considerando os esforços da comunidade internacional de *Setaria* em estabelecer esta planta como organismo modelo para estudos genéticos da fotossíntese C_4 e devido às características desta gramínea (possuir estatura pequena, condições de cultivo favoráveis, curto tempo de geração e genoma sequenciado), ela se apresenta como um modelo adequado para o estudo molecular das interações entre BPCP-gramíneas.

FIGURA 2 - Foto de exemplares de *S. viridis* (esquerda) e *S. italica* (direita) mostrando a diferença do porte físico entre as duas espécies FONTE: BRUTNELL et al., 2010.

2 OBJETIVOS

O objetivo geral deste trabalho foi estudar os mecanismos moleculares envolvidos na interação entre *Herbaspirillum seropedicae*-planta. Para tanto, foi caracterizado o perfil transcritômico de *H. seropedicae* colonizando raízes de trigo, através da construção de bibliotecas de mRNA e sequenciamento utilizando a técnica de sequenciamento de nova geração na plataforma SOLiD 4® (Capítulo I). Além disto, um modelo vegetal para o estudo da promoção do crescimento em plantas foi desenvolvido através de uma seleção de vários genótipos de *Setaria viridis* responsivos a inoculação com as bactérias promotoras do crescimento vegetal, *H. seropedicae* SmR1 e *A. brasilense* FP2 (Capítulo II).

3 CAPÍTULO I

3.1 RNA-seq Transcriptional Profiling of *Herbaspirillum seropedicae* Colonizing Bread Wheat (*Triticum aestivum*) Roots.

Abstract

Herbaspirillum seropedicae is a diazotrophic and endophytic bacterium that associates with economically important grasses promoting plant growth and increasing productivity. To identify genes related to bacterial ability to colonize plant, wheat seedlings growing hydroponically in Hoagland's medium were inoculated with *H. seropedicae* and incubated for 3 days. Total mRNA from the bacteria present in the root surface and in the plant medium were purified, depleted from rRNA and used for RNA-seq profiling. RT-qPCR analyses were conducted to confirm regulation of selected genes. Comparison of RNA profile of root attached and planktonic bacteria revealed extensive metabolic adaptations to the epiphytic life style. These adaptations include expression of specific adhesins and cell wall re-modeling to attach to the root. Additionally, the metabolism was adapted to the microxic environment and nitrogen-fixation genes were expressed. Polyhydroxyalkanoate (PHA) synthesis was activated, and PHA granules were stored as observed by microscopy. Genes related to plant growth promotion, such as auxin production were expressed. Many ABC transporter genes were regulated in the bacteria attached to the roots. The results provide new insights into the adaptation of *H. seropedicae* to the interaction with the plant.

Keywords: Biological nitrogen fixation, *H. seropedicae*, RNA-seq profiling, rhizosphere, PGPB-plant growth promoting bacteria.

Acknowledgments

The Brazilian Program of National Science and Technology Institute/INCT for Biological Nitrogen Fixation supported this work. We would like to acknowledge Dr. Eliane Vendrusculo (UFPR-Palotina) for kindly providing the wheat seeds. We are also thankful to Roseli Prado, Marilza D. Lamour and Valter Baura for technical assistance. V.C.S.P. was beneficiary of PhD fellowship from the Brazilian Research Council/ CNPq. G.W. was supported by Vice-presidency office of Education, Information and Communication (IOC/FIOCRUZ) and CAPES. F.P. acknowledges the support of Carlos Chagas Filho foundation for research from Rio de Janeiro State (FAPERJ) and Brazilian Research Council (CNPq). F.P. and the Bioinformatics Unit acknowledge the support of the Cancer Foundation and the Ministry of Health of Brazil.

Introduction

Endophytic bacteria are able to colonize plant internal tissues without injuring the host. Among these bacteria, the nitrogen fixers are considered promising biotechnological tools to improve agricultural productivity through biological nitrogen fixation (BNF) and plant growth promotion (Pankievicz et al. 2015). *Herbaspirillum seropedicae* is a useful model to study such a group of bacteria, since this organism is easily cultivated, has its genome sequenced (Pedrosa et al. 2011) and genetic manipulation techniques were developed. *H. seropedicae* is a diazotrophic and endophytic *Betaproteobacterium* found colonizing the rhizoplane and internal tissues of crops such as wheat, sugarcane, rice and maize (for a review see Monteiro et al. (2012)). *H. seropedicae* was also found colonizing diverse plants such as banana and pineapple (Magalhães Cruz et al. 2001). Under laboratory conditions *H. seropedicae* was able to colonize *P. vulgaris* plantlets (Schmidt et al. 2011) and micropropagated sugarcane (Muthukumarasamy et al. 2006). The inoculation of micropropagated sugarcane with *Herbaspirillum spp*. resulted in 17% increase of the rhizome presumably due to BNF (Oliveira et al. 2002). Other studies demonstrated that the inoculation of rice with *H. seropedicae* promoted a yield increase equivalent to the treatment with 40 kg N/ha (Baldani and Baldani 2005; Pereira and Baldani 1995).

Wheat is one of the main sources of calories and proteins of the human diet. Increase in wheat productivity and protein content depends on improved absorption of inorganic nitrogen, thus this crop may benefit from endophytic nitrogen-fixing bacteria (Saubidet and Barneix 1998). Nitrogen derived from biological nitrogen fixation was demonstrated in wheat inoculated with *Azospirillum brasilense* Sp7 (Rennie et al. 1983) and yield increase in *T. aestivum* inoculated with *Azospirillum lipoferum* Sp108 was observed under field and greenhouse conditions (Mertens and Hess 1984). Furthermore, El-Komy and collaborators (2003) showed that the inoculation of wheat with *H. seropedicae* led to an increase in grain productivity and shoot dry weight up to 31% and 23%, respectively.

The colonization process of a plant host by *H. seropedicae* begins with the attachment of the bacteria to the surface of the root, followed by entry at the

emergence of lateral roots and injuries, and spread through intercellular spaces with later occupation of the xylem (revised by Monteiro et al. (2012). Although many authors have shown that *H. seropedicae* colonizes graminaceous plants (Elbeltagy et al. 2001; James et al. 2002; Roncato-Maccari et al. 2003), much of these observations were made at a microscope level and little is known concerning the molecular factors that affect the interaction of *H. seropedicae* with grasses. Genome analyses indicated that *H. seropedicae* has several genes related to bacterial ability to colonize and promote plant growth, including: a) *nif* genes; b) type IV *pili* (T4P); c) auxin biosynthesis (IAA production); d) ACC deaminase gene; e) genes for attachment to surfaces (hemagglutinins/hemolysin) and biofilm formation (EPS) (Pedrosa et al. 2011). In addition, other studies showed that lipopolysaccharides (LPS) are required for *H. seropedicae* attachment to maize root and internal colonization of plant tissues (Balsanelli et al. 2010) and that the flavonoid naringenin regulates the expression of genes involved in cell wall synthesis of *H. seropedicae* (Tadra-Sfeir et al. 2011). On the other hand, mutagenesis of an EPS biosynthesis cluster indicated that exopolysaccharides are necessary for biofilm formation on abiotic surface but not for plant colonization (Balsanelli et al. 2014).

In this study we aimed to determine the global gene expression of *H. seropedicae* associated to wheat roots through transcriptional profiling of the bacteria in the planktonic state compared to the cells attached to the wheat roots (Online Resource 1). RNA-seq libraries were sequenced using next generation sequencing technology and the sequences obtained were mapped on the *H. seropedicae* genome. Analyses of the up-regulated mRNA in attached cells indicated activation of nitrogen fixation, polyhydroxyalkanoate metabolism, cell wall re-modeling and adhesion molecules such as adhesin, suggesting specific metabolic adaptations of the bacteria to the rhizospheric environment. Our results provide a panorama of gene expression of *H. seropedicae* during colonization of wheat roots, and reveal insights into pathways involved in plant-bacteria interaction.

Material and Methods

H. seropedicae growth conditions

The *H. seropedicae* strain SmR1 (Souza et al. 2000) was grown routinely at 30° C with shaking at 120 rotations per minute (rpm) in NFbHP-malate medium (Klassen et al. 1997) with 20 mM of NH_4Cl added (NFbHPN-Malate). Streptomycin was added as needed at the concentrations of 80 µg/mL. The bacterial strains for plant inoculation were pre-cultured overnight in 5 mL of NFbHPN-malate medium and antibiotic was added as needed. An overnight culture was used to inoculate 10 mL NFbHPN-malate medium, which was grown as previously described to an OD_{600} = 1.0. The bacterial cells were collected by quick centrifugation (14.000 rpm for 20 seconds at room temperature) to ensure that the bacteria remained viable. Cell pellets were re-suspended in the same volume of Hoagland's medium (Hoagland 1950) to a cell density of 10^7 cells/mL. 250 µL of this bacterial suspension was added to glass tube containing 25 mL of Hoagland's medium and two wheat plantlets to give 10^5 bacteria per mL.

Germination, inoculation and growth of plantlets

Seeds of *Triticum aestivum* (cv. CD104) were disinfected as described previously (Camilios-Neto et al. 2014; Döbereiner et al. 1995). *In vitro* plant cultivation was done under hydroponic and axenic conditions. Surface sterilized seeds were pre-germinated in water-agar petri dishes in the dark for 24 h at 30°C. The plantlets were then transferred to glass tubes (two plantlets in each tube) containing 25 mL of Hoagland's medium and polypropylene spheres (about 10 mL in volume) were added to each tube to serve as support for the plantlets (Online Resource.1). On the second day after germination, plantlets were inoculated with *H. seropedicae* to a final density of 10^5 *H. seropedicae* cells per mL of Hoagland's medium. The inoculated wheat plantlets were incubated at 26°C with a light cycle of 14/10 h (light/dark) for 72 hours.

Bacterial counting

To evaluate bacterial colonization, roots were sampled 6 to 126 hours post inoculation (h.p.i.). For total bacterial counting the roots were washed and crushed in 0.9% NaCl (m/v) using mortar and pestle. Homogenates were serially diluted and plated on NFbHPN-malate medium containing streptomycin (80µg/µL). Bacterial growth in the hydroponic medium was determined by plating serial dilutions as

described above. Colonies were counted after 2 days of incubation at 30°C and expressed as colony forming units (CFU) per mL of medium or per gram of fresh root tissue.

RNA purification, RNA-seq library construction and sequencing

Attached bacteria were recovered, in 5 mL of RNA latter solution (Ambion®), by vigorous vortexing (1 minute) of roots from 80 plantlets, 3 days after inoculation, followed by centrifugation (4,000 rpm; 40 min; 4ºC). Planktonic cells were collected by centrifugation (10,000 rpm; 1 min; 4ºC.) from the hydroponic medium at the same time. The pellet was resuspended in RNA latter solution (Ambion®) and stored at -20ºC. The total RNA of *H. seropedicae* cells (approximately 10^6 CFU/g of fresh wheat root) was extracted using Trizol (Invitrogen®) and treated with DNAseI (Ambion®) following the manufacturer instructions. The rRNA was depleted using the Microbe Express kit (Ambion®), the libraries for sequencing were prepared using the Whole Transcriptome Analysis kit (Life Technologies) and sequenced in next generation sequencing platform SOLiD 4 (Life Technologies) using the ToP Sequencing Kit (1x50 bp). Two independent samples were processed in parallel of each type of bacterial cell, resulting in 4 sequencing libraries, and each library was sequenced at least twice.

Transmission electron microscopy and scanning electron microscopy

Wheat roots colonized with *H. seropedicae* three days after inoculation were fixed with modified Karnovsk's fixative (Karnovsk 1965), post-fixed with 2% OsO_4 in 0.1 M cacodylic acid buffer (pH 7.2) for 1 h and embedded in Epon 812 (Luft 1961). Subsequently the colonized wheat roots were contrasted with 2% uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963), and examined with a JEOL-JEM 1200 EX II transmission electron microscope. For scanning electron microscopy, the colonized roots were fixed with modified Karnovsk's fixative (Karnovsk 1965), washed in 0.1 M cacodylic acid buffer (pH 7.2) and dehydrated in ethanol. Critical-point dryness was obtained with a Bal-Tec CPD – 030, the samples were coated with gold using a Balzers SCD – 030 sputter coater and examined with a JEOL-JSM 6360 LV scanning electron microscope.

Data analyses

The sequences obtained were mapped on the *H. seropedicae* genome using the software CLC Genomics Workbench (v. 6.5.1). The following parameters were used: reads were trimmed to minimum of 40 bp, 90% alignment to the reference sequence and 80% identity required for inclusion as a mapped read, number of hits equal to 1, number of additional bases down and upstream of the CDS equal to 50 bp. Expressed genes were those that had more than 3 times of coverage. The differential gene expression analyses was performed using DESeq (DESeq using R-package from Bioconductor's project) and were considered regulated those with a fold-change greater than or equal to two and p-value ≤ 0.05. RPKM values (Mortazavi et al. 2008) were calculated using CLC Genomics Workbench (v. 6.5.1). Aiming to evaluate the impact of up and down-regulated genes in *H. seropedicae* metabolic pathways, we performed a gene set enrichment analysis (GSEA) using the Bioconductor's Project (version 2.14) (Reimers and Carey 2006) package Model-based Gene Set Analysis (MGSA) (version 1.13) (Bauer et al. 2010) in statistical environment R (version 3.1.0) (R Core Team, 2014). MGSA calculate the posterior probability of a pathway being affected by up or down-regulated genes. Pathways from KEGG database (Kanehisa et al. 2014) were employed for this analysis and only differentially expressed genes with FDR ≤ 0.05 were used.

Reverse Transcription-quantitative PCR

Total RNA from planktonic and attached bacteria was obtained in independent experiments under the same conditions used for RNA-Seq. Total RNA (300 ng) was used to synthesize cDNA using the high-capacity cDNA reverse transcription kit (Life technologies®). The 16S rRNA of *H. seropedicae* was used as reference. The primers used for the analyses were for the genes *nifH*, *ntrC*, *amtB*, *fixP*, *fnr2*, *ttuC*, *Hsero_2999*, *Hsero_0639*, *pilS*, *pilT*, *adhA*, *narG*, *nirD*, *narU*, *nasF* and *narX*, and their sequences are shown in online resource 3. Primer efficiency was calculated through cDNA dilution curve over at least 5 orders of magnitude. The RT-qPCR experiments were performed using the Power SYBR green master mix (Life technologies®) and the reaction run on a Step One Plus real-time PCR system.

Relative gene expression was determined by the $2^{\Delta\Delta CT}$ (threshold cycle) method (Livak and Schmittgen 2001).

Results

Wheat plantlets colonization

A hydroponic model to study the interaction between wheat and diazotrophic bacteria was setup under axenic conditions. Bacterial counts showed that the number of *H. seropedicae* cells colonizing wheat roots and free-living (hereafter called planktonic) increased up to 10^8 cells/ g of fresh root and 10^7 cells per mL of cultivation media, respectively, after 24 hours post inoculation (h.p.i), and then remained constant until 168 h.p.i (Fig. 1).

To check whether wheat exudates could support bacterial growth, *H. seropedicae* was inoculated in filter-sterilized conditioned Hoagland's medium (obtained from three days hydroponic culture of wheat plantlets in the presence or absence of *H. seropedicae*). The results showed that wheat exudates indeed support *H. seropedicae* growth, as seen by the increase in bacterial population in conditioned Hoagland's medium (Online Resource. 2). Root exudation includes the secretion of ions, enzymes, mucilage, and many different carbon-containing primary and secondary metabolites, and these nutrients attract microbes to the plant root as well as sustain their proliferation (Bais et al. 2006). In addition to the nutrients, molecules exuded by the plant can influence gene expression and the ability of the microbe to colonize the plant root.

RNA-seq profiling of *H. seropedicae* interacting with wheat plants

For the RNA-seq transcriptional analysis, wheat plantlets inoculated with *H. seropedicae* were incubated at 26 °C with a light cycle of 14 hours for 72 hours. Free-living or planktonic (PLANK) and wheat root-attached *H. seropedicae* cells (WRA) were collected and used for RNA extraction. Two independent samples (biological replicates, containing 80 plantlets [WRA] or from 1 L of Hoagland's cultivation medium [PLANK]) of each condition were used to construct 4 sequencing libraries; each PLANK library was sequenced twice, whereas WRA libraries were

sequenced three times. Pearson's correlation coefficient of the transcriptome data (RPKM values) for PLANK and WRA libraries were r=0.98 and r=0.99, respectively, indicating the biological and technical replicates had high reproducibility. On the other hand, the correlation coefficient when the two conditions were compared was r = 0.32 (Table 1).

A total of 99,842,318 reads were obtained for the PLANK libraries and 46,343,321 reads for the WRA libraries; after quality trimming to a minimum of 40 bp, 90% of alignment and 80% of identity to reference, about 97,800,104 (98.0%) and 45,712,110 (98.6%) reads remained, respectively. The latter set was mapped against the *H. seropedicae* genome and wheat sequence database (Table 1).

The planktonic libraries had the highest percentage of sequences mapped to the *H. seropedicae* sequences (55 to 65%) and the lowest percentage mapping to wheat sequences (7.2 to 7.8%), while 23% of the reads from the root-attached cells mapped to wheat sequences and only 12.6 to 15.2% to *H. seropedicae* sequences. The different proportion of reads mapped to wheat sequences between the treatments may be due to detachment of epidermal cells from the roots during harvesting procedure, which required vigorous vortexing to recover the attached bacteria.

For gene expression analyses, we first mapped the reads to the *H. seropedicae* genome using the CLC genomics workbench and performed a DESeq analyses with the reads mapped to the reference genome. For the PLANK libraries we obtained 3,004,584 reads unambiguously mapped and for the WRA libraries 413,969 reads. Our analyses revealed 985 expressed genes (with 3X or higher coverage) in the WRA sample and 2,130 in the PLANK sample; out of these 403 were differentially expressed (fold-change greater than 2 and p-value ≤ 0.05), 152 up regulated and 251 down regulated (Table 2). Differentially expressed genes represent about 8.3% of the *H. seropedicae* genome, indicating that ample changes in *H. seropedicae* gene expression are required to switch from free-living to attached life style.

In order to extract more information from the transcriptomic data we performed a gene set enrichment analysis (GSEA). Analysis of the up-regulated gene set in WRA samples permitted the identification of three overrepresented KEGG pathways,

as follows: oxidative phosphorylation (10 genes); chloroalkene and chloroalkene degradation (5 genes); and pyruvate metabolism (5 genes) (Table 3). A total of five KEGG pathways were overrepresented among the down-regulated genes set. Ribosome pathway had the highest posterior probability of being overrepresented (43 genes were identified associated with this pathway) among down-regulated genes in root-attached cells. RNA degradation (6 genes); RNA synthesis (3 genes); plant-pathogen interaction (3 genes) and protein export (5 genes) had their posterior probability also higher than 0.5 of being overrepresented in the sub-set of down-regulated genes (Table 4). Down regulation of processes involved in proteins biosynthesis pathway (RNA metabolism) indicates that the attached bacteria have a lower duplication rate compared with those in the planktonic state. Nitrogen fixation genes were also overrepresented (posterior probability of 1) in the up-regulated genes dataset of root-attached cells.

Nitrogen fixation and bacterial adaptation to microoxic environment

The overall expression of genes from bacteria attached to the plant roots compared to the planktonic cells suggested that the root surface environment requires specific adaptation for bacterial survival. In this environment, bacteria seem to face deprivation of some nutrients, including oxygen and ammonium. Oxygen sensing is very important for bacterial survival and successful colonization of the plant. The bacterial respiratory shift from oxic to microoxic condition relies on a strategy that ensures that cells can sense and respond to changes. *H. seropedicae* can sense oxygen through Fnr-like proteins, which act as intracellular redox sensors and regulate gene expression according to changes in oxygen levels (Batista et al. 2013). We found that expression of *fnr1* (Hsero_3197) increased 16.8-fold in attached cells, and *fnr2* (Hsero_2381) 38.9-fold. Up-regulation was confirmed by RT-qPCR assay (Fig. 5). However *fnr3* (Hsero_2538) expression had only a minor change. Batista et al. (2013) observed that *fnr1* expression was apparently regulated by Fnr3 and oxygen, while *fnr3* expression was only marginally affected. They also showed that Fnr1 and Fnr3, not only regulate expression of the *cbb3*-type terminal oxidase, but also control the cytochrome content and other components required for the cytochrome *c*-based electron transport. *fixNOPQ* coding for *cbb3-type* oxidase

were up-regulated in attached cells, suggesting that Fnr proteins are activated. The role of Fnr2 could not be identified yet. The finding that *fnr2* is not regulated by oxygen (Batista et al. 2013) and that is up-regulated in root-attached cells may indicate involvement of Fnr2 protein in plant-bacteria interaction.

Regulation of nitrogen fixation and N assimilation in *H. seropedicae* is well characterized (Chubatsu et al. 2012). On the other hand, little is known about nitrogen fixation during grass-bacterial interactions. Although expression of *H. seropedicae nif* genes was shown in roots, stems and leaves of rice, sorghum and maize (James et al. 2002; Roncato-Maccari et al. 2003) here the expression of the whole nitrogen fixation cluster was observed during the plant-bacterial association, strongly indicating active nitrogen fixation by the bacteria. NifH was among the most abundant protein expressed during interaction of *H. seropedicae* and rice seedlings (Alberton et al. 2013).

nif genes were expressed by both *H. seropedicae* colonizing wheat roots and planktonic cells. However, the expression of the *nifHDKENXHsero_2847Hsero_2846fdxB* operon was more prominent in attached bacteria (Fig. 2). In this condition, nitrogenase structural genes *nifHDK* (Hsero_2853; Hsero_2852; Hsero_2851) were up-regulated 34- to 67-fold compared to planktonic cells, and similar levels of up-regulation occurred for the other genes in the operon. Up-regulation of the *nifH* in attached bacteria was confirmed by quantitative real-time PCR with a fold-change comparable to that of RNA-seq (Fig. 5).

Expression of the *nif* transcription activator gene, *nifA* (Hsero_2871), *ntrC* (Hsero_3125) and *glnA* (Hsero_3127) were 60.8-, 7.4- and 2.7-fold up-regulated, respectively, in the WRA libraries. The NtrC-activated operon *nlmAglnKamtB* (Hsero_0083; Hsero_0084; Hsero_0085) (Huergo et al. 2010; Noindorf et al. 2011) was also up-regulated in the WRA libraries with fold-changes of 14.8, 38.9 and 23.8, respectively. Up-regulation of *ntrC* and *amtB* was confirmed by RT-qPCR with a fold-change of 5.3 and 9.3-fold, respectively (Fig. 5). Taken together the results show that cells attached to wheat roots were nitrogen deprived, since the Ntr system was up-regulated. Higher expression of *nif* may also be partially explained by lower oxygen levels since *H. seropedicae* NifA protein is inactivated by O_2 (Souza et al. 1999).

H. seropedicae also contains genes encoding an assimilatory nitrate

reductase (NAS) (*nasAHsero_2899*), a nitrite reductase (*nirBD*) and a respiratory nitrate reductase (NAR) (*narGHJI*) (Pedrosa et al. 2011). Interestingly, genes related to nitrate assimilation (*nasAHsero_2899* and *nirBD* genes) were up-regulated in the attached cells, whereas genes related to the respiratory nitrate reductase were down-regulated. The up-regulation and down-regulation in the attached cells of *nirD* and *narG* genes, respectively, was confirmed by RT-qPCR (Fig. 5). Additionally, RT-qPCR also confirmed the up-regulation in attached cells of *nasF* gene, which encodes to a nitrate transporter related to the NAS, and the down-regulation of *narU* gene, encoding to a nitrate transporter related to NAR (Fig. 5).

The regulation of genes related to nitrate assimilation are well studied in *Klebsiella pneumoniae*, in which *nasA* gene was found to be induced in response to nitrate (Lin and Stewart 1998). Expression analyses of *nasF* promoter in *H. seropedicae* showed that its expression is highly induced in response to nitrate (Bonato, unpublished data). Since no source of nitrogen was added during cultivation of wheat plantlets, the data suggests that wheat roots can exudate nitrate. This result is in agreement with precious work, which shows the up-regulation of *H. seropedicae* genes encoding NAS in maize roots in response to nitrate exudate from maize (Balsanelli et al. 2015). Therefore, nitrate may be a source of nitrogen in *Herbaspirillum*-plant interaction, although the bacteria rely mainly on nitrogen fixation (Pankievicz et al. 2015).

Adhesion

The first step of the colonization process is the attachment of bacteria to the plant cell, a process mediated by bacterial and host surface constituents, such as lipopolysaccharides (LPS), adhesins and lectins (Balsanelli et al. 2010; Balsanelli et al. 2013). Two genes coding for filamentous hemagglutinins proteins were up-regulated in cells attached to the plant roots. Hsero_1294 and *fhaB* (Hsero_3251) had a fold-change of 2.8 and 2.3-fold, respectively (p-value=0.01 and 0.04, respectively). Filamentous hemagglutinin proteins (FHA) are important adhesins involved in mediating the attachment of the bacteria to the host. In the phytopathogen *Xanthomonas axonopodis pv. citri* mutation in *fhaB* gene impaired virulence and adhesion of the bacteria *in planta* assays (Gottig et al. 2009).

