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CARACTERIZAÇÃO DE ISOLADOS DE *STREPTOMYCES* SPP. PROVENIENTES DE RAÍZES DE FABACEAE COMO RIZOBACTÉRIAS PROMOTORAS DE CRESCIMENTO E INDUTORAS DE RESPOSTAS DE DEFESA EM SOJA [*GLYCINE MAX* (L.) MERRILL]

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul como requisito para obtenção do título de mestre.

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" Nos alicerces das grandes descobertas existem grandes falhas, nos alicerces das grandes falhas existem grandes sonhos de superação. Realizar os sonhos implica riscos, riscos implicam escolhas, escolhas implicam erros. Quem sonha não encontra estradas sem obstáculos, lucidez sem perturbações, alegrias sem aflição. Mas quem sonha voa mais alto, caminha mais longe. Toda pessoa, da infância ao último estágio de vida precisa sonhar. " **Augusto Cury**

RESUMO

As rizobactérias promotoras de crescimento de plantas (PGPR) podem aumentar a produtividade agrícola, atuando através da promoção de crescimento vegetal por meio de fitormônios reguladores de crescimento, facilitando a captação de nutrientes e de compostos químicos no solo, bem como inibindo fatores de estresse vegetal. Bactérias do gênero *Streptomyces* spp. (Stm) apresentam grande potencial biotecnológico, pois além de promoverem o crescimento e a indução de defesa vegetal, também são conhecidas pela grande produção de metabólitos secundários, incluindo antibióticos e fenazinas. A soja [*Glycine max* (L.) Merrill] é uma das principais leguminosas cultivadas no mundo, sendo o Brasil o segundo maior produtor. Sua produção é afetada por inúmeras doenças, como a Pústula bacteriana causada pelo fitopatógeno *Xanthomonas axonopodis* pv. *glycines* (Xag). O objetivo deste trabalho foi avaliar 11 isolados de *Streptomyces* spp. oriundos da rizosfera de plantas de Fabaceae quanto às características de PGPR, bem como, à capacidade de modulação das vias de defesa de plantas de soja em resposta à fitobactéria patogênica Xag. Os isolados foram avaliados quanto às características de PGPR pela produção de sideróforos, de ácido indolacético (AIA), da enzima 1-aminociclopropano-1-ácido carboxílico (ACC) desaminase e de fenazinas. Para a identificação taxonômica e a avaliação da promoção de crescimento de plantas de soja foram selecionados três isolados com características de PGPR. O ensaio de promoção de crescimento ocorreu em casa de vegetação por meio da microbiolização das sementes pelos isolados selecionados e o controle com água destilada estéril. Os parâmetros avaliados foram: comprimento, massa fresca e seca, de parte aérea e raiz, aos 15, 30 e 45 dias de cultivo. Para a avaliação da capacidade de indução dos mecanismos de defesa de plantas de soja, foi selecionado o isolado que obteve o melhor desempenho no ensaio de promoção de crescimento. Sementes de soja, de cultivar sensível e resistente à Xag, foram microbiolizadas com o isolado de Stm selecionado e cultivadas em casa de vegetação. As plantas obtidas foram desafiadas com Xag, 15 dias após a sua emergência. Os tratamentos consistiram de (a) sementes tratadas com água destilada estéril (controle absoluto); (b) sementes microbiolizadas com StmCLV45 (Stm45); (c) sementes tratadas com água destilada estéril e plantas desafiadas com Xag (Xag); e (d) sementes microbiolizadas StmCLV45 e plantas desafiadas com Xag (Stm45+Xag). As respostas enzimáticas relacionadas às vias de defesa foram avaliadas bioquimicamente, pela análise da atividade da fenilalanina amônia liase (PAL) e pela produção dos compostos fenólicos, nos tempos 0, 24, 48, 72 e 144 horas pós inoculação (hpi) da Xag. A expressão dos genes relacionados à defesa das plantas de soja desafiadas com

Xag foi determinada pela expressão relativa de *JAZ*, *ERF5*, *PAL* e *PR1* por qPCR, nos tempos 0, 12, 24 e 48 hpi. Os resultados da análise bioquímica indicaram os isolados CLV42, CLV44 e CLV46 como maiores produtores de sideróforos e os isolados CLV41, CLV45 e CLV46 com maior atividade de ACC desaminase. Todos os isolados foram capazes de produzir AIA, com destaque para o isolado CLV45, que produziu 398,53 µg AIA g^{-1} de células. A fenazina piocianina (PYO) também foi detectada em todos os isolados, entretanto o mesmo não ocorreu para a fenazina 1-ácido carboxílico (PCA), somente produzida por CLV41, CLV43 e CLV45. Os isolados CLV42, CLV44 e CLV45 foram selecionados por suas características de PGPR para o ensaio de promoção do crescimento de plantas de soja em casa de vegetação e caracterizados taxonomicamente como espécies do gênero *Streptomyces*. Nenhum dos isolados avaliados no ensaio causou déficit de crescimento em plantas de soja. O isolado CLV45 promoveu significativamente o crescimento de parte aérea de plantas soja, em 36,63%, corroborado pela maior massa seca, 17,97%, em relação ao grupo controle, sendo selecionado para avaliação nas vias de defesa da soja. A expressão do gene *PAL* foi moderadamente aumentada em plantas suscetíveis Stm45+Xag em 12 hpi, seguido por aumento da atividade da enzima PAL de 48 a 144 hpi, embora o acúmulo correspondente de compostos fenólicos não tenha sido registrado. Na cultivar resistente, a expressão de *PAL* em plantas Stm45+Xag resultou em alta atividade desta enzima. A expressão aumentada de *ERF5* e a diminuição de expressão do gene *JAZ* em 12 hpi em plantas Stm45+Xag de ambas as cultivares sugeriram que etileno e ácido jasmônico desempenharam função na defesa sistêmica induzida por *Streptomyces* sp. CLV45 contra Xag em plantas de soja.

Palavras-chave: PGPR, Defesa vegetal, ISR, Jasmonato, *Xanthomonas axonopodis* pv. *glycines*

ABSTRACT

The plant growth promoting rhizobacteria (PGPR) can increase agricultural productivity by promoting growth through production of plant hormones, facilitating the uptake of nutrients and chemicals on the soil, as well as inhibiting plant stress factors. *Streptomyces* spp. (Stm) are bacteria with great biotechnological potential, because in addition to its growth promotion and plant defense induction, they are also known as great producers of secondary metabolites, including antibiotics and phenazines. Soybean [*Glycine max* (L) Merrill] is one of the main legume crop grown around the world and Brazil is the second largest producer. Its production is affected by many diseases, among which bacterial pustule caused by the pathogen *Xanthomonas axonopodis* pv. *glycines* (Xag). The objective of this project was to evaluate isolates of *Streptomyces* spp. obtained from the rhizosphere of Fabaceae plants regarding characteristics of PGPR, as well as the modulation capacity of soybean defenses in response to the phytopathogen Xag. Eleven isolates of *Streptomyces* spp. were screened for PGPR traits by siderophores production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole-3-acetic acid (IAA) and phenazines. For a taxonomic identification and growth evaluation of soybean plants, three isolates were selected for their biochemical characteristics. The growth promoting assay was performed in greenhouse using bacterized seeds with the selected isolate and sterile distilled water was use for the control. Length, fresh and dry weight from shoot and root at 15, 30 and 45 days of cultivation were the evaluated parameters. For evaluation of the induction capacity of defense mechanisms of soybean plants, the isolate that obtained the best performance in the growth promotion test was selected. Seeds of soybeans, from sensitive cultivars and resistant to Xag, were bacterized with the selected Stm isolate and grown under greenhouse conditions. The plants were challenged with Xag 15 days after emergence. The treatments consisted of (a) plants treated with sterile distilled water (absolute control); (b) plants bacterized with Stm CLV45 (Stm45); (c) water-treated and Xag challenged plants; and (d) plants bacterized with StmCLV45 and challenged with Xag (Stm45+Xag). The enzymatic responses related to the defense pathways were evaluated biochemically, by analyzing the activity of phenylalanine ammonia lyase (PAL) and by the production of phenolic compounds at times 0, 24, 48, 72 and 144 hours post infection (hpi) of Xag. The expression of the genes related to the defense in Xag challenged soybean plants was determined by the relative expression of the genes *PAL, JAZ, ERF5* and *PR1* by qPCR at times 0, 12, 24 and 48 hpi. The results of the biochemical analysis indicated the isolates CLV42, CLV44 and CLV46 as the major producers of siderophores and CLV41, CLV45 and

CLV46 isolates with higher ACC deaminase activity. All isolates were able to produce IAA, highlighting the isolate CLV45, which produced 398.53 μg AIA g-1 cell. Phenazine pyocyanin (PYO) was also detected in all isolates, but the same did not occur for the 1-carboxylic acid phenazine (PCA), only produced by CLV41, CLV43 and CLV45. The isolates CLV42, CLV44 and CLV45 were selected for their PGPR characteristics for the growth promotion trial of greenhouse soybean plants and taxonomically characterized as species of the genus Streptomyces. None of the isolates evaluated in the trial caused a growth deficit in soybean plants. The CLV45 isolate significantly promoted the growth of soybean shoots in 36.63%, corroborated by the highest dry mass, 17.97%, in relation to the control group, being selected for soybean defense pathways induction. Expression of *PAL* gene was moderately enhanced in susceptible Stm45+Xag plants at 12 hpi, followed by increase of PAL enzyme activity from 48 to 144 hpi, although corresponding accumulation of phenolic compounds was not recorded. In the resistant cultivar, the highlighted expression of *PAL* in Stm45+Xag plants resulted in high activity of this enzyme. Enhanced expression of *ERF5* and decrease on *JAZ* gene at 12 hpi in Stm45+Xag plants from both cultivars suggested that ET and JA play a concert role on induced systemic defense by *Streptomyces* sp. CLV45 against Xag in soybean.

Key words: PGPR, Plant defense, ISR, Jasmonate, *Xanthomonas axonopodis* pv. *glycines*.

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LISTA DE ABREVIATURAS E SIGLAS

AACPD – Área abaixo da curva de progressão da doença

AIA – Ácido indol-3-acético

ACC – Enzima 1-aminociclopropano-1-carboxilato desaminase

AJ – Ácido jasmônico

AS – Ácido salicílico

CLAE – Cromatografia Líquida de Alta Eficiência

DAMPs – Padrões moleculares associados a danos (do inglês, *Danger-associated molecular patterns*)

DPI – Dias pós inoculação (dpi)

ET – Etileno

ETI – Imunidade desencadeada por efetores (do inglês, *Effector-triggered immunity*)

ETS – Suscetibilidade desencadeada por efetores (do inglês, *Effector-triggered susceptibility*)

Hpi – Horas pós inoculação

HR – Reação de hipersensibilidade (do inglês, *Hypersensitive reaction*)

UFC – Unidade formadora de colônia

ISP – Projeto internacional *Streptomyces* (do inglês, *International Streptomyces Project*)

ISR – Indução de resistência sistêmica (do inglês, *Induced systemic resistance*)

LB – Meio de cultura Luria-Bertani

MAMPs – Padrões moleculares associados a microrganismos (do inglês, *Microorganism-associated molecular patterns*)

NPR1 – Proteína não expressora do gene PR1 (do inglês, *nonexpresser of pathogenesis-related gene 1 protein*)

PAL – Fenilalanina amônia liase (do inglês, *Phenylalanine ammonia lyase*)

PAMPs – Padrões moleculares associados a patógenos (do inglês, *Pathogen-associated molecular patterns*)

PCR – Reação em cadeia da polimerase (do inglês, *Polymerase chain reaction*)

PGPRs – Rizobactérias promotoras de crescimento vegetal (do inglês, *Plant growth promoting rhizobacteria*)

PRPs – Proteínas relacionadas à patogênese (do inglês, *Pathogenesis-related proteins*)

PRRs – Receptores de reconhecimento padrão (do inglês, *Pattern recognition receptors*)

PTI – Imunidade desencadeada por PAMP (do inglês, *PAMP-triggered immunity*)

R – Resistência

SAR – Indução de resistência adquirida (do inglês, *Induced acquired resistance*)

Stm – *Streptomyces* spp.

Xag – *Xanthomonas axonopodis* pv. *glycines*

SUMÁRIO

CAPÍTULO I: DA INTRODUÇÃO AOS OBJETIVOS

1. INTRODUÇÃO

1.1. A rizosfera e as rizobactérias promotoras de crescimento vegetal (PGPR)

A porção de solo que envolve as raízes e está sob sua influência é chamada de rizosfera (Kloepper et al. 1980b; Hartmann et al. 2008). A composição da rizosfera é fundamental para a sanidade vegetal, sendo a região de maior interação de plantas com o solo e com a vasta gama de microrganismos ali presentes (Bais et al. 2006; Bulgarelli et al. 2013). A microbiota que habita a rizosfera é específica e distinta da existente no solo do entorno (Raaijmakers et al. 2009), consequência da concorrência direta por nutrientes exsudados nas raízes (Bais et al. 2006; Raynaud et al. 2008; Bulgarelli et al. 2013; Chaparro et al. 2013).

As bactérias que se estabelecem neste ambiente competitivo são referidas como rizobactérias. Elas formam uma numerosa e significativa comunidade rizosfera-competente, abrangendo de 10^5-10^7 UFC por grama de raiz fresca (Bulgarelli et al. 2013). Diversos trabalhos têm sido realizados a fim de conhecer a diversidade e compreender a atividade bacteriana na rizosfera, permitindo um aprofundamento no conhecimento das características dessas linhagens, de tal modo que se alcance uma maior compreensão do seu papel na interação com a planta e com o meio ambiente (Zehnder et al. 2001; Berg e Smalla 2009; Chaparro et al. 2013; Forte et al. 2015; Salla et al. 2016).

Dentre as rizobactérias, um grupo se destaca por exercer efeitos positivos sobre o desenvolvimento das plantas, denominadas rizobactérias promotoras de crescimento vegetal (PGPR, do inglês, *Plant Growth-Promoting Rhizobacteria*). As PGPRs são microrganismos não patogênicos (endofíticos e/ou vida livre), cuja interação com as plantas é benéfica e simbiótica (Suslow et al. 1979; Kloepper et al. 1980b; Bulgarelli et al. 2013). Elas possuem alta capacidade de adaptação à ampla variedade de ambientes, com elevada taxa de reprodução e grande versatilidade bioquímica para metabolizar uma série de compostos naturais e xenobióticos (Palaniyandi et al. 2013c). Além disso, as PGPRs exercem efeitos benéficos sobre a planta, promovendo direta e indiretamente o crescimento vegetal (Lugtenberg e Kamilova 2009; Glick 2012; Palaniyandi et al. 2013c; Vacheron et al. 2013; Gupta et al. 2015).

De forma direta, a promoção de crescimento por PGPRs envolve a produção de compostos que regulam o balanço hormonal, suplementam e disponibilizam elementos nutricionais chave, antes indisponíveis para as plantas. Tais compostos envolvem a síntese de sideróforos (Kloepper et al. 1980a; Rajkumar et al. 2010; Sharma et al. 2013) e a produção de fitormônios reguladores de crescimento vegetal, tal como ácido indol-3-acético (AIA) (Patten

e Glick 1996; Spaepen e Vanderleyden 2011). Desta forma, as PGPRs atuam como biofertilizantes e fitoestimuladoras na rizosfera em que se estabelecem (Vessey 2003; Siddiqui 2006; Spaepen e Vanderleyden 2011; Gupta et al. 2015).

O Ferro (Fe) é um micronutriente essencial para o desenvolvimento da planta, sendo sua concentração na rizosfera extremamente baixa (van Loon e Bakker 2005; Gupta et al. 2015). A forma de Fe mais comumente encontrada no ambiente aeróbico é do íon férrico (Fe^{3+}), que nestas condições ele está inacessível para as plantas e alguns microrganismos (Vessey 2003; Rajkumar et al. 2010; Palaniyandi et al. 2013b; Gupta et al. 2015). PGPR produtoras de sideróforos podem reduzir o Fe^{3+} a Fe^{2+} e assim torná-lo disponível para a planta. Gupta *et al.* (2015) demonstraram a relação positiva da produção de sideróforos sobre o crescimento de plantas, utilizando sideróforos férricos radiomarcados como única fonte de Fe, mostrando que plantas podem utilizar o Fe disponibilizado por PGPRs, incluindo rizobactérias do gênero *Streptomyces*. (Gupta et al. 2015).

As plantas, assim como outros seres vivos, têm seus processos metabólicos mediados por hormônios, tais como, auxinas, citocininas, giberelinas, ácido abscísico e etileno (Sanches et al. 2009; Berg 2009; Bhattacharyya e Jha 2012). Quando associadas às plantas, as bactérias produtoras de auxinas podem oferecer equilíbrio no balanço hormonal vegetal (Berg 2009). Cerca de 80% das rizobactérias (incluindo PGPRs) sintetizam AIA (Patten e Glick 1996; Barazani e Friedman 1999; Vessey 2003; Vacheron et al. 2013), sendo este o hormônio auxínico mais bem caracterizado (Sanches et al. 2009; Mabood et al. 2014; Gupta et al. 2015). O AIA está relacionado a muitos processos de desenvolvimento vegetal (Spaepen e Vanderleyden 2011). Em baixas concentrações, o AIA pode estimular o alongamento da raiz primária, enquanto que em níveis elevados pode estimular a formação de raízes laterais, estabilizar o comprimento da raiz primária e induzir a formação de tricomas radiculares (Vacheron et al. 2013). A inoculação de plantas com PGPRs produtoras de AIA estimula a mudanças na estrutura radicular, que proporcionam o aumento da superfície de contato da raiz com o solo, levando à maior absorção de minerais e exsudação, o que estimula a colonização por mais PGPRs, promovendo, assim, o crescimento vegetal (Vessey 2003; Spaepen et al. 2007; Mabood et al. 2014).

