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Isolamento e caracterização de bactérias promotoras de crescimento vegetal de lavouras experimentais de arroz sob diferentes níveis de fertilização

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Resumo

A fertilização química é amplamente utilizada para aumento da produtividade de plantações, mas sua produção e uso levaram a severos danos ambientais. Para reduzir o uso de fertilizantes, pode-se fazer uso de bactérias promotoras de crescimento vegetal (*Plant Growth Promoting Rhizobacteria*, PGPR), que são bactérias associadas a plantas e que melhoram a sua saúde, tamanho e produtividade através de vários mecanismos. A eficiência das PGPR sabidamente flutua com as condições ambientais; porém, há poucos estudos abordando os efeitos da fertilização química nas características de promoção de crescimento dessas bactérias. Neste trabalho foram analisados os efeitos da fertilização a longo prazo na diversidade de 190 linhagens bacterianas isoladas do solo rizosférico e de raízes de arroz, além da ocorrência e níveis de expressão de algumas características de promoção de crescimento vegetal. Os resultados obtidos demonstraram que a fertilização tem pequeno efeito na diversidade bacteriana, mas um grande efeito nas habilidades de solubilização de fosfato e produção de compostos indólicos. Propõe-se que, em condições de ausência de nutrientes, as plantas selecionam bactérias que apresentem boa capacidade de solubilização de fosfato para uma íntima associação com suas raízes, ao invés de bactérias que sejam boas produtoras de compostos indólicos. Quando em condições de disponibilidade moderada de nutrientes as plantas selecionam bactérias que sejam boas produtoras de compostos indólicos, ao invés de boas solubilizadoras de fosfato. Em condições de abundância de nutrientes essa preferência seletiva parece estar desativada. Após sete linhagens bacterianas terem sido testadas para promoção de crescimento vegetal *in vivo* de arroz em casa de vegetação, as previsões descritas acima foram avaliadas em um experimento a campo. De fato observou-se que bactérias eficientes em solubilizar fosfato promoveram o crescimento das plantas apenas em condições limitadas de nutrientes e que bactérias produtoras de compostos indólicos promoveram o crescimento vegetal apenas em condições de disponibilidade moderada de nutrientes. Quando a disponibilidade de nutrientes foi abundante, a solubilização de fosfato e a produção de compostos indólicos não foram fatores chave para promover o crescimento vegetal. Essas observações podem ser utilizadas para uma prospecção direcionada de PGPRs e seleção antecipada de candidatas à promoção de crescimento vegetal, de acordo com as necessidades da planta e os interesses dos agricultores.

Abstract

Chemical fertilization is widely used for increased crop productivity, but its production and use lead to serious environmental damage. To reduce the use of fertilizers, one can make use of plant growth promoting rhizobacteria (PGPR), which are plant-associated bacteria that increase plant health, size and yield through various mechanisms. PGPR effectiveness is known to fluctuate with environmental conditions; however, studies regarding the effect of chemical fertilization on plant growth promoting (PGP) traits of PGPR are scarce. In this work, the effects of long-term fertilization on the diazotrophic diversity, occurrence and expression levels of PGP traits from 190 bacterial strains isolated from rhizospheric soil and roots of rice were analyzed. We found that fertilization had a limited effect on diversity but had a major effect on phosphate solubilization and indolic compounds (IC) production abilities. We propose that plants select bacteria that present good phosphate solubilization ability for intimate root association in lieu of good IC production under nutrient-poor conditions and select good IC producers in lieu of good phosphate solubilizers under nutrient-moderate conditions. In nutrient-rich conditions, this selection preference seems to be deactivated. After testing seven selected isolates for effective *in vivo* plant growth promotion in greenhouse conditions, our predictions were tested in the field. We found that good phosphate solubilizers only promoted growth at nutrient-poor conditions and that good IC producers only promoted growth at nutrient-moderate conditions. In nutrient-rich conditions, phosphate solubilization and IC production were not key factors to promote plant growth. These findings may be used for directed PGPR prospection and anticipated PGPR candidate selection, according to plant needs and farmer interests.

Sumário

1. Introdução Geral

1.1. Questões ambientais no uso e produção de fertilizantes

O modelo de produção de alimentos em lavouras comerciais vai mudar em médio a longo prazo. Essa mudança se dará devido à falta de sustentabilidade econômica, energética e ambiental do atual modelo, levando a uma grave crise mundial na produção de alimentos, ou devido a tecnologias que permitam maior eficiência e menor custo na produção destes. Este trabalho visa explorar uma das tecnologias que podem facilitar a produção de alimentos.

A insustentabilidade do sistema atual de produção de alimentos ocorre devido ao uso abusivo de fertilizantes químicos para incrementar a produção agrícola. Nitrogênio (N), fósforo (P) e potássio (K) são nutrientes limitantes no solo para o desenvolvimento vegetal: qunado há escassez de um desses nutrientes, a planta não consegue desenvolver ao máximo o seu potencial de produção. Utilizando fertilizantes químicos com esses elementos (NPK) ocorre um salto na produção agrícola, como foi observado nos séculos XIX e XX (Evenson & Gollin, 2003).

Em 1850 a humanidade já enfrentava uma crise na produção de alimentos para alimentar 1,5 bilhão de pessoas. A fertilidade dos campos cultivados foi caindo à medida que os nutrientes eram recolhidos das lavouras mas não eram suficientemente repostos com as práticas agrícolas daquele tempo, como adição de matéria orgânica nas plantações (Dawson & Hilton 2011). Era a escassez do P que estava limitando o desenvolvimento vegetal. Sem a tecnologia da fertilização por fosfato rochoso (fonte de P), descoberta em 1840, os países industrializados não conseguiriam alimentar sua população. Com o P abundante via fertilizantes fosfatados, o N passou a ser o próximo nutriente limitante. A tecnologia para a produção sintética de sulfato de amônio (fonte de N) a partir do nitrogênio atmosférico surgiu em 1909 (Dawson & Hilton 2011). O processo conhecido como Haber-Bosch para produção de N reativo, a ser implementado na agricultura (e também na produção de munição balística na 1º Guerra Mundial, que estava prestes a eclodir), levou a um premio Nobel em 1918 e aumentou enormemente a produção agrícola no decorrer dos anos (Dawson & Hilton 2011). Não existiam (e não existem) outras fontes viáveis de nitrogênio reativo: ele não pode ser minerado, extraído ou cultivado para, então, ser aplicado na agricultura. A sua única fonte é a síntese química. Políticas de incentivo ao aumento da produção agrícola foram implementadas

após a fome experimentada pela Europa na 2º Guerra Mundial, incentivando o uso e produção de fertilizantes. Nos anos 60, novas variedades de arroz e trigo, desenvolvidas por institutos internacionais de pesquisa, apresentavam maior produtividade e melhor resposta aos fertilizantes químicos. Essas variedades foram rapidamente adotadas, principalmente em países em desenvolvimento, por sua lucratividade (Evenson & Gollin, 2003). Por ser comercialmente favorável, a produtividade extra provida pelo fertilizante se espalhou com força em todo o mundo com o passar dos anos. O custo dessa produtividade extra, porém, foi muito maior do que o preço do fertilizante.

Grande parte dos nutrientes que são aplicados nas lavouras através do uso de fertilizantes não são absorvidos pelas plantas. Dependendo de algumas características dos solos, 60 a 90% dos nutrientes adicionados à lavoura acabam sendo desperdiçados (Adesemoye & Kloepper, 2009b). Estes nutrientes acabam por poluir o ambiente ao serem lixiviados para corpos d'agua ou volatizam para a atmosfera. Nos corpos d'água, assim como nos sistemas terrestres, N e P são nutrientes limitantes que impedem que o desenvolvimento vegetal atinja o máximo de produtividade. Quando N e P provenientes dos fertilizantes escoam para a água, eles são utilizados por cianobactérias e algas que crescem descontroladamente, configurando uma situação de eutrofização de corpos hídricos. A eutrofização tem diversos aspectos negativos no contexto econômico, ambiental e de saúde pública. Entre os danos gerados pela eutrofização estão a hipóxia (falta de oxigênio na água) que leva à mortandade de peixes, alterações na rede trófica, perda de diversidade, bloqueio da luz solar para além da superfície do corpo dágua, proliferação de algas tóxicas, entupimento de redes de captação de água, alterações na ciclagem de nutrientes, alterações na qualidade de água para consumo humano, atrapalham o tráfego náutico e outros (Carpenter et al., 1998; Lau & Lane, 2002). Estes danos ocorrem em rios, lagos e áreas costeiras de todo o mundo, criando "zonas mortas" de diversidade em importantes recursos hídricos (Good & Beatty, 2011; Adesemoye & Kloepper, 2009b)

Os derivados de N também podem volatilizar para a atmosfera na forma de gases de efeito estufa, através da nitrificação e denitrificação promovida por micro-organismos, gerando grandes danos em escala global. Os gases volatilizados podem, também, retornar ao solo, incorporando nutrientes inadvertidamente em áreas naturais, o que leva a taxas de crescimentos anormais, desequilíbrios na ciclagem de nutrientes e alterações na biodiversidade (UNEP, 2007). Cerca de 75% do N_2O de origem antrópica provém da agricultura (Inselsbacher et al., 2010) e a fertilização também reduz a

capacidade de solos aerados em absorver CH_4, outro importante gás de efeito estufa. O N_2O é um dos gases de efeito estufa mais perigosos, pois ele é muito estável (dura em média 100 anos na atmosfera) e muito potente (1 g de N_2O tem um potencial de aquecimento global de 300 g de C_2O) (UNEP, 2007). Estes tipos de danos são de difícil controle, uma vez que ocorrem por todo o mundo e se expressam longe das fontes poluidoras. Ao mesmo tempo, o agente poluidor (o agricultor) recebe benefícios financeiros diretamente por agir dessa forma, pois sua produção agrícola fica alta devido ao uso de fertilizantes sem que ele seja responsabilizado pelos danos que causa no ambiente e em populações humanas. (Good & Beatty, 2011)

Outro problema do uso de fertilizantes se refere a sua produção. A principal fonte de N dos fertilizantes químicos é a amônia (NH_3), que, para ser sintetizada, precisa de N e H. A fonte de N usada nos fertilizantes é a nossa atmosfera, composta por mais de 70% de dinitrogenio (N_2). O dinitrogênio atmosférico é biologicamente inerte e não é absorvido ou utilizado por animais, fungos ou plantas, pois a ligação tripla dos dois átomos de nitrogênio é muito forte e é necessária uma grande quantidade de energia para sua quebra. Para suprir o H da amônia sintética, gás natural (CH_4) é consumido em grandes quantidades (FAO, 2011). A queima de CH_4 libera H livre que irá reagir com o N livre, formando NH_3. O consumo de gás natural é a única forma comercialmente viável de produção de H livre (a hidrólise da água para formação de H livre consome três vezes mais energia). O gás natural, porém, é um combustível fóssil não-renovável e limitado. O gasto energético para a produção de fertilizantes químicos é de cerca de 1,1% de toda a energia produzida no planeta, e, apesar do processo atual de produção ser cerca de seis vezes mais eficiente que o inicial, ele ainda é energeticamente ineficiente. Cerca de 90% da energia consumida na produção de fertilizantes é usada na síntese de nitrogênio reativo (Dawson & Hilton 2011; FAO, 2011).

