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PROTECTION AGAINST BIOTIC AND ABIOTIC STRESSES IN COMMON BEAN BY RHIZOBACTERIA

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/ Fitopatologia, área de concentração em Bacteriologia/Controle biológico, para obtenção do título de Doutor.

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LAVRAS – MG
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RESUMO GERAL

Estresses bióticos e abióticos podem interferir com o desempenho de rizobactérias. Com este estudo objetivou-se a: (a) avaliar o efeito dos compostos orgânicos voláteis (VOCs) de *Bacillus amyloliquefaciens* e *B. subtilis* ALB629 UFLA285 no controle da antracnose (*Colletotrichum lindemuthianum* - *Cl*); (b) avaliar a eficácia de ALB629 na promoção de crescimento, absorção de nutrientes e na contribuição da suplementação de Mg nas taxas fotossintéticas, assimilação de CO_2, teor de clorofila e controle da murcha-de-curtobacterium (MCB) causada por *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) na presença de ALB629; (C) descobrir se os exsudados provenientes de sementes são capazes de interferir na formação de biofilme e no crescimento de ALB629; (d) avaliar o desempenho do ALB629 na sanidade da planta sob estresse hídrico. Experimentos *in vitro* e *in vivo* foram desenvolvidos para avaliar o número de esporos e o crescimento micelial de *Cl*, assim como o controle da antracnose na presença dos VOCs de ALB629 e UFLA285. Plantas provenientes de sementes tratadas com ALB629 foram avaliadas quanto às variáveis de crescimento, fotossíntese e controle de MCB em solo com diferentes teores de Mg (0-50 mg kg^{-1}). Alternativamente, ALB629 foi avaliado quanto a formação de biofilme e quanto às variáveis relacionadas às plantas sob estresse hídrico. ALB629 reduziu o número de esporos (31%), e UFLA285 e ALB629 inibiram o crescimento micelial de *Cl* (16-18%), respectivamente. Os VOCs controlaram a antracnose *in vivo* (79-85%) e foram identificados como sendo 3-hidroxi-2-butanona, ácido 3-metilbutanoico e ácido 2-metilbutanoico. Na dose de 25 mg kg^{-1} de Mg, foi verificado um aumento na acumulação de CO_2 no mesofilo da folha para ALB629 e controle, indicando baixa fixação de CO_2 e baixa atividade da Rubisco. As maiores doses de Mg causaram um aumento no teor de clorofila e fotossíntese em plantas tratadas com a rizobacteria. Além disso, a 25 mg kg^{-1} de Mg, houve um aumento no teor de clorofila para ALB629 (30%) e uma redução na severidade da MCB (51%). Além disso, a fotossíntese foi negativamente correlacionada com a doença ($r = -0,53$, $p<0,01$). Exsudato de sementes aumentou o biofilme e o crescimento de ALB629 tanto *in vitro* e na superfície da semente. Houve um aumento na expressão em ALB629 de *TASA* e *EPSD*, ca. 2- e 6 vezes, respectivamente. Plântulas de sementes tratadas com $ALB629^{rif\text{-}Nal}$ mostraram uma maior concentração da bactéria quando o ácido málico estava presente, promoveu o crescimento da planta e maior tolerância à seca. Este estudo mostrou que UFLA285 e ALB629 desempenham um papel importante na melhoria da sanidade ao feijoeiro contra estresses bióticos e abióticos.

Palavras-chave: Biocontrole. *Colletotrichum lindemuthianum. Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. Fotossíntese. Estresse abiótico.

GENERAL ABSTRACT

Biotic and abiotic stresses may interfere with the performance of plant-associated rhizobacteria. The objectives of this study were: (a) to evaluate the effect of the volatile organic compounds (VOCs) of *Bacillus amylolicefaciens* ALB629 and *B. subtilis* UFLA285 in anthracnose (*Colletotrichum lindemuthianum* - *Cl*) control; (b) to evaluate the effectiveness of ALB629 in promoting plant growth, nutrient uptake and the contribution of Mg supplementation to photosynthetic rates, CO_2 assimilation, chlorophyll content, and bacterial wilt (BW) control caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) at ALB629 presence; (c) to find out whether exudates from seeds are able to interfere with ALB629 biofilm formation and growth; (d) to check the performance of ALB629 on plant health under drought stress. *In vitro* and *in vivo* tests were set up to assess the spore numbers and the mycelial growth of *Cl* as well as anthracnose control in the presence of ALB629 and UFLA285 VOCs. Additionally, bean plants from seeds treated with ALB629 were assessed for growth promotion-related variables, photosynthetic-related variables, and BW control when plants were grown in soil with different Mg contents (0–50 mg kg^{-1}). Alternatively, ALB629 was tested for biofilm formation and for its effect on the plant-related variables under drought stress. ALB629 reduced spore numbers (31%), while UFLA285 and ALB629 inhibited mycelial growth of *Cl* (16–18%), respectively. Both bacterial volatiles controlled anthracnose *in vivo* (79–85%) and were identified as 3-hydroxy-2-butanone, 3-methylbutanoic acid, and 2-methylbutanoic acid. At 25 mg kg^{-1} Mg, an increased accumulation of CO_2 was found in the leaf mesophyll of the ALB629 and control, indicating low CO_2 fixation and low Rubisco activity. Higher doses of Mg caused an increase in chlorophyll content and in photosynthetic rates in rhizobacterium-treated plants. Furthermore, at 25 mg kg^{-1} Mg, there was an increase in chlorophyll content in ALB629 (30%) and a reduction in BW severity (51%). Moreover, photosynthesis was negatively correlated with BW ($r = -0.53$, $p<0.01$). Seed exudates increased ALB629 biofilm and the ALB629 cell counts both in culture and on the bean seed surface. Furthermore, seed exudates up-regulated biofilm operons in ALB629 *TasA* and *EpsD* by about 2- and 6-fold, respectively. Seedlings from seeds treated with $ALB629^{rif\text{-}nal}$ showed a higher concentration of the bacteria when the malic acid was present, showed a promotion in plants growth and imparted drought tolerance. This study showed that UFLA285 and ALB629 play a major role in improving common bean health against biotic and abiotic stresses.

Keywords: Biocontrol. *Colletotrichum lindemuthianum. Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. Photosynthesis. Abiotic stress.

SUMÁRIO

PRIMEIRA PARTE

1. INTRODUÇÃO GERAL

O feijão comum (*Phaseolus vulgaris* L.) é a leguminosa de cultivo mais difundido no mundo, e tem grande importância econômica e social para o Brasil. A cultura está implantada em praticamente todo o território nacional, ocupando lugar de destaque na constituição da dieta do brasileiro, por ser, reconhecidamente, excelente fonte de proteínas, possuir carboidratos complexos, fibras, vitaminas e micronutrientes (CENTRO INTERNACIONAL DE AGRICULTURA TROPICAL - CIAT, 2002).

Segundo dados da Companhia Nacional de Abastecimento - CONAB (2016), o Brasil é o maior produtor e consumidor mundial, com a produção na safra de 2014/2015 de 3.151,2 mil toneladas e uma área plantada de 2.977,5 mil hectares. Minas Gerais é o segundo maior produtor do país contribuindo com 527,1 mil toneladas o que representa 18% da produção nacional.

A cultura, tradicionalmente conduzida por pequenos agricultores na safra de verão nos últimos anos teve alta rentabilidade, passando a ser cultivada em diversas épocas do ano, principalmente em cultivos irrigados no inverno, por grandes produtores e com emprego de alta tecnologia. Entretanto, apesar de todo o desenvolvimento tecnológico a cultura é considerada de risco econômico. Dentre as causas deste risco está o estresse abiótico, como a seca (AMMAR et al., 2015).

Além do estresse hídrico, as doenças representam uma das principais causas da sua baixa produtividade no Brasil, podendo causar, dependendo das condições de ambiente, perdas totais ou, inviabilizar determinadas áreas para o cultivo (LIMA et al., 2010). Além do mais, no país existe uma tradição do uso de sementes próprias (HERBES et al., 2008), que é um dos grandes entraves ao incremento da produtividade devido à disseminação de doenças. Em Minas Gerais, por exemplo, apenas 10% da área são cultivadas com sementes fiscalizadas (SENA et al., 2008).

Dentre as doenças que ocorrem na cultura do feijoeiro, a antracnose, causada pelo fungo *Colletotrichum lindemuthianum* é considerada mundialmente uma das doenças de maior importância à cultura (GILLARD; RANATUNGA, 2013), podendo causar perdas na produção superiores a 90%, sob condições favoráveis (BARDAS et al., 2009). Além da antracnose, as doenças de origem bacteriana têm causado sérios prejuízos em função da facilidade de transmissão por sementes, rápida disseminação e carência de ferramentas eficazes de controle. A semente é o principal veículo de disseminação e introdução de bactérias fitopatogênicas em novas áreas de cultivo, sendo essa, a principal forma de transmissão de *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (COLLINS; JONES, 1983; HEDGES, 1922, 1926) (*Cff*), agente da murcha-de-curtobacterium no Brasil.

Por outro lado, as rizobactérias apresentam um potencial para serem usadas no manejo de doenças e sua aplicação via tratamento de sementes pode controlar o progresso de doenças que são disseminadas por sementes (MARTINS et al., 2013), como é o caso da antracnose do feijoeiro e a murcha-de-curtobacterium. Além do controle da doença, benefícios adicionais pelas rizobactérias são proporcionados à cultura, como é o caso da promoção de crescimento (KLOEPPER; LIFSHITZ; ZABLOTOWICZ, 1989; ORHAN et al., 2006), da bioacumulação de nutrientes, como por exemplo o Ferro (FREITAS et al., 2015) e da maior tolerância da planta a estresses abióticos (MARTINS et al., 2014).

2 REFERENCIAL TEÓRICO

2.1 Antracnose do feijoeiro

Entre as doenças que ocorrem na cultura do feijoeiro, a antracnose, causada pelo fungo *Colletotrichum lindemuthianum* (*Cl*) é considerada mundialmente uma das doenças de maior importância à cultura (GILLARD; RANATUNGA, 2013). Atualmente, a doença pode ser encontrada em todos os continentes (DISCOVERLIFE, 2016), como mostra a Figura 1.

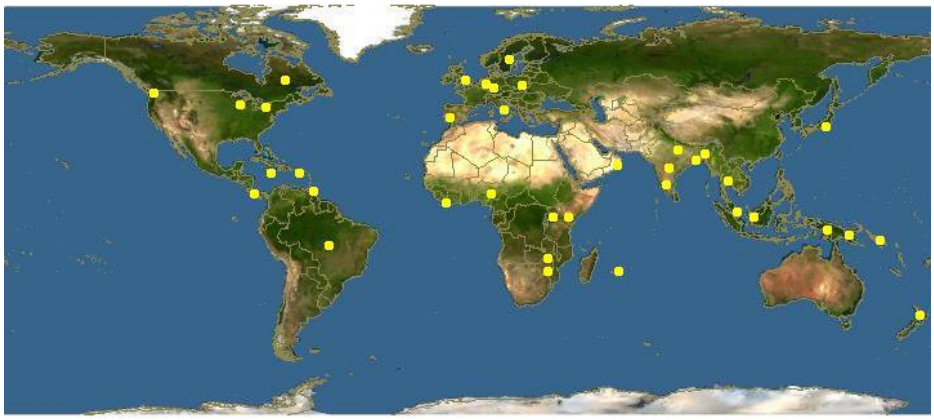

Figura 1 Distribuição geográfica de *Colletotrichum lindemuthianum*, agente etiológico da antracnose do feijoeiro. Fonte: Discoverlife (2016)

As condições favoráveis à doença são temperaturas amenas, precipitação frequente e alta umidade relativa. Sob essas condições, as perdas podem superar 90% da produção. Nas folhas os sintomas da doença iniciam-se na face abaxial, ao longo das nervuras, como pequenas manchas de cor pardo-avermelhada que se tornam de coloração café-escura a negra. Os sintomas podem também estar presentes no pecíolo, caule e vagem como lesões enegrecidas, ovaladas e deprimidas. Nas sementes os sintomas se manifestam por manchas empardecidas e deprimidas (REY et al., 2009), sendo a semente o principal meio de disseminação do patógeno a longas distâncias (SILVA; POZZA; MACHADO, 2013).

Em relação às medidas de controle da doenças atualmente empregadas estão, entre as principais, o uso de cultivares resistentes e a aplicação de fungicidas. Entretanto, a ocorrência de quebra de resistência pelo patógeno já tem sido reportada (MELOTTO; BALARDIN; KELLY, 2000; RODRIGUEZ-GUERRA et al., 2003), devido principalmente a alta variabilidade genética do patógeno; mais de 100 raças de *Cl* já foram identificadas mundialmente (MELOTTO; BALARDIN; KELLY, 2000). Além do efeito prejudicial do controle químico para o ambiente e a microorganismos não-alvo, uma melhor eficiência no controle requer aplicações frequentes (CONNER et al., 2004) e uso de mais de um produto (GILLARD; RANATUNGA; CONNER, 2012), o que aumenta o risco de resistência a doenças.

2.2 Murcha-de-curtobacterium

No Brasil há registros de ocorrência de diferentes bacterioses no feijoeiro: crestamento bacteriano comum, crestamento bacteriano aureolado, fogo-selvagem e murcha-de-curtobacterium que tem como agentes etiológicos respectivamente, *Xanthomonas axonopodis* pv. *phaseoli* (= *X. campestris* pv. *phaseoli*) (ROBBS, 1954); *Pseudomonas savastonoi* pv. *phaseolicola* (= *P. syringae* pv. *phaseolicola*) (COSTA; PARADELA, 1972); *P. syringae* pv. *tabaci* (RIBEIRO et al., 1980) e *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) (MARINGONI; ROSA, 1997). Dentre essas doenças, a murcha-de-curtobacterium é uma doença emergente e de alto risco à produção de feijão.

A doença, apesar de ser quarentenária em muitos países, atualmente encontra-se amplamente disseminada. O patógeno é capaz de causar severas perdas na produção como já ocorreu nos Estados Unidos (COYNE; SCHUSTER, 1979; DOWSON, 1957; THOMAS; GRAHAM, 1952; VENETTE; LAMPRA; GROSS, 1995), onde há relatos de até 90% de queda na produção (HEDGES, 1926). A murcha-de-curtobacterium foi detectada pela primeira vez em Dakota do Sul, EUA (HEDGES, 1922), sendo

posteriormente constatada em outros estados como Michigan, Virgínia, Maryland, Montana e Columbia (COYNE; SCHUSTER, 1979; DOWSON, 1957; HEDGES, 1922, 1926; VENETTE; LAMPRA; GROSS, 1995). A doença também foi detectada em alguns países europeus, bem como na Austrália, Canadá, México e Colômbia (COMITE DE SANIDADE VEGETAL DEL CONO SUR - COSAVE, 201).