Genes coding for exopolysaccharides biosynthesis (*eps* genes) were not expressed by *H. seropedicae* in neither condition. The transcriptomic analyses of epiphytic cells of *H. seropedicae* colonizing the maize rhizosphere also demonstrated the absence of *eps* genes expression (Balsanelli et al. 2015), corroborating the finding that EPS production by *H. seropedicae* is not necessary for colonization of maize roots (Balsanelli et al. 2014) and, apparently, nor for wheat roots. LPS seems involved in maize colonization by *H. seropedicae* (Balsanelli et al. 2010). The cluster for LPS synthesis comprehending CDS Hsero_4197 to Hsero_4222 was expressed in both planktonic and attached bacteria cells (Online Resource 4). On the other hand, *lpxB* (Hsero_2191) encoding a lipid-A-disaccharide synthase was 10.9-fold up-regulated in attached cells. Hsero_2761, encoding a Wza-like outer membrane polysaccharide export protein, and Hsero_4222, encoding a CapD polysaccharide biosynthesis protein, were also up-regulated in attached cells, although the difference in expression was not statistically significant. These genes (Hsero_2191, Hsero_2761 and Hesero_4222) were also up-regulated in *H. seropedicae* attached to maize root surface (Balsanelli et al. 2015). Four genes coding for membrane porins were up-regulated in attached cells: Hsero_0185, Hsero_1344, Hsero_4260 and Hsero_4295 with 6.1- 34.4- 13.1- and 2.8-fold change, respectively (Online Resource 4). Balsanelli et al. (2015) observed that the porin genes Hsero_0677, Hsero_1043 and Hsero_2393 were up-regulated in *H. seropedicae* attached to maize root and suggested that the expression of specific membrane porin genes might be involved in colonization. Interestingly, Hsero_4260 and Hsero_4295 were repressed in maize root attached cells further supporting that the set of porin genes expressed depends on the host plant and possibly on the stage of colonization.

Cell motility

Flagellar motility guides bacteria movement in response to diverse stimuli towards a desired environment. The main flagella gene cluster of *H. seropedicae* attached to the plant cells were down-regulated, however with a fold-change lower than 2 (Online Resource 4). Some flagellar related genes were significantly repressed: the flagellins *fliC* (Hsero_2070) and *flaG* (Hero_2069) had expression decreased by 1.9 and 3.0 –fold in the attached cells, respectively. Also, *fliP*

(Hsero_2049), *flgK* (Hsero_2042) and *motB* (Hsero_2985) that encode structural proteins of the flagella were 6.7, 3.4 and 2.5 fold down-regulated. In addition, two genes coding for transcription activator proteins, *flhC* (Hsero_2988) and *flhD* (Hsero_2989), were repressed in attached cells with fold-changes of 2.4 and 2.7, respectively (Online Resource 4). *H. seropedicae* cells cultivated in presence of the flavonoid naringenin showed a marked repression of flagellar genes (Tadra-Sfeir et al. 2015). Flavonoids are signal plant molecules that can influence the bacterial colonization process, and may be a signal modulating motility of bacteria in the rhizosphere.

Repression of flagellar genes suggests a shift from free-swimming to sessile lifestyle upon attaching to the root surface. In this new state *H. seropedicae* cells could be using twitching motility guided by type IV *pili*, since the expression of the system ATPase, encoded by *pilT* (Hsero_0816) increased 1.6-fold in the attached cells, and *pilI* encoding the twitching motility protein, increased 2.5-fold. Although, with low significance level in the RNA-seq (p-value=0.29 and 0.25, respectively) the RT-qPCR experiments showed a slight up-regulation of *pilT* gene (1.3-fold). In addition, the gene *pilS* (Hsero_0812), coding for a putative type IV *pilus* assembly protein, was repressed in the RNA-seq (24.5-fold) (Online Resource 4) and also down-regulated in the RT-qPCR analyses (Fig. 5). The exact function of PilS in various organisms is not clear yet; in *P. aeruginosa* PilS2 is not required for twitching motility, but may be involved with the negative transcriptional regulation controlling the expression or activity of the type IV *pili* (Mattick 2002)

Electronic microscopy of wheat root colonized by *H. seropedicae* revealed structures in attached bacterial cells similar to type IV *pili* (Fig. 4B and 4C). These structures were 5 times longer than the bacterial cell as measured by the software ImageJ. Type 4 *pili* genes are widely distributed in both Gram-negative and Gram-positive pathogenic bacteria and play essential roles in host colonization and virulence in susceptible host targets. Comparisons between various endophytic strains suggest that twitching motility by PilT-mediated retraction of type IV *pili* is likely an important feature for bacterial colonization of root surfaces (Reinhold-Hurek and Hurek 2011). The results suggest that the type IV *pili* of *H. seropedicae* might be related to the bacterial colonization of root plants.

Chemotaxis

The bacterial ability of sensing the environment is another important trait involved in bacterial colonization. Methyl-accepting chemotaxis proteins or MCPs seems to be important elements in this process (Grebe and Stock 1998). The RNA-seq analyses revealed that gene expression of several methyl-accepting chemotaxis proteins (MCPs) were highly up-regulated in the WRA libraries (Hsero_2914, Hsero_2915 and Hsero_2723 increased 17.6- 18.1- and 5.9- fold, respectively). Balsanelli and collaborators (2015) also found Hsero_2723 up-regulated when *H. seropedicae* were attached to maize roots. Indeed, these authors suggested that another *H. seropedicae* gene, Hsero_3720, is a key MCP regulator responsible for sensing the rhizosphere environment. The Hsero_3720 mutant was less efficient colonizer and this gene was expressed in the planktonic cells in contact with maize roots (Balsanelli et al. 2015). Hsero_3720 was also highly expressed in planktonic cells in contact with wheat roots and its expression compared to attached cells was 55-fold decreased (RPKM (WRA)=106; RPKM (PLANK)=4856), suggesting that the protein coded by this gene is important for cells in the planktonic state.

Transporters and Stress related genes

ATP-binding cassette (ABC) transporters catalyze the translocation of various substrates across bacterial membrane. They enable the uptake of nutrients and important molecules (importers) or facilitate the extrusion of toxins (exporters) (Dawson and Locher 2006). The RNA-seq analyses showed an expressive regulation of ABC transporters in *H. seropedicae*, with 10 ABC transporters genes up-regulated and 19 down-regulated in the wheat root attached bacteria compared to planktonic cells. Among the down-regulated genes, 4 were for branched-chain amino acid transporters, one proline/glycine transporter, two aspartate/glutamate transporters, two nitrate/sulfate transporters, two sugar transporters, with sorbitol or maltose as possible substrates, one ion transporter, one metal transporter and three transporters of unknown substrates. Interestingly, two toluene tolerance ABC transporter genes (Hsero_4079 and -4077) related to bacterial defense and a multidrug ABC transporter gene (Hsero_4073) were down-regulated by 2.4 (p-value=0.03), 1.4 (p-

value=0.48) and 6.5-fold (p-value=0.003) in wheat root attached cells, respectively (Online Resource 4). These transporters may be related to bacterial defense.

Among the up-regulated transporters were found genes for three ABC transporters of glutamate/aspartate and one dipeptide/oligopeptide transporter. An oligopeptide transporter operon (Hsero_1130-1131-1132-1133) had expression increased by 25-fold to 43-fold. In many Gram-negative bacteria this kind of transporters have a role in nutrition, recycling cell-wall peptides or adhesion to host cells (Higgins and Linton 2004). Also were up-regulated ABC transporters for sugars, branched-chain amino acids and nitrate/sulfate molecules (Online Resource 4).

The expression of one ABC exporter operon increased by 12 to 97 fold (Hsero_2386 -2387 -2388 -2389; p-value ≤ 0.05). This transporter is probably related to efflux of antimicrobial compounds. The ABC transport for putrescine was also up-regulated (*potCHDA* Hsero_1078-1079-1080-1081; p-value= 0.55; 0.80; 0.02; 0.0004, respectively). Putrescine or 1,4-diaminobutane is a biogenic polyamine present in nearly all living cells and is involved with the bacterial response to osmotic stress (Miller and Wood 1996). It is expected that the osmolarity of the rhizoplane is higher than the regions located away from the roots. The rapid adaptation of bacteria to the osmolarity increase would facilitate the colonization of the rhizosphere (Miller and Wood 1996). Also, the amount of putrescine in plant cells increase in response to the osmotic stress (Flores and Galston 1982). The genomic analyses made by Mitter et al. (2013) showed that all studied endophytes had putrescine carrier genes (e.g.. *Azospirillum sp*. B510, *Burkholderia phytofirmans* PsJN, *Klebsiella pneumoniae* 342, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619, *Pseudomonas stuzeri* A1501 and *Enterobacter sp*. 638). However, it is still not clear if these transporters are involved in protection from the putrescine produced by the plant or to the adaptation of bacteria to hyperosmolarity in the colonized environment.

Polyhydroxyalkanoates metabolism

The genome of *H. seropedicae* contains several genes that are likely involved in polyhydroxyalkanoates (PHA) metabolism. The genes encoding enzymes related to PHA biosynthesis were up-regulated in the *H. seropedicae* attached cells. First, *phaA2* (Hsero_0239) encoding a 3-ketothiolase, which condenses two acetyl-CoA to

form acetoacetyl-CoA, had a 2.7-fold increase in expression (p-value = 0.0001). Second, the expression of *phaB* (Hsero_2998) encoding a NADPH-dependent acetoacetyl-CoA reductase (PhaB), which reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA, increased 1.6 times (p-value = 0.40). Third, mRNA of the *phaC1* (Hsero_2999) encoding the PHA synthase had a 1.5-fold increase (p-value = 0.33) (Fig. 3). RT-qPCR experiments also showed *phaC1* activation (1.9-fold increase) in attached cells compared to planktonic cells (Fig. 5).

In other bacteria poly-hydroxyacids production is stimulated under low concentrations of nitrogen and oxygen (Anderson and Dawes 1990; Jendrossek 2009; Madison and Huisman 1999), which prevail in the environment of both root-attached and planktonic *H. seropedicae* cells. Furthermore, carbon excess or high carbon/nitrogen ratio also induces PHA synthesis (Hervás et al. 2008). Since cells attached to roots show higher expression of PHA biosynthesis genes, it is likely that these cells have more access to carbon source supplied by the root exudates compared to the planktonic bacteria.

H. seropedicae genome encodes three proteins associated to the PHA granule, the phasin PhaP1 (Hsero_1639) with a 2.7-fold increase in WRA library (p-value=0.0001), and two phasins related proteins PhaP2 (Hsero_4759) whose expression were also increased by 2.4-fold in the WRA library (p-value=0.04) and PhaP3 (Hsero_2401) which was not expressed in the PLANK nor WRA libraries (Fig. 3). In contrast, expression of *phbF* (Hsero_2997) gene encoding the regulatory protein associated to the PHA granule (PhbF), which may act as a transcriptional repressor of genes of PHA metabolism in *H. seropedicae* (Kadowaki et al. 2011), decreased by 1.8-fold in the attached bacteria (p-value=0.09). The down-regulation of *phbF* in the bacteria attached to the wheat roots is consistent to increased expression of PHA biosynthesis genes and large amount of PHA granules observed in the transmission electron micrograph of *H. seropedicae* colonizing wheat roots 3 days post inoculation (Fig. 4D).

PHA production appears to be an important feature for root colonization and plant growth promotion by other rhizobacteria. For example, field experiments showed that *A. brasilense* strains that produce PHA are more efficient as inoculants (Fibach-Paldi et al. 2012). In the rhizobacteria *A. brasilense,* accumulation of

polyhydroxyalkanoate was shown to support chemotaxis, motility, and cell multiplication (Kadouri et al. 2005). *A. brasilense* attached to wheat root plantlets cultivated in Hoagland's medium also expressed genes related to the PHA biosynthesis (Camilios-Neto et al. 2014). To further support the role of PHA metabolism in root colonization, it was shown recently that knockout of *phaC1* impaired maize colonization by *H. seropedicae* (Balsanelli et al. 2015).

The genome of *H. seropedicae* contains two additional genes, *phaC2* and *phaC3,* encoding putative PHA synthases, which are located distant from *phaC1*. The *phaC2* is located on the operon with *pta-ackA* genes encoding phosphotransacetylase and acetate kinase enzymes. The mRNA levels of *phaC2* (Hsero_2405) and *phaC3* (Hsero_0265) were 36.7- (p-value=0.01) and 12.4- fold (p-value=0.05) increased, respectively, in the bacteria attached to the roots while *phaC1* was expressed but not regulated (Online Resource 4). The average fold change increase for the operon *phaC2-fab1-pta-ackA* (Hsero_2405-Hsero_2406-Hsero_2407-Hsero_2408) was greater than 6 fold, but the significance was higher than 0.05 only for Hsero_2408 (p-value=0.43). This result suggests the activation of the phosphate acetyltransferase-acetate kinase pathway, which converts acetyl-CoA to acetate and produces ATP. In cyanobacteria, acetyl-phosphate seems to play a role controlling PHA synthase. The organization of the operon and co-expression of the *pta-ackA* genes with *phaC2* in *H. seropedicae* suggests a role for acetyl-phosphate in PHA metabolism during plant colonization. Mutation of *phaC1* of *H. seropedicae* abolished PHA synthesis *in vitro*, suggesting that PhaC1 is the main gene responsible for PHA synthesis (Tirapelle et al. 2013). The induction of PhaC2 in the rhizoplane suggests specific role for this putative PHA synthase. The results suggest that colonization of the root surface require active PHA metabolism in *H. seropedicae*.

The conversion of acetyl-CoA to acetate may be activated to supply energy in an oxygen-deprived environment. Under this condition PHA biosynthesis may function to recycle NAD+. Interestingly, a gene for an alcohol dehydrogenase, *adhA* (Hsero_0964), was also found 10-fold up-regulated (p-value ≤ 0.05), which may suggest oxidation of ethanol to acetate to recycle reduced co-enzymes. The induction of the *adhA* was confirmed by RT-qPCR (2.5-fold increase in the cells

attached to the root) (Fig. 5). Krause et al. (2011) showed that *Azoarcus sp*. strain BH72 expressed different alcohol dehydrogenases during the interaction with rice. Proteomic analyses of *H. seropedicae* SmR1 colonizing rice also found the expression of the AdhA enzyme in root-colonized samples (Alberton et al. 2013), suggesting an important role of this enzyme in the rhizosphere colonization by bacteria.

Phytohormones related genes

Auxins are the major class of phytohormones involved in regulation of plant growth and development, being indole-3-acetic acid (IAA) the most studied hormone of this class (Spaepen et al. 2007; Went and Thimann 1937). *H. seropedicae* possesses genes to produce IAA from four different possible pathways (Bastián et al. 1998; Pedrosa et al. 2011). Among these possible pathways there are two with up-regulated genes found in the WRA libraries: 1) conversion of indolepyruvate into indole-acetic acid through the indolepyruvate ferredoxin oxidoreductase activity (enzyme encoded by Hsero_4278) and 2) conversion of indole-3-acetonitrile to indolacetate through the activity of the nitrilase (enzyme encoded by Hsero_1422). The increase in expression of Hsero_4278 and Hsero_1422 was 3.1- (p-value=0.04) and 2.7- fold (p-value=0.12) in attached cells may suggest that IAA synthesis by *H. seropedicae* is regulated by the plant.

Discussion

Beatty and Good (2011) suggested three main approaches to enhance plant nitrogen nutrition, and one of them is the utilization of diazotrophic endophytic bacteria as biofertilizers. More recently, Gutiérrez (2012) emphasized the importance to increase the understanding of N-use efficiency by using systems biology approaches concerning N-regulatory networks and molecular mechanisms to control growth and development of plants. Indeed, the use of 13N labeled N_2 showed that *Setaria viridis* incorporated N-fixed by the diazotrophic bacteria *H. seropedicae* and *A. brasilense* (Pankievicz et al. 2015), opening new avenues for utilization of plant growth promoting bacteria.

H. seropedicae is the best characterized specie of *Herbaspirillum* genera and has been shown to play an important role in the nitrogen fixation systems (Bonatto et al. 2012; Chubatsu et al. 2012; James et al. 1997; Pedrosa et al. 2011). In addition, this bacterium is a model of grass-associative endophytic nitrogen fixing bacteria and can potentially be used as biofertilizer. However, molecular cues and signals that trigger plant colonization are largely not understood.

Global transcriptional analysis of *H. seropedicae* during plant-bacterial interaction using two different types of bacterial cells, i.e., cells attached to wheat roots or planktonic, uncovered new facets of the endophyte-graminea interaction. Genes expressed and comparative analysis between the two conditions revealed a consistent global profile and a high correlation was observed for the two biological replicates in each condition. The expression data obtained by RNA-seq was supported by RT-qPCR of selected genes.

Many studies showed that bacteria change their gene expression to adapt to specific environment or host interaction. Under the used conditions *nif* gene expression, mainly in *H. seropedicae* attached to plant roots, was high. Activation of *fixNOP* in attached cells indicated that the root surface environment is microaerophilic, and this may be more appropriate for nitrogen fixation. In addition attached cells potentially have access to carbon source facilitated. We also observed that the shift from free-swimming to attached lifestyle seems to lead to a reduction in flagella gene expression and up-regulation of *pilT* type 4 *pili* gene which probably

enables adhesion to wheat roots and twitching motility. Also, the up-regulation of specific genes coding for methyl-accepting chemotaxis proteins suggests that the bacteria are sensing environmental signals. Indeed, two specific genes coding for MCPs found up-regulated during the wheat interaction were also found in *H. seropedicae* colonizing maize (Hsero_2723 and Hsero_3720) (Balsanelli et al. 2015).

Among the metabolic changes undergone by *H. seropedicae*, up-regulation in attached cells of polyhydroxyalkanoate synthesis and degradation genes was prominent. An array of transporters was also induced suggesting exudation of several carbon sources by the plant. Activation of *nif* genes and other Ntr-regulated genes indicated N-limitation. The production of polyhydroxyalkanoates occurs under low nitrogen, oxygen or phosphate. PHA production appears to be an important feature for root colonization and plant growth promotion by *azospirilla* and field experiments showed that strain that produces PHA are more likely to be effective as inoculant (Fibach-Paldi et al. 2012).

In summary, this study presents a panorama of the wheat - *H. seropedicae* interaction, and allowed identification of candidate genes and pathways potentially involved with attachment, metabolic adaptations and interaction with plants.

References

Alberton D et al. (2013) Comparative proteomics analysis of the rice roots colonized by *Herbaspirillum seropedicae* strain SmR1 reveals induction of the methionine recycling in the plant host J Proteome Res 12:4757-4768 doi:10.1021/pr400425f

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates Microbiol Rev 54:450-472

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms Annu Rev Plant Biol 57:233-266 doi:10.1146/annurev.arplant.57.032905.105159

Baldani JI, Baldani VL (2005) History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience. An Acad Bras Cienc 77:549-579 doi:S0001-37652005000300014 [pii]/S0001-37652005000300014

Balsanelli E, de Baura VA, Pedrosa Fde O, de Souza EM, Monteiro RA (2014) Exopolysaccharide biosynthesis enables mature biofilm formation on abiotic surfaces by *Herbaspirillum seropedicae* PLoS One 9:e110392 doi:10.1371/journal.pone.0110392

Balsanelli E et al. (2010) *Herbaspirillum seropedicae* rfbB and rfbC genes are required for maize colonization. Environ Microbiol doi:EMI2187 [pii]10.1111/j.1462-2920.2010.02187.x

Balsanelli E et al. (2015) Molecular adaptations of *Herbaspirillum seropedicae* during colonization of the maize rhizosphere Environmental microbiology doi:10.1111/1462-2920.12887

Balsanelli E et al. (2013) Maize root lectins mediate the interaction with *Herbaspirillum seropedicae* via N-acetyl glucosamine residues of lipopolysaccharides PLoS One 8:e77001 doi:10.1371/journal.pone.0077001

Bastián F, Cohen A, Piccoli P, Luna V, Baraldi R, Bottini R (1998) Production of indole-3-acetic acid and gibberellins A1 and A3 by Acetobacter diazotrophicus and Herbaspir- illum *seropedicae* in chemically-defined culture media. Plant Growth Regul 24:7-11

Batista MB et al. (2013) The *Herbaspirillum seropedicae* SmR1 Fnr orthologs controls the cytochrome composition of the electron transport chain Sci Rep 3:2544 doi:10.1038/srep02544

Bauer S, Gagneur J, Robinson PN (2010) GOing Bayesian: model-based gene set analysis of genome-scale data Nucleic Acids Res 38:3523-3532 doi:10.1093/nar/gkq045

Beatty PH, Good AG (2011) Plant science. Future prospects for cereals that fix nitrogen Science 333:416-417 doi:10.1126/science.1209467

Bonatto AC, Souza EM, Oliveira MA, Monteiro RA, Chubatsu LS, Huergo LF, Pedrosa FO (2012) Uridylylation of *Herbaspirillum seropedicae* GlnB and GlnK proteins is differentially affected by ATP, ADP and 2-oxoglutarate in vitro Arch Microbiol 194:643-652 doi:10.1007/s00203-012-0799-9

Camilios-Neto D 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

Chubatsu LS et al. (2012) Nitrogen fixation control in H*erbaspirillum seropedicae* Plant Soil 356:197-207 doi:DOI 10.1007/s11104-011-0819-6

Dawson RJ, Locher KP (2006) Structure of a bacterial multidrug ABC transporter Nature 443:180-185 doi:10.1038/nature05155

Döbereiner J, Baldani VLS, Baldani JI (1995) Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas. Brasília: EMBRAPA –SPI: Itaguaí, RJ: EMBRAPA-CNPAB

el-Komy HM, Saad OA, Hetta AM (2003) Significance of *Herbaspirillum seropedicae* inoculation and/or straw amendment on growth and dinitrogen fixation of wheat using 15N-dilution method Folia Microbiol (Praha) 48:787-793

Elbeltagy A et al. (2001) Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species Appl Environ Microbiol 67:5285-5293 doi:10.1128/AEM.67.11.5285-5293.2001

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

Flores HE, Galston AW (1982) Polyamines and plant stress: activation of putrescine biosynthesis by osmotic shock Science 217:1259-1261 doi:10.1126/science.217.4566.1259

Gottig N, Garavaglia BS, Garofalo CG, Orellano EG, Ottado J (2009) A filamentous hemagglutinin-like

protein of Xanthomonas axonopodis pv. citri, the phytopathogen responsible for citrus canker, is involved in bacterial virulence PLoS One 4:e4358 doi:10.1371/journal.pone.0004358

Grebe TW, Stock J (1998) Bacterial chemotaxis: the five sensors of a bacterium Curr Biol 8:R154-157

Gutiérrez RA (2012) Systems biology for enhanced plant nitrogen nutrition Science 336:1673-1675 doi:10.1126/science.1217620

Hervás AB, Canosa I, Santero E (2008) Transcriptome analysis of Pseudomonas putida in response to nitrogen availability J Bacteriol 190:416-420 doi:10.1128/JB.01230-07

Higgins CF, Linton KJ (2004) The ATP switch model for ABC transporters Nat Struct Mol Biol 11:918-926 doi:10.1038/nsmb836

Hoagland DRA, D.I. (1950) The water-culture method for growing plants without soil. California Agricultural Experiment Station, Circular -- 347, 1 edition edn. College of Agriculture, University of California, Berkeley, Calif

Huergo LF et al. (2010) Proteomic analysis of *Herbaspirillum seropedicae* reveals ammonium-induced AmtB-dependent membrane sequestration of PII proteins FEMS Microbiol Lett 308:40-47 doi:10.1111/j.1574-6968.2010.01986.x

James EK et al. (2002) Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Mol Plant Microbe Interact 15:894-906 doi:10.1094/MPMI.2002.15.9.894

James EK, Olivares FL, Baldani JI, Döbereiner J (1997) *Herbaspirillum*, an endophytic diazotroph colonizing vascular tissue 3Sorghum bicolor L. Moench Journal of Experimental Botany 48:785-798 doi:10.1093/jxb/48.3.785

Jendrossek D (2009) Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes) J Bacteriol 191:3195-3202 doi:10.1128/JB.01723-08

Kadouri D, Jurkevitch E, Okon Y, Castro-Sowinski S (2005) Ecological and agricultural significance of bacterial polyhydroxyalkanoates Crit Rev Microbiol 31:55-67 doi:10.1080/10408410590899228

Kadowaki MA et al. (2011) Identification and characterization of PhbF: a DNA binding protein with regulatory role in the PHB metabolism of *Herbaspirillum seropedicae* SmR1 BMC Microbiol 11:230 doi:10.1186/1471-2180-11-230

Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M (2014) Data, information, knowledge and principle: back to metabolism in KEGG Nucleic Acids Res 42:D199-205 doi:10.1093/nar/gkt1076

Karnovsk M (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy J Cell Biol 27:A137