De forma indireta, o crescimento vegetal é promovido por meio do antagonismo exercido pelas PGPRs a microrganismos nocivos à planta, dito antagonismo 'rizobactéria-fitopatógeno' (Berg 2009). Tal antagonismo pode ocorrer através da competição por nicho e exsudatos radiculares (Bais et al. 2006; Verma et al. 2011); da antibiose (Pal et al. 2006; Sarma et al. 2015); do aumento da tolerância ao estresse (Palaniyandi et al. 2014); e/ou

induzindo a resistência vegetal (van Loon et al. 1998; Lugtenberg e Kamilova 2009; Vacheron et al. 2013; Burketova et al. 2015).

Na rizosfera, as plantas atraem e estimulam o metabolismo e o crescimento microbiano através de seus exsudatos radiculares (Mabood et al. 2014). Para as rizobactérias obterem sucesso, é necessário que elas sejam efetivas na competição por nicho e nutrientes da planta hospedeira (Palaniyandi et al. 2013c). A competição ocorre quando um mesmo recurso ambientalmente limitado é disputado (Kloepper et al. 1980a), sendo o resultado dessa interação a diminuição de metabolismo, reprodução e crescimento de pelo menos um dos organismos envolvidos (Pal et al. 2006). Neste caso, quando uma PGPR exerce antagonismo a um fitopatógeno e o último sofre uma redução populacional e/ou perda de nicho, ocorre o chamado controle biológico, ou biocontrole (Pal et al. 2006), garantindo o desenvolvimento do vegetal. Kloepper et al. (1980b) foram os primeiros a mostrar um dos mecanismos de antagonismo bacteriano através da produção de sideróforos por *Pseudomonas fluorescens* e a sua importância na competição por micronutrientes essenciais como o Fe, demonstrando *in vitro* o controle biológico de *Erwinia carotovora*, fitopatógeno de plantas de tomate. Também foi relatada a supressão de fitopatógenos por estreptomicetos produtores de sideróforos inibindo a germinação de esporos do fungo *Moniliophthora perniciosa* (Macagnan et al. 2008).

O estresse vegetal pode ser consequência de fatores abióticos como alagamentos, seca e contaminação, e de fatores bióticos, como danos mecânicos sofridos por pragas ou por microrganismos patogênicos (Siddiqui 2006; Berg 2009). O etileno (ET) é o hormônio relacionado às repostas de estresse vegetal, também conhecido como hormônio de senescência (Pierik et al. 2006). Em concentrações elevadas, o ET leva à desfolha e à diminuição do crescimento vegetal, afetando o desempenho das culturas (Bhattacharyya e Jha 2012). O 1-aminociclopropano-1-carboxilico (ACC) é o precursor da via de síntese do ET em plantas. Por ocupar a rizosfera, rizobactérias produtoras da enzima ACC desaminase utilizam o ACC como fonte de nitrogênio, mantendo a concentração do precursor de ET baixa, promovendo maior resistência ao estresse, assim como o crescimento radicular (Glick 2005).

A antibiose se caracteriza pela interação desarmônica entre diferentes espécies (Odum 2004). Na rizosfera, algumas PGPR podem exercer o antagonismo através da produção de compostos nocivos, tais como antibióticos, compostos orgânicos voláteis e enzimas hidrolíticas (Mazzola 2002; Shilev 2013). Atuando direta ou indiretamente sobre outros organismos, como vírus, bactérias, fungos, nematódeos e insetos, inibindo o seu desenvolvimento e reprodução, e conferindo vantagem competitiva em relação à colonização radicular (Shilev 2013; Zhang et al. 2015).

1.2. *Streptomyces* spp.

Streptomyces spp. (Stm) são Actinobactérias, Gram-positivas, aeróbicas, filamentosas e produtoras de esporos (Waksman e Henrici 1943). Seu crescimento ótimo ocorre entre temperaturas de 25 a 35 °C e valores de pH de 6,5 a 8,0. Morfologicamente, diferem de outros actinomicetos pela formação de hifas aéreas (ou esporóforos), no final da fase reprodutiva, que se diferenciam em uma cadeia de esporos unicelulares (Õmura et al. 2001; Madigan et al. 2016), possibilitando a sobrevivência em condições ambientais desfavoráveis. Elas compreendem cerca de 10% da microbiota do solo e desempenham importantes associações na comunidade rizosférica (Schrey e Tarkka 2008; Palaniyandi et al. 2013b). Possuem a capacidade de degradar lignocelulose e quitina (Bentley et al. 2002), assim como agroquímicos e metais pesados (Francis et al. 2010), sendo, portanto, fundamentais para a reciclagem da matéria orgânica.

Mecanismos diretos de promoção de crescimento vegetal foram relatados para muitos estreptomicetos, tais como a produção de sideróforos (Hamdali et al. 2008; Gupta et al. 2015), a solubilização de fosfatos (Palaniyandi et al. 2013b; Jog et al. 2014), e a produção do fitormônio auxínico AIA (Dalmas et al. 2011; Gopalakrishnan et al. 2015). Stm também foram relatadas atuando indiretamente sobre o desenvolvimento vegetal, por meio da indução de respostas de defesa (Hamdali et al. 2008; Lehr et al. 2008; Berg 2009; Jog et al. 2014); da diminuição da síntese de ET pela síntese de ACC-desaminase (Kloepper et al. 1980a; Macagnan et al. 2008; El-Tarabily 2008; Jog et al. 2014; Palaniyandi et al. 2014); do antagonismo a agentes patogênicos (Franco-Correa et al. 2010); e/ou pela antibiose, através da produção de compostos antimicrobianos (Liu et al. 2013; Shilev 2013; Zhang et al. 2015) garantindo a fitossanidade. A produção de antibióticos também é considerada uma característica fundamental para a seleção de agentes de controle biológico, incidindo diretamente na supressão de patógenos da rizosfera (Melo 1998; Shilev 2013; Gupta et al. 2015).

Nesse sentido, há um grande interesse no estudo das Stm como agentes de controle biológico (Palaniyandi et al. 2013a; Salla et al. 2014; Kumar et al. 2015; Gopalakrishnan et al. 2015). Stm são produtoras de uma ampla gama de metabólitos secundários de interesse biotecnológico (Õmura et al. 2001), que incluem compostos voláteis (Wang et al. 2013), enzimas hidrolíticas (Macagnan et al. 2008; Palaniyandi et al. 2013b) e compostos antimicrobianos (Liu et al. 2013). Comercialmente, as Stm destacam-se pela vasta produção de antibióticos, sendo fonte de mais da metade dos produtos comercializados atualmente, como por exemplo a estreptomicina (Õmura et al. 2001; Liu et al. 2013).

Dentre os compostos antimicrobianos produzidos por Stm, encontram-se as fenazinas, que compreendem um grande grupo de compostos heterocíclicos nitrogenados (Laursen e Nielsen 2004; Pierson e Pierson 2010). Elas são produzidas exclusivamente por actinobactérias, sendo muitas Stm utilizadas como fonte natural deste composto (Luo et al. 2015). As fenazinas exibem atividade antimicrobiana, antiparasitária, antitumoral (Laursen e Nielsen 2004). A 1-Hidroxi-fenazina (1-OH-PHZ), isolada de *Streptomyces griseoluteus* P510, inibiu o crescimento de fitopatógenos, tais como *Alternaria solani, Sclerotinia sclerotiorum, Fusarium oxysporium* e *F. graminearum* (Luo et al. 2015). Recentemente, a fenazina-1-ácido carboxílico (PCA) foi atestada e registrada pelo Ministério da Agricultura da China devido à sua alta eficiência contra vários fitopatógenos, baixa toxicidade aos seres humanos e compatibilidade ambiental (Xu et al. 2015). Além da atividade antimicrobiana, as fenazinas são relatadas como eficientes indutores de resistência sistêmica de plantas (de Vleesschauwer et al. 2006).

1.3. Mecanismos de defesa vegetal

Na natureza, são muitos os agentes bióticos que podem causar doenças em plantas, tais como bactérias, fungos, nematódeos e insetos. Todavia, milhões de anos de pressão seletiva favoreceram o desenvolvimento do complexo sistema de defesa vegetal, sendo o estado resistente uma regra e o suscetível uma exceção (Agrios 2005). A resistência vegetal é definida, fisiologicamente, como a capacidade da planta em atrasar ou evitar a entrada e/ou a subsequente atividade do patógeno em seus tecidos (Goodman et al. 1986). Nesse sentido, as bases da resistência de plantas se alicerçam em mecanismos estruturais e bioquímicos pré-formados (ou constitutivos), os quais fornecem proteção de forma não específica e independente de estímulos externos (Trigiano 2010). Além destes, as plantas também possuem mecanismos latentes de resistência, que são ativados por elicitores biológicos ou químicos, atuando de maneira coordenada e adaptativa, constituindo a resistência induzida (Barros et al. 2010; Kumar et al. 2012; Pieterse et al. 2014).

Atualmente, existem duas linhas bem caracterizadas de indução de defesa no sistema imunológico vegetal (Jones e Dangl 2006). A primeira linha, denominada imunidade desencadeada por PAMPs (PTI), ocorre via receptores de reconhecimento padrão (PRRs) transmembranares, que reconhecem padrões moleculares associados a patógenos e microrganismos (PAMPs e MAMPs), ou sinais endógenos às células vegetais derivados de danos, chamados de padrões moleculares associados a danos (DAMPs) (Jones e Dangl 2006; Zipfel 2009; Boller e Felix 2009). Apesar da PTI ser considerada uma resposta de defesa

eficiente contra uma ampla gama de microrganismos, patógenos bem-sucedidos adaptaram-se de forma a minimizar as defesas do hospedeiro e sintetizar moléculas efetoras que contribuem para sua virulência e interferem na PTI, resultando em suscetibilidade desencadeada por efetores (ETS) (Jones e Dangl 2006; Dodds e Rathjen 2010). Por outro lado, as plantas desenvolveram uma segunda linha de defesa em que a resistência (R) ocorre através do reconhecimento específico de moléculas efetoras patogênicas (ou produtos do gene *Avr*) por proteínas R do vegetal (como as proteínas NB-LRR), resultando em imunidade desencadeada por efetores (ETI) (Jones e Dangl 2006; Dodds e Rathjen 2010).

Após o reconhecimento do eliciador pelo hospedeiro, inicia-se rapidamente a ativação local da defesa primária, através dos processos de transdução de sinal e ativação de genes de defesa, levando a planta a uma resposta sistêmica (Cordeiro e de Sá 1999). A transdução de sinal caracteriza-se, inicialmente, pelo influxo de íons e pelo processo oxidativo, que envolve o acúmulo citoplasmático de peróxido de hidrogênio (H_2O_2) e progride até a ativação de genes para a síntese de novas proteínas (Levine et al. 1994; Shirasu et al. 1997; Jones e Dangl 2006). Envolve também o processo de fosforilação e desfosforilação de proteínas, tais como fosfolipases, envolvidas na formação do ácido linolênico, precursor do ácido jasmônico (AJ) e de seu éster metílico (MeJA). Além do AJ, a biossíntese de outros dois hormônios envolvidos nas respostas de defesa vegetal, o ácido salicílico (AS) e o etileno (ET), é estimulada nesta etapa (Cordeiro e de Sá 1999). Enzimas relacionadas com a síntese de compostos secundários, como por exemplo, a fenilalanina amônia liase (PAL), são rapidamente induzidas, contribuindo com o processo de defesa relacionado à inibição da infecção pelo patógeno (Dixon e Paiva 1995). Outros genes são ativados tardiamente no processo de defesa e estão relacionados à produção de compostos como lignina e calose (Kauss 1987; Underwood 2012), enzimas hidrolíticas (Yarullina et al. 2016) e síntese de proteínas relacionadas à patogênese (PR) (Jain e Kumar 2015).

A resposta celular gerada pela ETI é similar a PTI, somente mais acelerada e ampliada, resultando em resistência do vegetal à doença e, geralmente, em morte celular programada no local da infecção por reação de hipersensibilidade (HR). A HR visa impedir a multiplicação do patógeno nas células infectadas, limitando ou interrompendo o processo da disseminação da doença e prevenindo a entrada adicional de patógenos biotróficos que poderiam prosperar no tecido vivo do hospedeiro (Jones e Dangl 2006; Pieterse et al. 2014). Embora a PTI e a ETI sejam, em grande parte, efetivas contra agentes estressores, o reconhecimento de moléculas elicitoras no sítio de indução geralmente induz a resistência sistêmica do vegetal (Hammerschmidt e Kuć 1995; van Loon e Bakker 2005; Pieterse et al. 2014), envolvendo um ou mais sinais locais que se propagam a

tecidos distais, permitindo assim, uma capacidade defensiva melhorada em partes da planta ainda não afetadas (Dempsey e Klessig 2012; Shah e Zeier 2013).

A resistência sistêmica vegetal induzida por patógenos é chamada de resistência sistêmica adquirida (SAR) (Vlot et al. 2009; Spoel e Dong 2012). A sinalização da SAR é dependente do acúmulo de AS nos tecidos sistêmicos e envolve a ativação coordenada de genes PR, muitos dos quais codificam proteínas PR, como PR-1 e PR-2, frequentemente utilizadas como marcadores dessa via (Vernooij et al. 1994; van Loon et al. 2006). A PR-1 é a proteína de resistência mais abundante em tecidos infectados e os genes PR-1 são expressos em resposta a diferentes fatores, tais como hormônios, radiação UV, ferimentos e patógenos (Brederode et al. 1991). Já a PR-2 é uma β-beta glucanase que tem atividade antimicrobiana, agindo sobre a parede celular do patógeno. O acúmulo de proteínas PR produz uma resposta HR como mecanismo de defesa, podendo resultar em alterações visuais no hospedeiro, como por exemplo, lesões necróticas (van Loon et al. 1998).

Apesar do AS ser imprescindível para a SAR, ele não é o sinal translocado pelos tecidos (Vernooij et al. 1994). Metabólitos como o éster metílico do AS (MeSA), ácido azeláico (AzA) e ácido pipecólico (Pip) são alguns dos sinalizadores da SAR no sistema vascular (Pieterse et al. 2014). O início da SAR requer a ativação da enzima FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1) responsável pela transdução e amplificação da sinalização sistêmica (Mishina e Zeier 2006; Pieterse et al. 2014). O aumento dos níveis de AS está relacionado à alteração no potencial redox celular, acarretando na redução da proteína não-expressora de genes PR-1 (NPR1) à sua forma monomérica ativa. Após a sua ativação, a proteína NPR1 se encaminha para o núcleo, onde atua como co-ativadora de transcrição, interagindo com membros das famílias TGA e WRKY de fatores de transcrição, ativando um grande conjunto de genes PR responsivos ao AS (Vlot et al. 2009; Spoel e Dong 2012; Pieterse et al. 2012). O funcionamento adequado da NPR1 requer a regulação pelas suas proteínas parálogas, NPR3 e NPR4, com diferentes afinidades por AS, funcionando como adaptadores no processo de ubiquitinação dessa proteína, regulando assim, a atividade da NPR1 por meio da sua degradação (Spoel et al. 2009; Fu et al. 2012). Em baixos níveis de AS, tal como durante a PTI ou em tecidos distais da infecção, a NPR4 estabiliza a NPR1, ativando a expressão de genes PR. Em níveis elevados de AS, como na ETI, a NPR3 intermedia a degradação de NPR1, resultando em morte celular programada local. A SAR resulta em imunização temporária contra futuras infecções e confere resistência contra um amplo espectro de patógenos (Pieterse et al. 2014).

Diferentemente da SAR, a indução de resistência sistêmica (ISR) é desencadeada por microrganismos não patogênicos (como PGPRs) ou por fatores abióticos (Barros et al. 2010;

Pieterse et al. 2014). A sinalização da via de ISR independe de AS, sendo o AJ e ET os principais hormônios de regulação desta via (Pieterse et al. 1996, 1998, 2000; Van Wees et al. 1997; Mabood et al. 2014). O AJ é sintetizado na via da oxilipina e induzido tanto por estresses bióticos como abióticos. A elucidação do envolvimento do AJ nas respostas de defesa iniciou com o descobrimento da proteína JASMONATE ZIM DOMAIN (JAZ), a qual é reguladora negativa da expressão de genes induzidos por AJ (Yan et al. 2007; Thines et al. 2007). A degradação proteossômica de proteínas JAZ ocasiona a liberação de fatores transcrição como os da família MYB, que se ligam aos promotores de genes de defesa de resposta ao AJ (Song et al. 2013).

A proteína NPR1 demonstrou estar envolvida na expressão da ISR mediada por AJ/ET (Pieterse et al. 1998). A localização nuclear de NPR1, que é essencial para a expressão de genes de defesa mediados por AS, não é necessária para a sinalização do AJ, possuindo uma função citosólica independente da ativação de genes PR na ISR (Pieterse et al. 2012), mostrando que a comunicação antagônica entre AS e AJ é modulada pela NPR1 no citosol (Spoel et al. 2003; Pieterse e Van Loon 2004; Pieterse et al. 2005; Bulgarelli et al. 2013).

Em baixas concentrações o AS e AJ atuam em sinergismo nos tecidos distantes do local de infecção, promovendo uma resposta de defesa inespecífica e de amplo espectro de ação. Ao entrar em contato com diferentes tipos de patógenos (biotróficos ou necrotróficos), a planta passa a aumentar os níveis de AS (estratégia contra biotróficos) ou de AJ (estratégia contra necrotróficos) no tecido infectado, desencadeando a resposta de defesa (Thaler et al. 2012; Sarma et al. 2015). Em altas concentrações, estes hormônios passam a agir de forma antagônica, promovendo uma resposta de defesa específica, voltada para o tipo de patógeno detectado (Fu e Dong 2013).