O P inserido nos fertilizantes precisa ser minerado de rochas e seus estoques também são limitados. Cerca de 90% do fosfato rochoso minerado hoje no mundo é utilizado pela agricultura, principalmente para a produção de fertilizantes. Entretanto, apenas 20% do P do fosfato rochoso chega aos alimentos consumidos pela população, com os outros 80% sendo perdidos na mineração, produção e aplicação do fertilizante. De acordo algumas estimativas, o pico de produção de fosfato rochoso de qualidade deve ser alcançado perto de 2033. A partir desse ponto, a produção mundial de fosfato rochoso irá cair, enquanto o preços seguirão a subir. A mineração de fosfato rochoso no

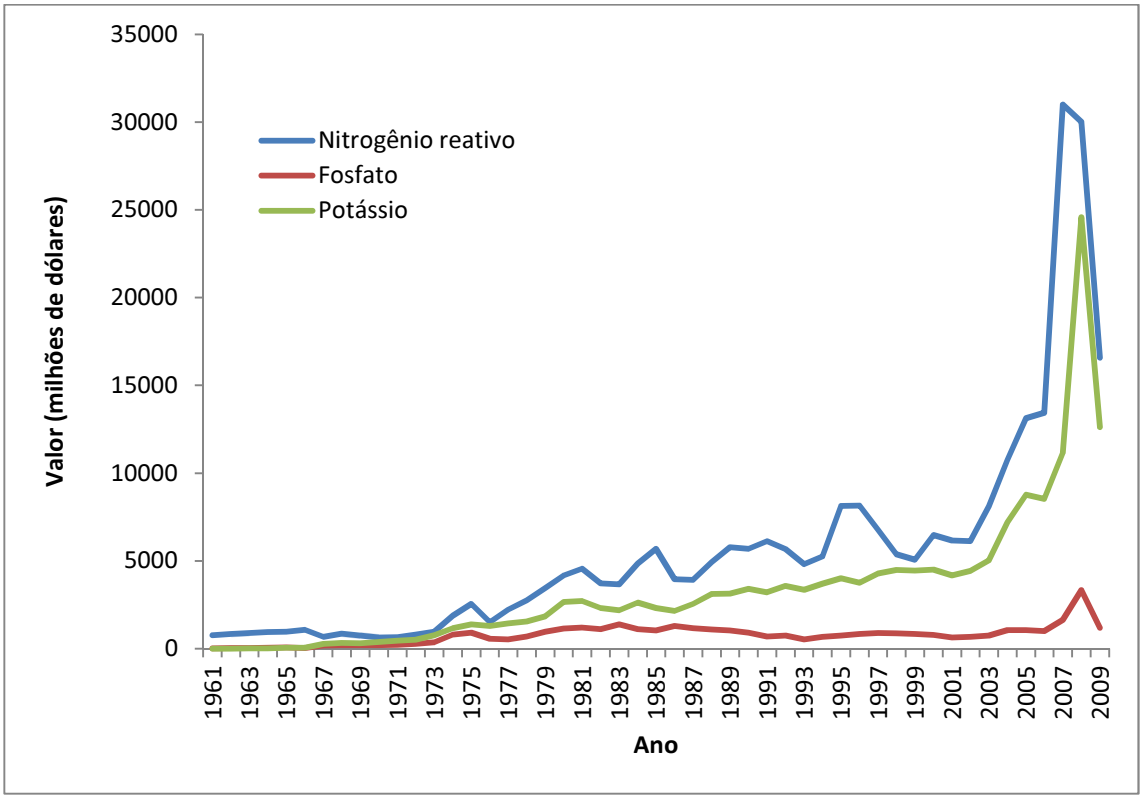
futuro pode complicar-se ainda mais devido a questões geopolíticas: 90% das reservas mundiais de fosfato estão em cinco países, sendo 77-85% dessas reservas no Marrocos. Uma parte significativa da reserva do Marrocos está no oeste do Saara, em uma região que o Marrocos ocupa contrariando resoluções da ONU. Na crise de alimentos de 2008, os preços do fosfato rochoso subiram 800% em 18 meses (Neset & Cordell, 2012). O valor global dos fertilizantes nitrogenados passou de U$32 bi, em 1987, para U$80 bi atualmente e deve chegar a U$150 bi em 2030 (Good & Beatty, 2011).

Assim como P, K também é minerado, mas causa menores preocupações. As principais fontes de K para uso na agricultura são os depósitos de antigos mares e lagos intracontinentais soterrados. Como fonte secundária, é possível remover o potássio de lagos salgados, como do mar Morto (Römheld & Kirkby, 2010). O potássio não volatiza para a atmosfera e não induz episódios de eutrofização. Altas concentrações dele no solo e na água não são prejudiciais à saúde humana nem ameaçam a fauna e a flora (Ramasamy et al., 2005), além de ocorrerem naturalmente em altas concentrações nas águas oceânicas (400 mg/L de K contra 0.06 mg/L de P) (Dawson & Hilton 2011). Em geral, o K não causa danos detectáveis no ambiente.

A disponibilidade do K é muito influenciada por algumas propriedades do solo, pois ele está estocado em compartimentos de diferente disponibilidade e volume. Noventa a 98% do K do solo está em formas inertes que lentamente são dissolvidas, mas que eventualmente podem ser esgotadas (como ocorre hoje na Índia) se a remoção do K pela colheita for maior que a adição por fertilizantes ou adubos (Römheld & Kirkby, 2010). Quando essas reservas se tornarem muito baixas, os produtores podem se tornar absolutamente dependentes da fertilização química (processo similar ao que ocorreu com o P na Europa no século XIX). A absorção de K está altamente relacionada ao crescimento das raízes, principalmente as laterais (Römheld& Kirkby, 2010; Spiess, 2011).

Ocorre que com a atual demanda de alimentos, nossa sociedade passou a ser absolutamente dependente da produtividade extra gerada pelos fertilizantes químicos, já que cerca de 40% da população mundial é alimentada a partir da produtividade extra gerada por fertilizantes (UNEP, 2007). O preço e o uso dos fertilizantes têm aumentado com o tempo, à medida que as fontes que o produzem vão se esgotando. Obviamente, isso mostra um cenário absolutamente insustentável: não podemos contar indefinidamente com um recurso limitado. Se continuarmos seguindo o modelo atual, essas fontes irão acabar (ou ficar tão escassas que seu uso para produção de fertilizantes

ficará inviável), a produtividade agrícola vai baixar e a população mundial enfrentará uma grave crise de falta de alimentos. O gasto mundial anual com a importação de fertilizantes e o consumo anual de fertilizantes pode ser visto na Figura 1.



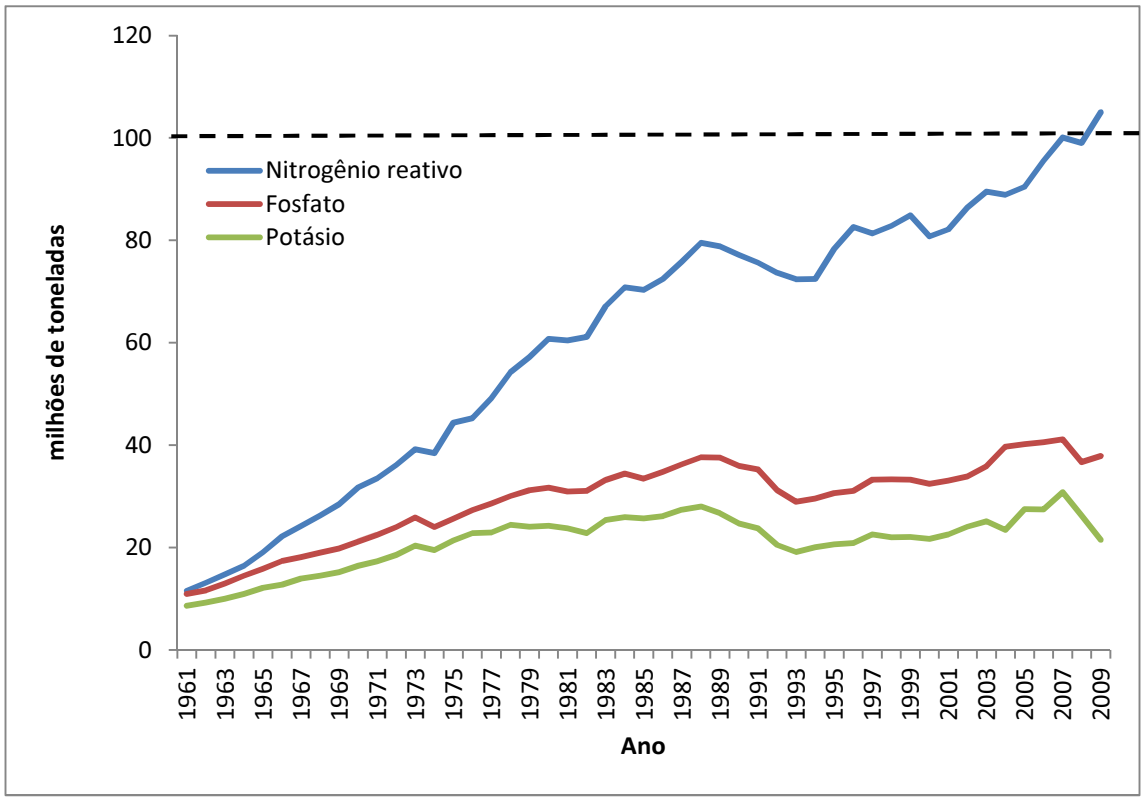


Figura 1. Gastos na importação (parte superior) e consumo (parte inferior) de fertilizantes no mundo, de 1961 até 2009. A linha preta tracejada representa a média da fixação biológica de nitrogênio, por ano, no mundo (FAO, 1999; UNEP, 2007)

Existem muitos outros problemas relacionados às práticas atuais da agricultura industrial. As áreas de alta produtividade agrícola, com os melhores solos e maior potencial produtivo, têm diminuído devido à expansão urbana. É comercialmente muito mais favorável transformar uma área de produção agrícola próxima a um centro urbano em um complexo habitacional ou comercial, mesmo que as melhores técnicas sejam aplicadas para produtividade máxima: a agricultura não consegue competir com os preços do mercado imobiliário alavancados pela expansão urbana (Singh et al., 2011). O uso de inseticidas e herbicidas, muitas vezes aplicados de forma impensada, também leva a graves problemas ambientais ao escaparem para o ambiente, além dos efeitos nocivos à saúde do consumidor. Traços residuais desses agrotóxicos se acumulam com o tempo em organismos no topo da cadeia alimentar e levam a uma série de danos à saúde.

1.2. Bactérias promotoras de crescimento vegetal

Evidentemente, este modelo de produção precisa de novas técnicas, tecnologias e políticas para fazer um uso mais racional dos recursos disponíveis. Uma das tecnologias mais promissoras, principalmente para a redução do uso de fertilizantes químicos, é o uso de bactérias promotoras de crescimento vegetal (PGPR, de *Plant Growth Promoting Rizobacteria*), micro-organismos capazes de aumentar a produção agrícola ao interagirem com as plantas (Sokolova *et al.*, 2011). As PGPR ocorrem naturalmente em todos os tipos de plantas, normalmente associadas às raízes dos vegetais. Essas bactérias podem estar associadas intimamente às raízes, estando dentro das células vegetais, em espaços intracelulares, na superfície das raízes ou, então, mais afastadas, no solo adjacente às raízes (denominado de rizosfera). Elas utilizam uma série de mecanismos diretos e indiretos para promover o crescimento vegetal, como a produção de hormônios de crescimento vegetal, solubilização de nutrientes do solo, proteção contra patógenos e estresses abióticos, fixação de nitrogênio atmosférico e outros (Glick et al., 1998; Babalola, 2010; Hayat et al., 2010). Em troca, a planta libera exudatos pelas suas raízes: fontes de carbono que permitem, de forma seletiva, o

desenvolvimento bacteriano (Ambrosini et al., 2011; Farina et al., 2012). O nível de especificidade dessas interações varia muito de acordo com a planta e a bactéria, havendo bactérias de vida livre, que podem ou não se associar a plantas de genótipos distintos, até relações simbióticas restritas a certas linhagens de bactérias com determinadas linhagens de plantas. O estudo para incrementar a produção agrícola através das PGPR começou pelos anos 80, e a eficiência desses organismos já foi demonstrada em muitos estudos. De maneira geral, o uso de PGPRs permite uma redução na aplicação de fertilizantes de até 50%, sem haver reduções na produtividade (Baldani et al., 1986; Alves et al. 2003; Adesemoye et al, 2009a; Hayat et al., 2010; Good & Beatty, 2011; Miransare, 2011). Duas características presentes nas PGPRs tem interações diretas na aplicação de fertilizantes: a capacidade delas em fixar nitrogênio atmosférico e de solubilizar nutrientes do solo.

Algumas PGPRs, entre muitas outras bactérias de diversos nichos e gêneros, conseguem fixar nitrogênio atmosférico através da enzima nitrogenase (Nase) (Affourtit et al., 2001). Estas bactérias são capazes de quebrar a forte ligação covalente tripla do dinitrogênio (N_2) atmosférico, e, então, incorporar esse nitrogênio a biomoléculas, por isso são denominadas de *diazotróficas*. Bactérias pertencentes aos gêneros *Herbaspirilum, Azospirillum*, *Pseudomonas, Bradyrhizobium* e *Rhizobium* podem ser fixadoras de nitrogênio (Baldani et al., 1986). Este processo é extremamente importante para a vida na terra, já que o nitrogênio é um dos elementos chave na constituição dos seres vivos, estando presente em aminoácidos e ácidos nucléicos. Se o nitrogênio atmosférico não fosse fixado por micro-organismos, a única fonte natural de nitrogênio biologicamente reativo na Terra seria aquela liberada por descargas elétricas na atmosfera: a passagem de relâmpagos rompe a ligação do dinitrogênio e este reage com outras moléculas, para aí, então, poder ser incorporado pelos organimos. A quantidade de nitrogênio fixada dessa forma, porém, representa apenas 2,5% do nitrogênio fixado naturalmente (Borucki & Chameides, 1984).