Klassen G, Pedrosa F, Souza E, Funayama S, Rigo L (1997) Effect of nitrogen compounds on nitrogenase activity in *Herbaspirillum seropedicae* SMR1 Canadian Journal of Microbiology 43:887-891

Krause A, Bischoff B, Miché L, Battistoni F, Reinhold-Hurek B (2011) Exploring the function of alcohol dehydrogenases during the endophytic life of Azoarcus Sp. strain BH72 Mol Plant Microbe Interact 24:1325-1332 doi:10.1094/MPMI-05-11-0139

Lin JT, Stewart V (1998) Nitrate Assimilation by Bacteria Advances in Microbial Physiology 39:1-30

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 25:402-408 doi:10.1006/meth.2001.1262

Luft JH (1961) Improvements in epoxy resin embedding methods J Biophys Biochem Cytol 9:409-414

Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic Microbiol Mol Biol Rev 63:21-53

Magalhães Cruz L, de Souza EM, Weber OB, Baldani JI, Döbereiner J, Pedrosa FO (2001) 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (M*usa s*pp.) and pineapple (A*nanas comosus (*L.) Merril). Appl Environ Microbiol 67:2375-2379 doi:10.1128/AEM.67.5.2375-2379.2001

Mattick JS (2002) Type IV pili and twitching motility Annu Rev Microbiol 56:289-314 doi:10.1146/annurev.micro.56.012302.160938

Mertens T, Hess D (1984) Yield increases in spring wheat (Triticum aestivum L.) inoculated with*Azospirillum lipoferum* under greenhouse and field conditions of a temperate region Plant and Soil 82:87-89 doi:10.1007/BF02220773

Miller KJ, Wood JM (1996) Osmoadaptation by rhizosphere bacteria Annu Rev Microbiol 50:101-136 doi:10.1146/annurev.micro.50.1.101

Mitter B et al. (2013) Comparative genome analysis of Burkholderia phytofirmans PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants Front Plant Sci 4:120 doi:10.3389/fpls.2013.00120

Monteiro RA et al. (2012) *Herbaspirillum*-plant interactions: microscopical, histological and molecular aspects Plant and Soil 356 doi:10.1007/s11104-012-1125-7

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5:621-628 doi:nmeth.1226 [pii] 10.1038/nmeth.1226

Muthukumarasamy R, Govindarajan M, Vadivelu M, Revathi G (2006) N-fertilizer saving by the inoculation of Gluconacetobacter diazotrophicus and *Herbaspirillum* sp. in micropropagated sugarcane plants Microbiol Res 161:238-245 doi:10.1016/j.micres.2005.08.007

Noindorf L et al. (2011) Role of PII proteins in nitrogen fixation control of *Herbaspirillum seropedicae* strain SmR1 BMC Microbiol 11:8 doi:10.1186/1471-2180-11-8

Oliveira ALM, Urquiaga S, Dobereiner J, Baldani JI (2002) The effect of inoculating endophytic N-2-fixing bacteria on micropropagated sugarcane plants Plant and Soil 242:205-215 doi:Doi 10.1023/A:1016249704336

Pankievicz VC et al. (2015) Robust biological nitrogen fixation in a model grass-bacterial association The Plant journal : for cell and molecular biology 81:907-919 doi:10.1111/tpj.12777

Pedrosa FO et al. (2011) Genome of H*erbaspirillum seropedicae* Strain SmR1, a Specialized Diazotrophic Endophyte of Tropical Grasses Plos Genetics 7 doi:e100206410.1371/journal.pgen.1002064

Pereira JAR, Baldani JI (1995) Selection of A*zospirillum* spp. and H*erbaspirillum seropedicae* strains to inoculate rice and maize plants. Paper presented at the INTERNATIONAL SYMPOSIUM ON SUSTAINABLE AGRICULTURE FOR THE TROPICS: THE ROLE BIOLOGICAL NITROGEN FIXATION, Angra dos REIS, RJ, Brazil.

Reimers M, Carey VJ (2006) Bioconductor: an open source framework for bioinformatics and computational biology Methods Enzymol 411:119-134 doi:10.1016/S0076-6879(06)11008-3

Reinhold-Hurek B, Hurek T (2011) Living inside plants: bacterial endophytes. Curr Opin Plant Biol doi:S1369-5266(11)00054-9 [pii]10.1016/j.pbi.2011.04.004

Rennie RJ, deFreitas JR, Ruschel AP, Vose PV (1983) 15N isotope dilution to quantify dinitrogen (N2) fixation associated with Canadian and Brazilian wheat Canadian Journal of Botany 61:1667-1671

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy J Cell Biol 17:208-212

Roncato-Maccari LD et al. (2003) Endophytic *Herbaspirillum seropedicae* expresses nif genes in gramineous plants. FEMS Microbiol Ecol 45:39-47 doi:FEM39 [pii]10.1016/S0168-6496(03)00108-9

Saubidet MI, Barneix AJ (1998) Growth stimulation and nitrogen supply to wheat plants inoculated with *Azospirillum brasilense* Journal of Plant Nutrition 12:2565-2577 doi:10.1080/01904169809365588

Schmidt MA, Souza EM, Baura V, Wassem R, Yates MG, Pedrosa FO, Monteiro RA (2011) Evidence for the endophytic colonization of Phaseolus vulgaris (common bean) roots by the diazotroph *Herbaspirillum seropedicae* Braz J Med Biol Res 44:182-185

Souza EM, Pedrosa FO, Drummond M, Rigo LU, Yates MG (1999) Control of *Herbaspirillum seropedicae* NifA activity by ammonium ions and oxygen J Bacteriol 181:681-684

Souza EM, Pedrosa FO, Rigo LU, Machado HB, Yates MG (2000) Expression of the ni*fA ge*ne of H*erbaspirillum seropedicae:* role of the NtrC and NifA binding sites and of the -24/-12 promoter element Microbiology-Uk 146:1407-1418

Spaepen S, Vanderleyden J, Remans R (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling FEMS Microbiol Rev 31:425-448 doi:10.1111/j.1574-6976.2007.00072.x

Tadra-Sfeir MZ et al. (2015) Genome wide transcriptional profiling of *Herbaspirillum seropedicae* SmR1 grown in the presence of naringenin Front Microbiol 6:491 doi:10.3389/fmicb.2015.00491

Tadra-Sfeir MZ et al. (2011) Naringenin regulates expression of genes involved in cell wall synthesis in H*erbaspirillum seropedicae A*ppl Environ Microbiol 77:2180-2183 doi:AEM.02071-10 [pii]10.1128/AEM.02071-10

Tirapelle EF et al. (2013) Identification of proteins associated with polyhydroxybutyrate granules from H*erbaspirillum seropedicae Sm*R1--old partners, new players PLoS One 8:e75066

doi:10.1371/journal.pone.0075066
Watson ML (1958) Staining of tissue sections for electron microscopy with heavy metals J Biophys Biochem Cytol 4:475-478
Went FW, Thimann KV (1937) Phytohormones. New York

Table and Figure Legends

Table 1: Number of reads mapping to different reference databases.

Biological Replicate Samples	Total # of reads	Reads remaining after trimming	r*	r**	Reads mapped to Hs*** tRNA genes	Reads mapped to Hs rRNA genes	Unique Reads mapped to Hs CDS	% reads mapped to Hs sequences	Reads mapped to wheat chloroplast genome	Reads mapped to wheat mitochondrial genome	Reads mapped to wheat EST	% reads mapped to wheat sequences
WRA1	25,836,672	25,491,158	0.99	0.32 (1) 0.29 (2)	15,106	2,992,011	224,627	12,68%	2,244,649	114,332	3,607,494	23.41%
WRA2	20,506,649	20,220,952			14,509	2,864,544	189,357	15,2%	1,444,488	111,663	3,139,598,	23.22%
Plank 1	46,532,584	45,581,780	0.98		654,192	22,881,973	1,532,127	55,0%	461,309	213,704	2,636,029	7.26%
Plank 2	53,309,734	52,218,324			484,975	31,969,105	1,474,565	65,0%	615,928	140,866	3,319,709	7.81%

* r = Pearson correlation factor

** Pearson correlation factor between biological replicates from different conditions

*** *Herbaspirillum seropedicae*

Table 2: Number of expressed, regulated or differentially expressed genes.

	PLANK	WRA
Technical replicates	2	3
Biological replicates	2	2
Total Expressed genes	2,167	
Regulated genes	403	
Up-regulated genes	152	
Down-regulated genes	251	

Table 3: Gene set enrichment analysis of up-regulated genes with FDR ≤ 0.05

Pathway Description	KEGG id	Identified Genes	Probability	std.error
Oxidative phosphorylation	ko00190	10	1	1.72E-003
Chloroalkene and chloroalkene degradation	ko00625	5	0.91	3.98E-003
Pyruvate metabolism	ko00620	5	0.58	6.24E-003

Table 4: Gene set enrichment analysis of down-regulated genes with FDR ≤ 0.05

Pathway Description	KEGG id	Identified Genes	Probability	std.error
Ribosome	ko03010	43	1	2.02E-004
RNA degradation	ko03018	6	0.99	6.93E-004
RNA polymerase	ko03020	3	0.97	2.11E-003
Plant-pathogen interaction	ko04626	3	0.66	7.34E-003
Protein export	ko03060	5	0.57	1.34E-002

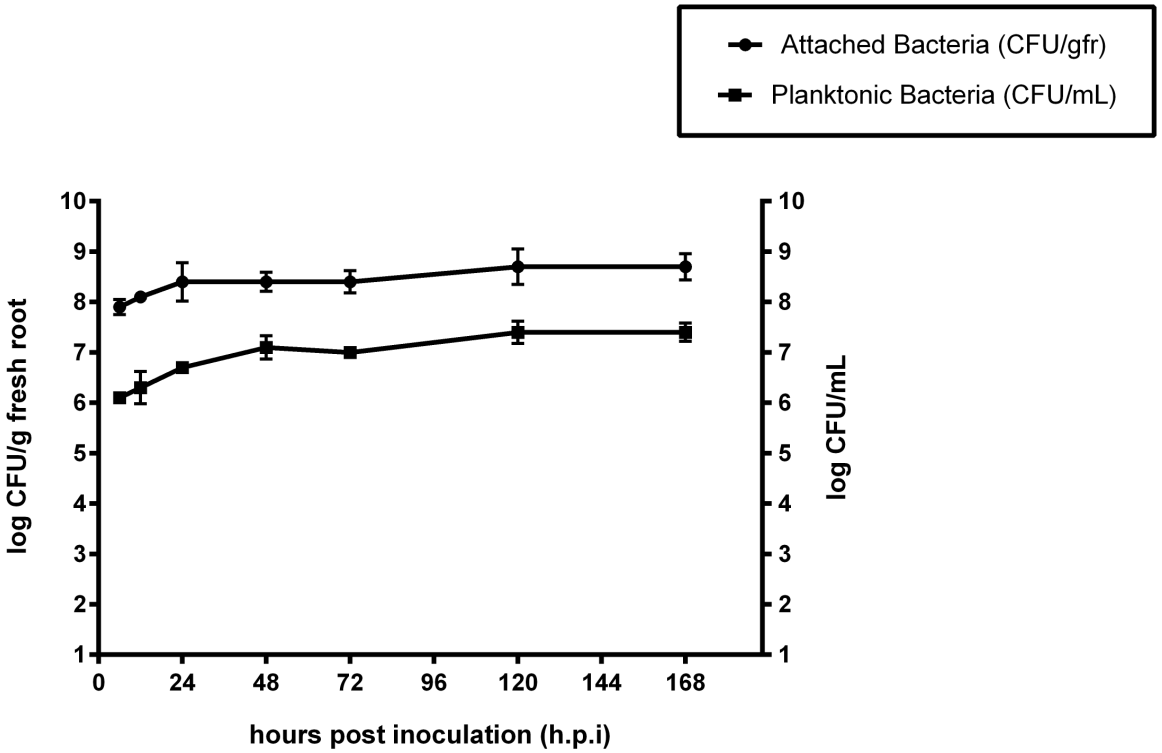


Fig. 1 Time course colonization of wheat roots by *H. seropedicae*. (n) Number of bacteria attached to wheat roots. (□) Number of bacteria in Hoagland's medium. Each point is the average of three independent replicates and each replicate was made of 3 biological replicates. Error bars are standard deviation between the biological replicates. CFU= Colony forming units.

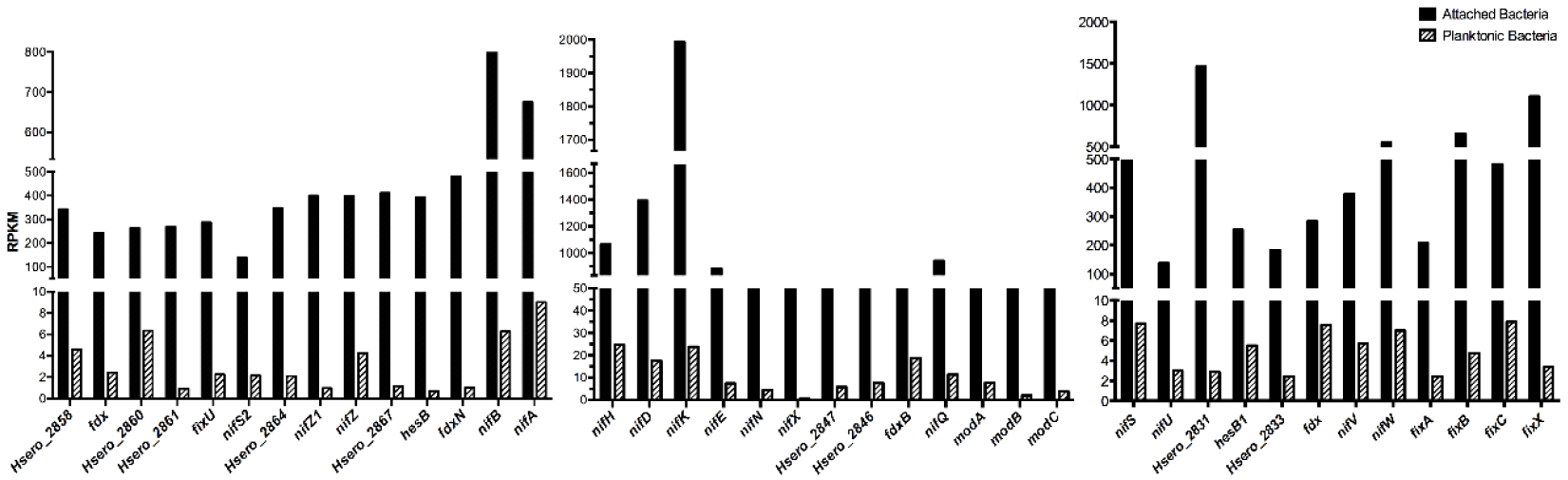


Fig. 2 Structural and regulatory *nif* genes activated in the bacteria attached to the wheat seedlings roots. Expression is represented in RPKM and the conditions are cells attached to wheat root or planktonic.

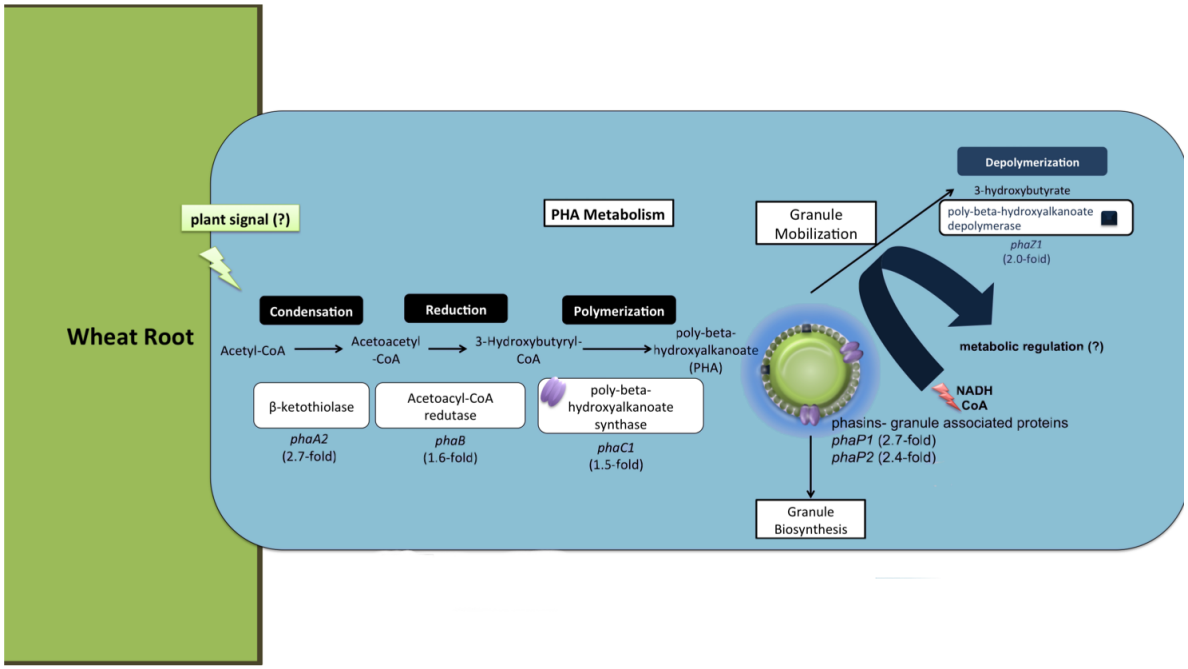


Fig. 3 PHA metabolic pathway adaptations of *H. seropedicae* attached to wheat root. Schematic representation of the PHA biosynthesis and degradation pathways. Fold-change values of genes up-regulated are shown.

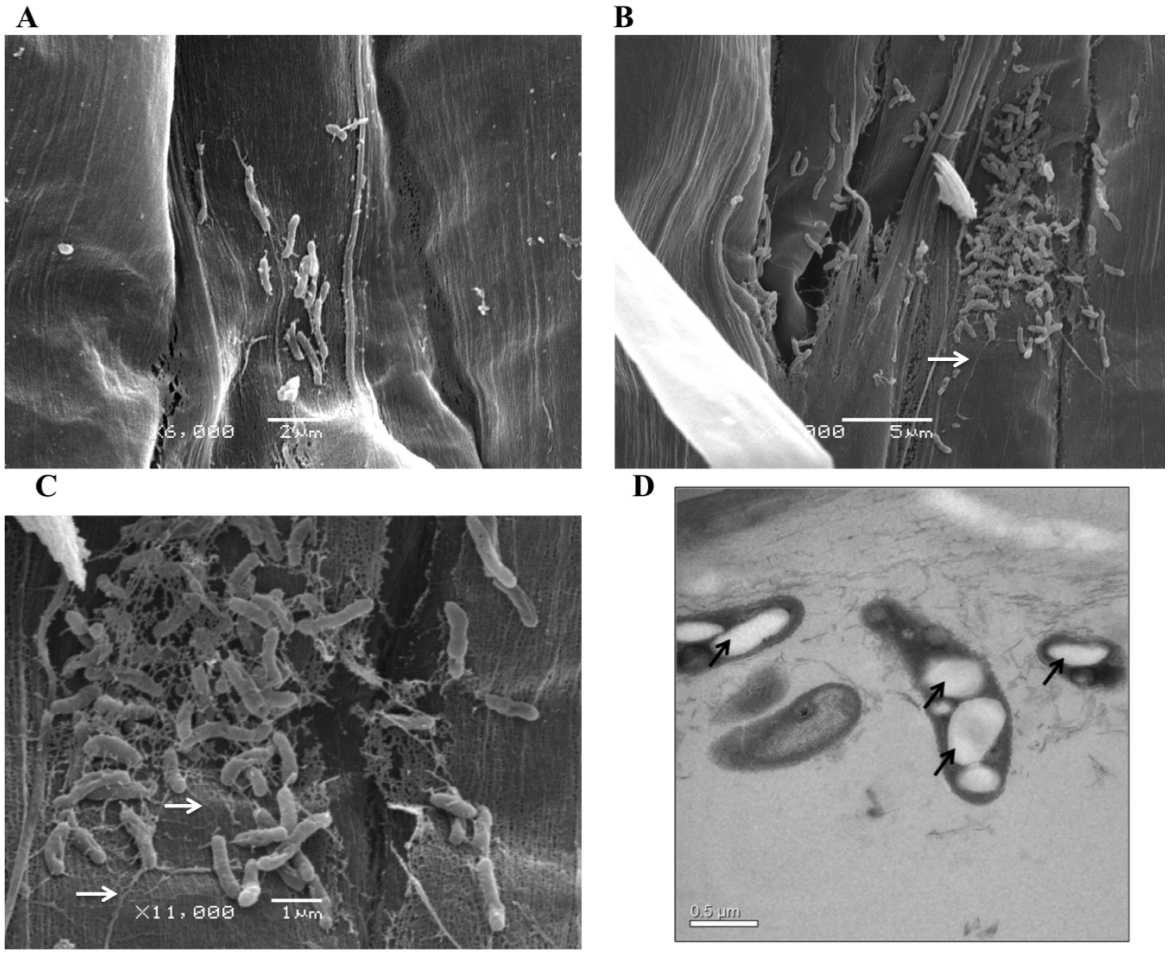


Fig. 4 Electronic Microscopy of *H. seropedicae* attached to wheat roots 3 days after inoculation. Scanning electronic microscopy showing bacteria (A) colonizing wheat root. (B) and (C) show formation of a microcolony. White arrows indicate structures similar to type 4 *pili*. In (D) transmission electron micrograph of *H. seropedicae* attached to the wheat roots 3 days after inoculation. Black arrows indicate PHA granules.

