JOSIANE FUKAMI

BACTÉRIAS PROMOTORAS DO CRESCIMENTO DE PLANTAS:

ESTRATÉGIAS DE COLONIZAÇÃO E PARA A MITIGAÇÃO DE ESTRESSES ABIÓTICOS EM MILHO

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Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

Fukami, Josiane.
Bactérias Promotoras do Crescimento de Plantas : Estratégias de colonização e para a mitigação de estresses abióticos em milho / Josiane Fukami. - Londrina, 2018.
114 f.

Orientador: Mariangela Hungria.
Tese (Doutorado em Biotecnologia) - Universidade Estadual de Londrina, Centro de Ciências Exatas, , 2018.
Inclui bibliografia.

1. *Quorum sensing* - Tese. 2. Estresse oxidativo - Tese. 3. Genes de defesa de plantas - Tese. 4. Estresse salino - Tese. I. Hungria, Mariangela. II. Universidade Estadual de Londrina. Centro de Ciências Exatas. . III. Título.

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Londrina, 20 de fevereiro de 2018.

AGRADECIMENTOS

Agradeço à Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) pela concessão da bolsa de doutorado e doutorado sanduíche, tornando possível a realização dos trabalhos.

À pós-graduação em Biotecnologia da Universidade Estadual de Londrina (UEL), Empresa Brasileira de Pesquisa Agropecuária (Embrapa Soja) e Universidad de Sevilla, Espanha, pelo conhecimento e infraestrutura cedidos.

Agradeço também à minha orientadora Mariangela que nessa longa jornada de vida acadêmica, proporcionou-me tantas oportunidades de conhecimento, e constante aprendizado. Por entender as necessidades de seus alunos, sabendo reconhecer o potencial de cada um.

Ao professor Javier e Manolo, da Universidade de Sevilla, Espanha, pelo aporte e orientação na condução dos ensaios realizados. E, também, a Isamar, Clara, Pablo, Charo, Ramón e todos que me deram suporte e me ajudaram na minha estadia alí. Além das amizades que ali fiz e guardo na lembrança dos bons momentos, Jadson, Louise, Joana, Graci,

Roberto, Valesca. A todos os amigos que fiz no Laboratório de Biotecnologia, que de alguma forma tornaram minha passagem ali mais alegre e feliz, seja no aprendizado científico, seja como de vida.

E, por fim, e não menos importante, minha família; por todo o aprendizado e amor, e que me fazem levantar todos os dias e agradecer à Deus, pela vida, pela família e pelo amor Gustavo. E, em especial, à minha vozinha Sonoko, que graças à Deus tive a oportunidade de conviver tantos anos na sua presença e companhia.

“ Deus não escolhe os capacitados, mas capacita os escolhidos. Fazer ou não fazer algo, só depende de nossa vontade e perseverança.”

(Albert Eisten)

FUKAMI, Josiane. **Bactérias promotoras do crescimento de plantas:** estratégias de colonização e para mitigação de estresses abióticos em milho. 2018. 114 f. Tese (Doutorado em Biotecnologia) – Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

Os inoculantes à base de *Azospirillum brasilense* estirpes Ab-V5 e Ab-V6, conhecidos pelo seu efeito benéfico às plantas, principalmente em gramíneas e, mais recentemente, pela coinoculação com os rizóbios em leguminosas, vêm sendo utilizados de modo exponencial no Brasil. Este trabalho teve por objetivo estudar essas estirpes de *Azospirillum*, a fim de elucidar os mecanismos de *quorum sensing* (QS) na colonização de milho (*Zea mays* L.) (Artigo A); estratégias de inoculação para mitigar os efeitos dos agrotóxicos utilizados no tratamento de sementes, com ênfase no estudo da indução de genes relacionados ao estresse oxidativo e defesa de plantas (Artigo B); avaliar os efeitos pela inoculação e coinoculação com *Rhizobium tropici* em plantas de milho, com o intuito de atenuar os efeitos do estresse salino (Artigo C); e investigar a indução de mecanismos de defesa no milho por diferentes métodos de inoculação ou em condições de estresse salino coinoculados com *Rhizobium tropici* (Artigo D). No que concerne ao Artigo A, foi evidenciado que as estirpes estudadas apresentam comportamentos distintos referentes ao sistema QS, uma vez que a estirpe Ab-V5 responde à adição externa de moléculas de N-acil homoserinas lactonas (AHL), enquanto que a estirpe Ab-V6 não apresentou qualquer resposta. Esses resultados foram comprovados quando as estirpes foram inoculadas em plantas de milho, empregando-se a estratégia de *quorum quenching* (QQ). No artigo B, foram relatados os benefícios pela inoculação com *Azospirillum*, bem como de seus metabólitos, na promoção de crescimento de plantas de milho, independentemente da estratégia de inoculação empregada, podendo ser aplicada pelo método tradicional – via semente – ou, por pulverização foliar, ainda que, aparentemente, a sobrevivência das bactérias na folha seja baixa. Ademais, foi evidenciado que o uso dessas bactérias pode conferir maior proteção às plantas, em consequência da indução de genes relacionados à defesa vegetal. No artigo C foram demonstrados efeitos positivos da inoculação com *A. brasilense* e da coinoculação com *R. tropici* em milho. Deve ser dada ênfase aos resultados obtidos com a estirpe Ab-V6 de *A. brasilense*, isolada ou coinoculada com *R. tropici* CIAT 899, identificadas como promissoras para mitigar o efeito de estresse salino em milho. No artigo D, observou-se que o *A. brasilense* confere proteção às plantas de milho por indução simultânea via ácido jasmônico (AJ) e ácido salicílico (AS) e, em condições de estresse salino via AS e ácido abscísico (ABA). Deste modo, o conhecimento mais aprofundado do modo de colonização, bem como de resposta das plantas frente à inoculação ou, ainda, de plantas submetidas a condições de estresse salino, pode auxiliar na definição de diferentes estratégias de uso de *A. brasilense*, de modo a ampliar a adoção e os benefícios oriundos do uso dessas bactérias.

Palavras-chave: *Quorum sensing*. Estresse oxidativo. Genes de defesa de plantas. Estresse salino. Coinoculação.

FUKAMI, Josiane. **Plant growth-promoting bacteria:** strategies of colonization and mitigation of abiotic stresses in maize. 2018. 114 p. Thesis (Doctoral Degree in Biotechnology) – Universidade Estadual de Londrina, Londrina, 2018.

ABSTRACT

Inoculants with *Azospirillum brasilense* strains Ab-V5 and Ab-V6, known for their beneficial effects in plants, especially grasses, and more recently co-inoculated with rhizobia in legume plants, have been exponentially used in Brazil. This study had the objective of evaluating these strains of *Azospirillum* in order to elucidate quorum sensing (QS) mechanisms in the colonization of maize (*Zea mays* L.) (Paper A); the strategies of inoculation to mitigate the effects of pesticides used for seed treatment, with an emphasis on the induction of genes related to oxidative stress and plant defense (Paper B); evaluate the effects of single or coinoculation with *Rhizobium tropici* in maize plants to attenuate the effects of saline stress (Paper C). and investigate the induction of defense mechanisms in maize by different methods of inoculation or in conditions of saline stress coinoculated with *Rhizobium tropici* (Paper D). Concerning the Paper A, the strains showed different behavior regarding the QS system, as strain Ab-V5 responded to the addition of exogenous molecules of N-acyl homoserine lactones (AHL), whereas the strain Ab-V6 did not show any response. These results were confirmed in inoculated maize plants, using a quorum quenching (QQ) strategy. In Paper B, benefits of inoculation of *Azospirillum* and their metabolites in the growth promotion of maize plants were observed, independently of the strategy of inoculation, either via seeds, or by leaf spray, despite an apparently low bacterial survival in the last case. These bacteria can provide beneficial protection to plants, due to the induction of genes related to plant defense. In Paper C the beneficial effects of co-inoculation of *A. brasilense* and *R. tropici* in maize were demonstrated, with emphasis on *A. brasilense* Ab-V6 single or co-inoculated with *R. tropici* CIAT 899 mitigating saline stress in maize plants. In article D, we demonstrated that *A. brasilense* confers protection to maize plants by simultaneous induction of jasmonic acid (JA) and salycilic acid (SA) pathways, and, under saline stressing conditions, by SA and abscisic acid (ABA) pathways. Thus, a better understanding of colonization mechanisms, as well as the response of plants inoculated, or in conditions of saline stress, can help in the definition of different strategies for use of *A. brasilense*, spreading the adoption and benefits resulting from these bacteria.

Keywords: *Quorun sensing*. Oxidative stress. Plant defense genes. Salinity stress. Coinoculation.

SUMÁRIO

1 INTRODUÇÃO

Consoante ao aumento da população nos países emergentes e com a expectativa de vida que cresce a cada ano no mundo, a demanda de alimentos, tanto para o consumo humano, quanto para a alimentação animal, aumenta na mesma proporção. A consequência lógica disso tudo é a necessidade de mais produção de alimentos, o que nos remete a cultivos cada vez mais intensos. Em diversas situações, os cultivos são realizados sob monocultivos e são altamente dependentes do uso extensivo de insumos agrícolas, tais como agrotóxicos, fertilizantes, corretivos, entre outros. Existe, portanto, a necessidade de se buscar práticas agrícolas mais sustentáveis, de modo a preservar os agroecossistemas, utilizando para isso práticas conservacionistas, e tendo como resultado final uma produção ecologicamente e economicamente mais sustentável.

Desde os primeiros relatos do gênero *Spirillum*, e anos mais tarde reclassificado como *Azospirillum* devido à sua capacidade de fixar nitrogênio (N) atmosférico descoberta pela Dra. Johanna Döbereiner e colaboradores na década de 1970, este é o gênero mais estudado e comumente conhecido de bactérias promotoras de crescimentos de plantas (BPCP), beneficiando às plantas mediante a produção de fitormônios vegetais, por exemplo, as auxinas. Ademais, são capazes de colonizar diversas plantas hospedeiras em condições edafoclimáticas adversas, incluindo gramíneas como a cultura do milho (*Zea mays* L.), de alta expressão econômica mundial.

No Brasil, a partir da recomendação comercial das estirpes Ab-V5 e Ab-6 de *Azospirillum brasilense* em 2009, tem-se intensificado a prática de inoculação em gramíneas. Mais recentemente, a prática de coinoculação de leguminosas com rizóbios e aumentou ainda mais o uso de *A. brasilense*, saindo de pouco mais de 400 mil doses comercializadas em 2009 para cerca de 4,5 milhões de doses comercializadas na safra 2016/17. Contudo, existe uma limitação de compatibilidade das bactérias com os produtos químicos usados no tratamento de sementes, que pode reduzir a eficiência de inoculação via sementes – método mais empregado – e, por consequência, o efeito benéfico às plantas. Neste sentido, outras estratégias de inoculação que evitem o contato direto das bactérias com os produtos químicos podem ser mais eficazes em promover a sobrevivência das mesmas e para que possam promover seus efeitos benéficos. Como estratégias alternativas, estão a aplicação de inoculantes no sulco de semeadura e a pulverização da parte aérea após a emergência das plântulas.

Estudos prévios realizados em nosso laboratório com *A. brasilense* indicam que a infecção dessas bactérias pode ocorrer via estômatos, porém, são necessárias técnicas mais refinadas para obtenção de resultados conclusivos, visando delinear a melhor estratégia de inoculação, de modo a maximizar os benefícios à planta hospedeira. Ademais, o uso de metabólitos produzidos por bactérias em plantas também poderia ser mais uma estratégia alternativa, como já foi constatado em nosso laboratório com a aplicação de metabólitos de rizóbios em plantas de milho.

Apesar dos avanços obtidos nas últimas décadas em aspectos bioquímicos e moleculares da interação planta-*Azospirillum*, e dada à complexidade do sistema *quorum sensing* (QS) de *A. brasilense* nas etapas e formas de colonização, bem como a resposta das plantas frente à inoculação com essas estirpes, os resultados ainda são inconsistentes.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Estudar os efeitos benéficos da inoculação de *Azospirillum brasilense* em plantas de milho, com ênfase nas estratégias de inoculação e na mitigação de estresses abióticos das plantas inoculadas.

2.2 OBJETIVOS ESPECÍFICOS

i) Estudar os possíveis mecanismos de *quorum sensing* (QS) de *A. brasilense* estirpes Ab-V5 e Ab-V6 envolvidos na colonização de plantas de milho;

ii) Confirmar a expressão de genes de defesa e de estresse oxidativo em plantas de milho inoculadas via sementes ou pulverização foliar com *A. brasilense* ou seus metabólitos;

iii) Verificar os efeitos da coinoculação de *A. brasilense* e *Rhizobium tropici* na mitigação do estresse salino sofrido pela planta de milho;

iv) Investigar os efeitos do *Azospirillum brasilense* estirpes Ab-V5 e Ab-V6 na indução de mecanismos de resistência sistêmica adquirida (RSA) e de resistência sistêmica induzida (RSI) em plantas de milho.

3 REVISÃO DA LITERATURA

3.1 CULTURA DO MILHO

Reconhecida mundialmente, a cultura do milho (*Zea mays* L.) possui importante papel na agricultura, desde seu uso na alimentação animal, até na indústria de alta tecnologia, representando a sua versatilidade. O milho é uma cultura monocotiledônea, pertencente à família Poaceae, anual com sistema radicular fasciculado, caule ereto (BENSON; PEARCE, 1987), monoica com polinização cruzada, ou seja, possui as estruturas sexuais na mesma planta em inflorescências diferentes, as masculinas situadas no pendão e as femininas nas espigas axilares.

No cenário nacional, o milho é a segunda cultura de maior importância econômica no agronegócio brasileiro. Somente na safra 2015/2016 – primeira e segunda safras – a produção no país correspondeu a 67 milhões de toneladas (CONAB, 2017a). Já na evolução mundial de produção, o milho é a cultura de maior expressão – 968 milhões de toneladas em 2015/2016 (USDA, 2017). O Brasil destaca-se como o terceiro maior produtor mundial, superado apenas pelos Estados Unidos, com 346 milhões de toneladas e pela China, com 224 milhões de toneladas (USDA, 2017).

O milho é umas das culturas mais versáteis, haja vista sua alta capacidade de adaptação, consequência da alta variabilidade genética para essa adaptação às mais variadas condições agroclimáticas (MAHESH et al., 2015). Nos últimos anos, o Brasil vem passando por importantes mudanças tecnológicas na cultura, o que resultou em aumentos significativos de produtividade e da produção (MACHADO et al., 2013), sem aumentar significativamente a área cultivada. Desse modo, enquanto a área cultivada aumentou apenas 15,7% nos últimos 20 anos, a produção aumentou 105,4% e o ganho de produtividade foi de 78% (CONAB, 2017b), que passou de 2.356 kg ha^{-1} na safra agrícola 1995/1996, alcançando patamares de 4.181 kg ha^{-1} na safra 2015/2016, chegando a 7.330 kg ha^{-1} no estado de Santa Catarina (CONAB, 2017b).

Esses fatores estão atrelados ao uso de técnicas adequadas de cultivo, sementes mais produtivas. No entanto, de maneira geral, no Brasil, o milho é cultivado em solos de baixa fertilidade, necessitando, de altas doses de fertilizantes nitrogenados (MARTINS et al., 2017). Assim, o uso de técnicas biológicas a fim de melhorar a eficiência do uso dos fertilizantes nitrogenados pode representar uma alternativa sustentável para auxiliar no cultivo desse cereal.

3.2 BACTÉRIAS DIAZOTRÓFICAS

As bactérias diazotróficas são capazes de fixar o N atmosférico (N_2), tornando-o disponível para as plantas por meio da conversão enzimática de N_2 em íons amônio (NH_4^+), processo comumente conhecido como Fixação Biológica de Nitrogênio (FBN). Essas bactérias são classificadas em três grupos: *i)* bactérias simbióticas, com a formação de estruturas típicas, os nódulos; *ii)* bactérias associativas, que contribuem para o crescimento dos vegetais, porém, sem a formação de estruturas diferenciadas, não estabelecendo, assim, uma simbiose e; *iii)* bactérias de vida livre, que são capazes de fixar N_2 para o seu próprio uso (EVANS; BURRIS, 1992; MARIN et al., 1999).

Indubitavelmente, o mais eficiente dentre os três grupos é o das bactérias simbióticas, também denominadas rizóbios, um termo geral usado para descrever uma variedade de gêneros de bactérias que estabelecem uma estreita relação com as plantas, provocando nas raízes das leguminosas o desenvolvimento de estruturas diferenciadas, os nódulos. Os rizóbios mais estudados e utilizados na agricultura pertencem aos gêneros *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* e *Bradyrhizobium* (WEYENS et al., 2010). Um dos exemplos clássicos é o de bactérias do gênero *Bradyrhizobium*, que formam nódulos nas raízes da soja (*Glycine max* (L.) Merr.), conseguindo suprir a planta com N suficiente para sustentar altas produtividades (HUNGRIA; CAMPO; MENDES, 2001).

Para que ocorra a FBN, é necessário o estabelecimento da simbiose entre planta-bactéria, com a coordenação na expressão de genes da planta hospedeira e da bactéria, que são regulados pela troca mútua de sinais moleculares (VIEIRA et al., 2010). As raízes jovens são de grande importância para a simbiose, sendo chave para a infectividade dos rizóbios na maioria das leguminosas, representando o sítio de maior aderência das bactérias nas raízes, notadamente nos pelos radiculares, possibilitando a penetração do rizóbio na raiz e a formação dos nódulos (HUNGRIA, 1994).

O gênero *Azospirillum* compreende predominantemente bactérias colonizadoras da rizosfera, enquanto que somente algumas estirpes são aptas a colonizar o interior de raízes (STEENHOUDT; VANDERLEYDEN, 2000), ou seja, logo após a inoculação, as bactérias aderem-se às raízes das plantas, proliferam-se e, em alguns casos, posteriormente, invadem e colonizam o tecido interno das raízes (PEREG; LUZ; BASHAN, 2016). Esse é o caso, por exemplo, de *A. brasilense* estirpe Sp245, que coloniza o interior das raízes, ao passo que *A. brasilense* estirpe Sp7 coloniza apenas as superfícies das raízes

(ASSMUS et al., 1995; SCHLOTER; HARTMANN, 1998; ROTHBALLER; SCHMID; HARTMANN, 2003).

3.2.1 Gênero *Rhizobium*

Os rizóbios são abundantemente encontrados em solos tropicais, e conhecidos principalmente por sua capacidade de estabelecer simbiose com diversas leguminosas, sendo objeto de estudos básicos e práticos há mais de 120 anos (ORMEÑO-ORRILLO et al., 2012). A cultura de maior expressão econômica no Brasil que se beneficia da FBN em simbiose com o gênero *Rhizobium* é a do feijoeiro (*Phaseolus vulgaris* L.). Essa leguminosa é bastante promíscua, sendo capaz de estabelecer simbiose com diversas espécies rizobianas, como *R. etli, R. leucaenae, R. freirei* e *R. tropici*. Cabe comentar que o gênero *Rhizobium* hoje conta com quase 100 espécies (BACTERIO, 2017).

Os rizóbios pertencem à subclasse α das Proteobactérias, são Gram-negativas (HUMANN; KAHN, 2015), com 0,5-0,9 μm de diâmetro por 1,2 – 3,0 μm de comprimento, em forma de bastonetes móveis. Contêm grânulos de poly-β-hidroxibutirato e tornam-se pleiomórficas (bacteroides) em simbiose (DATTA et al., 2014). São considerados de crescimento rápido, visível em 2-3 dias de incubação, acidificando o meio de cultivo, com ótimo de temperatura variando entre 25 a 42 ºC e pH na faixa de 4 a 9, dependendo da espécie (DATTA et al., 2014).

Atualmente, no Brasil, as estirpes de *R. tropici* CIAT 899 (=SEMIA 4077) e H 12 (=SEMIA 4088) e PRF 81 (=SEMIA 4080) (essa última reclassificada como *R. freirei*, DALL'AGNOL et al., 2013), são autorizadas pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA) para o uso em inoculantes comerciais para a cultura do feijoeiro. Várias outras propriedades indicam que bactérias da espécie *R. tropici* exibem características de alta eficiência da FBN, são competitivas em solos ácidos em relação a rizóbios indígenas, são geneticamente estáveis, ou seja, não perdem facilmente os genes relacionados à FBN, e apresentam tolerância intrínseca a diversos estresses abióticos, como altas temperaturas, acidez, estresses salino e hídrico (ORMEÑO-ORRILLO et al., 2012; 2016; GOMES; ORMEÑO-ORRILLO; HUNGRIA, 2015; GUASCH-VIDAL et al., 2013).

Estudos realizados com o feijoeiro inoculado com as estirpes CIAT 899, H 12 e PRF 81, em condições não limitantes para a FBN, indicaram que as plantas produziram tanto quanto as plantas que receberam 60 kg ha^{-1}de fertilizantes nitrogenados (GOMES;

ORMEÑO-ORRILLO; HUNGRIA, 2015), sendo capazes de propiciar rendimentos superiores a 3.000 kg/ha (HUNGRIA et al., 2000; PELEGRIN et al., 2009). No entanto, para a recomendação adequada do uso de inoculantes no feijoeiro, a fim de obter produtividades desejadas, vale ressaltar que a substituição total ou parcial de fertilizantes nitrogenados vai depender das condições edafoclimáticas da área cultivada, bem como das práticas de manejo utilizadas.

A coinoculação com rizóbios e BPCPs têm sido uma prática para aumentar a sustentabilidade dos sistemas de produção agrícola, especialmente na cultura da soja, aumentando a eficiência da FBN pelos rizóbios (HUNGRIA; NOGUEIRA; ARAUJO, 2013; PÉREZ-MONTAÑO et al., 2014; CHIBEBA et al., 2015; CEREZINI et al., 2016). Dentre os benefícios relacionados ao *Azospirillum* em coinoculação, estão o estímulo ao crescimento de raízes laterais e de pelos radiculares (JUGE et al., 2012), aumentando a possibilidade de efetiva infecção e formação de nódulos precocemente (CHIBEBA et al., 2015).

No Brasil, foram constatados efeitos positivos da coinoculação com *A. brasilense* e *Bradyrhizobium* na cultura da soja, podendo resultar em incrementos adicionais da ordem de 8% na produtividade de grãos, em comparação com o tratamento inoculado exclusivamente com *B. japonicum* (HUNGRIA; NOGUEIRA; ARAUJO, 2013). Alguns estudos demonstram o melhor desenvolvimento da soja e do milho, além de acelerar a germinação de sementes e a formação dos nódulos em soja; os autores atribuíram os benefícios à produção de fitormônios por *Azospirillum*, como o AIA, ácido giberélico (GA_3) e zeatina (CASSÁN et al., 2009).

Há relatos positivos também de coinoculação de *R. tropici* e *A. brasilense* na cultura do feijoeiro, em que foram observados efeitos sinergísticos na nodulação, crescimento de plantas, rendimento e FBN (HUNGRIA; NOGUEIRA; ARAUJO, 2013). Além de sua capacidade de fixar N_2 atmosférico, algumas estirpes de *Rhizobium* também são capazes de sintetizar fitormônios (YANNI; DAZZO, 2015; IMADA et al., 2017), uma propriedade que está ampliando seu uso como BPCP também em não-leguminosas (GARCÍA-FRAILE et al., 2012; YANNI; DAZZO, 2015). Como exemplo, tem-se o estudo de Askary e colaboradores (2009), com relatos de benefícios pela coinoculação com *Rhizobium* e *A. brasilense* em trigo.

3.2.2 Gênero *Azospirillum*

As espécies do gênero *Azospirillum* são amplamente distribuídas na natureza, sendo encontradas em solos tropicais, subtropicais e regiões temperadas de todo o globo (SANT'ANNA et al., 2011). Atualmente existem 17 espécies descritas: *A. agricola*, *A. brasilense*, *A. canadense*, *A. doebereinerae*, *A. fermentarium*, *A. formosense*, *A. halopraeferens*, *A. humicireducens*, *A. largimobile*, *A. lipoferum*, *A. melinis*, *A. oryzae*, *A. picis*, *A. rugosum*, *A. soli, A. thiophilum* e *A. zeae* (BACTERIO, 2017). *A. agricola* foi a última espécie descrita (LIN et al., 2016), enquanto que as espécies *A. amazonese* e *A. irakense* foram reclassificadas como *Nitrospirillum amazonense* e *Niveispirillum irakense*, respectivamente (LIN et al., 2015). Dentre as espécies descritas até o momento, *A. brasilense* e *A. lipoferum* são as com maior número de estudos (BALDANI et al., 2005; FIBACH-PALDI; BURDMAN; OKON, 2012), em termos de fisiologia e genética (REIS; PEDRAZA; TEIXEIRA, 2010).

Bactérias do gênero *Azospirillum* pertencem à subclasse α das Proteobactérias, são Gram-negativas (LIN et al., 2012), em forma de bastonetes, ligeiramente curvadas, com 1,0 μm de diâmetro por 2,0 a 3,0 μm de comprimento, que sob algumas condições podem formar espirais (JOFRÉ et al., 2008) e com alta mobilidade (ALEXANDRE, 2015). A temperatura ótima de crescimento desses microrganismos varia entre 28 e 41 ºC, dependendo da espécie (ECKERT et al., 2001). São aeróbicos típicos quando supridos com fonte de N combinado, como amônio, nitrato, nitrito, aminoácidos, sendo essas as fontes de N preferenciais (STEENHOUDT; VANDERLEYDEN, 2000) e microaerofílicos quando crescem dependentes da fixação de N_2 (WEBER; BALDANI; DOBEREINER, 2000).

Os *Azospirillum* têm sido amplamente estudados devido à sua capacidade de promoção de crescimento às culturas de grande importância agrícola, tais como milho, trigo (*Triticum aestivum* L.) e arroz (*Oryza sativa* L.), entre outras culturas e, mais recentemente, também estudados em pastagens como *Brachiaria* (=*Uruchloa*) (CASSÁN; OKON; CREUS, 2015; HUNGRIA; NOGUEIRA; ARAUJO, 2016; PEREG; LUZ; BASHAN, 2016). Do mesmo modo, estudos extensos com diversas estirpes de *A. brasilense e A. lipoferum* foram conduzidos no Brasil, inclusive resultando na recomendação das primeiras estirpes de *A. brasilense* para produção de inoculantes comerciais (HUNGRIA et al., 2010). Atualmente, as estirpes Ab-V5 e Ab-V6 são largamente utilizadas no país (HUNGRIA; NOGUEIRA; ARAUJO, 2013).

Dentre os principais benefícios relatados para esse gênero está a biossíntese de auxinas, fitormônio relacionado ao crescimento de plantas. No entanto, estudos relatam a produção de óxido nítrico (NO) por *A. brasilense*, atuando como molécula sinalizadora na cascata hormonal, levando à formação de raízes laterais (PAGNUSSAT et al., 2002; CORREA-ARAGUNDE et al., 2006). Além disso, outros estudos atribuem os efeitos positivos no trigo à capacidade do *A. brasilense* de solubilizar fósforo (P) inorgânico, tornando-o disponível às plantas e promovendo maior rendimento de grãos, com 19% de incremento em relação às plantas não inoculadas (TURAN et al., 2012).

Indiscutivelmente, o gênero *Azospirillum* promove o crescimento de plantas. No entanto, por conta de diversos mecanismos já estudados até o momento, Bashan e de-Bashan (2010), propuseram a "teoria de múltiplos mecanismos", na qual essas bactérias poderiam atuar em um padrão cumulativo ou sequencial de efeitos, isto é, pequenos efeitos de mecanismos ocorrendo simultânea ou consecutivamente, desencadeando um efeito final maior na planta.

Neste contexto, os mecanismos de promoção do crescimento de maior magnitude propostos para o *Azospirillum* são: a melhoria na captação de água e nutrientes do solo (ARDAKANI et al., 2011); a produção e liberação de fitormônios e outras moléculas, tais como, auxinas (SPAEPEN; VANDERLEYDEN, 2015), citocininas (TIEN; GASKINS; HUBBELL, 1979), giberelinas (BOTTINI et al., 1989), ácido abscísico (COHEN; BOTTINI; PICCOLI, 2008), etileno (STRZELCZYK; KAMPERT; LI, 1994) e ácido salicílico (SAHOO et al., 2014); FBN (MARQUES et al., 2017); solubilização de fosfato (RODRIGUEZ et al., 2004) e mitigação de estresses abióticos como salinidade e seca (CREUS; SUELDO; BARASSI, 2004; RODRÍGUEZ-SALAZAR et al., 2009), fitorremediação de solos contaminados (TUGAROVA et al., 2014), proteção contra alta intensidade luminosa (BASHAN et al., 2006), etc.

As auxinas representam uma classe importante de fitormônios envolvidos na coordenação do crescimento e desenvolvimento das plantas (ABEL; THEOLOGIS, 2010). A síntese de auxinas não se restringe somente às plantas, haja vista a diversidade de espécies de bactérias e fungos conhecidos por produzir auxinas, sendo o ácido indol-3-acético (AIA) a molécula mais documentada (SPAEPEN; VANDERLEYDEN; REMANS, 2007). As BPCPs possuem diferentes rotas para a biossíntese de AIA; atualmente, para o gênero *Azospirillum* são propostas quatro rotas diferentes, sendo três dependentes de triptofano (Trp) e uma rota independente de Trp. Um exemplo amplamente estudado é o da síntese em *A. brasilense* Sp245 com a via do ácido indol-3-pirúvico (IPA) [Triptofano (Trp) → IPA → indol-3-

acetaldeído (IAId) → AIA)], o mais abundante na presença do precursor Trp (COSTACURTA; KEIJERS; VANDERLEYDEN, 1994).

Muitos outros compostos indólicos, intermediários das vias de biossíntese AIA, têm sido identificados em sobrenadantes de *Azospirillum* sp., por exemplo, o ácido indol-3-etanólico e o ácido indol-3-lático, os quais, embora suas funções fisiológicas permaneçam desconhecidas, podem ser compostos de armazenamento intermediários das vias de biossíntese do AIA (CASSÁN; VANDERLEYDEN; SPAEPEN, 2014). São conhecidos poucos exemplos de biossíntese de AIA pela via independente de Trp, sendo o *A. brasilense* SpM7918 o mais estudado, no qual mais de 90% do AIA produzido ocorre pela via independente de Trp e os restantes 10% são sintetizados utilizando o Trp como precursor (PRINSEN et al., 1993; GOSWAMI et al., 2016). No entanto, o caminho exato e as enzimas utilizadas para a síntese de AIA por esta rota ainda não são conhecidas (SPAEPEN; VANDERLEYDEN; REMANS, 2007; JHA; SARAF, 2015).

Outros mecanismos de promoção de crescimento propostos para o *Azospirillum* são: produção de óxido nítrico (CASSÁN; VANDERLEYDEN; SPAEPEN, 2014); controle biológico de patógenos de plantas, de fungos como *Colletotrichum acutatum* no morangueiro (*Fragaria ananasa*) (TORTORA; DÍAZ-RICCI; PEDRAZA, 2011), como contra bactérias, por exemplo, *Xanthomonas campestris* pv. *vesicatoria* no tomateiro (*Solanum lycopersicum* L.) (ROMERO et al., 2003) e a nematoides, como *Meloidogyne incognita* (KHAN; KOUNSAR; HAMID, 2002). Tais bactérias também produzem substâncias que prejudicam ou inibem outros microrganismos e parasitas, mas não as plantas. Como exemplos de mecanismos de inibição, têm-se a produção de sideróforos (TORTORA; DÍAZ-RICCI; PEDRAZA, 2011) com a limitação da disponibilidade de ferro para os fitopatógenos, ou através de alterações no metabolismo da planta hospedeira, aumentando a sua resistência contra a infecção de patógenos, em um processo denominado de indução de resistência sistêmica (IRS) (VAN LOON; BAKKER, 2005).

Contudo, para que esses múltiplos mecanismos ocorram, são necessários alguns requisitos, cuja resposta depende da planta hospedeira, das estirpes de *Azospirillum* e, ainda, das condições ambientais para o estabelecimento efetivo da interação planta-bactéria.

3.3 INTERAÇÃO PLANTA-BACTÉRIA

A aderência das bactérias às raízes representa um passo necessário para o estabelecimento de uma interação eficiente entre planta-bactéria, ocorrendo inicialmente, por meio de compostos produzidos pelas plantas, que em seguida são transportados para além da membrana celular e secretados na rizosfera circundante (BAIS et al., 2006), principal nicho colonizado pelo *Azospirillum.* A alta mobilidade desse gênero é mediada por um flagelo polar sob a forma de *swimming* (permite a bactéria se locomover em meio aquoso) e diversos flagelos laterais sob a forma *swarmming* (permite a bactéria se locomover em superficie) (ALEXANDRE, 2015). E, ainda, aliada à quimiotaxia positiva para certos ácidos orgânicos, açúcares e aminoácidos constituintes da rizosfera (BABALOLA, 2010) faz com que essas bactérias possam aderir à superfície das raízes (CROES et al., 1993).