O ET está envolvido na defesa contra patógenos como um transdutor de sinal sistêmico, sendo fundamental em nível radicular para o estabelecimento da ISR (Knoester et al. 1999; Verhagen et al. 2004). O gene MYB72, da família de fatores de transcrição MYB, foi identificado como um dos genes amplamente expressos na epiderme da raiz e nas células corticais em plantas estimuladas à ISR após o tratamento com PGPRs (Verhagen et al. 2004). Além disso, o MYB72 é expresso especificamente em raízes associadas a rizobactérias indutoras de ISR sob condições limitadas de ferro (Pieterse et al. 2014). A indução do gene MYB72 nas raízes pode ocorrer por estresse causado por PGPRs através da produção de sideróforos ou por compostos orgânicos voláteis (Zhang et al. 2007). Assim, a ativação do gene de resposta à deficiência de ferro, que é fundamental para a expressão da ISR, ocorre mesmo que as plantas não sofram fisicamente com a limitação de Fe (Pieterse et al. 2014). O modelo de resposta ao ET também inclui a integração de genes PR e fatores de transcrição específicos, como o *ETHYLENE INSENSITIVE 3* (EIN3) e *ETHYLENE RESPONSE FACTOR 1*

(ERF1) (Ecker 1995; Van Der Ent et al. 2009). Os hormônios ET, AJ e MeJA têm ação sinérgica na expressão de proteínas PR (Xu et al. 1994) e todos eles encontram-se envolvidos com a ativação de defensinas (Penninckx et al. 1996) e de enzimas de síntese de fitoalexinas (Ecker e Davis 1987; Gundlach et al. 1992; Cordeiro e de Sá 1999).

Trabalhos com plantas estimuladas à ISR por bactérias benéficas mostraram que não houve um aumento na produção de AJ/ET, nem a indução da expressão de genes responsivos a eles, sugerindo que a ISR está mais relacionada com o aumento da sensibilidade destes hormônios (van Wees et al. 1999; Pieterse et al. 2000). Análises transcriptômicas em plantas de *Arabidopsis* estimuladas à ISR mostraram que, após o desafio com um agente patogênico, houve uma superexpressão dos genes VSP e PDF1.2, regulados por AJ, e do gene HEL, regulado por ET (van Wees et al. 1999; Verhagen et al. 2004; Van Oosten et al. 2008). Este aumento na sensibilidade, responsável por preparar a planta para melhor combater o ataque de agentes estressores, é chamado de "*priming*" e é caracterizado por uma ativação mais rápida e/ou mais potente das defesas celulares após a invasão, resultando no aumento do nível de resistência (Conrath et al. 2006).

A resistência sistêmica mediada por PGPR está relacionada com a expressão de vários genes que codificam enzimas relacionadas ao metabolismo secundário vegetal (Pieterse et al. 1998; Choudhary e Johri 2009; Sarma et al. 2015). Dentre as diversas vias metabólicas envolvidas nas respostas de defesa, a atividade da enzima PAL está intimamente relacionada à síntese de compostos de defesa e de interação da planta com microrganismos. A PAL é a primeira enzima da via dos fenilpropanóides que catalisa a transformação de L-fenilalanina em ácido *trans*-cinâmico (Dixon e Paiva 1995). O ácido *trans*-cinâmico é o principal intermediário na biossíntese de compostos fenólicos, participando também, da biossíntese do AS.

A ISR desencadeada por PGPR aumenta a resistência da parede celular vegetal e altera as respostas fisiológicas e metabólicas da planta hospedeira, tendo sido constatado um aumento na síntese de compostos de defesa em plantas logo após o desafio com agentes patogênicos (Compant et al. 2005). O acúmulo de substâncias de natureza fenólica parece ser consequência do aumento da atividade da PAL. Os compostos fenólicos produzidos, além da atividade antimicrobiana direta, também participam como substrato para síntese de lignina, no reforço da parede celular do vegetal (Singh et al. 2013). Podem, além disso, ser tóxicos à célula vegetal, participando da HR no processo de defesa. O aumento da concentração de compostos fenólicos resulta em aumento na atividade de outras enzimas como, polifenoloxidases e peroxidases, também envolvidas em respostas de defesa (Gaspar et al. 1985; Choudhary e Johri 2009).

1.4. Soja [*Glycine max* (L.) Merrill]

A soja [*Glycine max* (L.) Merrill] é uma leguminosa pertencente à Classe Magnoliopsida (Dicotiledônea), da família Fabaceae. Originária da costa leste asiática, principalmente ao longo do Rio Yangtzé, na China, a soja é considerada um dos cultivos mais antigos do mundo, datando de mais de cinco mil anos atrás (Embrapa 2013). Embora seja planta originária de clima temperado, a soja adapta-se bem em uma ampla faixa de temperatura (20 – 30 ºC). Assim, seus cultivares aclimatados desenvolvem-se bem nos climas tropical e subtropical. A espécie evoluiu muito a partir de cruzamentos naturais entre as espécies que foram domesticadas, estando atualmente muito diferente das suas ancestrais (Embrapa 2013).

A soja é a cultura agrícola que mais cresceu nas últimas três décadas e corresponde a mais de 56% da área plantada em grãos no Brasil (Conab 2016). Na safra de 2016/2017, a produção atingiu 105.558,2 mil toneladas, correspondendo a um incremento de 10,61% em relação à safra anterior (Conab 2017).

O Brasil é o segundo maior produtor mundial, atrás apenas dos EUA (Conab 2016). A indústria nacional produz, por ano, 5,8 milhões de toneladas de óleo comestível e 23,5 milhões de toneladas de farelo proteico (MAPA). Além disso, os grãos e o farelo de soja brasileira possuem alto teor de proteína e padrão de qualidade *Premium*, o que permite sua entrada em mercados extremamente exigentes como da União Europeia e do Japão. Seu grão é muito versátil e serve de matéria prima para a agroindústria, indústria química e de alimentos. Na alimentação humana, a soja entra na composição de vários produtos embutidos, em chocolates, temperos para saladas, óleos, entre outros. A soja também é utilizada para a fabricação do biodiesel, combustível capaz de reduzir em 78% a emissão dos gases causadores do efeito estufa na atmosfera (MAPA).

O ciclo de desenvolvimento da soja é dividido em duas fases, a vegetativa (V) e a reprodutiva (R). A fase V é primeiramente subdividida em fase vegetativa de emergência (VE) e fase vegetativa de cotilédone (VC). O nó da folha simples é o primeiro nó, utilizado como ponto de referência a partir do qual inicia-se a contagem para identificar o número de nós foliares superiores. As subdivisões subsequentes da fase V são designadas numericamente conforme o número de nós foliares como V1, V2, V3, até Vn, sendo “n” o número relativo ao último nó vegetativo formado pelo cultivar antes do florescimento, variando em função das diferenças varietais e ambientais. Já a fase R apresenta oito subdivisões bem definidas, que iniciam com o florescimento (R1) e vão até a maturação plena das vagens (R8) (Ritchie 1985).

1.5. *Xanthomonas axonopodis* pv. *glycines* e a pústula bacteriana

As doenças da soja estão entre os principais fatores que limitam a sua produção, com perdas anuais estimadas em 15 a 20% (CISoja; Embrapa 2013). No Brasil, foram identificadas aproximadamente 40 doenças que afetam a safra. E esse número continua aumentando com a expansão da soja para novas áreas, a intensificação da monocultura, a adoção de práticas inadequadas de manejo e a mudança do quadro de cultivares (Lima e Carneiro 2011).

Dentre as principais doenças de etiologia bacteriana em países produtores de soja encontra-se a pústula bacteriana (Wrather et al. 2001), causada pelo patógeno necrotrófico *Xanthomonas axonopodis* pv. *glycines* - Xag (sinonímia: *Xanthomonas campestres* pv. *glycines*) (Vauterin et al. 1995). Em regiões tropicais, onde as condições climáticas são favoráveis à doença, perdas na produção chegaram a 40% (Kaewnum et al. 2005).

A doença é típica de folhas, mas pode ocorrer de forma menos evidente em outros órgãos da planta (como pecíolos, hastes e vagens) em variedades suscetíveis (Hedges 1924). Os sintomas iniciam com pequenas manchas cloróticas arredondadas (não translúcidas), com centros elevados em ambas as faces da superfície foliar (Jones 1987; Narvel et al. 2001). No centro das lesões se desenvolvem as pústulas (Figura 1A), de coloração parda e opaca, principalmente na face abaxial, sendo circundadas por um estreito halo amarelado (Jones 1987). Com o desenvolvimento da doença, as lesões se transformam em áreas necróticas e irregulares (Figura 1B) que podem coalescer (Lanna 2015). A doença leva à desfolha prematura, resultando na perda de produtividade e qualidade dos grãos (Wolf 1924).

A Xag penetra na planta por estômatos ou ferimentos (Jones 1987) e, nos espaços intercelulares do tecido vegetal, sintetiza compostos que alteram o metabolismo das células parenquimáticas, causando hipertrofia e hiperplasia celular (Hedges 1924; Sain e Gour 2013), com consequente ruptura da epiderme e exposição do parênquima, resultando na formação da pústula (Lanna 2015).

A transmissão primária do patógeno pode ocorrer através de sementes e restos culturais (Henning et al. 2014). Infecções secundárias são favorecidas por precipitações e ventos, aliados às condições de umidade elevada (>70%) e altas temperaturas (>28 °C) (Narvel et al. 2001; Henning et al. 2014). Fatores como implementos agrícolas e o manejo nas lavouras, também são importantes para a disseminação secundária (Lanna 2015). A bactéria pode também se estabelecer na rizosfera de plantas voluntárias, como o trigo e o feijão-de-vagem, mantendo o inóculo viável para a seguinte lavoura de soja (Kennedy e Sinclair 1989; Meyer et al. 2013; Henning et al. 2014). Dessa forma, estratégias que visem reduzir a população desse

patógeno na rizosfera são de grande importância, pois evitam que fontes do inóculo persistam nas entressafras (Lanna 2015).

O uso de cultivares resistentes é o principal método de controle da doença (Hartwig e Lehman 1951; Manjaya e Pawar 1999), sendo a quase totalidade das cultivares utilizadas no Brasil classificadas como resistentes (Henning et al. 2014). No entanto, o uso continuado de um único genótipo resistente à doença pode favorecer o aumento de populações da fitobactéria adaptadas a infectar indivíduos de soja com tal característica (Lanna 2015).

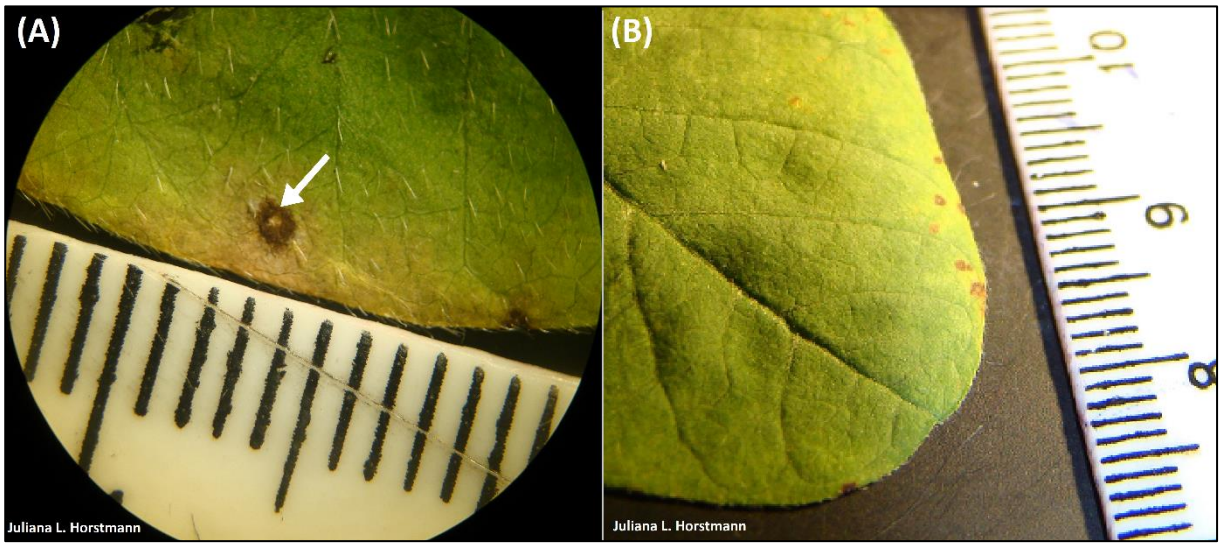

Figura 1 Doença da pústula bacteriana. (A) A pústula em detalhe: halo opaco clorótico em processo de necrose com centro elevado característico. (B) Pústulas no limbo foliar. (Imagens do autor).

2. JUSTIFICATIVA

A soja se estabeleceu como um dos produtos mais destacados da agricultura nacional e na balança comercial. A crescente demanda de mercado, principalmente no uso da soja em setores ainda não convencionais como o biodiesel e o seu beneficiamento pela valorização do dólar têm estimulado os produtores a aumentar a produção avançando com a oleaginosa sobre novas áreas (Conab 2016). A cultura da soja também é responsável por utilizar mais de 40% do total de fertilizantes no país (Ferreira e Vegro 2015). Além disso, as práticas convencionais de plantio levam ao uso excessivo de agroquímicos nas lavouras. Todos esses produtos químicos utilizados acabam gerando a contaminação dos mananciais hídricos, causando sérios danos ao meio ambiente e à saúde (Gupta *et al.* 2015).

As PGPRs apresentam um grande potencial como ferramenta biotecnológica para o desenvolvimento sustentável da agricultura (Rodríguez et al. 2006). De acordo com seus mecanismos de ação, elas podem ser classificadas como: (I) biofertilizantes, aumentando a disponibilidade de nutrientes para a planta; (II) fitoestimuladoras, atuando sobre a promoção direta de crescimento vegetal, geralmente via fitormônios; e (III) biopesticidas, através do controle de fitopatógenos pela produção de antibióticos, metabólitos antifúngicos e indução de resistência (Bhattacharyya e Jha 2012).

Dado o impacto ambiental negativo dos fertilizantes químicos e os seus custos crescentes, o uso de PGPRs como fertilizantes naturais é vantajoso para o desenvolvimento sustentável da agricultura (Rodríguez et al. 2006). Além disso, a produção de fitormônios por PGPR é considerada de grande valor na agricultura uma vez que afetam diretamente a fisiologia e o crescimento de plantas cultivadas (Mabood et al. 2014). Uma vantagem no uso de PGPRs como biofertilizantes em relação aos hormônios sintéticos, são que os fitormônios naturais são mais eficazes, visto que tem a sua liberação de forma lenta e contínua (Gupta et al. 2015).

O tratamento de plantas com PGPRs tem sido considerado uma potencial estratégia para o biocontrole de doenças (Zehnder et al. 2001; Compant et al. 2005). Quando comparados aos agroquímicos/pesticidas e fertilizantes sintéticos, inoculantes microbianos têm várias vantagens: (I) são mais seguros para a saúde e para o meio ambiente, (II) mostram atividade muito mais direcionada, (III) são eficazes em pequenas quantidades, (IV) se multiplicam e são controlados pelas plantas, bem como pela comunidade rizosférica, (V) decompõem-se muito mais rapidamente que os agroquímicos, (VI) podem ser integrados em cultivos orgânicos ou convencionais (Berg 2009).

Atualmente diversos inoculantes microbianos já estão no mercado e, nos últimos anos, a sua popularidade aumentou substancialmente, assim como as pesquisas na área, fato que tem melhorado sua consistência e eficácia (Bhattacharyya e Jha 2012). Levantamentos recentes de produtores convencionais e orgânicos mostram o interesse de ambos na utilização de inoculantes microbianos, sugerindo um aumento no mercado desses produtos nos próximos anos (Rzewnicki 2000; Mabood et al. 2014).

Nesse contexto, a caracterização de *Streptomyces* spp. como PGPRs e indutoras de respostas de defesa em plantas de soja, pode culminar na fabricação de um inoculante capaz de incrementar a produção, promovendo a redução do uso de fertilizantes e agroquímicos utilizados.

3. HIPÓTESES

I. Os isolados de *Streptomyces* spp. obtidos de solos com plantas de Fabaceae apresentam características de PGPR;

II. Dentre os 11 isolados de *Streptomyces* spp. analisados, pelo menos um promove o crescimento de plantas de soja;

III. Dentre os 11 isolados de *Streptomyces* spp. analisados, pelo menos um estimula as respostas de defesa de soja contra o agente patogênico *Xanthomonas axonopodis* pv. *glycines*.

4. OBJETIVOS

4.1. Objetivo geral

Avaliar os isolados de rizobactérias *Streptomyces* spp. oriundos da rizosfera de plantas de Fabaceae quanto às características de PGPR, e à modulação do metabolismo de defesa de plantas de *Glycine max* (L.) Merrill em resposta à fitobactéria patogênica *X. axonopodis* pv. *glycines*.

4.2. Objetivos específicos

I. Caracterizar bioquimicamente 11 isolados de *Streptomyces* spp. quanto à produção de AIA, sideróforos, ACC desaminase e fenazinas;

II. Selecionar três isolados de *Streptomyces* spp. com, no mínimo, duas características de PGPR para avaliar a promoção de crescimento em plantas de soja;

III. Identificar taxonomicamente os três isolados de *Streptomyces* spp. selecionados com características de PGPR, através de métodos moleculares;

IV. Avaliar o crescimento de plantas de soja tratadas com os três isolados selecionados, elencando um isolado eficiente;

V. Avaliar a resposta de defesa em plantas de soja pré-tratadas com o isolado de *Streptomyces* spp. (previamente selecionado) e desafiadas com o patógeno *Xanthomonas axonopodis* pv. *glycines*, através de alterações no seu metabolismo secundário e à expressão de genes relacionados as vias de defesa vegetal.