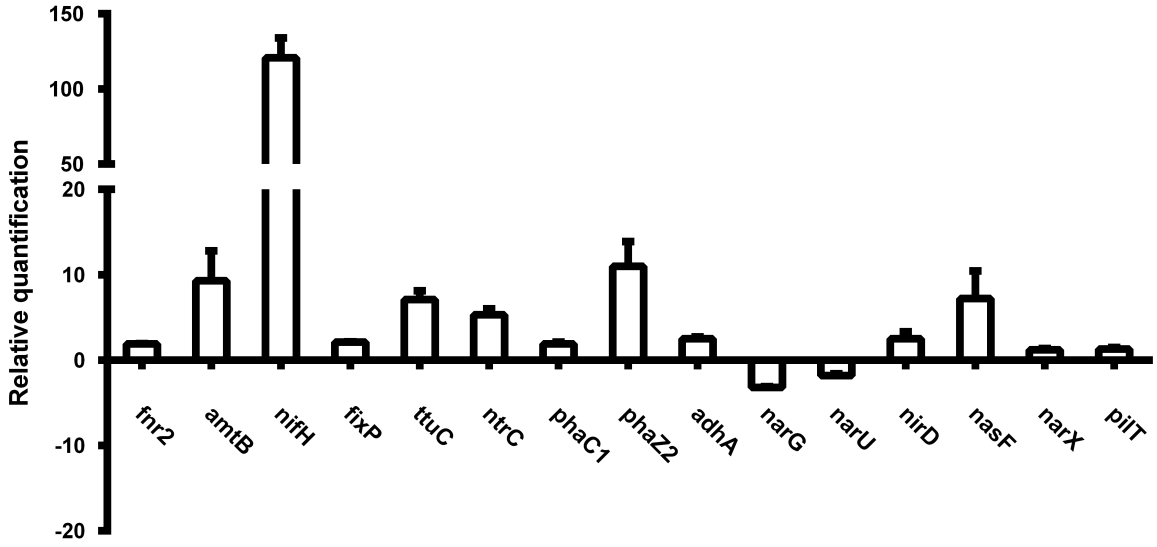
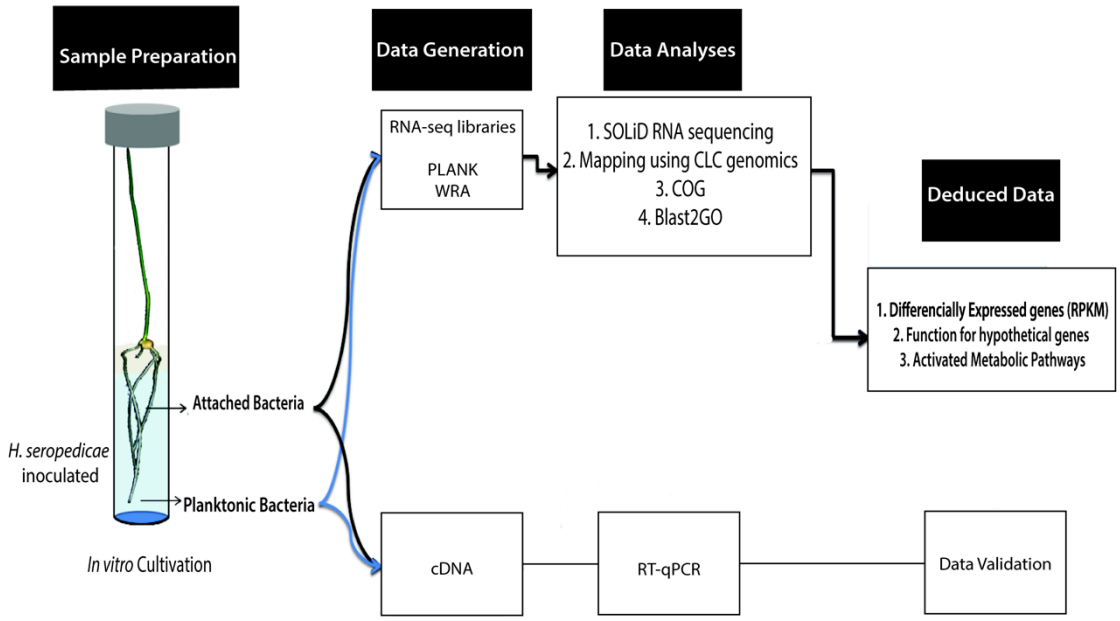
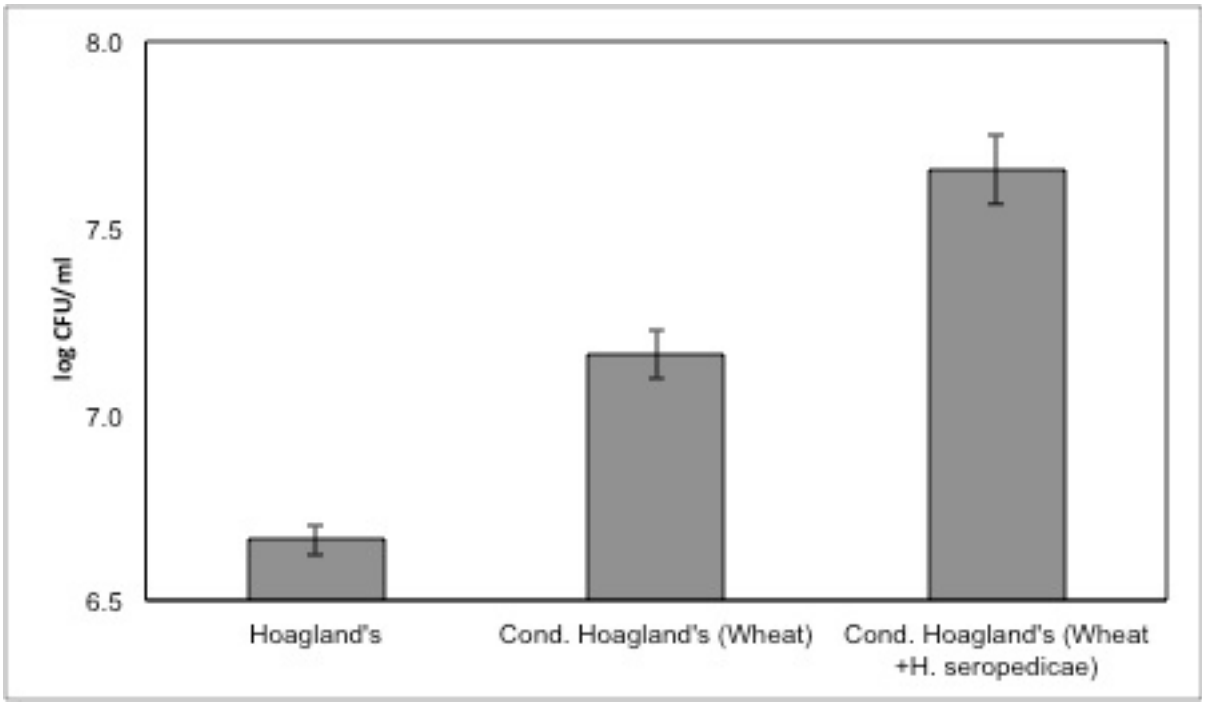


Fig. 5 Quantification of expression of selected genes of *H. seropedicae* colonizing wheat roots by reverse transcription-quantitative PCR. Relative gene expression of 16 candidate genes of *H. seropedicae* attached to the wheat root surface compared with cells in planktonic state. Regulation in RT-qPCR data is in agreement with RNA-seq data although the fold change varied. Data represents the mean of 3 technical replicates and 2 biological replicates. Error bar are standard deviation between the biological replicates.

Electronic Supplementary Material



Supplemental figure 1: Workflow of the RNA-seq experiment. The samples were prepared by harvesting the attached bacteria or planktonic bacteria from an *in vitro* axenic condition. Samples for RNA-seq libraries were independent from RT-qPCR assays. Differential expression was analyzed in terms of sequence numbers of expressed, up-regulated and down-regulated genes. GSEA was performed to find metabolic activated KEGG pathways.



Supplemental figure 2: *H. seropedicae* growth in medium containing wheat root exudates. *H. seropedicae* was inoculated in filter-sterilized conditioned Hoagland's medium, obtained from three days hydroponic culture of wheat seedlings growing in the presence (Hoagland's Wheat + *H. seropedicae*) or in absence of *H. seropedicae* (Hoagland's Wheat).

Supplemental table 1: Primers used for reverse transcription-quantitative PCR

Name	Sequence	Reference
RT16Sfor	5´- AAGCCTACCAAGGCGACGACGAT-3´	Tadra-Sfeir et al. (2015)
RT16Srev	5´- AGGAGTCTGGGCCGTGTCT-3´	Tadra-Sfeir et al. (2015)
RTnifHfor	5´- AACAAGGCGCAGGAAATCTACA-3´	Balsanelli et al. (2015)
RTnifHrev	5´- ATGCCCTTGGAGATGTTGTTG-3´	Balsanelli et al. (2015)
RTntrCfor	5´- CAAGGTCGCCCTGAAGCATA-3´	Balsanelli et al. (2015)
RTntrCrev	5´- CTGGATCTTGCGGGTGATG-3´	Balsanelli et al. (2015)
RTamtBfor	5' - TCGGGTGTGGTCTCCTTCAT - 3'	This work
RTamtBrev	5' - AGGCCTTCGCGTTCTTCTTC - 3'	This work
RTfixPfor	5' – GGCGCCCCCAATCTGT - 3'	This work
RTfixPrev	5' – GGTATTGTCGCGACCCTTGT - 3'	This work
RTfnr2for	5' – GGCGACTGAGCAATGAAGTCA - 3'	This work
RTfnr2rev	5' – CCAGCAAGAAGAACGCAAATC - 3'	This work
RTttuCfor	5´- CTGGGCCCACTGCAACTACT - 3'	This work
RTttuCrev	5´- TCGCTTCGAATCCCTTCAACT - 3'	This work
RTHsero2999for	5´- GGTGCGTTCCATCCATATCG - 3'	This work
RTHsero2999rev	5´- CAGAAGCCCAAGGCATTGA - 3'	This work
RTHsero0639for	5' – CGATACGCGGGTCAATCC - 3'	This work
RTHsero0639rev	5' – GGTGCTGATCATGTTCTTTTCG	This work
RTpilSfor	5´- GCACCGGGTTCACCATCA-3´	Balsanelli et al. (2015)
RTpilSrev	5´- CATGCTCACGCAGACATCCT-3´	Balsanelli et al. (2015)
RTpilTfor	5´- TGGCGAACACCATGTGATG - 3'	This work
RTpilTrev	5´- CGATCTGCTGGTTGTTGTTGA - 3'	This work
RTadhAfor	5´- TGACCGTCTACAAGGGCTTGA - 3'	This work
RTadhArev	5´- GCCAATGCCGGAGATGAC - 3'	This work
RTnarGfor	5' - AAGTCGGGCGCTGTCAAC - 3'	P. Bonato, unpublished
RTnarGrev	5' - GGAACGCCAGACGAACAT - 3'	P. Bonato, unpublished
RTnirDfor	5' - TCGGCAACCTGGGTGAAC -3'	P. Bonato, unpublished
RTnirDrev	5' - GTTGGCGGGCTCTTCCA - 3'	P. Bonato, unpublished
RTnarUfor	5' - CGTGTGGATGCTCTTCATTACCTA - 3'	P. Bonato, unpublished
RTnaUrev	5' - CGTAGTAGATGGCAGCGATGTT 3'	P. Bonato, unpublished
RTnasFfor	5' - CCGACAAGCGCGAATACAC- 3'	P. Bonato, unpublished
RTnasFrev	5' - CCAGCCAGTAATACAGCCACATC- 3'	P. Bonato, unpublished
RTnarXfor	5' - GTCCAACATCCGCAAGCAT- 3'	P. Bonato, unpublished
RTnarXrev	5' - TCGTCGGACACGGTCATG- 3'	P. Bonato, unpublished

4 CAPÍTULO II

4.1 *Setaria viridis*: A Model Grass to Explore Bacterial Plant Growth Promotion and Associative N_2-Fixation.

Summary: Nitrogen-fixing rhizobacteria can promote plant growth; however, it remains controversial whether biological nitrogen fixation (BNF) contributes to growth promotion. Mechanistic studies are needed that could be facilitated by adoption of a plant model in which BNF was well documented. Thirty genotypes of *Setaria viridis,* a model C_4 grass, were evaluated for their growth response to bacterial inoculation but only three responded strongly. Bacteria colonized both internal and epidermal root tissue and expressed nitrogenase, the enzyme for nitrogen fixation. $^{13}N_2$ tracer studies provided direct evidence for ^{13}N incorporation into host plants inoculated with *Azospirillum brasilense* and *Herbaspirillum seropedicae* bacteria. Furthermore, inoculation with an ammonium excreting-mutant strain of *A. brasilense* was able to meet all the plant's nitrogen demands for healthy growth under nitrogen limitation. 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.

Key Words: *Setaria*, nitrogen fixation, *Azospirillum*, *Herbaspirillum*, plant growth promotion, radioisotope, endophyte, rhizosphere

Introduction

Plant growth promoting bacteria (PGPB) colonize roots and engage in associative symbiosis with various host plants, including bioenergy grass species (Santi, Bogusz and Franche, 2013). In most cases, the mechanism of plant growth promotion is unknown. In selected cases, plant growth promotion is attributed to antagonism toward phytopathogens (Raaijmakers *et al.*, 2009) and/or the induction of plant resistance (Verhagen *et al.*, 2004). Other PGPB may act mostly by phytostimulation (e.g., release of phytohormones (Richardson *et al.*, 2009)). Several nitrogen-fixing PGPB have been identified as endophytes of grass species, including *Azoarcus* spp. in Kallar grass and rice (Reinhold *et al.*, 1986; Hurek, Reinhold-Hurek, Van Montagu*, et al.*, 1994), *Herbaspirillum seropedicae* in sugarcane (James and Olivares, 1998) and sorghum (James *et al.*, 1997), and *Gluconacetobacter diazotrophicus* in sugarcane (James *et al.*, 1994). Unlike rhizobia that form an intimate intracellular symbiosis with their legume hosts, PGPB do not induce the formation of observable plant structures. They are also usually not major components of the soil microflora. These nitrogen-fixing endophytes infect at the emergence of lateral roots and in the zone of elongation and differentiation above the root tip. After infection, the bacteria colonize the outer root cell layers and the root cortex. They can also gain access to the vascular tissue. These associations are strictly defined by the lack of any evidence of intracellular infection (James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998). However, very high numbers of PGPB in roots have been reported (i.e., $\leq 10^8$/ gram root dry weight) and with no observable disease symptoms (Reinhold *et al.*, 1986; Barraquio, Revilla and Ladha, 1997).

Many PGPB are capable of biological nitrogen fixation. 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 in endophytes, for example *Azoarcus* sp. in rice (Hurek, Reinhold-Hurek, Turner*, et al.*, 1994; Egener, Hurek and Reinhold-Hurek, 1999) and *Herbaspirillum* spp. in sugarcane and rice (Olivares *et al.*, 1997; James *et al.*, 2002). 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 (Sevilla *et al.*, 2001), *Azoarcus* sp. strain BH72 and Kallar grass (Hurek *et al.*, 2002), *Klebsiella* sp. and wheat (Iniguez, Dong and Triplett, 2004) or *Herbaspirillum seropedicae* Z67 and rice (Gyaneshwar *et al.*, 2002) and *Azospirillum brasilense* in *Setaria italica* (Okon *et al.*, 1983). However, even in these cases, the estimates are that the amount of nitrogen fixed would provide little or no contribution to the overall nitrogen demand. In contrast, some studies conducted in the field with wild grass species suggest that 30% or more of the plant nitrogen demands can be provided by BNF (Boddey and Victoria, 1986; Morais *et al.*, 2012), 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.

Results and Discussion

Plant response to inoculation is genotype specific

We screened over 30 genotypes of *Setaria viridis* for their response to inoculation with *Herbaspirillum seropedicae* and *Azospirillum 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* group. In contrast, *A. brasilense* is a member of the *Alphaproteobacteria* and its colonization is confined to the plant root surface (Tarrand Jj, Krieg Nr and J., 1978; Dobbelaere, Sofie *et al.*, 2001; Bashan, Holguin and De-Bashan, 2004). We identified three genotypes (EstepME034, EstepE017 and A10.1) that responded strongly to inoculation with increased growth, biomass, seed production and root growth. The *S. viridis* response to bacterial inoculation was quite variable depending on the genotype. 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, seeds 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 supplementary Figures S1-6. In general, a stronger response to inoculation was seen under the no-nitrogen conditions. Based on the results obtained, the genotype that responded most strongly to inoculation was *S. viridis* A10.1 (Figure 1), 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 for *S. viridis* (Brutnell *et al.*, 2010; Li and Brutnell, 2011). For these reasons, we focused more detailed analysis on this genotype.

The parameters analyzed for the *S. viridis* A10.1, plant height, inflorescence length, root fresh and dry weight did not show significant differences between uninoculated and inoculated plants when 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% (Figure 2A). 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 non-inoculated control plants. To check if the growth promotion effect of bacterial inoculation was occurring in the early developmental stages, the total root length and the lateral root number 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 non-inoculated plants at 17 d.a.i., whereas no increase was observed at 11 d.a.i. (Figure 2B).

Bacteria colonized both the exterior and interior of *S. viridis* roots.

Bacterial colonization of plant internal tissue is well described in many plant species. For example, nitrogen-fixing organisms from the genera *Burkholderia*, *Acetobacter* and *Pseudomonas* were found to colonize internal plant tissues (Lodewyckx *et al.*, 2002). *Herbaspirillum seropedicae* has also been shown to be

an endophyte of a variety of plant species (Baldani *et al.*, 1986; Magalhães Cruz *et al.*, 2001; Schmidt *et al.*, 2011). In order to examine whether *H. seropedicae* could colonize *S. viridis* root tissues, we used two strains, RAM4 expressing a DsRed and RAM10 expressing GFP. *H. seropedicae* colonizes both the exterior and interior of the root, including the root vascular tissue (Figure 3, Figure S7-S8). In order to examine colonization by *A. brasilense*, we used strain FP2-7 expressing a *nifH:gusA* gene. The use of this strain also allowed documentation of the expression of NifH, which corresponds to the small subunit of nitrogenase. *A. brasilense* colonized only the root surface of *S. viridis* at the base and tip of lateral roots, and along the elongation zone of parent roots, but clearly expressed nitrogenase under these conditions (Figure S9).

In these same experiments, we used bacterial plating to follow the time course of plant colonization by the two bacteria, taking advantage of the ability to uniquely select for each using their antibiotic resistance patterns (Figure S10). The *H. seropedicae* population reached 10^8 CFU/g of fresh 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* in some plants species is well described and initiates by crack-entry, usually at the site of lateral root emergence (James, *et al.*, 1997; Monteiro *et al.*, 2008). After 4 d.a.i. *H. seropedicae* DsRed expressing cells were found attached to the root hair cells (Figure S7). At 7 d.a.i., the bacteria were found in the internal tissues, colonizing the intercellular spaces of the plant cells (Figures S8). Using the software Axiovision, we were able to integrate the serial optical images obtained from the confocal microscope and observe the bacteria in different parts of the root tissue. This approach was used to clearly document the presence of *H. seropedicae* cells in the interior of roots (Figure 3). Measurements derived from these images showed endophytic colonization of *S. viridis* roots 9, 10 and 18 µm below the root surface, indicating the endophytic growth habit of this bacterium.

The expression of the *nifH* gene in *A. brasilense* was examined in roots 24 d.a.i. The root was analyzed in two parts, the upper, at 3 cm down to the hypocotyl zone, and the lower part, at 10 cm down to the hypocotyl, at the lateral root

emergency points and maturation zone. The roots were incubated in cacodylate buffer with 0.5 mg/mL of X-gluc (Hurek, Reinhold-Hurek, Van Montagu *et al.*, 1994) and observed in a Leica stereoscope. The *nifH* expression from *A. brasilense* was observed after 72h of incubation and the blue spots appeared mostly in the lower part of the root system, including lateral root cracks (Figure S9A), root tips (Figure S9B) and in the root elongation zone (Figure S9C). A roughly similar localization of *A. brasilense* on wheat roots was reported (Vande Broek *et al.*, 1993). These authors suggested that the lower part of the root might provide a microaerobic environment conducive to *A. brasilense* colonization.

Inoculated *S. viridis* plants support nitrogen fixation and incorporate the fixed N to support growth.

Demonstration of nitrogenase gene expression is not sufficient to conclude that BNF is occurring or, more importantly, the plant is using that fixed nitrogen. Hence, to address these issues, we measured the fixation and incorporation of $^{13}N_2$ gas by plants either mock inoculated or inoculated with *A. brasilense* (FP2-7) and *H. seropedicae* (RAM4) (Figure S11). Radiographic images of these inoculated plants revealed that the root systems acquired a measurable amount of radioactivity though it was not possible to ascertain the nature of the isotopic signature from the images (Figure S12). We also noted a significant amount of radioactivity fixed in photosynthetically active tissues that were unavoidably captured beneath the stem flange (see Figure S13). Follow-up decay analysis (Figure 4A-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 (Figure 4A) 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 $^{13}N_2$ gas (Kasel, Schueller and Ferrieri, 2010). Roots from the same inoculated plants, however, revealed a mixture of nitrogen-13 and carbon-11 isotopes when subjected to this analysis immediately following the tracer pulse (Figure 4B). Isotopic identification was based on a strong correlation between the experimentally derived half-lives and the published values. To verify that this nitrogen-13 signature was the result of bacterial N_2 fixation, roots from non-

inoculated control plants were subjected to this analysis. Results showed only a carbon-11 signature (Figure 4D). 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 min after the pulse, during which time the plant was subjected to high illumination (1500 μmol m^{-2} sec^{-1}) as a means to promote the plant's 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 minutes time course (Figure S13). Furthermore, decay analyses performed on aerial tissues after 15 minutes (Figure 4C) revealed a strong nitrogen-13 signature along with carbon-11 suggesting that some portion of the fixed $^{13}N_2$ ends up in biological transport within the host plant.

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 minus strains of *A. brasilense* (FP10) (Pedrosa and Yates, 1984) and *H. seropedicae* (SmR54) (Souza *et al.*, 2000) and inoculated with the ammonium-excreting mutant strain of *A. brasilense* (strain HM053) (Machado *et al.*, 1991), which is constitutively de-repressed for nitrogenase expression, fixing *in vitro* approximately 2X more N than the wild-type strain (Machado *et al.*, 1991; Vitorino *et al.*, 2001). Results from these studies, as well as those using the reporting wild-type 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. Data in this table was normalized for plant variability in dried tissue mass, as well as for the variability in the daily delivered tracer dose. Results showed that in plants inoculated with reporting wild-type strains of PGPB, $^{13}N_2$ fractional fixation was 747 ± 141 ppt 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. No evidence was seen of ^{13}N incorporation into tissue in either uninoculated plants or of plants inoculated with the minus N_2-fixing strains (FP10 and SmR54). Additionally, our studies using plants inoculated with the ammonium-excreting mutant strain (HM053) of *A. brasilense* showed 16-fold higher levels of $^{13}N_2$ fixation to 12,231 ± 5922 ppt on a root dry mass basis

compared to plants inoculated with wild-type strains. This level is sufficient to provide the plant's daily N demand, which was reflected by the healthy growth phenotype depicted in Figure 1. 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.

We also note that $^{11}CO_2$ fixation was strongly correlated with the BNF levels (Table 1). Inoculation with the ammonium-excreting mutant strain, HM053, resulted in the assimilation of 14-times more $^{11}CO_2$ fixed than seen with the wild-type strains, which in turn fixed 3-times more $^{11}CO_2$ than the uninoculated 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 $^{14}CO_2$ (Rosendahl, Vance and Pedersen, 1990), which was explained by enhanced activity of phosphoenol pyruvate 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 an inoculated plant 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 nitrogen-13 and carbon-11 signatures (Table 1). In the second study, we compared the effect of the light and dark cycles on the isotopic composition fixed within the root systems of plants inoculated with a mixture of the wild-type reporting strains of *H. seropedicae* (RAM4) and *A. brasilense* (FP2-7) and grown under low nitrate, as well as within roots of non-inoculated plants grown under high nitrate. Both $^{13}N_2$ and $^{11}CO_2$ fixation was apparent in detached roots. Furthermore, $^{13}N_2$ fixation was independent of diurnal effects, which is similar to what is observed in legumes (Weisz and Sinclair, 1988). 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.

Conclusions

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., (Jiang and Gresshoff, 1997; Cannon *et al.*, 2006; Sandal *et al.*, 2006; Oldroyd and Downie, 2008). These model legumes were chosen based on a variety of parameters, including rate of growth, fecundity (number of seeds produced), genetics (both are diploids), genome size, ease of genetic transformation, etc. Following this example, it would be useful to develop a plant model system that could provide a detailed systems-level, mechanistic understanding of PGPB-plant associations. Given the relevance of the work to biofuels, ideally such a model should be a diploid, C_4 grass species and have a sequenced genome. This suggests maize, sorghum, or *Setaria viridis*. Of these, only *Setaria* is a small plant, easily grown under greenhouse conditions, with a rapid growth cycle (Li and Brutnell, 2011). *Setaria* spp. are morphologically similar to the *Panicoideae* grasses, including major biofuel feedstocks, switchgrass (*Panicum virgatum*) and *Miscanthus* (*Miscanthus giganteus*). Of plants with sequenced genomes, *S. viridis* and *S. italica* (http://www.phytozome.net/foxtailmillet.php) are the closest relatives of the biofuel feedstock switchgrass. The data presented here suggest that, in addition, to using *S. viridis* as a model for other physiological processes, this plant also holds significant promise as a model for the study of associative nitrogen fixation and plant growth promotion.

Experimental Procedures

Bacteria growth conditions

H. seropedicae strain RAM4 (Nif+, Sm^R, Km^R, *dsRed*) expressing the DsRed fluorescent protein (Monteiro *et al.*, 2008) and *H. seropedicae* RAM10 (Nif+, Sm^R, Km^R, *gfp*; Monteiro, R., unpublished) expressing GPF were grown in liquid NFbHP-malate medium (Klassen, G. *et al.*, 1997) 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 (Machado *et al.*, 1991). 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 (Monteiro et al., 2008; Santos, K. unpublished results). *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,* fixes nitrogen constitutively in the presence of high ammonium concentrations and excretes NH_4^+ derived from nitrogen fixation (Machado et al., 1991). *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 species. 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.

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), Katryn Devos (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 (Hoagland and Arnon, 1950) 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.

***S. viridis* seedling inoculation and growth**

The *S. viridis* plantlets were inoculated with both *H. seropedicae* RAM4 and *A. brasilense* FP2-7. The cultures were growth 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 KNO_3 depending upon the treatment.

Plant growth promotion assay

To assess the phytostimulatory effect of *H. seropedicae* and *A. brasilense* in different *S. viridis* genotypes, the plantlets were inoculated as described above and control plants were inoculated with boiled cultures (non-inoculated), 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: leaf length, 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).

Bacterial counting assay

To evaluate the ability of the bacteria to colonize the selected *S. viridis* genotypes, the roots and 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 and leaves 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/µL of streptomycin and 200 µg/µL of kanamycin was used. *A. brasilense* selection used the NFbHPN-lactate medium supplemented with 80 µg/µL of streptomycin and 10 µg/µL of 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* (Roncato-Maccari *et al.*, 2003).

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 (Monteiro *et al.*, 2008), 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).

Statistical analyses

Statistical analyses of the growth promotion parameters were done using the *Wilcox* test (Wilcoxon Matched-Pairs; Wilcoxon signed-ranks test), which is a non-parametric method to compare two paired samples. A different number of samples were applied for each genotype or parameter due to seed availability, germination rate and percentage of survival in soil. Analyzed samples with p-value ≤ 0.05 were considered statistically significant.

[^{13}N]N_2 Production

[^{13}N]N_2 was produced *via* the $^{14}N(p,pn)^{13}N$ reaction leveraging 18 MeV protons from the BNL Ebco TR-19 Cyclotron (Ebco Industries Ltd, Richmond, BC, Canada). These protons were tightly 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 $^{14}N(p, \alpha)^{11}C$ reaction (Ferrieri e Wolf,

1983). 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}N]N_2 at the end-of-bombardment. This radioactive gas was immediately processed through a stripping trap (Ferrieri *et al.*, 2005); see Figure S11) which was comprised of 135 mg of silica supported Ni(0) catalyst (Shimadzu, Inc., Kyoto, Japan) mixed with 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).