A fixação biológica de nitrogênio é um processo muito custoso para a célula e é fortemente regulada. Além de um grande aparato enzimático especializado para realizar a fixação de nitrogênio, a célula precisa gastar 16 moléculas de ATP para cada molécula de dinitrogênio quebrada. Esse aparato enzimático também é altamente sensível ao oxigênio atmosférico, de forma que a fixação de nitrogênio costuma ocorrer em ambientes anaeróbicos ou com a enzima nitrogenase sendo protegida pelo metabolismo bacteriano para reduzir a chance de reação com o oxigênio. Sendo a Nase muito

sensível ao oxigênio, perdendo a sua função caso reaja com ele, uma bactéria diazotrófica não pode permitir que todo o gasto energético da produção das enzimas que reduzem o N seja desperdiçado pela interação com oxigênio. A célula também não irá fixar nitrogênio atmosférico se ela dispuser de outras fontes de nitrogênio em seu ambiente, mais fáceis de serem capturadas e metabolizadas (Zehr et al, 2000). A fertilização química, por conter N reativo em seus compostos, altera o metabolismo de nitrogênio das bactérias diazotróficas, de tal forma que a bactéria irá absorver o nitrogênio reativo distribuído no solo, ao invés de fixar o nitrogênio atmosférico por conta própria. Dessa forma, o fertilizante adicionado pelo agricultor pode ser consumido em grande parte por bactérias e não apenas pelas plantas. Não apenas o agricultor estará desperdiçando os recursos que aplicou, facilitando o desenvolvimento de bactérias ao invés do de plantas, como estará negligenciando um recurso natural do seu solo que, caso manejado, pode trazer benefícios econômicos e ambientais.

O exemplo de maior sucesso do uso de PGPRs para redução de fertilizantes e fixação de nitrogênio é o da soja brasileira. Cultivares de soja, desenvolvidas pela EMBRAPA, permitem que 100% do nitrogênio necessário para o desenvolvimento e produção máxima da cultura seja de origem bacteriana. Nenhum nitrogênio adicionado via fertilizante é necessário para produtividade máxima nas lavouras com essas linhagens. A economia gerada dessa forma foi de 2,5 bilhões de dólares em 2002, e é essa economia que permite que o Brasil entre de forma competitiva no mercado internacional da soja (Alves et al, 2003).

A solubilização de nutrientes como fosfato, ferro e potássio também interfere diretamente com a fertilização. A maior parte do fosfato assimilável pelas plantas que é adicionada via fertilizante rapidamente se torna indisponível ao reagir com complexos metálicos presentes no solo. Assim, pode ser que apenas 10% do fosfato adicionado sejam realmente utilizados pelas plantas, enquanto os outros 90% escorrem para o ambiente ou ficam retidos no solo em formas não-assimiláveis (Adesemoye & Kloepper, 2009b). Bactérias solubilizadoras de fosfato, como as dos gêneros *Bacillus, Pseudomonas, Enterobacter, Erwinia* e *Ochrobactrum* (Babalola, 2010), podem solubilizar esses complexos de fosfato pela secreção de ácidos orgânicos. Uma vez na forma solúvel, esses fosfatos poderão ser absorvidos pela bactéria ou pela planta. Não é possível que uma bactéria solubilizadora de fosfato substitua completamente a aplicação do fertilizante, uma vez que cultivos sucessivos sem adição de P externo irão eventualmente esgotar o solo, como aconteceu na Europa no século XIX (Dawson &

Hilton 2011). Porém, uma bactéria solubilizadora de fosfato pode aumentar drasticamente a eficiência do fertilizante que for adicionado à lavoura: é possível adicionar quantidades menores de fosfato enquanto a planta aumenta a biomassa, comparado a um controle não-inoculado e completamente fertilizado (Panhwar et al, 2011).

PGPRs também podem ajudar a reduzir o uso de agrotóxicos, uma vez que elas conseguem reduzir o ataque de patógenos nas lavouras. Uma das formas com que fazem isso é pela ocupação do nicho em que se encontram: como a rizosfera já está povoada por micro-organismos, é mais difícil que patógenos consigam atingir as raízes. Algumas PGPRs também são capazes de produzir antibióticos, ou induzir a planta a reforçar seus sistemas de resistência a patógenos (Mansoor et al., 2007; Wei et al., 1991; Guo, 2003). Outra característica de PGPR muito interessante é a produção de compostos indólicos, tais como o ácido indol acético. Estas substâncias, excretadas pela bactéria, agem como hormônios de crescimento vegetal e induzem ao aumento das raízes e folhas da planta (Hayat et al., 2010; Babalola 2010). Bactérias dos gêmeros *Pseudomonas, Bacillus, Arthrobacter, Enterobacter* e *Agrobacterium*, entre outras, podem produzir compostos indólicos (Yuan et al., 2010).

A eficiência das PGPRs pode ser afetada de várias maneiras, de tal forma que nem sempre os mesmos resultados são obtidos em diferentes condições com a mesma bactéria. Um dos principais fatores é a compatibilidade do micro-organismo com a planta, pois a especificidade pode ser dependente das linhagens de uma mesma cultura. A eficiência de uma PGPR pode ser alterada pela quantidade de micro-organimos inoculados, o veículo usado na inoculação, o estágio de desenvolvimento da planta no momento da inoculação, propriedades físico-químicas do solo, interações com a microbiota nativa, interações com outras PGPRs, o estado de saúde das plantas, o clima da região e muitos outros fatores. Evidentemente, a adição massiva de nutrientes no solo através da fertilização também tem o potencial de alterar a eficiência dessas interações.

A existência de tantos fatores que possam alterar a eficiência de uma PGPR para determinada cultivar pode tornar sua aplicação mais restrita a certas condições. Por isso a pesquisa nessa área sempre busca novos organismos que possuam uma maior eficiência ou inclusão de determinados nichos, como resistência à salinidade ou a um metal tóxico. Muitos estudos mostraram que uma determinada PGPR responde de forma diferente de acordo com o nível de fertilização das plantas. Poucos estudos, porém,

investigaram como determinados níveis de fertilização afetam a população de PGPRs em termos de diversidade e capacidade de exercer o crescimento vegetal.

2. Objetivos

a) Isolamento de bactérias diazotróficas que se associam ao arroz cultivado sob diferentes níveis de fertilização e análise das suas características promotoras de crescimento vegetal, para futura utilização em produtos inoculantes para essa lavoura;

b) Comparar as populações bacterianas isoladas de lavouras de arroz sob diferentes condições de fertilização, a fim de se verificar o quanto a presença de fertilizantes químicos interfere na composição destas populações (diversidade bacteriana) e em suas capacidades de promover o crescimento vegetal.

Objetivos Específicos

a) Isolar bactérias diazotróficas aeróbicas e anaeróbicas facultativas (bacilos) através de isolamento seletivo;

b) Avaliar a capacidade dos isolados quanto à fixação biológica de nitrogênio, solubilização de fosfatos, produção de fito-hormônios e sideróforos e comparar essas características para cada nível de fertilização entre as bactérias intimamente associadas à raíz e à rizosfera;

c) Diferenciar as linhagens isoladas por meio de PCR-RFLP do gene *nifH* e identificá-las por meio do sequenciamento parcial do gene do 16S rRNA;

d) Testar *in vivo* as linhagens bacterianas mais eficientes como PGPR e avaliar o potencial destas no aumento do crescimento do arroz, visando a posterior utilização destas em inoculantes para as lavouras.

3. Manuscrito a ser submetido ao periódico Environmental Microbiology

(Fator de impacto = 5,537)

The effects of different fertilization conditions on bacterial plant growth promoting traits: guidelines for directed bacterial prospection and testing

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Abstract

Chemical fertilization is widely used for increased crop productivity, but its production and use lead to serious environmental damage. To reduce the use of fertilizers, one can make use of plant growth promoting rhizobacteria (PGPR), which are plant-associated bacteria that increase plant health, size and yield through various mechanisms. PGPR effectiveness is known to fluctuate with environmental conditions; however, studies regarding the effect of chemical fertilization on plant growth promoting (PGP) traits of PGPR are scarce. In this work, the effects of long-term fertilization on the diazotrophic diversity, occurrence and expression levels of PGP traits from 190 bacterial strains isolated from rhizospheric soil and roots of rice were analyzed. We found that fertilization had a limited effect on diversity but had a major effect on phosphate solubilization and indolic compounds (IC) production abilities. We propose that plants select bacteria that present good phosphate solubilization ability for intimate root association in lieu of good IC production under nutrient-poor conditions and select good IC producers in lieu of good phosphate solubilizers under nutrient-moderate conditions. In nutrient-rich conditions, this selection preference seems to be deactivated. After testing seven selected isolates for effective *in vivo* plant growth promotion in greenhouse conditions, our predictions were tested in the field. We found that good phosphate solubilizers only promoted growth at nutrient-poor conditions and that good IC producers only promoted growth at nutrient-moderate conditions. In nutrient-rich conditions, phosphate solubilization and IC production were not key factors to promote plant growth. These findings may be used for directed PGPR prospection and anticipated PGPR candidate selection, according to plant needs and farmer interests.

Introduction

The reduction of fertilizer inputs into crop fields without decreasing productivity is a feasible but difficult challenge that requires attention from the scientific community. It is well known that a large fraction of the millions of tons of nutrients added to soils every year are not taken up by plants, as up to 50% of added nitrogen (N) and 90% of added phosphorus (P) can run off from crop fields (Simpson et al, 2011). Instead of running their intended course, these nutrients escape to the atmosphere or lixiviate to water sources, causing all manners of environmental, economic and public health problems through eutrophication and greenhouse gas generation at a global scale (Good & Beatty, 2011; Inselsbacher et al., 2010; Miransari, 2011). Fertilizer requires the consumption of non-renewable fossil fuels, and, currently, we are completely dependent on the extra productivity that fertilizers provide to feed the world population. This dependence generates a long term issue for food security because we simply cannot rely on a limited resource for an infinite time (FAO, 2011), especially because both the usage and the price of fertilizers are currently increasing (Good & Beatty, 2011). This delicate situation can be alleviated by employing plant growth promoting rhizobacteria (PGPR), which are beneficial soil and root-associated microorganisms capable of increasing plant health and size while reducing fertilizer inputs by up to 50% without any yield loss compared to fully fertilized controls (Baldani et al., 1986; Alves et al., 2003; Good & Beatty, 2011; Hayat et al., 2010; Miransare, 2011).

PGPR provide several possible strategies to enhance plant growth, such as the production of antibiotics, the solubilization and recycling of nutrients, the production of plant growth hormones, nitrogen fixation, the induction of enhanced plant defenses, soil detoxification and others (Glick et al., 1998; Babalola, 2010; Hayat et al., 2010). It is well know that plants affect the indigenous microbial populations in soil, and each plant species is thought to select for specific microbial populations that contribute most to their fitness, creating a selective environment and limited diversity (Berg and Smalla 2009). Plants continually communicate and interact with these bacteria mainly through carbon-rich root exudates that shape the activities and the bacterial community that inhabit the rhizosphere (Ambrosini et al., 2012; Farina et al., 2012). A plant might, for example, exude l-tryptophan (Ahmad et al., 2005), which can be used by some bacteria to produce indolic acetic acid, a phytohormone known to increase shoot and root length (Sokolova et al., 2011; Yuan et al., 2011).

Because PGPR are possible tools to reduce fertilizer usage, they should be thoroughly investigated. Indeed, the use of PGPR is one of the most promising sustainable choices for modern agriculture (Sokolova et al., 2011). Research papers focused on the enhanced use of fertilizer inputs through bacterial inoculants have been increasing in recent years, and there are several reports related to the responses of various selected PGPR to different fertilization regimes in different crops (Carlier et al., 2008; Adesemoye et al, 2009a, b; Zabihi et al., 2010). There are, however, few reports on the effects of fertilization levels on the indigenous diazotrophic population diversity and plant growth promoting (PGP) traits. Because plant root exudates change bacterial populations and the exudates can change according to plant health and nutrient status, fertilization levels should directly shape the plant-associated bacterial community. It would be interesting to know how fertilization interferes with PGP traits because most PGPR screening studies take samples from crops under fully fertilized conditions. This unilateral approach on PGPR prospection may induce researchers to bias samples and results, leaving portions of the PGPR community unexplored.

The aims of this work were to evaluate how different levels of N, P, and potassium (K) fertilization act on different PGP characteristics of rhizospheric and root-associated diazotrophs and how this can be used for situation-specific PGPR prospection and use. In addition to in-depth description and the comparison of PGP traits according to fertilization levels, we attempted to anticipate the most successful PGPR according to their PGP traits and plant fertilization condition in field trials.

Methodology

Sampling site description

Soil and rhizosphere samples were taken in February 2010 from an experimental rice field from Instituto Riograndense do Arroz (IRGA) in Cachoeirinha (29º56′51.9″S, 51º06′46.3″W), Rio Grande do Sul State, Brazil. This field has been under the same three fertilization conditions for seven years: in the unfertilized condition (Z), NPK fertilization levels were zero; in the light fertilization condition (L), 60 kg N, 20 kg P_2O_5 and 60 kg K_2O were added; and in the heavy fertilization condition (H), 120 kg N, 40 kg P_2O_5 and 100 kg K_2O were added. The plant screened for PGPR was *Oryza sativa* variety IRGA 424, which is a cold-resistant, regionally developed cultivar widely used by farmers in the state. The rice plants were all in a flowering stage. Soil from the fields had a pH of 5.0, a clay content of 21% and an organic matter content of 2.6%. The

climate in Rio Grande do Sul is considered to be humid subtropical, with average temperatures between 15 and 18ºC and large variations between -10 and 40°C.