CAPÍTULO II: MANUSCRITOS A SEREM SUBMETIDOS A PERIÓDICOS CIENTÍFICOS

Manuscrito 1

Streptomyces spp. from Fabaceae rhizosphere promotes growth of soybean plants

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Nota: Os autores optaram por incluir Tabelas e Figuras ao longo do texto para facilitar a leitura e interpretação pelos avaliadores da dissertação. No entanto, no momento da submissão do artigo, as normas serão seguidas e as tabelas e figuras serão apresentadas separadamente.

Streptomyces spp. from Fabaceae rhizosphere promotes growth of soybean plants

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Running headline: *Streptomyces* spp. promote soybean growth

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Abstract

Aim This study characterized isolates of the rhizobacteria *Streptomyces* spp. obtained from rhizosphere soil of Fabaceae plants regarding their plant growth promoting traits.

Methods and Results Eleven isolates of *Streptomyces* spp. were screened for potential PGPR characteristics such as siderophores production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole-3-acetic acid (IAA) and phenazines. Soybean seeds were bacterized with three isolates for testing growth promotion and plants were evaluated by length, fresh and dry masses of roots and shoots at 15, 30 and 45 days. All isolates were able to produce siderophores, whereas CLV41, CLV45 and CLV46 showed colony growth in presence of ACC, indicating the activity of ACC deaminase. All isolates produced IAA, although CLV45 was the most efficient reaching 398.53 µg of IAA g^{-1} cell. Pyocyanin was detected in all isolates and CLV26 was the most productive. CLV42, CLV44 and CLV45 were selected by their PGPR traits for the growth promotion assay. CLV45 significantly increased shoot growth in 36.63% and dry mass in 17.97% when compared to the control plants.

Conclusions The characterized *Streptomyces* spp. demonstrated different plant growth promoting traits and CLV45 effectively promoted growth of soybean.

Significance and Impact of Study *Streptomyces* spp**.** isolates could be used to increase plant growth being a candidate for a biofertilizer formulation.

Keywords: ACC deaminase, Actinomycetes, IAA, Siderophores, Phenazine

Introduction

Soybean [*Glycine max* (L.) Merrill], a dicotyledonous plant from Fabaceae family, is one of the most important crop for seed protein and oil content (Mingma *et al.* 2014). It is originated from temperate climate and it is currently well adapted to the tropical and subtropical conditions, between 20-30 °C (Embrapa 2003). Brazil is the second largest producer of soybean in the world, making use of 56% of the country's agricultural area for this crop cultivation (Conab 2016). In addition to the fact that new areas are added annually to meet market demand (Conab 2016) and the use of agrochemicals used for pest control (Bhattacharyya and Jha 2012), soybean is also the culture responsible for the greater demand for fertilizers, utilizing more than 40% of the Brazilian consumption (Ferreira and Vegro 2015). The excessive use of agrochemicals to crop fields has been reported to increase nitrate, nitrite, ammonium and phosphate, as well as other reactive chemical species in groundwater and surface water bodies, which may potentially cause serious environmental and health hazards, resulting in great damages to the environment (Gupta *et al.* 2015). In this context, the use of rhizobacteria as microbial inoculants has a high potential for sustainable development of agriculture (Rodríguez *et al.* 2006; Bhattacharyya and Jha 2012). Among rhizobacteria, there is a diverse group of species that promote plant development and health, named Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth 1978; Glick *et al.* 1998). The PGPR act directly and indirectly on plant growth (Berg 2009; Palaniyandi *et al.* 2013a; Gupta *et al.* 2015). Directly, they play a role on the production and availability of key elements for plant development, through solubilization of metal molecules, such as iron, by the synthesis of siderophores (Rajkumar *et al.* 2010); and by production of phytohormones such as indoleacetic acid (IAA) (Gopalakrishnan *et al.* 2015). Moreover, they indirectly help plants to withstand stress (biotic or abiotic) by reducing the synthesis of the hormone ethylene, through production of the enzyme 1-aminocyclopropane-1-carboxylic acid deaminase (ACC desaminase) (Glick *et al.* 1998; El-Tarabily 2008; Palaniyandi *et al.* 2014); by the antagonism to phytopathogenic agents, through production of antimicrobial compounds (Liu *et al.* 2013; Luo *et al.* 2015); as well as by the induction of systemic resistance (ISR) through plant recognition of molecular patterns associated with microorganisms, the MAMPs (Pal *et al.* 2006). Indeed, PGPRs may use one or a combination of some of these mechanisms and act as biofertilizers (Vessey 2003), phytostimulators (Spaepen and Vanderleyden 2011) and biocontrol agents (Pal *et al.* 2006, Palaniyandi *et al.* 2013a) in the rhizosphere in which they are established (Bhattacharyya and Jha 2012; Bulgarelli *et al.* 2013; Gupta *et al.* 2015).

Acidobacteria, Actinobacteria, Bacterioidetes, Choloroflexi, Firmicutes and Proteobacteria are some bacterial phyla reported as containing species of PGPR (Bulgarelli *et al.* 2013). Actinobacteria are widely distributed in the soil (10 - 50%) under the most varied conditions (Alexander 1977). Moreover, they are known for the great production of secondary metabolites with wide diversity of metabolic functions, such as enzymes and antibiotics

(Adegboye and Babalola 2012). *Streptomyces* spp. are Gram-positive filamentous actinobacteria that comprise about 10% of the total bacteria found in the soil (Janssen 2006). Many *Streptomyces* spp. are considered PGPR due to their ability to stimulate plant growth and induce defense responses in plants colonized by these microorganisms (Chater *et al.* 1996). There is also great interest in these bacteria due to their potential to produce antimicrobial metabolites such as phenazines (Wang *et al.* 2011b; Luo *et al.* 2015). Isolates of *Streptomyces* spp. originating from Araucaria Forest were characterized as PGPR, based on their capacity of IAA production, phosphate solubilization, antibiosis and production of siderophores (Dalmas *et al.* 2011; M.P. Dias, pers. comm.). Furthermore, there are studies showing the positive effect of *Streptomyces* spp. on growth of different crop species, such as wheat (Aldesuquy *et al.* 1998; Sadeghi *et al.* 2012) and tomato (Hamdali *et al.* 2008; Palaniyandi *et al.* 2014; M.P. Dias, pers. comm.). In eucalyptus plants, inoculation of roots with *Streptomyces* sp. PM9 resulted in significant increase of secondary roots and modulation of secondary metabolism (Salla *et al.* 2014). Likewise, inoculation with *S. filipinensis* significantly reduced the levels of endogenous ACC and promoted the growth of tomato plants (El-Tarabily 2008).

Although research with streptomycetes as PGPR and its use as bioinoculants has increased in recent decades, it is still necessary to improve the knowledge on the diversity of metabolites produced by different strains of *Streptomyces* sp., in order to exploit this group for maximum commercial potential in high impact crops such as soybeans (Berg 2009; Bhattacharyya and Jha 2012; Gupta *et al.* 2015). In this context, since much of the chemical signals involved in plant-rhizobacteria associations are species-specific (Bais *et al.* 2006), the isolation of *Streptomyces* sp. strains from the rhizosphere of plants from Fabaceae family is a strategy that may facilitate the selection of microorganisms adapted to the exudates of soybean plants, effectively colonizing this legume.

Given the above, the aim of this study was to characterize biochemically 11 strains of *Streptomyces* spp. isolated from soil and roots of Fabaceae plants, regarding their capacity of producing siderophores, indoleacetic acid (IAA), phenazines and the enzyme ACC deaminase. Based on such characteristics, the effect of these *Streptomyces* isolates on the growth of soybean plants was analyzed.

Material and Methods

Microorganisms and culture conditions

Eleven isolates of rhizobacteria *Streptomyces* spp. were previously obtained from rhizosphere of different Fabaceae plants collected in Rio Grande do Sul, Brazil (Table 1), selected with appropriated antibiotic and antifungal, identified through their morphology, and stored at -80 °C in a 20% glycerol solution. The isolates were

grown in ISP_2 liquid medium (Shirling and Gottlieb 1966), under agitation at 100 rpm for 5 days at 26 ± 2 °C. Each suspension was centrifuged (2,500 g, 15 min, room temperature), resuspended in sterile distilled water, and adjusted to final concentration of 10^7-10^8 CFU ml^{-1} for use as inoculum for the assays.

Table 1 List of *Streptomyces* spp. isolates, their rhizospheric origin and collection sites

Isolates	Fabaceae Species	Site Coordinates	
CLV21	*Mimosa scabrella* Benth.	29° 29.407S	50° 13.823W
CLV26	*Mimosa scabrella* Benth.	29° 28.815S	50° 10.506W
CLV38	*Inga* Mill.	29° 29.624S	50° 11.374W
CLV39	*Mimosa scabrella* Benth.	29° 29.625S	50° 11.083W
CLV40	*Inga* Mill.	29° 28.263S	50° 09.911W
CLV41	*Inga* Mill.	30° 84.068S	51° 130.211W
CLV42	*Inga* Mill.	30° 84.068S	51° 130.211W
CLV43	*Inga* Mill.	30° 84.068S	51° 130.211W
CLV44	*Phaseolus vulgaris* L.	30° 300.510S	50° 905.089W
CLV45	*Phaseolus vulgaris* L.	30° 300.510S	50° 905.089W
CLV46	*Phaseolus vulgaris* L.	30° 300.510S	50° 905.089W

Biochemical characterization of the isolates

All the 11 isolates were evaluated for PGPR characteristics such as production of siderophores, ACC deaminase, IAA and phenazines (Table 1). For the assessment of siderophore production ability, CAS-LB agar plates (Chrome Azurol S) were prepared according the cup plate method (5 mm-well) (Dingle *et al.* 1953). Isolates were grown in ISP_2 medium (10 ml), for 5 days at 26 ± 2 ºC. After incubation, 100 µl of each culture was inoculated in three plates CAS-LB agar with three wells, totalizing nine samples per isolate. Plates were incubated at 28 ± 2 °C for 7 days. As Distilled water and pyrocatechol (1 mol l^{-1}) were used as negative and positive controls, respectively. When siderophores are released by the bacteria, a change in the medium color from blue to yellowish-orange is observed (Figure 1). Measurements were taken considering the diameter of the halo zone (mm) after incubation. Data were expressed as mean of the halo boundary ± standard error (SE).

Production of the ACC deaminase (E.C. 4.1.99.4) by the *Streptomyces* spp. isolates was determined following Glick *et al.* (1995), adapted by Cattelan *et al.* (1999), with modifications. Briefly, the isolates were previously cultured in 10 ml liquid ISP_2 medium for 5 days at 26 ± 2 °C, centrifuged (20 min; 2,500 g) and washed twice with DF salts minimal medium (Dworkin and Foster 1958) without glucose and nitrogen (N) salts. The pellet was then resuspended in 10 mL of liquid DF salts medium without N and agitated for 2 days at 100 rpm and 26 ± 2 °C. For the evaluation of the production of ACC deaminase, three treatments with different N source media were prepared. In one 24-well plate, 500 µl of semi-solid DF salts medium with ACC (6 mg ml^{-1}) as the only N source was aseptically dispensed (DF+ACC). In another multiwell plate, the same medium was prepared without any source of

N (DF-N). For positive control, an identical plate was prepared with DF salts medium with N (DF complete; DF+N). Isolates aliquots (5 µl; 10^8 cfu ml^{-1}) were cultured in duplicate per treatment and the control was done with sterile distilled water. Plates were incubated at 28 ± 2 °C for 5 days. The qualitative assessment was based on the growth of each isolate, and it was considered positive for ACC deaminase production the isolates that grew in the medium with ACC, and showed no growth on DF–N (Cattelan *et al.* 1999).

The auxin production was analyzed by Salkowski's method (Salkowski 1885), modified by Dalmas *et al.* (2011). The isolates were previously grown in 10 ml liquid ISP2 medium for 5 days, at 26 ± 2 °C under agitation at 100 rpm. Bacterial suspensions were centrifuged for 15 min at 2,500 g at room temperature. The supernatant was combined with the Salkowski's reagent (1:1; v/v) and incubated for 30 min at room temperature in the dark. Absorbances were read in a spectrophotometer at 530 nm from five replications per isolate, and IAA levels were estimated in relation to the standard calibration curve of the hormone. The mass of the bacterial pellet was measured to calculate the IAA concentration per gram of cells.

In order to identify and quantify phenazines, *Streptomyces* spp. isolates were grown in 10 ml liquid ISP_2 medium, at 100 rpm and 26 ± 2 °C for 7 days (decline phase). Extraction and characterization procedures followed Meghraj *et al.* (2013) and Cezairliyan *et al.* (2013) with modifications. For extraction of phenazines, 20 ml of each isolate suspension was centrifuged and supernatant was equally divided in two flasks. Pellet with bacterial cells was weighted. Purification of pyocyanin (PYO) was performed by addition of chloroform (1:1 v/v) to the supernatant, gently mixed, maintained for 30 min, and then acidified to ca. pH 2 with concentrated HCl. Organic phase was used for analysis at 520 nm. For purification of phenazine-1-carboxylic acid (PCA) and 1- hydroxyl-phenazine (OH-Phe), supernatant was acidified with HCl 6 mol l^{-1} (1:1 v/v) followed by addition of ethyl acetate (1:1 v/v). Mixture was gently agitated and kept for 30 min for phase separation. Organic phase was evaporated under air stream and then residue was resuspended with 200 µL of 1 mol l^{-1} NaOH. Same volume of methanol was added to the solution and analysis was performed at 367 nm. The analytical HPLC system comprised a Sikam Chromatography TM S 600, and UV/VIS detector Mod. 3345 DAD. Phenazine separation was done in a MetaSil ODS column (5 µm; 250 x 4.6 mm), with a C_{18} guard column. Temperature of the column oven was set at 40 °C. Chromatographic data were processed by Clarity Chromatography Software. Mobile phase A consisted of water, and mobile phase B, acetonitrile. Both eluents were acidified by 2.5 % formic acid. The linear gradient consisted of 0-15% of eluent B from 0 to 2 min; 15-83% of B from 2 to 14 min; 83-0% of B from 14 to 16 min, and 0% of B until 20 min under flow rate of 1 mL min^{-1} (modified from Kern and Newman 2014). Quantification was based on calibration curves of the standards obtained from manufacturers as follow: 1-Hydroxyphenazine was obtained from Tokyo Chemical Industry Co. (Japan), pyocyanin

from Sigma Chemical Co. (USA), and phenazine-1-carboxylic acid from iChemical (China) and concentration was expressed as µg of phenazine g^{-1} cells. Two experimental replicates were used for each standard and bacterial sample.

Molecular identification of the isolates

Isolates selected in terms of their biochemical PGPR characteristics were subjected to taxonomic identification by using 16S rRNA gene sequencing. Pure cultures in 10 ml liquid ISP_2 medium were prepared and incubated at 28 ºC for 3 days (exponential phase). An aliquot of 100 µl of each bacterial suspension was plated in semisolid medium ISP_2 for 4 days. For the DNA extraction process, protocol was adapted from Singh *et al.* (2003) and Jain *et al.* (2006). The colonies were resuspended in 1 ml of saline solution 1 mol l^{-1} and 500 µl of guanidine isothiocyanate (Invitrogen, USA) was added for cell lysis (Wang *et al.* 2011a). Two extractions with chloroform:isoamylic alcohol (24:1) were performed, DNA was precipitated in isopropanol, washed with ethanol, and resuspended in Tris-EDTA buffer (Tris-HCl 10 mmol l^{-1} pH 8; EDTA 1 mmol l^{-1}). The quality and integrity of the DNA were determined by electrophoresis in 0.8% agarose gel and visualized by UV light. The amplification of 16S rRNA gene was done using universal primers 9F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1542R (5'- AGAAAGGAGGTGATCCAGCC-3') following the protocol described by Park *et al.* (2005). The PCR product was sequenced at Macrogen Inc., Republic of Korea. The sequences obtained were compared to those from the GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Promotion of plant growth by *Streptomyces* spp.

Soybean seeds (TMG-7262 RR INOX), without fungicide treatment, were provided by Ballagro AgroTecnologia Ltda., Brazil. For their use in the experiments, seeds were surface disinfested with sodium hypochlorite solution (2%, v/v) for 2 min, rinse three times with sterile distilled water and treated with the isolates. The procedure was carried out as follow: three selected isolates (CLV42, CLV44 e CLV45) were grown in 10 ml liquid ISP_2 medium, under agitation (100 rpm), at 28 ºC. After 5 days of cultivation, bacterial suspension was adjusted to the final concentration of 10^7-10^8 CFU ml^{-1} (OD_{600nm}= 1). For the bacterization, 30 seeds were immersed in 5 ml of bacterial suspension per treatment (CLV42, CLV44 and CLV45) for 5 min and partially dried for 1 h in uncovered petri dishes in a laminar flow hood. For the control, 30 seeds were treated with sterile distilled water. Seeds were sown into commercial substrate (clay, hummus and ground calcareous rock) in polypropylene bags (15x25 cm) and transferred to greenhouse. The experiment was set up with 30 plants per treatment, including the control group. Irrigation was carried out when necessary. Nutrient solution [10 mL of macronutrients salt solution; (0.41 g l^{-1} NH_4NO_3, 0.47 g l^{-1} KNO_3, 110 g l^{-1} $CaCl_2 \cdot 2H_2O$, 0.09 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.04 g l^{-1} KH_2PO_4)] was supplied to the plants every 15 days. Soybean plants growth was evaluated by

length (cm), as well as fresh and dry mass (g) of the shoot and roots at 15, 30 and 45 days after sowing. Dry mass was determined from oven-drying root and shoots at 40 ºC, until constant mass was reached.