Plant Growth for [^{13}N]N_2 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 5 mM (for non-inoculated control plants) or 0.5 mM (for bacterial inoculated plants and non-inoculated control plants).

Tracer Administration

Prior to a [^{13}N]N_2 experiment a planting pot containing a single study plant would be sealed into the tracer receiving chamber (see Supplemental Figure S11). 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}N]N_2 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.

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 (see Supplemental Figure S12). 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).

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. 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 (see Figure 4 A-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.

Acknowledgements

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 Ciências Sem Fronteiras Program, Brazil (supporting V.C.S. Pankievicz, F. P. Amaral and K.F Santos fellowships) 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 would also acknowledge the technician support of Dr. Aleksandr Jurkevic from the molecular cytology core from University of Missouri, and Dr. Carolina Galvão from UEPG, Brazil for the *Azospirillum brasilense* strain FP2-7.

Figures and Legends

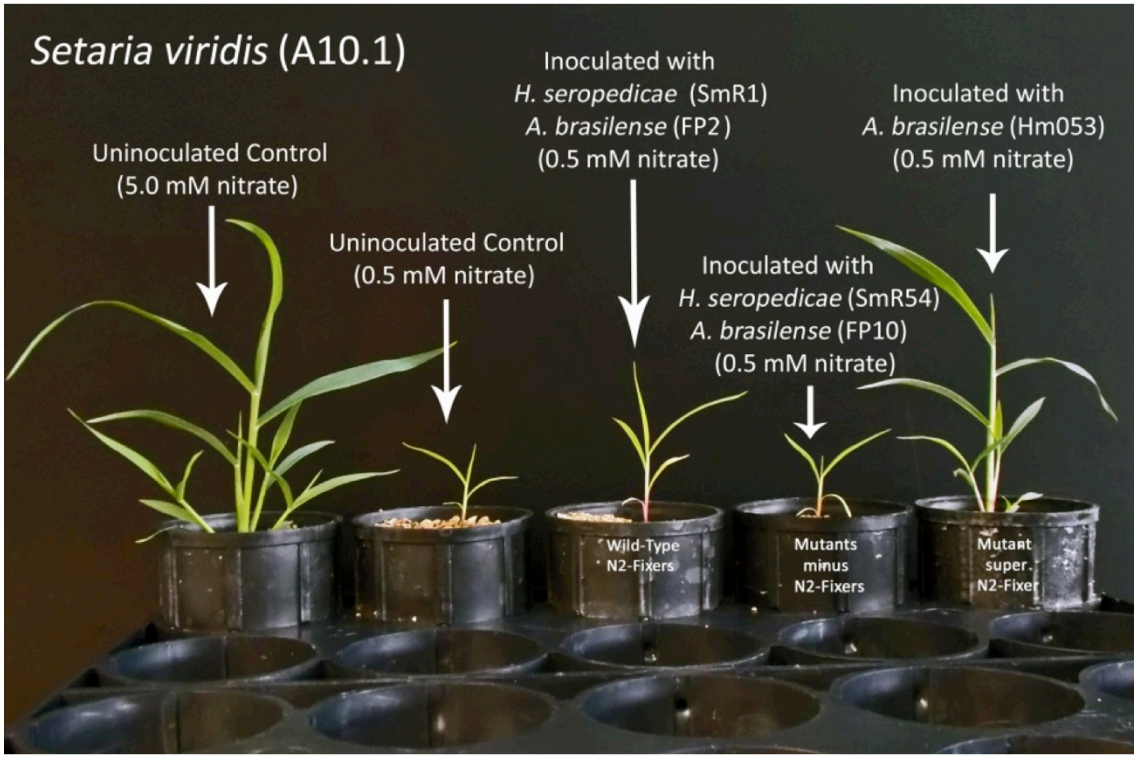


Figure 1: Representative pictures of *S. viridis* grown under different nitrogen regimes with or without inoculation. SmR1 and FP2 are wild-type strains of *H. seropedicae* and *A. brasilense*, respectively. SmR54 and FP10 are mutants of the same bacteria that are unable to fix nitrogen. Strain HM053 is an ammonium-excreting mutant strain of *A. brasilense*. We measure 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.

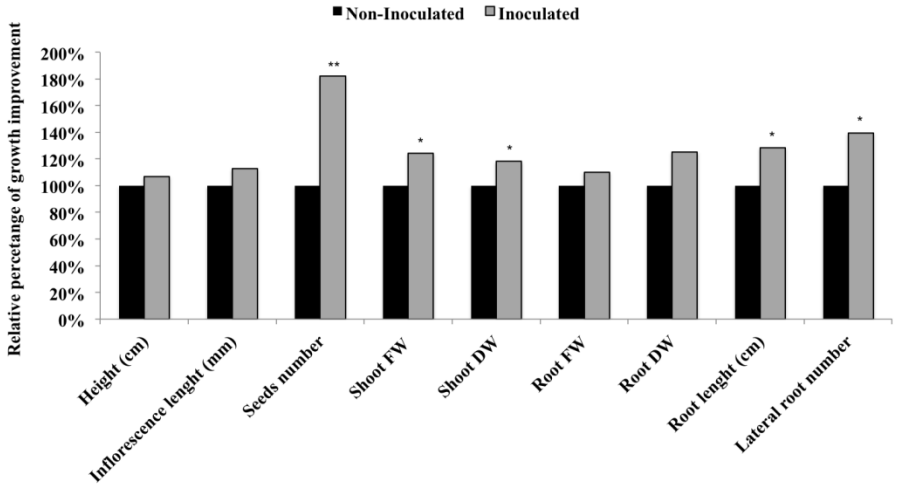


Figure 2A: Relative percentage of growth improvement by bacterial inoculation for the *S. viridis* A10.1 genotype grown without added nitrogen. In comparison to non-inoculated plants, plants inoculated with a mixture of *A. brasilense* and *H. seropedicae* showed significant growth promotion in seed number, shoot fresh weight (shoot FW), shoot dry weight (shoot DW), total root length, and total lateral root number. Each asterisk denotes a statistically significant different with p-value < 0.05. © 2015 The Authors The Plant Journal © 2015 John Wiley & Sons Ltd

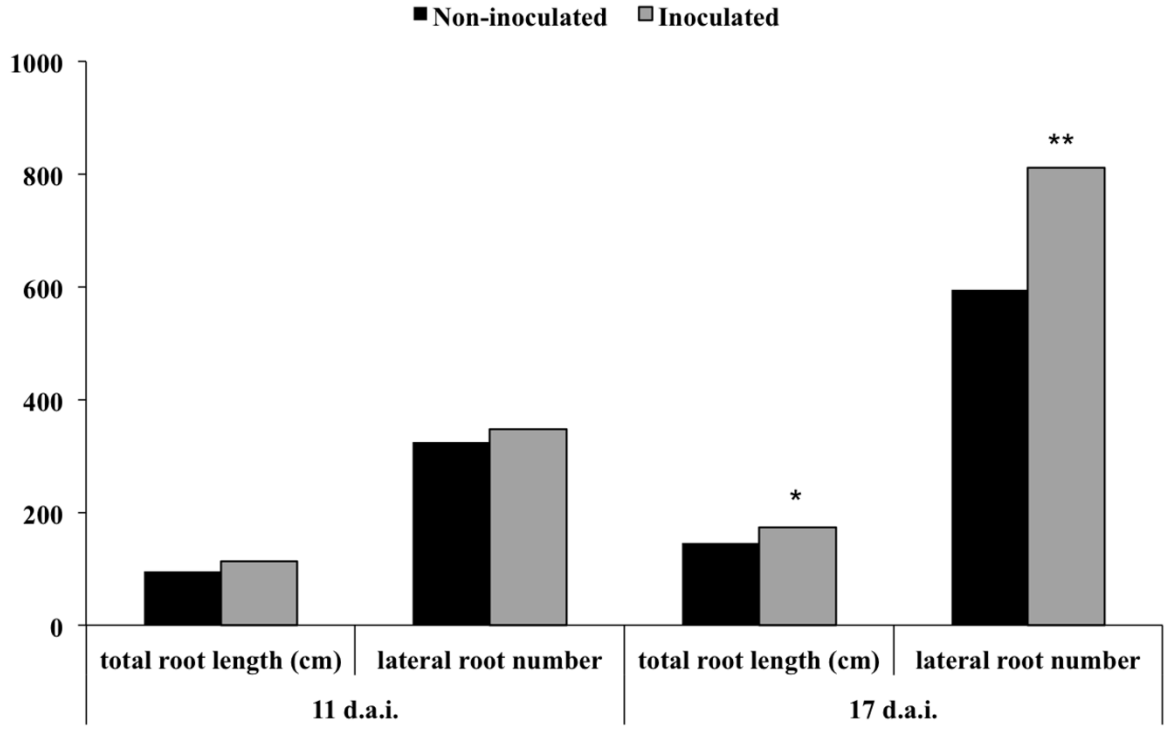


Figure 2B: Growth promotion effects by bacterial inoculation on early stages of root growth of the *S. viridis* A10.1 genotype. Plants were inoculated with a mixture containing equal CFUs of *A. brasilense* and *H. seropedicae* (total CFU 1 x 10^8 per plant). Inoculated plants showed a total root length 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. © 2015 The Authors The Plant Journal © 2015 John Wiley & Sons Ltd

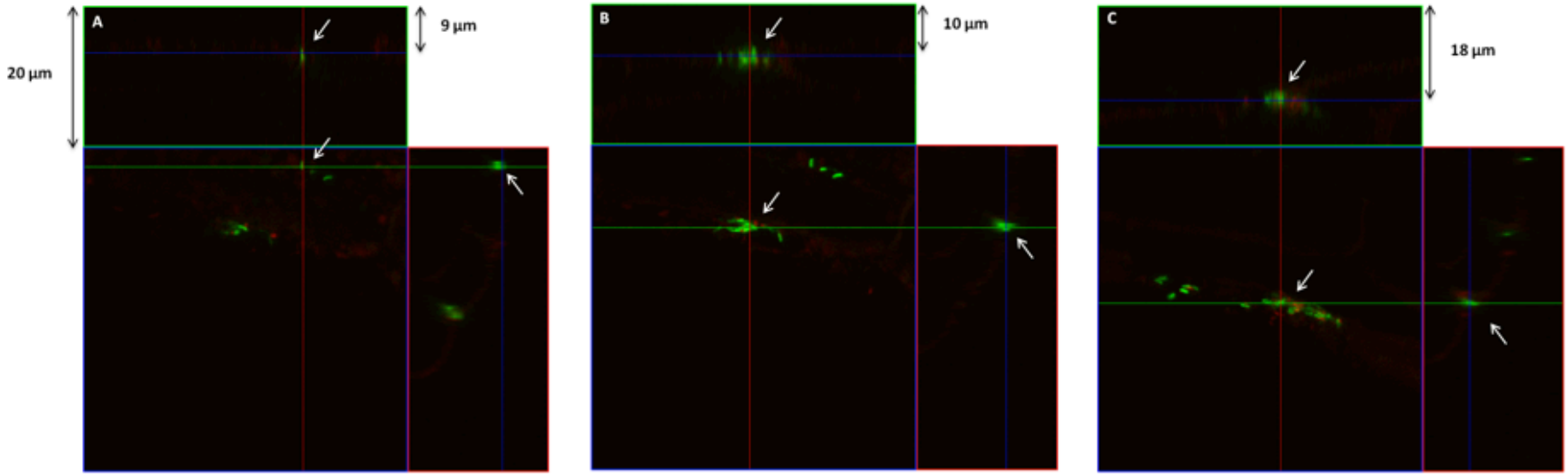


Figure 3: Orthogonal optical sections from cortical cells of *S. viridis* colonized by *H. seropedicae.* The *H. seropedicae* cells expressing GFP (green) in the red background of the root stained with propidium iodide. The central views framed in blue show x–y focal plans from the z-stacks. The red 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.

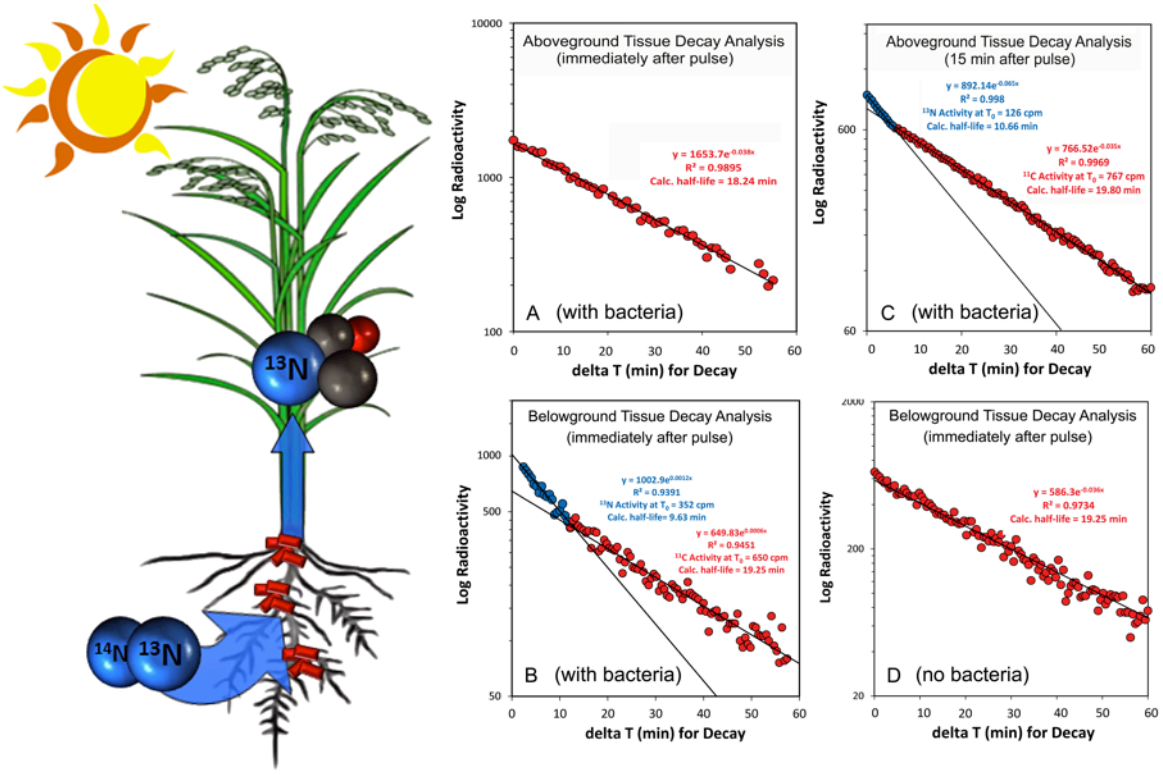


Figure 4: Comparative decay analyses of aboveground (Panels A & C) and belowground tissues (Panels B & D) immediately after the ^{13}NN pulse and 15 minutes after the pulse during high illumination. In this treatment, a logarithmic plot of the radioactivity level against time will yield a straight line for single isotope decay, and two straight lines for a mixture of two isotopes with differing decay rates. In the plots above, 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 minutes 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.

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 (non-inoculated)	Light	0.0 ppt	0.0072
Control (non-inoculated)	Light	0.0 ppt	0.0116
Control (non-inoculated)	Light	0.0 ppt	0.0101
Mean ± SD	Light	0.0 ppt	0.009.7 ± 0.0022[d.]
Control (non-inoculated)	Dark	0.0 ppt	0.0068
Control (non-inoculated)	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
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

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 bacteria 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 non-inoculated control plants.

d. Similar levels of $^{11}CO_2$ fixation were seen in the uninoculated plants as that in plants inoculated with reporting wild-type 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 ammonium-excreting 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).

Legends for supporting information

Figure S1: Shoot growth promotion parameters of *S. viridis* genotypes grown without added nitrogen.

Figure S2: Root growth promotion parameters of *S. viridis* genotypes grown without added nitrogen.

Figure S3: Number of seeds produced and inflorescence length for the *S. viridis* genotypes grown without added nitrogen.

Figure S4: Shoot growth promotion parameters of *S. viridis* genotypes grown with low-nitrogen addition (i.e., 0.5 mM KNO_3)**.**

Figure S5: Root growth promotion parameters of *S. viridis* genotypes grown with low-nitrogen addition (i.e., 0.5 mM KNO_3)**.**

Figure S6: Number of seeds produced and inflorescence length for *S viridis* grown with low-nitrogen addition (i.e., 0.5 mM)**.**

Figure S7: Confocal laser scanning microscope images of *H. seropedicae* on roots of *S. viridis* A10.1 four days after inoculation.

Figure S8: Confocal laser scanning microscope images of *H. seropedicae* on roots of *S. viridis* A10.1 genotype seven days after bacterial inoculation.

Figure S9. Epiphytic colonization and *nifH:gusA* expression by *A. brasilense* FP2-7 on the root surface of *S. viridis* A10.1 genotype*.*

Figure S10: Pattern of bacterial colonization of the roots of *S. viridis* A10.1 genotype.

Figure S11: Schematic drawing of the BNL ^{13}NN plant pulsing station.

Figure S12: Photograph and autoradiography image.

Figure S13: Time-activity trace showing transport of root activity aboveground.

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

References

Baldani, J.I., Baldani, V.L.D., Seldin, L. and Döbereiner, J. (1986) Characterization of *Herbaspirillum seropedicae*, a root-associated nitrogen fixing bacterium. *Intl. J. Sys. Bact.*, **36**, 86-93.

Barraquio, W.L., Revilla, L. and Ladha, J.K. (1997) Isolation of endophytic diazotrophic bacteria from wetland rice. *Plant and Soil*, **194**, 15-24.

Bashan, Y., Holguin, G. and de-Bashan, L.E. (2004) *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). *Canadian Journal of Microbiology*, **50**, 521-577.

Boddey, R.M. and Victoria, R.L. (1986) Estimation of biological nitrogen fixation associated with *Brachiaria* and *Paspalum* grasses using 15N labelled organic matter and fertilizer. *Plant and Soil*, **90**, 265-292.

Brutnell, T.P., Wang, L., Swartwood, K., Goldschmidt, A., Jackson, D., Zhu, X.G., Kellogg, E. and Van Eck, J. (2010) *Setaria viridis*: a model for C4 photosynthesis. *The Plant cell*, 22, 2537-2544.

Cannon, S.B., Sterck, L., Rombauts, S., Sato, S., Cheung, F., Gouzy, J., Wang, X., Mudge, J., Vasdewani, J., Schiex, T., Spannagl, M., Monaghan, E., Nicholson, C., Humphray, S. J., Schoof, H., Mayer, K., Rogers, J., Quartier, F., Oldroyd, G.E., Debellao, F., Cook, D., Retzel, E., Roe, B., Town, C., Tabata, S., Van de Peer, Y. and Young, N. (2006) Legume genome evolution viewed through the *Medicago truncatula* and *Lotus japonicus* genomes. *Proceedings of the National Academy of Sciences*, 103, 14959-14964.

Dobbelaere, S., Croonenborghs, A., Thys, A., Ptacek, D., Vanderleyden, J., Dutto, P., Labandera-Gonzalez, C., Caballero-Mellado, J., Aguirre, J.F., Kapulnik, Y., Brener, S., Burdman, S., Kadouri, D., Sarig, S. and Okon, Y. (2001) Responses of agronomically important crops to inoculation with *Azospirillum*. *Functional Plant Biology*, 28, 871-879.

Egener, T., Hurek, T. and Reinhold-Hurek, B. (1999) Endophytic Expression of nif Genes of *Azoarcus sp*. Strain BH72 in Rice Roots. *Molecular Plant-Microbe Interactions*, **12**, 813-819.

Ferrieri, R.A., Gray, D.W., Babst, B.A., Schueller, M.J., Schlyer, D.J., Thorpe, M.R., Orians, C.M. and Lerdau, M. (2005) Use of carbon-11 in *Populus* shows that exogenous jasmonic acid increases biosynthesis of isoprene from recently fixed carbon. *Plant Cell and Environment*, **28**, 591-602.

Ferrieri, R.A. and Wolf, A.P. (1983) The chemistry of positron emitting nucleogenic (hot) atoms with regard to preparation of labeled compounds of practical utility. *Radiochimica Acta* **34**, 69-83.

Gyaneshwar, P., James, E.K., Reddy, P.M. and Ladha, J.K. (2002) *Herbaspirillum* colonization increases growth and nitrogen accumulation in aluminium-tolerant rice varieties. *New Phytologist*, **154**, 131-145.

Hoagland, D.R. and Arnon, D.I. (1950) The water-culture method for growing plants without soil. *Circular. California Agricultural Experiment Station*, **347**, 32 pp.

Hurek, T., Handley, L.L., Reinhold-Hurek, B. and PichÃ©, Y. (2002) *Azoarcus* Grass Endophytes Contribute Fixed Nitrogen to the Plant in an Unculturable State. *Molecular Plant-Microbe Interactions*, **15**, 233-242.

Hurek, T., Reinhold-Hurek, B., Turner, G.L. and Bergersen, F.J. (1994a) Augmented rates of respiration and efficient nitrogen fixation at nanomolar concentrations of dissolved O2 in hyperinduced *Azoarcus sp.* strain BH72. *J.Bacteriol.*, **176**, 4726.

Hurek, T., Reinhold-Hurek, B., Van Montagu, M. and Kellenberger, E. (1994b) Root colonization and systemic spreading of *Azoarcus sp*. strain BH72 in grasses. *J.Bacteriol.*, **176**, 1913.

Iniguez, A.L., Dong, Y. and Triplett, E.W. (2004) Nitrogen Fixation in Wheat Provided by *Klebsiella pneumoniae* 342. *Molecular Plant-Microbe Interactions*, **17**, 1078-1085.

James, E.K., Gyaneshwar, P., Mathan, N., Barraquio, W.L., Reddy, P.M., Iannetta, P.P.M., Olivares, F.L. and Ladha, J.K. (2002) Infection and Colonization of Rice Seedlings by the Plant Growth-Promoting Bacterium *Herbaspirillum seropedicae* Z67. *Molecular Plant-Microbe Interactions*, **15**, 894-906.

James, E.K. and Olivares, F.b.L. (1998) Infection and Colonization of Sugarcane and Other Graminaceous Plants by Endophytic Diazotrophs. *Critical Reviews in Plant Sciences*, **17**, 77 - 119.

James, E.K., Olivares, F.L., Baldani, J.I. and Döbereiner, J. (1997) *Herbaspirillum*, an endophytic diazotroph colonizing vascular tissue in leaves of *Sorghum bicolor* L. Moench. . *J. Exp. Bot.*, **48**, 785-797.

James, E.K., Reis, V.M., Olivares, F.L., Baldandi, J.I. and Döbereiner, J. (1994) Infection of sugarcane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. *J. Exp. Bot.*, **45**, 757-766.

Jiang, Q.Y. and Gresshoff, P.M. (1997) Classical and molecular genetics of the model legume *Lotus japonicus*. *Mol.Plant Microbe.Interaction.*, **10**, 59.

Kasel, M.C.K., Schueller, M.J. and Ferrieri, R.A. (2010) Optimizing [13N]N2 radiochemistry for imaging nitrogen-fixation in root nodules of legumes. *J. Label Cmpds & Radiopharm.*, **53**, 592-597.

Klassen, G., Pedrosa, F.O., Souza, E.M., Funayama, S. and Rigo, L.U. (1997) Effect of nitrogen compounds on nitrogenase activity in *Herbaspirillum seropedicae* SMR1. *Canadian journal of microbiology*, **43**, 887-891.

Li, P. and Brutnell, T.P. (2011a) *Setaria viridis* and *Setaria italica*, model genetic systems for the Panicoid grasses. *Journal of experimental botany*, **62**, 3031-3037.

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S. and Van der Lelie, D. (2002) Endophytic bacteria and their potential applications. *Crit Rev Plant Sci*, 6, 583-606.

Machado, H.B., Funayama, S., Rigo, L.U. and Pedrosa, F.O. (1991) Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine. *Can J Microbiol*, **37**, 549-553.

Cruz M. , L., de Souza, E.M., Weber, O.B., Baldani, J.I., Döbereiner, J. and Pedrosa, F.e.O. (2001) 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa spp.*) and pineapple (*Ananas comosus* (L.) Merril). *Appl Environ Microbiol*, **67**, 2375-2379.

Monteiro, R.A., Schmidt, M.A., Baura, V.A.d., Balsanelli, E., Wassem, R., Yates, M.G., Randi, M.A.F., Pedrosa, F.O. and Souza, E.M.d. (2008) Early colonization pattern of maize (*Zea mays* L. Poales, Poaceae) roots by

Herbaspirillum seropedicae (Burkholderiales, Oxalobacteraceae). *Genetics and Molecular Biology*, 31, 932-937.

Morais, R., Quesada, D., Reis, V., Urquiaga, S., Alves, B.R. and Boddey, R. (2012) Contribution of biological nitrogen fixation to Elephant grass (*Pennisetum purpureum Schum*.). *Plant and Soil*, **356**, 23-34.