Bacterial isolation and storage procedures

Rhizospheric (R) and root-associated (A) putative diazotrophic bacteria were isolated according to Döbereiner (1995), using the nitrogen-free semi-solid NFb, LGI, and LGI-P media, with the modifications described by Farina et al. (2012). Bacilli were isolated according to Seldin et al. (1983) with the modifications described by Beneduzi et al. (2008), except that only rhizospheric soil was used for the bacterial isolation. Pure colonies were stored at -20ºC in 50% glycerol.

Bacterial identification

Bacterial DNA extraction, PCR amplification, RFLP analysis, and the partial sequencing of bacterial 16S rRNA genes were performed according to Ambrosini et al. (2012) with the use of MspI instead of TaqI in the RFLP procedure. Individual diversity analysis was completed for each fertilization condition. Sequencing was performed on a Megabace 1000 automatic 117 sequencer using the DY Enamic^{TM} ET Dye Terminator Cycle Sequencing Kit (GE HealthCare). The Chromas Lite software (v. 2.01, http://www.technelysium.com.au/chromas_lite.html) was used to verify the quality of the sequences and to check for possible chimeric origins. DNA sequences (approximately 450 bp) were compared with those available in the GenBank database using the BLASTN algorithm (Altschul, 1997; available on http://blast.ncbi.nlm.nih.gov/). The nucleotide sequences of the 190 partial 16S rRNA gene segments determined in this study have been deposited in the GenBank database under accession numbers JQ795087-JQ795181

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Evaluation of plant growth promotion abilities

Indolic compound (IC) production assay was performed according to Glickmann & Dessaux (1995), except that spectrophotometric measurements were performed only after 72 h of incubation, and isolates with IC concentrations below 15 µg IC ml^{-1} were considered non-producers. Siderophore production and phosphate solubilizing assays were performed as described by Glickmann & Dessaux (1995) and Schwyn & Neilands (1987). A colony solubilization index (SI) was used as an indirect measure to estimate

how much phosphate was solubilized (Ahemad & Khan, 2011). The SI was measured as [(zone size including colony diameter – colony diameter)/colony diameter], and it was measured 72 h after inoculation. The same procedure was used for siderophore analysis to estimate how much iron was chelated by the bacterial colonies (Ahemad & Khan, 2011). Nitrogenase (Nase) activity was evaluated as described by Boddey (1987) and Burris (1972), and only isolates able to reduce more than 1 µmol of acetylene mg protein^{-1} were considered nitrogen fixers.

Pot and field trials

Seven PGPR candidates were selected based on their PGP traits and were tested on rice plants at greenhouse conditions. These isolates were identified by partial 16S rDNA sequences as belonging to the following groups: 45 (*Burkholderia* sp.), 49 (*Enterobacter* sp.), 68 (*Enterobacter* sp.), 69 (*Enterobacter* sp.), 73 (*Pantoea* sp.), 86 (*Azorhizobium* sp.), and 148 (*Rhizobium* sp.). Rice seeds (variety IRGA 424) were surface sterilized in 70% ethanol for 2 min and 1.2% sodium hypochlorite for 10 min and rinsed 10 times in sterile tap water. Pots (500 ml) were sterilized with 70% ethanol and filled with sterile vermiculite substrate. Five seeds were planted 2.0 cm below the surface in each pot, with 10 pots per bacterial treatment in addition to a non-inoculated control. Bacterial isolates were grown in King B medium at 28ºC until they reached a final concentration of 10^9 cfu ml^{-1}. Subsequently, 5 ml of this suspension was applied in each pot for bacterial inoculation. All treatments were arranged in a randomized design and grown at 26ºC with a 12 h daylight period for 31 days. After emergence, the seedlings were thinned for three plantlets per pot. At day 11 from planting, all of the treatments and the control received Hoagland's nutrient solution diluted to 25% concentration. After the 31-day growth period, shoot and root lengths were measured in fresh material. The dry weight of the shoots and the roots was measured after drying for 7 days at 60ºC. The three plantlets per pot composed a single average per pot, which was subsequently used for statistical analysis.

For the field trial, isolates 45, 68, and 86 and a non-inoculated control were tested on rice at three different fertilization conditions, which corresponded to the same Z, L and H conditions originally applied. These bacteria were selected according to the bacterial genera to which they belong, the different PGP traits they displayed, and the effect observed on plant growth promotion during the greenhouse trial. The field trial was conducted at an experimental station of IRGA located in Cachoeira do Sul (52° 53’

42'' S 52° 53' 42'' W), RS, Brazil. The experimental design consisted of an entirely randomized block with four repeats, each plot with size of 5 m X 1.5 m. Bacteria were grown in the same manner as the greenhouse trials, and the rice seeds were inoculated with a bacterial suspension of 10^6 cfu ml^{-1} for 30 min before planting. Seeds were sown in late October of 2010, and fertilizer was added just before flooding (V4 stage). Harvest occurred in early March of 2011. Shoot length measurements were taken at pre-flowering and pre-harvesting stages. The field soil had a pH of 4.8, a clay content of 24% and an organic matter content of 1.7%.

Statistical analysis

A goodness-of-fit test was used to determine if the number of isolates able to present PGP traits was affected by fertilization conditions. The chi-square test for heterogeneity in 3 x 2 cells was used to determine if the three fertilization conditions affected in different ways the occurrence of PGP traits in A or R bacteria. For the number of nitrogen fixers the exact p-value was obtained due to low expected values. Quantitative PGP traits, pot trial results and field trials results were checked for normal distribution by histogram analysis and the Shapiro-Wilk test, and checked for equality of variance by Levene's test. For all PGP traits (phosphate SI n=111, IC production n=127, siderophore SI n=149 and Nase activity n=29), multiple comparisons were performed by the Kruskal-Wallis test with the SNK post-hoc test because most of the data were non-Gaussian or had significantly different variances, despite mathematical transformations. First, a single test was performed considering each isolate positive for each assay in each fertilization condition. Subsequently, another test was performed separating R and A bacteria, according to fertilization and bacterial source, for a total of six categories: unfertilized rhizospheric bacteria (ZR), unfertilized root-associated bacteria (ZA), light fertilization rhizospheric bacteria (LR), light fertilization root-associated bacteria (LA), heavy fertilization rhizospheric bacteria (HR) and heavy fertilization root-associated bacteria (HA). The correlation between phosphate SI and IC production was tested with Spearman's correlation test (n=76) because the data lacked the requerements for Pearson's correlation.

Pot trial results for shoot and root length and shoot dry weight (all with n=70, 10 triplicates per treatment) were tested with one-way ANOVA and Fisher's least significant difference (LSD) post-hoc test. Root dry weight was tested by the Kruskal-Wallis test with the SNK post-hoc test because variances of the treatments were

significantly different. Field trial results for shoot length from pre-flowering and pre-harvest stages were tested by a 3 x 2 factorial ANOVA, testing for fertilizer condition, bacterial treatment and their interaction (five measurements per plot, four plots per bacterial treatment and fertilization condition, n=240). Simple main effect analysis was used to test if there was any difference between bacterial treatments within a single fertilization treatment.

All ANOVA and exact p-value tests were conducted with SPSS 19.0, and all non-parametric tests were conducted with Bioestat 5.0. Differences were considered significant if $p<0.05$.

Results

Bacterial isolation

Sixty putative diazotrophic bacteria were taken from each fertilization condition using three different nitrogen-free media in aerobic conditions. Thirty rhizospheric and 30 root-associated bacterial isolates were obtained for each fertilization condition. Although we were able to isolate several putative diazotrophic bacteria in anaerobic conditions (bacillus-specific isolation), only 10 isolates survived at laboratory conditions (four in the Z condition and six in the H condition). In total, 190 putative diazotrophic bacteria were successfully stored, identified and tested for PGPR abilities.

Effect of fertilization on bacterial community composition and diversity

The most common bacterial genera found in all of the fertilization conditions (Z, L, and H) were *Burkholderia*, representing 22, 20, and 15% of the isolates per condition, respectively, and *Enterobacter*, representing 16, 22, and 18% of the isolates per condition, respectively. Five other bacterial genera (*Pseudomonas, Stenotrophomonas, Agrobacterium, Achromobacter,* and *Citrobacter*) were also ubiquitous in all conditions, representing together 33, 22, and 21% of the isolates per condition, respectively. The 13 genera outside this ubiquitous core represented 29, 36 and 46% of the isolates in Z, L and H condition, respectively. The Shannon diversity indices were 2.660, 2.150, and 3.070 per condition, respectively, and failed to be associated with fertilization conditions. The dominance of the ubiquitous bacterial genera decreased as the fertilization levels increased, but even for the H condition, the ubiquitous genera represented over 50% of the bacterial diversity (Figure 1).

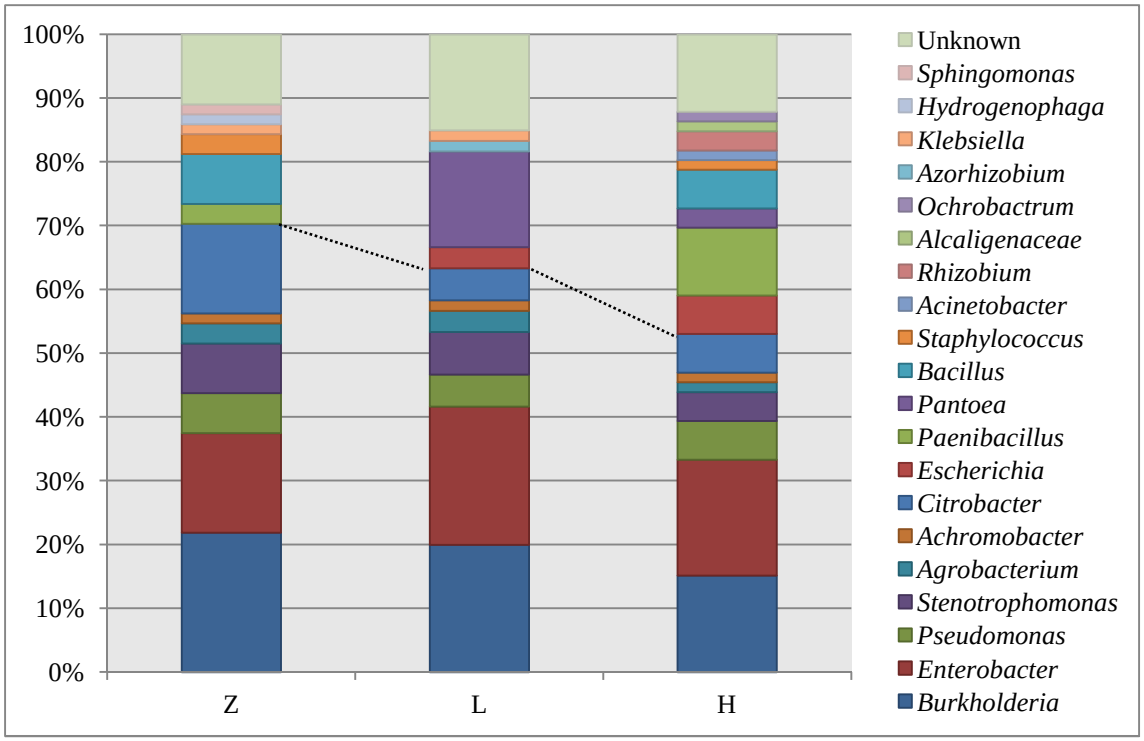

Figure 1. Genus composition for each fertilization condition. Over 50% of the bacteria found in each condition belonged to the same seven ubiquitous genera (represented under the dashed line). Overall microbial diversity did not change with fertilization conditions, but there was a steady decrease in the dominance of the ubiquitous genera. Z = unfertilized, L = light fertilization, H = heavy fertilization.

Effects of fertilization on the occurrence of PGP traits

The number of bacterial isolates able to produce IC, to produce siderophores, to solubilize phosphate, and to fix nitrogen (measured as the ability to reduce acetylene) are shown in Table 1. The different fertilization conditions changed the number of nitrogen fixers ($p=0.002$) but not the total number of IC producers ($p=0.551$), phosphate solubilizers ($p=0.784$), or siderophore producers ($p=0.845$); these results revealed more nitrogen-fixing bacteria in the higher fertilization condition.