Após essa etapa, inicia-se o processo de colonização pela formação de biofilme, que consiste na formação de uma matriz de exopolissacarídeos (EPS) produzida pelos próprios microrganismos aderidos às raízes, cuja função é garantir sua proteção dentro dessa matriz contra fatores ambientais externos. Os EPSs são formados principalmente por polissacarídeos, proteínas, ácidos nucleicos e lipídeos (FLEMMING; WINGENDER, 2010). Do mesmo modo, a formação de flóculos ou agregados, ambos relacionados à produção de EPS em *Azospirillum*, também é considerada indispensável para a firme aderência na superfície radicular, sugerindo que o efeito sobre as plantas não seja apenas pela aderência às raízes, mas também pela presença dessas estirpes na rizosfera (PEREG; LUZ; BASHAN, 2016).

Estudos conduzidos em nosso laboratório com *A. brasilense* indicam que a infecção também pode ocorrer via estômatos (FUKAMI et al., 2016), porém, são necessárias técnicas mais refinadas para obtenção de resultados conclusivos. Avaliar a possibilidade de infecção via foliar é importante porque existe uma limitação de compatibilidade das bactérias com os produtos químicos usados no tratamento de sementes que reduz a eficiência de inoculação via semente – método mais empregado – e, por consequência, o efeito benéfico às plantas. Desse modo, outras estratégias de inoculação que evitem o contato direto das bactérias com os produtos químicos podem ser mais eficazes à sobrevivência das bactérias inoculadas. Como estratégias alternativas, está a aplicação de inoculante via pulverização da parte aérea após a emergência das plântulas.

Os primeiros relatos de bactérias que colonizavam as plantas via estômatos foram constatados com as fitopatogênicas, como por exemplo, em *Pseudomonas syringae* pv.

anenae em arroz (*Oryza sativa* L.) (MATSUDA; SATO, 1983) e *Xanthomonas campestris* em maçã (*Pyrus malus* L.) (MAAS et al., 1985). Inicialmente, as bactérias próximas ou sobre os estômatos, rapidamente se multiplicam e infectam a câmara subestomática e, em seguida, os espaços intercelulares das células do mesofilo (KAKU, 2004). Existem outras portas de entradas desses patógenos, mas os estômatos dominam em número a parte aérea da planta e, portanto, representam uma das mais importantes possíveis vias de entrada do patógeno (MELOTTO; WILLIAM; HE, 2008).

A densidade de estômatos na superfície foliar varia entre as espécies de plantas, podendo haver até 300 estômatos mm^{-2}, que podem ocupar até 2% da superfície foliar (LAKE et al., 2001). No milho, o número de estômatos em condições ideais está em torno de 60 estômatos mm^{-2} na face adaxial, e 80 estômatos mm^{-2} na face abaxial (ZHENG et al., 2013), sendo considerada anfihipoestomática, ou seja, com presença de mais estômatos da face abaxial (FERRI, 1984). Além das bactérias fitopatogênicas, a infecção dos estômatos por bactérias endofíticas também foi relatada, como por exemplo, *Pantoea aglomerans* em associação com trigo (RUPPEL; WACHE, 1990) e *Bacillu*s sp. e *Corynebacterium* sp., em milho (SOUZA et al., 2004). No entanto, esses estudos não foram específicos para *Azospirillum*.

3.3.1 Sistema *quorum-sensing*

Na rizosfera, as bactérias usam um sistema de comunicação denominado *quorum-sensing* (QS) para coordenar e sincronizar vários comportamentos em diferentes ambientes, incluindo interações bactéria-bactéria e planta-bactéria, desempenhando um papel fundamental no estabelecimento de interações benéficas ou na patogênese (MARK et al., 2005; ZÚÑIGA et al., 2013; GRANDCLÉMENT et al., 2015;). O QS é um sistema de comunicação célula-célula, que controla a expressão de genes mediado por pequenas moléculas – autoindutores – capazes de induzir respostas dependentes da densidade populacional (FUQUA; WINANS; GREENBERG, 1994; BOYER; WISNIEWSKI-DYÉ, 2009).

As moléculas de sinalização mais estudas são as N-acil homoserinas lactonas (AHLs), diferindo em comprimento na porção de cadeia acil e na substituição na posição C-3, que podem ser parte de um grupo metil ou carregar um grupo oxi ou hidroxila (SUBRAMONI; VENTURI, 2009). As AHLs são sintetizadas pelas proteínas do tipo LuxI,

acumulando dentro e fora da membrana celular de forma equilibrada (HUDAIBERDIEV et al., 2015). À medida que a densidade populacional vai aumentando, a síntese de AHLs aumenta na mesma proporção até alcançar uma concentração limite e, então, essas moléculas se ligam ao receptor de sinais – proteínas reguladoras LuxR – que por sua vez irão regular a transcrição tanto do gene *luxI*, quanto de genes alvos e, assim, ativá-los (HUDAIBERDIEV et al., 2015). Os processos incluem: a formação de biofilme, a mobilidade, a produção de EPS, a síntese de fatores de virulência e de compostos antimicrobianos, entre outros (BOYER; WISNIEWSKI-DYÉ, 2009), resultando em fenótipos que são essenciais para a competição e a interação com o hospedeiro.

Para que uma bactéria tenha um sistema QS completo é indispensável ao menos uma proteína do tipo LuxI e uma LuxR. No entanto, essa proporção LuxI/LuxR nem sempre é equivalente. Uma grande quantidade de genomas bacterianos, além de possuírem o sistema QS completo, também possuem proteínas LuxR "extras" (CASE; LABBATE; KJELLEBERG, 2008). Esse é o caso, por exemplo, do patógeno *Pseudomonas aeruginosa*, que possui dois sistemas QS (LasI/LasR e RhlI/RhlR), além de dois homólogos LuxR "extras" (QscR e VqsR) (LEE; ZHANG, 2014), bem como de *Rhizobium leguminosarum* bv. viciae, que possui três sistemas QS (CinI/CinR, RaiI/RaiR e TraI/TraR), com um homólogo LuxR "extra" (BisR) e mais três homólogos LuxR não descritos (CASE; LABBATE; KJELLEBERG, 2008).

Embora os genes *luxR* geralmente sejam encontrados ao lado ou próximos do gene *luxI* no cromossomo bacteriano, muitos genes *luxR* têm sido descritos por não possuírem genes *luxI* nas proximidades de seu cromossomo ou, ainda, não posuírem qualquer gene homólogo *luxI*, sendo conhecidos como genes *luxR* 'solo' (PATEL et al., 2013). Esses genes 'solo' podem responder a sinais internos de AHL produzidos por um *luxI* não adjacente no cromossomo ou responder a sinais externos. No primeiro caso, pode-se citar o exemplo mencionado acima de *P. aeruginosa* com a proteína QscR (homólogo LuxR). No segundo, estão as bactérias patogênicas *Salmonela typhimurium* e *Escherichia coli*, que não possuem o sistema completo de QS, mas possuem um gene *sdiA* (homólogo *luxR*) que é capaz de responder a AHLs exógenas (AHMER et al., 1998; MICHAEL et al., 2001; AHMER, 2004; YAO et al., 2006), regulando, no caso de *S. typhimurium*, a virulência e, em *E. coli*, a formação de biofilme.

O sistema completo de QS mediado pelas AHLs parece não ser comumente encontrado no gênero *Azospirillum*, haja vista um estudo realizado por Vial e colaboradores (2006), demonstrou que, dentre 40 estirpes de *Azospirillum lipoferum*, apenas quatro

produziram moléculas de AHLs. Embora o método avaliado nesse estudo, com bactérias bioindicadoras, seja rápido, vale salientar que ele detecta apenas concentrações e AHLs específicas. Já com o sequenciamento do genoma é possível a busca de genes do sistema QS por todo seu genoma, possibilitando um resultado mais fidedigno.

A partir do sequenciamento e a publicação de estudos envolvendo quatro diferentes espécies de *Azospirillum* nos últimos anos, *A. lipoferum* 4B, *Azospirillum* sp. B510, *A. amazonense* Y2, e *A. brasilense* estirpes Sp245, CBG497 e Az39 (KANEKO et al., 2010; SANT'ANNA et al., 2011; WISNIEWSKI-DYÉ et al., 2011; RIVERA et al., 2014), estudos realizados por Wisniewski-Dyé e Vial (2015) puderam verificar que as três estirpes de *A. brasilense* e a estirpe *A. lipoferum* 4B não possuem o gene *luxI* em seus genomas, mas possuem o gene *luxR*, o que possibilita a essas bactérias responderem a sinais externos, sejam eles relacionados à comunicação entre comunidades distintas de microrganismos, ou na interação planta-bactéria.

Sabe-se também que as plantas produzem compostos de baixa massa molecular que imitam os sinais QS capazes de atuar como ativadores ou repressores dos sistemas QS de bactérias (TEPLITSKI; ROBINSON; BAUER, 2000; GAO et al., 2003; BAUER; MATHESIUS, 2004; DEGRASSI et al. 2007; PATEL et al., 2013). Esse é o caso da expressão de genes associados à virulência, tendo como consequência a infecção de fitopatógenos em plantas. O patógeno vascular *Xanthomonas oryzae* pv. *oryzae* de trigo sintetiza a proteína OryR (homólogo LuxR), que por sua vez responde aos sinais das plantas e ativa a expressão dos genes de mobilidade (FERLUGA et al., 2007; FERLUGA; VENTURI, 2009; GONZÁLEZ; VENTURI, 2013). Já *Xanthomonas axonopodis* pv. *glycines* – patógeno da cultura da soja [*Glycine max* (L.) Merr.] – causador da pústula bacteriana (CHATNAPARAT et al., 2012), sintetiza a proteína XagR (homólogo LuxR).

Em contrapartida, compostos produzidos pelas plantas também podem interagir com as bactérias, beneficiando as plantas. Como exemplo, *Pseudomonas fluorescens* sintetiza a proteína PsoR (homólogo LuxR), que responde a compostos produzidos por plantas de trigo, induzindo a expressão de genes na transcrição de compostos relacionados ao controle biológico, incluindo um gene de quitinase, genes envolvidos no metabolismo de ferro e na sínstese de compostos anti-fúngicos (SUBRAMONI et al., 2011). Outro exemplo é o da proteína NesR em *Sinorhizobium meliloti*, associada à adaptação a condições de estresse, atuando também na competição para a nodulação de plantas de alfafa (*Medicago sativa*) (PATANKAR; GONZÁLEZ, 2009).

Boyer e colaboradores (2008), em um estudo realizado com duas estirpes de *A. lipoferum*, B518 e TVV3, introduziram um gene que expressa uma enzima lactonase AttM de *Agrobacterium tumefaciens*, atuando na degradação das moléculas de AHLs sintetizadas pelas estirpes de *A. lipoferum*. Na estirpe B518, a degradação de AHL inibiu a atividade da pectinase, aumentou a síntese de sideróforo e reduziu a produção de AIA. Já na estirpe TVV3 nenhum dos fenótipos avaliados apresentou qualquer alteração pela inibição do QS mediado por AHL. Esses autores concluíram que o sistema QS de *Azospirillum* é específico de cada estirpe e regula as funções relacionadas à rizosfera e adaptação às raízes das plantas.

O mecanismo utilizado no estudo de Boyer e colaboradores (2008) é conhecido como *quorum-quenching* (QQ) e refere-se a todo processo inibidor ao sistema QS (DONG et al., 2001; GRANDCLÉMENT et al., 2015). O QQ é considerado um mecanismo natural desenvolvido por bactérias capaz de degradar suas próprias móleculas de AHL ou, ainda, usado como um mecanismo de competição entre distintos microrganismos (GRANDCLÉMENT et al., 2015). A enzima AHL-lactonase AiiA foi a primeira identificada em espécies de *Bacillus* para atenuar a virulência de *Erwinia carotovora* em solanáceas (DONG, 2000; LI; DU; CHEN, 2008). Já a bactéria *A. tumefaciens*, que causa a doença conhecida como galha da coroa, sintetiza a enzima lactonase AttM, que degrada sua própria molécula AHL autoindutora (ZHANG; WANG; ZHANG, 2002). A estratégia do sistema QQ pode ser empregada em estudos que envolvam o entendimento do sistema QS na interação planta-bactéria.

3.3.2 Mecanismos de defesa das plantas

3.3.2.1 Estresse oxidativo

As plantas sintetizam uma variedade de metabólitos secundários em resposta aos estresses bióticos e abióticos, que estão envolvidos em vários processos fisiológicos, cujas principais funções consistem no aumento da tolerância ao estresse e na defesa contra patógenos (SUDHA; RAVISHANKAR, 2002). Os fatores ambientais adversos ocasionam danos oxidativos nas plantas em decorrência do aumento de espécies reativas de oxigênio (ROS), isto é, desencadeiam um dos mecanismos iniciais da resposta de defesa da planta contra o ataque de patógenos (FINKEL, 2000; LEÓN; MONTESANO, 2013).

As moléculas de ROS abrangem os radicais livres resultantes do metabolismo do oxigênio, como radicais superóxido (O_2^-), radicais de hidroxila (OH^-),

peróxido de hidrogênio (H_2O_2) e oxigênio singleto ($^1O_2$) (BOWLER; MONTAGU; INZÉ, 1992; GILL; TUTEJA, 2010). O acúmulo de ROS pode danificar diretamente os componentes celulares, causando danos às membranas pela peroxidação lipídica (SMIRNOFF, 1993) e/ou pelo o acúmulo de solutos, tais como prolina e betaína, que possuem a função de proteção celular contra níveis aumentados de ROS (CHEN; MURATA, 2002).

O estresse oxidativo é aliviado nas plantas pela ação das enzimas antioxidantes, tais como a superóxido dismutase (EC 1.15.1.1; SOD), a catalase (EC 1.11.1.6; CAT) e a ascorbato peroxidase (EC 1.11.1.11; APX) (WISNIEWSKI-DYÉ et al., 2012; OZYIGIT et al., 2016). A enzima SOD constitui a primeira linha de defesa contra ROS, convertendo o radical superóxido (O_2^-) para H_2O_2 que, por sua vez, é removido pelas enzimas CAT e APX pela conversão de H_2O_2 em água e oxigênio (LAMB; DIXON, 1997; ASADA, 1999). Em geral, os sistemas de detoxificação de ROS variam com as espécies de plantas, cultivares e idade, bem como com o tipo e duração do estresse (HODGES et al., 1996).

Os genes que codificam as isoenzimas (enzimas que diferem na sequência de aminoácidos, mas catalisam a mesma reação química) atuam em diferentes compartimentos de células vegetais, como as SOD2 e SOD4 citossólicas, constituintes da classe Cu/ZnSOD, isto é, utilizam cobre e/ou zinco como cofator (JUNG; KERNODLE; SCANDALIOS, 2001); as isoformas citossólicas APX1 e APX2 são as mais importantes da família APX em proteção antioxidante (SHIGEOKA; MARUTA, 2014), induzidas principalmente sob condições extremas de luz ou estresse por calor (DAVLETOVA et al., 2005), enquanto CAT1 é encontrada em peroxissomos, glioxissomos e também no citossol (SCANDALIOS; GUAN; POLIDOROS, 1997; JUNG; KERNODLE; SCANDALIOS, 2001).

Apesar dos estudos iniciais terem sido focados em fitopatógenos, há indícios de que as BPCP e as simbióticas podem induzir o estresse oxidativo nas plantas como resposta inicial de defesa, pois percebem esses microrganismos como uma ameaça potencial, o que confere proteção contra o ataque subsequente de patógenos, mecanismo conhecido como resistência sistêmica. No caso das simbióticas, *S. meliloti* induz a produção de ROS no estágio inicial de infecção em plantas de alfalfa (SANTOS et al., 2001).

Embora *Azospirillum* aparentemente apresente falta de especificidade na promoção do crescimento de praticamente todos os gêneros de plantas e espécies investigadas até o momento (PEREG; LUZ; BASHAN, 2016), também há indícios de que as espécies e estirpes podem variar em determinantes da adaptação específica do nicho rizosférico que afetam as interações planta-bactéria (WISNIEWSKI-DYÉ et al., 2012). Como exemplo, a adaptação de *A. lipoferum* 4B à rizosfera de arroz parece envolver genes relacionados à

detoxificação de ROS (DROGUE et al., 2014), e também para o *A. brasilense* Sp245 em *Arabidopsis thaliana* (SPAEPEN et al., 2014).

3.3.2.2 Resistência sistêmica induzida

As plantas possuem vários mecanismos de defesa induzíveis para se protegerem do ataque de patógenos. Um exemplo clássico é a resistência sistêmica adquirida (RSA), que é ativada após a infecção por um patógeno necrotrófico, e confere resistência às plantas contra um amplo espectro de agentes patogênicos, isto é, protege as plantas contra infecções secundárias por semanas a meses (FU; DONG, 2013). Algumas BPCP podem também induzir o mecanismo de defesa das plantas, o que tem sido denominado como RSI (resistência sistêmica induzida), que corresponde a uma resposta de defesa da planta, permitindo que a mesma expresse resistência a algumas bactérias patogênicas, vírus e fungos (LUGTENBERG; KAMILOVA, 2009).

A RSI desencadeada por microrganismos não patogênicos inicia-se nas partes primárias infectadas, estendendo-se a outros tecidos (DUTTA; MISHRA; DILEEP KUMAR, 2008), isto é, espalham-se sistemicamente por toda a planta e aumentam a capacidade defensiva de tecidos distantes contra a infecção por agentes patogênicos (VAN LOON; BAKKER, 2005). Uma vez induzidas, as plantas podem permanecer protegidas por períodos prolongados (VAN LOON, 2007). Esse fenômeno foi descrito pela primeira vez por Van Peer, Niemann e Schippers (1991) em cravo (*Dianthus caryophyllus* L.), que foi protegido contra o fungo *Fusarium oxysporum* f. sp. *dianthi* através da síntese e acúmulo de fitoalexina, após a inoculação com *Pseudomonas* sp. WCS417r. O mecanismo também foi descrito por Wei, Kloepper e Tuzun (1991) em pepino (*Cucumis sativus* L.), em seis das 94 estirpes de BPCP avaliadas, sendo cinco espécies de *Pseudomonas* e uma *Serratia*, que protegeram as folhas contra a antracnose causada por *Colletotrichum orbiculare.*

Os mecanismos de RSI no hospedeiro estão relacionados a diversos fatores como: *i)* de desenvolvimento, relacionado à promoção de crescimento de plantas; *ii)* fisiológico, ou seja, a planta apresenta tolerância aos fitopatógenos, reduzindo os sintomas da doença por eles causada; *iii)* ambiental, associado ao antagonismo microbiano na rizosfera, podendo também alterar a interação planta-inseto; *iv)* bioquímico, conferindo resistência às plantas pela indução do fortalecimento da parede celular, da indução de síntese de fitoalexinas antimicrobianas, da indução de proteínas relacionadas à patogênese (Proteínas-PR) e do pré-

condicionamento (*priming*), que fornece à planta uma maior capacidade de ativar rápida e efetivamente as respostas de defesa celulares que são induzidas somente pelo contato com o patógeno (VAN LOON, 2007).

As proteínas-PR possuem diferentes funções, como as proteínas codificadas por PR1 (um membro de uma família de multigenes) (MORRIS et al., 1998), PR-2 (sintetiza uma β-1-3-glucanase) (KAUFFMANN et al., 1987) e PR4 (membro da família quitinase) (NASSER et al., 1988). A proteína PR1 ainda tem sua função bioquímica desconhecida (VAN LOON; REP; PIETERSE, 2006). Já as PR-2 e PR4 são enzimas hidrolíticas, incluindo as quitinases e glucanases, que sintetizadas pelas plantas através do mecanismo RSI atuam na inibição do crescimento fúngico, uma vez que os principais constituintes estruturais da parede celular dos fungos patogênicos são compostos por quitina e β-glucana.

A RSI também se caracteriza por uma interação específica planta-BPCP, isto é, uma BPCP que é capaz de desencadear a RSI em uma determinada espécie de planta pode não induzir em outra (VAN LOON, 2007). O principal grupo descrito de BPCP que desencadeia a RSI inclui estirpes dos gêneros *Azospirillum*, *Pseudomonas* e *Bacillus* (PÉREZ-MONTAÑO et al., 2014). Um estudo transcriptômico de *Azospirillum* sp. B510 (isolado da cv. Nipponbare) inoculado nas cultivares de arroz (Nipponbare e Cigalon) induziu um gene PR e reprimiu cinco genes PR na cultivar Nipponbare, enquanto que *A. lipoferum* 4B (isolado da cv. Cigalon), induziu mais genes relacionados à defesa na cultivar Nipponbare que na Cigalon (DROGUE et al., 2014). Em outro estudo com *Arabidopsis thaliana* os genes PR foram induzidos quando a planta foi inoculada com *A. brasilense* Sp245 (SPAEPEN et al., 2014). Estirpes de *B. subtilis* (SG JW.03) inoculadas em plantas de milho induziram genes relacionados à patogênese, incluindo PR-1 e PR-4 (GOND et al., 2014).

A RSA está associada à síntese e o acúmulo de ácido salicílico (AS) na planta, desencadeando uma expressão coordenada de genes que codificam proteínas PR (KAWAGOE et al., 2015). A proteína NPR1 (*"nonexpressor of PR genes1"*, relacionada ao sistema de defesa de plantas) é um regulador essencial no mecanismo RSA, que é transportada para o núcleo celular em resposta ao AS, onde atua como um coativador transcricional de um conjunto de genes PR (PIETERSE et al., 2012; PAJEROWSKA-MUKHTAR; EMERINE; MUKHTAR, 2013; PIETERSE et al., 2014). No caso das RSI, estudos sobre diferentes espécies de BPCP e plantas estabeleceram que a natureza da resistência sistêmica induzida, na maioria dos casos, é independente de AS (YAN et al., 2002; DE VLEESSCHAUWER et al., 2008; SEGARRA et al., 2009) e, geralmente, está associada a

moléculas sinalizadoras, como o jasmonato e o etileno (AHEMAD; KIBRET 2014; GLICK 2012).

Essas evidências corroboram os resultados de Yasuda e colaboradores (2009), quando inocularam plantas de arroz com *Azospirillum* sp. B510, o que aumentou a resistência da planta contra o fungo patogênico *Magnoporthe oryzae* e pelo patógeno virulento bacteriano *Xanthomonas oryzae,* por mecanismos independentes da sinalização AS, não havendo acúmulo de AS e proteínas PR nas plantas. Resultados similares também foram encontrados por Vleesschauwer e colaboradores (2008) para *P. fluorescens* WCS374r.

Vários estudos demonstraram que aplicações exógenas de AS (BARI; JONES, 2009) e ácido jasmônico (AJ) (BARI; JONES 2009; WASTERNACK 2007; LORENZO; SOLANO 2005) nas plantas induzem genes PR, tendo como consequência o aumento da resistência a vários agentes fitopatogênicos. Agrawal e colaboradores (2000) relataram a primeira evidência da aplicação exógena de AJ como indutor efetivo da família PR1 em arroz.

Há ainda relatos da eficiência da aplicação de produtos químicos indutores de RSI – como AJ ou AS – na redução de incidência de doenças em arroz. No entanto, quando foi aplicado EPS purificados de *Azospirillum*, as plantas, além de conferirem resistência contra o fungo *Pyricularia oryzae*, também apresentaram maior desenvolvimento, revelando claramente o duplo efeito de EPS de *Azospirillum* (SANKARI; DINAKAR; SEKAR, 2011). Esses resultados sugerem que metabólitos produzidos por *Azospirillum* também podem ser uma alternativa de indução de RSI, além de conferir promoção de crescimento de plantas.

3.4 Estresse Salino

As plantas estão expostas a diversos estresses ambientais como altas e baixas temperaturas, seca, salinidade, alcalinidade, raios UV e infecção por patógenos (SHARMA et al., 2012). Estima-se que cerca de 30% da produção mundial das culturas seja perdida como resultado de estresses abióticos (GOSWAMI et al., 2016). Entre os fatores limitantes, a salinidade é considerada um dos maiores estresses abióticos, impactando a produtividade agrícola e a sustentabilidade devido a reduções na fotossíntese, respiração e síntese proteica (AHMAD; PRASAD, 2012; DWIVEDI et al., 2015).

A salinidade provoca distúrbios nutricionais em plantas que levam a

deficiência de vários nutrientes e aumento dos níveis de Na^+ (ZAHEDI et al., 2012). Inicialmente, ocorre o estresse osmótico, devido à alta concentração de sal em torno das raízes, dificultando a absorção de água; em um segundo momento, ocorre o estresse iônico, que em altas concentrações no interior da planta podem ser tóxicas, resultando em inibição de muitos processos fisiológicos e bioquímicos, como a absorção e assimilação de nutrientes (HASEGAWA et al., 2000; MUNNS; TESTER, 2008).

Existem muitos mecanismos adaptativos que as plantas usam para lidar com os efeitos adversos da salinidade, como a síntese e acúmulo de solutos, tais como: aminoácido (prolina), álcoois de açúcar (manitol) e amônio quaternário (glicinebetaína), que retêm a água dentro das células para combater a desidratação (NUCCIO et al., 1999). Outro mecanismo reside no incremento da síntese de ROS nas células (conforme descrito anteriormente) (GURURANI et al., 2013).

O uso de BPCPs confere tolerância aos estresses abióticos, como a salinidade, a seca e extremos de temperatura (SARMA et al., 2012; Cerezini et al., 2016), que podem provocar a "tolerância sistêmica induzida" (TSI), envolvendo várias mudanças fisiológicas e bioquímicas nas plantas (YANG; KLOEPPER; RYU, 2009). Os mecanismos relacionados a TSI incluem defesa antioxidante (HEIDARI; GOLPAYEGANI, 2012; WANG et al., 2012); ajuste osmótico (SARMA; SAIKIA, 2014); produção de fitormônios como AIA (SPAEPEN; VANDERLEYDEN, 2015); estratégias de defesa como a expressão de genes PR (KIM et al., 2014) e a indução de proteínas de choque térmico (*heat shock proteins*, HSPs) (LIM; KIM, 2013).

Dentre as BPCP, o gênero *Azospirillum* – principalmente *A. brasilense* – é provavelmente o mais estudado para mitigar o estresse salino, com ensaios de inoculação realizados com milho (HAMDIA; SHADDAD; DOAA, 2004), trigo duro (*Triticum durum* var. Waha) (NABTI et al., 2009), trigo (ALAMRI; MOSTAFA, 2009), canola (*Brassica napus* L.) (BANIAGHIL et al., 2013), pimenta doce (*Capsicum annuum* L.) (AMOR; CUADRA-CRESPO, 2012) e trevo branco (*Trifolium repens*) (KHALID et al., 2017). Os autores relataram a melhora na captação de nutrientes e maior crescimento vegetativo.

É importante ressaltar que existem espécies incluídas no grupo simbiótico que são utilizadas em coinoculações com diferentes BPCP e podem estar envolvidas na TSI (HAN; LEE, 2005; BANO; FATIMA, 2009). Por exemplo, as bactérias do gênero *Rhizobium* apresentam resistência intrínseca à salinidade, e por isso foram conduzidos estudos com diferentes espécies vegetais em condições salinas, incluindo: ervilha (*Pisum sativum* L.), feijão fava (*Vicia faba* L.) (CORDOVILLA et al., 1999) e alface (*Lactuca sativa* L.) (HAN;

LEE, 2005). Além disso, relatos anteriores mostram benefícios em feijoeiro coinoculado com *R. tropici* CIAT 899 e *A. brasilense* Cd em condições de estresse salino (DARDANELLI et al., 2008).

Contudo, vale salientar que estudos mais aprofundados acerca dos mecanismos de ação dessas bactérias, bem como a resposta das plantas frente ao estresse são de fundamental importância para a compreensão e uso desses conhecimentos como estratégias futuras a fim de mitigar os efeitos de estresse abiótico nas plantas.

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4 ARTIGO A – Revelando estratégias de *quorum sensing* nas estirpes de *Azospirillum brasilense* Ab-V5 e Ab-V6

RESUMO

Azospirillum brasilense é uma importante bactéria promotora de crescimento de plantas (BPCP) que requer várias etapas para a colonização das raízes, incluindo a síntese de biofilme e exopolissacarídeo (EPS) e a mobilidade celular. Em diversas bactérias, esses mecanismos são mediados pelo sistema *quorum sensing* (QS) que regulam a expressão de genes específicos percebidos por moléculas auto-indutoras, as N-acil-homoserinas lactonas (AHLs). Foram investigados os mecanismos de QS em *A. brasilense* estirpes Ab-V5 e Ab-V6, que são amplamente utilizadas em inoculantes comerciais no Brasil. Nenhuma dessas estirpes possuem um gene *luxI*, mas possuem vários genes *luxR* 'solos' que podem detectar moléculas de AHL. Ao adicionar moléculas de AHLs sintéticas, verificou-se que a produção de biofilmes e EPS, e a mobilidade celular (*swimming* e *swarmming*) foram reguladas pelo QS em Ab-V5, mas não em Ab-V6. As diferenças foram observadas não apenas entre as estirpes, mas também na especificidade dos receptores do tipo LuxR para as moléculas de AHL. No entanto, a Ab-V6 sintetiza altas concentrações de ácido-indol-acético (AIA) e estas moléculas podem imitar sinais de AHL. Também foi utilizada a estratégia de *quorum-quenching* (QQ), obtendo transconjugantes de Ab-V5 e Ab-V6 carregando um plasmídeo com acil-homoserina lactonase. Assim, quando o milho (*Zea mays* L.) foi inoculado com as estirpes e transconjugantes, o crescimento da planta diminuiu com o transconjugante do Ab-V5 – confirmando a importância de um sistema QS mediado por AHL – mas não afetou a promoção de crescimento de plantas pela Ab-V6.

Palavras-chave: AHL. Biofilme. EPS. AIA. genes *lux*. Mobilidade celular. *N*-Acyl-homoserina lactona. QQ. QS. *Zea mays*

Arch Microbiol
DOI 10.1007/s00203-017-1422-x

ORIGINAL PAPER

Revealing strategies of quorum sensing in *Azospirillum brasilense* strains Ab-V5 and Ab-V6

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Received: 5 July 2017 / Revised: 21 July 2017 / Accepted: 30 July 2017

Abstract *Azospirillum brasilense* is an important plant-growth promoting bacterium (PGPB) that requires several critical steps for root colonization, including biofilm and exopolysaccharide (EPS) synthesis and cell motility. In several bacteria these mechanisms are mediated by quorum sensing (QS) systems that regulate the expression of specific genes mediated by the autoinducers *N*-acyl-homoserine lactones (AHLs). We investigated QS mechanisms in strains Ab-V5 and Ab-V6 of *A. brasilense*, which are broadly used in commercial inoculants in Brazil. Neither of these strains carries a *luxI* gene, but there are several *luxR* solos that might perceive AHL molecules. By adding external AHLs we verified that biofilm and EPS production and cell motility (swimming and swarming) were regulated via QS in Ab-V5, but not in Ab-V6. Differences were observed not only between strains, but also in the specificity of LuxR-type receptors to AHL molecules. However, Ab-V6 was outstanding in indole acetic acid (IAA) synthesis and this molecule might mimic AHL signals. We also applied the quorum quenching (QQ) strategy, obtaining transconjugants of Ab-V5 and Ab-V6 carrying a plasmid with acyl-homoserine lactonase. When maize (*Zea mays* L.) was inoculated with the wild-type and transconjugant strains, plant growth was decreased with the transconjugant of Ab-V5—confirming the importance of an AHL-mediated QS system—but did not affect plant growth promotion by Ab-V6.

Keywords AHL · Biofilm · EPS · IAA · *lux* genes · Cell motility · *N*-Acyl-homoserine lactones · QQ · QS · *Zea mays*

Communicated by Erko Stackebrandt.

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Introduction

The mechanism known as quorum sensing (QS) allows cell-to-cell communication to control the expression of certain genes at high cell density; the process is mediated by small diffusible signal molecules called autoinducers (Fuqua et al. 1994, 2001; Boyer and Wisniewski-Dyé 2009). QS systems are found in Gram-negative bacteria and the most studied autoinducers are the *N*-acyl-homoserine lactones (AHLs), synthesized by LuxI-type and detected by LuxR-type proteins, which, in turn, activate the expression of target genes (Hudaiberdiev et al. 2015). QS systems are used to coordinate and synchronize several responses to the environment (Grandclément et al. 2015), such as bioluminescence,

Published online: 05 August 2017

biofilm formation, cell motility, exopolysaccharide (EPS) production, synthesis of virulence factors and antimicrobial compounds, among others, which usually are essential for the successful establishment of a symbiotic or a pathogenic relationship with a eukaryotic host (Boyer and Wisniewski-Dyé 2009; Pérez-Montaño et al. 2014).

In some bacteria, the numbers of LuxI- and LuxR-family proteins are not always the same and several cases of 'extra' LuxR-type proteins have been reported (Case et al. 2008). Genome sequencing has also revealed that many bacteria have AHL/QS-related *luxR*-type genes which are unpaired to a cognate *luxI* gene; these genes may either reply to internal AHL signals produced by a non-adjacent *luxI* gene in the chromosome, or may reply to exogenous signals. Such unpaired *luxR*-type genes have been called *luxR* solos (Subramoni and Venturi 2009).