No Brasil, *Cff* era considerada praga quarentenária, sendo que em 1995 sua ocorrência foi relatada no estado de São Paulo por Maringoni e Rosa (1997) e, a partir daí, tornou-se de importância emergente para o cultivo do feijoeiro em diferentes regiões. Há relatos de até 46,7% de perdas na produção (MIRANDA FILHO, 2006). Atualmente, há relatos de que a *Cff* tem ocorrido com frequência em lavouras de feijoeiro nos Estados de São Paulo, Minas Gerais, Paraná, Santa Catarina, Distrito Federal e Goiás causando grandes problemas à cultura (LEITE JÚNIOR et al., 2001; MARINGONI, 2002; UESUGI; FREITAS; MENEZES, 2003; THEODORO; MARINGONI, 2006) (Figura 2).

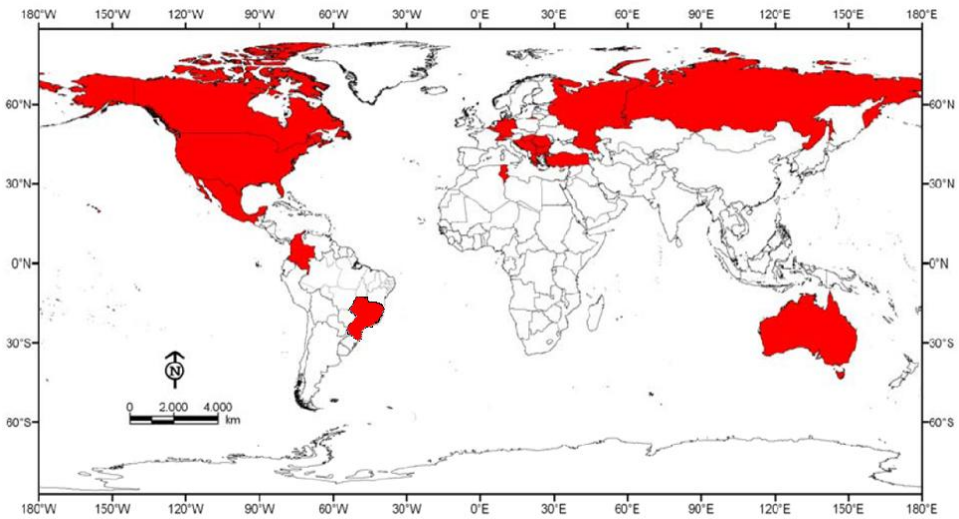

Figura 2 Modificado de Distribuição geográfica de *Curtobacterium flaccumfaciens* pv. *Flaccumfaciens*. Fonte: Bradbury (1986), Commonwealth Mycological Institute (1992) e Maringoni e Rosa (1997)

Entretanto, os sintomas podem ser erroneamente diagnosticados como murcha de fusarium ou de esclerócio. Segundo Maringoni e Rosa (1997), a murcha-de-curtobacterium pode estar ocorrendo há muito tempo na cultura do feijão no Brasil e, devido à similaridade de sintomas com a murcha-de-fusarium, causada por *Fusarium oxysporum* f.sp. *phaseoli*, ter sido confundida e não percebida anteriormente. De acordo com Hedges (1922, 1926), em alguns casos, pode-se também confundir as colônias amarelas de *Cff* com as de *X. axonopodis* pv. *phaseoli* o que dificulta o correto diagnóstico da doença, importante etapa para o controle.

Curtobacterium flaccumfaciens pv. *flaccumfaciens* é uma bactéria Gram-positiva e as colônias apresentam formato circular, bordos lisos, planas ou levemente convexas e de aspecto brilhante, com coloração variando de amarela a laranja, conforme as características descritas para o gênero (ROMEIRO, 2005).

A principal forma de disseminação de *Cff* é por sementes contaminadas oriundas de plantas doentes, não sendo disseminada por chuva e água de irrigação por ficar internamente nos tecidos vasculares. A bactéria pode penetrar na ausência de chuvas, não sendo observada a penetração via estômato.

A doença inicia-se com a seca de folíolos apicais, com posterior amarelecimento e murcha total das folhas (BIANCHINI; CARNEIRO; LEITE JÚNIOR, 2000). Uma vez na planta, *Cff* coloniza os tecidos vasculares e causa murcha e flacidez das folhas (HEDGE, 1926). Ocasionalmente, esse sintoma típico de murcha pode progredir para manchas amareladas e posteriormente necróticas, muito semelhantes ao crestamento bacteriano comum causado por *X. axonopodis* pv. *phaseoli*, no entanto a lesão marginal é mais irregular em *Cff*.

Além de sobreviver em semente mantida à temperatura ambiente por até 24 anos (BURKHOLDER, 1995), a bactéria também é capaz de sobreviver no solo. Miranda Filho (2006) verificou que pelo menos durante

dez meses, a bactéria se manteve viável e foi capaz de infectar as plantas de feijoeiro.

Poucos relatos sobre práticas de controle dessa doença foram publicados até o momento. Ainda não há cultivar imune à bactéria, embora já se tenha encontrado cultivares com diferentes graus de resistência (KRAUSE et al., 2009; SOUZA et al., 2006; VALENTINI et al., 2010). Quanto ao controle químico, não há até o momento produto registrado contra o patógeno no pais (BRASIL, 2016).

Atualmente, medidas de controle disponíveis recomendadas para o controle da murcha-de-curtobacterium incluiu o uso de sementes sadias e a prática da rotação de culturas (HERBES et al., 2008; VENETTE; LAMPRA; GROSS, 1995). Por outro lado, outros métodos como o controle biológico apresentam um potencial para serem usadas no manejo de doenças bacterianas e sua aplicação via tratamento de sementes pode controlar o progresso dessa doença (HUANG; ERICKSON; HSIEH, 2007; MARTINS et al., 2013).

2.3 Rizobactérias promotoras de crescimento

Os isolados endofiticos LRC 8311 de *Pantoea agglomerans* (HSIEH et al., 2005) e *Rhizobium leguminosarum* bv. *viceae* R21 (HUANG; ERICKSON; HSIEH, 2007), reduziram efetivamente a incidência e severidade da murcha-de-curtobacterium e promoveram aumento no crescimento de mudas de feijão. Ambos os trabalhos de prospecção de agentes de controle biológico da murcha-de-curtobacterium não resultaram em produtos disponíveis aos agricultores e, portanto ainda não são uma tecnologia recomendável para o manejo da doença. O uso de bactérias endosporogênicas para o manejo de doenças como é o caso das rizobactérias promotoras de crescimento (PGPRs) tem mais chances de resultar em um produto disponível aos agricultores pela maior facilidade de sobrevivência sob condições ambientais adversas e maior facilidade na obtenção de um

bioproduto com uma maior vida de prateleira (CHOUDHARY; JOHRI, 2009; HAYAT et al., 2010).

PGPRs são bactérias que podem estar associadas às raízes na rizosfera de várias espécies de plantas podendo atuar como promotoras de crescimento e/ou como agentes de biocontrole de doenças quando aplicadas às sementes ou raízes (KLOEPPER; LIFSHITZ; ZABLOTOWICZ, 1989). O controle biológico por PGPRs pode ser o resultado de uma combinação de mecanismos dos quais incluem a resistência induzida por compostos orgânicos voláteis microbianos (FIALHO et al., 2010; KAI et al., 2007). Indução de resistência é definida como um aumento da capacidade de defesa da planta contra uma ampla gama de patógenos e pragas, a qual é adquirida após uma adequada estimulação (RAMAMOORTHY et al., 2001).

Além do emprego das PGPRs como promotoras de crescimento e como agentes de controle biológico (tolerância a estresses bióticos), essas bactérias também podem atuar aumentando a capacidade da planta em acumular elementos essenciais, como verificado por Freitas et al. (2015), onde a aplicação de *Bacillus subtilis* GBO3 pôde aumentar a concentração de Fe em folhas de mandioca. Além da bioacumulação, outro benefício das PGPRs é o aumentando da tolerância da planta a estresses abióticos (GURURANI et al., 2013). Dentre as possíveis explicações que favorecem a atuação das PGPRs no aumento da tolerância a estresses abiótico está a formação de biofilm pelo microrganismo benéfico. Srivastava et al. (2008) verificaram que o isolado NBRI0987 de *Pseudomonas putida* pôde tolerar o estresse de 40 °C por 5 dias devido a formação de biofilme pela bactéria. Os biofilmes bacterianos são agregados multicelulares aderidos a um substrato biótico ou não que e composto de uma matriz polimérica de substâncias, como exopolissacarídeos, proteínas e às vezes DNA.

Perdas na produção devido à antracnose e à murcha-de-curtobacterium podem ser severas (BARDAS et al., 2009; MIRANDA FILHO, 2006), podendo ser maiores quando a infecção ocorre no início do cultivo. Deste modo, estratégias de controle que são empregadas no início

dos estádios da cultura podem apresentar maiores chances de controle principalmente quando se trata de patógenos transmitidos por sementes, como é o caso do *Cl* e da *Cff*. Considerando a importância das sementes na transmissão de patógenos e a necessidade de reduzir a quantidade de fungicidas aplicado no meio ambiente, o tratamento de sementes pode resultar em uma estratégia prática e de baixo custo para reduzir patógenos associados à semente.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Rhizobacterial volatiles in the control of anthracnose in common bean

Rhizobacterial volatiles in the control of anthracnose in common bean

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Rhizobacterial volatiles in the control of anthracnose in common bean

Abstract - Microbial volatile organic compounds (mVOCs) have been shown recently to be toxic to plant pathogens under *in vitro* conditions. However, there is a lack of information about its effect *in vivo*. We aimed at evaluating the effect of volatiles from rhizobacterial strains: *Bacillus amyloliquefaciens* ALB629 and UFLA285 on anthracnose (*Colletotrichum lindemuthianum*) disease control, one of the main diseases of dry bean (*Phaseolus vulgaris* L.). Primary, an *in vitro* test in bipartite Petri dish was set up to assess spore numbers and the pathogen mycelial growth in the presence of mVOCs. Also, in the absence of physical contact with plant roots, mVOCs were tested *in vivo* under growth chamber conditions to verify its effect on bean plants inoculated with *C. lindemuthianum*. The randomized complete block design with 5 and 4 replication was used for the *in vitro* and *in vivo* tests, respectively. Data were submitted to ANOVA and Tukey's multiple range tests (P=0.05) applied for significant means. ALB629 reduced spore numbers (31%), while UFLA285 and ALB629 inhibited mycelial growth by (16 and 18%), respectively. Additionally, both bacterial volatiles controlled anthracnose in the *in vivo* test (79–85%). The volatiles from bacteria were identified by solid phase micro extraction (SPME) coupled to gas chromatography with mass spectrometric detection (GC–MS) as 3-hydroxy-2-butanone, 3-methylbutanoic acid, and 2-methylbutanoic acid. This study showed that rhizobacteria volatiles have the potential to be used against common bean anthracnose and may represent a new tool for disease management.

Keywords: PGPR; *Phaseolus vulgaris*; Biocontrol; Plant disease; VOC

1 INTRODUCTION

Among the dry bean diseases, anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cav. is considered one of the most important for dry bean production worldwide (GILLARD; RANATUNGA, 2013). The disease is spread and can be found in all the continents of the world (LOWE; STAPLES; WALCOTT, 2014). Yield losses caused by this disease can be higher than 90% under favorable conditions for the pathogen, such as mild temperatures, frequent precipitation, and high relative humidity (BARDAS et al., 2009; SCHWARTZ et al., 2005). The main disease symptoms are discolored leaf veins and black sunken cankers on stems, petioles and pods.

Although resistant cultivars and chemical control are the main approaches used to manage this disease, the pathogen has been reported to have overcome the resistence in some commercial cultivars (MELOTTO; BALARDIN; KELLY, 2000; RODRIGUEZ-GUERRA et al., 2003) due its highly variable nature; more than one hundred pathotypes (races) of *C. lindemuthianum* have been identified worldwide (MELOTTO; BALARDIN; KELLY, 2000). Additionally, besides the harmful effect of chemical control to the environment and to no-target microorganisms, for a better chemical control effectiveness it is necessary frequent applications (CONNER et al., 2004) and use of more than one product (GILLARD; RANATUNGA; CONNER, 2012). In this background, biological control of plant disease using beneficial microorganisms has risen as a feasible alternative to replace chemical applications for being a safer and eco-friendly approach (MEDEIROS et al., 2012).

Biological control agents may suppress plant diseases mainly by (1) resistance induction through triggering plant defenses; (2) competition for nutrients and space; and (3) antibiosis thought the release of antibiotics and volatiles. Among these modes of actions, the role of volatiles still remain to be investigated regarding their effect on plant disease. Furthermore, microbial volatile organic compounds (mVOCs) have been shown to be

toxic to plant pathogens under *in vitro* conditions. However, there is a lack of information about its effect *in vivo*, especially in cultivated plants such as common bean. In Brazil common bean cv. "Pérola" is the most grown one, even though it is the highly susceptible cultivar to the anthracnose.

In this work we aimed to investigate the role of volatiles produced by two rhizobacterial strains: *Bacillus amylolicefaciens* ALB629 and UFLA285 on anthracnose (*Colletotrichum lindemuthianum*) control. Also, we aimed at identifying the microbial volatile organic compounds (mVOCs) produced by both ALB629 and UFLA285 by using solid phase micro extraction (SPME) coupled to gas chromatography with mass spectrometric detection (GC–MS).

2 MATERIALS AND METHODS

2.1 Pathogen and rhizobacterial cultivations

The monosporic culture was obtained according to Serra, Coelho e Menezes (2008). For inoculum production, *C. lindemuthianum* isolate Lv165, which was obtained from Laboratório de Resistência de Plantas from Department of Biology of Universidade Federal de Lavras, Brazil was grown on potato dextrose agar (PDA) in Petri dishes at 21ºC, in darkness for 15 days. After, 5 mL of sterile distilled water (SDW) was poured onto the medium surface and thoroughly scraped with a Drigalski spatula. The suspension was measured in Neubauer chamber to be 1×10^5 CFU mL^{-1}.

Selected rhizobacteria were obtained from cotton rhizosphere (UFLA285) and endophytically from healthy cacao trees (ALB629) (MEDEIROS et al., 2008, 2009). The bacteria are deposited respectively at the Mars Center for Cocoa Science, Itajuípe, BA and at Bacteriology laboratory of Universidade Federal de Lavras (UFLA), Brazil. The preserved bacteria in peptone glycerol at -80ºC were cultivated in agar nutrient (AN) medium in Petri dishes and incubated at room temperature (28ºC) for 48h before every experiment. Cells were transferred to the nutrient broth and

cultivated for 48h on a shaker at 150rpm at room temperature (28°C). The cells concentration was adjusted in Neubauer chamber to be 1×10^8 cells mL^{-1}.