There was no significant association between type of bacteria (R and A) and fertilization condition in relation to IC production ($p = 0.909$), phosphate solubilization ($p = 0.123$), siderophore production ($p = 0.979$) and nitrogen fixing ($p = 0.084$). This result shows that the proportion of A and R bacteria able to present a PGP trait was not significantly different among fertilization condition.

Table 1. Number of bacterial isolates able to express PGPR characteristics according to each fertilization condition and PGP trait.

Fertilization condition	IC producers			Phosphate solubilizers			Siderophore producers			Nitrogen fixers		
	A	R	Total	A	R	Total	A	R	Total	A	R	Total*
Z	24	20	44	12	22	34	24	24	48	0	3	3
L	19	19	38	20	20	40	27	26	53	6	2	8
H	23	22	45	22	15	37	25	23	48	11	7	18

Asterisk (*) shows statistical significance for the goodness of fit chi-square test ($p<0.05$). Z = unfertilized, L = light fertilization, H = heavy fertilization, A = root-associated bacteria, R = rhizospheric bacteria.

Effects of fertilization on the phosphate solubilization index and indolic compounds production

Mean scores for the phosphate SI and IC production according to bacterial source and fertilization condition are presented in Figure 2. Fertilization conditions altered the phosphate SI ($p<0.0001$). When the SI of real phosphate solubilizers were considered (discarding non-solubilizers), isolates from condition Z exhibited higher SI values than isolates from the L ($p<0.0001$) or H conditions ($p<0.0001$), whereas the SI from the L and H conditions were similar ($p=0.933$) (data not shown). SI data also differed amoung defined by type of bacteria (A or R) and fertilization levels, in a single Kruskal-Wallis test with six treatments ($p<0.0001$). ZA bacteria presented the highest mean rank for SI of all of the conditions, slightly higher than the ZR bacterial SI ($p=0.12$) but significantly higher than in any other fertilized condition (all $p<0.018$). In the L condition, LR bacteria presented a higher SI compared to LA bacteria ($p=0.004$), which the lowest SI mean rank among all 6 conditions. In the H condition, HA and HR bacteria were similar ($p=0.929$). These results are especially interesting because IC production showed exactly the opposite trend.

Fertilization conditions also altered IC production ($p=0.023$). From the isolates able to produce IC above 15 µg IC ml^{-1}, production was higher in the L condition compared to the Z condition ($p=0.007$), whereas in the H condition, IC production was similar to Z ($p=0.382$) and L ($p=0.061$). When the IC production was compared in the 6

groups based on A and R bacteria and fertilization levels, we observed the opposite results of those found for phosphate solubilization ($p<0.007$).

ZA bacteria presented the lowest mean rank for IC production of all of the conditions, which was significantly lower than ZR ($p=0.04$), LA ($p<0.0001$), HA ($p=0.034$) and not significantly but lower than LR ($p=0.052$). In the L condition, LA bacteria presented a higher but non-significant IC production compared to LR bacteria ($p=0.078$), and the highest mean rank for IC production was found in the LA bacteria. In the H condition, HA and HR bacteria were similar ($p=0.30$).

Therefore, the results from both the SI and IC production showed an interaction effect between fertilization and bacterial source.

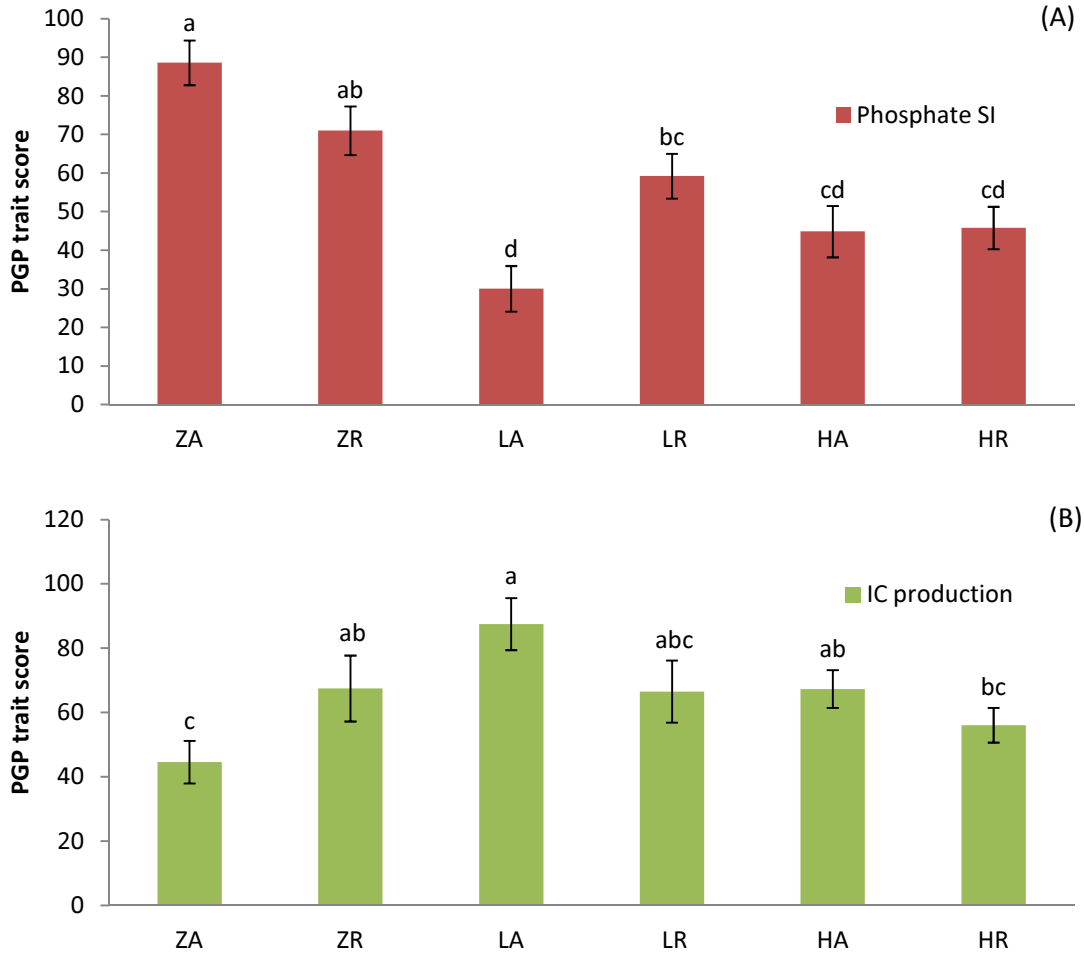

Figure 2. Mean score for the phosphate SI (A) and IC production (B) according to bacterial source and fertilization condition. Means (±1SE) with different letters are significantly different ($p<0.05$ in the Kruskal-Wallis SNK test). ZR = unfertilized rhizospheric bacteria, ZA = unfertilized root-associated bacteria, LR = light fertilization rhizospheric bacteria, LA = light fertilization root-associated bacteria, HR = heavy fertilization rhizospheric bacteria, and HA = heavy fertilization root-associated bacteria

A negative Spearman correlation (rs=-0.498) was found between the phosphate SI and IC production in the studied isolates (p< 0.0001 n=78, Figure 3). Apparently, a single bacterium may not able to produce high levels of IC while being able to solubilize large amounts of phosphate. This correlation has not been reported in the literature until now. This result corroborates the finding that ZA and LA bacteria show contrasting PGP traits. ZA presented the highest SI and lowest IC production, whereas LA presented the lowest SI and highest IC production.

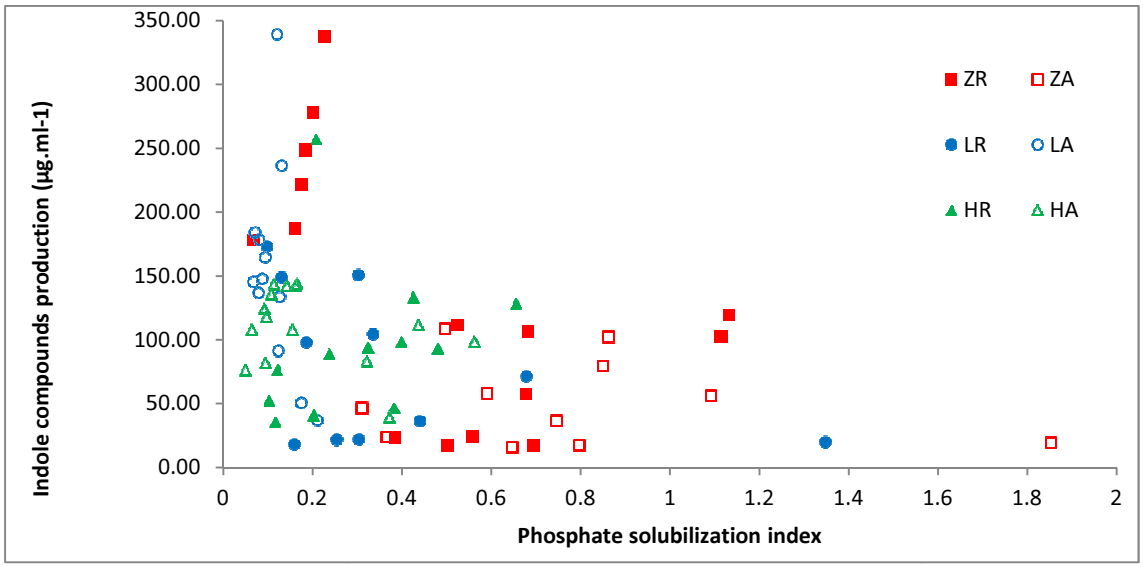

Figure 3. Scatter graph of IC production and phosphate solubilization by the isolates. Each point represents a single isolate able both to solubilize phosphate and produce IC above 15 µg IC ml^{-1}. ZR = unfertilized rhizospheric bacteria, ZA = unfertilized root-associated bacteria, LR = light fertilization rhizospheric bacteria, LA = light fertilization root-associated bacteria, HR = heavy fertilization rhizospheric bacteria, and HA = heavy fertilization root-associated bacteria

Effects of NPK fertilization on siderophore production and acetylene reduction

Surprisingly, siderophore production was affected by NPK fertilization (p=0.010), despite the fact that the fertilizer applied did not contain iron inputs and that iron is typically available in flooded soil conditions, such as those for paddy rice cultivation. Considering only the actual siderophore producers (n=149), the H condition showed a higher siderophore SI than the L condition (p=0.002), but the Z condition did not differ from either L or H conditions (p=0.155 and 0.130, respectively). When the

siderophore SI data from A and R bacteria were analyzed separately and according to fertilization levels, the siderophore SI was significantly higher for HR compared to all other conditions (all $p<0.023$), which were similar to each other (all $p>0.19$).

The acetylene reduction capacity of the isolates able to reduce over 1 µmol acetylene mg protein^{-1} did not change with fertilization ($p=0.283$). There was no significant difference in acetylene reduction when data from A and R bacteria were analyzed separately and according to fertilization levels 6 (groups), similarly to the results obtained for the other PGP traits ($p=0.916$).

Pot trials under greenhouse conditions

Shoot and root length and dry mass data from *in vivo* pot greenhouse trials are presented in Figure 4. The PGP traits for each isolate tested are presented in Table 2. Shoot length was affected by bacterial inoculation ($p=0.027$), with significantly longer shoots for isolates 49 ($p=0.021$), 68 ($p=0.036$) and 86 ($p=0.007$), compared to the control. Root length was also affected by bacterial inoculation ($p=0.001$), with significantly longer roots for isolates 45 ($p=0.035$), 68 ($p<0.001$), 69 ($p<0.001$) and 148 ($p=0.021$), compared to the control. Shoot dry weight was also affected by bacterial inoculation ($p=0.002$), with significantly heavier dry shoots for isolate 49 ($p=0.008$), compared to the control. Finally, root dry weight was unaffected by bacterial inoculation ($p=0.136$). Isolate selection for field trials was based on the occurrence of significant plant growth abilities for at least one plant measurement and divergent PGP traits of the isolates.