Azospirillum is a genus comprising versatile plant-growth-promoting bacteria (PGPB) (Cassán et al. 2014), with several reports of strains showing remarkable capacity to benefit plant growth in a variety of plant species, including important cereals such as maize (*Zea mays* L.) (Bashan and de-Bashan 2010; Hungria 2011; Hungria et al. 2010; Cassán et al. 2015; Pereg et al. 2016). Plant-growth promotion by *Azospirillum* relies on an array of mechanisms (Bashan and De-Bashan 2010), including enhanced uptake of nutrients and water (Ardakani et al. 2011), synthesis of phytohormones, such as auxins (Spaepen and Vanderleyden 2015), induction of plant-stress tolerance and defense genes (Fukami et al. 2017) and biological nitrogen fixation (BNF) (Marques et al. 2017). *Azospirillum* may regulate several processes by QS mechanisms. For example, *Azospirillum* is highly motile, by means of swimming and swarming (Alexandre 2015), and these key processes for root colonization may be controlled by QS. However, the complete AHL-mediated QS system seems unusual in *Azospirillum*; for example, Vial et al. (2006) found that only 4 out of 40 strains evaluated produced AHL molecules, all of which belonged to the species *A. lipoferum*. Nevertheless, plants may also produce low-molecular-weight compounds that mimic QS signals, acting as activators or repressors of bacterial AHL-mediated QS systems (Teplitski et al. 2000; Bauer and Mathesius 2004; Patel et al. 2013; Pérez-Montaño et al. 2013).

Quorum quenching (QQ) refers to all processes involved in the disturbance of QS (Dong et al. 2001; Grandclément et al. 2015). QQ is a natural microbial mechanism, either to degrade their own QS signals, or to establish competitive relationships with other microorganisms (Grandclément et al. 2015). The QQ enzyme AHL-lactonase—AiiA—was first identified in *Bacillus* in a process to attenuate the virulence of *Erwinia carotovora* (Li et al. 2008). Both QS and QQ play strategic roles in the bacterial interactions, including bacterium–plant associations.

In Brazil, *A. brasilense* strains Ab-V5 and Ab-V6 have been exponentially used in commercial inoculants for grain crops including maize and wheat (*Triticum aestivum* L.) and also for other legumes and non-legumes (Hungria et al. 2010, 2015, 2016; Hungria 2011; Marks et al. 2015; Fukami et al. 2016). Despite the importance of *Azospirillum*, few studies have been performed on QS in that genus and none with these two important strains. We examined bacterial mechanisms related to the QS and QQ mechanisms in Ab-V5 and Ab-V6, investigating possible impacts on interactions with maize.

Materials and methods

Strains, growth curves and IAA quantification

Azospirillum brasilense strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084) were obtained from the "Collection of Diazotrophic and Plant Growth Promoting Bacteria of Embrapa Soja" (WFCC # 1213, WDCM # 1054).

Growth curves were evaluated in two independent experiments. In experiment 1, strains were grown in DYGS liquid medium (Rodrigues Neto et al. 1986) (glucose, 2 g; malic acid, 2 g; bacto-peptone, 1.5 g; yeast extract, 2 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; glutamic acid, 1.5 g, pH 6.8), and in experiment 2 the DYGS medium was supplemented with 500 µg mL^{-1} of tryptophan (TRP, precursor of the synthesis of indole acetic acid-IAA) (DYGS-TRP). In experiment 2, IAA was also evaluated. In both experiments, pre-inocula were obtained by growth of each strain in 10 mL of liquid medium at 120 rpm, 28 ± 2 °C, in the dark, for 24–36 h, until OD_{600} 0.8 was reached. An aliquot of 1 mL of each pre-inoculum was transferred to flasks containing 100 mL of DYGS or DYGS-TRP. The flasks were incubated under the same conditions for 78 and 48 days, for experiments 1 and 2, respectively. The experiment was performed with triplicates. Cell counting was performed by the drop plate method (Miles et al. 1938), on Petri plates containing RC medium (Rodríguez-Cáceres 1982). A total of 22 counts were performed in experiment 1, as follows: T 0 h, T 8 h, T 14 h, T 20 h, T 36 h (~1 day), T 52 h (~2 days), T 76 h (~3 days), T 170 h (~7 days), T 194 h (~8 days), T 242 h (~10 days), T 290 h (~12 days), T 338 h (~14 days), T 386 h (~16 days), T 434 h (~18 days), T 506 h (~21 days), T 554 h (~23 days), T 602 h (~25 days), T 674 h (~28 days), T 1394 h (~58 days), T 1562 h (~65 days), T 1874 h (~78 days). In experiment 1, bacterial growth was also evaluated by measuring OD at $\lambda = 600$ nm at each evaluation, until 194 h. In experiment 2, 16 counts were performed, as follows: T 0 h, T 4 h, T 8 h, T 14 h, T 24 h (1 day), T 30 h (~1 day), T 36 h (~1 day), T 72 h (3 days), T 240 h (10 days), T 336 h (14 days), T 504 h

(21 days), T 576 h (24 days), T 672 h (28 days), T 840 h (35 days), T 1008 h (42 days), T 1152 h (48 days).

IAA-like compounds synthesized by *A. brasilense* strains Ab-V5 and Ab-V6 in experiment 2 were quantified in vitro by the Salkowski's colorimetric method (Glickmann and Dessaux 1995). One-mL of each culture in each time was centrifuged to remove cells, and used the supernatants for quantification of IAA.

Biofilm formation assay

The assays for biofilm evaluation were performed in 96-well polystyrene microtiter dishes, using the method of O'Toole and Kolter (1998), modified as described by del Cerro et al. (2015). Strains Ab-V5 and Ab-V6 were grown in DYGS medium supplemented with different autoinducer molecules (200 ng mL^{-1}) (Sigma), as follows: *N*-butanoyl-L-homoserine lactone (C4-AHL), *N*-hexanoyl-L-homoserine lactone (C6-AHL), *N*-octanoyl-L-homoserine lactone (C8-AHL), *N*-decanoyl-L-homoserine lactone (C10-AHL), *N*-dodecanoyl-L-homoserine lactone (C12-AHL), *N*-tetradecanoyl-L-homoserine lactone (C14-AHL), *N*-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-AHL) and *N*-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-AHL). Bacteria were grown for 7 days with gentle rocking at 28 ± 2 °C, in the dark. Every experiment was performed three times with six replicates each time.

Extracellular polysaccharides

The quantification of exopolysaccharides (EPSs) in the supernatants of *A. brasilense* strains Ab-V5 and Ab-V6 was performed with the anthrone-H_2SO_4 method (Tomlinson et al. 2010; del Cerro et al. 2016). Each bacterium was grown in 5 mL of DYGS medium supplemented with different autoinducer molecules, as described in item 2.2, at 120 rpm, 28 ± 2 °C, in the dark, for 48 h. Aliquots of 1 mL were then centrifuged to remove cells, and the supernatants analyzed for the EPS production. The experiment was performed with three replicates.

Motility assays

Bacteria were first grown in 5 mL of TY liquid medium (Beringer 1974) on an orbital shaker at 120 rpm, 28 ± 2 °C, in the dark, for 48 h. Swimming and swarming plates were prepared with TY medium containing 0.28 or 0.4% of Bacto Agar, respectively, and supplemented with the following autoinducer molecules (200 ng mL^{-1}) (Sigma): C4-AHL, C6-AHL, C8-AHL, C10-AHL, C12-AHL, C14-AHL, 3-oxo-C6-AHL and 3-oxo-C8-AHL. Aliquots (2 µL) of culture suspensions were drop-inoculated (swarming assay) or sink-inoculated (swimming assay) onto plates and air-dried in a laminar-flow cabinet. The plates were wrapped with parafilm to prevent dehydration and incubated at 28 ± 2 °C for 24, 48 and 72 h. The experiment was performed with four replicates.

Obtaining transconjugants of *A. brasilense*

The plasmid pME6863 from *Escherichia coli* (Reimmann et al. 2002) was transferred to *A. brasilense* strains Ab-V5 and Ab-V6 by conjugation, following the methodology described by Simon (1984), and using the helper plasmid pRK2013 (Figurski and Helinski 1979). The plasmid pME6863 contains the *aiiA* gene from *Bacillus* sp. strain A24 that encodes an *N*-acyl-homoserine lactonase, capable of inactivating AHL molecules. To select the transconjugants of *A. brasilense,* DYGS agar plates were supplemented with nalidixic acid (40 µg mL^{-1}) and tetracycline (20 µg mL^{-1}). Ab-V5 and Ab-V6 exhibit intrinsic resistance to the nalidixic acid, whereas *E. coli* containing the transfer plasmid shows resistance only to tetracycline. To confirm the presence of *aiiA* in the transconjugants, the DNA was extracted with the PureLink™ Quik Plasmid Miniprep kit (Invitrogen™). The *aiiA* gene was amplified by PCR with the primers aiiA-F (5 'GCAGGTCGTTGTTGGA 3′) and aiiA-R (5 'CAGGGAACACTTTACATCCC 3′), designed based on the gene sequence (AF397400) of *Bacillus* sp. A24, retrieved from the GenBank database (Reimmann et al. 2002). The amplification conditions consisted of an initial denaturation step of 95 °C for 2 min, followed by 45 cycles of 95 °C for 45 s, 59 °C for 3 s, 72 °C for 1 min and 30 s, and a final extension cycle of 72 °C for 7 min; reactions were performed in a DNA Engine® Thermal Cycler (Research INC, USA). The stability of the plasmid was continuously monitored with the antibiotics nalidixic acid and tetracycline in DYGS medium, and PCR reactions. The Ab-V5 and Ab-V6 *Azospirillum* strains harboring the pME6863 plasmid were called Ab-V5-QS and Ab-V6-QS, respectively.

Greenhouse experiment

One experiment was performed under greenhouse conditions using modified Leonard jars (Vincent 1970) containing sterilized substrate, consisting of a mixture of sand and pulverized coal (3:1, v/v), that received sterilized nutrient solution (Hoagland and Arnon 1950). Jars were arranged in a completely randomized design with five treatments and four replicates. Hybrid maize seeds (DKB350 YG) were surface-sterilized with 70% ethanol and 3% sodium hypochlorite. The treatments included a non-inoculated control with N-fertilizer (90 kg N ha^{-1}, corresponding to 75% of the N dose recommended for the maize crop in Brazil), inoculation with *A. brasilense* strains Ab-V5 and Ab-V6, and inoculation with the transconjugants Ab-V5-QS and Ab-V6-QS. For

seed inoculation, inocula were adjusted to supply 1.2×10^5 cells of *Azospirillum* seed^{-1}. Two seeds were sown per jar and thinned to one plant 3 days after seedling emergence (DAE); sterilized nutrient solution was applied as needed.

Plants were collected at 35 DAE. Before harvesting, chlorophyll content (CC) was determined according to Kaschuk et al. (2010), based on the SPAD (soil plant analysis development) index, with readings taken from the lowermost third of the +3 leaf. Plant height (PH, cm) and culm diameter (CD, mm) were determined with the aid of a digital caliper. Shoot fresh weight (SFW) was evaluated and the material was dried at 60 °C for approximately 72 h to determine shoot dry weight (SFW). Dry shoots were ground (18 mesh) and total N evaluated by the salicylate green method (Searle 1984).

Statistical analyses

Data obtained from the experiments were first evaluated for normality and variance homogeneity, followed by the analysis of variance (ANOVA). For the in vitro assays and greenhouse experiments, the Tukey's and Duncan's tests were used, respectively, to compare means when statistical significance was detected by the ANOVA *F* test ($p \leq 0.05$). For all analyses, the Statistica version 7.0 software was employed.

Results

Growth and synthesis of IAA by *A. brasilense* strains Ab-V5 and Ab-V6

Similar growth curves were obtained for *A. brasilense* strains Ab-V5 and Ab-V6 in DYGS liquid medium (Fig. 1a). The logarithmic phase occurred up to 36 h and was followed by a long stationary phase, lasting up to 674 h, and then a death phase. Cell growth of Ab-V5 in DYGS medium went from 1.43×10^9 to about 1.69×10^9 colony forming units (CFU) mL^{-1} and of Ab-V6 from 7.18×10^8 to 1.13×10^9 CFU mL^{-1} between 194 and 674 h of growth. We also monitored bacterial growth by evaluating OD_{600} up to 194 h (Fig. 1b), when the formation of aggregates started to affect the readings. Based on the growth curves shown in Fig. 1b, we established that at 48 h of growth in DYGS medium (late exponential phase) both strains reached the maximum growth yield, with an OD_{600} of 1.5. This condition was used for the other experiments.

Strains Ab-V5 and Ab-V6 differed in growth in DYGS medium supplemented with tryptophan (TRP). In Ab-V5 there was an initial delay in growth, observed between 4 and 24 h, whereas the lag phase of strain Ab-V6 lasted only 4 h. There was an initial logarithmic growth phase (μ1) lasting until 36 h, followed by a quick step of the stationary phase until 72 h, and a second step phase of growth (μ2) lasting 540 and 336 h for Ab-V5 and Ab-V6, respectively (Fig. 2a). Interestingly, at the final evaluation, the addition of TRP resulted in higher cell concentration, of about ten times, of both Ab-V5 and Ab-V6 (Fig. 2a), when compared to the growth without TRP (Fig. 1a).

IAA accumulation in the DYGS + TRP medium started to increase around 72 h and continued to increase up to the last evaluation at 48 days for both strains (Fig. 2b). The synthesis of IAA was far greater in Ab-V6 than in Ab-V5, and in the last evaluation resulted in about 90 and 50 μg IAA mL^{-1}, respectively.

Quorum-sensing system of *A. brasilense* and responses to autoinducer molecules

The genomes of *A. brasilense* strains Ab-V5 and Ab-V6 were obtained (data not shown) in a MiSeq™ and assembled as described before (Ribeiro et al. 2015). The genome was submitted to RAST (Rapid Annotations using Subsystems Technology) (Aziz et al. 2008) and searched for *lux*-type genes. We found 15 *luxR* genes; in Ab-V5, we found 14 putative genes that could be classified as AHL-dependent transcriptional regulators (LuxR homologues) and one that would fit better as a *luxR* regulator protein, while in Ab-V6 the 15 genes were classified as AHL-dependent transcriptional

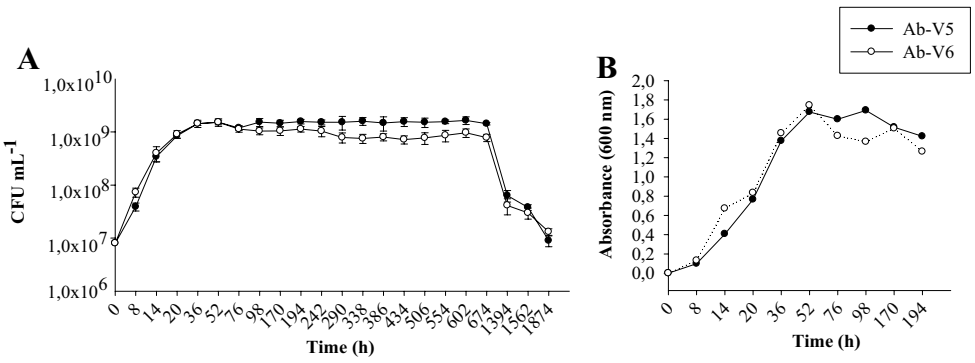

Fig. 1 Growth curves of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 in DYGS liquid medium. **a** Growth curve obtained by evaluation of colony forming units (CFU mL^{-1}). **b** Growth curve obtained by absorbance at 600 nm. *Black symbols* Ab-V5 strain; *open symbols* Ab-V6 strain

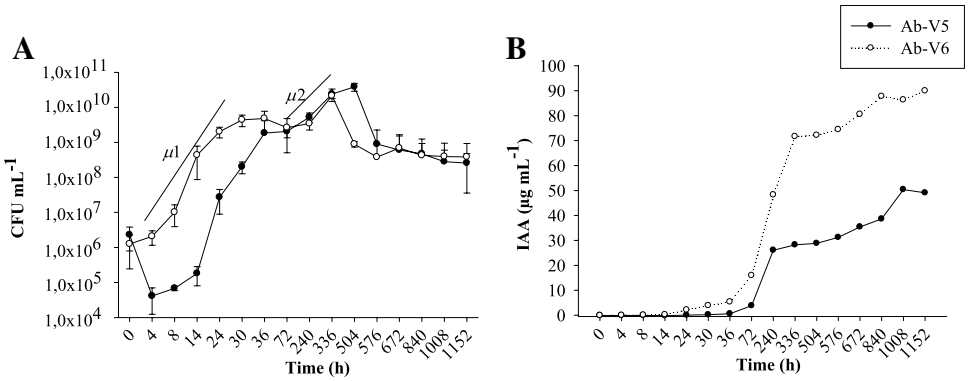

Fig. 2 Growth curves of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 in DYGS liquid medium supplemented with tryptophan. **a** Growth curve obtained by evaluation of colony forming units (CFU mL^{-1}). **b** Growth curve obtained by evaluation of absorbance at 600 nm. *Black symbols* Ab-V5 strain; *open symbols* Ab-V6 strain

regulators (according to this classification, a transcriptional regulator activates or represses the transcription of a gene by binding to a promoter of this gene, while a regulator can be a transcriptional regulator, but it can also act in other ways, such as by phosphorylating one protein). In both genomes *luxR* is replicated throughout the genome, resulting in a total of eight different LuxR homologues and one *luxR* regulator. Searching at the NCBI database we detected that there are highly similar genes in other *A. brasilense* strains, Sp245, Sp7 and Azo39. As none of the genomes had any *luxI* genes, we performed bioassays to verify bacterial responses to the addition of exogenous AHL autoinducers.

In the biofilm assay, the amount of biofilm produced by strain Ab-V5 was significantly increased when the medium was supplemented with C8-AHL and C10-AHL, in comparison to the control without any AHL molecule (Fig. 3a). However, for strain Ab-V6 supplemented with 3-oxo-C8-AHL, the production of biofilm decreased significantly in comparison to the control (Fig. 3a).

The production of EPS by strain Ab-V5 was significantly increased with the addition of C6-AHL and 3-oxo-C8-AHL, by 73 and 214%, respectively (Fig. 3b). As for the biofilm, EPS production by Ab-V6 was not stimulated by any of the molecules (Fig. 3b).

Swimming and swarming phenotypes were evaluated at 24, 48 and 72 days of incubation in the presence of different AHL molecules. Swimming motility by strain Ab-V5 was significantly increased, at 72 h of incubation, in the presence of the molecules C4-AHL, C6-AHL, C10-AHL (this one statistically not significant), C14-AHL, 3-oxo-C6-AHL and 3-oxo-C8-AHL, while in Ab-V6 none of the molecules showed any effect (Fig. 4a, b). In the swarming analysis, the C14-AHL molecule induced significantly the motility of Ab-V5 in the first 24 h of incubation, while inducing activity was observed for the C10-AHL and C12-AHL molecules at 48 h and for C8-AHL, 3-oxo-C6-AHL and 3-oxo-C8-AHL at 72 h (Fig. 4c). For the Ab-V6 strain, swarming motility was significantly induced only by C12-AHL and C14-AHL at 72 h of incubation (Fig. 4d).

AHL-mediated QS system inactivation in *A. brasilense* Ab-V5 and Ab-V6 and effects on maize-growth promotion

Transconjugants of Ab-V5 and Ab-V6 strains containing the pME6863 plasmid carrying the *aiiA* gene from *Bacillus* sp. strain A24—that encodes an *N*-acyl-homoserine lactonase capable of inactivating AHLs molecules—were obtained.

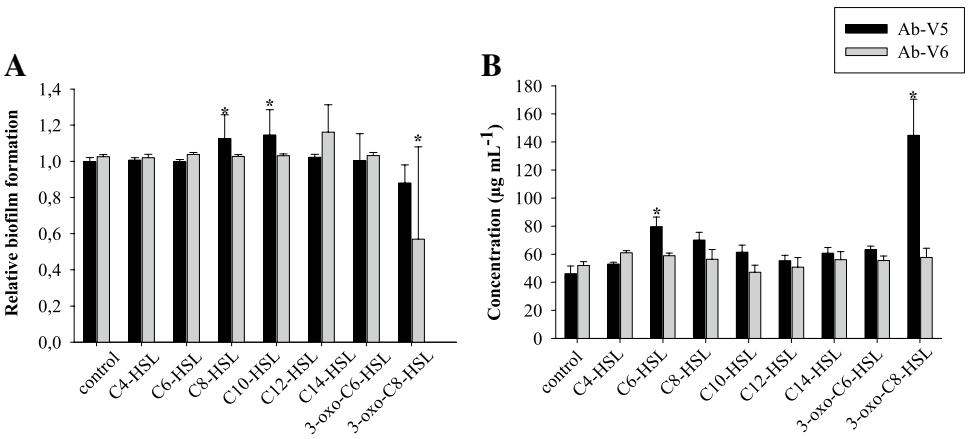

Fig. 3 Biofilm formation assay and production of exopolysaccharides (EPS) by *Azospirillum brasilense* strains Ab-V5 and Ab-V6. Bacteria were grown in DYGS medium supplemented with different *N*-acyl-homoserine lactone (AHL) molecules. **a** Relative biofilm formation. **b** Production of EPS. The *asterisks* indicate statistical difference in relation to the control treatment by the Tukey's test at the level $\alpha = 5\%$ probability. *Dark bars* Ab-V5 strain, *gray bars* Ab-V6 strain

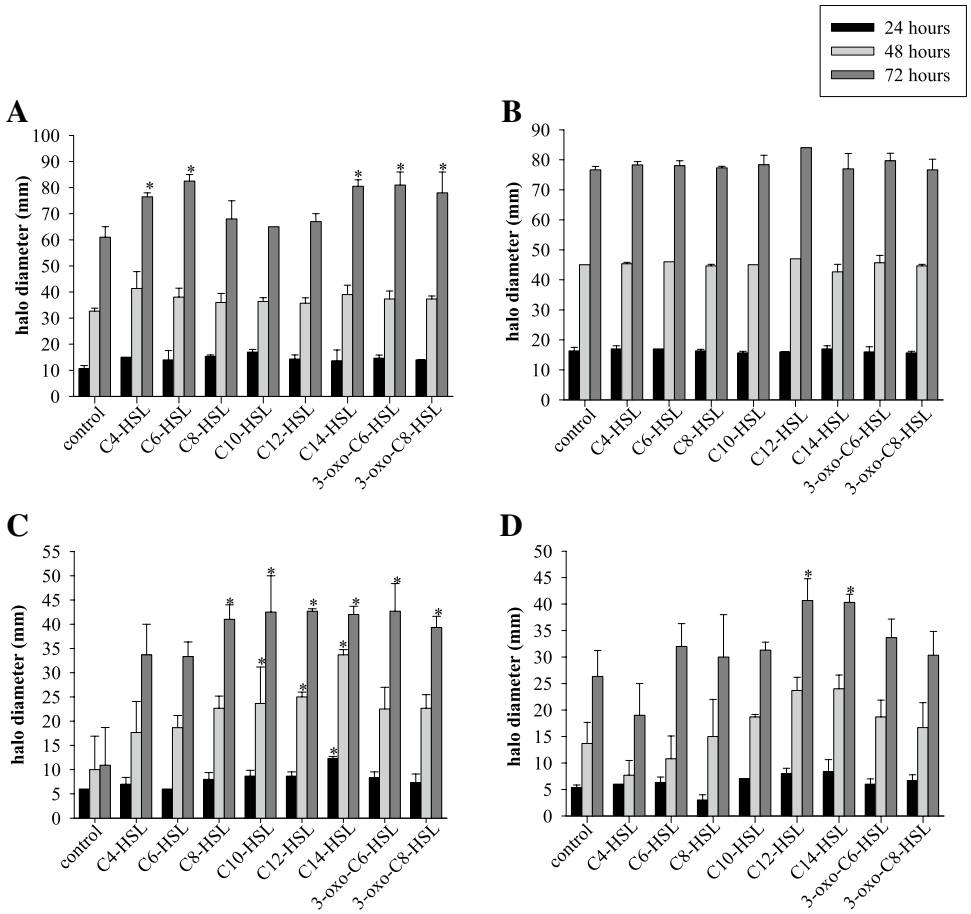

Fig. 4 Swimming and swarming motility phenotypes of the *Azospirillum brasilense* strains Ab-V5 and Ab-V6. Bacteria were grown in TY medium with 0.28 or 0.4% agar, supplemented with different acyl-homoserine lactone (AHL) molecules and quantified swim or swarm ring diameters of strains. **a** Swimming motility of Ab-V5. **b** Swimming motility of Ab-V6. **c** Swarming motility of Ab-V5. **d** Swarming motility of Ab-V6. The asterisks indicate statistical difference by the Tukey's test at the level $\alpha = 5\%$ probability in relation to the control. *Dark bars* 24 h, *light gray bars* 48 h, *dark gray bars* 72 h

Wild-type and transconjugant strains were inoculated in maize under greenhouse conditions. In general, PGPB properties of both wild-type strains were confirmed, but statistical differences were observed only by inoculation with Ab-V5 (Table 1). Shoot fresh weight (SFW), N content (NC) and total N accumulated in shoots (TNS) were significantly reduced in plants inoculated with the transconjugant Ab-V5-QS in comparison to the wild-type strain; shoot dry weight (SDW) was also reduced, but without statistical difference. To a lesser extent, we have also observed that NC and TNS decreased in plants inoculated with the Ab-V6-QS strain compared to the wild-type Ab-V6, but these differences did not reach the statistical significance set for this study (Table 1).

Table 1 Growth parameters in greenhouse experiment performed with hybrid maize DKB 350 YG inoculated with *Azospirillum brasilense* (Ab-V5, Ab-V6, Ab-V5-QS or Ab-V6-QS)

Treatment	CC ($\mu g\ cm^{-2}$)	PH ($cm\ pL^{-1}$)	CD ($mm\ pL^{-1}$)	SFW ($g\ pL^{-1}$)	SDW ($g\ pL^{-1}$)	NC ($mg\ N\ g^{-1}$)	TNS ($mg\ N\ pL^{-1}$)
Control	12.87^{ns}	81.0^{ns}	15.32^{ns}	93.36^{b}	12.71^{ns}	14.08^{b}	179.74^{b}
Ab-V5	16.27	91.75	15.71	117.29^{a}	15.09	20.53^{a}	326.90^{a}
Ab-V5-QS	13.44	82.25	15.70	94.51^{b}	12.98	11.47^{b}	149.03^{b}
Ab-V6	13.65	92.25	15.69	108.12^{ab}	15.76	15.50^{b}	235.42^{b}
Ab-V6-QS	14.67	92.25	16.09	113.70^{a}	15.09	14.46^{b}	221.61^{b}
p value	0.0978	0.1546	0.9252	0.0268	0.0779	0.0037	0.0031

Fresh weight (SFW), chlorophyll content (CC), N content (NC), total N accumulated in the shoots (TNS), plant height (PH) and culm diameter (CD). Parameters determined 35 days after seedling emergence

Means (four replicates) followed by the same letter on the same column are not significantly (ns) different according to Duncan's test ($p \leq 0.05$)

Discussion

Although *A. brasilense* strains Ab-V5 and Ab-V6 have been broadly used in commercial inoculants in Brazil since 2009 (Hungria et al. 2010, 2015, 2016; Hungria 2011; Marks et al. 2015; Fukami et al. 2016), with more than 3 million doses commercialized in 2016, the kinetics of their growth remained to be determined. In DYGS medium, both strains presented similar performances (Fig. 1), but differences were observed when the medium was supplemented with tryptophan (TRP, 500 μg mL^{-1}); for example, Ab-V6 grew faster than did Ab-V5 during the logarithmic phase, but, after that, Ab-V5 had the capacity to resume growth (Fig. 2a). Interestingly, the final cell concentrations for both strains were tenfold increased with the addition of tryptophan (Fig. 2a). Ona et al. (2005) reported similar results with *A. brasilense* strain Sp245.

Ab-V5 and Ab-V6 also differed in relation to the amount of IAA synthesized in DYGS + TRP medium. Low concentrations were detected at 36 h, but after that Ab-V6 synthesized far greater amounts of IAA than did Ab-V5, accumulating twofold more at the final evaluation (1152 h, 48 days) (Fig. 2a). In a study with *A. brasilense* Sp245, IAA biosynthesis was also cell-density dependent, occurring only after carbon (C) sources were consumed; furthermore, IAA was not detected in fed batch, when C was continuously supplied, indicating that C stress is required for IAA biosynthesis (Ona et al. 2005). Comparisons under different growth conditions are limited, but, in N-free medium (NFb) supplemented with 100 μg mL^{-1} of TRP, Masciarelli et al. (2013) reported that two strains of *A. brasilense* accumulated 41.5 μg mL^{-1} (Yu62) and 12.9 μg mL^{-1} (Az39) of IAA, far less than Ab-V6 and Ab-V5. Strain Sp245 synthesized even less IAA in the study performed by Ona et al. (2005), reaching a maximum of 3.8 μg mL^{-1} of IAA after 18 days in medium supplied with 50 μg mL^{-1} of TRP, and the IAA concentration was decreased with 200 μg mL^{-1} of TRP. Interestingly, we have recently identified the main molecules synthesized by both strains in the same medium enriched with TRP as IAA, indole-3-ethanol (TOL), indole-3-lactic acid (ILA) and salicylic acid (SA) (Fukami et al. 2017), but only now, in this study, we have recognized that the differences between the strains were quantitative, with far greater amounts of IAA synthesized by Ab-V6.

The complete quorum sensing (QS) system in Gram-negative bacteria requires at least one LuxI and the homologous LuxR, but in the chromosome there are also *luxR* genes lacking *luxI* genes close to them, called *luxR* solos (Case et al. 2008; Hudaiberdiev et al. 2015). These additional genes compose an incomplete QS system that may either reply to internal AHL signals produced by a non-adjacent *luxI* in the chromosome, or may reply to exogenous signals (Subramoni and Venturi 2009). A recent survey of prokaryotic genomes in the NCBI database detected that 2698 of the 3550 *luxR* genes found were solos (Hudaiberdiev et al. 2015). Important roles have been attributed to LuxR solos, such as plant-growth promotion, nodulation, cell motility, virulence, plasmid transfer, antibiotic synthesis and regulation of QS (Subramoni et al. 2015).

The complete AHL-mediated QS system is not typical of the *Azospirillum* genus; Vial et al. (2006) found that only 4 out of 40 strains produced AHL molecules, all of which belonged to the species *A. lipoferum*. The AHL molecules are structurally identified for two strains: *A. lipoferum* TVV3 produces 3-oxo-C8-AHL, C8-AHL, 3-oxo-C10-AHL, 3-OH-C10-AHL (*N*-3-hydroxy-decanoyl-homoserine lactone) and C10-AHL, whereas strain B518 produces 3-oxo-C6-AHL, C6-AHL, 3-oxo-C8-AHL, 3-OH-C8-AHL (*N*-3-hydroxy-octanoyl-homoserine lactone) and C8-AHL (Vial et al. 2006). As in other strains of *A. brasilense* analyzed by Vial et al. (2006), we did not detect AHL molecules synthesized by Ab-V5 and Ab-V6 (data not shown), and in their genomes we found no *luxI* gene; however, both strains carried several *luxR* solos. As the selective pressure to maintain LuxR solos probably relies on the detection and response to exogenous QS signals (Case et al. 2008), we investigated AHL-mediated QS processes as biofilm formation, EPS synthesis and cell motility in response to the addition of exogenous AHLs. We included molecules with different hydrophobicities, polarities and chain lengths, properties that may affect their diffusion.

In a study of biofilm formation in *Pseudomonas aeruginosa*, the diffusion of AHL (3-oxo-C12-AHL, *N*-3-oxo-dodecanoyl-L-homoserine lactone) was limited by the presence of hydrophobic EPS in the biofilm matrix (Charlton et al. 2000; Boyer and Wisniewski-Dyé 2009). In the same study, C4-AHL, with shorter chain length and lower hydrophobicity, showed lower interaction with the matrix, and became the main factor of the signaling process (Singh et al. 2000; Boyer and Wisniewski-Dyé 2009). In our study, *A. brasilense* Ab-V5 produced twofold more EPS in the presence of a longer chain molecule (3-oxo-C8-AHL), reducing biofilm formation, although the difference was not statistically significant. Effects were strain specific, as in strain Ab-V6 the synthesis of EPS was not affected by the addition of any of the AHL molecules, and biofilm formation was only reduced in the presence of 3-oxo-C8-AHL.

Motility is critical for bacterial survival, allowing the movement towards favorable conditions such as nutrients availability. *Azospirillum* possesses peritrichous flagella used for swarming and a polar flagellum used for swimming (Saikia et al. 2010). Once more, strain Ab-V5 responded to the addition of exogenous AHL molecules for both swimming and swarming phenotypes, whereas Ab-V6 responded only for swarming motility with the specific molecules C12-AHL and C14-AHL at 72 h of incubation (Fig. 4). As

in Ab-V6, other studies indicate that LuxR-type receptors may show specificity towards one or a few molecules, conferring responses to self or non-self signals (Riedel et al. 2001; Boyer et al. 2008; Subramoni et al. 2011). Attachment of bacteria to the root surface is critical for root colonization and involves bacterial motility, biofilm formation, EPS production, and the participation of surface proteins and the polar flagellum (Croes et al. 1993; Danhorn and Fuqua 2007). EPS is also related to the formation of aggregates in *Azospirillum*, also suggesting requirement for attachment to the root surface (Pereg et al. 2016).