Statistical analysis

Experiments were performed in a fully randomized design, tested for variance homogeneity by Levene's test ($\alpha \leq 0.05$). Data from experiments of biochemical characterization of *Streptomyces* spp. were analyzed by One-way ANOVA and mean differences were determined by Duncan Test at a significance level of $\alpha \leq 0.05$. The results obtained in the growth promotion assay were analyzed by Test T ($\alpha \leq 0.05$). All statistical analyses were performed using the software SPSS Statistics v. 22. Data from the experiments were expressed as mean ± SE.

Results and Discussion

Biochemical characterization

In this work, 11 bacterial isolates, previously obtained from the rhizosphere of Fabaceae plants and morphologically identified as *Streptomyces* sp., were biochemically characterized regarding their potential as PGPRs. The data obtained from the biochemical assays indicated that all isolates presented at least one of the characteristics of PGPR, evidencing the potential of this bacterial genus in promotion of plant growth (Gopalakrishnan *et al.* 2015).

The capability to produce siderophore under iron-limiting conditions was recorded in all isolates (Table 2). The CLV42 isolate showed the highest siderophore production, followed by CLV46, CLV44 and CLV41 (Figure 1; Table 2). Production of siderophores by species of the *Streptomyces* has been previously reported (Imbert *et al.* 1995; Rajkumar *et al.* 2010; Verma *et al.* 2011; Sadeghi *et al.* 2012; Lee *et al.* 2012; Palaniyandi *et al.* 2013b). This result is also in agreement with the literature in which the synthesis of siderophores, reported as an important feature in PGPR (Miethke and Marahiel 2007; Rajkumar *et al.* 2010; Lee *et al.* 2012), is commonly found in actinobacteria. Siderophores are small peptidic molecules containing side chains and functional groups that can form a ferric-siderophore complex (Crosa and Walsh 2002), returned to the cell surface by diffusion, allowing solubilization and extraction of iron from most mineral or organic complexes (Wandersman and Delepelaire 2004).

PGPR that possess ACC deaminase are capable of metabolizing ACC and therefore regulating the ethylene levels in the plant. ACC deaminase-producing PGPRs can increase root elongation and plant growth by reducing plant stress levels caused by ethylene (Glick *et al.* 1998; Siddikee *et al.* 2010). In this study, 5 out 11 isolates showed growth on the medium supplemented with ACC as the only source of N, indicating activity of the enzyme ACC deaminase (Table 2). Different intensities of enzyme activity were observed among these *Streptomyces* spp. isolates and CLV41, CLV45 and CLV46 were the most efficient in growing during the culture period of 5 days (Table 2). According to Jackson (1991),

elevated ethylene levels in legumes can inhibit root elongation and symbiotic fixation of nitrogen during germination. Therefore, the ACC deaminase activity present in these isolates could promote germination and root growth of soybean (Glick *et al.* 1995; Cattelan *et al.* 1999). Additionally, the presence of ACC deaminase in the *Streptomyces* sp. strain PGPA39 was suggested to participate in alleviation of salt stress in 'Micro Tom' tomato plants (Palaniyandi *et al.* 2014). Similarly, strains of the rhizobacteria *Pseudomonas* and *Serratia* sp. containing ACC-deaminase improved growth and yield of wheat plants under salt-stressed conditions (Zahir *et al.* 2009).

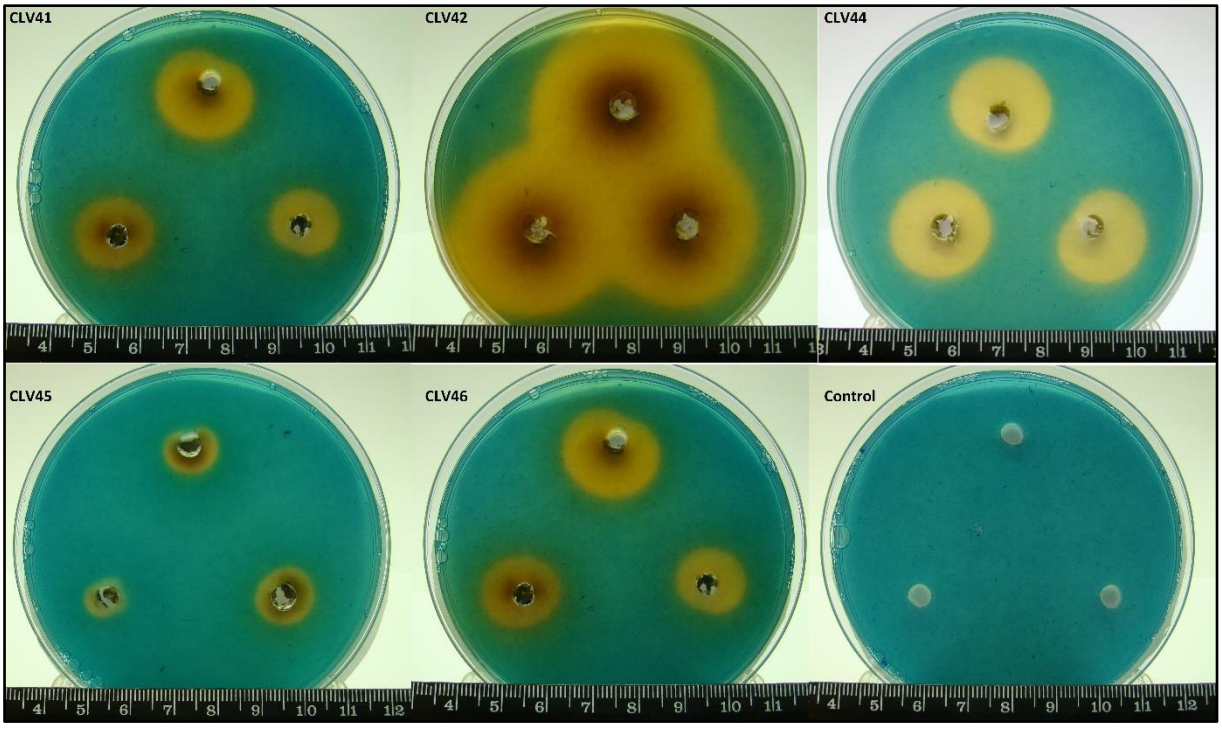

Figure 1 Siderophore production by *Streptomyces* spp. isolates CLV41, CLV42, CLV44, CLV45, CLV46 and negative control on Chrome Azurol agar (CAS) plates. The orange halo surrounding the colony indicates the release of siderophores by these rhizobacteria.

The indole acetic acid (IAA) is the main auxinic hormone produced by approximately 80% of known rhizobacteria (Patten and Glick, 1996; Barazani and Friedman 1999), being synthesized mainly in tryptophan-dependent biochemical pathways (Manulis *et al.* 1994). The production of IAA is an important mechanism exhibited by PGPR, being associated to the remodeling and development of the roots, increasing the absorption of nutrients by the plant, and thus, promoting its growth (van Loon 2007; Palaniyandi *et al.* 2013a). Plant growth responses related to IAA-*Streptomyces* spp. producers have been reported in important crops such as rice (Gopalakrishnan *et al.* 2014), wheat (Aldesuquy *et al.* 1998; Sadeghi *et al.* 2012) and eucalyptus (Mafia *et al.* 2009; Salla *et al.* 2014). In the present work, all isolates of *Streptomyces* spp. analyzed were capable of producing IAA (Table 2). Isolates widely differed on the capacity of IAA production (6.48 - 398.53 μg g^{-1} of cells), and such differences in performance of IAA-producing rhizobacteria can be attributed to the inherent individual properties

of each bacterium (Sarwar *et al.* 1992). The *Streptomyces* spp. CLV45 presented the highest IAA production, followed by CLV43, CLV44 and CLV40 isolates (Table 2). Similarly, *Streptomyces* strains producing high amounts of IAA were reported as PGPR in tomato and eucalyptus plants (El-Tarabily 2008; Verma *et al.* 2011; Salla *et al.* 2014). The other isolates analyzed in this study showed an IAA production of less than 50 µg g^{-1} of cells, concentration that has been used by some companies to formulate their fertilizing products (PuraKelp; Omnia Nutriologia, Brazil). Thus, the isolates with low production of IAA were considered as inefficient producers. The CLV 41 isolate was the one that showed the lowest production of this hormone (Table 2).

Table 2 PGPR characteristics of *Streptomyces* spp. isolates

Isolates	Siderophore production (cm)[a]	ACC deaminase activity [b]	IAA concentration (µg g^{-1} of cells)[c]
CLV21	0.26 ±0.02 d	-	10.25 ±2.25 h
CLV26	0.26 ±0.01 d	-	19.37 ±1.98 g
CLV38	0.39 ±0.01 d	-	22.03 ±0.34 g
CLV39	0.36 ±0.01 d	-	30.88 ±1.12 f
CLV40	0.34 ±0.01 d	-	105.65 ±4.59 c
CLV41	0.59 ±0.08 c	++	6.48 ±1.26 h
CLV42	1.71 ±0.04 a	-	41.68 ±0.72 e
CLV43	0.29 ±0.02 d	+	121.05 ±1.06 b
CLV44	0.63 ±0.08 c	+	109.04 ±1.06 c
CLV45	0.30 ±0.03 d	++	398.53 ±1.20 a
CLV46	0.75 ±0.04 b	++	46.87 ±0.37 d

[a] Values are the average width of the halo boundary (cm), in triplicates.
[b] ACC deaminase activity: (-) Undetectable colony, (+) Weak growth, (++) Strong growth, evaluated in duplicate.
[c] IAA was determined with at least three replicates.
Data are presented as mean ±SE. Different letters indicate significant difference at P≤ 0.05 by Duncan's Test.

Production of phenazines PYO, PCA, and 1-OH Phe was evaluated in the supernatants of suspension cultures of *Streptomyces* spp. isolates (Figure 2A). All isolates were able of producing PYO. The most productive isolate was CLV26, followed by the isolates CLV21 and CLV43 (Figure 2B). On the other hand, production of PCA was very limited, and only three isolates could produce this phenazine (Figure 2B). The highest concentration of PCA was found in the supernatant of the CLV43 culture, although the level was 23.6 times lower than the production of PYO by the same isolate. 1-OH Phenazine was not produced by any of the isolates tested. Phenazines are a diverse class of heterocyclic secondary metabolites and have been studied for many years due to their antibiotic properties and role in virulence (Pierson and Pierson 2010). They are best known by its effects on microorganisms, such as *Staphylococcus aureus*, *E. coli* and *Vibrio cholera* (Grahl *et al.* 2013), although phenazines can also impact multicellular organisms in both positive and negative ways. *Pseudomonas fluorescens* and *P. chlororaphis* are examples that beneficial phenazine producers play

a role on suppression of fungal diseases in plants (Pierson and Thomashow 1992). Most plants greatly benefit from phenazine production by bacteria, since these molecules can inhibit fungal and bacterial pathogens, as reported in tomato where PYO was involved in induced systemic resistance against *Botrytis cinerea* (Audenaert *et al.* 2002). Similarly, PYO secreted by *P. aeruginosa* 7NSK2 triggered systemic resistance to *Magnaporthe oryzae* in rice (Vleesschauwer *et al.* 2006). PCA, an antibiotic that is primarily produced by *Pseudomonas* spp., also exhibited biological effects on controlling *Xanthomonas oryzae* pv. *oryzae* on rice (Xu *et al.* 2015). El-Sayed *et al.* (2008) reported PCA from *P. putida* isolates that reached its maximum rate in isolate no. 103 (40 µg ml^{-1}) followed by isolate no. 102 (35.8 µg ml^{-1}).

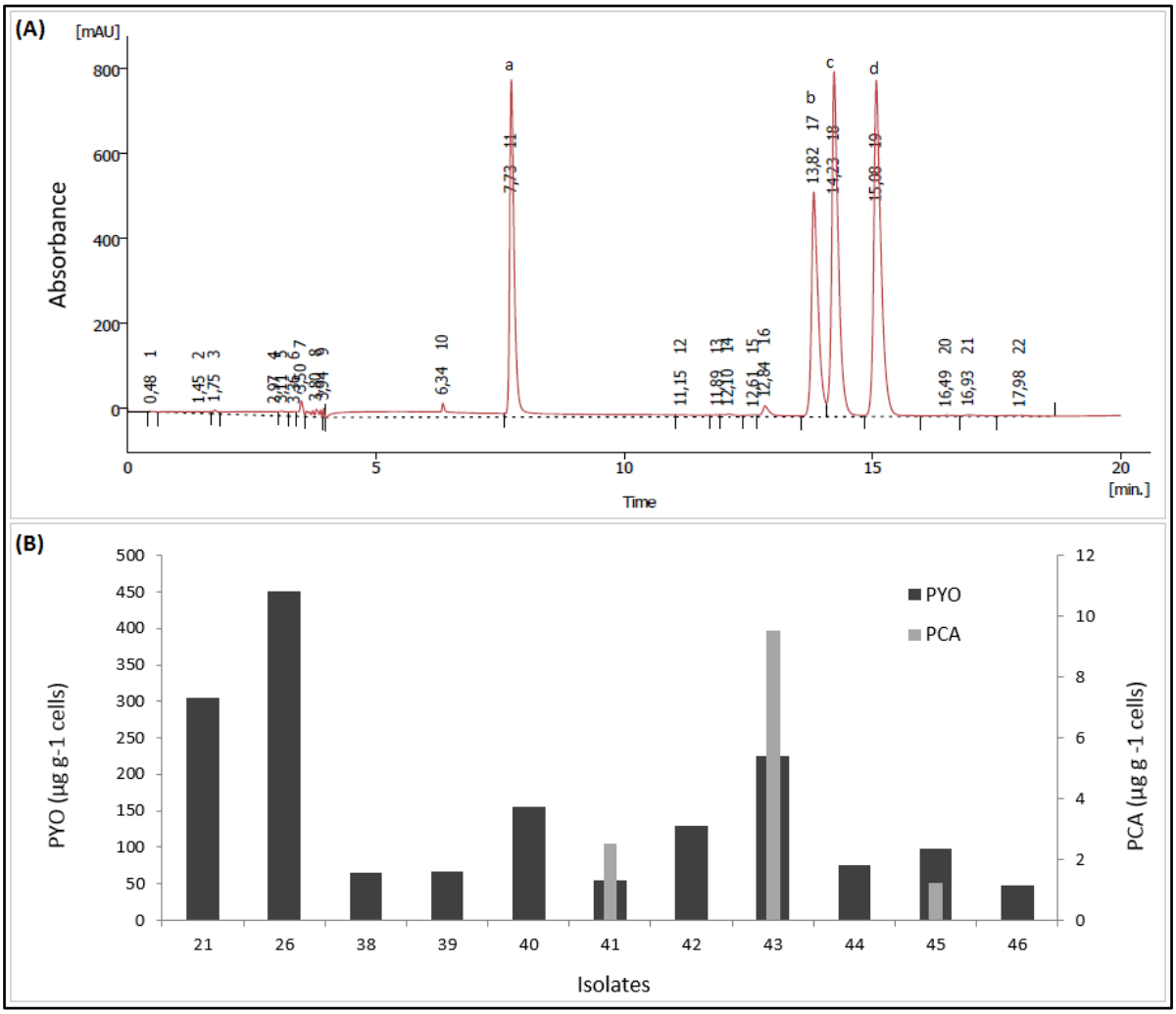

Figure 2 Phenazine production. (A) Chromatogram of phenazines: a. PYO (Pyocyanin); b. PCA (Phenazine-1-carboxylic acid); c. OH-Phenazine; d. Phenazine (basic molecule); (B) Quantification of phenazines, PYO and PCA, in the supernatant of *Streptomyces* spp. isolates cultures. Samples were evaluated in duplicates by HPLC.

Selection of isolates for molecular analysis and growth promotion assay

Promotion of plant growth mediated by PGPR has been related to the presence of several biochemical characteristics that affect plant metabolism, such as siderophores and hormone production (Kumar and Dube 1992; Arruda *et al.* 2013). In addition, the combination of the production of ACC deaminase and IAA by rhizobacteria has been strongly associated with the promotion of plant growth (Glick *et al.* 1994; El-Tarabily 2008; Siddikee *et al.* 2010). On the other hand, phenazine production by rhizobacteria is usually associated with plant defense against pathogenic organisms or nematodes and may not be directly related to growth promotion. According to the performance of our *Streptomyces* spp. isolates in regard of biochemical characterization, it was observed that CLV44 showed the highest biochemical versatility, presenting all the characteristics of PGPR analyzed, whereas CLV45 showed strong growth in presence of ACC and high production of IAA. The CLV42 isolate was prominent in the production of siderophores in relation to the other isolates. Thus, these three isolates were selected for the establishment of the experiment to evaluate the ability of *Streptomyces* spp. in promoting the growth of soybean plants.

Molecular identification of *Streptomyces* isolates

The selected isolates (CLV42, CLV44 and CLV45) were confirmed as belonging to the genus *Streptomyces* based on the 16S rRNA gene partial sequences. The DNA sequences are deposited in GenBank under the following access numbers: *Streptomyces* spp. CLV42–KY704165, CLV44–KY704108, and CLV45–KY704164.

Promotion of plant growth by *Streptomyces* spp.