Oldroyd, G.E.D. and Downie, J.A. (2008) Coordinating Nodule Morphogenesis with Rhizobial Infection in Legumes. *Annual Review of Plant Biology*, **59**, 519-546.

Olivares, F.L., James, E.K., Baldani, J.I. and DÖBereiner, J. (1997) Infection of mottled stripe disease-susceptible and resistant sugarcane varieties by the endophytic diazotroph *Herbaspirilium*. *New Phytologist*, **135**, 723-737.

Pedrosa, F.O. and Yates, M.G. (1984) Regulation of nitrogen fixation (*nif*) genes of *Azospirillum brasilense* by *nifA* and *ntr* (*gln*) type gene products. *FEMS microbiology letters*, **23**, 95-101.

Raaijmakers, J., Paulitz, T., Steinberg, C., Alabouvette, C. and Moënne-Loccoz, Y. (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil*, **321**, 341-361.

Reinhold-Hurek, B. and Hurek, T. (1998) Life in grasses: diazotrophic endophytes. *Trends in microbiology*, **6**, 139-144.

Reinhold, B., Hurek, T., Niemann, E.-G. and Fendrik, I. (1986) Close Association of *Azospirillum* and Diazotrophic Rods with Different Root Zones of Kallar Grass. *Appl. Environ. Microbiol.*, **52**, 520-526.

Richardson, A., Barea, J.-M., McNeill, A. and Prigent-Combaret, C. (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*, **321**, 305-339.

Roncato-Maccari, L.D.B., Ramos, H.J.O., Pedrosa, F.O., Alquini, Y., Chubatsu, L.S., Yates, M.G., Rigo, L.U., Steffens, M.B.R. and Souza, E.M. (2003) Endophytic *Herbaspirillum seropedicae* expresses *nif* genes in gramineous plants. *Fems Microbiology Ecology*, 45, 39-47.

Rosendahl, L., Vance, C.P. and Pedersen, W.B. (1990) Products of dark CO_2 fixation in pea root nodules support bacteroid metabolism. *Plant physiology*, **93**, 12.

Sandal, N., Petersen, T.R., Murray, J., Umehara, Y., Karas, B., Yano, K., Kumagai, H., Yoshikawa, M., Saito, K., Hayashi, M., Murakami, Y., Wang, X.W., Hakoyama, T., Imaizumi-Anraku, H., Sato, S., Kato, T., Chen, W.L., Hossain, M.S., Shibata, S., Wang, T.L., Yokota, K., Larsen, K., Kanamori, N., Madsen, E., Radutoiu, S., Madsen, L.H., Radu, T.G., Krusell, L., Ooki, Y., Banba, M., Betti, M., Rispail, N., Skot, L., Tuck, E., Perry, J., Yoshida, S., Vickers, K., Pike, J., Mulder, L., Charpentier, M., Muller, J., Ohtomo, R., Kojima, T., Ando, S., Marquez, A.J., Gresshoff, P.M., Harada, K., Webb, J., Hata, S., Suganuma, N., Kouchi, H., Kawasaki, S., Tabata, S., Hayashi, M., Parniske, M., Szczyglowski, K., Kawaguchi, M. and Stougaard, J. (2006) Genetics of symbiosis in *Lotus japonicus*: Recombinant inbred lines, comparative genetic maps, and map position of 35 symbiotic loci. *Molecular Plant-Microbe Interactions*, 19, 80-91.

Santi, C., Bogusz, D. and Franche, C. (2013) Biological nitrogen fixation in non-legume plants. *Annals of Botany*, pp. 743-767.

Schmidt, M.A., Souza, E.M., Baura, V., Wassem, R., Yates, M.G., Pedrosa, F.O. and Monteiro, R.A. (2011) Evidence for the endophytic colonization of *Phaseolus vulgaris* (common bean) roots by the diazotroph *Herbaspirillum seropedicae*. *Braz J Med Biol Res*, **44**, *182-185*.

Sevilla, M., Burris, R.H., Gunapala, N. and Kennedy, C. (2001) Comparison of Benefit to Sugarcane Plant Growth and $15N_2$ Incorporation Following Inoculation of Sterile Plants with *Acetobacter diazotrophicus* Wild-Type and *nifA*¯ Mutant Strains. *Molecular Plant-Microbe Interactions*, **14**, 358-366.

Souza, E.M., Pedrosa, F.O., Rigo, L.U., Machado, H.B. and Yates, M.G. (2000) Expression of the nifA gene of *Herbaspirillum seropedicae*: role of the NtrC and NifA binding sites and of the −24/−12 promoter element. *Microbiology*, **146**, 1407-1418.

Tarrand JJ, Krieg NR and J., D. (1978) 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**, 967-980.

Vande Broek, A., Michiels, J., Van Gool, A. and Vanderleyden, J. (1993) Spatial-Temporal Colonization Patterns of *Azospirillum brasilense* on the Wheat Root Surface and Expression of the Bacterial *nifH* Gene during Association. . *Molecular Plant Microbe Interaction*, 6, 592-600.

Verhagen, B.W., Glazebrook, J., Zhu, T., Chang, H.S., van Loon, L.C. and Pieterse, C.M. (2004) The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Molecular Plant-Microbe Interactions*, **17**, 895-908.

Vitorino, J.C., Steffens, M.B.R., Machado, H.B., Yates, M.G., Souza, E.M. and Pedrosa, F.O. (2001) Potential roles for the *glnB* and *ntrYX* genes in *Azospirillum brasilense*. *FEMS microbiology letters*, **201**, 199-204.

Weisz, P.R. and Sinclair, T.R. (1988) Soybean nodule gas permeability, nitrogen fixation and dirunal cycles in soil temperature. *Plant and Soil*, **109**, 227-234.

Supplementary Material

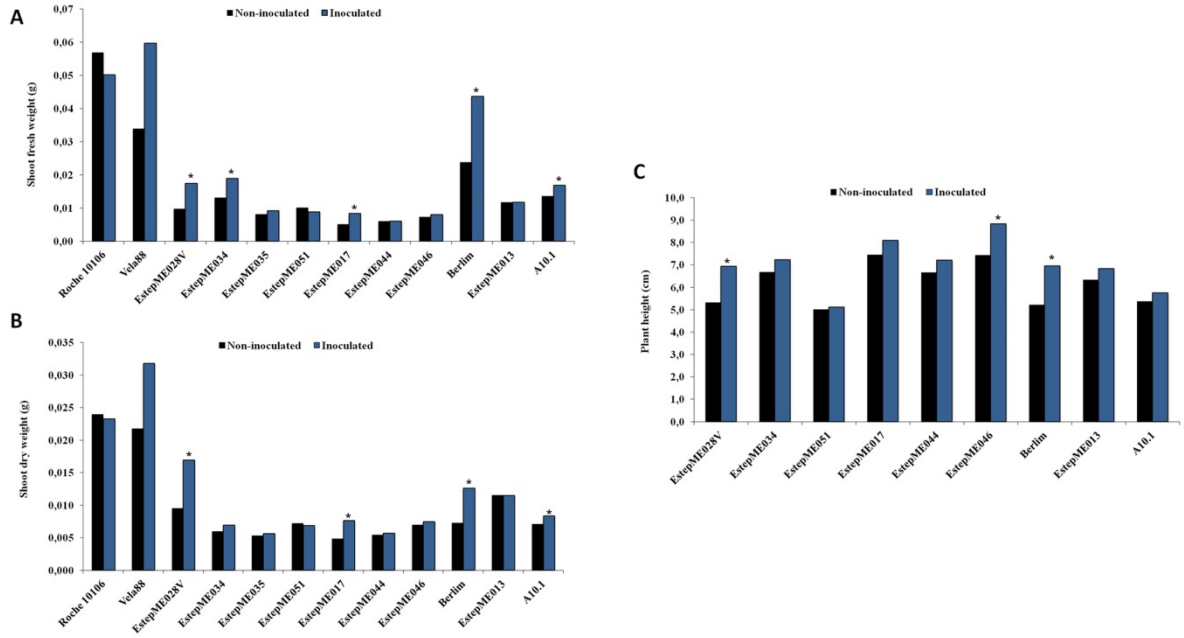


Figure S1: Shoot growth promotion parameters of *S. viridis* genotypes grown without added nitrogen. (A) Shoot fresh weight of the *S. viridis* genotypes, EstepME028V, EstepME034, EstepME017, Berlim, and A10.1 showed a significant increase when the plants were inoculated with *A. brasilense* and *H. 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.

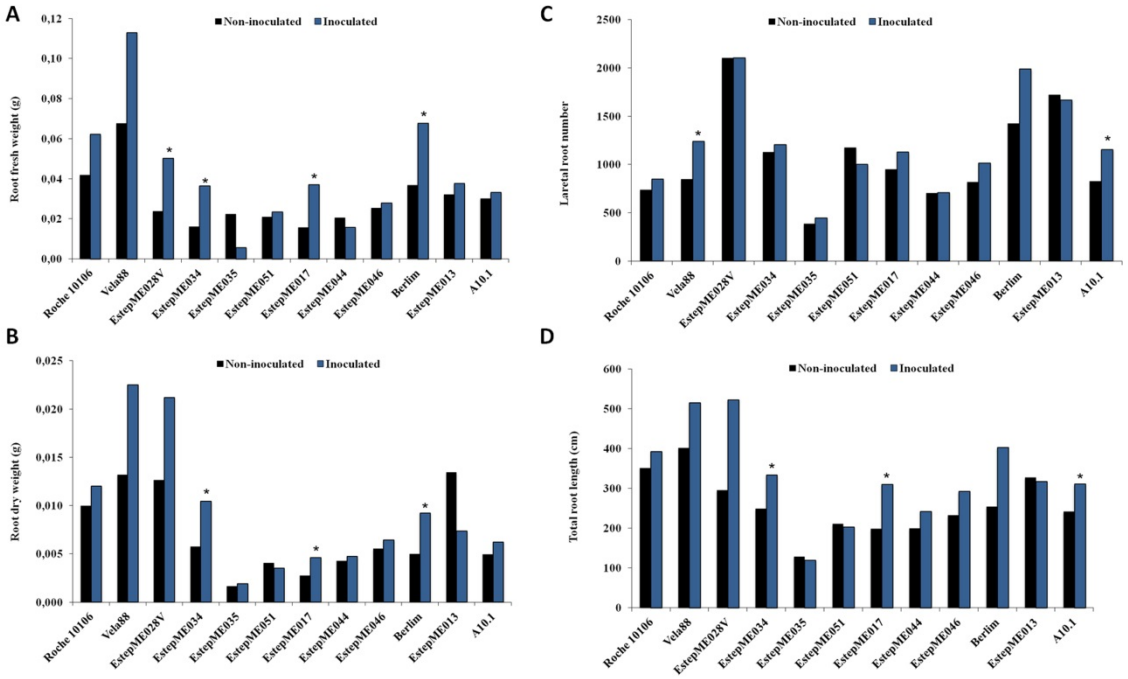


Figure S2: Root growth promotion parameters of *S. viridis* genotypes grown without added nitrogen. (A) Genotypes EstepME028V, EstepME034, EstepME017 and Berlim showed a significant increase in the root fresh weight when plants were inoculated with *A. brasilense* and *H. seropedicae.* (B) Genotypes, 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 non-inoculated and inoculated plants.

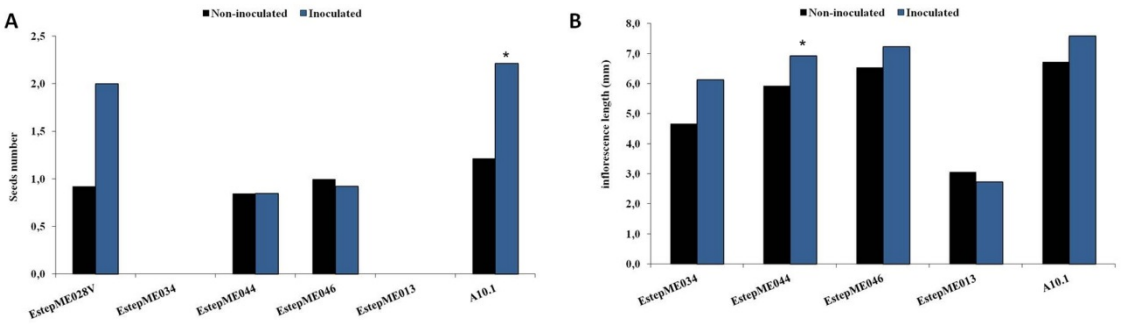


Figure S3: Number of seeds produced and inflorescence length for the *S. viridis* genotypes grown without added nitrogen (A) Only genotype A10.1 showed a significant increase in seed number when plants were inoculated with *A. brasilense* and *H. seropedicae,* while (B) the EstepME044 and EstepME034 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 non-inoculated and inoculated plants.

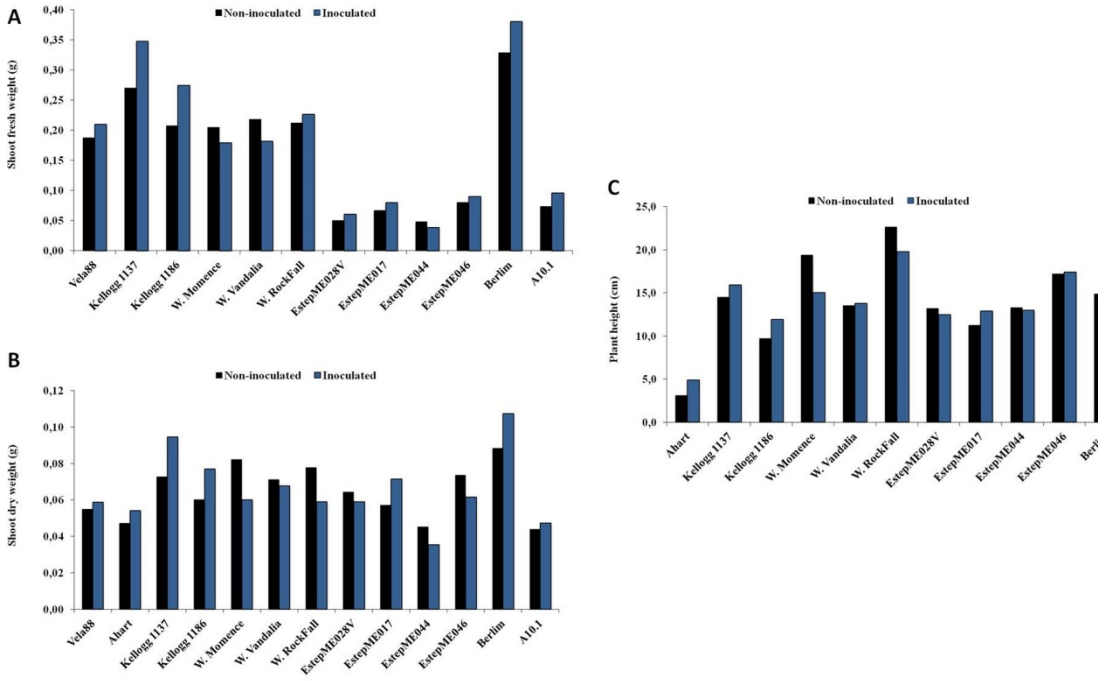


Figure S4: Shoot growth promotion parameters of *S. viridis* genotypes grown with low-nitrogen addition (i.e., 0.5 mM KNO_3). None of the genotypes showed a significant increase in shoot fresh or dry weight and plant height when plants were inoculated with *A. brasilense* and *H. seropedicae*. No statistically significant differences were observed.

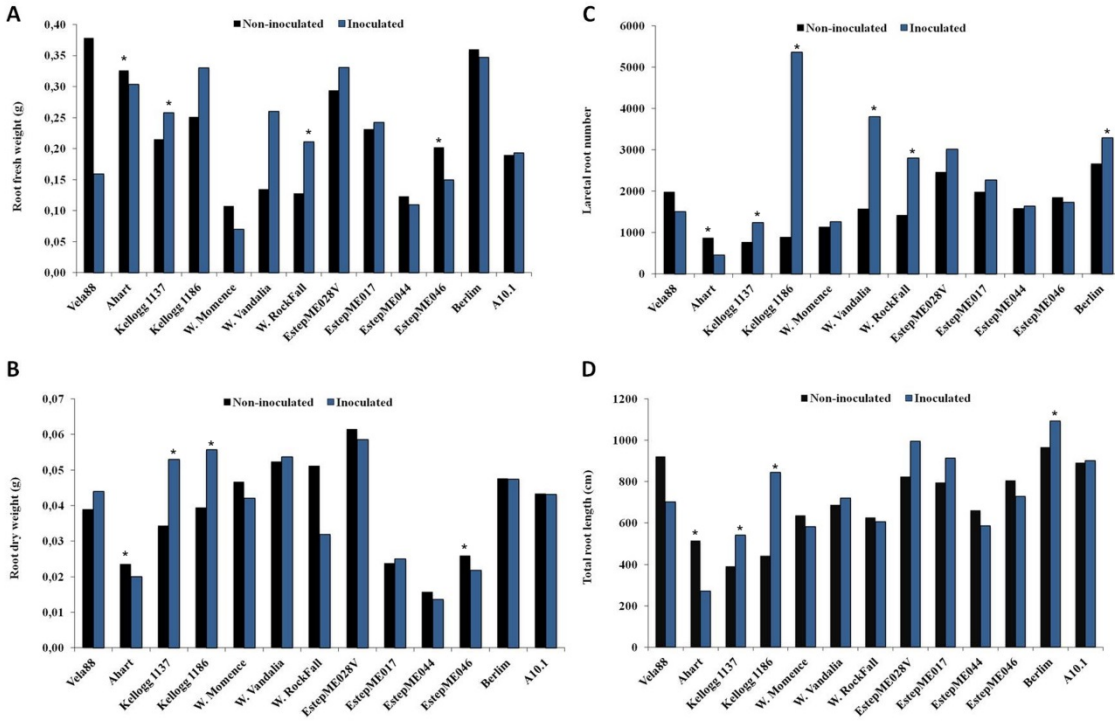


Figure S5: Root growth promotion parameters of *S. viridis* genotypes grown with low-nitrogen addition (i.e., 0.5 mM KNO_3). (A) Genotypes Kellogg1137 and W. Rockfall showed a significant increase in the root fresh weight when plants were inoculated with *A. brasilense* and *H. seropedicae*, while the genotypes Ahart and EstepME046 showed a decrease 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 genotype Ahart had a decreased lateral root number upon inoculation. (D) Genotypes Kellogg1137, Kellogg1186 and Berlim showed a significant increase in the root length when plants were inoculated, while the genotype Ahart showed a decreased total root length upon the inoculation with bacteria. Asterisks indicate a statistically significant difference with p-value ≤ 0.05 between non-inoculated and inoculated plants.

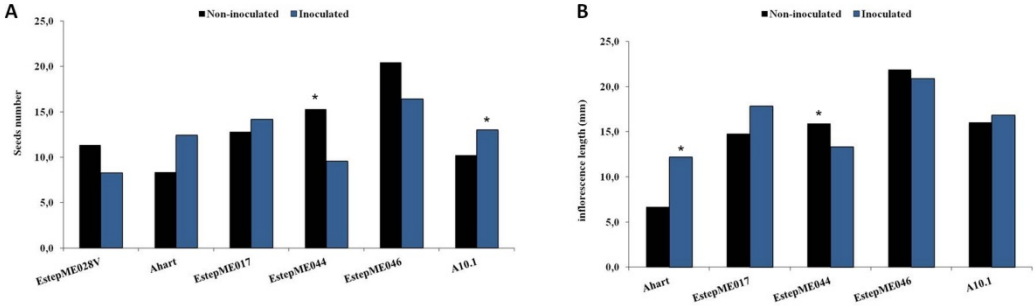


Figure S6: Number of seeds produced and inflorescence length for the genotypes of *S. viridis* grown with low-nitrogen addition (i.e., 0.5 mM KNO_3). The genotype A10.1 showed a significant increase in seed number (A) when plants were inoculated with *A. brasilense* and *H. seropedicae*, while the accession EstepME044 showed a decreased number of seeds and inflorescence length. (B) The genotype Ahart showed increased inflorescence length. Asterisks indicate a statistically significant difference with p-value ≤ 0.05 between non-inoculated and inoculated plants.

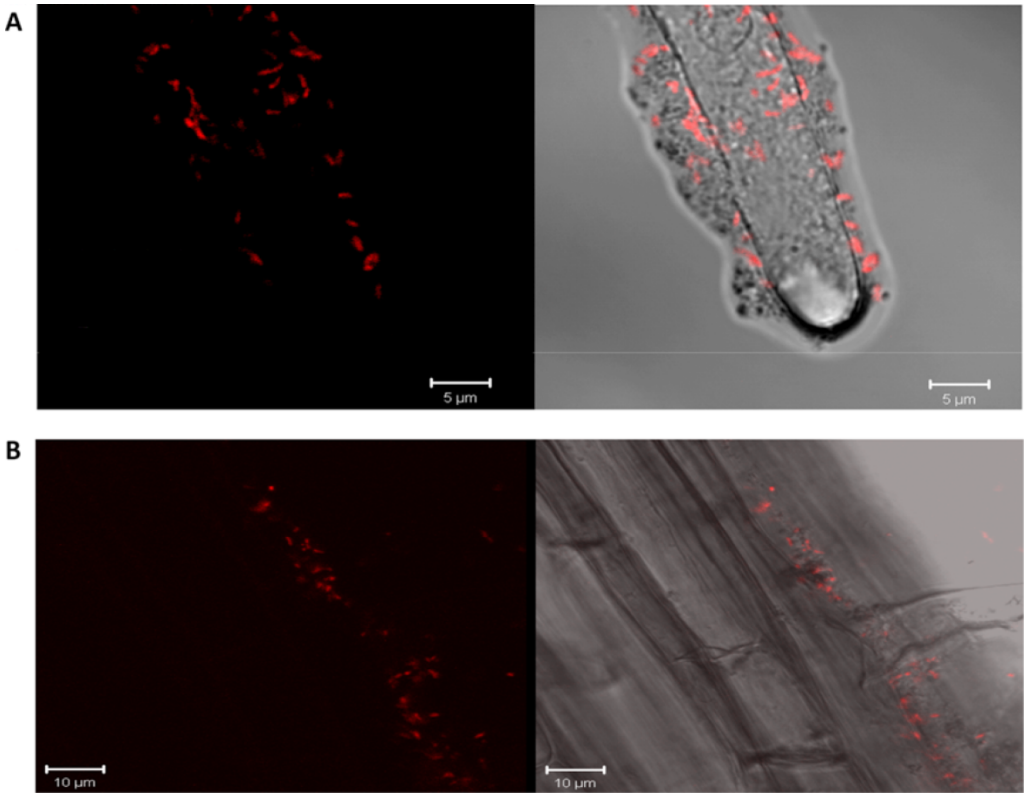


Figure S7: Confocal laser scanning microscope images of *H. seropedicae* on roots of *S. viridis* A10.1 after four days of inoculation. A root hair (A) and the base of a root hair (B) colonized by *H. seropedicae* RAM4 expressing DsRed shown in the left panels and light gray backgrounds shown in the 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.

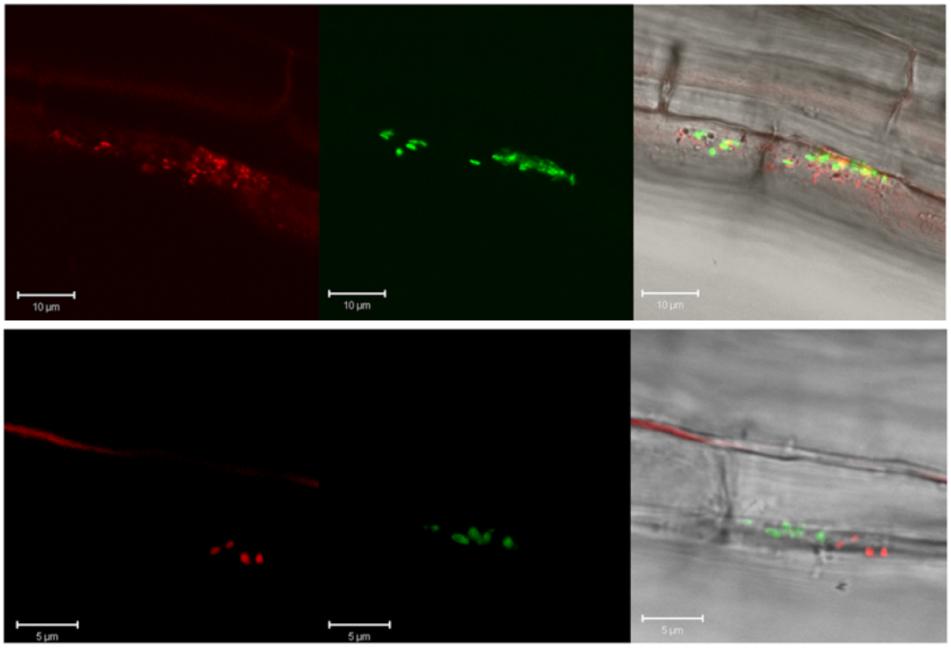


Figure S8: Confocal laser scanning microscope images of *H. seropedicae* on roots of *S. viridis* A10.1 genotype at seven days after bacterial inoculation. Bacterial cells colonize the intercellular spaces, delimited by the membranes of the plant cells. *H. seropedicae* RAM10 expressing GFP protein are 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.