Our results indicate that in strain Ab-V5 an AHL-mediated QS system controlled by *luxR* solo regulates biofilm formation and EPS production, as well as cell swimming and swarming motility, which are features that may impact plant–microbe interactions. Plants may also interfere in bacterial QS by producing low-molecular-weight compounds that mimic QS signals, acting as activators or repressors (Teplitski et al. 2000; Bauer and Mathesius 2004; Patel et al. 2013). For example, in *A. brasilense* Sp245 swarming is stimulated by wheat seedling exudates (Borisov et al. 2009). Therefore, we have also investigated the role of the *Azospirillum* QS system in maize-growth promotion, by using the quorum quenching (QQ) strategy with the lactonase *aiiA*. Our results indicate that the inhibition of the AHL-mediated QS system in *A. brasilense* strain Ab-V5 affected maize growth negatively, which may be attributed, among other factors, to an inefficient attachment of the strain to the roots. The results obtained in the greenhouse experiment were also in agreement with the analyses in vitro with strain Ab-V6 strain; growth parameters were similar with the wild-type and the transconjugant strain. This set of data suggests poor participation of the AHL-mediated QS system in Ab-V6 during root colonization.

We should also mention that, although signal communication in bacteria has not yet been completely elucidated, it has been suggested that IAA might act as a bacterial signal, analogous to a QS or autoactivation mechanism (Spaepen et al. 2007; Kim et al. 2011). Apparently Ab-V6 does not use a QS system in processes such as biofilm formation, EPS production or cell motility, but it synthesizes significant amounts of IAA, which might play a role in signaling. In addition, the high amount of IAA synthesized by Ab-V6 can help to explain the higher plant-growth-promoting capacity of this strain, in comparison to Ab-V5. Interestingly, in a previous study, the application of the metabolites of Ab-V6 by foliar spray—probably carrying phytohormones—were more effective in promoting maize growth than the metabolites of Ab-V5 (Fukami et al. 2017).

In conclusion, we report here that two important strains of *A. brasilense*, Ab-V5 and Ab-V6, do not possess a complete AHL-mediated QS system, but that both carry several *luxR* solos, which may recognize external molecules. Strain Ab-V5 perceived exogenous AHL molecules in QS mechanisms that affected biofilm formation, EPS synthesis, as well as cell-swarming and -swimming phenotypes. Contrarily, Ab-V6 apparently does not use the QS system, but rather produces high amounts of IAA and might utilize mechanisms mediated by IAA to mimic a QS signal. In addition to differences between the strains, we have also shown differences in the specificity of LuxR-type receptors to AHL molecules. Finally, by using a QQ strategy we were also able to confirm that QS affects maize-growth promotion by strain Ab-V5, but not by Ab-V6.

Acknowledgements Funded by Embrapa (02.13.08.003.00.00), INCT-Plant-Growth Promoting Microorganisms for Agricultural Sustainability and Environmental Responsibility (465133/2014-2)-Fundação Araucária; Ministerio de Economía y Competitividad (MINECO, AGL2016-77163-R) and CNPq-Universal (400468/2016-6). Authors thank Dr. Allan R.J. Eaglesham for English suggestions, J. Fukami acknowledges a PhD fellowship and a sandwich fellowship to Spain from CAPES and P. del Cerro a Ph.D. fellowship from MECD of the Spanish government (FPU14/00160). M.A. Nogueira and M. Hungria are also research fellows from CNPq.

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5 ARTIGO B – Fitormônios e a indução de genes de tolerância a estresse e de defesa pela inoculação de sementes e foliar com células e metabólitos de *Azospirillum brasilense* promovem o crescimento de milho

RESUMO

Azospirillum spp. são bactérias promotoras de crescimento de plantas mundialmente utilizadas como inoculantes em diversas culturas. Entre os mecanismos benéficos associados ao *Azospirillum*, estão o processo de fixação biológica de nitrogênio e a síntese de fitormônios. No Brasil, a aplicação de inoculantes contendo as estirpes Ab-V5 e Ab-V6 de *A. brasilense* em cereais está crescendo exponencialmente, e neste estudo investigou-se os efeitos da inoculação no milho com essas duas estirpes aplicadas nas sementes ou por pulverização foliar no estágio de crescimento V2.5 – como estratégia para mitigar a incompatibilidade com os pesticidas usados no tratamento de sementes. Investigaram-se também os efeitos da pulverização foliar dos metabólitos das duas estirpes em V2.5. A promoção de crescimento do milho ocorreu tanto pela inoculação das bactérias quanto por seus metabólitos. Quando inoculadas via foliar, embora a sobrevivência de *A. brasilense* nas folhas tenha sido confirmada por microscopia confocal e recuperação celular, poucas células foram detectadas após 24 h, indicando que os efeitos da pulverização foliar também podem estar relacionados aos metabólitos da bactéria. As principais moléculas detectadas nos sobrenadantes de ambas as estirpes foram ácido indol-3-acético, indol-3-etanólico, ácido indol-3-láctico e ácido salicílico. O RT-PCR dos genes relacionados ao estresse oxidativo (*APX1, APX2, CAT1, SOD2, SOD4*) e defesa de plantas (relacionados à patogênese *PR1, prp2* e *prp4*) foi avaliado em folhas e raízes de milho. As diferenças foram observadas de acordo com o gene, tecido vegetal, estirpe e método de inoculação, mas, em geral, a inoculação com *Azospirillum* resultou em *"up regulation"* dos genes de estresse oxidativo nas folhas e *"down-regulation"* nas raízes; ao contrário, em geral, os genes-PR foram *"down-regulated"* nas folhas e *"up-regulated"* nas raízes. Deve-se dar ênfase na aplicação de metabólitos, especialmente Ab-V5 + Ab-V6 que, em geral, resultou em maior *"up-regulation"* dos níveis de expressão dos genes de estresse oxidativo e genes PR, tanto nas folhas quanto nas raízes. Nós levantamos a hipótese de que os benefícios da inoculação de *Azospirillum* nas sementes ou via foliar, bem como a pulverização foliar de metabólitos de *Azospirillum*, estão fortemente relacionado com a síntese de fitormônios e com a indução de genes relacionados à tolerância ao estresse doxidativo e à defesa contra

patógenos.

Palavras-chave: *Azospirillum brasilense.* Pulverização foliar. Estresse oxidativo. Indução de resistência sistêmica. *Zea mays* L.

Fukami *et al. AMB Expr (2017) 7:153*
DOI 10.1186/s13568-017-0453-7

AMB Express

ORIGINAL ARTICLE

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Phytohormones and induction of plant-stress tolerance and defense genes by seed and foliar inoculation with *Azospirillum brasilense* cells and metabolites promote maize growth

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Abstract

Azospirillum spp. are plant-growth-promoting bacteria used worldwide as inoculants for a variety of crops. Among the beneficial mechanisms associated with *Azospirillum* inoculation, emphasis has been given to the biological nitrogen fixation process and to the synthesis of phytohormones. In Brazil, the application of inoculants containing *A. brasilense* strains Ab-V5 and Ab-V6 to cereals is exponentially growing and in this study we investigated the effects of maize inoculation with these two strains applied on seeds or by leaf spray at the V2.5 stage growth—a strategy to relieve incompatibility with pesticides used for seed treatment. We also investigate the effects of spraying the metabolites of these two strains at V2.5. Maize growth was promoted by the inoculation of bacteria and their metabolites. When applied via foliar spray, although *A. brasilense* survival on leaves was confirmed by confocal microscopy and cell recovery, few cells were detected after 24 h, indicating that the effects of bacterial leaf spray might also be related to their metabolites. The major molecules detected in the supernatants of both strains were indole-3-acetic acid, indole-3-ethanol, indole-3-lactic acid and salicylic acid. RT-PCR of genes related to oxidative stress (*APX1*, *APX2*, *CAT1*, *SOD2*, *SOD4*) and plant defense (pathogenesis-related *PR1*, *prp2* and *prp4*) was evaluated on maize leaves and roots. Differences were observed according to the gene, plant tissue, strain and method of application, but, in general, inoculation with *Azospirillum* resulted in up-regulation of oxidative stress genes in leaves and down-regulation in roots; contrarily, in general, *PR* genes were down-regulated in leaves and up-regulated in roots. Emphasis should be given to the application of metabolites, especially of Ab-V5 + Ab-V6 that in general resulted in the highest up-regulation of oxidative-stress and *PR* genes both in leaves and in roots. We hypothesize that the benefits of inoculation of *Azospirillum* on seeds or by leaf spray, as well as of leaf spraying of *Azospirillum* metabolites, are strongly correlated with the synthesis of phytohormones and by eliciting genes related to plant-stress tolerance and defense against pathogens.

Keywords: *Azospirillum brasilense*, Leaf spray, Oxidative stress, Induced systemic resistance, *Zea mays* L.

Introduction

Inoculation with *Azospirillum* spp. has been the subject of several studies (Bashan and Holguin 1998) due to their remarkable capacity of promoting growth of important cereals, i.e. maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.), in addition to several grasses (e.g. Hungria et al. 2010, 2016; Cassán et al. 2015; Pereg et al. 2016). The benefits in plant growth have been attributed to a variety of single or combined mechanisms that act either accumulatively or in cascade (Bashan and de-Bashan 2010), including: enhanced uptake of nutrients and water (Ardakani et al. 2011); production and

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secretion of phytohormones and other signaling molecules such as auxins (Spaepen and Vanderleyden 2015), cytokinins (Tien et al. 1979), gibberellins (Bottini et al. 1989) and salicylic acid (Sahoo et al. 2014); biological nitrogen fixation (Marques et al. 2017); and phosphate solubilization (Rodriguez et al. 2004). However, although *Azospirillum* spp. seem remarkable in their apparent lack of specificity in promoting growth of practically every plant genus and species investigated so far (Pereg et al. 2016), there are also indications that species and strains may vary in determinants of niche-specific adaptation to the rhizosphere that affect plant–microbe interactions (Wisniewski-Dyé et al. 2012). Examples of determinants of adaptation include reactive oxygen species (ROS) as shown with *A. lipoferum* strain 4B in the rice rhizosphere (Drogue et al. 2014). ROS molecules encompass free radicals resulting from oxygen metabolism such as superoxide radicals (O_2^-), hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Bowler et al. 1992; Gill and Tuteja 2010). The most important ROS detoxification mechanism is represented by the activity of superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*) and catalase (*CAT)* enzymes responsible for the scavenging of H_2O_2 by its conversion to water and O_2 (Lamb and Dixon 1997; Asada 1999). In general, ROS detoxification systems vary with plant species, cultivar, and age, and also with the type and duration of abiotic and biotic stress (Hodges et al. 1996).

Another intriguing feature of *Azospirillum* spp. is that although the species comprise non-pathogenic bacteria, they are also able to induce plant-defense mechanisms that may help against further pathogen attacks (Cassán et al. 2014). This property is called 'induced systemic resistance' (ISR), in which the bacterium triggers a plant reaction by emitting signals—the pathogenesis-related proteins (PRs)—that spread systemically throughout the plant and enhance the defensive capacity of distant tissues against infection by pathogens (Van Loon and Bakker 2005). Once induced, plants may remain protected for prolonged periods (Van Loon 2007). For example, there are reports of *Azospirillum* helping protection against *Colletotrichum acutatum* (anthracnose) in strawberry (*Fragaria ananassa* Duch.) (Tortora et al. 2011), and resistance to *Clavibacter michiganensis* subsp. *michiganensis* (bacterial canker), *Xanthomonas campestris* pv. *vesicatoria* (Romero et al. 2003) and *Rhizoctonia solani* (damping-off disease) (Gupta et al. 1995) in tomato plants (*Lycopersicon esculentum* Mill).

Reports of plant-growth improvement by the exogenous application of synthetic growth regulators (e.g. auxins, gibberellins, cytokinins) have long been the subject of studies (e.g. Halmann 1990); more recently, emphasis has also been given to their effect in increasing tolerance of abiotic and biotic stresses (Robert-Seilaniantz et al. 2011). Similar effects on stresses have been reported with the application of jasmonic acid (Bari and Jones 2009; Wasternack 2007; Lorenzo and Solano 2005) and salicylic acid (Bari and Jones 2009), which might induce *PR* (pathogenesis-related) genes and, consequently, enhance resistance to several pathogens.

The commercial use of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 on maize (*Z. mays* L.) and wheat (*T. aestivum* L.) crops in Brazil has grown exponentially since 2010 (Hungria et al. 2010; Hungria 2011). Our research group has started to investigate the effects of foliar-spray inoculation of *Azospirillum*, with the main practical purpose of avoiding the contact of the bacteria with harmful pesticides that are heavily applied to the seeds (Fukami et al. 2016). In this study we confirmed benefits to plant growth by seed and foliar applications of *Azospirillum*, but also verified responses to the application of their metabolites. We then investigated phytohormone production and the response of antioxidant systems with different methods of application of *Azospirillum* strains and their metabolites.

Materials and methods

Bacterial strains and inoculation methods

Bacteria consisted of strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084) of *Azospirillum brasilense* (from the "Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja", WFCC # 1213, WDCM # 1054). Both strains were derived from an *Azospirillum* selection program (Hungria et al. 2010) and are currently employed in commercial inoculants in Brazil (Hungria 2011).

The inoculants were initially prepared in DYGS medium (Rodrigues Neto et al. 1986) and, after growth for 48 h, cell concentrations were adjusted to 10^8 mL^{-1}. For the production of metabolites, inoculants were produced under the same conditions and up to the same concentration and were centrifuged at 5000 rpm for 15 min. By plating the supernatants obtained on DYGS medium we confirmed that they were free of *Azospirillum* cells.

Three methods of inoculation were compared: (i) standard seed inoculation (SI)—considered as the control; (ii) inoculation by leaf spray (ILS) at the V2.5 stage of the maize growth cycle (Hickman and Shroyer 1994); and (iii) application with metabolites from *A. brasilense* strains Ab-V5 and Ab-V6 by leaf spray (MLS) at the V2.5 stage (about 7 days after transplanting) (Hickman and Shroyer 1994).

Seeds were inoculated 1 h before sowing by thoroughly coating them to provide a final concentration of 1.6×10^5 cells $seed^{-1}$. For leaf-spray inoculation, an

aerograph atomizer was employed to mimic the action of a spraying apparatus. The soil surface was covered with aluminum foil to prevent the inoculant reaching it. The final volume of liquid for leaf-spray inoculation was 1 mL (water + inoculant) per pot containing a single plant, and inoculants were diluted with sterile distilled water at 1:1000 (v:v) for spraying, to achieve an application rate of 1.6×10^5 cells plant^{-1}. For leaf spray of metabolites, bacterial exudate corresponding to the same cell concentration as the seed inoculant used for leaf spray was used, with the application of 1 mL per plant corresponding to 1.6×10^5 cells plant^{-1}. Foliar-spray inoculations of pots containing maize plants were performed 7 days after transplanting.

Greenhouse experiment

The experiment was performed under greenhouse conditions, using modified Leonard jars (Vincent 1970) containing sterilized substrate, consisting of a mixture of sand and pulverized coal (3:1, v/v) with application of sterile nutrient solution (Fahraeus 1957). Jars were arranged in a completely randomized design with nine treatments, a non-inoculated control, and six replicates. Each treatment received 60 kg N ha^{-1} (50% of the N application recommended for the crop). Inoculation treatments consisted of mineral-N fertilizer (50% N) and different methods of inoculation: SI (standard seed inoculation at sowing), ILS (inoculation by leaf spray, at the V2.5 stage of maize growth) and MLS (inoculation with metabolites by leaf spray of *A. brasilense* strains Ab-V5 and Ab-V6 at the at the V2.5 stage).

Hybrid maize seeds (DKB330 VT PRO2) were surface-sterilized with 70% ethanol and 3% sodium hypochlorite (Vincent 1970). They were pre-geminated for 48 h at 25 °C in Petri plates containing 1% (v/v) water agar. After germination, two seedlings were transplanted per jar and thinned to one plant after 3 days. Temperature at the greenhouse in controlled by means of air conditioners and average of day and night temperatures were of 28 ± 2.3/23 ± 1.9 °C (day/night); the experiment was performed at the summer growing season, where light intensity is the most adequate for maize growth. Sterile nutrient solution was applied as needed.

At 30 days after transplanting, leaf-chlorophyll contents (CC) were determined according to Kaschuk et al. (2010), based on the "Soil Plant Analysis Development" (SPAD) index, with readings taken from the lowermost third of the +3 leaf (Trani et al. 1983). Biometric parameters of plant height (cm; PH) and culm diameter (mm; CD) of plants were determined with the aid of a digital caliper. Plants were harvested, separating leaves and roots, with three biological replicates. Fresh weight was determined and 2 g of fresh material of each sample were dried at 60 °C for approximately 72 h, until constant weights were achieved; tissues were weighed to estimate the factor for conversion from fresh to dry weight of each replicate. The remaining sampled tissues were frozen in liquid nitrogen and stored at −80 °C until further analyses.

Data obtained were first evaluated for normality and variance homogeneity, followed by the analysis of variance (ANOVA). Tukey's test was employed to compare means in cases where statistical significance had been detected by the ANOVA F test ($p \leq 0.05$). Statistica software version 7.0 was employed.

Identification of phytohormones produced by *A. brasilense* by UHPLC-HRMS/MS

The identification of phytohormones produced by *A. brasilense* strains Ab-V5 and Ab-V6 was performed by ultrahigh-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS/MS). Strains Ab-V5 and Ab-V6 were grown separately in DYGS medium (Rodrigues Neto et al. 1986) without tryptophan (TRP) or in DYGS supplemented with 500 µg mL^{-1} tryptophan (DYGS-TRP medium). Liquid bacterial inocula were incubated at 28 ± 2 °C with orbital shaking at 120 rpm for 14 days. The bacterial cultures were then filtered through nitrocellulose-membrane filters Millipore HA 0.45 µm to obtain the supernatants. The samples were filtered again in a microfiltration membrane, and 5-µL aliquots of each sample were analyzed. Hormones were identified by mass/charge ratio (*m/z*) values and by the retention times of the standard compounds indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-ethanol (TOL), indole-3-lactic acid (ILA), indole-3-pyruvic acid (IPyA), indole-3-propionic acid (IPA), kinetin (Kin), gibberellic acid (GA3), salicylic acid (SA) and jasmonic acid (JA); tri-methyl-indole-3-acetic acid (TmIAA) was used as internal standard.

RNA extraction, cDNA synthesis and quantitative RT-PCR

RNAs of leaves and roots were extracted with TRIzol® (Life Technologies/Thermo Fisher Scientific), and the concentration and purity were evaluated in a NanoDrop® ND1000 spectrophotometer (NanoDrop-Technologies, Inc.), while the integrity was evaluated by gel electrophoresis. Genomic DNA was removed with DNAseI (Invitrogen™) and the first strand of cDNA was synthesized using SuperscriptIII™ reverse transcriptase (Invitrogen™), according to the manufacturer's protocol.

Primers for the RT-qPCR targets were designed using primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) (Table 1) to obtain amplicons of 110–150 bp. The endogenous control genes of maize used were UBCE and UBCP, corresponding to the ubiquitin-conjugating enzyme and the ubiquitin carrier protein,

Table 1 Primers sequences used in the RT-qPCR analyses and sizes of the PCR products obtained

Target gene	Primer sequences (5′–3′)	Amplicon size (pb)
CAT1	*CAT1*F: ACAGCGATGAGTTGTGACGT	113
	*CAT1*R: ATCCTTGCTGCATCTGTCCG	
SOD2	SOD2F: GAGCACCTCAGGATGTTGCT	133
	*SOD2*R: CAGGTGCGCAACATTGTTCA	
SOD4	*SOD4*F: CGTCACCAGCAGGCTAGAAT	139
	*SOD4*R: AGCCAACAGTCCAACACAGT	
APX1	*APX1*F: GATCTTGTGGCTGCAGCATG	111
	*APX1*R: GGTGGACTCGAATTGCAGGA	
APX2	*APX2*F: ACGAAGATGTGATGAACTCAGC	138
	*APX2*R: GGCATTGGCATCGTTAATCAGT	
PR1	*PR1*F: ACTGCAAGCTGATCCACTCC	134
	*PR1*R: TGTTGGTGTCGTGGTCGTAG	
prp2	*prp2*F: ATTCATCGACGCGTCACAGT	117
	*prp2*R: CAGAGACAAGGACACGGACC	
prp4	*prp4*F: TACGACCACGACACCAACAG	143
	*prp4*R: GCTGCAGATGATGAAGACGC	

respectively (Manoli et al. 2012). These genes were used for data normalization of the cycle threshold (Ct) of RT-qPCR amplifications.

RT-qPCR reactions were performed in a 7500 RT-qPCR thermocycler (Applied Biosystems/Life Technologies). The reactions were performed in triplicate for each of the three biological replicates. The Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen™) was used following the manufacturer's instructions. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 2 min, 60 °C for 30 s and 72 °C for 30 s, in 45 cycles.

The data obtained were submitted to the Rest2009 software package (Pfaffl et al. 2002), providing a robust statistical analysis ($p \leq 0.05$).

Confocal laser scanning microscopy of *A. brasilense* on maize leaves

Maize leaf colonization by *A. brasilense* strains Ab-V5 and Ab-V6 expressing the *egfp* (enconding for enhanced green fluorescent protein) and *eyfp* (encoding for enhanced yellow fluorescent protein) reporter genes were analyzed by Confocal Laser-Scanning Microscopy (CLSM). First, plasmids pMP4655 (*egfp*) and pMP4658 (*eyfp*) (Bloemberg et al. 2000) were transferred by conjugation to *A. brasilense* Ab-V5 and Ab-V6. To select the transconjugants of *A. brasilense,* plates with DYGS agar medium (Rodrigues Neto et al. 1986) were supplemented with nalidixic acid (final concentration 40 μg mL^{-1}) and tetracycline (final concentration 20 μg mL^{-1}). The *Azospirillum* strains exhibit intrinsic resistance to the antibiotic nalidixic acid, whereas *Escherichia coli* containing the transfer plasmid shows only tetracycline resistance. Transconjugants were obtained for both strains of *Azospirillum*.

Seeds of maize (hybrid DKB330 VT PRO2) were surface-sterilized (Vincent 1970). Pre-germinated seeds (2 days) were transplanted to test tubes containing 70 mL of sterilized nutrient solution (Fahraeus 1957), and were grown under controlled greenhouse conditions. Mean temperatures during the experiment were of 25/18 °C (day/night) and relative humidity of 70%. At the V2.5 stage of maize growth, plants were singly inoculated by leaf spray with either *A. brasilense* strain Ab-V5 or Ab-V6 harboring the reporter plasmids expressing *egfp* and *eyfp* genes, respectively. Inoculant concentrations applied to the leaves were estimated at 3×10^5 and 7×10^5 cells cm^{-2} of leaf, for strains Ab-V5 and Ab-V6, respectively. At 1 h, 1 and 2 days after leaf spraying, the leaves were examined for the presence of fluorescent bacteria using CLSM equipped with an Ar–Hg laser (Leica TCS SP2, Leica, Wetzlar, Germany); the filter sets for fluorescence microscopy consisted of a 458-nm band-pass excitation and a 520–560 nm emission. Microscopy analyses were performed on intact alive plant tissues. Simultaneously to the analysis by microscopy, the presence of the bacteria on the leaves surface was verified by evaluation of colony-forming units evaluated by the drop plate method (Miles et al. 1938) 1 h, 1 and 2 days after leaf spraying.

Results

Effects of inoculation of *Azospirillum brasilense* and their metabolites on plant-growth parameters

In the greenhouse experiment performed to evaluate effects of inoculation on plant growth, it is worth mentioning that all treatments received the same amount of N-fertilizer, corresponding to 50% (60 kg of N ha^{-1}) of the dose recommended for the maize crop in Brazil. When different methods (via seed—SI at sowing or by leaf spray—ILS at the V2.5 stage) of inoculation of *A. brasilense* strains Ab-V5 and Ab-V6, in single or combined mixtures, or foliar-spray application of their metabolites (MLS), also at the V2.5 stage, were evaluated, statistically significant increases in chlorophyll content (CC) in relation to the non-inoculated control were observed in all treatments except for the SI with Ab-V5; the highest increases were observed in the treatments with MLS of Ab-V6 and MLS of Ab-V5 + Ab-V6, of 109 and 143%, respectively (Table 2). No statistical differences were observed for the parameters of plant high (PH) and culm diameter (CC). Shoot dry weight (SDW) was also improved by all inoculation treatments, except for MLS of Ab-V5. The best inoculation treatment of MLS of strains Ab-V5 and Ab-V6 increased SDW by 72%. In relation to

Table 2 Effects of inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 applied via seeds (seed inoculation, SI, at sowing) or by foliar application (inoculation by leaf spray, ILS, at the V2.5 stage) and of application of their metabolites (MLS) at the V2.5 stage on the chlorophyll content (CC), plant height (PH), culm diameter (CD) and shoot dry weight (SDW) of maize plants (DKB330 VT PRO2)

Treatment	CC (μg cm^{-2})	PH (cm)	CD (mm)	SDW (g pl^{-1})
T1: non-inoculated control	4.45 e[a]	57.33 a	12.22[ns]	3.24 c
T2: SI Ab-V5	5.03 e	57.00 a	13.46	4.56 ab
T3: SI Ab-V6	7.00 d	63.60 a	13.35	5.71 a
T4: SI Ab-V5 + Ab-V6	8.51 c	59.17 a	12.84	4.85 ab
T5: ILS Ab-V5	6.80 d	58.40 a	13.23	4.84 ab
T6: ILS Ab-V6	8.04 c	63.00 a	12.74	4.67 ab
T7: ILS Ab-V5 + Ab-V6	7.07 d	59.67 a	12.97	4.67 ab
T8: MLS Ab-V5	7.08 d	65.40 a	12.16	4.15 bc
T9: MLS Ab-V6	9.30 b	60.50 a	12.49	5.39 ab
T10: MLS Ab-V5 + Ab-V6	10.80 a	64.67 a	13.34	5.57 a
p value	<0.0001	0.03915	0.2609	<0.0001
CV (%)	10.11	8.50	8.69	13.81

All treatments received the equivalent of 60 kg of N ha^{-1} at sowing, plants were grown under greenhouse conditions and harvested at 30 days after transplanting

[a] Means (six replicates) followed by the same letter on the same column are not statistically different according to the Tukey's test ($p \leq 0.05$); [ns] statistically non-significant

this best treatment (T10), we should mention that the effect might be attributed mainly to the metabolites of Ab-V6, as the single metabolites of Ab-V6 (T9), but not of Ab-V5 (T8), resulted in increases in SDW (Table 2).

Identification of phytohormones produced by *A. brasilense* by UHPLC-HRMS/MS

UHPLC-HRMS/MS results obtained in the analysis of the supernatants from *Azospirillum* strains grown in DYGS or DYGS + TRP (tryptophan) media are presented in Table 3. For all samples, supplemented or not with tryptophan, the following main compounds were detected in the metabolites: indole-3-acetic acid (IAA), indole-3-ethanol (TOL), indole-3-lactic acid (ILA) and salicylic acid (SA). Other compounds have also been identified, but in relatively low amounts. In the supernatant of Ab-V5 grown without TRP, we detected gibberellic acid (GA_3) and jasmonic acid (JA) and, when supplemented with TRP, we detected indole-3-propionic acid (IPA). In the supernatant of strain Ab-V6 supplied with TRP we detected GA_3 (Table 3).

Expression of genes related to defense mechanisms in maize

Effects of inoculation with *Azospirillum* or their metabolites on the expression of genes encoding for antioxidant and *PR* proteins were determined by RT-qPCR (Figs. 1, 2, 3). When compared to the non-inoculated control (T1), the gene of the cytosolic isoform *APX1* in maize leaves was significantly up-regulated by inoculation in all treatments except for with the inoculation with strain Ab-V5 by leaf spray (T5) (Fig. 1a). The highest expression was achieved with treatment T10, with inoculation of metabolites of both strains, with an increase of 2.8-fold in comparison to the non-inoculated control. Contrarily, the expression of *APX1* in roots was down-regulated in all treatments (Fig. 1a). The expression of the *APX2* gene in leaves was up-regulated in all treatments, and statistically significant in six out of the nine inoculation treatments (Fig. 1b). Contrarily to *APX1* gene-expression in roots, *APX2* was significantly up-regulated with the metabolites of Ab-V5 (T8), and the metabolites of Ab-V5 + Ab-V6 (T10) (Fig. 1b). The same trend as for *APX* genes was observed with *CAT1* (Fig. 1c). The highest expression in leaves was achieved by seed inoculation with Ab-V5 (T2, 5.5-fold) and spraying of the metabolites of the same strain (T8, 6.9-fold). CAT1 was down-regulated in roots, except for the seed inoculation with Ab-V5 + Ab-V6 (T4) and the metabolite-spray treatments (T8, T9, T10) (Fig. 1c). When *SOD* genes were

Table 3 Identification by ultrahigh-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS/MS) of phytohormones produced by *A. brasilense* strains Ab-V5 and Ab-V6 after 14 days of growth on DYGS medium supplemented or not with tryptophan (TRP, 500 μg mL^{-1})

Treatment	IAA[a]	IBA	TOL	ILA	IPyA	IPA	Kin	GA_3	JA	SA
Ab-V5	+[b]	−	+	+	−	−	−	*	*	+
Ab-V5 + TRP	+	−	+	+	−	*	−	−	−	+
Ab-V6	+	−	+	+	−	−	−	−	−	+
Ab-V6 +TRP	+	−	+	+	−	−	−	*	−	+

[a] Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-ethanol (TOL), indole-3-lactic acid (ILA), indole-3-pyruvic (IPyA), indole-3-propionic acid (IPA), kinetin (Kin), gibberellic acid (GA_3), jasmonic acid (JA), salicylic acid (SA)

[b] + detected; − no detected; * low relation

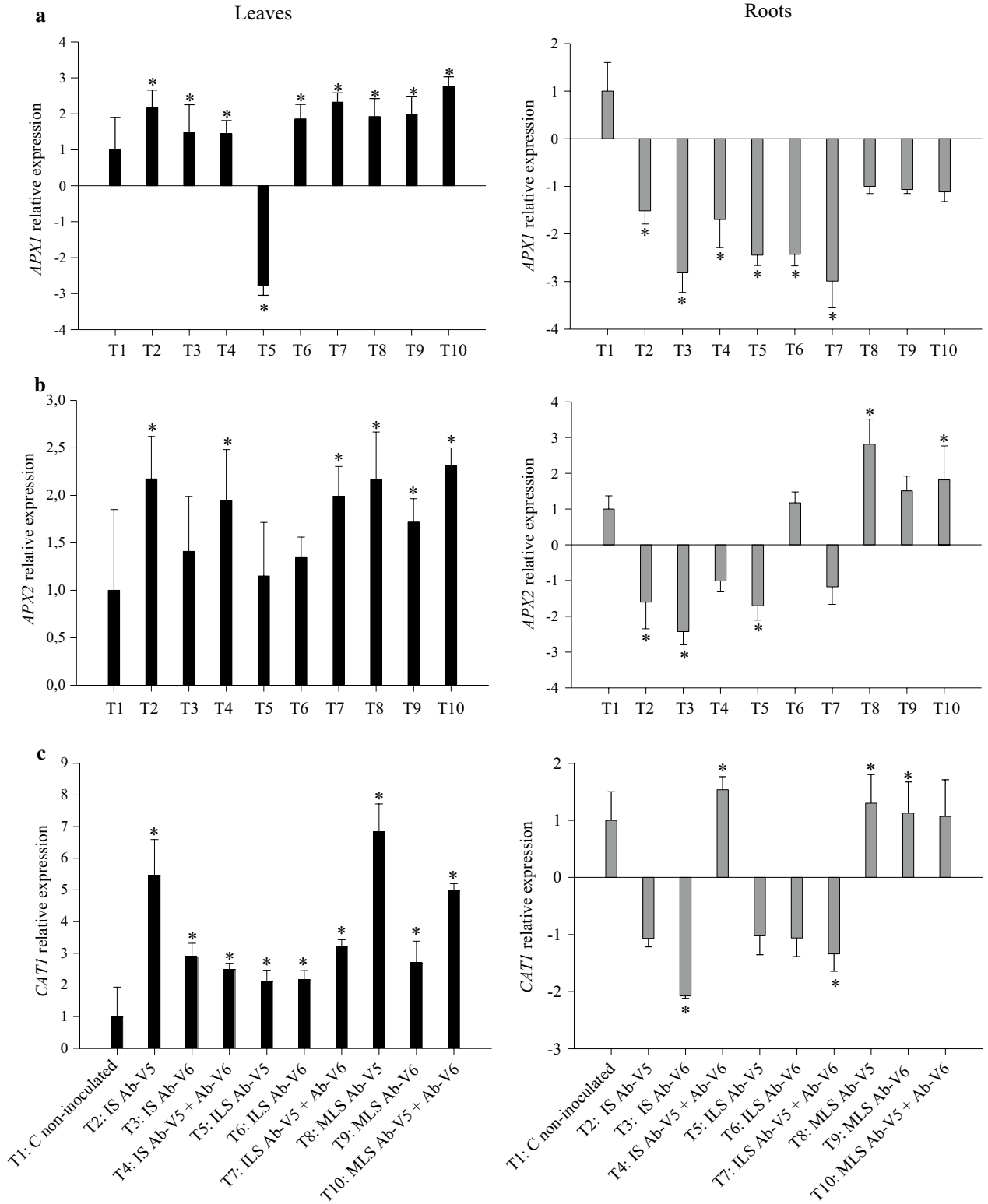

Fig. 1 RT-qPCR analysis of the expression of **a** *APX1*, **b** *APX2* and **c** *CAT* genes in maize leaves and roots when induced by *Azospirillum brasilense* strains Ab-V5 and Ab-V6 inoculated on seeds or by foliar spray, and also buy their metabolites applied by foliar spray. Data ± standard deviation from three biological replicates, each with three technical replicates. Data were normalized in relation to the endogenous control (UBCE and UBCP). The *asterisks* indicate statistically significant expression at the level α = 5%, determined by REST2009 software. *Black bars* leaf and *dark gray bars* root