2.2 Inhibition of Colletotrichum lindemuthianum growth by mVOCs

In this first experiment, an *in vitro* test was set up to analyze the effect of mVOC on pathogen spore numbers as well as mycelial growth. On one side of the bipartite Petri dish a 100 µL of *C. lindemuthianum* suspension was grown in PDA. On another plate side, a 100 µL of rhizobacteria suspension was grown in NA composed of 3.0 g L^{-1} meat extract, 3.5 g L^{-1} meat peptone, 5.0 g L^{-1} NaCl, 20.0 g L^{-1} agar. In the control treatment water was applied. Plates were sealed in order to retain the volatiles released by the bacteria and then incubated at room temperature (21°C) for 11 days. Mycelial growth was assessed at 3, 5, 7, 9, and 11 days after plating (DAP) and data expressed as cm. At 11 DAP, the number of spore numbers were also recorded.

2.3 Control of anthracnose by rhizobacterial volatiles in vivo

For the *in vivo* experiment, seeds of bean cv. 'Pérola' were initially disinfested in alcohol (70% ethanol) for 30 s, sodium hypochlorite (0.5% active chloride) for 10 min, washed thoroughly with SDW, air-dried in a low cabinet for 8h. Disinfested seeds were sown in one liter glass bottles containing a mixture of soil and sand (2:1), with 4 seeds per pot. Bottles were wrapped with aluminium foil to maintain roots in the dark. Four replicates for each treatment were used and arranged in a randomized complete block design. Seedlings were kept in a growth chamber until it reached seedlings with primary leaves fully expanded and watered daily to field capacity. Seedlings were thinned to have two seedlings per pots and, then three 1.5 mL eppendorfs containing rhizobacterial suspensions (1×10^8 cells mL^{-1}) or water (control) were inserted into pots in such way where there was no physical contact between plant roots and the bacterial

suspensions. Pots were covered with transparent plastic using an adhesive tape to seal the edges in order to retain the volatiles produced by the bacteria.

Seedlings remained for 72 h in contact with the volatiles and after pots were uncovered and a suspension of *C. lindemuthianum* was sprayed in bean leaves. After 7 days, seedlings were weekly rated for disease severity using a note scale from Godoy et al. (1997) with disease scores ranging from 0.1 to 24.0. With the values of this scale, data were transformed according to McKinney index (MCKINNEY, 1923) and used to calculate the area under the disease progress curve (AUDPC) (SHANER; FINNEY, 1977).

2.4 Volatiles identification

Rhizobacterial suspensions were obtained as described previously. Then, 100 µL of each bacterial suspension were added to a 20 mL septum-sealed SPME tubes and incubated at 21ºC for 11 days where the volatiles were analyzed. Tubes containing only NA medium were used as controls.

For volatile extractions 2 cm SPME fiber (Supelco Inc., Bellefonte, PA-USA) coated with divinylbenzene/polydimethylsiloxane/carboxen (DVB/PDMS/CAR) was used. SPME fiber was inserted into the tube through a silicone septa and exposed to the headspace for 35 min at 55ºC. After volatiles extraction the SPME fiber was inserted into the GC/MS injector for analyte desorption (2 min), separation, and detection. The GC-MS system consisted of a Shimadzu GCMS QP2010 Ultra (Shimadzu, Kyoto, Japan), equipped with a split-splitless injector, an AOC-5000 autoinjector (Shimadzu, Kyoto, Japan) and a HP-5MS fused-silica capillary column (30 m x 0.25 mm x 0.25 µm). Helium 5.0 grade was used as carrier gas at 1.0 mL min^{-1}. The injector was operated either in split 1:4 and splitless modes. The injector, the transfer line, as well as the ion source were kept at 250ºC, 240ºC and 200ºC, respectively. Oven temperature was programmed from 40ºC to 160ºC at 3ºC min^{-1} and then to 240ºC at 10ºC min^{-1}. MS scan range was set between 40 and 400 m/z.

Peak identification was performed using automated mass spectral deconvolution and identification system (AMDIS) v. 2.63 software and the NIST mass spectral search v. 1.7 software, both programs supplied by NIST (Washington—DC, USA). Peaks detected in bacterial samples and not found in blank samples were identified by comparing their spectrum against mass spectra library and confirmed by comparing experimental (RI Exp.) to literature (RI Lit.) retention indexes.

2.5 Experimental design and statistical analysis

The experiments were performed at the Universidade Federal de Lavras (UFLA), in Lavras, Minas Gerais, Brazil. The randomized complete block design with 5 and 4 replication was used for the *in vitro* and *in vivo* tests, respectively. Data were submitted to two-way and one-way variance analysis (ANOVA) for *in vitro* tests and *in vivo* test, respectively. Duncan's multiple range tests (P=0.05) were applied for significant means when necessary. For all analyses, the assumptions of normality of variance were checked by Shapiro-Wilk test and no transformation was necessary. SAS 9.3 was used for statistical analyses (SAS Institute, Cary NC).

3 RESULTS

There was no difference between experiments (P=0.5808) while we found a significant effect of treatments for mycelial growth at 11 DAP (P=0.0025), with a reduction by 16 and 18% for UFLA285 and ALB629 volatiles, respectively (Figure 1).

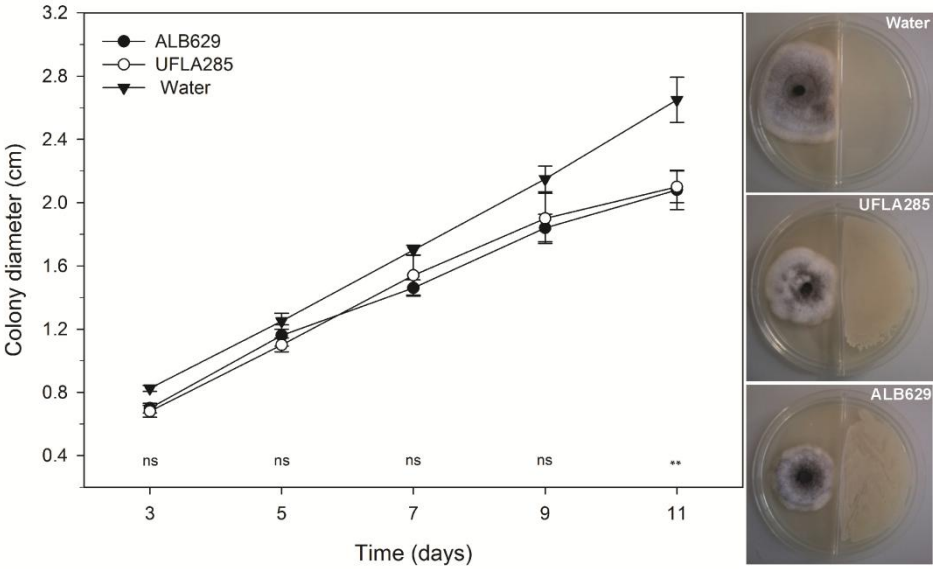

Figure 1 Effect of microbial volatile organic compounds (mVOCs) from *Bacillus amylolicefaciens* UFLA285 and ALB629 on *C. lindemuthianum* mycelial growth. **Significant at the 0.01 probability level by Tukey's multiple range tests. ns = not significative. Error bars represents ±SE. Average of two experiments

Regarding spore numbers, we found no statistically difference between experiments (P<0.55) but a decrease by the volatiles from ALB629 by 31%, (P<0.001) (Figure 2).

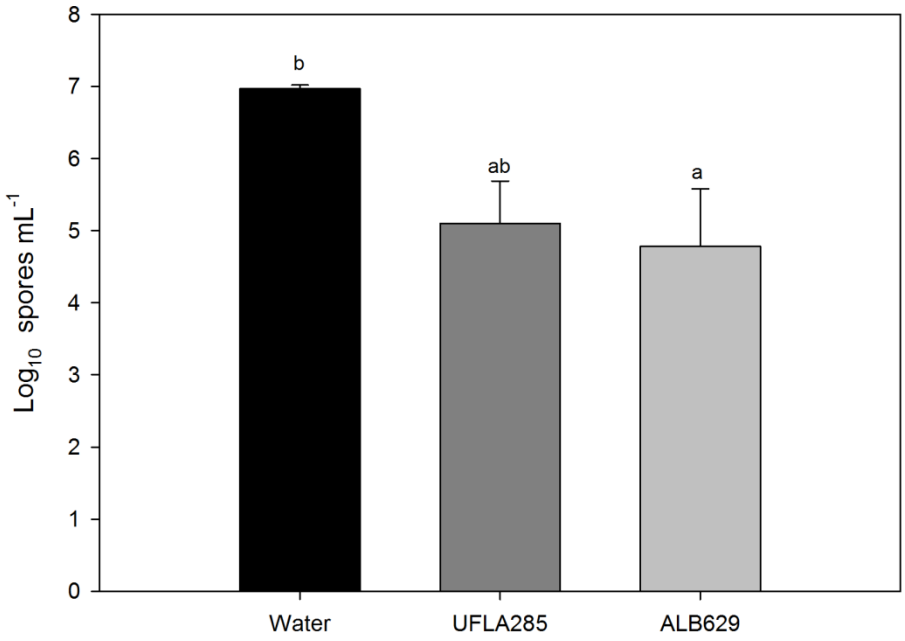

Figure 2 Effect of microbial volatile organic compounds (mVOCs) from *Bacillus amylolicefaciens* UFLA285 and ALB629 on the sporulation of *C. lindemuthianum*. **Significant at the 0.01 probability level by Tukey's multiple range tests. ns = not significative. Error bar represents ±SE

The mVOCs from both rhizobacteria statistically controlled anthracnose *in vivo* by reducing the AUDPC by 79–85%, respectively for ALB629 and UFLA285 ($P<0.001$) (Figure 3).

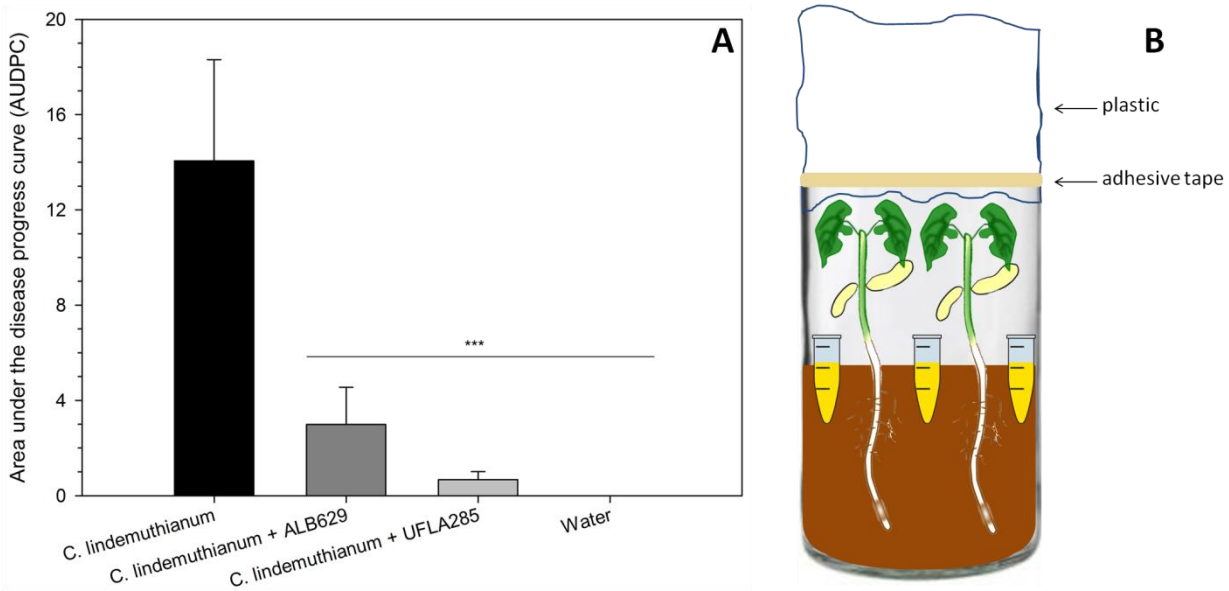

Figure 3 *In vivo* effect of microbial volatile organic compounds (mVOCs) from *Bacillus subtilis* UFLA285 and *B. amylolicefaciens* ALB629 in common bean cv. Pérola. (A) Area under disease progress curve (AUDPC) of Anthracnose caused by *C. lindemuthianum*; (B) Scheme of *in vivo* experiment. *** Significant at the 0.001 probability level according to Tukey's test. (Means of two experiments of four replicates of ten seedlings each). The line on each point represents ±SE

Also, the mVOCs produced by the Rhizobacteria were analyzed by SPME–GC–MS as shown in Table 1.

Table 1 Microbial volatile organic compounds (mVOCs) from *Bacillus amylolicefaciens* UFLA285 and ALB629 identified by SPME-GC-MS

				Samples[b]	
mVOCs	RI Exp.	RI Lit.[a]	Match. (%)	ALB629	UFLA285
3-hydroxy-2-butanone	713	710	92	√√	√√
3-methylbutanoic acid	867	876	90	√	√
2-methylbutanoic acid	878	884	92	√	√

[a]Machiels, D., Van Ruth, S.M., Posthumus, M.A., and Istasse, L. 2003. Gas chromatography-olfactometry analysis of the volatile compounds of two commercial Irish beef meats. Talanta. 60:755-764.
[b]Relative peak intensity on chromatogram: √ - low mVOC intensity; √√ - high mVOCs intensity.

4. Discussion

In this work we have indentified three different mVOCs produced by two rhizobacterial strains *Bacillus amylolicefaciens* ALB629 and UFLA285 that inhibited *Colletotrichum lindemuthianum* mycelia growth under both *in vitro* and *in vivo* conditions in common bean plants. Recent studies from our group have demonstrated that these same two rhizobacterial strains used in this study, could efficiently control two different important foliar diseases in common bean, bacterial wilt caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* even under abiotic stress (MARTINS et al., 2014, 2015; MARTINS, S. J. et al., 2013) as well as web blight under field conditions (MARTINS, S. A. et al., 2013).

Up to know, mVOCs have the potencial to promote plant growth (MINERDI et al., 2011; RYU et al., 2003) and *in vitro* inhibit plant pathogens (FIALHO et al., 2010; KAI et al., 2007). To the best of our knowledge, this was the first report of an *in vivo* test using mVOCs produced

by rhizobacteria against a plant pathogen in a grain crop, such as common bean.

Among the mVOCs identified in this work, 3-hydroxy-2-butanone also called acetoin, which was found in a higher concentration (Table 1), has also been found being produced by rhizobacterial strains with activity against plant pathogen (ARREBOLA; SIVAKUMAR; KORSTEN, 2010; RYU et al., 2004). Moreover, recently Magno-Pérez-Bryan et al. (2015) by sequencing the genomes of two strains of *Bacillus amylolicefaciens* CECT 8237 and CECT 8238, have shown that the bacteria have some functional genes related to the production of metabolite and volatile compounds, such as acetoin and 2-3-butanediol which can help plants to overcome plant diseases. Our group of work has also found that ALB629 is able to form biofilm (data not shown). Further studies will be conducted in order to find out what genes in *Bacillus amylolicefaciens* ALB629 and UFLA285 are responsible for the production of the volatile molecules we found in this work.