The influence of bacterization of soybean seeds with *Streptomyces* spp. isolates CLV42, CLV44 and CLV45 on plant growth promotion is shown in the Figure 3. In the first 15 days of the soybean growth promotion test, plants treated with CLV44 and CLV45 showed diminished root and shoot growth when compared to the control treatment (Figure 3A-B). Fresh and dry mass of roots was also significantly different from the control (Figure 3C and E). Treatment with the CLV42 isolate did not show a statistically significant difference in relation to the control group at 15 days. The initial performance of *Streptomyces* spp. CLV44 and CLV45 on soybean seedlings may be related to high production of IAA by these isolates (Table 2). This result might be explained by the role of auxin on the development of roots, where high concentrations of IAA inhibit the elongation and growth of tap root. As shown in the Table 2, CLV44 and CLV45 are very efficient on producing IAA and may have negatively affected the development of root during germination and early seedling development. Perhaps, the newly IAA produced by the *Streptomyces* isolates was uptaken by the root during initial development, most likely stimulating the activity of the enzyme ACC synthase, which convert S-adenosylmethionine to ACC. Consequently, the

increase of the substrate for ACC-oxidase, elevates the endogenous concentrations of ethylene (Glick *et al.* 1998), and ultimately inhibits root growth. Furthermore, the contact of the rhizobacteria with the developing roots can cause a delay in the initial plant growth, due to the deviation in the cellular metabolism for the recognition and establishment of the rhizobacteria-plant interaction, a trade-off often reported in this type of interaction (Berg 2009; Dalmas *et al.* 2011; Salla *et al.* 2014).

At 30 days from the onset of experiment, soybean plants bacterized with *Streptomyces* CLV44 showed significantly higher shoot length and dry mass when compared to the control group (Figure 3B and F). The observed increase is probably related to the CLV44 isolate's ability to produce IAA (El-Tarabily 2008). The soybean plants treated with *Streptomyces* CLV42 and CLV45 showed no statistical difference in relation to the control group at 30 days, regarding all parameters evaluated (Figure 3).

At 45 days, there was a significant effect of isolated CLV45 as PGPR on soybean plants, resulting in 36.63% increase of shoot growth, and 17.97% of shoot dry weight, in relation to control (Figure 3B and F). The coordinated production of IAA and the enzyme ACC deaminase by the CLV45 isolate was probably responsible by the growth stimulation of soybean plants (Glick *et al.* 1998; El-Tarabily 2008). Since the IAA secreted by a rhizobacterium can act directly on the root system, stimulating the elongation and cell division of plant cells (Patten and Glick 1996), the observed result is consistent with the model proposed by Glick *et al.* (1998), which describes a complex exchange of signals between IAA and ethylene in the promotion of plant growth by PGPR.

Soybean plants treated with the isolate CLV44 did not show any significant difference when compared to the control at 45 days, despite their performance at 30 days, when some level of growth promotion was observed (Figure 3B and F). The lack of correlation between the production of IAA by the isolate and the promotion of growth at 45 days may be related to other factors, such as low concentration of the hormone, due to the stabilization of the relation rhizobacteria-plant (Hsu, 2010), the presence of phytotoxins that mask the hormone effect, or the need for additional factors in the soil to promote growth (Palaniyandi *et al.* 2013a). At the same time point, the treatment of plants with *Streptomyces* spp. CLV42 also resulted in no statistical difference on growth when compared to the control group. Although it did not increase the growth of soybean plants, the isolate CLV42 was able to produce high levels of siderophores *in vitro*. The synthesized siderophores can not only solubilize iron but also other metals (Al, Cd, Cu, In, Pb e Zn), including radionuclides, such as Uranium and Neptunium (Kiss and Farkas 1998; Miethke and Marahiel 2007). This metabolic ability allows the efficient siderophores-producing bacteria to play an important role in bioremediation of soils contaminated by metals (Dimkpa *et al.* 2008; Khan *et al.* 2009). Moreover, siderophores may be part of promotion of antagonism against other microorganisms, limiting

their Fe availability in the rhizosphere (Rajkumar *et al.* 2010). They are also capable of triggering Induced Systemic Resistance, ISR (Nagarajkumar *et al.* 2004) and function as a biological control agent. Therefore, additional studies with the CLV42 isolate are necessary to characterize the type of produced siderophores and evaluate their potential as a biocontrol agent and bioremediation (Berg 2009; Verma *et al.* 2011).

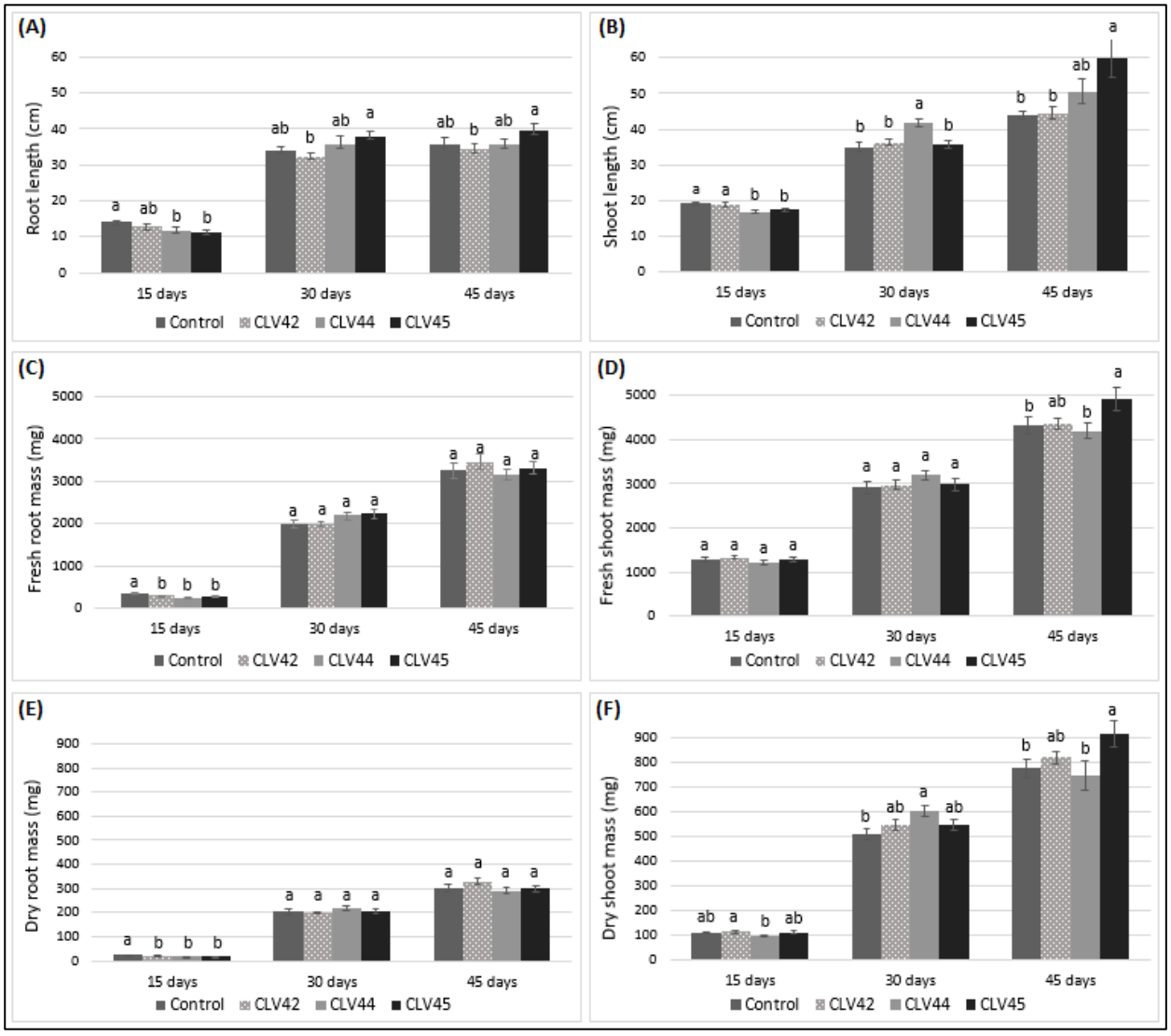

Figure 3 Evaluation of *Streptomyces* spp. CLV42, CLV44 and CLV45 isolates on the promotion of soybean plant growth. (A) Root length (cm); (B) Shoot length (cm); (C) Fresh root mass (mg); (D) Fresh shoot mass (mg); (E) Dry root mass (mg); (F) Dry shoot mass (mg). Different letters within each time of analysis mean significant difference by T Test ($\alpha \leq 0.05$).

Conclusion

The isolates of *Streptomyces* spp. obtained from rhizosphere of Fabaceae species demonstrated different plant growth promoting characteristics that can be used to improve plant growth. The greenhouse growth promotion trial showed that *Streptomyces* spp. CLV45 promoted growth of soybean plants and pointed it as a

strong candidate for the formulation of a biofertilizer. In addition, none of the isolates evaluated in greenhouse caused a growth deficit in soybean plants. Furthermore, a consortium combining different isolates of *Streptomyces* spp. may present a great potential as a plant biofertilizer, since combined PGPR characteristics of each isolate may benefit crops and environment by reducing the use of fertilizers and increasing productivity.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Manuscrito 2

Differential expression of defense-related genes in soybean plants elicited with *Streptomyces* sp. and challenged with *Xanthomonas axonopodis* pv. *glycines*

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Differential expression of defense-related genes in soybean plants elicited with *Streptomyces* sp. and challenged with *Xanthomonas axonopodis* pv. *glycines*

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Main conclusion Induction of systemic defense in soybean plants was recorded in *Streptomyces* sp. CLV45-elicited plants challenged with *X. axonopodis*. Ethylene and jasmonic acid participate on the defense mechanism.

Abstract

Streptomyces spp. (Stm) has been recognized as a microorganism capable of modulating and establishing the plant defense response. The isolate CLV45 (Stm45) was previously characterized as PGPR in soybean plants. Soybean is often infected by *Xanthomonas axonopodis* pv. *glycines* (Xag), a necrotrophic pathogen. The study aimed at evaluating the capability of Stm45 on inducing systemic defense response in soybean plants against Xag. Soybean seeds, from Xag susceptible and resistant cultivars, were bacterized with Stm45, and grown under greenhouse conditions. Treatments consisted of (i) plants raised from untreated seeds (absolute control); (ii) plants raised from seeds bacterized with Stm45; (iii) plants raised from non-bacterized seeds and challenged with *Xag* (Xag); and (iv) plants from seeds bacterized with Stm45 and challenged with Xag (Stm45+Xag). Inoculation with Xag was carried out 15 days after sowing. Expression of defense-related genes, activity of Phenylalanine ammonia lyase (PAL) enzyme and accumulation of phenolic compounds (Phe) were analyzed at different time points. Expression of *PAL1* gene was moderately enhanced in resistant Stm45+Xag plants at 12 hours post inoculation (hpi), followed by increase of PAL enzyme activity at 48 hpi, although corresponding accumulation of Phe was not recorded. Defense-related process pathway, such as the upregulation of *ERF-5* and downregulation of *JAZ*, were more rapidly observed in the Stm45+Xag in both resistant and susceptible plants at 12 hpi, respectively. This fast response might be associated with the *priming* phenomenon. Up-regulation of *PR1* gene was not observed in the Stm45+Xag treatment for both susceptible and resistant soybeans.

Key words: Ethylene, Induced systemic resistance, Jasmonate, Phenylalanine ammonia lyase, Plant defense, Salicylic acid

Introduction

The soybean [*Glycine max* (L.) Merrill] is a legume of the Fabaceae family adapted to the tropical and subtropical climate (Zhang et al. 1997). One of the main crops in the world economy, soybeans are marketed in the form of grains, bran and oil, and utilized for feedstock in several industrial sectors (Embrapa 2016). Brazil is the second largest producer in the world, behind only the USA (Conab 2016). Its production is heavily affected by pests and infectious diseases, which are responsible for decreased yields and economic losses. Bacterial diseases have an impact on soybean growing areas. The bacterial pustule, caused by the necrotrophic bacterium *Xanthomonas axonopodis* pv. *glycines* (Xag), is a common foliar disease in regions of high temperatures (≥ 30 ° C) and humidity (> 60% relative humidity) accompanied by rainfall, which favorers its development (Narvel et al. 2001). The bacterium penetrates the tissues through natural openings and wounds, leading to premature defoliation (Mingma et al. 2014). In resistant varieties, the reported symptoms are small, pale patches with no defined pustules. In susceptible cultivars, the symptoms include chlorotic patches with elevated pustules, which can evolve into large necrotic lesions (Narvel et al. 2001). Losses reported for this bacterial disease reached 50% of production worldwide (Prathuangwong et al. 1987; Narvel et al. 2001; Kaewnum et al. 2005; Hong 2011; Chatnaparat et al. 2012).

Rhizosphere microorganisms may provide a front-line defense against pathogen attack and are reported as biocontrol agents. Some rhizobacteria associated with crop plants are capable of exerting beneficial effects on their hosts and are referred as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper et al. 1980; Karthikeyan et al. 2010). PGPR improve plant growth directly by supplying nutrients (e.g. phosphorous, nitrogen), producing phytohormones [e.g. indoleacetic acid (IAA)], and/or decreasing the level of ethylene that is produced when the plant is stressed (Li et al. 2007; Lugtenberg and Kamilova 2009). PGPR can also elicit plant defenses (Van Loon and Glick 2004) and antagonize or prevent growth of phytopathogens or other deleterious microorganisms (Kloepper et al. 2004). Several microorganisms have been reported as candidates for biocontrol agents (Beneduzi et al. 2012). The Actinomycetes, a diverse group of rhizosphere microorganisms, are nowadays recognized as control agents of disease caused by several pathogens by either producing antimicrobial compounds and enzymes (Zhang et al. 2014; Couillerot et al. 2014) or by elicitation of induced systemic resistance (Choudhary and Johri 2009; Chithrashree et al. 2011).

Elicitation of plant defenses usually occurs through systemic resistance, the ISR (Induced Systemic Resistance) phenomenon, which resembles pathogen-induced systemic acquired resistance (SAR) under conditions where the inducing bacteria and the challenging pathogen remain spatially separated, excluding the possibility of a direct antagonistic effect of the biocontrol bacteria on the pathogen (Doornbos et al. 2012). Both types of induced resistance render uninfected plant sites more resistant to pathogens in several plant species (Beneduzi et al. 2012).

The induction of systemic resistance is usually triggered by non-pathogenic microorganisms (such as PGPRs) or by abiotic factors (Barros et al. 2010; Pieterse et al. 2014). Induced plant defenses are regulated by highly interconnected signaling networks in which the plant hormones such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) play a central role (Pieterse et al. 2000; Mabood et al. 2014). ISR mediated by PGPR is related to the expression of several genes coding for secondary metabolism-related enzymes (Choudhary and Johri 2009; Sarma et al. 2015). The elucidation of JA involvement in defense responses began with the discovery of the protein JASMONATE ZIM DOMAIN (JAZ), which is a negative regulator of JA-induced gene expression (Yan et al.

2007; Thines et al. 2007). The proteasomic degradation of JAZ proteins causes the release of transcription factors such as those from the MYB family, which bind to promoters of JA response genes (Song et al. 2013).

Studies have demonstrated that the NPR1 protein (NONEXPRESSER OF PR PROTEINS1) was necessary for ISR regulation mediated by JA/ET (van Loon et al. 1998; Pieterse et al. 2012). At low concentrations, SA and JA act in synergism in tissues distant from the site of infection, promoting a nonspecific defense response and broad-spectrum of action. SAR and ISR are effective for different pathogen lifestyles. Whereas SAR is generally considered efficient against biotrophic pathogens, ISR is generally efficient against necrotrophic pathogens (Thaler et al. 2012; Sarma et al. 2015). At high concentrations, these hormones start acting in an antagonistic way, promoting a specific defense response, focused on the type of pathogen (Fu and Dong 2013). Moreover, ET is involved in the plant defense against pathogens playing a role as systemic transductor, being fundamental at the root level for the establishment of ISR (Knoester et al. 1999; Verhagen et al. 2004). The ET response model also includes the integration of *PR* genes (Pathogenesis-Related genes) and specific transcription factors, such as *ETHYLENE INSENSITIVE 3* (EIN3) and *ETHYLENE RESPONSE FACTOR 1* (ERF1) (Ecker 1995; Van Der Ent et al. 2009). The hormones ET, JA and its ester Methyl jasmonate (MeJA) have synergistic action on the expression of PR proteins (Xu et al. 1994), although there are evidences that other plant hormones (IAA, GA, ABA and CK) are important modulators of plant defense against pathogens (Pieterse et al. 2009; Robert-Seilaniantz et al. 2011; Puga-Freitas and Blouin 2015).

Among the various metabolic pathways involved in defense responses, the activity of the enzyme phenylalanine ammonia lyase (PAL) is closely related to the synthesis of defense compounds and the interaction of the plant with microorganisms. PAL is the first enzyme in the phenylpropanoid pathway that catalyzes the transformation of L-phenylalanine into *trans*-cinnamic acid (Dixon and Paiva 1995), which is an important intermediate in the synthesis of phenolic compounds. Phenolics are recognized as defense compounds due to their activity as antimicrobial molecules and lignin precursors. Moreover, they participate in the hypersensitive response, which ultimately is the result of plant cell death (Singh et al. 2013). Phenolic compounds are also substrates for polyphenol oxidases (PPO) and peroxidases (POX), enzymes involved in the defense processes as well (Gaspar et al. 1985; Choudhary and Johri 2009).

The aim of this study was to evaluate the defense response in soybean plants elicited with the *Streptomyces* sp. CLV45 and challenged with the phytopathogen *X. axonopodis* pv. *glycines*, through analyses of gene expression and secondary metabolism related to plant defense.