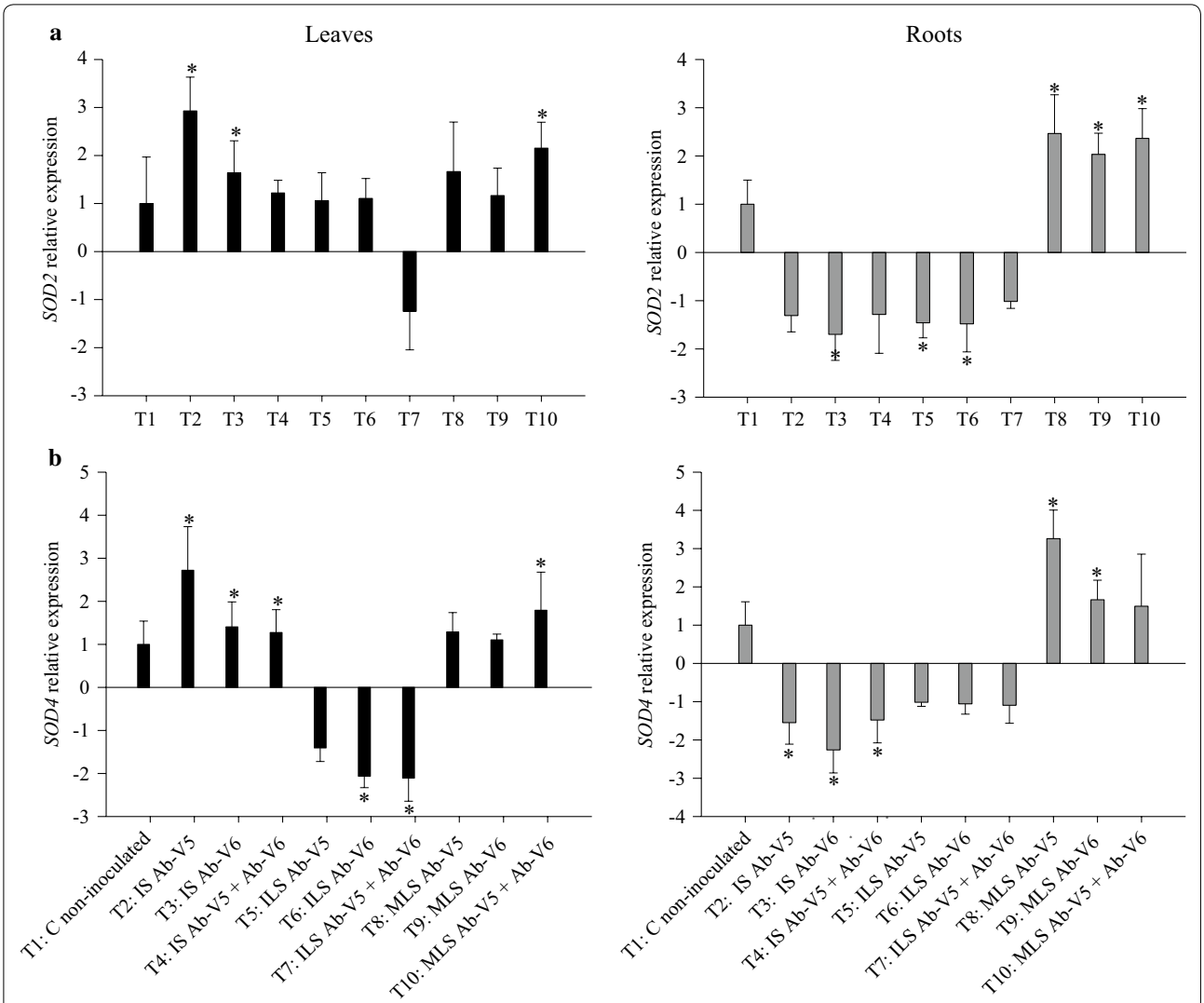

Fig. 2 RT-qPCR analysis of the expression of **a** *SOD2* and **b** *SOD4* genes, as described in the legend of Fig. 1

investigated, up-regulation in leaves was achieved in all treatments, except for when both strains were leaf sprayed for *SOD2* (Fig. 2a) and when *Azospirillum* cells were leaf sprayed for *SOD4* (Fig. 2b). Contrarily, both genes were down-regulated in roots when living cells were applied to seeds or sprayed, whereas the application of metabolites on leaves resulted in up-regulation, with the highest expression of 2.5-fold for *SOD2* and of 3.2-fold for *SOD4* with the metabolites of Ab-V5 (Fig. 2a, b).

Analyzing the *PR* group of genes (*PR1, prp2* and *prp4*) (Fig. 3a–c), in general seed inoculation with single strains (Ab-V5 or Ab-V6) up-regulated gene expression in leaves, whereas seed co-inoculation, and foliar inoculation with single strains down-regulated the genes. Seed inoculation with Ab-V5 (T2) increased by 10.4-fold the expression of *PR1* gene in leaves, whereas, with Ab-V6 (T3), up-regulation was of 5.1- and 5.5-fold for *PR1* and *prp2*, respectively. In relation to the effects of metabolite sprays on gene expression in leaves, emphasis should be given to the Ab-V5 + Ab-V6 treatment (T10), always showing up-regulation, in particular of *prp2* (6.4-fold). In relation to the gene expression in roots (Fig. 3a–c), in general all treatments resulted in up-regulation, but emphasis should be given to the co-inoculation of seeds on the expression of *PR1* (20.2-fold) and *prp4* (4.2-fold), respectively; down-regulation of *prp2* with the metabolites of Ab-V6 of Ab-V5 + Ab-V6 was not statistically significant (Fig. 3b).

Colonization of maize leaves by *A. brasilense*

In order to check whether *A. brasilense* cells are able to colonize maize leaves, strains Ab-V5 and

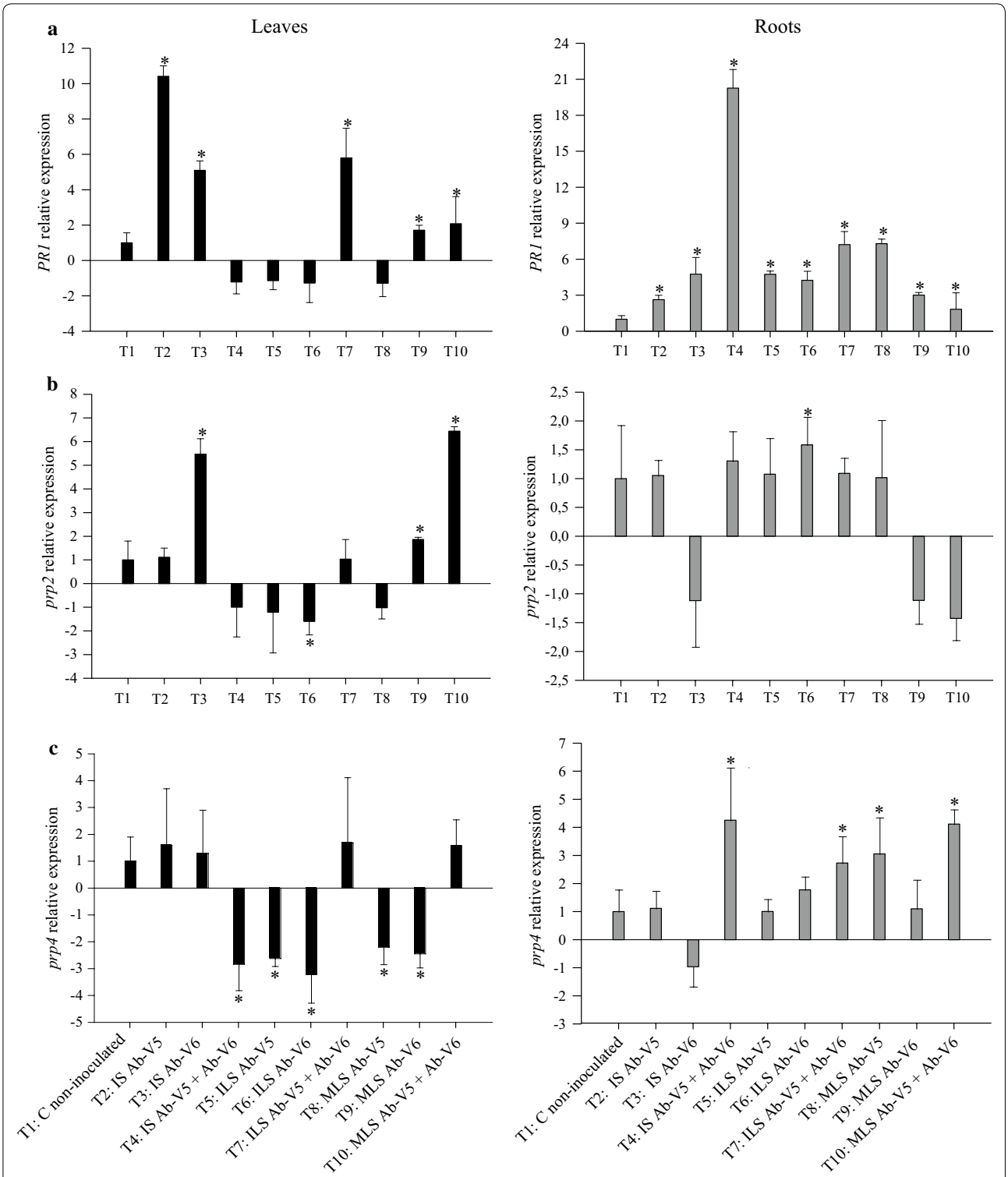

Fig. 3 RT-qPCR analysis of the expression of several **a** *PR1*, **b** *prp2* and **c** *prp4* genes, as described in the legend of Fig. 1

Ab-V6—harboring reporter plasmids expressing *egfp* and *eyfp* genes, respectively—were inoculated by leaf spray. After 1 h, 1 and 2 days of inoculation, the leaves were visualized by CLSM (Fig. 4). After 1 h of inoculation with both strains, (EGFP)-l and (EYFP)-labelled cells indicated that they were able to colonize leaves surface

(Fig. 4a, d), and the same was observed after 1 day of inoculation (Fig. 4b, c). However, after 2 days of inoculation, we were unable to detect the strains on the leaf surfaces. Simultaneously, bacteria counts on leaves surface were performed after 1 h, 1 and 2 days of leaf spraying. Values obtained for colony-forming units (CFUs) were as follows: 2×10^5, 1×10^5 and 5×10^2 CFUs cm^{-2} of leaf for strain Ab-V5 and 6×10^5, 5×10^5 and 5×10^2 CFU cm^{-2} of leaf for strain Ab-V6 after 1 h, 1 and 2 days, respectively. The low bacterial counts at 2 days after inoculation might explain why the bacteria were not visualized by CLSM.

Discussion

When maize growth was evaluated under greenhouse conditions, the benefits of inoculation with *A. brasilense* Ab-V5 and/or Ab-V6 applied to seeds or by foliar application at the V2.5 stage of plant growth were confirmed. The benefits of inoculation with *Azospirillum* at sowing, via seeds or in-furrow, have been demonstrated under greenhouse and field conditions in cereals, with an emphasis on maize (Dobbelaere and Okon 2007; Hungria et al. 2010; Hungria 2011; Okon et al. 2015; Fukami et al. 2016), and increasing use of strains Ab-V5 and Ab-V6 has been exponential in Brazil since 2010 (Hungria 2011). Improvements in grain yields of maize and wheat by foliar application of *Azospirillum* have also been reported (Clemente et al. 2016; Fukami et al. 2016), but the physiological and genetic basis of such improvements have yet to be elucidated.

Intriguing were the positive responses observed in our study to foliar application of metabolites of *Azospirillum*—especially with Ab-V5 + Ab-V6—at the V2.5 stage. Previously, we reported benefits to the maize crop by the

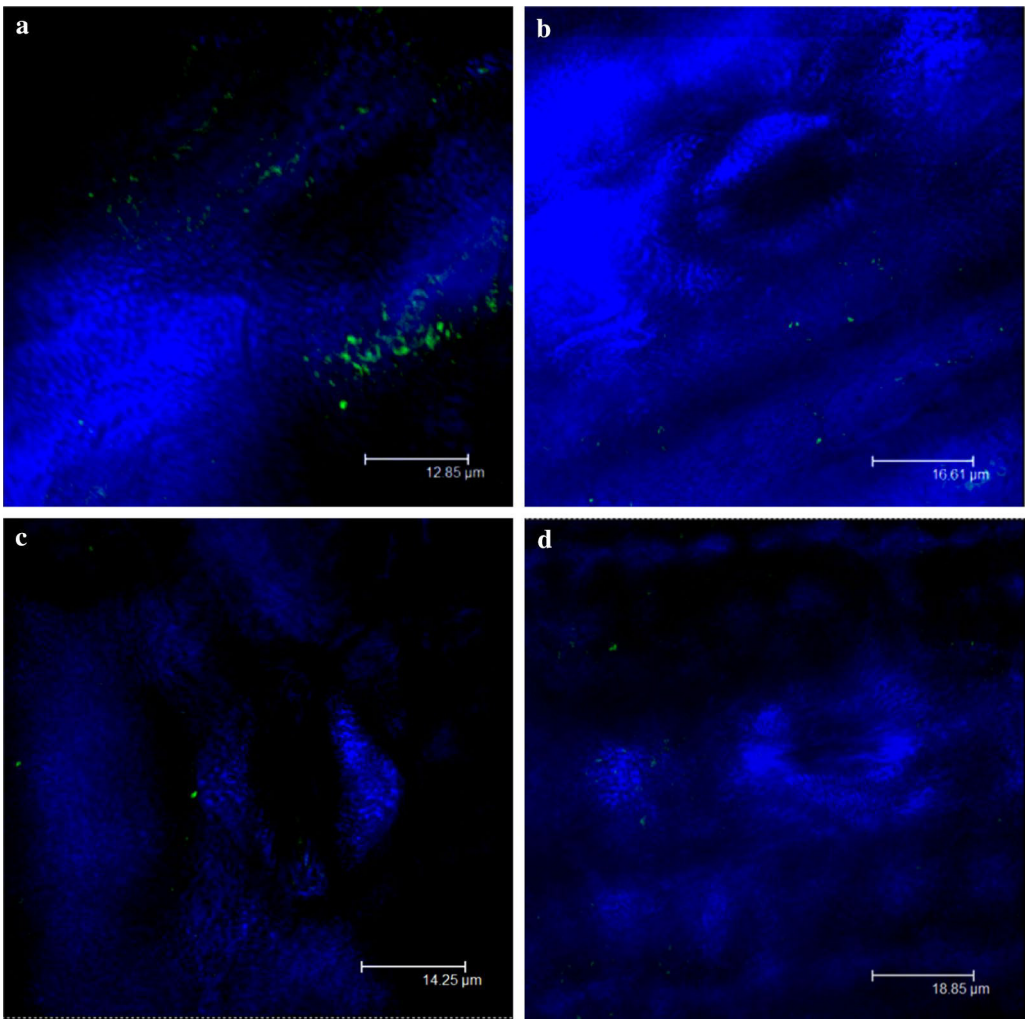

Fig. 4 Confocal laser scanning microscopy analysis of maize leaf surface colonization by *A. brasilense* expressing EGFP when inoculated by leaf spray. **a** *A. brasilense* Ab-V5 after 1 h, **b** *A. brasilense* Ab-V5 after 1 day, **c** *A. brasilense* Ab-V6 after 1 h, **d** *A. brasilense* Ab-V6 after 1 day of inoculation

application of metabolites of rhizobia, suggesting that the effects could be attributed to lipo-chitooligosaccharides (LCOs) or Nod factors (Marks et al. 2013, 2015) synthesized by the bacteria. Positive effects with application of Nod factors in maize, cotton (*Gossypium hirsutum*) and beet (*Beta vulgaris*) were also reported by Smith et al. (2015). However, as far as we are aware, this is the first scientific report of effects of *Azospirillum* metabolites on cereal growth.

To achieve a better understanding of the effects of leaf spraying with *Azospirillum* cells, we investigated the bacterial colonization of leaves by microscopy. Strains Ab-V5 and Ab-V6 were detected on leaves surfaces up to 24 h after inoculation, but the numbers of surviving cells (CFU) were markedly reduced, and, after 48 h, cells were not detected by microscopy. It is possible that the number of recovered cells after 24 h was too low to be detected by CLSM, but the mortality in 24 h was of the order of 1000-fold. Furthermore, we must bear in mind that our experiment was performed under controlled optimized conditions, and that mortality under stressful field conditions—UV light, desiccation, high temperature—would certainly be far higher. Therefore, it is reasonable to suggest that the benefits observed in our study from foliar spraying of *Azospirillum* cells resulted from metabolites present in the inoculant rather than from the living cells.

The first hypothesis to explain increased plant growth by spraying cells or metabolites of *A. brasilense* Ab-V5 and Ab-V6 relies on phytohormone production. We have identified the main molecules in the supernatants of the Ab-V5 and Ab-V6 strains, induced and non-induced with tryptophan, as being indole-3-acetic acid (IAA), indole-3-ethanol (TOL), indole-3-latic acid (ILA) and salicylic acid (SA). Although the physiological functions of TOL and ILA remain unknown, it is possible that intermediates of IAA biosynthesis pathways are converted into these storage compounds whenever necessary (Cassán et al. 2014). In addition, in some combinations of strains and tryptophan we detected traces of gibberellic acid (GA_3) and jasmonic acid (JA). The synthesis of phytohormones by *Azospirillum* has been broadly reported, and may differ between species and strains. The well studied *A. brasilense* strains Cd and Az39 produce IAA, zeatin, GA_3, abscisic acid and ethylene (Perrig et al. 2007), strain UAP154 produces IAA and indole-butyric acid (IBA) (Martínez-Morales et al. 2003), strain 703Ebc produces IAA, TOL, ILA and indole-3-methanol (Crozier et al. 1988), and Sp13t produces IAA, ILA, GA_3 and kinetin (Tien et al. 1979). Tien et al. (1979) also detected gibberellin-like molecules in the supernatants of *A. brasilense* Sp13t at low concentrations, of about 0.05 µg of GA_3 mL^{-1}. However, when applied at concentrations as low as 0.005 µg mL^{-1} to lettuce (*Lactuca sativa*), hypocotyls elongation was promoted and, in pearl millet (*Pennisetum americanum* L.), the number of lateral roots was increased. The benefits confirmed in our study of inoculation of seed with *Azospirillum* at sowing may be attributed to the effects of phytohormones in the rhizosphere, and we propose that these effects also occur from the application of cells and metabolites to the leaves.

Plants synthesize a variety of secondary metabolites that are involved in several physiological processes, and main functions of these compounds lie in providing stress tolerance and defense against pathogens (Sudha and Ravishankar 2002). Previous studies have reported that maize inoculation with *Azospirillum* results in significant changes in the secondary metabolic profiles of roots and shoots, suggesting the presence of finely-tuned interacting mechanisms (Walker et al. 2011). In addition, reactive oxygen species (ROS) in plants contribute to resisting biotic stresses such as pathogens and even symbiotic bacteria (before plant perceives benefit from the symbiosis) (Lamb and Dixon 1997; Santos et al. 2001), as well as to tolerating abiotic stresses (Ozyigit et al. 2016), such as saline conditions (Barakat 2011). However, ROS accumulation results in oxidative damage to cells such as lipid peroxidation with membrane destruction, protein inactivation or DNA mutation (García-Limones et al. 2002). Oxidative stress is relieved in plants by antioxidant enzymes such as catalase, superoxide dismutase and ascorbate peroxidase (Wisniewski-Dyé et al. 2012; Ozyigit et al. 2016). The genes encoding the isoenzymes are found in different plant-cell compartments, such as the cytosolic *SOD2*, *SOD4* (Jung et al. 2001), *APX1* and *APX2*, which are inducible mainly under extreme light or heat-stress conditions (Davletova et al. 2005), and *CAT1*, found in peroxisomes, glyoxysomes and also in the cytosol (Scandalios et al. 1997; Jung et al. 2001). We evaluated the effects of *Azospirillum* and its metabolites on the expression of genes related to the synthesis of the H_2O_2-generating enzyme (*SOD*), the H_2O_2-scavenging enzymes (*CAT* and *APX*) in maize leaves and roots. In general, inoculation of seeds with *A. brasilense* and by foliar spraying resulted in down-regulation transcription of oxidative stress genes (*APX1*, *APX2*, *SOD2*, *SOD4*) in roots, but genes were always up-regulated by leaf spray of metabolites, except for *APX1*. The results suggest that oxidative stress in roots persisted longer with the application of living cells than with their metabolites. Seed inoculation up-regulated all genes in leaves, but when cells were sprayed on leaves, *SOD4* with all strains and *APX1* with Ab-V5 were down-regulated. Similarly to the roots, when the metabolites were sprayed on the leaves the genes—now including *APX1*—were up-regulated. The up-regulation of *APX1* in leaves is particularly

interesting, as *APX* genes might be essential for chloroplast protection during light stress (Pnueli et al. 2003; Mittler et al. 2004; Davletova et al. 2005).

Another defense mechanism of the plants is mediated by ISR (induced systemic resistance), resulting in plant resistance to some pathogenic bacteria, viruses and fungi (Lugtenberg and Kamilova 2009). ISR is triggered by non-pathogenic microorganisms and starts in primary infected parts, extending to other plant tissues (Dutta et al. 2008). Biochemical or physiological changes in plants include induced accumulation of pathogenesis-related (PR) proteins that have different functions like the proteins encoded by *PR1* (a member of a multigene family) (Morris et al. 1998), *PR-2* (a β-1-3-glucanase) (Kauffmann et al. 1987), *PR4* (a chitinase family) (Nasser et al. 1988). Transcriptome studies of *PR* genes with *Azospirillum* sp. B510 applied as inoculum to rice (*O. sativa* L.) reported that one gene was up- and five were down-regulated (Drogue et al. 2014). In another study with *Arabidopsis thaliana* inoculated with *A. brasilense* Sp245, *PR* genes were also up-regulated (Spaepen et al. 2014). In our study, seed inoculation resulted in significant up-regulation of only one *PR* gene in roots, *PR1*, while foliar application in general resulted in up-regulation of *PR1*, *prp2* and *prp4* genes on roots. Up-regulation of *PR1* and *prp4* was also verified with metabolite spray. In relation to the gene expression in leaves, emphasis should be given to single-seed inoculation with both strains that up-regulated all *PR* genes. Interestingly, it has been shown that the use of more than one microorganism optimized ISR responses in pigeon pea (*Cajanus cajan*) (Dutta et al. 2008), similarly to our results with seed inoculation of Ab-V5 + Ab-V6 on roots. *Bacillus subtilis* also up-regulated *PR1* and *PR4*, but not *SOD2* genes in maize roots (Gond et al. 2015). It is also worth mentioning that ISR responses in different tissues from those where the microorganism is applied occurs, e.g. leaf spray with *Pseudomonas fluorescens* in rice induced ISR against the soil-borne plant pathogen *Rhizoctonia solani* (Vidhyasekaran and Muthamilan 1999).

ISR responses to a variety of plant pathogens usually have been associated with the signaling compounds jasmonate and ethylene (Glick 2012; Ahemad and Kibret 2014), the levels of which are increased in tissue independent of SA (Van Loon 2007); this mechanism has also been reported in the association of *Azospirillum* sp. B510 with rice (Yasuda et al. 2009). Indeed, several studies have demonstrated that exogenous applications of SA (Bari and Jones 2009) and JA (Agrawal et al. 2000; Lorenzo and Solano 2005; Wasternack 2007; Bari and Jones 2009) induce *PR* genes and consequently increase the resistance to several pathogens. In addition, exogenous applications of JA also increase the activities of *CAT* and *SOD* enzymes in soybean [*Glycine max* (L.) Merr.] plants stressed by cadmium (Noriega et al. 2012). The ISR might be related also to the reported effects of *A. brasilense* against soil-borne plant pathogens such as *Rhizoctonia* spp. (Russo et al. 2008) and *Fusarium oxysporum* f. sp. *matthiolae* (Somers et al. 2005).

It is worth considering that the exogenous application of synthetic growth regulators (e.g. IAA, GA, kin) has been broadly adopted by foliar spraying due to plant-growth promotion (Halmann 1990), but the commercial products are usually very expensive. However, in our study, the foliar spray of *Azospirillum* metabolites in general improved not only plant growth, but also up-regulated plant genes related to defense mechanisms, and might represent an alternative biological plant regulator.

In conclusion, we reported that, regardless of the method of inoculation—on seeds or by foliar application—the *A. brasilense* strains Ab-V5 and Ab-V6 promoted plant growth. Intriguingly, the foliar application of their metabolites also improved growth. The benefits of cell and metabolite application can be attributed both to the synthesis of phytohormones and to the induction of plant defense-related genes. Clearly, the application of biological low-cost inoculants containing *Azospirillum* cells or their metabolites, promoting plant growth and eliciting plant resistance to biotic and abiotic stresses, have important agronomic implications.

Authors' contributions
JF, FJO, MM and MH initiated and designed the study. FJO and MH contributed with reagents/materials. JF performed the experiments. JF, FJO, MM and MH analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Acknowledgements
Authors thank Dr. Allan R.J. Eaglesham for English suggestions, J. Fukami acknowledges a Ph.D. fellowship and a sandwich fellowship to Spain from CAPES. M. Hungria is also a fellow from CNPq.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data and materials cited on the manuscript are freely available for the scientific community.

Consent for publication
All authors gave the consented for publication.

Ethics approval and consent of participation
The study has not involved any human or animal participation or data.

Funding information
Financed by Embrapa (02.13.08.003.00.00), INCT-Plant-Growth Promoting Microorganisms for Agricultural Sustainability and Environmental Responsibility (465133/2014-2; Fundação Araucária) and Ministerio de Economía y Competitividad (Mineco, AGL2016-77163-R).

Publisher's Note

Received: 25 May 2017 Accepted: 11 July 2017
Published online: 17 July 2017

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6 ARTIGO C – Coinoculação de milho com *Azospirillum brasilense* e *Rhizobium tropici* como estratégia para mitigar estresse salino

RESUMO

As plantas são muito afetadas pela salinidade, mas algumas bactérias promotoras de crescimento de plantas (BPCP) podem desencadear a indução de tolerância sistêmica (ITS), conferindo proteção contra estresses abióticos. Investigaram-se mecanismos de tolerância sistêmica em condições de estresse salino (170 nM NaCl) em plantas de milho inoculadas com *Azospirillum brasilense* estirpes Ab-V5 e Ab-V6 e *Rhizobium tropici* CIAT 899, separadamente, ou co-inoculadas. Em condições de casa de vegetação, as plantas responderam positivamente à inoculação ou coinoculação, mas com diferenças entre estirpes. A inoculação afetou a atividade de enzimas que detoxificam as espécies reativas de oxigênio (ERO) – ascorbato peroxidase (APX), catalase (CAT) e superóxido dismutase (SOD) – principalmente nas folhas. A concentração de prolina nas folhas e raízes, e malondialdeído (MDA) nas folhas – moléculas marcadoras de estresse osmótico nas plantas – foram significativamente reduzidas devido à inoculação, indicando a redução do estresse. Diferenças significativas foram atribuídas à inoculação na expressão de genes relacionados à atividade antioxidante, em geral com *"up-regulation"* de *APX1, CAT1,SOD2* e *SOD4* nas folhas, e *APX2* nas raízes. Os genes relacionados à patogênese *PR1, prp2, prp4* e a proteína *"heat-shock" hsp70* foram *"down-regulated"* nas folhas e raízes. Considerando-se todos os genes avaliados, a inoculação com PBCP pode promover a proteção contra os efeitos negativos do estresse salino. No entanto, diferenças foram observadas entre estirpes, sendo que o *A. brasilense* Ab-V5 não promoveu tolerância ao estresse salino, enquanto que o melhor tratamento para mitigar o estresse salino foram Ab-V6 e coinoculação com Ab-V6+CIAT 899. Assim, a inoculação ou coinoculção com essas estirpes pode representar uma efetiva estratégia para mitigar o estresse salino em milho**.**

Palavras-chave: Estresse oxidativo. Estresse salino. Estresse abiótico. *Zea mays* L.

Functional Plant Biology
https://doi.org/10.1071/FP17167

Co-inoculation of maize with *Azospirillum brasilense* and *Rhizobium tropici* as a strategy to mitigate salinity stress

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Abstract. Plants are highly affected by salinity, but some plant growth-promoting bacteria (PGPB) may trigger induced systemic tolerance (IST), conferring protection against abiotic stresses. We investigated plant mechanisms under saline stress (170 mM NaCl) when maize was singly or co-inoculated with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 and *Rhizobium tropici* strain CIAT 899. Under greenhouse conditions, plants responded positively to inoculation and co-inoculation, but with differences between strains. Inoculation affected antioxidant enzymes that detoxify reactive oxygen species (ROS) – ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) – mainly in leaves. Proline contents in leaves and roots and malondialdehyde (MDA) in leaves – plant-stress-marker molecules – were significantly reduced due to the inoculation, indicating reduced need for the synthesis of these molecules. Significant differences were attributed to inoculation in the expression of genes related to antioxidant activity, in general with upregulation of *APX1*, *CAT1*, *SOD2* and *SOD4* in leaves, and *APX2 i*n roots. Pathogenesis-related genes *PR1*, *prp2*, *prp4* and heat-shock protein *hsp70* were downregulated in leaves and roots, indicating that inoculation with PGPB might reduce the need for this protection. Together the results indicate that inoculation with PGPB might provide protection from the negative effects of saline stress. However, differences were observed between strains, as *A. brasilense* Ab-V5 did not show salt tolerance, while the best inoculation treatments to mitigate saline stress were with Ab-V6 and co-inoculation with Ab-V6+CIAT 899. Inoculation with these strains may represent an effective strategy to mitigate salinity stress.

Additional keywords: abiotic stress, *Azospirillum* spp., oxidative stress, PGPB, *Rhizobium* spp., salinity stress, *Zea mays*.

Received 11 June 2017, accepted 9 September 2017, published online 10 October 2017

Introduction

It has been estimated that around 30% of the world's crop production is lost as a result of abiotic stresses (Goswami *et al.* 2016), including UV radiation, exposure to intense sunlight, drought, salinity, high temperature and chilling (Sharma *et al.* 2012). Among the limiting factors, salinity is considered as a major abiotic stress, impacting agricultural productivity and sustainability due to reductions in photosynthesis, respiration, and protein synthesis (Dwivedi *et al.* 2015). There is evidence that plant growth-promoting bacteria (PGPB) may enhance crop production under environmentally stressful conditions (Bashan and de-Bashan 2010; Dwivedi *et al.* 2015; Cerezini *et al.* 2016; Kaushal and Wani 2016). The genus *Azospirillum* – and the species *Azospirillum brasilense* – is certainly the most studied PGPB (Cassán *et al.* 2014), with reports of remarkable capacity to improve growth in a variety of plant species, including important cereals such as maize (*Zea mays* L.) (Bashan and de-Bashan 2010; Pereg *et al.* 2016). Plant growth promotion by *Azospirillum* relies on an array of mechanisms (Bashan and de-Bashan 2010), including enhanced uptake of nutrients and water (Ardakani *et al.* 2011), synthesis of phytohormones (indole-3-acetic acid, IAA) (Spaepen and Vanderleyden 2015), gibberellins (Bottini *et al.* 1989), cytokinins (Tien *et al.* 1979), salicylic acid (SA) (Sahoo *et al.* 2014), biological nitrogen fixation (BNF) (Marques *et al.* 2017) and solubilisation of phosphate (Rodriguez *et al.* 2004).

The genus *Rhizobium* has long been characterised by symbiotic BNF with a broad range of legumes. Noteworthy are the bacteria belonging to the *Rhizobium tropici* group, which show remarkable intrinsic tolerance to several abiotic

stresses (Ormeño-Orrillo *et al*. 2012; Gomes *et al*. 2015). In legumes, co-inoculation of *Rhizobium*/*Bradyrhizobium* and *Azospirillum* also increases crop yields (Hungria *et al*. 2013, 2015), and positive effects were reported by the co-inoculation of common bean (*Phaseolus vulgaris* L.) with *R. tropici* and *A. brasilense* (Hungria *et al*. 2013; de Souza and Ferreira 2017). In addition, co-inoculation of legumes may help in the mitigation of abiotic stresses, such as drought (Cerezini *et al*. 2016).