Seed coating formulation may contain active ingredients whether it be single or in combination of active ingredients in encapsulated form, e.g. as slow release capsules or microcapsules, as have been shown by some researches (HITCHCOCK et al., 2015; SCARFATO et al., 2007). This study provides perspectives to some mVOCs produced by rhizobacteria to be used in the future for the food production benefits.

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ARTIGO 2

Common bean growth and health promoted by rhizobacteria and the contribution of magnesium to the observed responses

Common bean growth and health promoted by rhizobacteria and the contribution of magnesium to the observed responses

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Common bean growth and health promoted by rhizobacteria and the contribution of magnesium to the observed responses

Abstract Abiotic effects, such as nutrient abundance in soil, may interfere with the performance of plant-associated rhizobacteria in terms of plant physiology as well as disease control. We aimed to evaluate the effectiveness of rhizobacteria in the promotion of bean growth and nutrient uptake and the contribution of magnesium (Mg) supplementation to photosynthetic rates, CO_2 assimilation, chlorophyll content, and bacterial wilt severity (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*). Bean plants from seeds treated with rhizobacteria were assessed for growth promotion-related variables, photosynthetic-related variables, as well as disease severity when plants were grown in soil with different magnesium contents (0–50 mg kg^{-1}). There was a 33%–45% increase in root dry weight (*Bacillus subtilis* UFLA168* and *B. amyloliquefaciens* ALB629) and a 24%–35% increase in relative growth index (*B. subtilis* UFLA285, UFLA168*, copper oxychloride, *Paenibacillus lentimorbus* MEN2). At 25 mg kg^{-1} Mg, although the plant continued to take up Mg from the soil, increased accumulation of CO_2 was found in the leaf mesophyll of both the ALB629 and control treatments, indicating low CO_2 fixation and low Rubisco activity. Higher doses of Mg caused an increase in chlorophyll content as well as in photosynthetic rates in rhizobacterium-treated plants. Additionally, at 25 mg kg^{-1} Mg, there was an increase in chlorophyll content in ALB629 (30%) and a reduction in bacterial wilt severity (51%). Moreover, photosynthesis was negatively correlated with disease severity ($r = -0.53$, $P<0.01$). Therefore, ALB629 is a promising bacterial strain to improve bean plant growth and nutrient uptake and reduce plant disease even under abiotic stress.

Key words: PGPR; *Phaseolus vulgaris;* Seed treatment; Biocontrol; Photosynthesis; Chlorophyll

1. Introduction

With increasing problems associated with the use of synthetic chemicals in agriculture (negative impacts on health and the environment), there has been ever-increasing interest in the use of beneficial microorganisms to improve plant health while ensuring that products are safe for human consumption and enabling protection of the environment (Zafar et al. 2011).

For instance, some of these beneficial microorganisms such as the endophytic bacterium *Pantoea agglomerans* (Beijerinck) Gavini et al. isolate LRC 8311 enhanced seedling growth when applied to common bean seeds (Hsieh et al. 2005). Additionally, *Rhizobium leguminosarum* bv. *viceae* R21 may increase seedling emergence and plant height (Huang et al. 2007).

While gram-negative bacteria have the potential to promote bean plant growth and control disease, bacteria belonging to the *Bacillus* genus produce endospores, which confer a higher tolerance to sudden environmental changes and are easier to formulate in a product with a long shelf life (Hayat et al. 2010; Saharan and Nehra; 2011). They are free-living bacteria in the soil and are known as plant growth-promoting rhizobacteria (PGPR). When applied to seeds or roots, certain strains may benefit crops by stimulation of plant growth (Kloepper et al. 1989; Orhan et al. 2006), suppression of plant diseases (Martins et al. 2013), enhancement of plant nutrient uptake (Remans et al. 2008; Saharan and Nehra, 2011), and/or by phytoremediation (Khan 2005).

Magnesium (Mg) is an essential element for plant growth and reproduction. It has noteworthy functions in plants including its role as enzyme co-factor for peroxidase (POX), an enzyme involved in plant defense, and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), a key enzyme for photosynthesis (Hawkesford et al. 2012). This element also has a central position in the chlorophyll molecule (Waraich et al. 2011). Therefore, magnesium plays an important role in photosynthesis, and its lack

in many weathered soils is a matter of concern. Increasing nutrient uptake is a plausible strategy to remediate contaminated soils (Khan, 2005) or sustain plant yield in nutrient-deficient soils (Zafar et al. 2011).

Many PGPR have also potential for disease control. Currently, bacterial wilt of the common bean caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) (Hedges) Collins and Jones (Hedges, 1922, 1926) is a serious threat because it is a seed-transmitted disease of the common bean, which is cultivated in Brazil and in another countries around the world (Corrêa et al., 2014; Huang, et al., 2007). Although no commercially resistant cultivars or chemical treatments are available to growers for management of bean bacterial wilt, we have shown that PGPR can be successfully used to manage this disease with up to 70% disease reduction (Martins et al., 2013) even when plants are incubated at different temperatures (Martins et al., 2014). Once *Cff* infects bean plants, it causes wilting and a reduction in Mg uptake (Maringoni, 2003), which results in reduced yield. Therefore, either supplementing the nutrient in the soil or increasing nutrient uptake by PGPR treatment enables plants to better tolerate the disease and sustains plant development. Furthermore, it is common sense that disease is an exception and not a rule (Staskawicz, 2001). Therefore, for commercial purposes, it is important to show growers that even in the absence of the disease, PGPR treatment may result in other benefits, such as growth promotion and enhanced nutrient uptake.

The aim of this work was to evaluate the contribution of PGPR strains to growth promotion, nutrient uptake and bacterial wilt control in the common bean at different Mg concentrations in the soil.

2. Materials and methods

2.1 PGPR strains

Experiments were conducted under greenhouse conditions (temperature ca. 30 °C, relative humidity ca. 63% and light intensity ca. 1000 µmol m^{-2} s^{-1}) at

the Universidade Federal de Lavras (UFLA), in Lavras, Minas Gerais, Brazil (915 m altitude, 21°13'34''S and 44°58'31''W). The PGPR strains used in this study were as follows: *Paenibacillus lentimorbus* MEN2, *Bacillus amyloliquefaciens* ALB629, *B. subtilis* UFLA285, and *B. subtilis* UFLA168*, which were obtained from rhizosphere soil and endophytically from roots of field-cultivated cotton plants or donated by research centers (Medeiros et al. 2008; Medeiros 2009) and selected based on their ability to provide biological control of bean bacterial wilt (Martins et al. 2013). The PGPR strains are deposited at the Mars Center for Cocoa Science, Itajuípe, BA (ALB629), UFRPE, Recife, PE (MEN2), and at Bacteriology laboratory of UFLA, MG (UFLA285 and UFLA168*).

2.2 Seed treatment with PGPR

Selected PGPR preserved in peptone glycerol at -80ºC were cultivated on nutrient agar medium in Petri dishes and incubated at 28ºC for 48 h prior to every experiment. Cells were transferred to nutrient-broth medium and cultivated for 48 h on a shaker at 150 rpm at 28ºC. The endospore concentration was adjusted to 1×10^8 CFU mL^{-1} in a Neubauer chamber and used for seed treatment.

Seeds of the common bean cv. 'Pérola' were initially disinfested in alcohol (70% ethanol) for 30 s and sodium hypochlorite (5% active chloride) for 10 min and subsequently washed thoroughly with sterile distilled water (SDW) and air-dried in a flow cabinet for 8 h. Disinfested seeds were soaked for 30 min in the antagonist's suspension (2 mL g^{-1} seed) at 10^8 CFU/mL, in the fungicide copper oxychloride or in water (2 g seed L^{-1}). They were dried overnight and sown (10 seeds per pot) in 5-L pots containing a mixture of soil and sand (2:1). The soil mixture had the following characteristics: $pH_{(H2O)}$: 5.6, Mg: 0.2 $cmol_c$ dm^{-3}, sum of bases (S value): 2.67 $cmol_c$ dm^{-3}, organic matter: 11.8 g kg^{-1} and clay content: 400 g kg^{-1}.

Plants were kept in a greenhouse. Four replicates of each treatment were performed and arranged in a randomized block design.

2.3 Assessment of analyzed variables

Seedling emergence from the 5th to the 12th day after sowing (DAS) was recorded daily and used to calculate the speed emergence index (SEI) according to Teixeira and Machado (2003) as well as the percent of seedling emergence (PSE) from the last evaluated period. At 12, 15, 18, 21, and 24 DAS, seedling height was recorded by measuring from the cotyledon insertion to the apical bud, and the obtained data were used to calculate the relative growth index (RGI) as RGI = (LnP2 - LnP1)/ (T2 - T1), where *Ln* = natural logarithm, and *P2* and *P1* = seedling height at times *T2* (end time) and *T1* (initial time).

All plants were harvested, and shoots were separated from roots at 24 DAS, a time set based on a previous work showing colonization of the common bean by ALB629 (Martins et al. 2014). Roots were thoroughly washed in tap water, and both shoots and roots were wrapped and oven-dried at 70 ºC for 72 h to a constant weight to obtain shoot (SDW) and root dry weight (RDW). This experiment was repeated three times.

2.4 Assessment of nutrient contents

The same experimental procedures were performed as described above, however the nutrient contents from the shoots used to determine SDW were analyzed to estimate the effect of the seed treatment with PGPR on nutrient uptake. Dried shoots of the common bean were weighted (0.5 g) and digested in a mix of nitric (4 mL) and perchloric (2 mL) acids. Later, the extract was diluted for nutrient determination by atomic absorption spectroscopy according to Malavolta et al. (1997). Data were expressed as g kg^{-1} and mg kg^{-1} of dry weight (DW) for macronutrients (N, P, K, Ca, Mg, and S) and mg kg^{-1} for micronutrients (B, Cu, Zn, Mn, and Fe) (Table 1). The experiment was repeated twice.

2.5 PGPR performance under increasing Mg levels

The bacterial strain that promoted the highest plant growth and nutrient uptake was selected for the following experiments, i.e., chlorophyll content, photosynthetic and CO_2 assimilation rates determined by the ratio of the intracellular (Ci) and ambient (Ca) CO2 concentrations (Ci/Ca), as well as magnesium uptake under conditions of different nutrient supplements to the soil. PGPR cultivation, seed treatment and seed sowing were performed as described previously. Soil fertilization was also performed as recommended for the common bean crop for all experiments, except for Mg, which was supplemented as a magnesium chloride hexahydrate (MgCl2 · 6H2O) p.a. salt, at the following doses: 0, 25, 35 and 50 mg kg^{-1}. Treatments consisted of seeds treated with ALB629 or water (control) sown in soil with the four described magnesium concentrations, which were calculated to lower the soil Ca:Mg ratio from 12:1 to 3:1, a proportion thought to be ideal for optimum plant growth (Da Silva et al. 2004; De Oliveira et al. 2013; Yeo et al. 2013). The experiment was repeated twice.

2.6 Photosynthetic capacity and Ci/Ca rate measurements

The photosynthetic capacity (μmol CO_2 m^{-2} s^{-1}) and CO_2 assimilation rate (μmol CO_2 mol air^{-1}) of plants were measured at the V2 phenological stage, i.e., seedlings with the first true leaves fully expanded. The ecophysiological measurements of all plants were performed with an Infra-Red Gas Analyzer (LI-6400XT Portable Photosynthesis System, LI-COR, Lincoln, USA) with a photon flux density of 300 μmol m^{-2} s^{-1} between 8:30 a.m. and 10:30 a.m. as described by Salvestro et al. (2012).

2.7 Chlorophyll determination and assessment of Mg content

At 24 DAS, two leaves per plant for each treatment were detached for chlorophyll determination. Approximately 140-150 mg tissue was macerated in 80% aqueous acetone (5 mL) followed by filtration through a paper filter. Aliquots were adjusted with 80% aqueous acetone to 25 mL,

and extract absorbances were read on spectrophotometer at 646.8 and 663.2 nm, respectively, to determine the levels of chlorophyll a and b. The total chlorophyll content was calculated as [(7.15 x A663) + (18.71 x A647)]/[1000x(fresh weight of leaves)] and was reported as mg Chl. per g FW, as described by Lichtenthaler (1987). The same procedure described for nutrient contents analysis in the previous experiment was performed for magnesium content measurement.

2.8 Biocontrol of bacterial wilt by ALB629

A fourth assay was set up using 25 mg kg^{-1} magnesium in the soil to determine the capacity of ALB629 to control bacterial wilt disease in this condition.

The *Cff* isolate used for this study was the yellow variant of *Cff* from Santa Catarina State, Brazil (*Cff* SC – Feij-2928, isolated in March 23rd 2003 at Campos Novos, Santa Catarina State, Brazil from the common bean *Phaseolus vulgaris* cv. Pérola), which was obtained from the culture collection of the plant bacteriology laboratory at the Universidade Estadual Paulista (Botucatu, Brazil), preserved in dried infected leaves from which the bacterium was reisolated and tested for pathogenicity before each experiment. The pathogen isolated from the dried leaves was grown on 523 medium (Kado and Heskett, 1970) in Petri dishes and incubated at ca. 28 °C for 48 h. Seeds of cv. 'Pérola' were initially disinfested as described previously and then artificially inoculated with *Cff* using the physiological conditioning technique (Deuner et al., 2011). Seed treatment with ALB629 or water (control) was performed as described above. Seeds were then dried overnight and sown (5 seeds per pot) in 3-L pots containing a mixture of soil and sand (2:1), with 5 seeds per pot. Plants were kept in the greenhouse and watered when necessary.

Disease severity was recorded based on a note scale (Hsieh et al. 2003), which ranges from 0 to 5, where 0 = no wilt symptoms; 1 = wilt on one of the primary leaves; 2 = wilt on both primary leaves but not on the first

trifoliolate; 3 = wilt on the first trifoliolate; 4 = death of seedling after development of primary leaves; and 5 = unmerged seedling or death of seedling before development of primary leaves. Using these values, data were transformed according to the McKinney index (McKinney, 1923) and used to calculate the area under the disease-progress curve (AUDPC) (Shaner & Finney, 1977). In addition to disease severity, all parameters evaluated in the second experiment were assessed in this assay as well. The experiment was repeated twice.