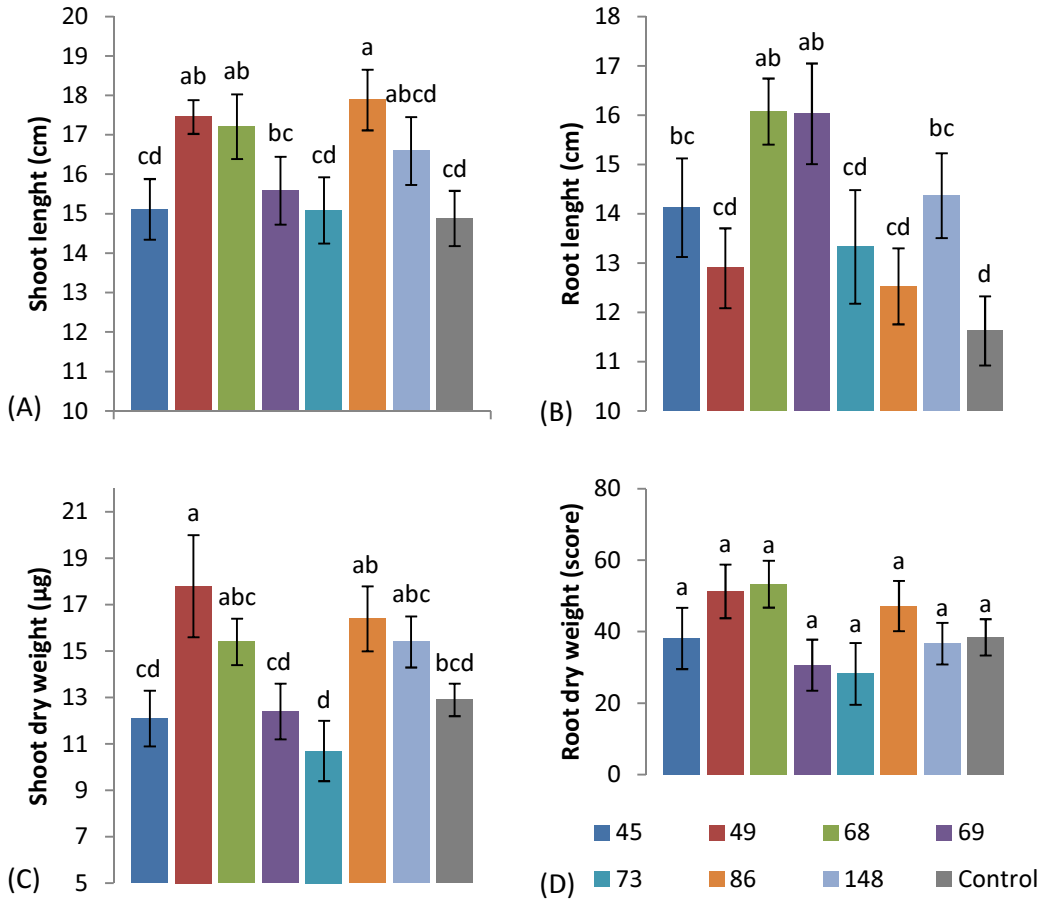

Figure 4. Shoot and root length (A and B) and dry mass (C and D) from *in vivo* pot trials under greenhouse conditions with bacterial inoculation of different isolates and a non-inoculated control. Means (±1 SE) with different letters represent statistical significance ($p<0.05$ Fisher's LSD test). Data from graph "d" was subjected to the Kruskal-Wallis test and represent mean scores.

Table 2. Bacteria tested as possible PGPR in greenhouse and field conditions.

Isolate name and source	Isolate species	IC production	Phosphate SI	Siderophore SI	Nase activity	Plant growth promotion	
						Pot trial	Field trial
45, ZR	*Burkholderia* sp.	119.45	1.13	3.31	48.953	root length	Only at Z condition
49, ZR	*Enterobacter* sp.	248.54	0.184	1.32	-	shoot length, shoot dry weight	nt
68, LA	*Enterobacter* sp.	339.08	0.120	0.333	-	root length, shoot length	Only at Lcondition
69, LA	*Enterobacter* sp.	248	0.131	0.231	30.291	root length	nt
73, LA	*Pantoea* sp.	133.67	0.126	1.00	1.324	none	nt
86, LA	*Azorhizobium* sp.	173.14	-	0.380	-	shoot length	Only at Lcondition
148, HA	*Rhizobium* sp.	139.89	-	0.351	2.337	root length	nt

Nase activity is expressed as µmol acetylene mg protein^{-1}, IC production as µg IC ml^{-1}, and phosphate and siderophore as their solubilization indexes. Plant growth promotion during the pot trials relative to significant differences from the control ($p<0.05$, Fisher LSD test). Plant growth promotion during the field trials relative to significantly longer shoots compared to any treatment within the indicated fertilizer conditions ($p<0.05$, simple main effect analysis). nt = not tested, - = did not show this PGP trait

Field trials under different fertilization conditions

Pre-flowering and pre-harvest shoot length measurements according to fertilization and bacterial treatment are shown in Figure 5. In the pre-flowering stage, there was significant interaction between bacterial and fertilization treatments for shoot length ($p=0.013$). In the Z condition, isolate 45, a good phosphate solubilizer, promoted higher shoot length compared to control ($p=0.042$), to isolate 68 ($p=0.017$) and to isolate 86 ($p=0.004$), the last two identified as good IC producers. There were no significant differences in shoot length between isolates for the L ($p=0.077$) or H conditions ($p=0.514$), although isolate 86 presented a remarkably higher mean shoot length compared to isolate 45 in the L condition. In the pre-harvest stage, there was again significant interaction between bacterial and fertilization treatments for shoot length ($p=0.001$). Bacteria affect shoot length in the L condition, isolate 45 presenting a lower shoot length compared to control ($p=0.023$), isolate 68 ($p<0.001$) and isolate 86

(p=0.001). There were no significant differences in shoot length between isolates for the Z (p=0.353) or H conditions (p=0.065), although isolate 86 presented again a lower mean shoot length compared to isolate 45 in the H condition. Table 2 summarizes which isolates were significantly different for other treatments in the same fertilization condition.

Both in pre-flowering and pre-harvesting stages, the shoot lenght in presence of isolates 86 and 68 at L and H conditions did not differ from control (p=0.119 and p=0.973 respectively). These results indicate that isolates 86 and 68 can promote plant growth and possibly save 50% of fertilizer input compared to a non-inoculated, fully fertilized control without plant shoot length reduction.

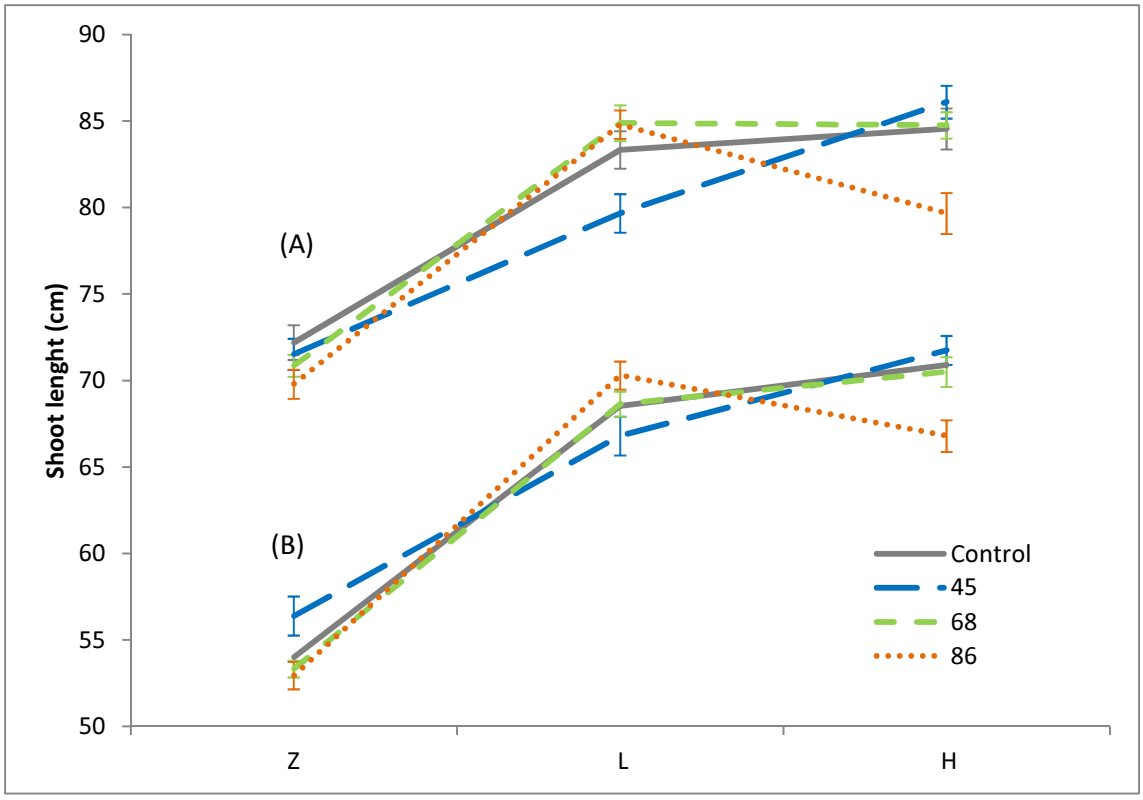

Figure 5. Pre-flowering (A) and pre-harvest (B) shoot length means (presented with ±1 SE) according to fertilization and bacterial treatment. There were significant interactions at the pre-flowering (p=0.013) and the pre-harvest stages (p=0.001), according to factorial two-way ANOVA, indicating that isolate plant growth promotion depends on fertilization conditions.

Discussion

Effect of fertilization on bacterial community composition and diversity

Our diversity analysis results suggest that fertilization condition did not profoundly affect rhizospheric and root-associated diazotrophic communities because over half of the diversity found for every condition shared the same genus composition in similar proportions, and the diversity indexes did not show either a continual increase or decrease in diazotrophic diversity according to fertilization conditions. The only fertilization-dependent alteration found was that the seven ubiquitous genera present in all of the conditions became less abundant. It would be expected to find greater diversity in fertilized soils because an increase in the nutrient availability through fertilization can sustain a larger diazotrophic population (Yim, et al., 2009; Islam et al., 2010; Pariona-Llanos et al., 2010). However, the Shannon diversity indexes was not associated with the fertilization conditions. Apparently, different fertilization conditions

presented only a minor impact on diazotrophic diversity, leaving the core of the community mostly undisturbed. Similar results were found both by lack of correlation of the Shannon diversity indexes according to fertilizer amendment (Liu et al., 2009) and the minor effect of fertilization practices on diazotrophic community composition (Piceno & Lovell, 2000; Wartiainen, 2008). In other bacterial groups, such as ammonia oxidizers and denitrifiers, the bacterial communities also suffered little change when interacting with fertilizers (Enwall et al., 2005).

Effects of fertilization on the occurrence of PGP traits

Several reports have shown an increase in the number of nitrogen-fixing bacteria in chemical fertilized conditions compared to unfertilized conditions (Yim et al., 2009; Islam et al., 2010; Pariona-Llanos et al., 2010). This increase likely occurs because of the greater plant exudation caused by the increase of available nutrients as greater root exudation increases rhizospheric and root-associated bacterial populations (Piceno & Lovell, 2000). Unfortunately, we were unable to assess if there were any differences in actual nitrogen fixation rates *in situ* according to the fertilization condition. The number of phosphate solubilizers, IC and siderophore producers were also unaffected by different fertilization conditions in other reports (Rodgers & Chaw, 2001; Hu et al., 2009; Yim et al, 2009; Yuan et al., 2010).

Effects of fertilization on the phosphate solubilization index and indolic compounds production

Together, these data suggest that in an unfertilized condition, plants are able to select the best phosphate solubilizers in intimate association with the roots in lieu of the best IC producers, present on the rhizosphere. When under light fertilization conditions, however, plants instead select the best IC producers in intimate association with the roots in lieu of the best phosphate solubilizers, also present on the rhizosphere. Apparently, plants select phosphate solubilizers in nutrient-poor conditions and IC producers in moderate nutrient conditions. However, in nutrient abundant conditions, there was no significant difference between the phosphate SI or IC production of root-associated and rhizospheric bacteria. Apparently, when under heavy fertilization conditions, plants no longer select associated bacteria based on their phosphate SI or IC production capacities. These findings may be important when testing for PGPR in different field conditions: a good IC producer may be unable to successfully colonize

plant roots (and thus promote plant growth) in nutrient-poor conditions because the plant selects for nutrient solubilizing bacteria. These findings may also have implications for PGPR prospection: if a good IC producer is sought, root-associated bacteria in lightly fertilized fields are promising targets. If phosphate starvation is an issue in the studied crops, PGPR in root-associated bacteria under unfertilized conditions are promising targets.

There are few reports of the effects of fertilization on IC producing and phosphate solubilizing plant-associated bacteria according to different fertilization conditions. Although Yuan et al. (2010) found similar levels of IC production under unfertilized and NPK fertilized conditions, we found that IC production was higher for isolates from light fertilization conditions. This difference could be due to sample size, which was three times larger in the present study. Also, unlike Yuan et al. (2010), we isolated root-associated bacteria, that produced more IC then soil bacteria in the L condition. Yim et al. (2009) also screened for IC producing bacteria under different fertilization conditions but did not study the effects of fertilizer on IC production levels. Hu et al. (2009) found less active phosphate solubilizers in soils fertilized with organic manure, while NPK fertilization was similar to an unfertilized control. In general, however, bacterial metabolic activities for phosphate solubilization and mineralization were lowered by fertilizers with high P input. In addition, Hu found that P-deficient soils resulted in higher metabolic activity of phosphate solubilizers compared to P-amended soils. Although Hu (2009) found no similar reports for his findings, we now support his conclusions with our own data, as the best phosphate solubilizers were found in the unfertilized condition.