In addition to their capacity to fix atmospheric N_2, some *Rhizobium* strains synthesise phytohormones, a property that is expanding their use as PGPB also in non-legumes (García-Fraile *et al*. 2012; Yanni and Dazzo 2015). The plant-growth-promoting role of *Rhizobium* in non-legumes is emphasised by reports of its isolation as endophytic bacteria in a broad-range of non-legumes and countries (Yanni and Dazzo 2015), indicating that these bacteria may play important roles in plant development that are not yet fully understood.

Abiotic stresses enhance the synthesis and accumulation of proline in plants (Molazem and Bashirzadeh 2015), as well as of toxic reactive oxygen species (ROS), including free radicals such as the superoxide anion ($O_2^{\bullet-}$), the hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) during the metabolism of oxygen (Bowler *et al*. 1992). Under normal conditions, ROS are rapidly removed by antioxidative defence mechanisms; however, the removal can be impaired by saline stress (Foyer and Noctor 2003), leading to cell damage. The most usual mechanism to detoxify ROS is by the induction of ROS-scavenging enzymes, such as the ascorbate peroxidase (APX), which converts H_2O_2 to water and oxygen (Lamb and Dixon 1997; Asada 1999). Another mechanism involves the superoxide dismutase (SOD), which converts the superoxide radical to H_2O_2 and is removed by catalase (CAT) (Gill and Tuteja 2010). Previous studies have shown that higher activity of antioxidant enzymes in plants, either constitutive or induced, confers resistance to oxidative damage (Yang *et al*. 2009; Gill and Tuteja 2010).

Some PGPBs can also play protective roles against biotic stresses by triggering reactions in plant roots, with the emission of signals – pathogenesis-related proteins (PRs) – that spread systemically in the plant, enhancing defences against pathogens, a mechanism known as 'induced systemic resistance' (ISR) (van Loon and Bakker 2005). Moreover, the PGPB may also elicit 'induced systemic tolerance' (IST), involving various physiological and biochemical changes in plants (Yang *et al*. 2009) that confer tolerance of abiotic stresses, such as salinity, drought, and high and low temperatures (Maheshwari 2012). Mechanisms related to IST include antioxidant defence (Wang *et al*. 2012), osmotic adjustment (Sarma and Saikia 2014), production of phytohormones such as IAA (Spaepen and Vanderleyden 2015), defence strategies such as the expression of *PR* genes (Kim *et al*. 2014), and the induction of heat-shock proteins (HSPs) (Lim and Kim 2013).

Co-inoculation of non-legumes with *Rhizobium* and *Azospirillum* represents a new strategy that might improve crop yields, but understanding the mechanisms activated by this innovative co-inoculation under saline stress requires further investigation. In our study, we investigated plant mechanisms related to tolerance of salinity in maize plants inoculated with *R. tropici* and *A. brasilense*.

Materials and methods

Bacterial strains and growth conditions

Bacteria consisted of *Azospirillum brasilense* strains Ab-V5 (= CNPSo 2083) and Ab-V6 (= CNPSo 2084), derived from an *Azospirillum* selection program (Hungria *et al*. 2010) and currently employed in commercial inoculants in Brazil (Hungria 2011) for both non-legumes and legumes crops (Hungria *et al*. 2010, 2013). Experiments also included *Rhizobium tropici* strain CIAT 899 (= CNPSo 142, = SEMIA 4077), employed in commercial inoculants for the common bean crop in Brazil (Ormeño-Orrillo *et al*. 2012). The strains are deposited at the Culture Collection of Diazotrophic and Plant-Growth-Promoting Bacteria of Embrapa Soja (WFCC # 1213, WDCM # 1054).

R. tropici was grown in liquid tryptone yeast (TY) medium (Beringer 1974) and *A. brasilense* in liquid DYGS medium (Rodrigues Neto *et al*. 1986), both at 28°C and 120 rpm for 48 h. Inocula containing strains Ab-V5, Ab-V6 and CIAT 899 were applied to the seeds to provide 3×10^5 cells seed^{-1} of each bacterium.

Greenhouse experiments

The experiment was performed under greenhouse conditions, using modified Leonard jars (Vincent 1970) containing sterilised substrate, consisting of a mixture of sand and pulverised coal (3 : 1, v/v) with application of sterile modified nutrient solution (Fahraeus 1957) supplied with 170 mM NaCl. Jars were arranged in a completely randomised design with seven treatments and six replicates. All treatments received 60 kg N ha^{-1} (50% of the recommended dose of nitrogen fertiliser for the maize crop in Brazil), supplied in the nutrient solution as 5 mM of KNO_3. The seven treatments consisted of a non-inoculated control and inoculation with each individual strain (*A. brasilense* strains Ab-V5, Ab-V6 and *R. tropici* CIAT 899) or co-inoculation in pairs (Ab-V5 + Ab-V6; CIAT 899 + Ab-V5; CIAT 899 + Ab-V6).

Seeds of hybrid maize (DKB330 VT PRO2) were surface-sterilised with 70% ethanol and 3% sodium hypochlorite (Vincent 1970). They were pre-geminated for 48 h at 25°C in Petri plates containing 1% (v/v) water agar. Two seedlings were then transplanted per jar and inoculated according to the treatments; plants were thinned to one plant per jar 3 days after transplanting. Mean temperatures during the experiments were of 28/23°C (day/night), and the sterilised nutrient solution was applied as needed.

Plants from all treatments were harvested 32 days after transplanting for measurements of plant components. Before the plants were harvested, chlorophyll content (CC) was determined according to Kaschuk *et al*. (2010) and based on the SPAD (Soil Plant Analysis Development) index, with readings taken from the lowermost third of the +3 leaf (Trani *et al*. 1983).

Biometric parameters of plant height (cm; PH) and culm diameter (mm; CD) were determined with the aid of a digital calliper. Plants were harvested, separating leaves and roots, with three biological replicates. Fresh weight was determined and 2 g of the fresh material of each sample were dried at 60°C for ~72 h, until constant weights were achieved; tissues were weighed to estimate the factor of conversion from fresh to dry weight. The remaining sampled tissues were frozen in liquid nitrogen and stored at −80°C until further analyses.

The experiment was performed twice.

Enzyme assays and lipid peroxidation

The APX activity (ascorbate peroxidase, EC: 1.11.1.11) was determined by monitoring the rate of H_2O_2-dependent oxidation of ascorbate (coefficient of extinction of 2.8 mM^{-1} cm^{-1}) in absorbance at 290 nm for 120 s (Hossain and Asada 1984). The reaction mixture consisted of 50 mM HEPES-NaOH buffer (pH 7.6), 0.2 mM ascorbate, 5 mM H_2O_2 and leaf and root extract, 50 and 100 µL respectively.

CAT activity (catalase peroxidase, EC 1.11.1.6) was evaluated as described by Beers and Sizer (1952), estimating the absorbance reduction at 240 nm for 120 s, as result of H_2O_2 utilisation (extinction coefficient of 39.58 m^{-1} cm^{-1}). The reaction mixture contained 60 mM potassium phosphate buffer (pH 7.0), 5 mM H_2O_2 and leaf and root extract, 50 and 100 µL respectively.

SOD activity (superoxide dismutase, EC: 1.15.1.1) was measured by monitoring spectrophotometrically the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich 1971). The assay mixture in a total volume of 2 mL contained 50 mM Na phosphate buffer (pH 7.8), 33 mM NBT, 10 mM L-methionine, 0.66 mM EDTA, 0.0033 mM riboflavin, and 20 µL of enzyme extract (Bor *et al.* 2003). Enzymatic activities were reported as units per mg of protein (Bradford 1976), using BSA (bovine serum albumin) as a standard.

Lipid peroxidation was performed as described by Hodges *et al.* (1999), with the trichloroacetic acid (TCA) and thiobarbituric acid (TBA), determining malondialdehyde (MDA) as the end product of lipid peroxidation. This method corrects the interference generated by other compounds, and the MDA was reported as nmol mL^{-1} g FW^{-1}.

Enzyme assays and lipid peroxidation were performed in three biological replicates, each with three replicates.

Determination of proline

Proline was determined by the method of Bates *et al.* (1973), modified by Chen *et al.* (2001). Plant tissues were homegeneised with 3% sulfosalicylic acid and filtered. The supernatant was submitted to the reaction with acetic acid and acid ninhydrin at 100°C for 1 h, and the reaction terminated in an ice bath. The absorbance was determined at 520 nm and the concentration of proline was expressed as µM g FW^{-1}. Evaluation was performed in triplicate for each of the three biological replicates.

Total RNA extraction, cDNA synthesis and quantitative RT-qPCR

RNAs of leaves and roots were extracted with TRIzol (Thermo Fisher Scientific), and the concentration and purity were evaluated in a NanoDrop ND1000 spectrophotometer (NanoDrop-Technologies Inc.) and the integrity was evaluated by gel electrophoresis. Genomic DNA was removed with DNAseI (Invitrogen) and the first strand of cDNA was synthesised using SuperscriptIII reverse transcriptase (Invitrogen), according to the manufacturer's protocol.

Primers for the RT-qPCR targets were designed using primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/, accssed 20 September 2017) (Table 1) to obtain amplicons of 110–150 bp. The endogenous control genes of maize used were UBCE and UBCP, corresponding to the ubiquitin-conjugating enzyme and the ubiquitin carrier protein respectively (Manoli *et al.* 2012).

RT-qPCR reactions were performed in a 7500 RT-qPCR thermocycler (Applied Biosystems). The reactions were performed in triplicate for each of the three biological replicates. The Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) was used following the manufacturer's instructions. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 2 min, 60°C for 30 s and 72°C for 30 s, in 45 cycles. The data obtained were submitted to the Rest2009 software package (Pfaffl *et al.* 2002).

Statistical analyses

Data obtained were first evaluated for normality and variance homogeneity, followed by the analysis of variance (ANOVA). Tukey's test was employed to compare means in cases where statistical significance was detected by the ANOVA *F*-test ($P \leq 0.05$). For all analyses, the Statistica ver. 7.0 software (StatSoft) was employed. In the case of RT-qPCR analysis, the Rest2009 software package (Technisch Universitat München) (Pfaffl *et al.* 2002) was used. The software allows the inclusion of more than one gene as endogenous controls for normalisation, improving the confidence of the results. Therefore, we added two genes (UBCE and UBCP) as endogenous controls for data normalisation of cycle threshold (Ct) of RT-qPCR amplifications. The analysis provided a robust statistical support ($P \leq 0.05$).

Results

Plant-growth parameters

Maize inoculation with *A. brasilense* (strains Ab-V5 and Ab-V6) and co-inoculation with *A. brasilense* and *R. tropici* (strain CIAT 899) of plants grown under saline conditions resulted in improved growth in comparison to the non-inoculated control (Table 2). Under greenhouse-controlled conditions and salt stress, shoot

Table 1. Primers sequences used in the RT-qPCR and sizes of the PCR products obtained

Target gene	Primer sequences (5′–3′)	Amplicon size
CAT1	*CAT1*F – ACAGCGATGAGTTGTGACGT	113 bp
	*CAT1*R – ATCCTTGCTGCATCTGTCCG	
SOD2	SOD2F – GAGCACCTCAGGATGTTGCT	133 bp
	*SOD2*R – CAGGTGCGCAACATTGTTCA	
SOD4	*SOD4*F – CGTCACCAGCAGGCTAGAAT	139 bp
	*SOD4*R – AGCCAACAGTCCAACACAGT	
APX1	APX1F – GATCTTGTGGCTGCAGCATG	111 bp
	*APX1*R – GGTGGACTCGAATTGCAGGA	
APX2	*APX2*F – ACGAAGATGTGATGAACTCAGC	138 bp
	*APX2*R – GGCATTGGCATCGTTAATCAGT	
PR1	*PR1*F – ACTGCAAGCTGATCCACTCC	134 bp
	*PR1*R – TGTTGGTGTCGTGGTCGTAG	
prp2	*prp2*F – ATTCATCGACGCGTCACAGT	117 bp
	*prp2*R – CAGAGACAAGGACACGGACC	
prp4	*prp4*F – TACGACCACGACACCAACAG	143 bp
	*prp4*R – GCTGCAGATGATGAAGACGC	
hsp70	*hsp70*F – TTGGTTAGTGTTCGAGGTTTGG	110 bp
	*hsp70*R – TTTCTGAGAAGTTCACCACAGG	

dry weight (SDW) was significantly increased by the inoculation with *A. brasilense* Ab-V6 and by co-inoculation with Ab-V6 and *R. tropici* CIAT 899, by 124 and 83%, respectively, in comparison to the non-inoculated control. Culm diameter (CD) was also increased by inoculation with Ab-V6, Ab-V5+Ab-V6 and Ab-V5+CIAT 899. However, no treatment effects were found in chlorophyll content (CC) or plant height (PH) (Table 2). The experiment was repeated and the data confirmed.

Enzyme assays, lipid peroxidation and determination of proline

Results regarding the activities of antioxidant enzymes (APX, CAT, SOD), lipid peroxidation (estimated by the MDA content) and proline accumulation were examined in maize leaves and roots. All treatments, except for the co-inoculation with Ab-V6 + CIAT 899 decreased significantly the activity of APX in leaves when compared with the non-inoculated control; activity in roots was not decreased with Ab-V6 and Ab-V6 + CIAT 899 (Fig. 1*a*). CAT activity in leaves was significantly increased by inoculation with Ab-V5 + Ab-V6, but was significantly decreased when strain Ab-V5 was used as single inoculum (Fig. 1*b*). In roots, CAT activity was significantly decreased by the inoculation with Ab-V5 + Ab-V6, and with CIAT 899 alone or co-inoculated with Ab-V5 or Ab-V6 (Fig. 1*b*). In relation to lipid peroxidation in plants (MDA), for leaves of plants grown under saline stress, for all treatments except when inoculated Ab-V5 and with Ab-V5 + CIAT 899 the MDA was significantly reduced (Fig. 1*c*). However, roots of plants co-inoculated with CIAT 899 + Ab-V6 exhibited higher lipid peroxidation (Fig. 1*c*). None of the inoculation treatments affected significantly SOD activity in leaves in relation to the non-inoculated control (Fig. 1*d*), and we were unable to detect SOD activity in roots.

Proline content in maize leaves was decreased in all inoculation treatments, more strongly in co-inoculation with CIAT 899 and Ab-V5 or Ab-V6, by 52 and 63% respectively (Fig. 2). Likewise, proline accumulation in roots was statistically decreased in all inoculated and co-inoculated treatments, except for when singly inoculated with Ab-V5. Proline contents were far higher in shoots than in roots (Fig. 2).

Table 2. Shoot dry weight (SDW), culm diameter (CD), plant height (PH) and chlorophyll content (CC) in a greenhouse experiment performed with hybrid maize DKB330 VT PRO2 grown under saline stressing conditions (170 mM NaCl) in response to inoculation with *Azospirillum brasilense* (Ab-V5 and Ab-V6) and *Rhizobium tropici* (CIAT 899)

Parameters determined 32 days after transplanting. Means (six replicates) followed by the same letter on the same column are not significantly different according to the Tukey's test ($P \leq 0.05$); ns, not significant ($P > 0.05$)

Treatment	SDW (g plant^{-1})	CD (mm)	PH (cm)	CC (μg cm^{-2})
T1: Control non-inoculated	2.26b	11.26b	51.50ns	4.61ns
T2: Ab-V5	3.56ab	12.09ab	53.50	5.84
T3: Ab-V6	5.07a	13.17a	54.50	5.33
T4: Ab-V5 + Ab-V6	3.80ab	13.08a	54.58	5.50
T5: CIAT 899	3.77ab	12.35ab	58.17	4.15
T6: CIAT 899 + Ab-V5	3.62ab	12.53a	57.90	5.30
T7: CIAT 899 + Ab-V6	4.13a	12.34ab	56.00	4.88
P-value	0.0021	0.0313	0.0358	0.3590
CV (%)	25.92	7.79	6.70	25.94

Expression of genes related to defence mechanisms in maize

PGPB and rhizobial treatments in maize plants under saline conditions led to differential regulation of the antioxidant expression of *APX*, *CAT* and *SOD* genes. The cytosolic isoform *APX1* in maize leaves was significantly upregulated by the inoculation with Ab-V6, CIAT 899 and Ab-V6+CIAT 899, but downregulated with Ab-V5 (Fig. 3*a*). Downregulation of *APX1* was observed in roots of all inoculated treatments (Fig. 3*a*). Contrary to *APX1*, the cytosolic *APX2* gene was downregulated in leaves of inoculated plants, but upregulated in roots, with an emphasis on the co-inoculation of Ab-V6+CIAT 899, with an increase of 14.6-fold (Fig. 3*b*). In relation to the expression of the *CAT1* gene, inoculation resulted in upregulation in leaves, except in the presence of Ab-V5, with significant increases with Ab-V6 and co-inoculation of CIAT 899 with either Ab-V5 or Ab-V6 (Fig. 3*c*). Contrarily, all inoculation treatments downregulated *CAT1* expression in roots (Fig. 3*c*).

For the expression of the *SOD2* gene in leaves, significant upregulation in leaves was observed by inoculation with CIAT 899 singly or co-inoculated with Ab-V5 or Ab-V6, whereas downregulation occurred in roots of all inoculated treatments (Fig. 4*a*). Inoculation with strains Ab-V5 or Ab-V6 and co-inoculation of Ab-V6 + CIAT 899 resulted in significant upregulation of *SOD4* in leaves, and co-inoculation with CIAT 899 along with either Ab-V5 or Ab-V6 resulted in upregulation in roots (Fig. 4*b*). However, the other inoculation treatments resulted in downregulation of *SOD4* expression in roots (Fig. 4*b*).

To summarise, in general the genes *APX1*, *CAT1*, *SOD2* and *SOD4* encoding antioxidant enzymes were upregulated in leaves and downregulated in roots of inoculated plants. However, single inoculation with Ab-V5 usually did not follow this pattern, while inoculation with Ab-V6 and co-inoculation of Ab-V6 + CIAT 899 showed stronger upregulation of genes related to antioxidant enzymes. These results, with an emphasis on the co-inoculation with Ab-V6 + CIAT 899 are in agreement with the lower accumulation of MDA and proline, indicating a protective effects in maize under saline stress by some inoculation treatments.

In relation to the group of PR genes, *PR1*, *prp2* and *prp4*, in general they were downregulated both in leaves and in roots of inoculated treatments (Fig. 5*a–d*). One exception was with the co-inoculation of Ab-V5 + CIAT 899 for the *prp2* gene in leaves. Likewise, both in maize leaves and roots all inoculation treatments resulted in downregulation of the *hsp70* gene (Fig. 5).

Discussion

A better understanding of the effects of saline stress in plants is critically needed, due to increasing problems of soil salinity in many parts of the world. The use of PGPBs has been proposed as a management practice to mitigate the effects of salinity on crops (Bashan and de-Bashan 2010; Dwivedi *et al.* 2015). *Azospirillum* – with an emphasis on the species *A. brasilense* – is probably the most studied PGPB to mitigate saline stress, with

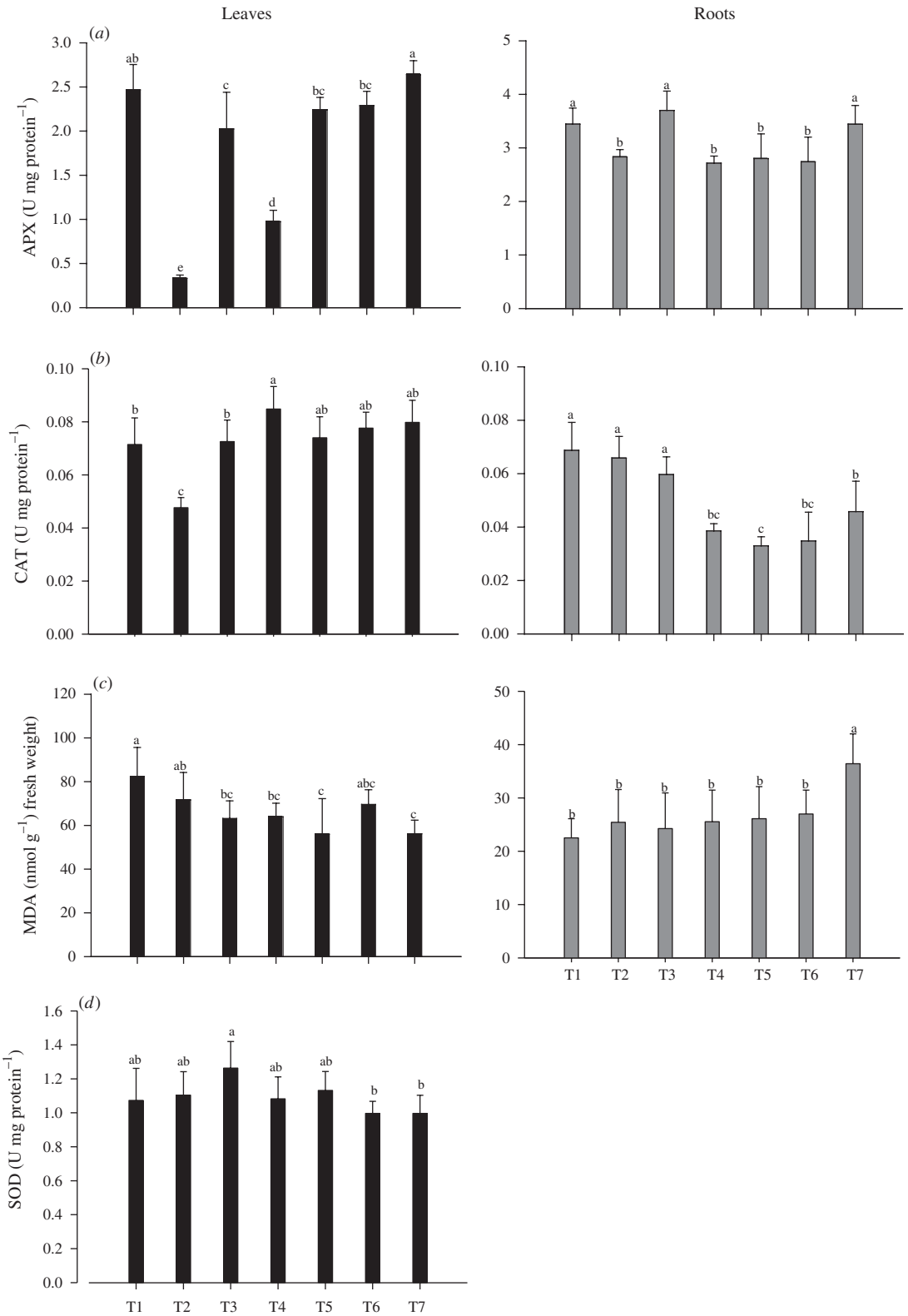

Fig. 1. Enzyme assays and lipid peroxidation in response to co-inoculation of *Azospirillum brasilense* and *Rhizobium tropici* in maize grown under saline stress conditions (170 mM NaCl). Data represent the means ± s.d. from three biological replicates, each with three further replicates. Means followed by the same letter on the same bars are not significantly different according to Tukey's test ($P \leq 0.05$). Black bars, leaf; dark grey bars, root. (*a*) Ascorbate peroxidase (APX); (*b*) catalase (CAT); (*c*) lipid peroxidation (MDA); (*d*) superoxide dismutase (SOD).

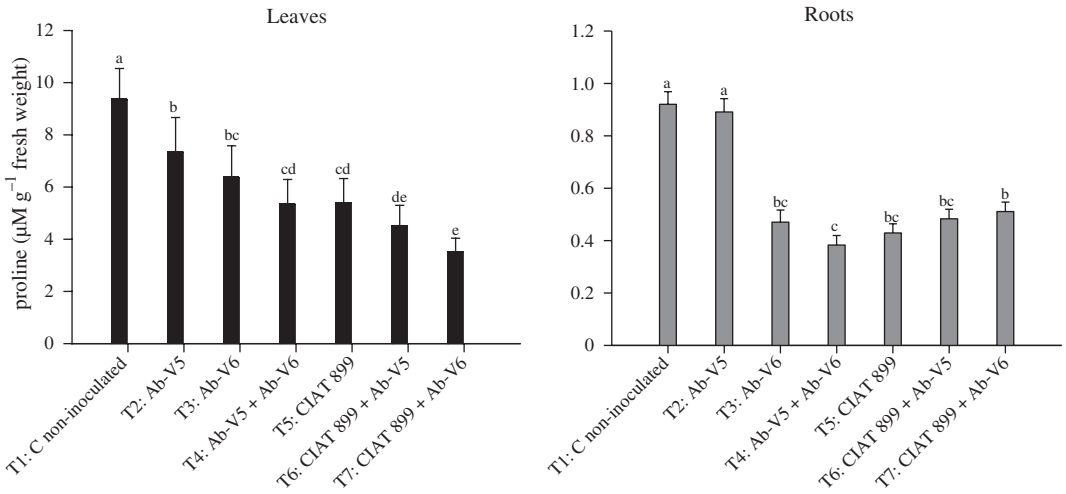

Fig. 2. Proline content in response to co-inoculation of *Azospirillum brasilense* and *Rhizobium tropici* in maize grown under saline stress conditions (170 mM NaCl). Data represent the means ± s.d. from three biological replicates, each with three further replicates. Means followed by the same letter on the same bars are not significantly different according to Tukey's test ($P \leq 0.05$). Black bars, leaf; dark grey bars, root.

inoculation experiments performed with several plant species, including maize (Hamdia *et al*. 2004). Further, recognition of rhizobia as PGPBs in non-legumes is increasing (García-Fraile *et al*. 2012; Yanni and Dazzo 2015), and many rhizobial strains are known to be highly stress-tolerant in comparison to their compatible host legumes (Vriezen *et al*. 2007). Indeed, *Rhizobium* has shown effectiveness in N_2-fixation or growth promotion of both legumes and non-legumes under saline conditions, e.g. with pea (*Pisum sativum*), faba bean (*Vicia faba*) (Cordovilla *et al*. 1999) and lettuce (*Lactuca sativa*) (Han and Lee 2005). There are also reports showing benefits of co-inoculation under salt stress, such as the relieve of the negative effects in common bean co-inoculated with *R. tropici* CIAT 899 and *A. brasilense* Cd (Dardanelli *et al*. 2008).

R. tropici CIAT 899 shows intrinsic tolerance of several abiotic stressing conditions (Ormeño-Orrillo *et al*. 2012; Gomes *et al*. 2015), and *A. brasilense* strains Ab-V5 and Ab-V6 have been successfully used in commercial inoculants in Brazil for maize, wheat, soybean and common bean crops (Hungria *et al*. 2010, 2013, 2015; Hungria 2011). Therefore, we have chosen strains *R. tropici* CIAT 899 and *A. brasilense* Ab-V5 and Ab-V6 to study their possible effects on plant mechanisms that may help in the mitigation of saline stress.

One of the mechanisms reported for PGPBs to enhance plant tolerance of abiotic stresses is known as induced systemic tolerance (IST) (Kaushal and Wani 2016), including antioxidant defence (Wang *et al*. 2012), osmotic adjustment (Sarma and Saikia 2014), synthesis of phytohormones such as IAA (Spaepen and Vanderleyden 2015) and expression of genes such as *PRs* (Kim *et al*. 2014) and HSPs (Lim and Kim 2013). Salt stress leads to increased reactive oxygen species (ROS) that result in plant cellular toxicity (Munns and Tester 2008) and in lipid peroxidation (Sunkar *et al*. 2003). Sophisticated mechanisms are required to act as signalling molecules regulating adaptive responses to salt stress (Shafi *et al*. 2015), including APX (ascorbate peroxidase), CAT (catalase) and SOD (superoxide dismutase) enzymes, that scavenge ROS molecules; therefore, these enzymes can be used as stress markers. In our study, most inoculation treatments did not affect APX, CAT or SOD activities in leaves; however, single inoculation of strain Ab-V5 resulted in the lowest protective effect against salt when APX and CAT in leaves were considered. Similar results of decreased APX and CAT activities were reported for wheat co-inoculated with *Bacillus subtilis* and *Arthrobacter* sp. (Upadhyay *et al*. 2012), and for APX activity in lettuce co-inoculated with *Serratia* sp. and *Rhizobium* sp. (Han and Lee 2005) under salinity stress. In contrast, the treatment with more positive responses was the co-inoculation of Ab-V6 + CIAT 899.

Malondialdehyde (MDA) is an end product of polyunsaturated fatty acid oxygenation and is commonly used as an indicator of oxidative stress for assessing lipid peroxidation (Hodges *et al*. 1999). We observed increased lipid peroxidation in leaves, but not in roots, associated with the MDA content in non-inoculated plants under salt stress, whereas plant inoculation decreased damage in leaves, except for the treatments with Ab-V5 and with Ab-V5 + CIAT 899. The best induced antioxidative defence was achieved with *A. brasilense* Ab-V6 + CIAT 899, reducing the need of the plant to synthesise MDA. Khalid *et al*. (2017) also reported that *A. brasilense* regulated oxidative stress, favouring cell membrane integrity.

Proline is another stress-marker molecule reported to accumulate in the tissues of plants under abiotic stresses, including salinity (Goswami *et al*. 2016); it accumulates rapidly after the onset of stresses (Carillo *et al*. 2008). In our study, proline decreased significantly in leaves and roots of all inoculated plants, except for the roots inoculated with Ab-V5. Similar results were reported in maize both in leaves and in roots inoculated with *A. brasilense* and grown under saline stress (Hamdia *et al*. 2004). Again, emphasis should be given to the best performance of plants co-inoculated with Ab-V6 + CIAT 899, with the lowest content in leaves. Our results suggest a protective effect of the inoculated or co-inoculated bacteria on the

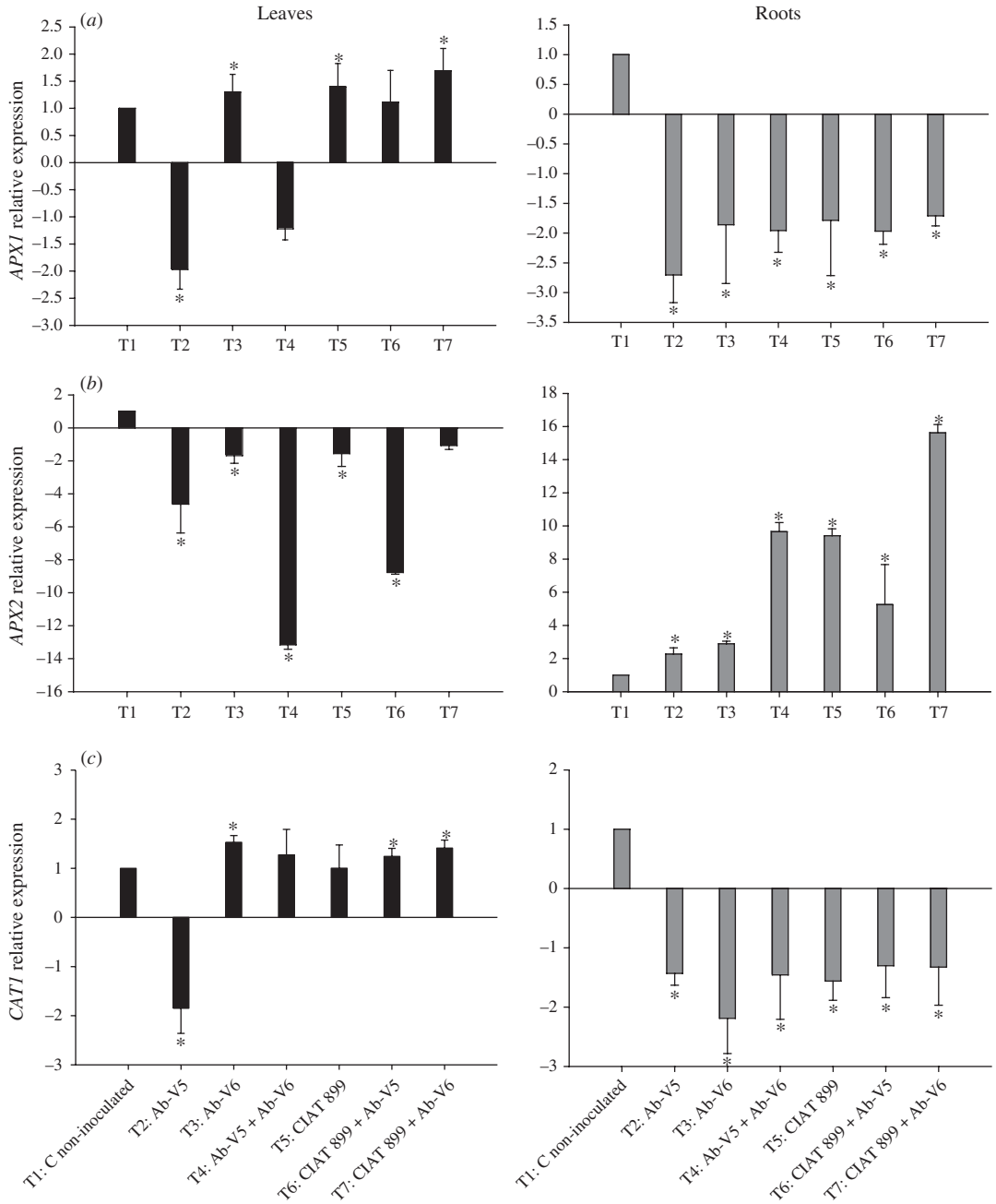

Fig. 3. RT-qPCR analysis of the expression of *APX* and *CAT1* genes in response to co-inoculation of *Azospirillum brasilense* and *Rhizobium tropici* in maize grown under saline stress conditions (170 mM NaCl). Data represent the means ± s.d. from three biological replicates, each with three further replicates. Data were normalised in relation to the endogenous controls UBCE and UBCP. Statistically significant expression at the level $\alpha = 5\%$ is indicated, *, determined by the REST2009 software. Black bars, leaf; dark grey bars, root. (*a*) *APX1* expression; (*b*) *APX2* expression; (*c*) *CAT1* expression.

plants, resulting in lower accumulation of this amino acid in tissues.