2.9 Experimental design and statistical analysis

For the first and second experiments, the experimental design was randomized blocks with six treatments and four replicates. Data were subjected to one-way variance analysis (ANOVA), and for significant means Tukey's multiple range test (P=0.05) was applied. In the third experiment, the experimental design was randomized blocks in a 2 x 4 factorial scheme (treatments x Mg doses) with 4 replications. Data were subjected to regression equations and a parallelism (F-test) test. The goodness of fit of the models was tested at 0.05 significance and evaluated by the coefficient of determination (R^2). For the fourth experiment, a randomized-blocks experimental design was used with 3 treatments and 4 replicates. Data were subjected to one-way variance analysis (ANOVA), and for significant means Tukey's multiple range test (P=0.05) was applied.

Additionally, Pearson's correlation coefficients (r) were determined to evaluate the strengths of the relationships between the following parameters: (1) AUDPD and photosynthetic rate; (2) AUDPD and Mg content in shoots; (3) AUDPD and chlorophyll content; (4) AUDPD and CO_2 assimilation rates; (5) Mg content in shoots and photosynthetic rate; (6) Mg content in shoots and chlorophyll content; (7) Mg content in shoots and CO_2 assimilation rates; (8) photosynthetic rate and CO_2 assimilation rates; (9) photosynthetic capacity and chlorophyll content; and (10) CO_2 assimilation rates and chlorophyll content.

For all analyses, the assumptions of normality of variance were evaluated, and no transformation was necessary. SAS 9.3 was used for statistical analyses (SAS Institute, Cary NC).

3. Results

The rhizobacterial strains MEN2, UFLA168*, and UFLA285, as well as copper oxychloride increased the relative growth index ($P<0.001$) while ALB629 and UFLA168* promoted a higher root dry weight ($P=0.007$) compared to the control (Figure 1). However, none of the means differed compared among themselves in terms of speed emergence index ($P=0.0591$), percent seedling emergence ($P=0.3502$), and shoot dry weight ($P=0.2911$) (supplementary data).

Data presented in Table 1 show the total nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, boron, copper, manganese, zinc, and iron contents in common bean shoots.

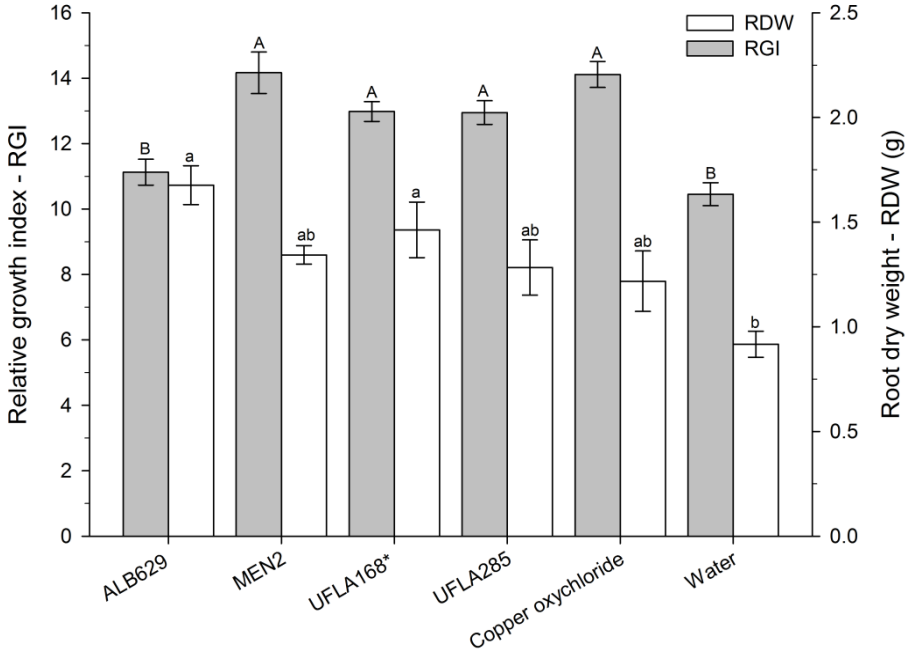

Fig. 1 Effect of seed treatment with PGPR, copper oxychloride or water on the relative growth index (RGI) measuring from the cotyledon insertion to the apical bud at 12, 15, 18, 21, and 24 DAS CV= 6.08% and on root dry weight (RDW) obtained from oven-dried at 70ºC for 72 h to a constant weight at 24 DAS CV=17.45%. Bars with the same letter are similar at the 5% level according to Tukey's multiple range test. The line on each bar represents ±SE. (Means of 3 experiments; 4 replicates of 10 seedlings per pot each.)

Table 1. Effect of seed treatment with PGPR on mineral nutrient concentrations in common bean shoots using regular fertilizer as recommended for the crop. Values of each column followed by the same letter(s) are not significantly different according to Tukey's test. (Means of 2 experiments of 4 replicates of 10 seedlings each).

Treatments	$^{**}N^{3-}$	$^{***}P^{3-}$	$^{**}K^{+}$	$^{ns}Ca^{2+}$	$^{**}Mg^{2+}$	$^{***}S^{2-}$	$^{**}B^{3+}$	$^{***}Cu^{2+}$	$^{ns}Mn^{2+}$	$^{ns}Zn^{2+}$	$^{***}Fe^{2+}$
	---------- g kg^{-1} ----------							---------- mg kg^{-1} ----------			
ALB629	48.8a	1.5a	24.2a	9.5	2.8a	3.8a	59.4a	6.5b	119.8	23.5	512.4a
MEN2	48.3a	1.3ab	24.2a	10.0	2.5b	3.7a	44.5ab	5.9b	118.1	20.9	394.6a
UFLA168*	45.6ab	1.3ab	23.8a	9.7	2.6ab	3.3ab	59.4a	5.7b	111.8	20.5	411.5a
UFLA285	47.5ab	1.3ab	24.3a	8.7	2.7ab	3.4ab	54.9a	6.0b	109.9	21.2	473.6a
Copper oxychloride	44.6b	1.1b	21.6b	6.3	2.5b	3.0b	30.3b	9.8a	104.1	20.1	216.1b
Water^{nc}	49.1a	1.3ab	24.2a	8.9	2.5b	3.6a	54.7a	5.9b	104.6	20.5	489.3a
CV (%)	5.1	10.3	5.9	31.7	7.6	9.9	31.1	11.7	13.4	10.8	19.1

ns = Not significant
** Significant at the 0.01 probability level
*** Significant at the 0.001 probability level
nc = negative control (seeds treated with sterile water)

Among the tested PGPR, only ALB629 promoted an increase in magnesium uptake (P=0.005) compared to the control. In contrast, copper oxychloride promoted a decrease in the contents of the analyzed nutrients (N, K, S, B, Fe) while causing an increase Cu content (P<0.001). Once among the PGPR tested, only ALB629 increased Mg in common bean shoots it was therefore used in the following assays.

A summary of the regression analysis results is presented in Figure (2abcd). An increase in Mg in the shoot was observed with increased Mg in the soil regardless of the seed treatment. However, for plants treated with ALB629 at the lower Mg doses, the nutrient was found in higher proportions (Figure 2a). At $mg.kg^{-1}$ Mg, a decrease in chlorophyll content was found for the control treatment while it remained steady in the ALB629 treatment (Figure 2b). Photosynthesis activity decreased with increased nutrient levels in the soil regardless of the seed treatment (Figure 2c). For the CO_2 assimilation rate, at low magnesium levels in the soil (0 and 25 $mg.kg^{-1}$), higher rates were observed in water-treated plants compared to ALB629-treated ones. At high doses, no difference between treatments was observed for this variable (Figure 2d).

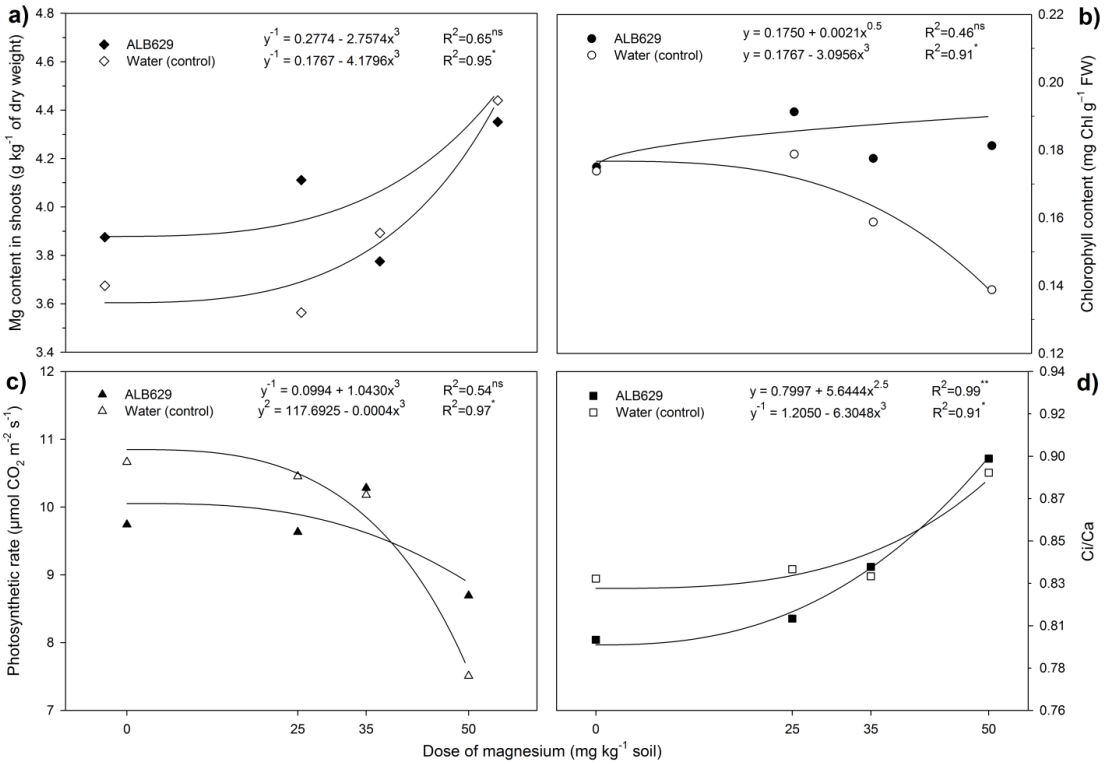

Fig. 2 The lines are regression fits used to describe the effect of bean seed treatment with *B. amyloliquefaciens* strain ALB629 or water (control) on A) Mg content in shoots; B) total chlorophyll contents (chlorophyll a + b); C) photosynthetic rates; D) CO_2 assimilation rates (based on the ratio of Ci and Ca intercellular and ambient CO_2 concentrations, respectively). *Angular coefficients differ significantly by the *F*-test. (Means of 2 experiments; 4 replicates of 5 seedlings per pot each.)

At 25 mg Mg kg^{-1} soil, the ALB629 seed treatment controlled bacterial wilt compared to the control water treatment (P<0.001) (Table 2) and increased the chlorophyll content (P=0.273). However, there was no significant effect of treatment regarding photosynthetic capacity (P=0.064), CO_2 assimilation rates (P=0.828) or Mg content in shoots (P=0.482).

Table 2. Effect of seed treatment with *Bacillus amyloliquefaciens* ALB629 on chlorophyll content (chlorophyll a + b) (mg Chl g^{-1} FW), photosynthetic capacity (µmol CO_2 m^{-2} s^{-1}), CO_2 assimilation rate (µmol CO_2 mol air^{-1}), Mg content in shoots (g kg^{-1} of dry weight), and bacterial wilt (AUDPC) control in the common bean grown in soil containing 25 mg of Mg kg^{-1} soil. (Means of 2 experiments; 4 replicates of 5 seedlings per pot each.)

Treatments	Chlorophyll*	Photosynthesis^{ns}	Ci/Ca^{ns}	Mg^{ns}	AUDPC***
ALB629	0.252a	8.967	0.725	4.5	0.0a
Cff + ALB629	0.248a	7.753	0.728	4.1	151.3b
Cff	0.194b	7.371	0.745	4.3	171.6c
CV (%)	15.9	16.1	8.6	14.3	61.4

*Significant at the 0.05 probability level.

***Significant at the 0.001 probability level.

ns = not significant by Tukey's multiple range test.

There was a significant negative correlation between bacterial wilt disease and photosynthetic capacity (Figure 3a). In addition, a significant positive correlation between Mg content and CO_2 assimilation rate (Figure 3b), Mg content and photosynthetic capacity (Figure 3c), and photosynthetic capacity and CO_2 assimilation rate (Figure 3d) was found. Conversely, there was no correlation between photosynthetic capacity and chlorophyll content (P=0.869), CO_2 assimilation rate and chlorophyll content (P=0.811), CO_2 assimilation rate and AUDPC (P=0.304), chlorophyll content and AUDPC (P=0.245), chlorophyll content and Mg content (P=0.645), and AUDPC and Mg content (P=0.081).

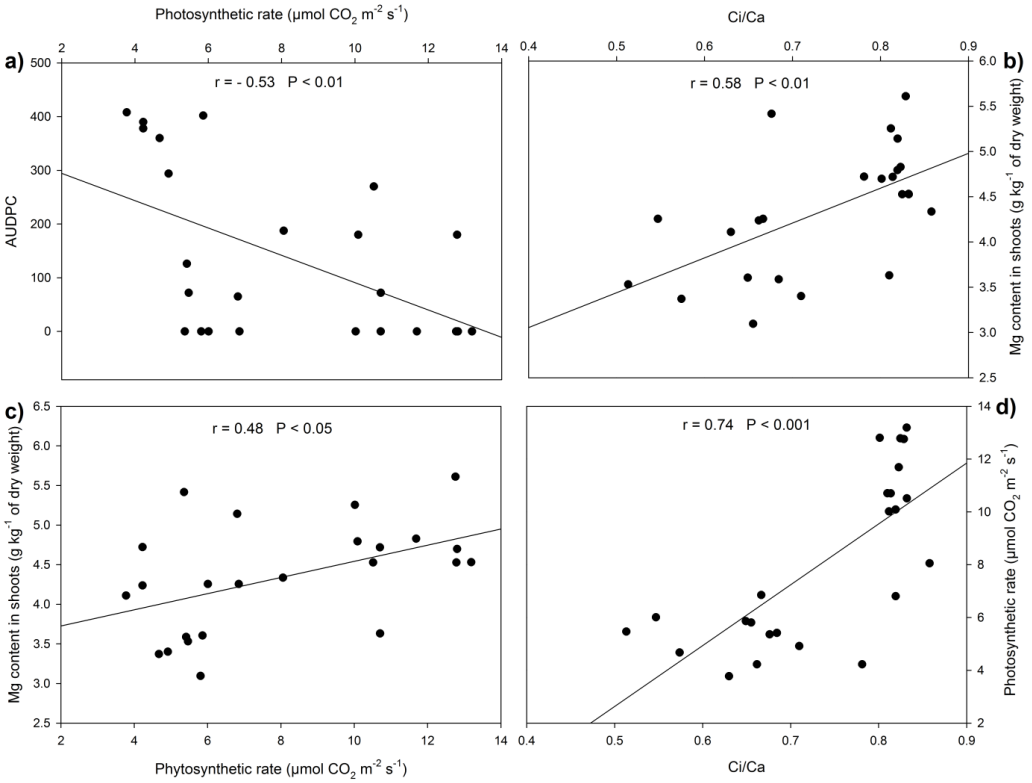

Fig. 3 Correlation coefficients between A) area under the disease progress curve (AUDPD) and photosynthetic rate; B) Mg content in shoots and CO_2 assimilation rate; C) Mg content in shoots and photosynthetic rate; D) photosynthetic rate and CO_2 assimilation rate. (Means of 5 seedlings per pot in 2 experiments with 4 replicates, *n*=24.)