It is well known that plant nutrition changes root exudates and thus associated bacteria, but the present study is the first report to show a preference for IC producing bacteria over phosphate solubilizing bacteria according to NPK fertilization. A moderate but significant ($p<0.0001$) inverse correlation between phosphate solubilization and IC production was found in the studied isolates. Figure 3 shows a lack of isolates able to both produce IC and solubilize phosphate above a certain limit, although there are bacteria able to present sufficient IC production to promote plant growth through this PGP trait while at the same time also solubilize enough phosphate to promote plant growth. Most importantly, this correlation supports our hypothesis that the plant selects either good IC producers or good phosphate solubilizers to associate with its roots, and apparently the selection occurs according to nutrient levels. The

reasons for this limitation still need to be studied, but it does not appear to be inducted by molecular mechanisms, as Bianco & Defez (2010) reports an increase in phosphate solubilization in an IC super producing, genetically engineered bacteria. Notably, our work is different because we took a sample from natural indigenous bacteria, whereas these authors artificially overexpressed IC production in a single strain. Thus, improvement of phosphate solubilization by high IC production does not appear to occur in nature.

Effects of NPK fertilization on siderophore production and acetylene reduction

The reasons behind the effect of heavy NPK fertilization on rhizospheric bacteria siderophore production still need to be studied because we were unable to explain it with our current data. A single report shows that the addition of straw or manure on crop soil does not change siderophore production compared to an untreated control (Rodgers & Chaw, 2001), but no other studies have been published regarding the effects of chemical fertilization on siderophore production. A siderophore-IC production correlation (n=108) was also analyzed using spearman's coefficient because the shape in the scatter graphic was similar to Figure 3. The correlation was significant ($p=0.03$), although ($rs= - 0.2$). However, this particular finding should be approached cautiously: the correlation is not significant depending on the formula used for the SI calculation, halo size measurements for siderophore production are not well established, and there are no data in the literature with which to even remotely compare this finding. Nase activity of the isolates was not affected by fertilization conditions. Similar results were found by Piceno & Lovell (2000), who observed that acetylene reduction rates were not uniformly affected by the presence of chemical fertilizers. Because diazotrophic diversity only slightly changed due to fertilization conditions, it is not surprising that the nitrogen fixing potential of these isolates was also unchanged.

Pot trials under greenhouse conditions

Pot trials under greenhouse conditions successfully allowed us to select PGPR candidates for field tests. Isolates 68 and 49 promoted growth in two plant measurements; isolates 45, 69, 86 and 148 promoted growth in one plant measurement; and isolate 73 did not promote plant growth. As isolates 68 and 49 (both *Enterobacter* sp.) presented similar PGP traits (high IC production and low phosphate SI), only isolate 68 was tested in field. Isolate 45 (*Burkholderia* sp.) was selected due to its high

phosphate SI ability. Isolate 86 (*Azorhizobium* sp.) was selected due to its high IC production and its inability to solubilize phosphate.

Field trials under different fertilization conditions

Field trials results support our hypothesis that good phosphate solubilizers should promote plant growth in condition Z and that good IC producers should promote growth in the L condition. The isolates showed better performance in the fertilization conditions that they were initially isolated from (Table 2), which reinforces our suggestion that PGPR prospection should take fertilization conditions into account when searching for PGPR candidates. Additionally, isolates 86 and 68 in the L condition were similar to the fully fertilized, non-inoculated controls and thus could be used to reduce fertilizer inputs without decreasing plant size. In the H condition, bacterial IC production and phosphate SI were not as important to drive plant growth; although both good IC producers promoted remarkably different growth rates, the good phosphate solubilizer presented a slightly higher growth rate when phosphate was readily available. Other factors, such as bacterial genome, unscreened PGP traits, and other members of the diazotrophic community, might better explain plant-PGPR responses in the H condition. Figure 5 shows the same visual pattern for all of the treatments under all of the fertilization conditions, except for a sharp decrease in isolate 45 in the Z condition at the pre-harvesting stage. This decrease in plant growth promotion according to plant maturity was unexpected, but an important point of our model still applies: good IC producers do not promote plant growth under Z conditions.

Concluding remarks

Although SI measurements may not be the best tool to quantify phosphate solubilization, sufficient data support our conclusions. Our statistical tests on phosphate solubilization were generally significant, even using nonparametric statistics, and used a large sample size. Despite the methodological differences, our overall phosphate solubilization result also confirms those of Hu (2009). Both SI and spectrophotometrically measured phosphate solubilization may decrease due to certain stress (Ahemad & Khan, 2011). Additionally, phosphate solubilization followed exactly the opposite path of IC production: ZA presented the highest SI and lowest IC production, whereas LA presented the lowest phosphate SI and highest IC production. Finally, we found a significant inverse correlation between phosphate SI and IC

production. Thus, despite phosphate SI measurements being an indirect method for phosphate solubilization quantification, we performed several statistical tests that independently corroborate the hypothesis that fertilization produces different effects on phosphate solubilization and IC production.

The calculation of the phosphate solubilization indexes, which is based on halo size measurements, differs between authors. To ensure that we were not using a biased calculation, we also calculated SI as [(zone size including colony diameter /colony diameter) x 100] (Hu X et al., 2009), and (zone size including colony diameter - colony size) (Chatli & Beri 2008; Nautiyal, 2009; Trivedi et al., 2007) and subsequently used this equations in all tests for phosphate solubilization, siderophore production and the correlation of both against IC production. All statistical outcomes remained unchanged despite the SI calculation formula change, except for the siderophore and IC production correlation for the (zone size including colony diameter - colony size) SI formula ($p=0.12$). These results reinforce the use of colony size as a suitable enough tool to quantify phosphate solubilization (at least at our sample size), but we hesitate to consider the siderophore correlation as a real biological event rather than a methodology artifact.

Our PGP traits analysis suggests that plants select for good phosphate solubilizers in the Z condition and good IC producers in the L condition. Our field results showed that good phosphate solubilizers promoted growth in Z conditions, and good IC producers promoted growth at L conditions, at one or another stage of plant maturity. Our hypothetical assumptions based on PGP trait screening from rice plants under different fertilization conditions were thus experimentally confirmed.

The fact that plants select for one PGP trait over another has interesting implications on PGPR prospector search for bacteria in the right location (roots or rhizosphere) at the right fertilization condition, according to researcher needs and interests. Moreover, effective PGPRs, such as isolates 68 and 86, might fail to promote plant growth under completely non-fertilized conditions. Additionally, isolates promoted more plant growth in the fertilization conditions that they were originally isolated from. This observation is especially interesting because most PGPR prospection searches for usable bacteria at fully fertilized field conditions and tests them at 50% fertilization conditions. Confirmation of these results with other crops and bacterial isolates would be of great importance for PGPR research.

References

Adesemoye, a O., Torbert, H. a, & Kloepper, J. W. (2009). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microbial ecology*, *58*(4), 921-9.

Adesemoye, A. O., & Kloepper, J. W. (2009). Plant-microbes interactions in enhanced fertilizer-use efficiency. *Applied microbiology and biotechnology*, *85*(1), 1-12.

Ahemad, M., & Khan, M. S. (2011). Effects of insecticides on plant-growth-promoting activities of phosphate solubilizing rhizobacterium Klebsiella sp . strain PS19. *Pesticide Biochemistry and Physiology*, *100*(1), 51-56.

Ahmad, F., Ahmad, I., & Khan, M. S. (2005). Indole Acetic Acid Production by the Indigenous Isolates of Azotobacter and Fluorescent Pseudomonas in the Presence and Absence of Tryptophan. *Turk J Biol*, *29*, 29-34.

Alves, B. J. R., Boddey, R. M., & Urquiaga, S. (2003). The success of BNF in soybean in Brazil. *Plant and Soil*, *252*(1) 1-9.

Ambrosini, A., Beneduzi, A., Stefanski, T., Pinheiro, F.G., Vargas, L.K. and Passaglia, L.M.P. (2011) Screening of Plant Growth Promoting Rhizobacteria Isolated from Sunflower (Helianthus annuus L.). *Plant and Soil*, in press.

Altschul SF,Madden TL, SchaefferAA, Zhang J, Zhang Z,Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*, 3389–3402

Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology letters*, *32*(11), 1559-70.

Baldani, J. I., Baldani, V. L. D., Seldin, L., & Dobereiner, A. N. D. J. (1986). a Root-Associated Nitrogen-Fixing Bacterium. *International Journal of Systematic Bacteriology*, *36*(1) 86-93.

Beneduzi, A., Peres, D., Beschoren, P., Helena, M., Zanettini, B., Maria, L., & Passaglia, P. (2008). Genetic and phenotypic diversity of plant-growth-promoting bacilli isolated from wheat fields in southern Brazil. *Research in Microbiology*, *159*, 244-250.

Berg G., & Smalla K. (2009). Plant species and soil type coopera- tively shape the structure and function of microbial com- munities in the rhizosphere. *FEMS Microbiol Ecology 68*, 1– 13.

Bianco, C., & Defez, R. (2010). Improvement of phosphate solubilization and Medicago plant yield by an indole-3-acetic acid-overproducing strain of Sinorhizobium meliloti. *Applied and environmental microbiology*, *76*(14), 4626-32.

Boddey, R.M. (1987) Methods for quantification of nitrogen fixation associated with gramineae. *Critical Reviews in Plant Sciences, 6*, 209–266.

Burris, R.H. (1972) Nitrogen fixation assay methods and techniques. *Methods in Enzymology, 24*, 415–431.

Carlier, E., Rovera, M., Rossi Jaume, A., & Rosas, S. B. (2008). Improvement of growth, under field conditions, of wheat inoculated with Pseudomonas chlororaphis subsp. aurantiaca SR1. *World Journal of Microbiology and Biotechnology, 24*(11), 2653-2658.

Chatli, A. S., & Beri, V. (2008). Isolation and characterisation of phosphate solubilising microorganisms from the cold desert habitat of Salix alba Linn . in trans Himalayan region of Himachal Pradesh. *Indian Journal of Microbiology*, 48(2), 267-273,

Döbereiner J (1988). Isolation and identification of root associated diazotrophs. *Plant and Soil, 110*(2):207–212

Enwall, K., Philippot, L., & Hallin, S. (2005). Activity and Composition of the Denitrifying Bacterial Community Respond Differently to Long-Term Fertilization Activity and Composition of the Denitrifying Bacterial Community Respond Differently to Long-Term Fertilization. *Applied and enviromental microbiology*, *71*(12).

Farina, R., Beneduzi, A., Ambrosini, A., de Campos, S. B., Lisboa, B. B., Wendisch, V., Vargas, L. K., et al. (2012). Diversity of plant growth-promoting rhizobacteria communities associated with the stages of canola growth. *Applied Soil Ecology*, *55*, 44-52.

Food and Agriculture Organization of the United Nations (FAO) & Sims, Ralph E.H.(2011) *"Energy-smart" food for people and climate : Issue paper / [Ralph E.H. Sims]* FAO , Rome. 78 pages.

Glick, B., Penrose, D., & Li, J. (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *Journal of theoretical biology*, *190*(1), 63-8.

Glickmann, E. & Dessaux, Y. (1995). A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Applied Environmental Microbiology 61*, 793–796.

Good, A. G., & Beatty, P. H. (2011). Fertilizing Nature: A Tragedy of Excess in the Commons. *PLoS Biology*, *9*(8), e1001124.

Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology*, *60*(4), 579-598.

Hu X.-juan, Li, . Z.-jia & Jun, Y.-cheng C. (2010). Isolation and identification of a phosphate-solubilizing bacterium *Pantoeastewartii* subsp. *stewartii* g6, and effects of temperature, salinity, and pH on its growth under indoor culture conditions. *Aquaculture International*, *18*(6) 1079-1091.

Hu, J., Lin, X., Wang, J., Chu, H., Yin, R., & Zhang, J. (2009). Population size and specific potential of P-mineralizing and -solubilizing bacteria under long-term P-deficiency fertilization in a sandy loam soil. *Pedobiologia*, *53*(1), 49-58.

Inselsbacher, E., Wanek, W., Ripka, K., Hackl, E., Sessitsch, A., Strauss, J., & Zechmeister-Boltenstern, S. (2010). Greenhouse gas fluxes respond to different N fertilizer types due to altered plant-soil-microbe interactions. *Plant and Soil*, *343*(1-2), 17-35.

Islam, R., Trivedi, P., Madhaiyan, M., Seshadri, S., Lee, G., Yang, J., Kim, Y., et al. (2009). Isolation, enumeration, and characterization of diazotrophic bacteria from paddy soil sample under long-term fertilizer management experiment. *Biology and Fertility of Soils*, *46*(3), 261-269.