At the transcription level, *APX1*, *CAT1 and SOD2* genes in leaves and roots and *SOD4* gene in roots were downregulated in maize inoculated with Ab-V5 compared with the non-inoculated control; similar responses were observed for plant growth, adding more evidences that the strain has low tolerance of salinity. Therefore, although *A. brasilense* Ab-V5 has been reported as an excellent maize-growth promoter under field conditions, in these reports the soils were not saline-limited (Hungria *et al*. 2010; Matsumura *et al*. 2015). These results confirm the importance of considering strain/plant genotype interactions when searching for tolerance of abiotic stresses, as pointed out by Dwivedi *et al*. (2015).

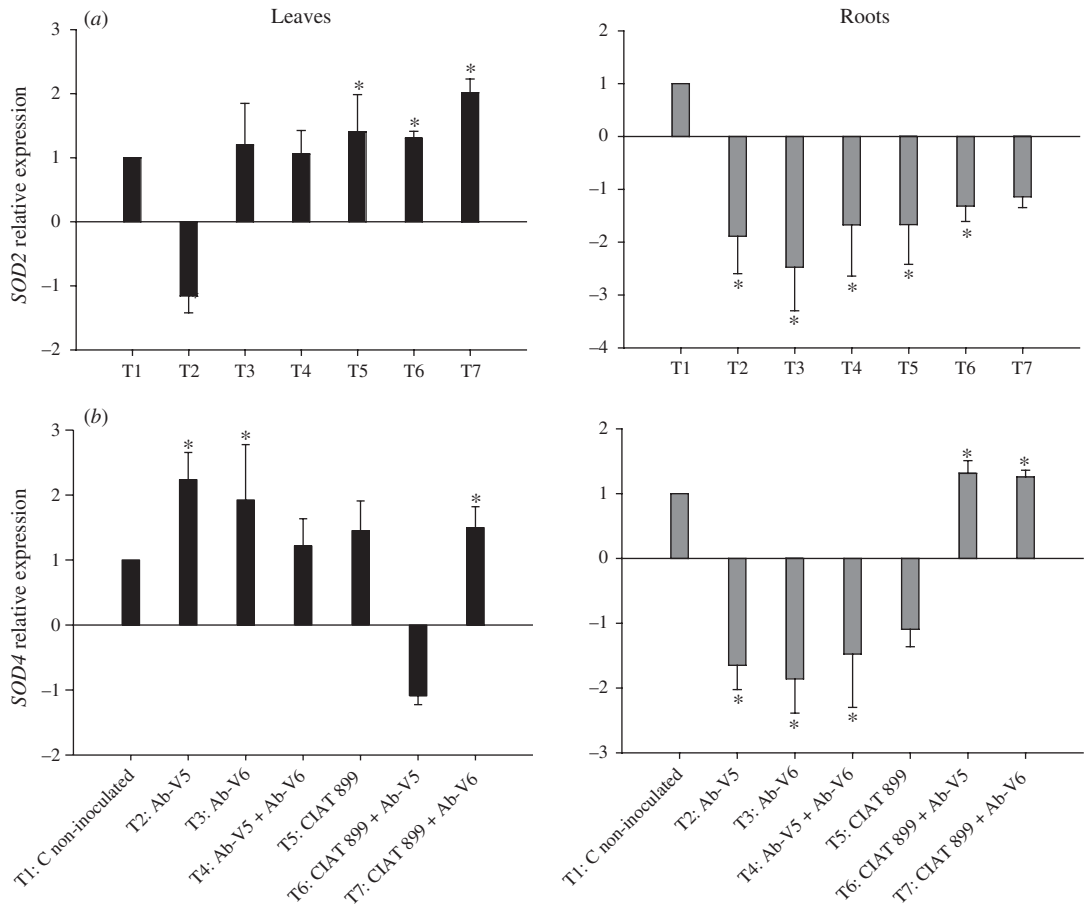

Fig. 4. RT-qPCR analysis of the expression *SOD* genes in response to co-inoculation of *Azospirillum brasilense* and *Rhizobium tropici* in maize grown under saline stress conditions (170 mM NaCl). Data represent the means ± s.d. from three biological replicates, each with three further replicates. Data were normalised in relation to the endogenous controls UBCE and UBCP. Statistically significant expression at the level $\alpha = 5\%$ is indicated, *, determined by the REST2009 software. Black bars, leaf; dark grey bars, root. (*a*) *SOD2* expression; (*b*) *SOD4* expression.

The transcriptions of *APX1*, *CAT1*, *SOD2* and *SOD4* genes were upregulated in leaves of plants inoculated with *A. brasilense* Ab-V6, with *R. tropici* CIAT 899 and with Ab-V6 + CIAT 899, suggesting that these inoculation treatments had the ability to modulate plant-defence responses against ROS. However, the same genes were downregulated in roots, except for *SOD4* in co-inoculation. The cytosolic genes *APX1* and *APX2* in plants are strongly involved in the protection against abiotic stresses (Shigeoka and Maruta 2014). We noted that in our study, *APX1* and *CAT1* were upregulated in leaves of inoculated plants, whereas *APX2* was downregulated; however, in roots, *APX1* and *CAT1* were downregulated and *APX2* was upregulated. A possible explanation may be related to the sensitivity of the *APX2* gene to the catalase, since both *APX1* and *CAT1* responded contrarily to *APX2*. In *Arabidopsis*, it has been shown that, under high light stress, the *APX2* gene is inhibited by catalase and is not relieved by the superoxide dismutase (Karpinski *et al*. 1999); furthermore, Jiang *et al*. (2016) showed that the expression of the *APX1* gene was higher in leaves and lower in roots. *CAT1* is localised in the peroxisomes and in the cytosol (Gill and Tuteja 2010), and expresses both in roots and in leaves (Scandalios *et al*. 1997). Medici *et al*. (2004) reported increased catalase activity in the roots of maize supplied with high N, and the downregulation of *CAT1* gene in our study might be explained by N deficiency due to the salinity. We also observed that *SOD2* and *SOD4* genes, which encode cytosolic Cu/Zn-SOD enzymes (Jung *et al*. 2001), in maize roots were downregulated in most inoculation treatments, similar to the results observed with Kentucky bluegrass (*Poa pratensis*) under abiotic stress (water restriction) (Bian and Jiang 2009).

Among the genes related to the defensive response, the pathogenesis related (*PR*) genes encode for proteins that have different functions, such as PR1 (a member of a multigene family (Morris *et al*. 1998)), *prp*-2 (a β-1-3-glucanase (Kauffmann *et al*. 1987)) and *prp4* (a chitinase (Nasser *et al*. 1988)). In our study, these genes were generally downregulated in both leaves and roots, what might indicate that the protection related to the PGPB might reduce the need for expression of these genes. Another interesting gene is the *hsp70*, which encodes for a member of the small heat-shock protein family of chaperones, and play important roles in plant-stress tolerance (Bartels and Sunkar 2005). In our study, in the treatments inoculated with

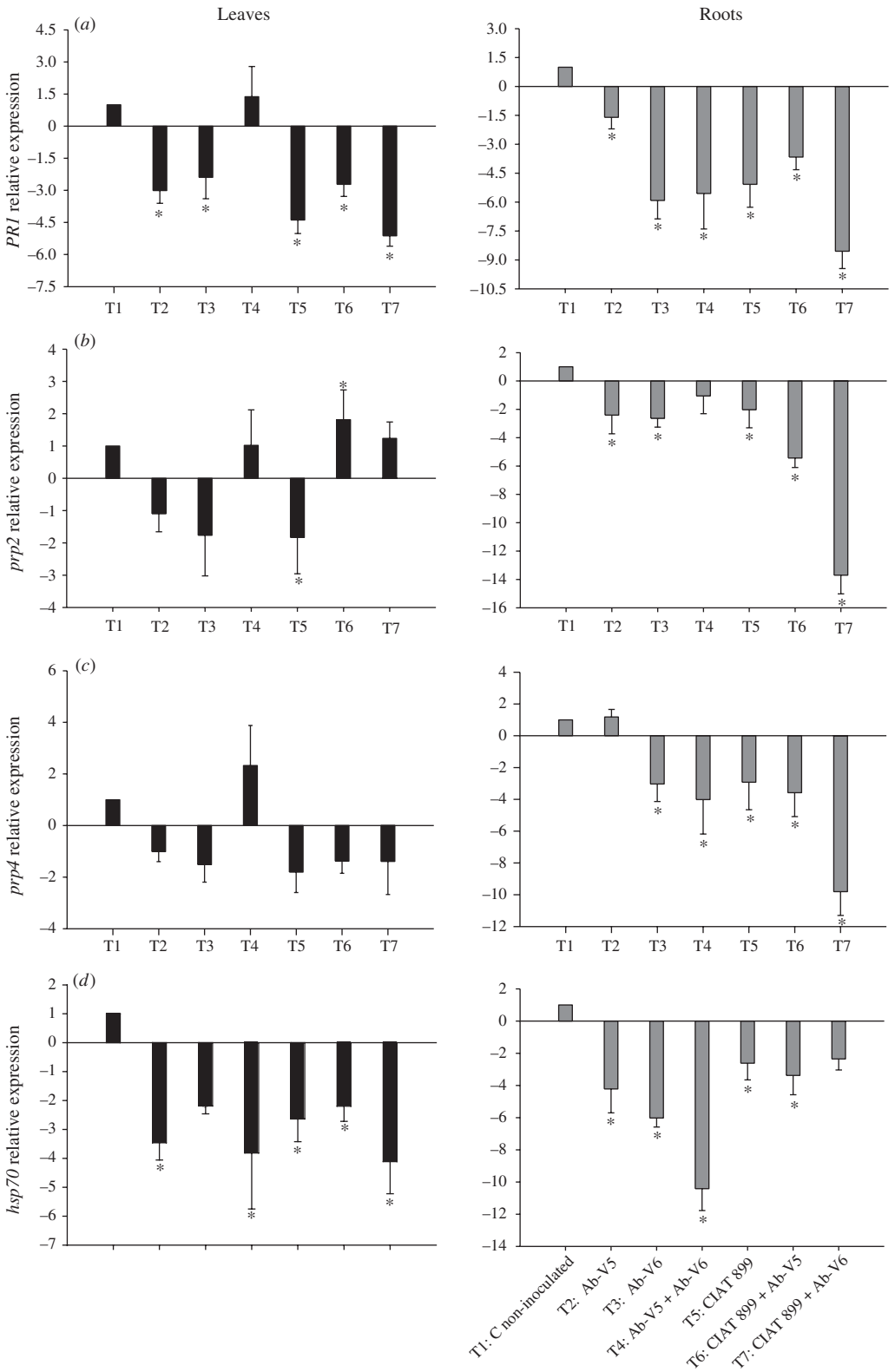

Fig. 5. RT-qPCR analysis of the expression of PR genes (*a–c*) and *hsp70* (*d*) in response to co-inoculation of *Azospirillum brasilense* and *Rhizobium tropici* in maize grown under saline stress conditions (170 mM NaCl). Data represent the means ± s.d. from three biological replicates, each with three further replicates. Data were normalised in relation to the endogenous controls UBCE and UBCP. Statistically significant expression at the level $\alpha = 5\%$ is indicated, *, determined by the REST2009 software. Black bars, leaf; dark grey bars, root.

A. brasilense alone or co-inoculated with *Rhizobium* under salt stress, *hsp70* was downregulated both in leaves and in roots, indicating that the plant might not need this protection. These results indicate that inoculation of maize with the different PGPBs might provide protection from the negative effects of saline stress. Similar results were reported for the *Arabidopsis-Azospirillum* interaction, with eight out of nine genes – among them *hsp70* – related to heat stress being downregulated after inoculation with *A. brasilense* strain Sp7 (Ahmed 2010).

In conclusion, we have shown that inoculation with *A. brasilense*, or with *R. tropici* alone or co-inoculated were able to alleviate the negative impacts of salt stress (170 mM NaCl) on maize, reflected in improved plant growth, and in the expression of stress-tolerance enzymes and gene transcription. However, differences at the strain level occurred, as *A. brasilense* strain Ab-V6, but not Ab-V5, was identified as promising as an agent to mitigate saline stress. *A. brasilense* Ab-V6 and *R. tropici* CIAT 899 also benefited IST mechanisms; however, expression of the studied genes varied with the gene and the plant tissue. Single inoculation of *A. brasilense* Ab-V6 and co-inoculation of Ab-V6 and *R. tropici* CIAT 899 were identified as the best treatments, and their use as inoculants may represent an effective strategy to enhance maize growth under salinity stress.

Conflicts of interest

The authors declare no conflicts of interests.

Acknowledgements

Funded by Embrapa (02.13.08.003.00.00), INCT-Plant-Growth Promoting Microorganisms for Agricultural Sustainability and Environmental Responsibility (CNPq 465133/2014-2/Fundação Araucária/CAPES), CNPq-Universal (400468/2016-6) and Ministerio de Economía y Competitividad (MINECO, AGL2016-77163-R). Authors thank Dr Allan RJ Eaglesham for English suggestions, J Fukami acknowledges a PhD fellowship and a sandwich fellowship to Spain from CAPES. M Hungria is also a fellow from CNPq.

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7 ARTIGO D – Atividade antioxidante e indução de mecanismos de resistência ao estresse com a inoculação de *Azospirillum brasilense*

RESUMO

Foi realizado um estudo para investigar os efeitos do *Azospirillum brasilense* estirpes Ab-V5 e Ab-V6 na indução de mecanismos de resistência sistêmica adquirida (RSA) e de resistência sistêmica induzida (RSI) em plantas de milho (*Zea mays* L.). Em condições normais de crescimento, os tratamentos consistiram de inoculação de células na semeadura, e por pulverização foliar de células ou seus metabólitos no estágio V2.5 de desenvolvimento; em condições de estresse salino (170 mM NaCl), os tratamentos consistiram de inoculação padrão com *A. brasilense* e coinoculação com *Rhizobium tropici*. Os principais compostos dos metabólitos de *Azospirillum* foram identificados como ácido indol-3-acético (AIA) e ácido salicílico (AS). Em condições normais, os tratamentos com células de *A. brasilense* na semeadura ou por pulverização foliar aumentaram a atividade das enzimas catalase (CAT), superóxido dismutase (SOD) e malondialdeído (MDA) nas folhas, e do ascorbato peroxidase (APX) nas raízes; no entanto, intrigantemente, no geral as atividades mais altas foram observadas com a pulverização foliar dos metabólitos. Em condições normais, os altos níveis de AS e ácido jasmônico (AJ) foram obtidos nas folhas por pulverização foliar dos metabólitos, de AS nas raízes por pulverização foliar de células, e de AJ nas raízes pela inoculação padrão e pulverização foliar de metabólitos. Em condições de estresse salino, a proteção da planta ocorreu via AS e ácido abscíco (ABA), mas não via AJ. Em geral, a inoculação aumentou AS nas folhas e raízes, e ABA nas folhas. Nós levantamos a hipótese de que o *A. brasilense* confere proteção às plantas de milho pela indução simultânea via AJ e AS e, em condições de estresse salino via AS e ABA.

Palavras-chave: *Azospirillum brasilense,* Resistência sistêmica induzida, Resistência sistêmica adquirida, Ácido jasmônico, Ácido salicílico, AIA, ABA, *Zea mays* L., enzimas antioxidantes.

Antioxidant activity and induction of mechanisms of resistance to stresses related to the inoculation with *Azospirillum brasilense*

Abstract

We investigated the effects of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 in the induction of mechanisms of systemic acquired resistance (SAR) and induced system resistance (ISR) on maize (*Zea mays* L.) plants. Under normal growth conditions, the treatments consisted of the standard inoculation of cells at sowing, and leaf spray of cells or their metabolites at the V2.5 growth stage; under saline stress (170 mM NaCl), the treatments consisted of standard single and co-inoculation of *A. brasilense* and *Rhizobium tropici*. The main compounds in the *Azospirillum* metabolites were identified as indole-3-acetic acid (IAA) and salycilic acid (SA). Under normal conditions, *A. brasilense* cells applied at sowing or by leaf spray increased the activities of catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) in leaves, and of ascorbate peroxidase (APX) in roots; however, interestingly, in general the highest activities were observed by leaf spray of metabolites. Under normal conditions, the highest levels of salycilic acid (SA) and jasmonic acid (JA) were achieved in leaves by leaf spray of metabolites, of SA in roots by leaf spray of cells, and of JA in roots by standard inoculation and leaf spray of metabolites. Under saline stress, plant protection occurred via SA and *abscisic acid* (ABA), but not JA. In general, inoculation resulted in further increases in SA in leaves and roots, and ABA in leaves. We hypothesize that *A. brasilense* confers protection to maize plants by simultaneous induction of JA and SA pathways, and, under saline stressing conditions, by SA and ABA pathways.

Keywords: Induced systemic resistance, Systemic acquired resistance, Jasmonic acid, Salicylic acid, AIA, ABA, *Zea mays* L, Antioxidant enzymes.

Introduction

Plants adopt mechanisms and produce molecules when submitted to biotic and abiotic stresses (de Wit 2007). One main mechanism relies on the accumulation of reactive oxygen species (ROS) in plants tissues, including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive and unstable hydroxyl radical ($^{\bullet}OH$), whose production by plants are increased in response to biotic and abiotic stresses (Gill and Tuteja 2010). The activity of enzymes related to the synthesis of antioxidants represent the most important mechanism of ROS detoxification in plants and include the superoxide dismutase (SOD), the ascorbate peroxidase (APX), and the catalase (CAT), being responsible for the scavenging of H_2O_2 by its conversion to water and O_2 (Lamb and Dixon 1997; Asada 1999).

Phytohormones also regulate processes and signaling networks involved in plant responses to biotic and abiotic stresses (Bari and Jones 2009), and salicylic acid (SA), jasmonic acid (JA) with its derivatives (such as jasmonate), and ethylene (ET) are recognized as key signaling molecules regulating resistance in plants (Glazebrook 2001; Browse 2009; Pieterse et al. 2012). The regulation occurs by means of different signaling pathways that are interlinked and are called as induced systemic resistance (ISR), and systemic acquired resistance (SAR) (Pozo et al. 2008).

A global demand for agriculture sustainability has promoted an increasing use of plant-growth-promoting bacteria (PGPB) by the farmers. Nowadays, rhizobia—commercially used for more than a century—in symbioses with legumes have also been called as PGPB. Besides rhizobia, the most studied and used PGPB is *Azospirillum* (Cassán et al. 2014), encompassing bacteria with remarkable capacity to benefit a variety of plant species, comprising important cereals such as maize (*Zea mays* L.) (Bashan and De-Bashan 2010; Hungria et al. 2010; Hungria 2011; Fukami et al. 2016; Pereg et al. 2016). Plant-growth promotion by

Azospirillum relies on an array of mechanisms (Bashan and De-Bashan 2010), including enhanced uptake of nutrients and water (Ardakani et al. 2011), biological nitrogen fixation (BNF) (Marques et al. 2017), and synthesis of phytohormones and other signaling molecules such as auxins (Spaepen and Vanderleyden 2015; Fukami et al. 2017b), cytokinins (Tien et al. 1979), gibberellins (Bottini et al. 2004), and salicylic acid (Sahoo et al. 2014; Fukami et al. 2017b).

Some PGPB can also play protective roles by eliciting 'induced systemic tolerance' (IST), involving various physiological and biochemical changes in plants (Yang et al. 2009) that confer tolerance of abiotic stresses (Maheshwari 2012). Mechanisms related to IST include antioxidant defense (Wang et al. 2012), osmotic adjustment (Sarma and Saikia 2014), production of phytohormones such as IAA (indole-3-acetic acid) (Spaepen and Vanderleyden 2015), and defense strategies such as the expression of pathogenesis-related (*PR*) genes (Kim et al. 2014).

PGPB also help plants to alleviate the deleterious effect of ROS by inducing in plants the activity of antioxidant enzymes (Upadhyay et al. 2012). In addition, PGPB activate ISR in plants by an array of biochemical pathways (Lugtenberg and Kamilova 2009); ISR is mediated, at least partially, by JA and ET signaling, inducing PR proteins such as PR-3, PR-4 (chitinase family), and PDF1.2 (a member of plant defensins) (van Loon and van Strien 1999). SAR is another induced defense mechanism mediated by SA synthesis, protecting the plants from pathogens, by the expression of *PR* genes (Durrant and Dong 2004), with an emphasis on *PR-1*, *PR-2* and *PR-5*, not only at the site of primary infection, but also systemically in the uninfected plant tissues (Malamy et al. 1990). It has been reported that the NPR1 protein "nonexpressor of *PR gene1"* is an essential regulator of the SAR mechanism, being transported to the cell nucleus in response to SA, where it acts as a transcriptional co-activator set of PR proteins (Pieterse et al. 2012, Pajerowska-Mukhtar et al. 2013). The role

of NPR1 in ISR is apparently different from its role in SAR (Pieterse and Van Wees 2015). In addition, in JA/ET signaling and in ISR there is evidence for a cytosolic function of NPR1 (Stein et al. 2008; Pieterse et al. 2012).

In this study, we investigated the role of *Azospirillum brasilense* strains Ab-V5 and Ab-V6—used in commercial inoculants for legumes and non-legumes in Brazil (Hungria et al. 2010; Hungria 2011; Hungria et al. 2013)—in eliciting mechanisms of plant defense in maize (*Zea may*s L.). We also investigated bacterial effects under saline stress conditions, when maize was co-inoculated with *A. brasilense* and *Rhizobium tropici* CIAT 899, one strain also well known for its high tolerance of abiotic stresses (Martínez-Romero et al. 1991; Gomes et al., 2015).

Materials and methods

Bacterial strains and preparation of inoculants and metabolites

Strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084) of *Azospirillum brasilense*, derived from an *Azospirillum* selection program performed in Brazil and currently used in commercial inoculants in the country for both non-legume and legume crops (Hungria et al. 2010; Hungria 2011 ; Hungria et al. 2013) were used in the studies. In addition, *Rhizobium tropici* strain CIAT 899 (=CNPSo 142, =SEMIA 4077), employed in commercial inoculants for the common bean (*Phaseolus vulgaris* L.) crop in Brazil and other countries of South America and Africa was also used (Gomes et al. 2015). The strains are deposited at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC Collection # 1213, WDCM Collection # 1054).

The inoculants containing *A. brasilense* were prepared in liquid DYGS medium (as described by Fukami et al. 2018) and *R. tropici* in liquid tryptone yeast (TY) medium

(Beringer 1974), and after growth for 48 h, cell concentrations were adjusted to 10^8 mL^{-1}. For the production of metabolites, bacteria were grown under the same conditions and were centrifuged at 5,000 rpm for 15 min. By plating the supernatants obtained in plates containing DYGS medium we confirmed that they were free of *Azospirillum* cells.

Quantification of phytohormones produced by *A. brasilense* by UHPLC-HRMS/MS

Bacterial growth conditions

Strains Ab-V5 and Ab-V6 were grown separately in DYGS medium (Fukami et al. 2018), with three biological replicate for each strain. Liquid bacterial inocula were incubated at 28 ± 2 °C with orbital shaking at 120 rpm for 14 days. The bacterial cultures were then filtered through nitrocellulose-membrane filters Millipore HA 0.45 µm to obtain the supernatants. The samples were filtered again in a microfiltration membrane, and 5-µL aliquots of each sample were analyzed.

UPLC-MS/MS analysis of SA and JA

Quantitative analyses of phytohormones produced by *A. brasilense* strains Ab-V5 and Ab-V6 was performed by ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS/MS). The analyses of salicylic acid (SA) and jasmonic acid (JA) were performed on a Waters Acquity UPLC coupled to a Waters Xevo TQS-micro (Waters Corporation, USA), consisting of a triple quadrupole mass spectrometer equipped with an electrospray ion-source operated in negative mode and controlled by MassLynx 4.1. UPLC analyses were carried out using a Waters Acquity BEH C18 column (1.7 µm particle size, 50 x 2.1 mm) at 40 ºC and 0.5 mL/min flow rate. A binary gradient consisting of (A) water and (B) methanol, both containing 0.1% formic acid, was used with the following

elution profile: 10% B (0.5 min), linear gradient to 100% B (3.4 min), 100% B (0.3 min) and 10% B (0.7 min). The injection volume was 5 µL.

Multiple Reaction Monitoring (MRM) was applied where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. The transitions employed for SA and JA were 136.9/92.9 and 136.9/64.9, and 209.0/58.9 and 209.0/108.9, respectively, choosing the first one for quantification and the second as confirmatory. For UPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: capillary voltage, 3.0 kV; desolvation source temperature, 550 °C; desolvation source gas flow, 600 L/h; and cone source gas flow, 50 L/h.

UHPLC-HRMS analysis of auxins

Auxins [indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-ethanol (TOL), indole-3-lactic acid (ILA), indole-3-pyruvic acid (IPyA), indole-3-propionic acid (IPA)], gibberellic acid (GA3), and kinetin (Kin) were analyzed with a Thermo Scientific Liquid Chromatography system consisting of a binary UHPLC Dionex Ultimate 3000 RS connected to a quadrupole-orbitrap Qexactive hybrid mass spectrometer (ThermoFisher Scientific, USA) with HESI ionization probe. Xcalibur software was used for instrument control and data acquisition. Separation was carried out using an Acquity BEH C18 column (1.7 µm particle size, 50 x 2.1 mm) (Waters) at 40 ºC at a flow rate of 0.5 mL/min. A binary gradient consisting of (A) water and (B) methanol, both containing 0.1% formic acid, was used with the following elution profile: 5% B (1 min), linear gradient to 100% B (9 min), 100% B (2 min) and 5% B (3 min). The injection volume was 5 µL.

A full scan (FS) acquisition method was used in positive mode and 70,000 of resolution at *m/z* 200 FWHM. HESI source parameters were: spray voltage, 3.5 kV; S lens level, 50; capillary temperature, 320 °C; sheath and auxiliary gas flow, 60 and 25 respectively (arbitrary

units); and probe heater temperature, 400 ºC. Quantification was carried out selecting the pseudomolecular ion of each auxin: *m/z* 162.09134 for TOL, 176.07061 for IAA, 190.08626 for IPA, 204.10191 for IBA, 206.08117 for ILA, 204.06552 for IPyA, 216, 08799 for kin, and 345.13326 for GA3. As confirmatory ions some ions produced in-source fragmentation were selected for each compound. TraceFinder 3.3 software was used for data treatment

Greenhouse experiments

Experiment 1: Study with A. brasilense cells and metabolites

In Experiment 1, three methods of inoculation were compared: *i*) standard inoculation (SI) at sowing, considered as the control; *ii*) inoculation by leaf spray (ILS) at the V2.5 stage of the maize growth cycle (Hickman and Shroyer 1994; about seven days after transplanting); and *iii*) application of metabolites of *A. brasilense* strains Ab-V5 and Ab-V6 by leaf spray (MLS) at the V2.5 stage. In total, the experiment had ten treatments, consisting of the combinations of strains and inoculation methods, as follows: 1) non-inoculated control; 2) SI with Ab-V5; 3) SI with Ab-V6; 4) SI with Ab-V5 + Ab-V6; 5) ILS with Ab-V5; 6) ILS with Ab-V6; 7) ILS with Ab-V5 + Ab-V6; 8) MLS of Ab-V5; 9) MLS of Ab-V6; 10) MLS of Ab-V5 + Ab-V6. The experiment was arranged in a completely randomized design with six replicates for each treatment.

The experiment was performed under greenhouse conditions, using modified Leonard jars (Vincent 1970) containing sterilized substrate, consisting of a mixture of sand and pulverized coal (3:1, v/v). Hybrid maize seeds (DKB330 VT PRO2) were surface-disinfected with 70% ethanol and 3% sodium hypochlorite (Vincent 1970) and were pre-geminated for 48 h at 25 °C in Petri plates containing 1% (v/v) water agar. After germination, two seedlings were transplanted per jar and thinned to one plant after three days.

For the SI treatment, seedlings were inoculated by adding inoculant to provide a final concentration of 1.6×10^5 cells plant^{-1}, immediately after transplanting. The concentration at the 10^5 level was based on our previous studies of maximization of the contribution of both strains (Hungria et al. 2010; Hungria et al. 2013). For the ILS treatments, an aerograph atomizer was employed to mimic the action of a spraying apparatus. The soil surface was covered with aluminum foil to prevent the inoculant to reach the soil. The final volume of liquid for leaf-spray inoculation was 1 mL (water + inoculant) per pot containing a single plant, and inoculants were diluted with sterile distilled water at 1:1000 (v:v) for spraying, to achieve an application rate of 1.6×10^5 cells plant^{-1}. For leaf spray of metabolites, the MLS treatments, bacterial exudates obtained from the culture cells with 10^8 mL^{-1} were used, with the application of 1 mL per plant.

All plants received sterile nutrient solution (Fahraeus 1957), and N-mineral corresponding to 60 kg N ha^{-1} (50% of the doses recommended for the maize crop in Brazil), supplied as a nutrient solution with 5 mM of KNO_3. Average day and night temperatures were of 28 ± 2.3/23 ± 1.9 °C (day/night); the experiment was performed at the summer growing season, where light intensity is the most adequate for maize growth.

Plants were harvested at 35 days, separating leaves and roots. Three biological replicates were used for evaluation of plant growth parameters, and the results are available elsewhere (Fukami et al. 2017b). The remaining three replicates were frozen in liquid nitrogen and stored at −80 °C until further analyses. The analyses performed included enzyme assays, lipid peroxidation, and quantification of endogenous phytohormones in leaves and roots by UHPLC-HRMS/MS, as described in later in this materials and methods section.

Experiment 2: Study with A. brasilense and R. tropici cells under saline stress

Experiment 2 consisted of inoculation with *A. brasilense* (strains Ab-V5 and Ab-V6) and co-inoculation with *R. tropici* (strain CIAT 899) under saline condition (170 mM NaCl). The experiment consisted of seven treatments, as follows: 1) non-inoculated control; and all others inoculated at sowing with 2) Ab-V5; 3) Ab-V6; 4) Ab-V5 + Ab-V6; 5) CIAT 899; 6) Ab-V5 + CIAT 899; 7) Ab-V6 + CIAT 899.

The experiment was arranged in a completely randomized design with six replicates for each treatment. The experiment was performed in modified Leonard jars, as described in Experiment 1. Seeds of hybrid maize seeds (DKB330 VT PRO2) were surface-disinfected and pre-geminated for 48 h at 25 °C in Petri plates containing 1% (v/v) water agar, as described in Experiment 1. After germination, two seedlings were transplanted per jar and thinned to one plant after three days. Inocula containing strains Ab-V5, Ab-V6 and CIAT 899 were applied to the seedlings and provided 3.0 x 10^5 cells $plant^{-1}$ of each bacterium. The concentration was slightly different from Experiment 1 because the inoculant had a slightly higher concentration, and cell count by plating was confirmed only five days later after inoculation.

As in Experiment 1, plants received sterile nutrient solution (Fahraeus 1957) and N-mineral corresponding to 60 kg N ha^{-1} (50% of the doses recommended for the maize crop in Brazil), supplied as a nutrient solution with 5 mM of KNO_3, but, in addition, they received 170 mM NaCl.

Growth conditions and harvest were as described in Experiment 1. Growth parameters obtained in this experiment are available elsewhere (Fukami et al. 2017a), Quantification of endogenous phytohormones in maize leaves and roots was analysed by UHPLC-HRMS/MS, as described in a following item of the materials and methods section.

Enzyme assays and lipid peroxidation

The enzymes assays and evaluation of lipid peroxidation were performed in leaves and roots of maize plants of Experiment 1. The APX activity (EC: 1.11.1.11) was determined by monitoring the rate of H_2O_2-dependent oxidation of ascorbate (extinction coefficient of 2.8 mM^{-1} cm^{-1}) in absorbance at 290 nm for 120 s (Hossain and Asada 1984). The reaction mixture consisted of 50 mM HEPES-NaOH buffer (pH 7.6), 0.2 mM ascorbate and 5 mM H_2O_2 and leaf and root extract, 50 and 100 µL, respectively.