4. Discussion

Although plant beneficial microorganisms such as PGPR are one of the most promising methods of improving plant health instead of or in synergistic combination with synthetic chemicals, the commercialization of biocontrol agents is still limited (Fravel, 2005; Medeiros et al. 2012). To improve the chance of having some of these PGPR strains become a well-accepted product in the future, we analyzed the performance of a PGPR selected for disease control on growth promotion, nutrient uptake, plant photosynthesis, CO_2 assimilation rate, and chlorophyll content in a common

bean crop. PGPR strains did not cause phytotoxicity or pathogenicity to bean plants but did promote plant growth, enhance root dry matter, and increase Mg uptake.

Both plant growth and nutrient uptake are energy-consuming processes. However, plants primed by PGPR invest less energy in activating such mechanisms (Niu et al. 2011), thus avoiding unnecessary consumption of energy so that plants have more energy to direct to other important metabolic processes such as reproduction and yield.

The threshold soil level at which ABL629 could sustain growth was 25 mg kg^{-1} Mg. From that point, although plants still increased their uptake of Mg from soil, CO_2 accumulation was found at a higher proportion in the leaf mesophyll for both ALB629 and control treatments, indicating low CO_2 fixation and slower Rubisco activity. At higher Mg doses a decrease in chlorophyll content as well as in photosynthetic rate was also found for the control treatment.

Furthermore, in the experiment with 25 mg kg^{-1} Mg, ALB629 enhanced the total chlorophyll content, with a shoot Mg content even higher than that recommended for optimal plant growth (1.5 – 3.5 g kg^{-1}) by Hawkesford et al. (2012).

Magnesium is essential for normal plant growth and development (Bose et al. 2011). Mg is an integral part of chlorophyll and, hence, essential for photosynthesis. It is also involved in regulation of ion transport and cation balance in plants and acts as an activator of more than 300 enzymes (e.g., RNA polymerases, ATPases, protein kinases, phosphatases), including peroxidase, an enzyme involved in plant defense against pathogens (Bose et al. 2011; Kim et al. 2013; Salehi and Tahamtani; 2012). To the best of our knowledge, the present study is the first report of a PGPR strain increasing Mg uptake in the common bean, which could cause better use of this nutrient in Mg-deficient soil or even reduce the Mg input in the soil considering the increased nutrient-uptake efficiency. Further investigations to explain the mechanisms by which ALB629 increases Mg uptake in plants under

conditions of low nutrient availability need to be performed. For other plants such as *Arabidopsis thaliana*, the mechanism by which iron uptake increases after *B. subtilis* GB03 colonization involves both gene regulation through upregulation of the iron transporter IRT1 as well as rhizosphere acidification, which in turn facilitates nutrient absorption (Zhang et al., 2009).

Our study also indicated a possible risk of overusing copper oxychloride as a seed treatment. Although the use of this product increased RGI and Cu shoot content because the fungicide contains copper (Cu) as one of its components ($CuCl_2.3Cu(OH)_2$), its use resulted in a decrease in the contents of almost all nutrients tested in this work, including N, K, S, B, and Fe. Furthermore, this reduction in nutrient content may have resulted in the reduction of PSE, SEI, SDW, and/or RSW. While widely recommended for bacterial disease control (Agrofit, 2014), copper oxychloride cannot reduce bacterial wilt severity (Martins et al. 2013). These results indicate a risk of using copper oxychloride-based fungicides in common bean production and reinforces the need to search for alternative sustainable disease-control strategies that have no negative on plants.

Common bean seed treatment with PGPR proved to promote plant growth, nutrient uptake and disease control, boosting the chance of these PGPR to become a commercial product for common bean production. Additionally, this study showed that at high doses of Mg, the PGPR strain *Bacillus amyloliquefaciens* ALB629 could sustain plant health by increasing chlorophyll content and controlling bacterial wilt.

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ARTIGO 3

Seed exudates from common bean (*Phaseolus vulgaris* L.) favor *Bacillus amyloliquefaciens* ALB629 biofilm formation and plant drought tolerance

Seed exudates from common bean (*Phaseolus vulgaris* L.) favor *Bacillus amyloliquefaciens* ALB629 biofilm formation and plant drought tolerance

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Abstract - *Bacillus amyloliquefaciens* ALB629 promotes common bean health and growth under normal and abiotic stress conditions. Understanding the mechanism imparting drought tolerance fosters the development of a seed coating to maximize ALB629 benefit to be used as a commercial biocontrol and biofertilizer agent. Seed exudates from common bean were tested *in vitro* for ALB629 biofilm formation and bacterial growth. Furthermore, the performance of ALB629 on plant-related variables under drought stress were also checked. Seed exudates (1 and 5% v/v) increased ALB629 biofilm formation regardless of the time point in which the exudates were collected. In addition, there was an increase in the ALB629 cell counts both in culture and on the bean seed surface when the bacteria were in contact with the exudates. Moreover, seed exudates from common bean up-regulated biofilm operons in ALB629 *TasA* and *EpsD* by about 2- and 6-fold, respectively. Seedlings from seeds treated with ALB629$^{rif\text{-}nal}$ and subjected to drought trials showed a higher concentration of the bacteria when malic acid was present. Additionally, we showed that seed treatment with ALB629 imparts drought tolerance and also promotes growth in plants under drought stress by increasing plant water holding capacity - fresh:dry weight ratio, seedling emergence index, plant fresh weight, as well as seedling stem size. Here, we showed that seed exudates, in particular malic acid, play a major role in improving plant health against drought stress in common bean.

Keywords: Seed treatment, PGPR, abiotic stress, sporulation, rhizobacteria

1 INTRODUCTION

Abiotic stress regimes, such as drought, may interfere on plant growth and development. Of late, drought occurrences have alarmed growers around the world. In Brazil, for instance, the agriculture recently faced the worst drought in the last 80 years (NEWSCIENTIST…, 2015). On the other hand, utilization of "biologicals" has been shown to be an eco-friendly alternative that can increase plant tolerance and reduce drought impact on plant development (KAVAMURA et al., 2013; VURUKONDA et al., 2016). Although the effect of beneficial microbes on plants is discussed in the literature, there has been comparatively much less progress in elucidating the mechanisms that are involved in beneficial microbes' effect(s) on plant performance against abiotic stress, with the exception of the best studied model systems (BARRIUSO; SOLANO; GUTIÉRREZ MAÑERO, 2008; STAUDINGER et al., 2016).

Among the plant beneficial microbes are the plant growth-promoting rhizobacteria (PGPR), which when applied to seeds or roots can colonize plant rhizosphere and then benefit plants in many ways (MARTINS et al., 2015; SPENCE et al., 2014). Various studies have shown that the secretions on rhizosphere can stimulate or inhibit the microorganism growth (HAO et al., 2010; LING et al., 2011; RANDY et al., 2009) and also play a major role in recruitment and colonization of beneficial rhizobacteria (DUTTA; PODILE, 2010; HUANG et al., 2014). Chen et al. (2012) showed that root exudates of tomato could increase Bacillus biofilm and also that malic acid was a major exudate component involved in this process.

Biofilm are ubiquitous communities of tightly associated bacteria encased in an extracellular matrix of polymeric substances, such as exopolysaccharides (EPS), proteins, and sometimes DNA. In *Bacillus subilis*, this extracellular matrix is composed mainly of EPS and the protein TasA, which polymerizes into amyloid-like fibers (BRANDA et al., 2006; ROMERO et al., 2010). Besides, Spo0A is a regulator DNA-binding protein for the *Bacillus subtilis* sporulation, which also has been found to governs

biofilm formation (LOPEZ; VLAMAKIS; KOLTER, 2009). A gain in biofilm formation may increase the potential of a beneficial bacterium to sustain the plant's health under abiotic stresses. Although information regarding root exudates mediating rhizospheric interactions has been extensively studied (BADRI et al., 2013; CHAPARRO; BADRI; VIVANCO, 2013; CHAPARRO et al., 2013), no information about the primary conditions, on seeds, regardless whether it is from legume or no-legume, which may or may not favor the rhizobacteria plant colonization is available. For commercial viability of biologicals to be tried as biofertilizer agents, it is important to understand how beneficial rhizobacteria interact with seeds, as the benign bacteria are usually applied through seed treatment. Seed treatment increases the time of contact between plant-microbials, besides being a cheaper alternative to maintain plant health compared to the aerial spraying or drenching method. In this study, we wanted to unravel the events which favor the seed-rhizobacteria interaction and which may interfere with the rhizobacteria colonization and growth on the spermosphere.

The objectives of this work were to determine the role of seed exudates on *Bacillus amylolicefaciens* ALB629 biofilm formation and plant growth under drought stress.

2 MATERIALS AND METHODS

2.1 Superficial seed sterilizations

Common bean seeds cultivar Lariat were surface sterilized by soaking in 4% sodium hypochlorite for 4 min followed by 3 washes with sterile water. Then, the seeds were swirled in 70% ethanol for 3 min and washed 3 times with sterile water.

Rice seeds cultivar MI04 were surface sterilized in 4% sodium hypochlorite for 10 min followed by 3 washes with sterile water. Then, seeds

were swirled in 70% ethanol for 10 min and extensively washed with sterile water.

2.2 Preparation of seed exudates

Since we were interested in studying malic acid possibly present in the seed exudates and once malic acid is a polar molecule we obtained seed exudates by soaking the seeds in $MgCl_2$ solution (0.2%) for 2h, 24h, and 48h.. Six seeds per 15 mL tubes (2 g seeds L^{-1}) with 6 repetitions were used. Seed exudates were passed through 0.22 μm pore filter membranes and checked for eventual contamination by plating an aliquot of 50 μL in LB medium. Plates were incubated at 28 °C for 24h and then checked for bacterial growth.

2.3 Effect of seed exudates on ALB629 biofilm formation

ALB629 was grown overnight in LB medium and washed in $MgCl_2$ as described above and re-suspended in MSGG medium to a final density of OD_{600} = 0.8.

To check the effect of seed exudates on the enhancement of rhizobacteria biofilm formation, three concentrations of seed exudates [0.4%, 1%, 5% v/v, or $MgCl_2$ solution (control)] were mixed with the bacterial suspension using the method of O'Toole and Kolter (1998). Samples of 100 μL of the diluted cells were aliquoted into sterile 96-well microtiter plates with 8 wells per treatment as replications. Plates were covered and incubated at 30 °C without agitation for 48h. Cells that had adhered to the well walls were treated with 0.1% crystal violet for 10 to 15 min at 25 °C without agitation; the plates were drained of liquid via pipet, gently rinsed several times with water, and allowed to dry at room temperature. The dye that had stained the cells was solubilized in 200 μL of 95% (v/v) ethanol. Biofilm formation was quantified by measuring the optical density at 630 nm for each well using Wallac 1420 Manager plate reader.

2.4 Effect of seed exudates on ALB629 growth

To check the ALB629 growth with bean seed exudate presence, the bacteria were grown as described previously and re-suspended in MSGG medium to get a final density of OD_{600} = 0.2. Then, bacterial suspensions were grown for 4 and 10 hours at 28 °C, 220 rpm with seed exudates at 1%. As a control, the bacteria were grown without seed exudates. At the referred time points, ALB629 suspensions were diluted and 10µL was poured on Petri dishes with LB media. Plates were incubated at 28 °C for 48h and cells were counted for the number of colony forming units (CFU). Six replicates (n=6) were used, three of which were biological replicates and two of which were technical replicates for treatment.

2.5 Effect of seed exudates on seed treatment with ALB629 seen under confocal microscope

ALB629 was grown overnight in LB medium, washed in $MgCl_2$ as described above, and re-suspended in $MgCl_2$ solution to a final density of OD_{600} = 0.8. Common bean seeds were treated with the bacterial solution for 2h. In one of the treatments 1% of bean seed exudate was added to the final bacterial solution. As a control, seeds were treated with water. Seeds were then fixed in 2% glutaraldehyde for 2h, washed in filtered PBS buffer and finally stained in SYTO® 13 Green Fluorescent Nucleic Acid Stain for 7 min. The seeds were analyzed in LSM 710 confocal microscopy.

2.6 RT-PCR and semi-quantification

For this experiment, 1% of seed exudate concentration from the 24h seed exudate was used to check the expression of the follow *TasA* (biofilm), *EpsD* (exopolysaccharide), *Spo0A* (sporulation) *RecA*, at 0h, 4h, and 10h of bacterial growth. The bacterium was grown overnight in LB medium and washed in $MgCl_2$ as described above and re-suspended in MSGG medium to a final density of OD_{600} = 0.2. Bean seed exudate at 1% was added into the bacterial suspension and grown for 0h, 6h, and 12h at 28 °C, 220 rpm. As a

control, the bacteria were grown without seed exudates. Total RNA was isolated using the MACHEREY-NAGEL kit from 1.5 mL bacterial suspension, following the manufacturer's instructions. For 0h, RNA extraction was performed from the bacterial suspension samples at the first time point of bacterial growth. For RT-PCR, cDNA was synthesized using M-MuLV reverse transcriptase (New England Biolabs) from 500 ng of RNA according to the Applied Biosystems protocol, followed by PCR amplification using DyNAzyme II DNA polymerase (DyNAzyme). The gene-specific primers for the genes RecA, TasA, EpsD, and Spo0A are listed in the Table 1. Three experimental replicates for each treatment were used in two different experiments. The band intensity was taken from agarose gel images and quantified by Image J 1.47v (National Institute of Health, USA).

Table 1 Gene-specific primers and annealing temperatures used for RT–PCR

Primer	Primer sequence (5′–3′)	Annealing temperature (°C)
RecA foward	AAAAAACAAAGTCGCTCCTCCG	55
RecA reverse	CGATATCCAGTTCAGTTCCAA	55
TasA foward	GGATTTCCTCAGCCAGTTTG	55
TasA reverse	TTTCGGAACTCCGTCGTACT	55
EpsD foward	TTTTCGGCAGCCATTCCTTC	55
EpsD reverse	TGTATCTGACATTGTGCGGTTT	55
Spo0A foward	GACGGACTTGCGGTTTTAGA	32
Spo0A reverse	GCCGATTTCATGGATAATGC	32

2.7 Identification analysis of malic acid in the seed exudates

Identification of malic acid was carried out using high-performance liquid chromatography (HPLC) Shimadzu, equipped with a DAD Shimadzu SPD-M20A detector. The sample separation was carried out on a C18 VP-ODS column (150 × 4,6 mm × 4.6 µm) with a GVP-ODS pre-column (10 x 4,6 mm). The injection volume was 20 µL, the UV detector wavelength was 210 nm, flow 0.2 mL min^{-1}, analysis temperature was 30 °C, and data were obtained by LCSolution software. The standard solution used was prepared by malic acid Supelco PA and Milli-Q water.