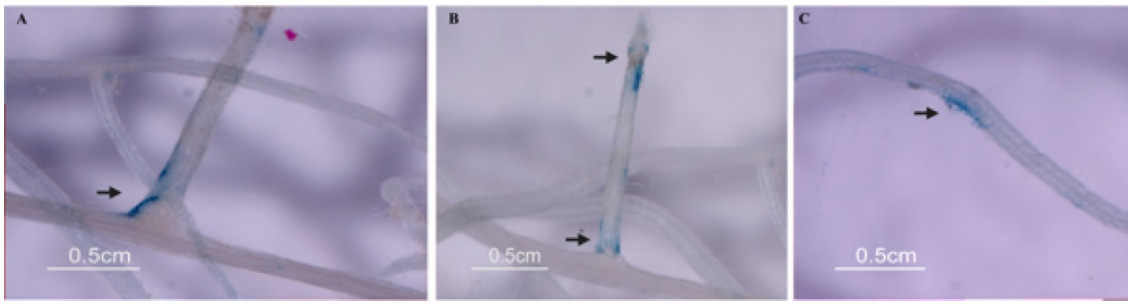


Figure S9. Epiphytic colonization on root surface of *S. viridis* A10.1 genotype by *A. brasilense* FP2-7. The plants were inoculated with *A. brasilense* FP2-7 expressing the *nifH:gusA* gene for 24 days, and then stained for GUS expression. Black arrows indicate regions of colonization at the lateral 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.

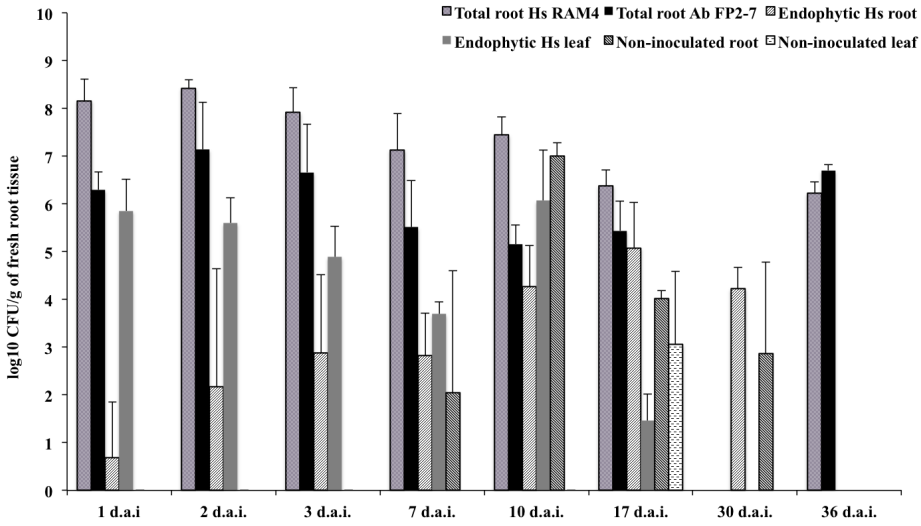


Figure S10: Pattern of bacterial colonization of the roots of *S. viridis* A10.1 genotype. Data are expressed in colony forming units (CFU) per gram of fresh tissue by the following sampling: *H. seropedicae* RAM4 recovered from total root tissue (Total root Hs RAM4); *A. brasilense* FP2-7 recovered from total root tissue (Total root Ab FP2-7), internal *H. seropedicae* recovered from surface sterilized root (Endophyte Hs root), internal *H. seropedicae* recovered from sterilized leaf tissue (Endophyte Hs leaf). No uninoculated plants presented natural contamination with *H. seropedicae* or *A. brasilense*, althought some natural colonizer were recovered from total roots (Control Root) and leafs (Control Leaf). Values are mean ± SE of at least five replicates. The absence of data for some conditions are due to the number of cells below the minimun level of detection.

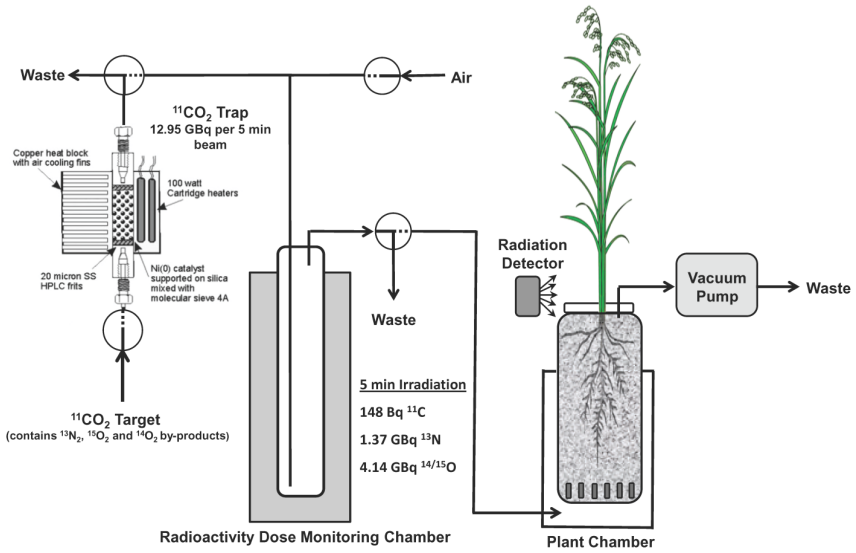


Figure S11: Schematic drawing of the BNL ^{13}NN plant pulsing station. This schematic depicts the setup by which doses of ^{13}NN were produced on the BNL cyclotron. ^{13}NN was generated from a gaseous target system that is designed to produce high levels of $^{11}CO_2$. ^{13}NN is a by-product of 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 $t_{½}$ 2 min, 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.

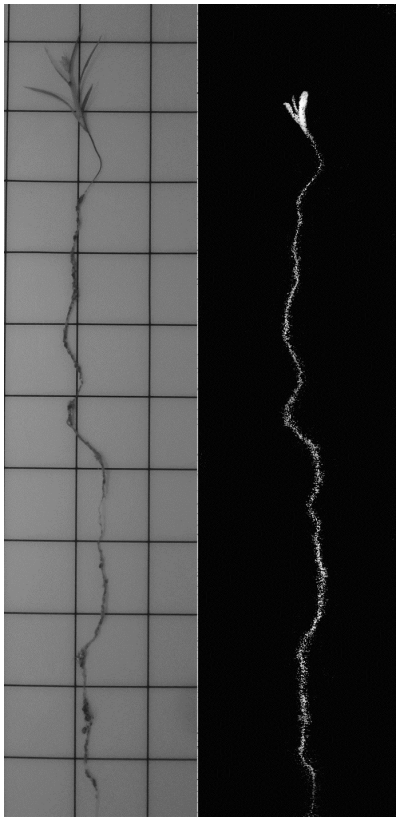


Figure S12: Photograph and autoradiography image of the plants. The photograph was taken after the roots were removed from the Turface soil column. Roots were washed using a phosphate buffered saline solution then laid out for radiographic imaging. It was not possible to disentangle the fine roots for this image. Images were acquired using GE Medical Systems Typhoon 7000 imager.

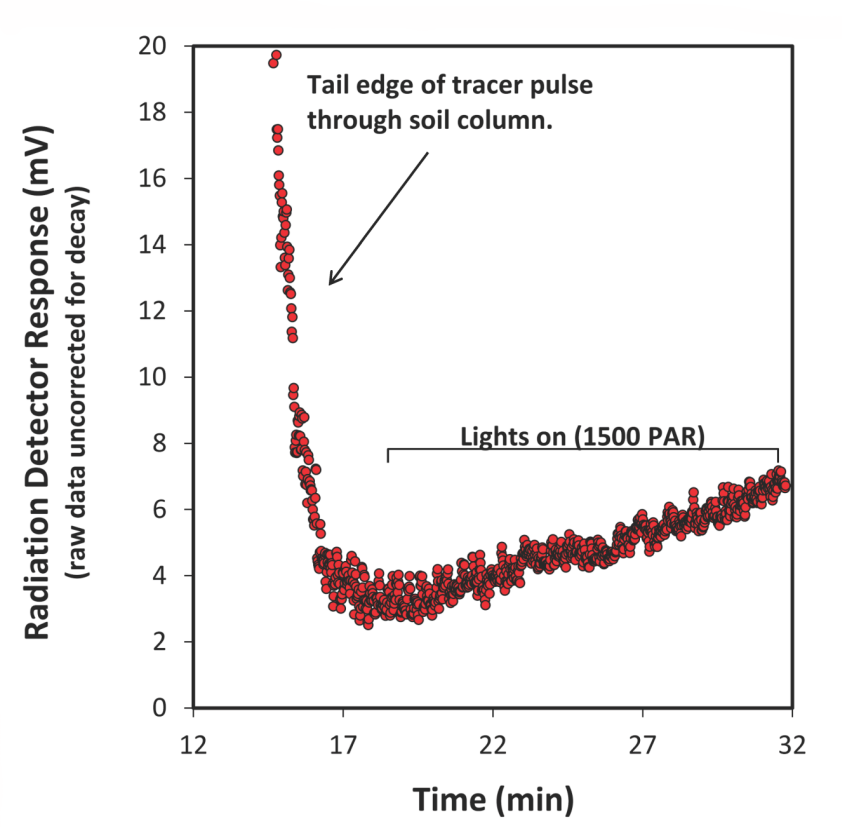


Figure S13: Time-activity trace showing transport of root activity aboveground. Data presented in this graph is the uncorrected millivolt response of the CsI (diode) radiation detector affixed to the upper aerial portions of the plant. The initial data shows the tail edge of the pulse. Shoot lights were 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.

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

		Root lenght (cm)		Lateral root number		Root fresh Weight (g)		Root dry weight (g)		Plant height (cm)		Seeds number		Inflorescence lenght (mm)		Shoot fresh weight (g)		Shoot dry weight (g)	
		Non-inoc	Inoc	Non-inoc	Inoc	Non-inoc	Inoc	Non-inoc	Inoc	Non-inoc	Inoc	Non-inoc	Inoc	Non-inoc	Inoc	Non-inoc	Inoc	Non-inoc	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
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
Waselkov RockFall	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

5 CONSIDERAÇÕES FINAIS

Durante este trabalho foram desenvolvidos estudos moleculares e fisiológicos para elucidar os mecanismos envolvidos na interação BPCP-gramíneas. O transcritoma de *H. seropedicae* aderido às raízes de trigo mostrou o panorama geral do perfil de transcritos expressos por esta bactéria durante a colonização de raízes de plântulas de *T. aestivum*. Enquanto que os estudos da interação entre *H. seropedicae* e *A. brasilense* com a planta *S. viridis* foram estabeleceu um novo modelo para estudar a interações benéficas entre planta e bactérias promotoras de crescimento.

Em *S. viridis* a resposta à inoculação variou de acordo com o genótipo da planta, uma vez que apenas 3 genótipos (EstepME034, EstepE017 e A10.1) mostraram uma resposta positiva à inoculação em quatro ou mais parâmetros de crescimento (Tabela S1 - Capitulo II). Dentre os parâmetros que mais chamaram atenção está o aumento radicular, uma vez que a raiz é o tecido vegetal em contato direto com o ambiente externo e com a microbiota em geral.

Para *S. viridis* genótipo A10.1 houve um aumento do comprimento total das raízes e do número de raízes laterais. Mas, além dos efeitos a nível radicular, as BPCP podem modificar a fisiologia e funcionamento de tecidos vegetais distantes do local de colonização, como nas folhas. No presente caso este efeito foi observado, pois juntamente com os benefícios causados às raízes, também a parte aérea da planta mostrou um aumento da massa seca e de comprimento e também houve aumento do número de sementes produzidas.

Transcritoma de *H. seropedicae* associado a raízes de trigo

O perfil transcricional de *H. seropedicae* aderido às raízes de trigo revelou uma extensa modificação no metabolismo da bactéria em resposta à interação com a planta. Já foi demostrado que os lipopolissacarídeos de parede celular (BALSANELLI *et al.*, 2010 e 2013) estão envolvidos nos processos iniciais de adesão de *H. seropedicae* às raízes de milho. Mas, o transcritoma de *H. seropedicae* aderido às raízes de trigo comparado com as células planctônicas não

apresentou expressão de genes envolvidos na síntese de lipopolissacarídeos. Foi observado, no entanto, a presença de genes de hemaglutininas/hemolisinas que possuem domínios proteicos de adesinas, e podem ser responsáveis pela adesão da bactéria em raízes de trigo. Nos transcritos expressos de *A. brasilense* aderido às raízes de trigo foram encontrados ativados 3 genes de proteínas de ligação à cálcio que possuem domínios de hemolisinas (CAMILIOS-NETO *et al.*, 2014); proteínas de ligação à cálcio também tem sido associados com o estabelecimento da colonização de *Rhizobium* na planta hospedeira (AMPE *et al.*, 2003).

Além disso, a expressão de genes da biossíntese de peptideoglicanos em *H. seropedicae* aderido à raiz sugere um rearranjo da parede celular bacteriana em resposta à adaptação para a interação com a planta.

Uma vez que a bactéria está aderida à superfície da raiz é plausível a repressão dos genes da maquinaria do flagelo, os quais são responsáveis pela motilidade em ambiente líquido. A baixa expressão de genes flagelares foi observada. No entanto, alguns genes relacionados com quimiotaxia foram ativados. A quimiotaxia é um processo de transmissão de sinal que guia o movimento de células móveis em direção a gradientes crescentes ou decrescentes de substâncias quimo-atrativas. Genes de três proteínas do tipo CheD aceptoras de grupo metil foram expressas na biblioteca de bactérias aderidas (Hsero_0394, Hsero_1251 e Hsero_2021). Além disso, a expressão da metiltranferase CheR (Hsero_0625) e CheB (Hsero_0626), que metila e desmetila, respectivamente, as proteínas aceptoras de metil tiveram os níveis de expressão aumentados 5,4 e 2,4 vezes em células aderidas. Em *P. aeruginosa* foi demonstrado que CheR1 não tem papel na motilidade dependente de flagelo, mas tem atividade essencial em processos iniciais de adesão à superfícies sólidas (SCHMIDT, J. *et al.*, 2011). Na bactéria *A. tumefaciens*, que coloniza o ambiente intracelular das plantas, a quimiotaxia é importante nos processos de adesão (MERRITT, DANHORN e FUQUA, 2007). Provavelmente a ativação do genes relacionados a quimiotaxia esteja envolvida com o processo de adesão de *H. seropedicae*.

É possível ainda, que o pili do tipo IV esteja envolvido nos processos de adesão de *H. seropedicae* à raiz. O sistema pili tipo IV é responsável também pela motilidade tipo *twitching*, que envolve a polimerização e despolimerização do pilus propulsionando a bactéria. Genes relacionados a este sistema, *pilT* e *pilI* encontram-

se ativados em *H. seropedicae* aderido à raiz de trigo.

Em relação aos mecanismos bacterianos que promovem o crescimento vegetal, os genes que codificam para as proteínas estruturais e regulatórias da nitrogenase estavam entre aqueles com maiores níveis de ativação. Resultados semelhantes de ativação da fixação de nitrogênio foram encontrados em *A. brasilense* colonizando raízes de trigo (CAMILIOS-NETO *et al.*, 2014). A bactéria *A. lipoferum* reconhece duas lecitinas vegetais (WGA-*wheat germ agglutinin* e DSA-*Datura stramonium agglutinin*) através do resíduos específicos de N-acetilglucosamina da superfície celular e esta ligação estimula a fixação de nitrogênio (KARPATI *et al.*, 1999). Alguns autores afirmam que esse comportamento é específico do gênero *Azospirillum*, já que o mesmo não é observado em *Azotobacter vinelandii* e *Klebsiella pneumoniae* (DROGUE *et al.*, 2014). Porém, já foi demonstrado que lecitinas de raiz de milho estão envolvidas na adesão de *H. seropedicae* via resíduos de N-acetilglucosaminoglicanos de LPS (BALSANELLI *et al.*, 2013). Portanto, é possível que de algum modo, o reconhecimento mutuo entre bactéria e planta influencie de forma específica a indução de genes diretamente relacionados com a interação planta-bactéria.

Ainda, foi observado que bactérias aderidas às raízes apresentam genes de diversos transportadores do tipo ABC diferencialmente regulados. Um total de 38 genes de transportadores regulados foram identificados. Dentre os ativados encontram-se vários *operons* incluindo sistemas de transporte de aminoácidos em geral e de cadeia ramificada, prolina/glicina e aspartato/glutamato. Além disso, estavam ativados transportadores de nitrato bem como o operon responsável pela assimilação e degradação de ureia. Em um estudo com *Pseudomonas putida*, foi observada a regulação destas mesmas classes de transportadores em uma condição limitante de nitrogênio, e mais ainda, foi demonstrado que estes genes são regulados por NtrC através da análise transcritômica do mutante *ntrC* de *P. putida* (HERVÁS, CANOSA e SANTERO, 2008). É importante para a resposta à limitação de nitrogênio a ativação de transportadores responsáveis pela assimilação de compostos contendo nitrogênio. Além disso, o transportador de amônio, codificado pelo gene *amtB,* está 9,3 vezes ativado nas bactéria aderidas, o que foi confirmado por RT-qPCR. Estes resultados indicam que as células aderidas estão mais limitadas em nitrogênio do que as células planctônicas.

Dentre os transportadores reprimidos, foram encontrados 3 transportadores de açucares, os quais podem ter como substrato maltose, sorbitol ou glicerol, segundo a análise feita no *transportDB* (www.membranetransport.org). Hervás e colaboradores (2008) encontraram transportadores de glicerol reprimidos em *P. putida* em condições limitantes de nitrogênio. A explicação dada para isso foi que o regulador NtrC contribui para a redução da entrada de carbono no ciclo dos ácidos tricarboxílicos quando a concentração de nitrogênio é baixa, sugerindo que NtrC pode controlar o catabolismo de carbono na bactéria. Para suportar essa hipótese, neste trabalho os autores encontraram dois genes da via de utilização de açúcares reprimidos: o gene *zwf-1* (glucose-6-fosfato desidrogenase) e o gene *gap-1* (gliceraldeido-3-fosfato desidrogenase).

Em *H. seropedicae* aderido ás raízes de trigo foram ativados transportadores de antibióticos e estavam reprimidos transportadores relacionados a secreção de toxinas e transportadores multidrogas. A inibição destes transportadores favorece o estabelecimento da bactéria, uma vez que não aciona o sistema de defesa do hospedeiro.

A produção de auxina e polifosfatos também é um possível mecanismo de promoção de crescimento vegetal utilizado por *H. seropedicae*. A biossíntese de auxina pela bactéria depende principalmente das vias dependentes de triptofano e a maior fonte de triptofano para as bactérias promotoras de crescimento vem do exsudato das raízes (SPAEPEN, VANDERLEYDEN e REMANS, 2007). É possível que *H. seropedicae* produza auxina por dois mecanismos diferentes, 1) pela conversão de indol-piruvato em ácido indol-acético através da enzima indol-piruvato oxidoredutase, codificada pelo gene Hsero_4278, que foi ativado 3 vezes mais na bactéria aderida do que na planctônica; ou 2) pela via de conversão de 3-indol-piruvato a indol-acetato pela enzima nitrilase codificada pelo gene Hsero_1422 que foi ativado 2,3 vezes nas células aderidas.

Outra via metabólica ativada em *H. seropedicae* durante a colonização foram as vias de biossíntese e degradação de polihidroxialcanoatos (PHA). A síntese de PHA é estimulada em condições onde há excesso de carbono ou privação de N, P e O (HERVÁS, CANOSA e SANTERO, 2008). Os dados do transcritoma sugerem que as bactérias aderidas as raízes de trigo estão utilizando ácidos orgânicos excretados pela planta para produção de PHA, sendo que as condições de baixo oxigênio e

nitrogênio em que a bactéria se encontra favorecem a ativação desta via. No entanto, genes que codificam para a PHA depolimerase que catalisa a despolimerização de PHA, foram ativados nessa condição. A biossíntese e degradação simultânea de polihidroxialcanoatos ocorre em outras bactérias, mas a razão desta regulação não é conhecida. Alguns autores sugerem que a síntese e degradação destes compostos é controlada pelas razões metabólicas de [acetil-CoA]/[CoA] e [NADH]/[NAD], os quais também controlam a via da β-oxidação (REN *et al.*, 2009).

As fotos de microscopia (Figura 5D, Capitulo I) comprovam que as bactérias aderidas estão produzindo grânulos de PHA. A produção de PHA é importante para o metabolismo adaptativo da bactéria em condições extremas, mas também para estabelecer interação com vegetais. Estirpe de *Azospirillum* capazes de produzir grandes quantidades de PHA são mais eficientes na promoção de crescimento de culturas de milho e trigo (DOBBELAERE, *et al.*, 2001). O estudos do metabolismo de polihidroxialcanoatos em *H. seropedicae* é promissor e parece ser importante para a interação planta-bactéria.

Os resultados da análise transcritômica devem direcionar futuros estudos sobre a interação *H. seropedicae*-trigo. Apesar de não ser possível atribuir funções específicas dos genes na adaptação metabólica da bactéria durante a interação com a planta, o conhecimento da expressão gênica diferencial permitirá o direcionamento das pesquisas. Durante a interação *H. seropedicae*-trigo foram regulados genes de transportadores do tipo ABC, fixação de nitrogênio, genes *fnr*, sistema pili do tipo IV, metabolismo de polihidroxialcanoatos, adesinas específicas, genes de quimiotaxia e da produção de fitohormônios. Todos são candidatos para estudos mais aprofundados revelados neste trabalho.

Setaria viridis: Um novo modelo para o estudo de interação diazotrofos-gramínea.

Apesar de estudos em sistemas biológicos diversos serem muito informativos, a adoção de sistemas modelos permite a padronização de protocolos e facilita a comparação de resultados levando a descobertas mais profundas acerca dos mecanismos moleculares do fenômeno estudado. Por exemplo, a adoção da planta *A. thaliana* impulsionou imensamente os estudos genéticos vegetais. O mesmo foi

alcançado com a adoção das plantas *Medicago truncatula* e *Lotus japonicus* nos estudos de nodulação em *Rhizobium.* Neste contexto, a planta *S. viridis* pode ser um novo modelo para os estudos de fixação biológica de nitrogênio em bactérias diazotróficas endofíticas. Neste trabalho foi demostrado que esta planta é capaz de responder positivamente à inoculação. A co-inoculação de *S. viridis* com *H. seropedicae* e *A. brasilense* estimulou um aumento significativo no comprimento e no número das raízes laterais, um efeito provavelmente devido a produção de fitohormônios. A produção de auxina por bactérias promotoras de crescimento está relacionada com o aumento da raiz ou do número de raízes laterais. A inoculação de mutantes de *Azospirillum* que tem a produção de auxina diminuída resultou em uma menor proliferação de raízes (DOBBELAERE *et al.*, 1999). Em *Pseudomonas putida* estirpe CR12-2 o aumento no comprimento da raiz também foi relacionado com o aumento da produção de auxina pela bactéria, durante a interação com plântulas de canola (XIE, PASTERNAK e GLICK, 1996).

Mais ainda, o trabalho em colaboração com o laboratório estrangeiro *Brookhaven National Laboratory* permitiu a utilização de experimentos modernos para a observação da contribuição da fixação biológica de nitrogênio na planta. Os dados sugerem que cerca de 7% do nitrogênio total da planta venha da fixação de nitrogênio das bactérias inoculadas, *A. brasilense* e *H. seropedicae*.

Desta forma, este trabalho propõe que além do uso já estabelecido de *S. viridis* nos estudos de fisiologia vegetal de gramíneas, esta planta seja utilizada como modelo para estudos de interação gramíneas C_4 - diazotróficas promotoras de crescimento de planta. As bactérias *H. seropedicae* e *A. brasilense* são modelos para interação endofítica e epifítica, respectivamente.

6 REFERENCIAS BIBLIOGRÁFICAS

AHMAD, F.; AHMAD, I.; KHAN, M. S. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. **Microbiol Res,** v. 163, n. 2, p. 173-81, 2008.

ALEXANDRE, G. Coupling metabolism and chemotaxis-dependent behaviours by energy taxis receptors. **Microbiology,** v. 156, n. Pt 8, p. 2283-93, Aug 2010.

ALEXANDRE, G.; GREER, S. E.; ZHULIN, I. B. Energy taxis is the dominant behavior in *Azospirillum brasilense*. **J Bacteriol,** v. 182, n. 21, p. 6042-8, Nov 2000.