Material and Methods

Cultivation of microorganisms

Streptomyces spp. CLV45 (Stm45 – GenBank accession number: KY704164) was previously selected from a pool of eleven native *Streptomyces* spp. CLV45 was chosen due to PGPR traits, such as auxin and siderophore production and improvement of soybean growth (data not shown).Culture was grown aerobically in 10 ml liquid ISP2 medium (Shirling and Gottlieb 1966), for 5 days at 28 ± 2 °C (stationary phase). Bacterial suspension was centrifuged (2,500 g, 15 min, room temperature), resuspended in sterile distilled water, and adjusted to final concentration of 10^7-10^8 cfu ml^{-1} (OD_{600nm}= 1.0) for use as inoculum for the experiment. The pathogenic bacterium *X. axonopodis* pv. *glycines* (Xag) was obtained from the Instituto Biológico de São Paulo, Brazil (#327, from *G. max*; NCPPB 3658). For inoculum preparation, Xag was cultivated in 10 ml LB medium under agitation at 28 ºC for 24 h, centrifuged at 2,500g for 15 min and the bacterial pellet was resuspended with sterile

water. Cell suspension was adjusted to optical density (OD_{600nm}) of 0.3 corresponding to approximately 2.5×10^8 cfu ml^{-1}.

Plant material and experimental conditions

Soybean seeds of two cultivars, one susceptible (BMX POTENCIA RR) and one resistant (TMG-7262 RR INOX) to Xag, without fungicide treatment, were provided by Ballagro AgroTecnologia Ltda. Brazil and Syngenta Brazil, respectively. For their use in the experiment, seeds were surface disinfested with sodium hypochlorite solution (2%, v/v) for 2 min, rinsed three times with sterile distilled water and treated with the Stm45. For the bacterization, 40 seeds were immersed in 6 ml of bacterial suspension by treatment for 5 min (Stm45, and control) and partial dried for 1 h in uncovered petri dishes in a laminar flow hood. For the control, the seeds were treated with sterile distilled water. Seeds were sown into commercial substrate (clay, hummus and ground calcareous rock) in polypropylene bags (15x25 cm) and maintained in the greenhouse. Experiment was set up with 20 plants per treatment, including the control group.

Treatments consisted of (i) plants raised from untreated seeds (absolute control, H_2O); (ii) plants raised from seeds bacterized with Stm45; (iii) plants raised from untreated seeds and challenged with *Xag* (Xag); and (iv) plants from seeds bacterized with Stm45 and challenged with *Xag* (Stm45+Xag). Inoculation with Xag was carried out 15 days after sowing the seeds by gently rubbing a brush dipped in bacterial suspension on abaxial face of the first true leaf at the third leaflet (Soares 2009). In treatments (i) and (ii) water was used instead of Xag to treat plants. Inoculated plants were grown at 100% relative humidity. Irrigation was carried out when necessary. Nutrient solution [10 ml of macronutrients salt solution; (0.41 g L^{-1} NH_4NO_3, 0.47 g L^{-1} KNO_3, 110 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.09 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.04 g L^{-1} KH_2PO_4)] was supplied to the plants every 15 days. Defense responses of soybean plants against Xag were evaluated by analysis of PAL activity and phenolic compounds at 0, 24, 48, 72 and 144 hours post Xag infection (hpi) and through the expression of defense-related genes at 0, 12, 24 and 48 hpi. Samples of leaves were collected for all analyses.

Quantification of defense-related genes

In order to investigate the mechanism of defense triggered by Stm CLV45 against Xag in soybean plants, leaf samples (150 mg) were collected at determined time points, immediately flash frozen in liquid nitrogen, and stored at –80°C. Total RNA was extract according CTAB method described by Gambino et al. (2008), with modifications. Briefly, leaf samples were ground in liquid nitrogen and RNA was extracted with CTAB buffer with 2% β-mercaptoethanol. Samples were subject to three extractions with chloroform:isoamylic alcohol (24:1 v/v), and precipitated by Lithium chloride 8M. The pellet was resuspended in SSTE buffer, precipitated in cold isopropanol, and resuspended in 20 µL H_2O RNase free. RNA integrity was evaluated by 1% agarose gel. Quantity and quality of total RNA was determined by NanoDrop Lite spectrophotometer (Thermo Scientific). Synthesis of the first-strand of complementary DNA (cDNA) was carried out by SuperScript® First-Strand Synthesis System for RT-PCR kit (Invitrogen™) following the manufacturer's guidelines. The reaction was incubated in a thermocycler (Amplitherm™) for 50 min at 42 °C for cDNA synthesis and at 70 °C for 15 minutes at the end of the reaction.

The expression of four soybean defense-related genes (*PR-1, JAZ, ERF5* and *PAL)* was quantified from each cDNA sample. The constitutively expressed gene *actin* was used to normalize the quantification of

expression of each target gene (Arfaoui et al. 2016; Zou et al. 2005). Specific primers (Table 1) were designed from their nucleotide sequences by referring to National Center for Biotechnology Information (NCBI) and synthetized by Macrogen (Seoul, Korea). The real-time polymerase chain reactions (RT-qPCR) for evaluating the relative expression of the genes were performed using StepOne™ Real-Time PCR System (Applied Biosystems®), using SYBR® Green I (Invitrogen™) as the fluorescent reporter signal and ROX (Invitrogen™) as the passive reference dye. The conditions were 5 min at 95 °C, 47 cycles of 95 °C for 15 seconds and 60 °C for 1 min. At the end of each cycle, fluorescence SYBR® Green indicator was measured. The specificity of the reaction and the formation of primer dimers was monitored by the presence of a single peak at Melting curve analysis. The relative expression of the genes was calculated according to Pfaffl (2001). The efficiency of the primers in the qPCR was evaluated by the LinRegPCR software, version 2014.6.

Table 1 Oligonucleotide primer pairs used for quantitative RT-qPCR analysis

Target gene	Forward/reverse primers	Target sequence*	Tm (ºC)†	Amplicon (bp)	Reference
PR-1	F: 5' TGATGTTGCCTACGCTCAAC R: 5' AAGCAGCAACCGTATCATCC	AF136636	62.6 62.5	167	Upchurch et al. 2010
ERF5	F: 5' ACAAGGCCAAGCTCAACTTCCCG R: 5' AGGCAGCGTTGCTTTTTGGAAGG	NM_001256464.1	60.0 58.6	79	Zhai 2012
JAZ	F: 5' TTGCGCAGAGCCACGAACAAAG R: 5' AACAACCCAAGTCTGCAATGCCC	XM_003544327.3	58.9 59.0	92	Predicted
PAL1	F: 5' AAGTGCTGAGCAACACAACC R: 5' GTCAATTGCTGGCAAAGTG	X52953.1	63.4 59.9	130	Arfaoui et al. 2016
β-actin	F: 5′ CAATCCCAAGGCCAACAGA R: 5' TGGCAGGCACATTGAAAGTC	AW350943	62.3 63.6	66	Arfaoui et al. 2016

*http://blast.ncbi.nlm.nih.gov

† Tm: primers pair melting temperature (ºC).

Determination of PAL activity and phenolic compounds content

Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) was colorimetric assayed by the method described by Navarre et al. (2013), with modifications. Leaf samples (250 mg) were homogenized in extraction buffer containing Tris-HCl (50 mM pH 8.8), EDTA (1 mM), Triton X-100 (0.2% v/v) and PVP-40 (1% w/v). Reactions consisted of 50 mM sodium borate buffer (pH 8.8), 10 mM of phenylalanine and leaf extract. They were incubated at 37 °C for 1 h and absorbancies were determined at 290 nm. The concentration of cinnamic acid formed in the reactions was determined from the calibration curve with commercial standard. Specific activity of PAL was expressed as nm of cinnamic acid ml^{-1} min^{-1} mg $protein^{-1}$. Enzyme assay was repeated three times. Protein estimation of the extracts was determined following Bradford (1976) using bovine serum albumin (BSA) as standard.

Quantification of total phenolics was performed following by Sartor et al. (2013). Leaf samples (125 mg) were subjected to extraction in 80% (v/v) methanol. The colorimetric reaction consisted of distilled water, 1N Folin Ciocalteu reagent, plant extract and sodium carbonate (Na_2CO_3), incubated at room temperature for 30 min

in the dark. Absorbances were read in a spectrophotometer at 765 nm. Colorimetric reactions were repeated three times. Gallic acid was use as the standard. Phenolic contents were expressed as mg g^{-1} fresh mass (FM).

Statistical Analysis

Experiments were performed in a fully randomized design, tested for variance homogeneity by Levene's test ($\alpha \leq 0.05$). Each treatment was replicated three times. Data from relative gene expression, enzyme activity and contents of phenolic compounds were expressed as means ± standard error (SE) and subjected to analysis of variance (ANOVA). Treatment means were compared by Tukey test at significance level of $P< 0.05$. All statistical analyzes were performed using SPSS software v. 22.

Results and Discussion

Streptomyces sp. CLV45 was previously evaluated and determined as PGPR in soybean plants, since it demonstrated ability of IAA and siderophore production, as well as the activity of the ACC deaminase, enzyme involved in the ethylene biosynthesis pathway (data not shown). In the same study, the production of phenazine-1-carboxylic acid (PCA) and pyocyanin (PYO) by this isolate was also detected. In this context, the use of *Streptomyces* sp. CLV45 as treatment for soybean seeds could be a beneficial component of integrated pest management.

Differential expression of defense-related genes in soybean plants treated with Stm45 and challenged with Xag was observed either on the susceptible or on the resistant cultivar (Fig. 1, Fig**.** 2). Phenylalanine ammonia lyase (PAL) is a key enzyme of phenylpropanoid metabolism in plants and catalyzes the biosynthesis of phenolic compounds (Yang et al. 2009). Up-regulation of *PAL* gene has been related to responses of plant defense against pathogens in many plant species (Conrath, 2011; Kurabachew et al., 2013). In this study, susceptible soybean cultivar showed increased expression of *PAL* gene in Stm45-treated plants at 12 hpi, whereas Xag-treated plants showed the maximum expression at 24 hpi (Fig. 1a), indicating some level of response when plants were treated either with the rhizobacterium or the pathogen. At 24 hpi, up-regulation of *PAL1* was detected in Stm45 and Stm45+Xag when compared to the untreated plants (Fig. 1a). On the other hand, on the resistant cultivar, expression of this key gene reached the maximum relative expression at 12 hpi in plants elicited with Stm45 and challenged with Xag (Fig. 2a). Although its expression in resistant plants had significantly decreased at 24 hpi, a positive difference was found at 48 hpi in Stm45+Xag plants when compared to either Stm45 or Xag plants.

Overall, *PAL1* gene expression was not clearly time-correlated to the enzyme activity in resistant plants. However, as result of the increase in the *PAL1* gene expression in Stm45+Xag resistant plants, a significant augment on PAL enzyme activity was recorded at 48 hpi, being superior to the other treatments at this time point (Fig. 3b). On the other hand, despite of the moderate variation in *PAL1* gene expression at 24 hpi in the susceptible cultivar (Fig. 1a), activity of this enzyme was increased at 48 hpi in Stm45+Xag plants (Fig. 3a). It seems that Stm45-root colonization was able to induce response in both soybean cultivars, up-regulating *PAL1* gene and stimulating PAL activity in the phenylpropanoid pathway when plants were later infected with pathogen (Fig. 3).

Related to PAL enzyme activity, accumulation of phenolic compounds differed within the time intervals in both cultivars analyzed. In the susceptible cultivar, a visible increase of accumulation of phenolics is noted in Stm45 plants at 24 hpi (Fig. 4a), simultaneously with the increase of PAL activity (Fig. 3a). Although an

increase of phenolic compounds in plants rose from bacterized seeds and challenged by pathogen was expected (Singh et al., 2002), the variation found in the activities of PAL enzyme along the time points was not observed in the phenolic accumulation. Our hypothesis is that the produced and accumulated phenolics were likely used as substrates for enzymes such as polyphenol oxidases and peroxidases, activated upon Xag inoculation (24 hpi). Ecker and Davis (1987) suggested that low accumulation of phenolic compounds in the susceptible cultivar of carrot, early in the defense process against pathogen, may be related to the susceptible phenotype of this cultivar.

Similarly, a transient stimulation of PAL activity was seen on Stm45+Xag resistant cultivar at 48 hpi (Fig. 3b), 36 hours after the increase on gene expression had been recorded (Fig. 2a). Contrary to what was observed on susceptible cultivar, phenolic compounds were early accumulated in plants which was infected with Xag (24 hpi) and maintained these high levels up to 144 hpi (Fig. 4b), most probably for maintaining the defense pressure against the pathogenic bacteria. The increase in phenolic compounds is well known as a defense mechanism in plants. Phenolics may be oxidized to quinones by polyphenol oxidases, producing more toxic antimicrobial molecules than the phenolic themselves (Lattanzio et al. 2006). Early and increased activity of defense enzymes PAL, POX and PPO leads to disease suppression in rice plants treated with PGPR in response to invasion by *X. axonopodis* pv. *oryzae* (Chithrashree et al. 2011). Plants of *Eucalyptus grandis* and *E. globulus* pre-treated with *Streptomyces* ap. PM9 and challenged with *Botrytis cinerea* also showed early alteration on PPO and POX activities, as well as on the levels of phenolic compounds, resulting in a delay of disease progression (Salla et al. 2016). Latha et al. (2009) suggested that the application of the rhizobacteria *Pseudomonas* spp. and Zimmu leaf extracts helped in reducing disease caused by *Alternaria solani* in tomato plants, through increased levels of POX, PPO and PAL, as well as phenolic compounds. Partially resistant soybean has shown an increment in phenylpropanoid pathway, compared to susceptible cultivar, when both were inoculated with *Fusarium solani* sp. *glycines,* indicating that these compounds may be involved in the partial resistance response (Lozovaya et al., 2004). Contrary to our results, reports have showed that PAL activity increased in resistant tomato cultivars more than in susceptible cultivars after inoculation with *X. axonopodis* pv. *vesicatoria* (Kavitha and Umesha 2008; Mandal et al. 2013).

The plant hormones, such jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) play important role in the signaling networks against biotic and abiotic stresses (Shigenaga and Argueso 2016). Wounding or necrotrophic pathogen attack is followed by significant up-regulation of JA-responsive genes (Yang et al. 2011; Wasternack and Hause 2013). The octadecanoid pathway is one of the best-known mechanisms in plant defense, and its final product, the jasmonic acid, induces the expression of several genes related to biotic stress (Shah et al. 2014). Our data indicated that in soybean, expression of *JAZ* gene showed differences along the time intervals analyzed (Fig. 1b, Fig**.** 2b). In the susceptible cultivar, difference was seen at 12 hpi in Stm45+Xag plants, showing a reduction of 2.6 fold compared to Xag-treated plants (Fig. 1b). On the other hand, differential response on *JAZ* expression in resistant cultivar was only registered at 48 hpi, and Stm45+Xag plants expressed *JAZ* gene ca. 5-fold higher than in Xag-plants (Fig. 2b). Upon infection with pathogen, JAZ protein, a transcriptional repressor of JA-responsive genes, is degraded through the 26S proteasome pathway. This results in the release of MYC transcription factors, which initiate transcription activity of JA-regulated genes such as *PDF1.2* (*PLANT DEFENSIN 1.2*), JA-responsive genes involved in defense against necrotrophic pathogens (Shigenaga and Argueso 2016). In our results, the previous inoculation with Stm CLV45 resulted in a fast downregulation of *JAZ* when plants were challenged with the necrotrophic Xag. Interestingly, at 0 h, Stm45-plants showed very low expression of *JAZ* gene in susceptible cultivar (Fig. 1b), contrasting

to its expression in resistant cultivar, which was approximately 4 times higher than the absolute control plants, at the same time point (Fig. 2b), suggesting that JA pathway was activated by CLV45 only in the susceptible cultivar.

Similar to JA, ethylene is known to regulate multiple physiological and developmental processes in plants, and is also involved in the reactions of plants to abiotic and biotic stresses (van Loon et al. 2006). The role of ET in plant defense is somewhat controversial since it is involved in plant resistance in some pathosystems but contributes to disease production in others (Kunkel and Brooks 2002). Expression of some members of ERF family can be regulated by plant defense inducers such as ET, JA, and SA. It has been reported that overexpression of *ERF2* gene in transgenic plants resulted on disease resistance in tobacco (Zhang et al. 2009). In our study, the highest expression of *ERF5* was promoted by both Stm45 and Stm45+Xag treatments on resistant plants at 12 hpi and with Stm45+Xag at 48 hpi (Fig. 2c). On the susceptible plants increase, on *ERF5* expression was recorded only on Stm45+Xag plant at 12 hpi (Fig. 1c). Enhanced ethylene production is an early, active response of plants to perception of pathogen attack and is associated with the induction of defense reactions (van Loon et al. 2006), and our result supports the role of ET-induced defense mediated by PGPR. It is known that JA and ET signaling pathways are also both required for induction of ISR, a form of systemic resistance that can be triggered by root-colonizing bacterium, for example *Pseudomonas fluorescens* (Pieterse and van Loon 1999). This observation provides evidence that JA and ET signaling pathways to modulate each other (Kunkel and Brooks 2002).

Ethylene can also induce certain types of pathogenesis-related proteins or phytoalexins, and, through stimulation of the phenylpropanoid pathway, trigger the strengthening of cell walls in various plant species (van Loon et al. 2006). Interestingly, an expected response was observed in the resistant plants treated with Stm45 or Stm45+Xag, where the upregulation of *ERF5* at 12 hpi preceded the increment on PAL activity at 48 hpi (Figs. 2c and 3b). Such response was not evidenced in the susceptible soybean treated with Stm45 or Stm45+Xag, where PAL activity was induced at 48 hpi (Fig. 3a) without significant changes in *ERF5* expression at 12 hpi, although low expression of *ERF5* was seen at 24 hpi (Fig. 3a).