2. 8 Time point for seed treatment

ALB629 was grown in 125 mL flasks with 20 mL of LB medium at 28 °C, 220 rpm for 24h, 48h, and 5 days. Cells were collected by centrifugation, washed with sterile 10 mM $MgCl_2$ and re-suspended in $MgCl_2$ solution to a final concentration of $1x10^5$ CFU mL^{-1}. Common bean seeds were soaked for 30 min, 2h, and 4h in ALB629 solution (2 mL g^{-1} seed) or water (control) under agitation at 28 °C. Ten seeds per treatment with 10 replicates were used. After the time exposures, seeds were dried at room temperature for 30 min in a cabinet. In each treatment, a volume of $MgCl_2$ solution 2 mL g^{-1} seed was added in 15 mL sterile tubes and submitted to sonicator bath for 5 min. A volume of 10 µL was plated in LB medium up to $1x10^5$ dilution. After 24 h, cells were counted and the number of colony-forming units (CFU) was assessed per g of seed (log_{10} CFU g^{-1}).

2. 9 Selection of the ALB629$^{rif\text{-}nal}$ mutant

To study the antagonist colonization of bean seedlings submitted to drought stress, a spontaneous rifampicin/nalidixic acid mutant (ALB629$^{rif\text{-}nal}$) was selected from a *B. amylolicefaciens* strain ALB629 based on Medeiros et al. (2009). To select the mutant, increasing amendments of both antibiotics were added to the LB medium up to 100 ppm at each bacterial culture, similar to a previously described method (MARTINS et al., 2014).

2. 10 In vivo drought stress experiment

In order to find out the capability of ALB629 in sustaining plant growth under abiotic stress, we selected the best time point regarding seed treatment in the previous assay to proceed with the seed treatment. A treatment using malic acid (0.5%) Kleijn et al. (2010) was used in order to find out its effect on biological seed treatment. For both treatments, pH was brought to 5.6 - 5.8. Bacterial suspensions were submitted to the seed treatment for 2 hours from a bacterial culture of 2-d-old as described before.

Pots with 1 seedling per 0.3L pot were kept under growth chamber conditions: 200 µE m^{-2} s^{-1} 12h of photoperiod of 23 °C/15 °C (day/night), and relative humidity of 55/70% (day/night) according to Shevyakova et al. (2013) for abiotic stress.

Seedling emergence was recorded daily and used to calculate the speed emergence index (SEI) as described by Teixeira and Machado (2003), as well as percentage of seedling emergence (PSE) from the last evaluated period. When the first fully expanded leaves appeared, irrigation was suspended for two weeks, the time point in which plants started showing signs of wilting. Then, plants were collected and checked for colony-forming units (CFU) and growth promotion parameters: fresh and dry weights, fw/dw ratio, stem size, leaf dimension (width and length).

2. 11 ALB629 visualization on seeds under SEM

After being subjected to the treatment with ALB629 as described above, the seeds were fixated in 2% of glutaraldehyde for 2h, washed in 1x PBS buffer three times for 5 min each, and immersed in 1% Osmium Tetroxide (OsO_4) for 1.5 hours. After fixation and post-fixation, the seeds were dehydrated with ethanol in the following concentrations: 25%, 50%, 75%, 95%, and 100% for 30 min each. Following dehydration, samples were critically point dried in a Tousimis Autosamdri-815B and sputter coated with gold palladium. Samples were visualized under a Hitachi S-4700 field emission scanning electron microscope (FE-SEM).

2. 12 Experimental design and statistical analysis

The randomized complete block design with 8 and 10 replications was used for the *in vitro* and *in vivo* tests, respectively. Data were submitted to one-way variance analysis (ANOVA) and for significant means Scott-Knott test, Tukey's multiple range tests, or Student's t test ($p<0.05$) were applied when necessary. For all analyses, the assumptions of normality of variance were checked by the Shapiro-Wilk test and no transformation was necessary.

3 RESULTS

Plates which were incubated for 24h to check an eventual contamination by seed exudates did not show any sign of bacterial growth, or even other microbials. However, when common bean seed exudates were added into the ALB629 culture, there was an increase of biofilm formation by the bacteria compared to the bacteria culture growth itself for the 1% and 5% of seed exudate concentrations, regardless of the time point in which the seed exudates were collected (Figure 1).

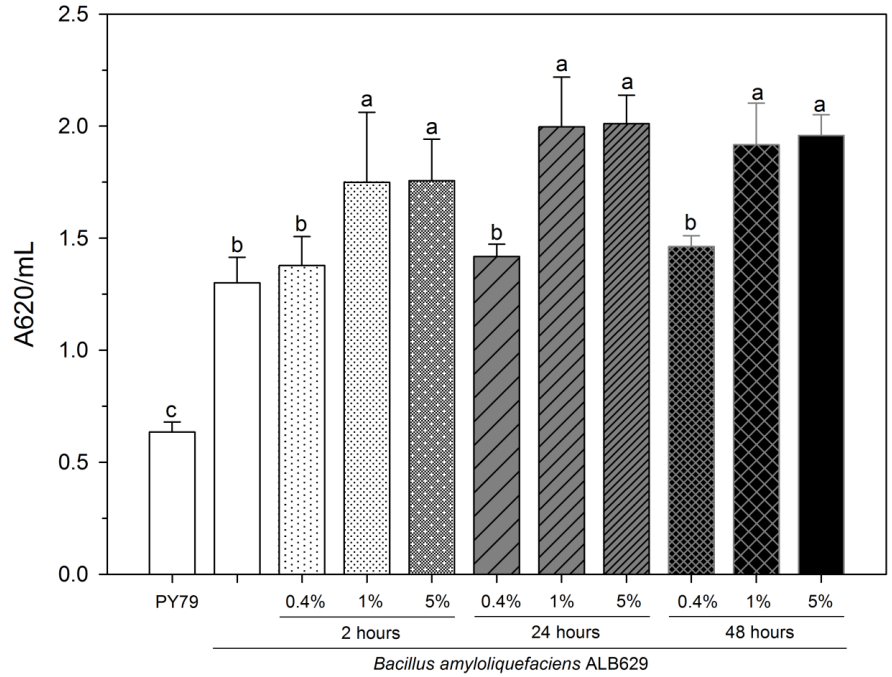


Figure 1 *In vitro* biofilm formation by *B. amylolicefaciens* ALB629 in 96 well plates with different concentrations of bean seed exudates from different time points (2h, 24h, and 48h). The black bar represents the control (ALB629 without seed exudate treatment) and PY79 represents a bacterial positive control. Bars with the same letter are similar at the 5% level according to Scott-Knott test. The line on each bar represents ±SE

There was a significantly higher growth for ALB629 in the presence of bean seed exudates for both 4 (p=0.0001) and 10 hours (p=0.0002) of growth (Figure 2). Additionally, seeds in contact with ALB629 and bean seed exudate (1%) showed more biofilm and/or bacteria on their surfaces when seeds were visualized on a confocal microscope (Figure 3).

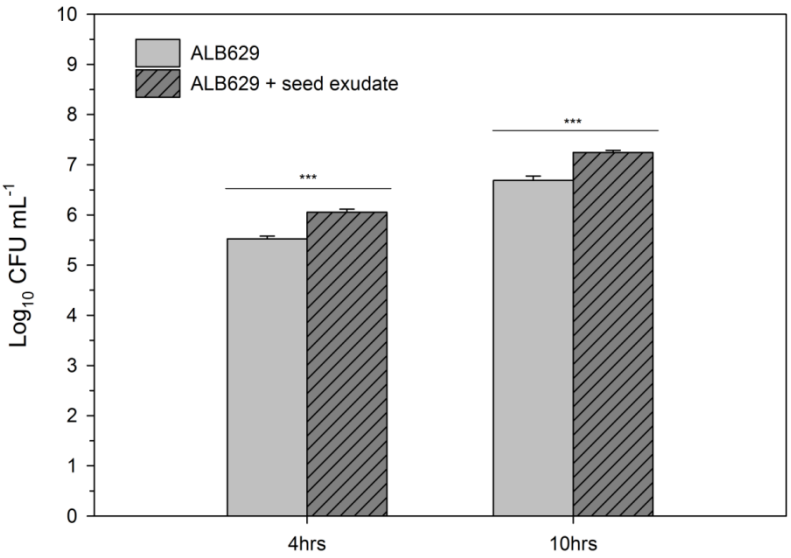


Figure 2 Seed exudates from common bean stimulate ALB629 growth when the nutrient agar medium was amended with (1%) (v/v) of the seed exudate and growth checked at 4 and 10 hours. ***Significant at the 0.001 probability level by Student's t test. The line on each bar represents ±SE

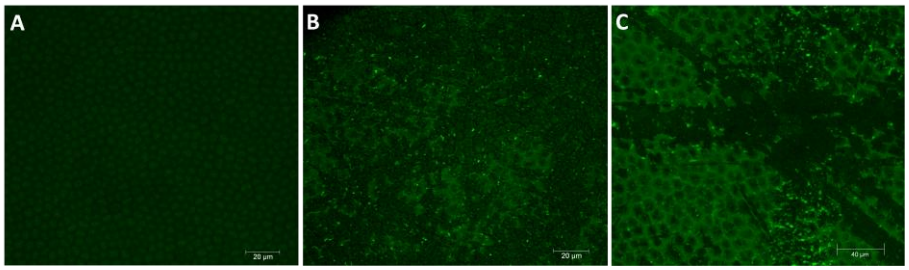


Figure 3 Surface of common bean seeds under confocal microscope. A) control (seed treated with water), B) seed treated with ALB629 only and, C) seed treated with ALB629 + seed exudate (1%) (v/v). The green punctate spots show an abundance of ALB629 on common seed coats. Each image is represented by a z stack of 115 images

Seed exudates from common bean up-regulated biofilm operons in ALB629 in a *TasA* and *EpsD* dependent manner at 4 hours by about 2- and 6-fold, respectively higher than the treatment 0 hour (Figure 4-A, B). On the other hand, in the absence of added seed exudate, ALB629 biofilm operons *TasA* and *EpsD* increased their expressions only at 10 hours by about 1.5-

and 4-fold, respectively. However, the difference between the treatment regarding *Spo0A* was not statistically significant regardless of the time point tested (Figure 4-C).

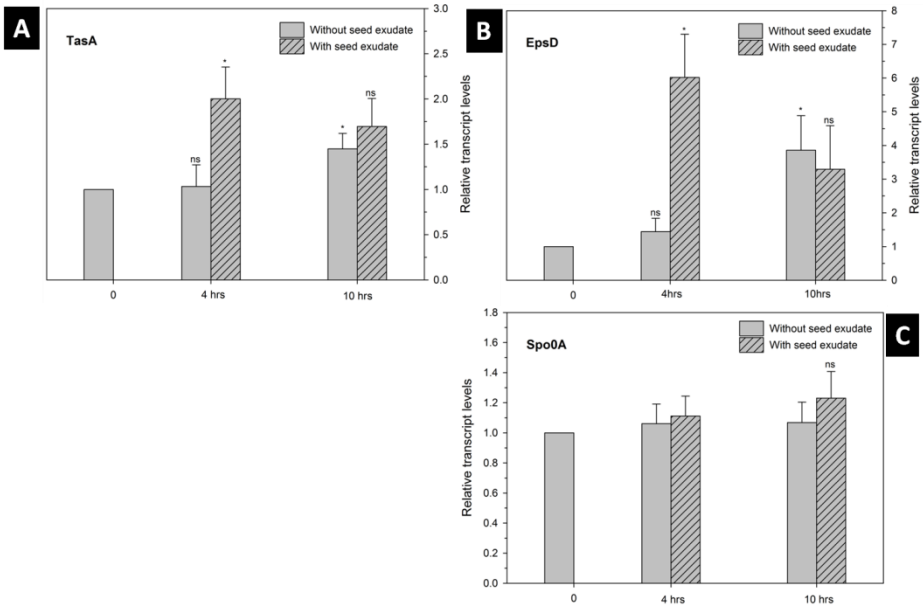


Figure 4 Seed exudates from common bean up-regulate ALB629 biofilm operons in ALB629. *Significant at the 0.05 probability level by Student's t test. ns=Not significant. The line on each bar represents ±SE

When subjected to drought trials for two weeks, seedlings from seeds treated with ALB629$^{rif\text{-}nal}$ and malic acid showed higher colonization by the rhizobacteria compared to the bacterium alone (Figure 5).

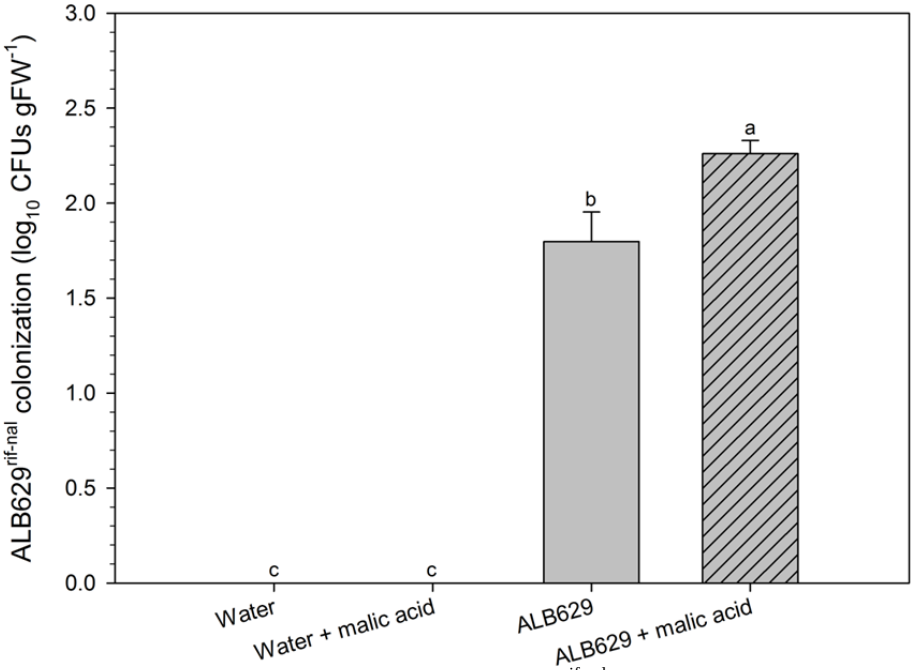


Figure 6 Effects of seed treatment of ALB629$^{rif\text{-}nal}$ with malic acid on root colonization. Bars with the same letter are similar at the 5% level according to Tukey's multiple range test. The line on each bar represents SE

In addition, the seed treatment with ALB629 and malic acid increased the seedling emergence index (Figure 6C), leaf length (Figure 6D), plant fresh weight, plant water holding capacity (fresh:dry weight ratio) (Figure 6B) compared to the water control or water associated with malic acid (Figure 6B, E). Moreover, ALB629 itself could increase the seedling stem size (Figure 6A). The time point used for the biological seed treatment was two hours of seed immersion in a two-day-old bacterial suspension (Supplementary data 1A, B, C).