Liu, X.-Z., Zhang, L.-M., Prosser, J. I., & He, J.-Z. (2009). Abundance and community structure of sulfate reducing prokaryotes in a paddy soil of southern China under different fertilization regimes. *Soil Biology and Biochemistry*, *41*(4), 687-694.

Miransari, M. (2011). Soil microbes and plant fertilization. *Applied microbiology and biotechnology*, *92*(5), 875-85.

Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters*, *170*(436), 265-270.

Pariona-Llanos, R., Ibañez de Santi Ferrara, F., Soto Gonzales, H. H., & Barbosa, H. R. (2010). Influence of organic fertilization on the number of culturable diazotrophic endophytic bacteria isolated from sugarcane. *European Journal of Soil Biology*, *46*(6), 387-393.

Piceno, Y., & Lovell, C. (2000). Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. *Microbial ecology*, *39*(1), 32–40.

Rodgers-Gray, B. S., & Shaw, M. W. (2001). The effect of incorporating straw or manure into the soil on the natural microflora of winter wheat. *Plant Pathology*, *50*(5), 537-545.

Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem 160*, 47–56.

Seldin, L., van Elsas, J.D., Penido, E.G.C. (1983) Bacillus nitrogens fixers from Brazilian soils. *Plant and Soil 70*, 243-255.

Simpson, R. J., Oberson, A., Culvenor, R. a., Ryan, M. H., Veneklaas, E. J., Lambers, H., Lynch, J. P., et al. (2011). *Strategies and agronomic interventions to improve the phosphorus-use efficiency of farming systems*. *Plant and Soil*, *342*(1-2), 89-120.

Singh, J. S., Pandey, V. C., & Singh, D. P. (2011). Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development. *Agriculture, Ecosystems & Environment*, *140*(3-4), 339-353.

Sokolova, M. G., Akimova, G. P., & Vaishlya, O. B. (2011). Effect of phytohormones synthesized by rhizosphere bacteria on plants. *Applied Biochemistry and Microbiology*, *47*(3), 274-278.

Trivedi, P., Kumar, B., Pandey, A., & Palni, L. M. S. (2007). Growth promotion of rice by phosphate solubilizing bioinoculants in a Himalayan location. *Developments in Plant and Soil Sciences, 102*, 291-299.

Wartiainen, I. (2008). Variation in the active diazotrophic community in rice paddy—nifH PCR-DGGE analysis of rhizosphere and bulk soil. *Applied Soil Ecology, 39*(1), 65-75.

Yim, W.-J., Poonguzhali, S., Madhaiyan, M., Palaniappan, P., Siddikee, M. a., & Sa, T. (2009). Characterization of plant-growth promoting diazotrophic bacteria isolated from field grown Chinese cabbage under different fertilization conditions. *The Journal of Microbiology, 47*(2), 147-155.

Yuan, C.-L., Mou, C.-X., Wu, W.-L., & Guo, Y.-B. (2010). Effect of different fertilization treatments on indole-3-acetic acid producing bacteria in soil. *Journal of Soils and Sediments, 11*(2), 322-329.

Zabihi, H. R., Savaghebi, G. R., Khavazi, K., Ganjali, a., & Miransari, M. (2010). Pseudomonas bacteria and phosphorous fertilization, affecting wheat (Triticum

aestivum L.) yield and P uptake under greenhouse and field conditions. *Acta Physiologiae Plantarum*, *33*(1), 145-152.

4. Considerações finais

Todos os resultados obtidos até o momento puderam ser inseridos no manuscrito a ser submetido à revista científica, mas há, ainda, outros resultados a serem incorporados na versão final.

Apenas um parâmetro (estatura da planta) foi considerado nos testes em campo. Outros critérios agronômicos importantes, como a análise de nutrientes no estágio de pré-florescimento, a produção por hectare, o número de grãos por panícula e o número de panículas por m², serão medidos, processados e incluídos no manuscrito antes da sua submissão à revista científica. A inclusão desses critérios permitirá a realização de uma MANOVA entre os diversos parâmetros estudados para melhor verificar a ação das bactérias.

Com os dados analisados até agora, podemos concluir que:

1 – A fertilização a longo prazo teve pequenos efeitos na diversidade de bactérias diazotróficas associadas ao arroz. Este resultado está de acordo com a literatura e mostra a resiliência da comunidade bacteriana diazotrófica diante dessa alteração ambiental.

2 – A fertilização altera profundamente a seleção da planta por bactérias que produzam compostos indólicos ou solubilizem fosfato, de tal forma que:

a) Em condições escassas de nutrientes a planta busca associar-se a bactérias que sejam boas solubilizadoras de fosfato.

b) Em condições de disponibilidade moderada de nutrientes a planta busca associar-se a bactérias que sejam boas produtoras de compostos indólicos.

c) Em condições de abundância de nutrientes, a seleção com base na solubilização de fosfato e produção de composto indólicos deixa de acontecer.

Estes resultados podem ser utilizados na prospecção de PGPRs, de tal forma que o investigador possa buscar bactérias com a caraterística de maior interesse no local certo e que esteja sobre as condições certas. Por exemplo, se ele busca bactérias solubilizadoras de fosfato, ele deve procurar por bacterias associadas a raízes em condições sem fertilizantes

3 – Há uma correlação inversa entre a solubilização de fosfato e a produção de compostos indólicos. Os resultados desse trabalho indicam que dificilmente será encontrada, na natureza, uma bactéria que seja capaz de solubilizar grandes quantidades

de fosfato e que, ao mesmo tempo, seja capaz de produzir grandes quantidades de compostos indólicos.

Este resultado reforça a proposta de que a planta faz uma seleção dualista das bactérias que vai se associar, tendo que escolher entre bactérias capazes de expressar uma ou outra característica de promoção de crescimento.

4 – Os testes em campo demonstraram que as bactérias apresentaram um desempenho melhor na promoção do crescimento das plantas quando testadas nas condições que foram originalmente isoladas, de forma que uma boa solubilizadora de fosfato promoveu o crescimento vegetal apenas na condição de ausência de nutrientes, e as boas produtoras de compostos indólicos promoveram o crescimento vegetal apenas em condições de disponibilidade moderada de nutrientes.

Estes resultados podem ser utilizados para tentar prever a eficência de PGPRs a campo, de acordo com as suas características de promoção de crescimento e os níveis de fertilização testados.

5. **Referências bibliográficas da Introdução Geral**

Adesemoye, A. O., Torbert, H. a, & Kloepper, J. W. (2009a). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microbial ecology*, *58*(4), 921-9.

Adesemoye, A. O., & Kloepper, J. W. (2009b). Plant-microbes interactions in enhanced fertilizer-use efficiency. *Applied microbiology and biotechnology*, *85*(1), 1-12.

Affourtit J, Zehr JP & Paerl HW (2001) Distribution of nitrogen-fixing microorganisms along the Neuse river estuary, North Carolina. *Microbial Ecology, 41,* 114-123.

Alves, B. J. R., Boddey, R. M., & Urquiaga, S. (2003). The success of BNF in soybean in Brazil. *Plant and Soil, 252*(1) 1-9.

Ambrosini, A., Beneduzi, A., Stefanski, T., Pinheiro, F.G., Vargas, L.K. and Passaglia, L.M.P. (2011) Screening of Plant Growth Promoting Rhizobacteria Isolated from Sunflower (Helianthus annuus L.). *Plant and Soil,* in press.

Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology letters*, *32*(11), 1559-70.

Baldani, J. I., Baldani, V. L. D., Seldin, L., & Dobereiner, A. N. D. J. (1986). a Root-Associated Nitrogen-Fixing Bacterium. *International Journal of Ssystematic Bacteriology*, *36*(1) 86-93.

Borucki, W. J., and W. L. Chameides (1984), Lightning: Estimates of the rates of energy dissipation and nitrogen fixation, *Rev. Geophys.*, 22(4), 363–372.

Carpenter, S.R., Caraco, N.F., Correl, D.L., Howarth, R.W., Sharpley, A.N.; Smith, V.H. (1998) Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications,8*(3), 559-568.

Dawson, C. J., & Hilton, J. (2011). Fertilizer availability in a resource-limited world: Production and recycling of nitrogen and phosphorus. *Food Policy*, *36*, S14-S22.

Evenson, R. E., & Gollin, D. (2003). Assessing the impact of the green revolution, 1960 to 2000. *Science*, *300*(5620), 758-62.

Farina, R., Beneduzi, A., Ambrosini, A., de Campos, S. B., Lisboa, B. B., Wendisch, V., Vargas, L. K., et al. (2012). Diversity of plant growth-promoting rhizobacteria communities associated with the stages of canola growth. *Applied Soil Ecology*, *55*, 44-52.

Food and Agriculture Organization of the United Nations (FAO) & Sims, Ralph E.H. (2011) *"Energy-smart" food for people and climate : Issue paper / [Ralph E.H. Sims]* FAO , Rome. 78 pages.

Food and Agriculture Organization of the United Nations (FAO). 1999 *FAOSTAT [electronic resource]* http://bibpurl.oclc.org/web/1250

Glick, B., Penrose, D., & Li, J. (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *Journal of theoretical biology*, *190*(1), 63-8.

Good, A. G., & Beatty, P. H. (2011). Fertilizing Nature: A Tragedy of Excess in the Commons. *PLoS Biology*, *9*(8), e1001124.

Guo, J. (2004). Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. *Biological Control*, *29*(1), 66-72.

Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology*, *60*(4), 579-598.

Inselsbacher, E., Wanek, W., Ripka, K., Hackl, E., Sessitsch, A., Strauss, J., & Zechmeister-Boltenstern, S. (2010). Greenhouse gas fluxes respond to different N fertilizer types due to altered plant-soil-microbe interactions. *Plant and Soil*, *343*(1-2), 17-35.

Lau, S.S.S., Lane, S.N. (2002) Biological and chemical factors influencing shallow lake eutrophication: a long-term study. *The Science of the Total Environment, 288* (3) 167-181.

Mansoor, F., Sultana, V., & Ehteshamul-haque, S. (2007). Enhancement of biocontrol potential of. *In Vitro*, *39*(6), 2113-2119.

Miransari, Mohammad. (2011). Soil microbes and plant fertilization. *Applied microbiology and biotechnology*, *92*(5), 875-85.

Neset, T.-S. S., & Cordell, D. (2012). Global phosphorus scarcity: identifying synergies for a sustainable future. *Journal of the science of food and agriculture*, *92*(1), 2-6.

Panhwar, Q. A., Othman, R., Rahman, Z. A., Meon, S., & Ismail, M. R. (2011). Effect of Phosphatic Fertilizer on Root Colonization of Aerobic rice by Phosphate-Solubilizing Bacteria. *Proceedings of International Conference on Food Engineering and Biotechnology*, *9*, 145-149.

Ramasamy, C. Ramanathan, S. and Dhakshinamoorthy, M. 2005. Perspectives of Agricultural Research and Development. A compilation of articles for centenary

Seminar on Recent Advances in Agricultural Research held on 5.9.2005 at Tamil Nadu Agricultural University, Coimbatore - 641 003, India.

Römheld, V., & Kirkby, E. a. (2010). Research on potassium in agriculture: needs and prospects. *Plant and Soil*, *335*(1-2), 155-180.

Simpson, R. J., Oberson, A., Culvenor, R. a., Ryan, M. H., Veneklaas, E. J., Lambers, H., Lynch, J. P., et al. (2011). *Strategies and agronomic interventions to improve the phosphorus-use efficiency of farming systems*. *Plant and Soil* (pp. 89-120).

Singh, J. S., Pandey, V. C., & Singh, D. P. (2011). Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development. *Agriculture, Ecosystems & Environment*, *140*(3-4), 339-353. Elsevier B.V.

Sokolova, M. G., Akimova, G. P., & Vaishlya, O. B. (2011). Effect of phytohormones synthesized by rhizosphere bacteria on plants. *Applied Biochemistry and Microbiology*, *47*(3), 274-278.

Spiess, E. (2011). Nitrogen, phosphorus and potassium balances and cycles of Swiss agriculture from 1975 to 2008. *Nutrient Cycling in Agroecosystems*, 351-365.

UNEP and WHRC. Reactive Nitrogen in the Environment: Too Much or Too Little of a Good Thing. *United Nations Environment Programme*, Paris, 2007.

Wei, G., Kloepper, J.W., & Tuzun, S. (1991). Induction of systemic resistence of cucumber to *Colletotrichum orbiculare* by select of strains of plant growth-promoting rhizobacteria. *Phytopatholog, 81*,1508-1512

Yuan, C.-L., Mou, C.-X., Wu, W.-L., & Guo, Y.-B. (2010). Effect of different fertilization treatments on indole-3-acetic acid producing bacteria in soil. *Journal of Soils and Sediments*, *11*(2), 322-329.

Zehr J.P., Carpenter E.J. e Villareal T.A. (2000). New perspectives on nitrogen-fixing microorganisms in tropical and subtropical oceans. *Trends in Microbiology*, *8*(2), 68-73.