AMPE, F. et al. Transcriptome analysis of *Sinorhizobium meliloti* during symbiosis. **Genome Biol,** v. 4, n. 2, p. R15, 2003.

BAIS, H. P. et al. The role of root exudates in rhizosphere interactions with plants and other organisms. **Annu Rev Plant Biol,** v. 57, p. 233-66, 2006.

BALDANI, J. I.; BALDANI, V. L. History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience. **An Acad Bras Cienc,** v. 77, n. 3, p. 549-79, Sep 2005.

BALDANI, J. I. et al. Characterization of *Herbaspirillum seropedicae*, a root-associated nitrogen fixing bacterium. **Intl. *J. Sys. Bact.*,** v. 36, p. 86-93, 1986.

BALDANI, V. L. D. et al. Identification and ecology of *Herbaspirillum seropedicae* and the closely related *Pseudomonas rubrisubalbicans*. ***Symbiosis*,** v. 19, p. 65–73, 1992.

BALDANI, V.L.D.; JAMES, E.K.; BALDANI, J.I.; DÖBEREINER, J.. Colonization of rice by the nitrogen-fixing bacteria *Herbaspirillum spp*. and *Azospirillum brasilense*. ***International Congress on Nitrogen Fixation*.** 9**:** 860 p. 1992.

BALSANELLI, E. et al. *Herbaspirillum seropedicae rfbB* and *rfbC* genes are required for maize colonization. **Environ Microbiol**, Mar 2010.

BALSANELLI, E. et al. Maize root lectins mediate the interaction with *Herbaspirillum seropedicae* via N-acetyl glucosamine residues of lipopolysaccharides. **PLoS One,** v. 8, n. 10, p. e77001, 2013.

BASHAN, Y.; DE-BASHAN, L. Fresh-weight measurements of roots provide inaccurate estimates of the effects of plant growth-promoting bacteria on root growth: a critical examination. **Soil Biology & Biochemistry,** v. 37, n. 10, p. 1795-1804, OCT 2005.

BASHAN, Y.; DE-BASHAN, L. E. How the plant growth-promoting bacterium

Azospirillum promotes plant growth – a critical assessment. **Adv Agron** v. 108 p. 77–136 2010.

BASHAN, Y.; HOLGUIN, G.; DE-BASHAN, L. E. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). **Canadian Journal of Microbiology,** v. 50, n. 8, p. 521-577, 2004/08/01 2004.

BENNETZEN, J. L. et al. Reference genome sequence of the model plant Setaria. **Nat Biotechnol,** v. 30, n. 6, june 2012

BERG, H. C. Chemotaxis in bacteria. ***Annu Rev Biophys Bioeng***, p. 119-136, 1975.

BLADERGROEN, M. R.; BADELT, K.; SPAINK, H. P. Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. **Mol Plant Microbe Interact,** v. 16, n. 1, p. 53-64, Jan 2003.

BLENCOWE, B. J.; AHMAD, S.; LEE, L. J. Current-generation high-throughput sequencing: deepening insights into mammalian transcriptomes. **Genes Dev,** v. 23, n. 12, p. 1379-86, Jun 2009.

BLOOM, A. J.; JACKSON, L. E.; SMART, D. R. Root growth as a function of rhizosphere ammonium and nitrate. . **Plant Cell Environ,** v. 16, p. 199-206, 1993.

BODDEY, R. M.; VICTORIA, R. L. Estimation of biological nitrogen fixation associated with *Brachiaria* and *Paspalum grasses* using15N labelled organic matter and fertilizer. **Plant and Soil,** v. 90, n. 1-3, p. 265-292, 1986/02/01 1986.

BOLWELL, G.; WOJTASZEK, P. Mechanisms for the generation of reactive oxygen species in plant defence – a broad perspective. **Physiol Molec Plant Pathol,** v. 51, n. 6, p. 347-366 1997

BOYER, F. et al. Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? **BMC Genomics,** v. 10, p. 104, 2009.

BRADER, G. et al. Metabolic potential of endophytic bacteria. **Curr Opin Biotechnol,** v. 27, p. 30-7, Jun 2014.

BRENCIC, A.; WINANS, S. C. Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. **Microbiol Mol Biol Rev,** v. 69, n. 1, p. 155-94, Mar 2005.

BRUTNELL, T. P. et al. *Setaria viridis*: a model for C4 photosynthesis. **The Plant cell,** v. 22, n. 8, p. 2537-44, Aug 2010.

BULGARELLI, D. et al. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. **Nature,** v. 488, n. 7409, p. 91-5, Aug 2012.

BURDMAN, S. et al. 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, n. 4, p. 555-61, Apr 2001.

BÖHM, M.; HUREK, T.; REINHOLD-HUREK, B. Twitching motility is essential for endophytic rice colonization by the N2-fixing endophyte *Azoarcus* sp. strain BH72. **Mol Plant Microbe Interact,** v. 20, n. 5, p. 526-33, May 2007.

CAMILIOS-NETO, D. et al. Dual RNA-seq transcriptional analysis of wheat roots colonized by *Azospirillum brasilense* reveals up-regulation of nutrient acquisition and cell cycle genes. **BMC Genomics,** v. 15, p. 378, 2014.

CANNON, S. B. et al. Legume genome evolution viewed through the *Medicago truncatula* and *Lotus japonicus* genomes. **Proceedings of the National Academy of Sciences,** v. 103, n. 40, p. 14959-14964, October 3, 2006

CATALAN, A. L. et al. Production of polyhydroxyalkanoates by *Herbaspirillum seropedicae* grown with different sole carbon sources and on lactose when engineered to express the l*acZlacY* genes. **Enzyme Microb Tech** v. 40 p. 1352–1357 2007

CHENG, Z.; PARK, E. e GLICK, B.R. 1-Aminocyclopropane-1-carboxylate deaminase from *Pseudomonas putida* UW4 facilitates the growth of canola in the presence of salt. **Can J Microbiol.** v. 53, n.7, p. 912-8, 2007.

CHOUDHARY, D. K.; JOHRI, B. N. Interactions of *Bacillus* spp. and plants with special reference to induced systemic resistance (ISR). **Microbiol Res,** v. 164, n. 5, p. 493-513, 2009.

CHUBATSU, L. S. et al. Nitrogen fixation control in *Herbaspirillum seropedicae*. **Plant Soil**, 2011.

CROES, C. L. et al. The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. **J. Gen. Microbiol.,** v. 139, p. 2261-2269, 1993.

DA SILVA, L. C.; MIGUENS, F. C.; OLIVARES, F. L. *Herbaspirillum seropedicae* and sugarcane endophytic interaction investigated by using high pressure freezing electron microscopy. **Braz J Microbiol,** v. 34(1), p. 69-71, 2003.

DIXON, R.; KAHN, D. Genetic regulation of biological nitrogen fixation. **Nat Rev Microbiol,** v. 2, n. 8, p. 621-31, Aug 2004.

DOBBELAERE, S. et al. Responses of agronomically important crops to inoculation with *Azospirillum*. **Functional Plant Biology,** v. 28, n. 9, p. 871-879, 2001.

DOBBELAERE, S. et al. Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. . **Plant and Soil,** v. 212, p. 155–164, 1999.

DROGUE, B. et al. Which specificity in cooperation between phytostimulating rhizobacteria and plants? **Res Microbiol,** v. 163, n. 8, p. 500-10, 2012 Sep-Oct, 2012.

DROGUE, B. et al. Genome wide profiling of *Azospirillum lipoferum* 4B gene expression during interaction with rice roots**. FEMS Microbiology Ecology**. v. 2, 87**:** p. 543-555, 2014.

DÖBEREINER, J.; PEDROSA, F. O. Nitrogen-fixing bacteria in nonleguminous crop plants. ***Science Tech publishers*/Springer-Verlag**, 1987.

DÖRR, J.; HUREK, T.; REINHOLD-HUREK, B. Type IV pili are involved in plant-microbe and fungus-microbe interactions. **Mol Microbiol,** v. 30, n. 1, p. 7-17, Oct 1998.

ECKERT, B. et al. *Azospirillum doebereinerae sp*. nov., a nitrogen-fixing bacterium associated with the C_4-grass *Miscanthus*. **International Journal of Systematic & Evolutionary Microbiology,** v. 51, n. Pt 1, p. 17-26, 2001.

FIBACH-PALDI, S.; BURDMAN, S.; OKON, Y. Key physiological properties contributing to rhizosphere adaptation and plant growth promotion abilities of *Azospirillum brasilense*. **FEMS Microbiol Lett,** v. 326, n. 2, p. 99-108, Jan 2012.

FUENTES-RAMIREZ, L. E.; CABALLERO-MELLADO, J. Bacterial biofertilizers. PGPR: Biological Control and Biofertilization. In: SADIQUI, Z. A. (Ed.). Springer-Verlag, Dordrecht p.143–172, 2005

GARCIA DE SALAMONE, I. E. et al. Biological nitrogen fixation in *Azospirillum* strain-maize genotype associations as evaluated by the 15N isotope dilution technique. **Biol Fertil Soils** v. 23, p. 249–256., 1996.

GIANNOUKOS, G. et al. Efficient and robust RNA-seq process for cultured bacteria and complex community transcriptomes. **Genome Biol,** v. 13, n. 3, p. R23, 2012.

GLICK, B.R. *et al.* Promotion of plant growth by ACC deaminase-producing soil bacteria. **European Journal of Plant Pathology**, v. 119, p. 329–339, 2007.

HAAS, B. J. et al. How deep is deep enough for RNA-Seq profiling of bacterial transcriptomes? **BMC Genomics,** v. 13, p. 734, 2012.

HAMEEDA, B. et al. Growth promotion of maize by phosphate solubilizing bacteria isolated from compost and microfauna. . **Micro- biol Res** v. 163, p. 234-242, 2008.

HAZELBAUER, G. L.; FALKE, J. J.; PARKINSON, J. S. Bacterial chemoreceptors: high-performance signaling in networked arrays. **Trends Biochem Sci,** v. 33, n. 1, p. 9-19, Jan 2008.

HELMAN, Y.; BURDMAN, S.; OKON, Y. Plant growth promotion by rhizosphere

bacteria through direct effects. In: ROSENBERG, E. e GOPHNA, U. (Ed.). **Beneficial Microorganisms in Multicellular Life Forms**. Berlin Springer- Verlag, p.89–103. 2011.

HERVÁS, A. B.; CANOSA, I.; SANTERO, E. Transcriptome analysis of *Pseudomonas putida* in response to nitrogen availability. **J Bacteriol,** v. 190, n. 1, p. 416-20, Jan 2008.

HIRSCH, P. R.; MAUCHLINE, T. H. Who's who in the plant root microbiome? **Nat Biotechnol,** v. 30, n. 10, p. 961-2, Oct 2012.

HORI, K.; MATSUMOTO, S. Bacterial adhesion: from mechanism to control. **Biochem. Engin. J.,** v. 48 p. 424-434, 2010

HUNGRIA, M. et al. Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. **Plant and Soil,** v. 331, p. 413-425, 2010.

HUREK, T. et al. 16S rRNA-targeted polymerase chain reaction and oligonucleotide hybridization to screen for *Azoarcus* spp., grass-associated diazotrophs. **Appl Environ Microbiol,** v. 59, n. 11, p. 3816-24, Nov 1993.

JAMES, E. K. et al. Infection And Colonization Of Rice Seedlings By The Plant Growth-Promoting Bacterium *Herbaspirillum Seropedicae* Z67. **Mol Plant Microbe Interact,** v. 15, n. 9, p. 894-906, Sep 2002.

JAMES, E. K.; OLIVARES, F. B. L. Infection and Colonization of Sugarcane and Other Graminaceous Plants by Endophytic Diazotrophs. **Critical Reviews in Plant Sciences,** v. 17, n. 1, p. 77 - 119, 1998.

JAMES, E. K. et al. *Herbaspirillum*, an endophytic diazotroph colonizing vascular tissue in leaves of *Sorghum bicolor* L. Moench. . **J. Exp. Bot.,** v. 48, p. 785-797, 1997.

JAMES, E. K. et al. Infection of sugarcane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. **J. Exp. Bot.,** v. 45, p. 757-766, 1994.

JIANG, H.; BARBIER, H.; BRUTNELL, T. Methods for performing crosses in *Setaria viridis*, a new model system for the grasses. **J Vis Exp**, n. 80, 2013.

JIANG, Q.Y. AND GRESSHOFF, P.M. Classical and molecular genetics of the model legume *Lotus japonicus*. **Mol.Plant Microbe.Interaction**., 10, 59, 1997

KADOURI, D. et al. Identification and isolation of genes involved in poly(beta-hydroxybutyrate) biosynthesis in *Azospirillum brasilense* and characterization of a *phbC* mutant. **Appl Environ Microbiol,** v. 68, n. 6, p. 2943-9, Jun 2002.

KADOURI, D.; JURKEVITCH, E.; OKON, Y. Involvement of the reserve material poly-b-hydroxybutyrate in *Azospirillum brasilense* stress endurance and root

colonization. **Appl Environ Microbiol** v. 69, p. 3244–3250 2003.

KARPATI, E. et al. Interaction of *Azospirillum lipoferum* with wheat germ agglutinin stimulates nitrogen fixation. **J Bacteriol,** v. 181, n. 13, p. 3949-55, Jul 1999.

KLOEPPER, J. W.; SCHROTH, M. N. Plant growth-promoting rhizobacteria on radishes. In: PHYTO-BACTERIOLOGIE, S. D. P. V. E., **Proceedings of the IVth International Conference on Plant Pathogenic Bacteria** p.879-882, 1978.

KUMAR, R. et al. Establishment of *Azotobacter* on plant roots: chemotactic response, development and analysis of root exudates of cotton (*Gossypium hirsutum L.*) and wheat (*Triticum aestivum L.*). **J Basic Microbiol,** v. 47, n. 5, p. 436-9, Oct 2007.

LERNER, A. et al. The *Azospirillum brasilense* Sp7 *noeJ* and *noeL* genes are involved in extracellular polysaccharide biosynthesis. **Microbiology,** v. 155, n. Pt 12, p. 4058-68, Dec 2009.

LOPEZ-DE-VICTORIA, G.; LOVELL, C. R. Chemotaxis of *Azospirillum* species to aromatic compounds. **Appl Environ Microbiol,** v. 59, n. 9, p. 2951-5, Sep 1993.

LUNDBERG, D. S. et al. Defining the core *Arabidopsis thaliana* root microbiome. **Nature,** v. 488, n. 7409, p. 86-90, Aug 2012.

MADISON, L. L.; HUISMAN, G. W. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. **Microbiol Mol Biol Rev,** v. 63, n. 1, p. 21-53, Mar 1999.

CRUZ, L. M. et al. 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa spp*.) and pineapple (*Ananas comosus* (L.) Merril). **Appl Environ Microbiol,** v. 67, n. 5, p. 2375-9, May 2001.

MANTELIN, S.; TOURAINE, B. Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake. **J Exp Bot,** v. 55, n. 394, p. 27-34, Jan 2004.

MARK, G. L. et al. Transcriptome profiling of bacterial responses to root exudates identifies genes involved in microbe-plant interactions. **Proc Natl Acad Sci U S A,** v. 102, n. 48, p. 17454-9, Nov 2005.

MATTICK, J. S. Type IV pili and twitching motility. **Annu Rev Microbiol,** v. 56, p. 289-314, 2002.

MEHNAZ, S.; LAZAROVITS, G. Inoculation effects of *Pseudomonas putida*, *Gluconacetobacter azotocaptans*, and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. **Microb Ecol** v. 51 p. 326–335., 2006.

MERRITT, P. M.; DANHORN, T.; FUQUA, C. Motility and chemotaxis in *Agrobacterium tumefaciens* surface attachment and biofilm formation. **J Bacteriol,** v.

189, n. 22, p. 8005-14, Nov 2007.

MITTER, B. et al. Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. **Front Plant Sci,** v. 4, p. 120, 2013.

MOLINA-FAVERO, C. et al. Aerobic nitric oxide production by *Azospirillum brasilense* Sp245 and its influence on root architecture in tomato. **Mol Plant Microbe Interact,** v. 21, n. 7, p. 1001-9, Jul 2008.

MULETA, D.; ASSEFA, F.; GRANHALL, U. In vitro Antagonism of Rhizobacteria Isolated from *Coffea arabica* L. against Emerging Fungal Coffee Pathogens **Engineering in Life Sciences** v. 7, n. 6. 577-586 p. 2007.

OKON, Y. *Azospirillum* as a potential inoculant for agriculture. **Trends Biotechnol** v. 3 p. 223–228 1985

OKON, Y.; LABANDERA-GONZALEZ, C. A. Agronomic applications of *Azospirillum*: An evaluation of 20 years worldwide field inoculation. **Soil Biology and Biochemistry,** v. 26, n. 12, p. 1591-1601, 1994.

OLIVARES, F. L. et al. Occurrence of the endophytic diazotrophs *Herbaspirillum spp*. in roots, stems and leaves predominantly of Gramineae. ***Fertil. Soils*****,** v. *21*, p. 197-200, 1996.

PATEL, D. K. et al. Repression of mineral phosphate solubilizing phenotype in the presence of weak organic acids in plant growth promoting fluorescent *Pseudomonads*. **Bioresour Technol,** v. 102, n. 3, p. 3055-61, Feb 2011.

PEDRAZA, R. O. et al. *Azospirillum* inoculation and nitrogen fertilization effect on grain yield and on the diversity of endophytic bacteria in the phyllosphere of rice rainfed crop. **European Journal of Soil Biology,** v. 45, n. 1, p. 36-43, 2009.

PEDROSA, F. O. et al. Genome of *Herbaspirillum seropedicae* Strain SmR1, a Specialized Diazotrophic Endophyte of Tropical Grasses. **PLoS Genet,** v. 7, n. 5, p. e1002064, May 2011.

POSTGATE, J. R.; CANNON, F. C. The molecular and genetics manipulation of nitrogen-fixation. **Philos Trans R Soc Lond B Biol Sci,** v. 292 p. 589-599, 1981

PUKATZKI, S.; MCAULEY, S. B.; MIYATA, S. T. The type VI secretion system: translocation of effectors and effector-domains. **Curr Opin Microbiol,** v. 12, n. 1, p. 11-7, Feb 2009.

PÉREZ-MONTAÑO, F. et al. Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. **Microbiol Res,** v. 169, n. 5-6, p. 325-36, May-Jun 2014.

RAMOS, M. G. et al. Quantification of the contribution of biological nitrogen fixation

to tropical green manure crops and the residual benefit to a subsequent maize crop using 15N-isotope techniques. **J Biotechnol,** v. 91, n. 2-3, p. 105-15, Oct 2001.

REES, D. C.; HOWARD, J. B. Nitrogenase: standing at the crossroads. **Curr Opin Chem Biol,** v. 4, n. 5, p. 559-66, Oct 2000.

REINHOLD, B.; HUREK, T.; FENDRIK, I. Strain-specific chemotaxis of *Azospirillum* spp. **J Bacteriol,** v. 162, n. 1, p. 190-5, Apr 1985.

REINHOLD-HUREK, B.; HUREK, T. Life in grasses: diazotrophic endophytes. **Trends Microbiol,** v. 6, n. 4, p. 139-144, 1998.
REINHOLD-HUREK B, HUREK T. Living inside plants: bacterial endophytes. Curr Opin Plant Biol, v.14 p.1-9 , 2011

REN, Q. et al. Simultaneous accumulation and degradation of polyhydroxyalkanoates: futile cycle or clever regulation? **Biomacromolecules,** v. 10, n. 4, p. 916-22, Apr 2009.

RICHARDSON, A. et al. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. **Plant and Soil,** v. 321, n. 1, p. 305-339, 2009.

RODRÍGUEZ, H.; FRAGA, R. Phosphate solubilizing bacteria and their role in plant growth promotion. **Biotechnol Adv,** v. 17, n. 4-5, p. 319-39, Oct 1999.

RODRÍGUEZ-NAVARRO, D. N.; DARDANELLI, M. S.; RUÍZ-SAÍNZ, J. E. Attachment of bacteria to the roots of higher plants. **FEMS Microbiol Lett,** v. 272, n. 2, p. 127-36, Jul 2007.

RONCATO-MACCARI, L. D. et al. Endophytic *Herbaspirillum seropedicae* expresses *nif* genes in gramineous plants. **FEMS Microbiol Ecol,** v. 45, n. 1, p. 39-47, ISSN 1574-6941. Jul 2003.

SCHMIDT, J. et al. The *Pseudomonas aeruginosa* chemotaxis methyltransferase CheR1 impacts on bacterial surface sampling. **PLoS One,** v. 6, n. 3, p. e18184, 2011.

SCHMIDT, M. A. et al. The type III secretion system is necessary for the development of a pathogenic and endophytic interaction between *Herbaspirillum rubrisubalbicans* and Poaceae. **BMC Microbiol,** v. 12, p. 98, 2012.

SHIDORE, T. et al. Transcriptomic analysis of responses to exudates reveal genes required for rhizosphere competence of the endophyte *Azoarcus* sp. strain BH72. **Environ Microbiol,** v. 14, n. 10, p. 2775-87, Oct 2012.

SPAEPEN, S.; VANDERLEYDEN, J.; OKON, Y. Plant growth- promoting actions of rhizobacteria. . **Adv Bot Res,** v. 51, p. 283–320, 2009

SPAEPEN, S.; VANDERLEYDEN, J.; REMANS, R. Indole-3-acetic acid in microbial

and microorganism-plant signaling. **FEMS Microbiol Rev,** v. 31, n. 4, p. 425-48, Jul 2007.

SPAEPEN, S. et al. Characterization of phenylpyruvate decarboxylase, involved in auxin production of *Azospirillum brasilense*. **J Bacteriol,** v. 189, n. 21, p. 7626-33, Nov 2007.

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, n. 4, p. 487-506, Oct 2000.

STEIN, T.; HAYEN-SCHNEG, N.; FENDRIK, I. Contribution of BNF by *Azoarcus* sp. BH72 in *Sorghum vulgare*. **Soil Biol Biochem,** v. 29, p. 969–971., 1997.

SUDHAKAR, P. et al. Effect of foliar application of *Azotobacter*, *Azospirillum* and *Beijerinckia* on leaf yield and quality of mulberry (*Morus alba*). **J Agric Sci** v. 134, p. 227-234, 2000.

SUN, Y.; CHENG, Z.; GLICK, B. The presence of a 1-aminocyclopropane-1-carboxylate (ACC) deaminase deletion mutation alters the physiology of the endophytic plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN. **Fems Microbiology Letters,** v. 296, n. 1, p. 131-136, JUL 2009.

TAGHAVI, S. et al. Genome sequence of the plant growth promoting endophytic bacterium *Enterobacter* sp. 638. **PLoS Genet,** v. 6, n. 5, p. e1000943, May 2010.

TJEPKEMA, J. D.; BURRIS, R. H. Nitrogenase activity associated with some Wisconsin prairie grasses. **Plant and Soil,** v. 45, n. 1, p. 81-94, 1976.

TORTO-ALALIBO, T. et al. Common and contrasting themes in host cell-targeted effectors from bacterial, fungal, oomycete and nematode plant symbionts described using the Gene Ontology. **BMC Microbiol,** v. 9 Suppl 1, p. S3, 2009.

TSENG, T. T.; TYLER, B. M.; SETUBAL, J. C. Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. **BMC Microbiol,** v. 9 Suppl 1, p. S2, 2009.

VON CAEMMERER, S.; QUICK, W. P.; FURBANK, R. T. The development of C_4 rice: current progress and future challenges. **Science,** v. 336, n. 6089, p. 1671-2, Jun 2012.

VRANOVA, V. et al. Dominant amino acids, organic acids and sugars in water-soluble root exudates of C_4 plants: a mini-review. **Acta Univ. Agric. Silvic. Mendel. Brun.**, p. 441-446, 2010

WALKER, V. et al. Host plant secondary metabolite profiling shows a complex, strain-dependent response of maize to plant growth-promoting rhizobacteria of the genus *Azospirillum*. **New Phytologist,** 189**:** p. 494-50, 2011.

WANG, X. et al. Transcriptome-wide identification of novel imprinted genes in neonatal mouse brain. **PLoS One,** v. 3, n. 12, p. e3839, 2008.

WESTERMANN, A. J.; GORSKI, S. A.; VOGEL, J. Dual RNA-seq of pathogen and host. **Nat Rev Microbiol,** v. 10, n. 9, p. 618-30, Sep 2012.

WITHOLT, B.; KESSLER, B. Perspectives of medium chain length poly(hydroxyalkanoates), a versatile set of bacterial bioplastics. **Current opinion in Biotechnology,** v. 10, n. 3, p. 279-285, 1999.

WU, H. Y. et al. Secretome analysis uncovers an Hcp-family protein secreted via a type VI secretion system in *Agrobacterium tumefaciens*. **J Bacteriol,** v. 190, p. 2841-2850, 2008.

XIE, H.; PASTERNAK, J. J.; GLICK, B. R. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* CR12-2 that overproduce indoleacetic acid. **Curr Microbiol** v. 32, p. 67–71, 1996.