Defense-related process pathway, such as the upregulation of *ERF-5* and downregulation of *JAZ*, were more rapidly observed in the Stm45+Xag in both resistant and susceptible plants at 12 hpi, respectively. Moreover, the early upregulation of P*AL1* at 12 hpi was observed in resistant soybean cultivar. This fast response might be associated with the *priming* phenomenon. Defense priming establishes in the tissue exposed to the stimulus and in the systemic or untreated parts of the plant. When primed, plants respond to very low stimulation with faster and stronger defense than unprimed plants, and this frequently comes with local and systemic immunity or abiotic stress tolerance (Reimer-Michalski and Conrath, 2016). Priming of defense responses is not solely confined to the SAR. It has also been demonstrated in rhizobacterium-mediated induced systemic resistance (ISR) (Conrath et al., 2002). In our study, protection as a result of microbial antagonism was excluded because the inducing Stm45 and the pathogen Xag were inoculated at, and remained confined to, spatially separated parts on the same plants, indicating the defense mediated by the plant.

Induced resistance also involves multiple mechanisms that include increased activity of pathogenesis-related (PR) proteins like chitinase, β-1,3-glucanase e peroxidase (Nisha et al. 2012). In our study, expression of *PR-1* gene in soybean plants varied among treatments. In both susceptible and resistant soybean cultivars, *PR-1* showed increased expression in Stm45-plants at 12 hpi (Fig. 1d, Fig**.** 2d). However, opposite response was observed in Xag- and Stm45+Xag- plants in both cultivars at the same time. While resistant cultivar showed no differences on *PR-1* expression at 24 hpi, the susceptible showed higher expression when plants were inoculated

with Xag, of *Streptomyces* compared to the treatment Stm45 (Fig. 1d, Fig. 2d). PR proteins are generally induced in response to a variety of pathogens' attempt of attack, and are produced following the recognition of pathogen-derived molecules by host plant cells and the activation of transduction pathways (Rivière et al. 2008). PRs play direct roles in conferring resistance because their expression coincides with the onset of the phenomenon of Systemic Acquired Resistance (SAR). PR-1 is a known marker of the SAR response and its expression is salicylic acid-responsive (Nisha et al. 2012; Vallejo-Reyna et al. 2015), a defense hormone that increases at the site of infection and induces the expression of PR-1 gene. Treatment of *A. thaliana* plants with SA results in marked transcriptional changes in more than 2,000 genes. This transcriptional reprogramming is largely dependent on the transcription cofactor NONEXPRESSER OF PR PROTEINS1 (NPR1), which activates a myriad of defense-related genes (such as PR genes) and genes encoding transcription factors that initiate further transcriptional cascades (Spoel and Dong 2012). As expected for plant resistance induced by rhizobacteria and mediated by ISR, we did not observe an upregulation of *PR1* gene in the Stm45+Xag treatment for both susceptible and resistant soybeans.

The defense mechanism proposed for Arabidopsis postulated that AS-dependent defenses are triggered against biotrophic microorganisms, whereas JA- and ET- mediate defenses against necrotrophics (Glazebrook 2005). However, this could be considered a simplification of the complex signaling events triggered after pathogen recognition (Nováková et al. 2014). Our results indicate that, at some extension, defense through *PR1* gene expression is occurring in plants infected with Xag, suggesting the SA pathway has been triggered by this necrotrophic pathogen.

Despite the presence of effective defense systems, many bacterial pathogens are able to evade or suppress the host defense or modulate the metabolism of the host to obtain nutrients for their colonization through virulence strategies (Choudhary and Johri 2009). Pathogenicity of *Xanthomonas* spp. and most other Gram-negative phytopathogenic bacteria depends on a conserved type III secretion (T3S) system which injects more than 25 different effector proteins into the plant cell. Some effectors, formerly designated as avirulence proteins, are specifically recognized in resistant plants containing corresponding resistance (R) genes. Such recognition triggers plant defense reactions often culminating in the hypersensitive response (HR), a rapid localized cell death, and restricting pathogen ingress (Kay and Bonas 2009). Susceptible plants do not recognize efficiently or in an appropriate time the effector system of pathogenic bacteria, and thus, seed bacterization with *Streptomyces* sp., in conjunction with other strategies will be a worthwhile approach to explore as an alternative to biocontrol of bacterial plant disease.

In conclusion, the activation of defenses in soybean plants treated with *Streptomyces* sp. CLV45 and challenged with the necrotrophic pathogen Xag was more evident as being part of a crosstalk between JA and ET, an expected response of ISR phenomenon. Since several microorganisms are been commercial used as biocontrol agent, *Streptomyces* sp. CLV45 rhizobacteria might be useful in formulating new inoculants aiming resistance-inducing action and consequently more efficient managing of crop systems.

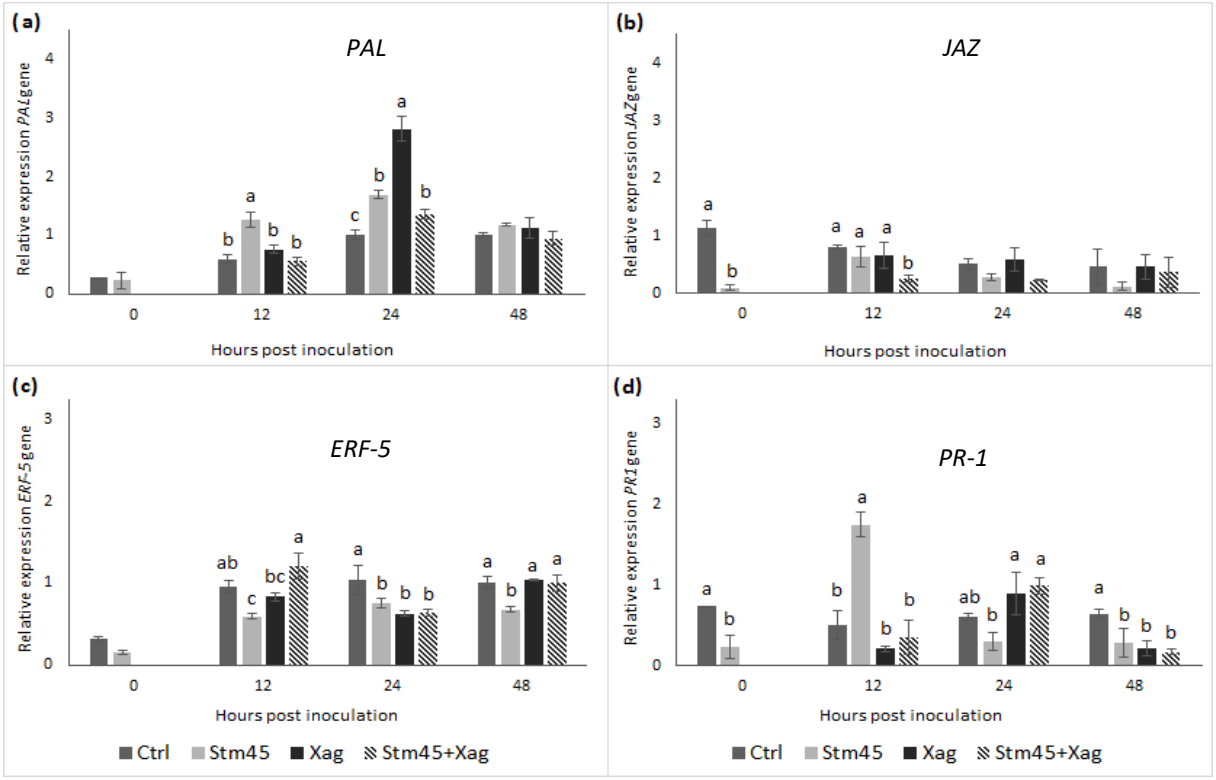

Fig. 1 Relative expression of defense-related genes in soybean cultivar susceptible to Xag. (a) *PAL* gene; (b) *JAZ* gene; (c) *ERF-5* gene; (d) *PR1* gene. Different letters within each time of analysis mean significant difference among treatments and unlabeled columns indicate no difference by Tukey Test ($\alpha \leq 0.05$).

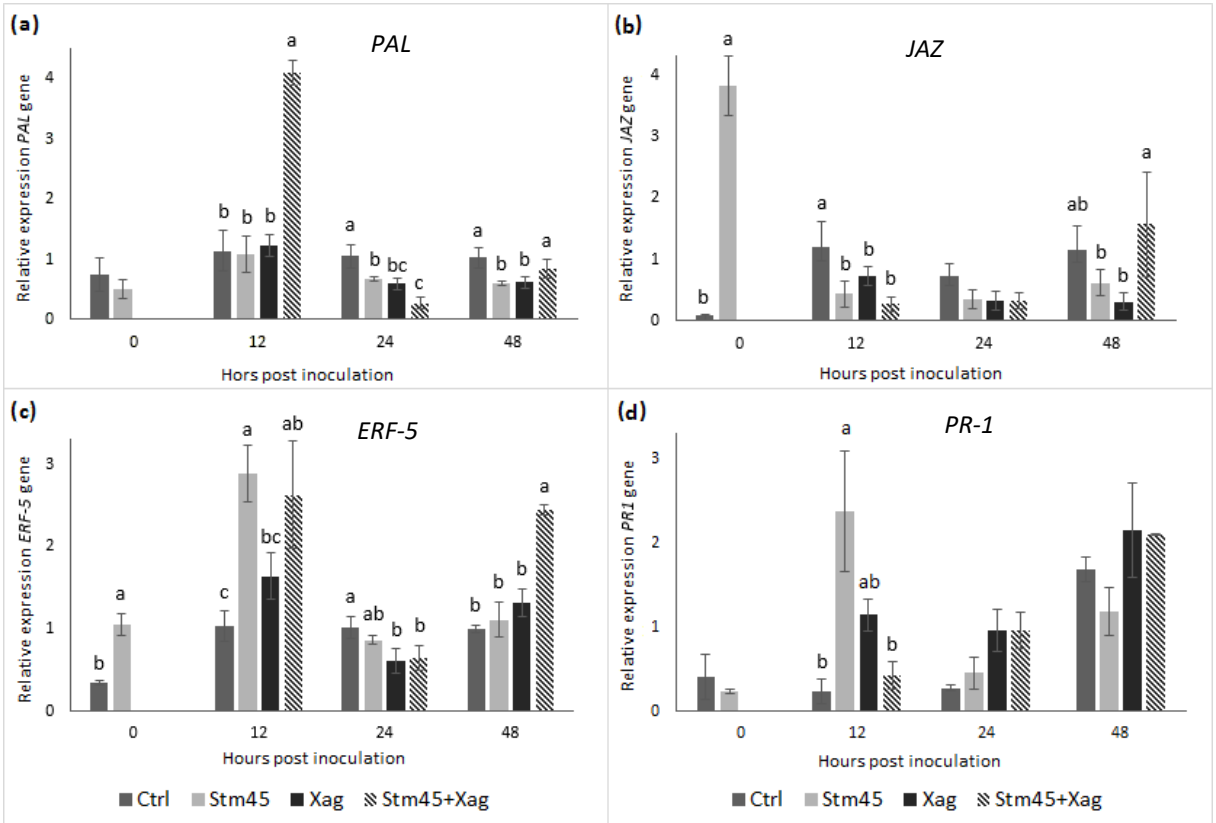

Fig. 2 Relative expression of defense-related genes in soybean cultivar resistant to Xag. (a) PAL gene; (b) JAZ gene; (c) ERF-5 gene; (d) PR1 gene. Different letters within each time of analysis mean significant difference among treatments and unlabeled columns indicate no difference by Tukey Test by Tukey Test ($\alpha \leq 0.05$)

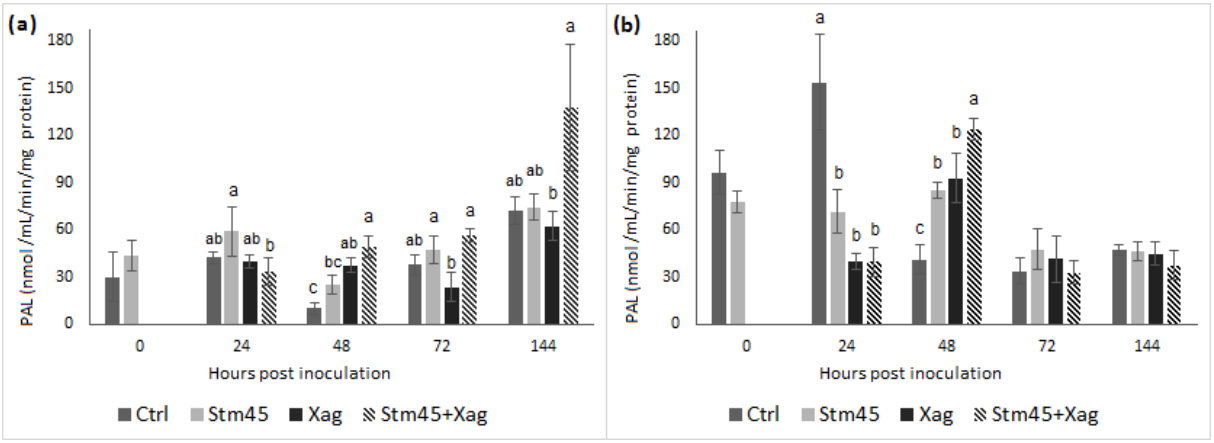

Fig. 3 Enzymatic activity of phenylalanine ammonia lyase (PAL). (a) Soybean susceptible cultivar. (b) Soybean resistant cultivar. The enzymatic activity of PAL was expressed in nm cinnamic acid ml^{-1} min^{-1} mg of $protein^{-1}$. Different letters within each time of analysis mean significant difference and unlabeled bars mean no significant difference among treatments by Tukey Test ($\alpha \leq 0.05$).

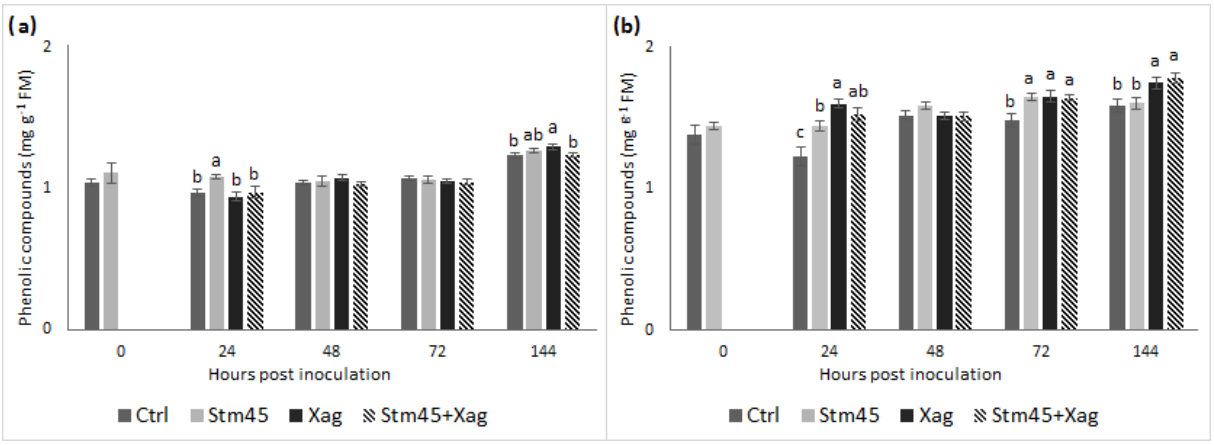

Fig. 4 Quantification of total phenols. (a) Soybean susceptible cultivar. (b) Soybean resistant cultivar. Phenolic compounds were expressed as mg g^{-1} fresh mass (FM). Different letters within each time of analysis mean significant difference and unlabeled bars mean no significant difference among treatments by Tukey Test ($\alpha \leq 0.05$)

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Author Contribution Statement

JLH, RMS and ERS conceived and designed research. JLH and FO conducted experiments. JLH, FO and ERS analyzed data. LVA and RMS contributed to data analyses and discussion of the results. JHL and ERS wrote the manuscript. All authors read and approved the manuscript.

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CAPÍTULO IV: CONSIDERAÇÕES FINAIS

CONSIDERAÇÕES FINAIS

Todos os 11 isolados de *Streptomyces* spp. avaliados foram capazes de produzir características desejáveis para sua utilização como PGPR, tais como sideróforos, enzima ACC desaminase e o fitormônio AIA e fenazinas (PYO e PCA).

Os resultados deste projeto de pesquisa permitiram estabelecer a interação eficiente de, pelo menos, um isolado de *Streptomyces* spp. com plantas de soja, promovendo o crescimento e induzindo respostas de defesa contra o fitopatógeno *X. axonopodis* pv. *glycines*.

A atividade promotora do crescimento de plantas de soja pela rizobactéria *Streptomyces* sp. CLV45 caracteriza este isolado como uma PGPR. Este isolado também foi capaz de induzir respostas de defesa de plantas de soja contra o fitopatógeno *X. axonopodis* pv. *glycines*, o caracterizando como biofertilizante e agente de biocontrole.

Além disso, um consórcio com os diferentes isolados de *Streptomyces* spp. avaliados em conjunto com a *Streptomyces* sp. CLV45 pode apresentar um grande potencial como bioinoculante vegetal, uma vez que as características de PGPR combinadas podem beneficiar a produtividade das culturas e minimizar os danos causados ao meio ambiente pelo uso de fertilizantes e agroquímicos.

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