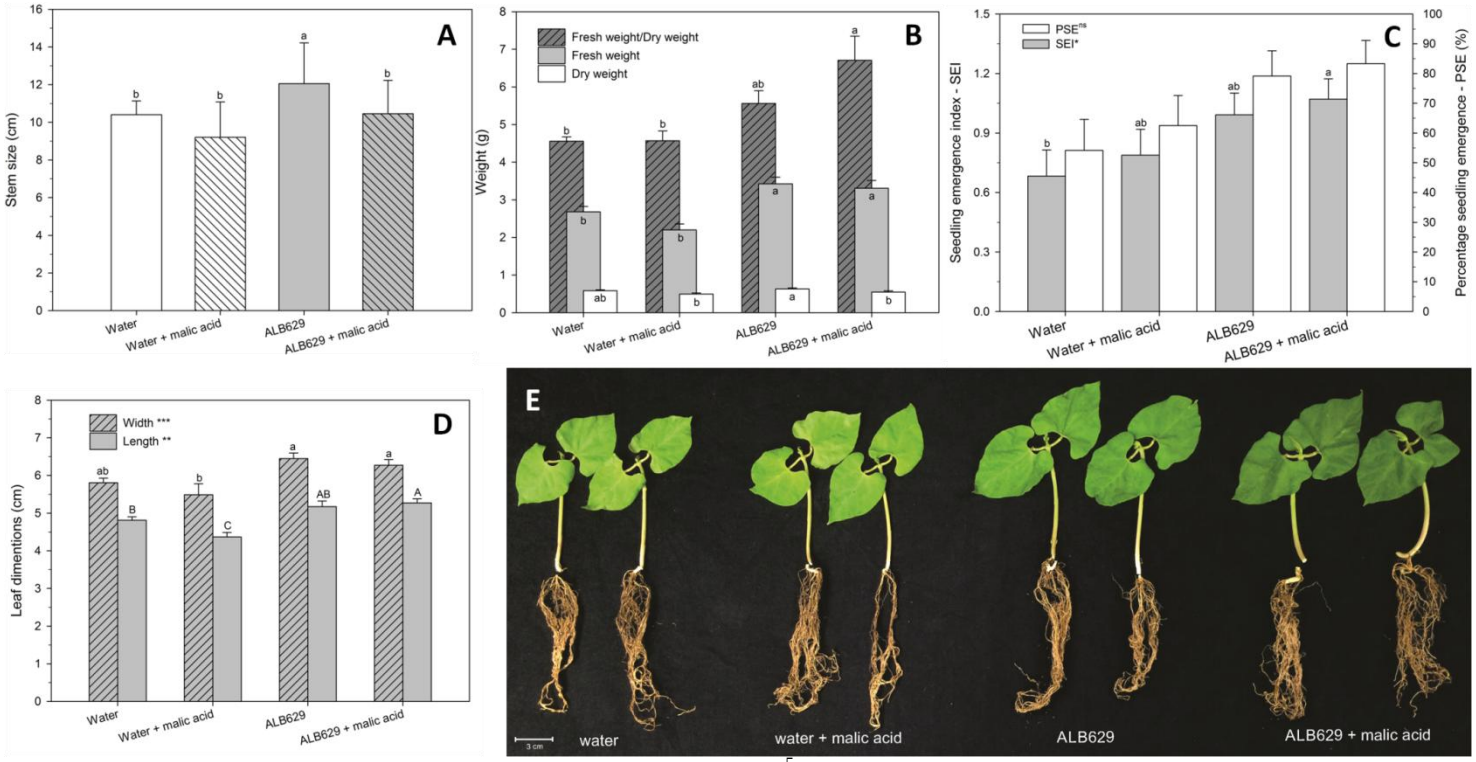


Figure 6 Effects of seed soaking (2mL suspension/g of seed) of ALB629 (10^5 CFU/mL) or water (control) amended or not with malic acid at the seed treatment (0.5% v/v) on A) stem size; B) biomass accumulation water holding capacity; C) seedling emergence index and percentage seedling emergence; D) leaf dimensions; E) bean seedlings after 2 weeks under drought stress. Bars with the same letter are similar at the 5% level according to Tukey's multiple range test. The line on each bar represents SE

4. Discussion

In this study, we have shown that *Bacillus amyloliquefaciens* ALB629 is capable of forming biofilm and that seed exudate could accelerate the bacterial growth and boost the biofilm formation in ALB629. By testing seed exudate from a no-legume we found out that the biofilm increase is not specifically related to a legume seed exudate, since rice seed exudate could trigger higher biofilm concentration as well, regardless of the tested seed exudate concentration (Supplementary data 2). Moreover, through confocal microscopic images we reinforced that extra seed exudates on the surface of common bean seed could recruit more bacterial cells and reinforce the biofilm.

As seeds imbibe water and germinate, they passively release exudates, forming a chemical gradient around seeds. The spermosphere is a primary colonization court of many kinds of microorganisms, including pathogens and beneficial microorganisms, such as rhizobacteria. Among the many advantages of beneficial microorganisms is that these microbials can trigger the plant defense *in priming* (CONRATH et al., 2015; POZO et al., 2008), which represents an ecological advantage factor against not only biotic competitors but also abiotic stresses, by which plants are constantly challenged. Here, we have shown that when ALB629 was grown in the presence of seed exudate there was a faster and stronger upregulation for biofilm operons (*TasA* and *EpsD*) than when seed exudate was not present. Chen and Nelson (2008) also have shown that a seed colonizing microbial community that develops within 8 hours can suppress *Pythium ultimum* on wheat and cucumber.

Although we did not find a statistical difference between treatments regarding the percentage seedling emergence (PSE), ALB629 could accelerate the seed germination by increasing seedling emergence index (SEI). Besides, SEI was higher when ALB629 was in contact with malic acid (Figure 6C).

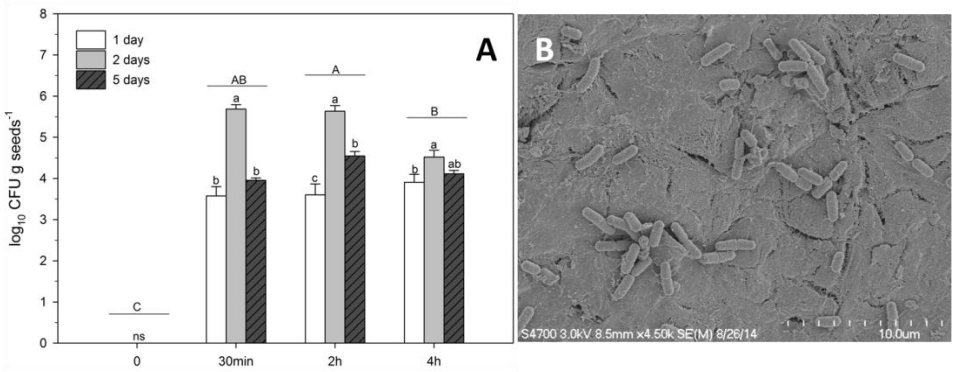
Under field conditions, a faster germination can avoid many damping off pathogen attacks and sustain a bigger plant stand (BECKSTEAD et al., 2007).

By HPLC analysis, we found a molecule in the seed exudate used in this work at the same retention time of malic acid (14.6 min), indicating a strong correlation between the molecule and the organic acid. In our drought stress test, seedlings from seeds treated with ALB629 and with a malic acid supplementation showed a promotion in growth and could tolerate the abiotic stress by increasing plant water capacity. Additionally, a higher ALB629 population was found on treated plants. In accordance with these findings, Rudrappa et al. (2008) have shown that the malic acid secreted from *Arabidopsis thaliana* roots could recruit *Bacillus subtilis* FB17, a beneficial bacterium from soil. The malic acid has also been shown to be the major tomato root exudate component involved in the process of increasing Bacillus biofilm exudates according to Chen et al. (2012).

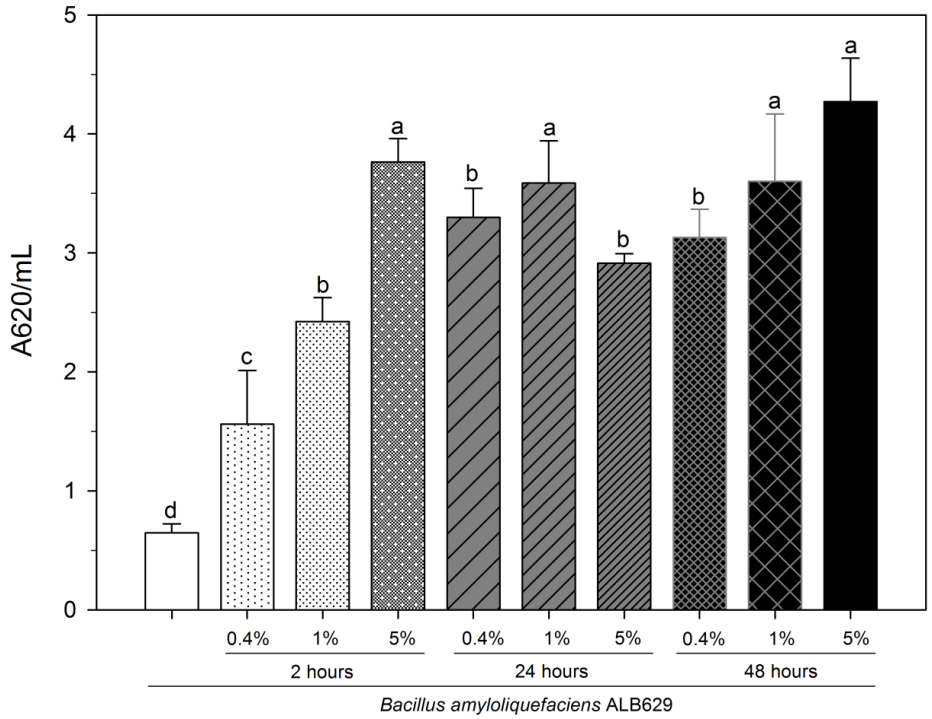
The utilization of "biologicals" for crop improvement is a sustainable strategy, but issues related to variation in efficacy are still an obstacle that must be overcome. The beneficial agents' survival rate, population density as well as its quality and effectiveness are the prerequisites for their effectiveness or specific functions in the rhizosphere (RAAIJMAKERS et al., 2009). Besides the benefits showed in this work by *B. amylolicefaciens* ALB629, in previous studies when ALB629 was applied in common bean through seed treatment, it offered a resistance to biotic stress by controlling the bacterial wilt, an emergent bacterial disease to common bean in Brazil (MARTINS et al., 2013), even under high temperature stress (MARTINS et al., 2014). This same rhizobacteria strain could also enhance common bean plants' nutrient uptake from soil and increase the plant's physiological activities (MARTINS et al., 2015). Another benefit of bacteria belonging to the Bacillus genus, such as ALB629, is that it can produce endospores, which confer resistance to abiotic stresses, stability during

formulation and a longer shelf life (CHOUDHARY; JOHRI, 2009; HAYAT et al., 2010).

In conclusion, we showed in this work that *Bacillus amyloliquefaciens* ALB629 was benefited by exudates in events even before seed germination. Also, the present study revealed that malic acid, an organic acid component of seed/root exudates, can be used as nutritional sources for ALB629's growth, to increase ALB629 biofilm formation, as well as to reinforce its population on common bean roots, besides offering the plant a tolerance to drought stress. To the best of our knowledge, this study was the first one that unraveled the mechanisms before root secretions that favored beneficial microorganisms such as the rhizobacteria in a cultivated crop (*Phaseolus vulgaris*).



Supplementary data 1 A) Time points and bacterial culture age for biological seed treatment with *B. amylolicefaciens* ALB629 expressed as the number of colony-forming units (CFU) per g of seed (log_{10} CFU g^{-1}). Bars with the same letter are similar at the 5% level according to the Scott-Knott's test. The line on each bar represents ±SE. B) Common bean seed colonization by *B. amylolicefaciens* ALB629 from seed treatment of two hours of seed immersion in a two-day-old bacterial suspension observed through a scanning electron microscope (SEM)



Supplementary data 2 *In vitro* biofilm formation by *B. amylolicefaciens* ALB629 in 96 well plates with different concentrations of rice seed exudates from different time points (2h, 24h, and 48h). The bar in red represents the control (ALB629 without seed exudate treatment). Bars with the same letter are similar at the 5% level according to the Scott-Knott test. The line on each bar represents ±SE

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CONSIDERAÇÕES FINAIS

As rizobactérias têm o potencial de controlar doenças de plantas e induzirem tolerância a estresses abióticos. Nesta tese, foi mostrado que as rizobactérias podem aumentar a absorção de magnésio (Mg), teor de clorofila e a taxa fotossintética. Aumentos em absorção do Mg podem ser conseguidos pelo aumento de seu teor no solo, mas níveis elevados do mineral podem ser tóxicos às plantas. Entretanto, se a planta é tratada com a rizobactéria *Bacillus amylolicefaciens* (ALB629) a planta mantém níveis normais de fotossíntese independente da concentração do mineral no solo, amenizando por meio de mecanismo (s) ainda não conhecido (s), os efeitos tóxicos de seu excesso.

Em muitas situações a indução de tolerância a estresses abióticos pode ser de amplo espectro e, foi também observado nesta tese que o tratamento com a mesma rizobactéria pôde aumentar a tolerância a estresse hídrico e este ser potencializado pela maior colonização de raízes e formação de biofilme bacteriano. Este biofilme por sua vez pode ser potencializado com a adição de exsudatos da semente ou simplesmente, um de seus componente, o ácido málico.

Além do mais, estas bactérias podem proteger o feijoeiro contra estresses bióticos como infecções fúngicas e bacterianas. As plantas de feijoeiro tratadas com a rizobactéria tiveram redução na murcha de curtobacterium e esta proteção foi independente do teor de magnésio do solo, ou seja, mesmo em níveis tóxicos do mineral, as plantas tratadas com a rizobactéria permaneceram sadias. Um dos mecanismos pelos quais estas bactérias podem proteger as plantas são os compostos orgânicos voléteis como a acetoína com ação tóxica direta a *Colletotrichum lindemuthianum* e que protegem o feijoeiro contra a antracnose.