CAT activity (EC 1.11.1.6) was evaluated as described by Beers and Sizer (1952), estimating the absorbance reduction at 240 nm for 120 s, as a result of H_2O_2 utilization (extinction coefficient of 39.58 m^{-1} cm^{-1}). The reaction mixture contained 60 mM potassium phosphate buffer (pH 7.0), 5 mM H_2O_2 and leaf and root extract, 50 and 100 µL, respectively.

SOD activity (EC: 1.15.1.1) was measured by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm (Beuchamp and Fridovich 1971). The assay mixture in a total volume of 2 mL contained 50 mM Na phosphate buffer (pH 7.8), 33 mM NBT, 10 mM L-methionine, 0.66 mM EDTA, 0.0033 mM of riboflavin, and 20 µL of enzyme extract (Bor et al. 2003). Enzymatic activities were reported as units per mg of protein (Bradford 1976), using BSA as a standard.

Lipid peroxidation was performed as described before (Hodges et al. 1999), with the trichloroacetic acid (TCA) and thiobarbituric acid (TBA), which determines malondialdehyde (MDA) as an end product of lipid peroxidation. This method corrects the interference generated by other compounds, and the MDA was reported as nmol mL^{-1} g FW^{-1}.

Enzyme assays and lipid peroxidation were performed in triplicate for each of the three biological replicates.

Quantification of endogenous phytohormones in maize plants by UHPLC-HRMS/MS

Extraction and purification of phytohormones in maize leaves and roots were performed by the method of t'Kindt et al. (2009), with some modifications. Fresh plants were cut and immediately frozen in liquid nitrogen. Following, tissues were ground on a mortar, with continuous additions of liquid nitrogen. The pulverized tissues were transferred to a Falcon tube and lyophilized, yielding a dry powder. Around 50 mg of powder were resuspended in 0.6 mL of a mixture of cold (-20 ºC) methanol:water 80:20 (v/v) and stirred with a Thermoshaker (Optic Ivymen System, TR100-G) for 15 min at 1,250 rpm (metal block previously cooled at -20 °C), and then sonicated for 5 min. After centrifugation at 15,000 *g* for 15 min, the supernatant was taken and evaporated in a Speed Vac (miVac DNA concentrator, Genevac) for 2 h at 30 °C. The extracts were dissolved in 0.3 mL of a mixture of methanol:water 10:90 (v/v) containing 0.1% formic acid, micro filtered with a 0.2 μm nylon filter, and analyzed using UPLC-MS/MS or UHPLC-HRMS.

Analyses of SA, *abscisic acid* (ABA) and JA were performed by UPLC-MSMS and analysis of auxins (TOL, IAA, IBA, IPA, ILA and IPyA) by UHPLC-HRMS, as described in the item of UHPLC-HRMS analysis of auxins. The transitions employed for ABA in MRM were of 263.1/153.0 and 263.1/204.0.

Statistical analyses of the greenhouse experiment

Data obtained from the greenhouse experiments were first evaluated for normality and variance homogeneity, followed by the analysis of variance (ANOVA). The Tukey's test was used to compare means when statistical significance was detected by the ANOVA F test ($p \leq 0.05$). For all analyses, the Statistica version 7.0 software was employed.

Results

Quantification of phytohormones produced by *A. brasilense* by UHPLC-HRMS/MS

The UHPLC-HRMS/MS results obtained in the analysis of the metabolites produced by *Azospirillum* strains grown in DYGS medium are presented in Table 1. The main compounds quantified were: indole-3-acetic acid (IAA), indole-3-ethanol (TOL), and salicylic acid (SA). Strain Ab-V6 synthesized 233 and 50% more IAA and SA than strain Ab-V5, respectively. Indole-3-lactic acid (ILA) and jasmonic acid (JA) were identified, but in relatively low amounts, of < 20.0 and <1.0 µg L^{-1}, respectively. In the supernatants of both strains, the indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPyA), indole-3-propionic acid (IPA), kinetin (Kin), and gibberellic acid (GA3) were not detected.

Table 1 Quantification (by UHPLC-HRMS/MS) of phytohormones[a] synthesized *in vitro* by *Azospirilum brasilense* strains Ab-V5 and Ab-V6 after 14 days of growth in DYGS medium.

Sample	IAA[a]	IBA	TOL	ILA	IPyA	IPA	Kin	GA_3	JA	SA
µg L^{-1} medium										
Ab-V5	180 ± 0.03	nd[b]	5.70 ±0.10	<20.0	nd	nd	nd	nd	<1.0	14.2 ±0.2
Ab-V6	600 ± 0.10	nd	4.53 ±0.04	<20.0	nd	nd	nd	nd	<1.0	21.3 ±0.4

[a] Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-ethanol (TOL), indole-3-lactic acid (ILA), indole-3-pyruvic (IPyA), indole-3-propionic acid (IPA), kinetin (Kin), gibberellic acid (GA3), jasmonic acid (JA), salicylic acid (SA).
[b] not detected.
Data ± standard deviation from three biological replicates.

Enzyme assays and lipid peroxidation

Enzymatic activities of different enzymes of the antioxidative defense system (APX, CAT, SOD) and of lipid peroxidation (MDA) were examined in maize leaves and roots grown under normal conditions, in Experiment 1, and the results are shown in Fig. 1.

In comparison to the non-inoculated control (T1), the activity of APX in leaves was not statistically affected neither by the method of inoculation, nor by the strains (Fig. 1A). In roots, the treatments with standard inoculation at sowing (SI) with Ab-V6 and Ab-V5 + Ab-V6 (T3, T4), and the inoculation of Ab-V5 by leaf spray (ILS) (T5) were statistically higher than the non-inoculated control (T1). In addition, noteworthy were the treatments receiving the metabolites leaf spraying (MLS), with an emphasis on those of Ab-V5 alone or in combination with Ab-V6 (T8, T10), resulting in the highest increases of APX in roots when compared to T1 (Fig. 1A).

CAT activity in leaves was significantly increased by leaf spray at the V2.5 stage of strain Ab-V6 (T6), and of the metabolites of Ab-V5 + Ab-V6 (T10); all inoculation treatments increased CAT activity in roots, with an emphasis on the leaf spray of strains Ab-V5 and Ab-V6 (T5 and T6) (Fig. 1B).

In relation to the MDA, all treatments increased the levels in leaves, but with an emphasis on the leaf spray of the cells and their metabolites at the V2.5 stage (T5 to T10) (Fig. 1C). However, the opposite was verified in roots, such that the standard inoculation at sowing with Ab-V5 was the only treatment to significantly increase MDA (Fig. 1C).

As expected, SOD activity was found exclusively in leaves, and the treatments with leaf spray of cells (T7) and metabolites of (T10) of Ab-V5 + Ab-V6, and of metabolites of Ab-V6 (T9) resulted in increases in the antioxidant activity (Fig. 1D).

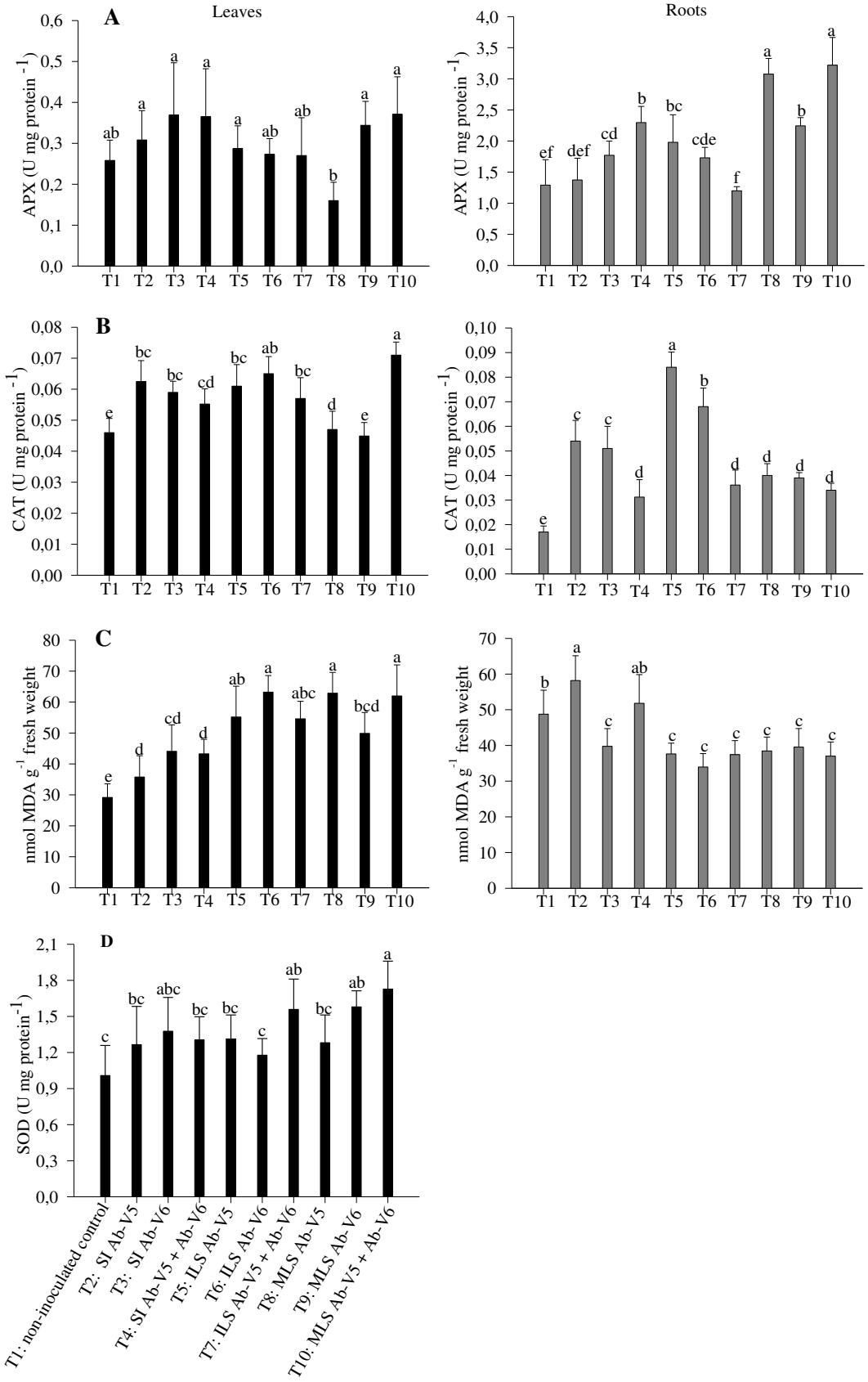
A
Leaves
Roots
APX (U mg protein -1)
B
CAT (U mg protein -1)
C
nmol MDA g -1 fresh weight
D
SOD (U mg protein-1)
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
T1: non-inoculated control
T2: SI Ab-V5
T3: SI Ab-V6
T4: SI Ab-V5 + Ab-V6
T5: ILS Ab-V5
T6: ILS Ab-V6
T7: ILS Ab-V5 + Ab-V6
T8: MLS Ab-V5
T9: MLS Ab-V6
T10: MLS Ab-V5 + Ab-V6

Fig. 1 Effects of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 on the enzyme activities and lipid peroxidation in maize plants. Treatments consisted of standard inoculation of the strains at sowing (SI), inoculation of strains by leaf spray (ILS) at the V2.5 maize growth stage, and when the cell-free metabolites were applied by leaf spray (MLS) at the V2.5 stage. Data represent the means ± standard deviation from three biological replicates, each with three technical replicates. Means followed by the same letter for each parameter, for leaves or roots, are not significantly different from one another according to Tukey's test ($p \leq 0.05$). Black bars: leaf; Dark gray bars: root. (A) Ascorbate peroxidase (APX); (B) Catalase (CAT); (C) Lipid peroxidation (MDA); D. Superoxide dismutase (SOD).

Quantification of endogenous phytohormones in maize plants by UHPLC-HRMS/MS

Quantification of phytohormones in maize roots and leaves in response to inoculation of *A. brasilense* cells and metabolites with different methods and growth under normal conditions (Experiment 1), and in response to inoculation of *A. brasilense* and co-inoculation with *R. tropici* under saline stress conditions (Experiment 2) was evaluated by UHPLC-HRMS/MS. In both experiments, IBA, TOL, ILA, IPyA, and IPA were not detected. IAA was detected in relatively low amounts (<30 ng g^{-1} dry extract), and in Experiment 1 ABA was very low; therefore, these results are not shown.

In relation to the SA levels in leaves of plants of grown under normal conditions (Experiment 1), emphasis should be given to the increase by leaf spray of the metabolites (T8, T9, T10), but also a light increment with respect to T1 was detected by leaf spray of cells of Ab-V5 + Ab-V6 strains (T7) (Fig. 2A). A reduction of the SA levels in leaves, with respect to the non-inoculated control, was observed with standard inoculation with Ab-V5 and Ab-V6 (T2 and T3) and by leaf spray of cells of Ab-V5 and Ab-V6 treatments (T5 and T6) (Fig. 2A). In roots, significant increases were verified with standard inoculation of Ab-V6 (T3) and Ab-V5 + Ab-V6 (T4), and by leaf spray of the metabolites of Ab-V5 and Ab-V6 (T8 and T9). Nevertheless, the highest levels of SA in roots were verified when cells of Ab-V5 and Ab-V6 were leaf sprayed (T5 and T6) (Fig. 2A).

Still when plants were grown under normal conditions (Experiment 1), JA in leaves was significantly decreased by all treatments with SI (T2, T3, T4), by leaf spray of Ab-V5 cells (T5), and by leaf spray of the metabolites of Ab-V6 (T9) (Fig 2B). Contrarily, leaf spray of Ab-V6 and of Ab-V5 + Ab-V6 (T6 and T7), and especially of the metabolites of Ab-V5 + Ab-V6 (T10) significantly increased JA in leaves when compared to T1. In roots, the treatments with the highest increases of JA in comparison to T1 were SI with Ab-V6 and with Ab-V5 + Ab-V6 (T3 and T4), and leaf spray of the metabolites of Ab-V6 (T9). Reduction of the JA levels in roots was detected with the standard (T2), cell leaf spray (T5), and metabolite leaf spray (T8) of Ab-V5, and by leaf spray of Ab-V5 + Ab-V6 (T7) and their metabolites (T10) (Fig. 2B).

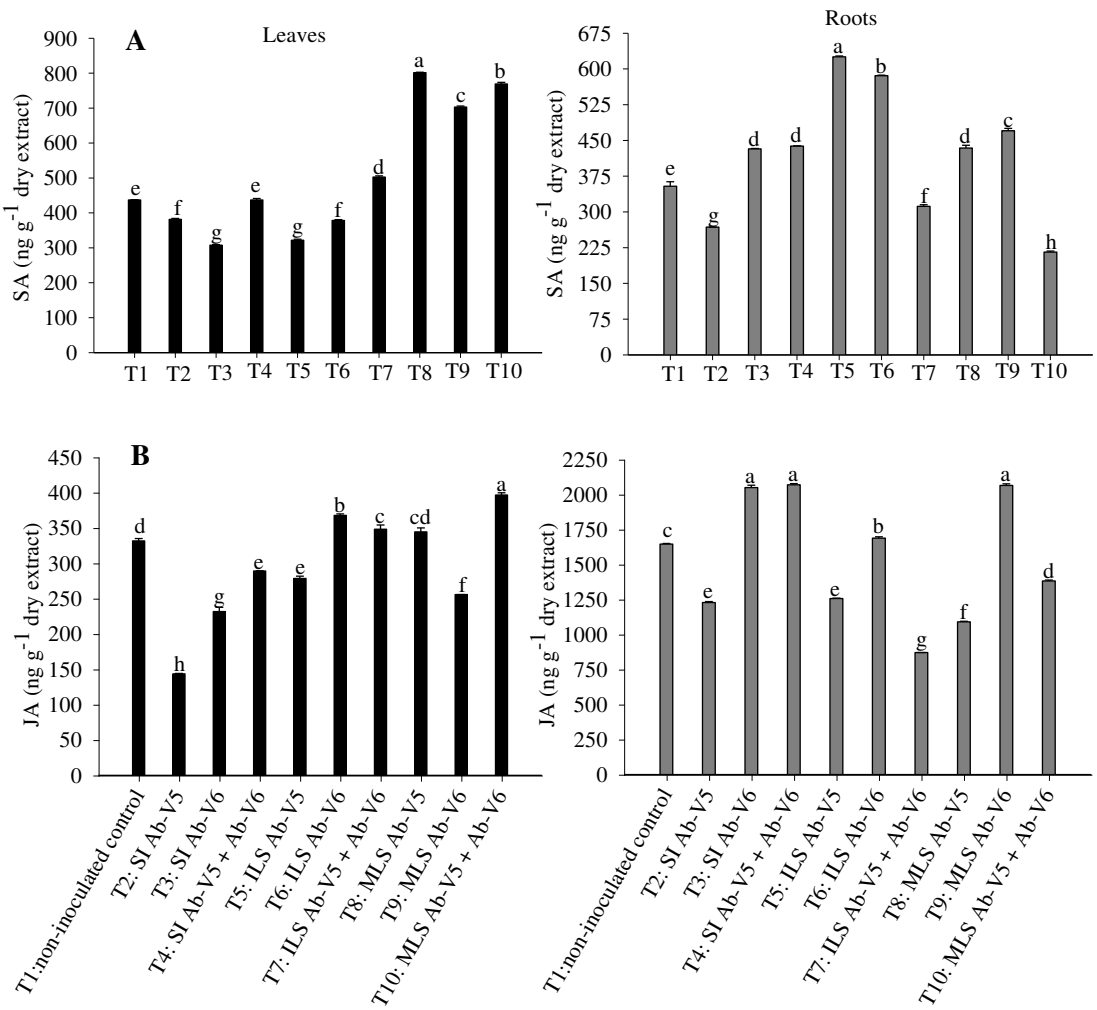

Fig. 2. Accumulation of salicylic acid (SA) and jasmonic acid (JA) in response to inoculation with *Azospirillum brasilense* Ab-V5 and Ab-V6, when the strains were standard inoculated at sowing (SI), inoculated by leaf spray (ILS) at the V2.5 maize growth stage, or when the cell-free metabolites of the strains were applied by leaf spray (MLS) at the V2.5 stage. Data represent the means ± standard deviation from three biological replicates, each with three technical replicates. Means followed by the same letter for each parameter, for leaves or roots, are not significantly different from one another according to Tukey's test ($p \leq 0.05$). Black bars: leaf; Dark gray bars: root. (A) Salycilic acid (SA); (B) Jasmonic acid (JA).

Under salinity stress (Experiment 2), treatments with single inoculation of Ab-V5, Ab-V6 and CIAT 899 (T2, T3 and T5) accumulated significantly more SA in leaves than the non-inoculated control (T1), but the highest accumulation, of 34% in relation to T1, was achieved with the co-inoculation of CIAT 899 + Ab-V6 (Fig. 3A). SA content in roots increased in all treatments, but with the inoculation with Ab-V5 (T2) and Ab-V6 (T3) increased by 56 and 62%, respectively, in comparison to T1 (Fig. 3A).

None of inoculation treatments affected significantly JA accumulation in leaves (Fig. 3B). However, JA in roots was statistically increased by inoculation with Ab-V5 + Ab-V6 (T4) and Ab-V5 + CIAT 899 (T6), by 140 and 82%, respectively, in comparison to T1, but it was significantly decreased in all other treatments except for the inoculation with CIAT 899 (T5) (Fig. 3B).

ABA in tissues was detected when plants were under saline stress conditions. In leaves, ABA increased when plants were inoculated with *A. brasilense* strains alone (T2, T3), or in combination with CIAT 899 (T6, T7), whereas a decrease occurred with the inoculation of Ab-V5 + Ab-V6 (T4), while CIAT 899 (T5) did not show difference from the control T1 (Fig. 3C). No statistical differences were observed in ABA in roots (Fig. 3C).

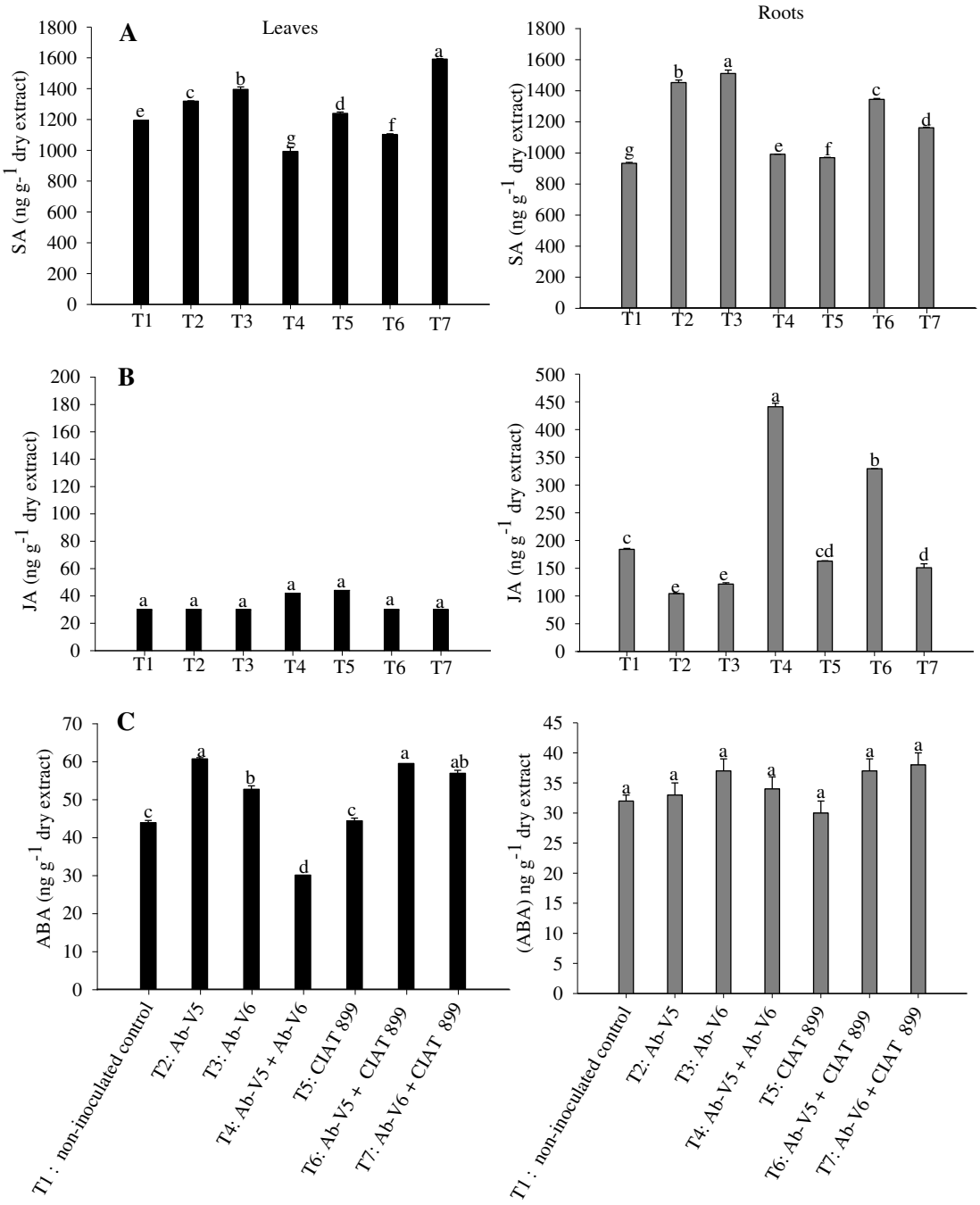

Fig. 3 Accumulation of salycilic acid (SA), jasmonic acid (JA) and abscicic acid (ABA) in response to inoculation of *Azospirillum brasilense* Ab-V5 and Ab-V6, and co-inoculation of *Azospirillum* with *Rhizobium tropici* CIAT 899 in maize grown under saline stress conditions (170 mM NaCl). Data represent ± standard deviation from each of the three replicates derived from the analysis of six biological replicates. Data represent the means ± standard deviation from three biological replicates, each with three technical replicates. Means followed by the same letter for each parameter, for leaves or roots, are not significantly different from one another according to Tukey's test ($p \leq 0.05$). Black bars: leaf; Dark gray bars: root. (A) Salycilic acid (SA); (B) Jasmonic acid (JA); (C) Abscisic acid (ABA).

Discussion

A. brasilense strains Ab-V5 and Ab-V6 have been used in commercial inoculants for non-legumes, as well as for co-inoculation of legumes in Brazil since 2009, and nowadays, more than 3 million doses of inoculants have been sold annually with these strains (Hungria et al. 2010; Hungria, 2011; Hungria et al. 2013). Interestingly, we have seen that Ab-V5 and Ab-V6 are also effective in promoting plant growth and increasing grain yield even with unusual ways of inoculation, more specifically, by leaf spray (Fukami et al. 2016). Following, benefits of leaf spray not only of *Azospirillum* cells, but also of their free-cells metabolites were reported, but surprisingly, when cells were leaf sprayed, recovery of bacteria in leaves was very low (Fukami et al. 2017b). Finally, it has also been reported that some of the growth-promoting effects of Ab-V5 and Ab-V6 could be related to the induction of plant-defense genes, conferring tolerance of abiotic stresses (Fukami et al. 2017a; Fukami et al. 2017b). Now, we proceeded with these studies, trying to elucidate the mechanisms related to the maize growth promotion, in response to different strains and methods of inoculation.

Under non-stressing conditions, plants synthesize low levels of potentially toxic oxygen metabolites, resulting in an appropriate balance between production and scavenging of ROS (Sharma et al. 2012). However, the equilibrium is unbalanced by several biotic and abiotic stresses, such as salinity, UV radiation, drought, extremes of temperature, nutrient depletion, and pathogen attacks (Gill and Tuteja 2010). These stresses result in ROS accumulation in plant tissues and, consequently, oxidative damages, such as lipid peroxidation with membrane destruction, protein inactivation, and DNA mutation (García-Limones et al. 2002). The oxidant enzymes operate in different subcellular compartments and respond when cells are exposed to oxidative stresses (Sharma et al. 2012), with an emphasis on the enzymes of catalase (CAT), superoxide dismutase (SOD) (Wisniewski-Dyé et al. 2012), and ascorbate

peroxidase (APX) (Ozyigit et al. 2016). In Experiment 1, with plants grown under normal conditions, we evaluated, in maize leaves and roots, the response of the H_2O_2–generating enzyme SOD, and of the H_2O_2–scavenging enzymes CAT and APX, and our results indicated that the inoculation with *A. brasilense* Ab-V5 and Ab-V6 resulted in considerable changes in the antioxidant status of maize. When cells were used in standard inoculation at sowing, or applied at the V2.5 stage by leaf spray, in general, the activities of CAT and APX were increased in roots, and CAT and SOD, but not APX increased in leaves. Interestingly, some of the treatments with spray of metabolites of *A. brasilense* at the V2.5 stage resulted in high activities of APX, CAT and SOD in leaves, and of APX in and roots (Fig. 1). It is worth mentioning that particularly for the application of *Azospirillum* metabolites by leaf spray, there was an outstanding increase in the APX activity in roots, and, as pointed out by Gill and Tuteja (2010), APX has higher affinity for H_2O_2 than CAT; consequently, its role might be more important in the management of ROS under stressing conditions. Altogether, these results suggest beneficial effects of inoculation of cells, applied at sowing or later by leaf spray, as well as of spraying metabolites of *Azospirillum*, promoting the activity of antioxidant scavenging-H_2O_2 enzymes, and conferring protection to plants against oxidative stresses.

Walker et al. (2011) reported that *Azospirillum* promoted significant changes in secondary metabolic profiles, mainly benzoxazinoids, suggesting the presence of fine-tuned interaction mechanisms. Malondialdehyde (MDA) is a product of lipid peroxidation by ROS and an important indicator of oxidative stress in plants exposed to stress conditions (Hodges et al. 1999). In Experiment 1, MDA in leaves was increased in all inoculation treatments, another evidence of the contribution of these inoculation treatments in activating plant mechanisms against oxidative stresses (Fig. 1C); however, except for SI inoculation with Ab-V5 AND Ab-V5 + Ab-V6, MDA was decreased in roots. The highest levels of MDA in

leaves were detected in maize plant inoculated with cells or metabolites by spray, and curiously, in these treatments the MDA levels were significantly lower in roots than in the control treatment. These results could suggest that the increase in MDA in leaves when the spray did not imply in translocation to other plant tissues, such as roots.

Plants also possess a variety of inducible defense mechanisms against pathogens. Systemic acquired resistance (SAR) is activated after pathogen attach, conferring resistance to against other pathogens (Fu and Dong 2013). In addition, for some PGPB, induced system resistance (ISR) has been reported as a mechanism of plant defense (Lugtenberg and Kamilova 2009). NPR1 (non-expressor of pathogenesis-related PR1) protein is the master transcriptional co-regulator of both defense pathways, activating the expression of either SA-dependent *PR* genes or SA-independent mechanisms, such as the JA- and the ET-dependent activation of defense-related genes for the establishment of SAR and ISR, respectively (Caarls et al. 2015; van Wees et al. 2000). Previously, we were able to detect SA, but not JA in Ab-V5 and Ab-V6 metabolites (Fukami et al. 2017b), and now we were able to quantify the SA synthesized by *Azospirillum* and to confirm that the strains produce very low amounts of JA (Table 1); in addition, we quantified both compounds in maize leaves and roots. Again, in plants grown under normal conditions, inoculation by leaf spray of *Azospirillum* metabolites was outstanding in increasing the SA levels in leaves, while the leaf spray of cells resulted in the highest levels in roots (Fig 2A). We may hypothesize that the increase in SA levels in roots due leaf spray of alive bacteria could be related to quorum-sensing (QS) mechanisms, that can induce resistance in distant plant tissues via SA (Choudhary and Johri 2009; Schenk et al. 2014). Furthermore, recent studies about the QS mechanisms in Ab-V5 and Ab-V6 raised the hypothesis that IAA molecules—produced in high amounts by Ab-V5 and mainly Ab-V6 (Table 1)—could also mimic AHL signals (Fukami et al. 2018), and these molecules might participate in the cross-talking defense mechanisms (Pieterse et al. 2012). It is worth

mentioning that several studies have demonstrated that the exogenous application of SA can induce *PR* genes, increasing the resistance to pathogens (Bari and Jones 2009; Lemarié et al. 2015); SA produced by *Azospirillum* strains could perform as a "priming" effect for the plant. In addition, as we have previously shown, inoculation with *Azospirillum* cells and metabolites, via seeds or leaf spray affect the expression of *PR* genes in maize leaves and roots (Fukami et al. 2017b).

In the case of JA, higher levels were detected in roots than in leaves, in both normal and stressing conditions. Under normal conditions, the highest increase in leaves was observed by leaf spray with the combined metabolites of Ab-V5 + Ab-V6, while in roots were obtained with standard inoculation with cells of Ab-V6 and of Ab-V5 + Ab-V6, and by spraying the metabolites of Ab-V6 (Fig. 2B). This is evidence that the molecules could function as signals transported within the plants. The JA results could be associated with the expression of *prp-4* gene in maize roots reported by Fukami et al. (2017b), resulting in ISR. The additive enhanced capacity of the simultaneous activation of SAR and ISR suggests that NPR1 may regulate and connect different hormone-dependent defense pathways (van Wees et al. 2000; Pieterse et al. 2012; Yang et al. 2015), altogether contributing to plant resistance against pathogens (Lemarié et al. 2015). Therefore, our results suggest that inoculation with *A. brasilense* cells and metabolites confer protection to maize plants by the simultaneous induction of JA and SA pathways. In addition, SA produced by *Azospirillum* might have the potential to alleviate oxidative stress, as confirmed in a previous study when exogenous SA was added to maize plants (Yadava et al. 2015).

PGPB have also been reported to enhance plant tolerance of abiotic stresses by mechanisms known as induced systemic tolerance (IST) (Kaushal and Wani 2016), including synthesis of phytohormones such as IAA (Spaepen and Vanderleyden 2015), antioxidant defense (Wang et al. 2012), osmotic adjustment (Sarma and Saikia 2014), and expression of

genes such as *PR* (Kim et al. 2014). Among the PGPB, the genus *Azospirillum*–mainly *A. brasilense*– is probably the most studied to mitigate salinity stress, with inoculation tests performed in several plant species, including maize (Hamdia et al. 2004; Fukami et al. 2017a). In Experiment 2, we investigated the effects of inoculation of *A. brasilense* Ab-V5 and Ab-V6, and also of *R. tropici* strain CIAT 889—known by its high tolerance of several abiotic stress (Martínez-Romero et al. 1991, Gomes et al. 2015)—in the mitigation of the effects of saline stress, with an emphasis on the defense responses related to the SA and JA pathways, triggering SAR and ISR, respectively. ABA was confirmed to play a critical role in saline stress, as it was not detected in Experiment 1, with plants grown under normal conditions, but was present in both leaves and roots of maize grown with saline stress. However, further increases in ABA in leaves but not in roots were observed by inoculation with Ab-V5 and Ab-V6 alone or co-inoculated with CIAT 899 (Fig. 3C). The accumulation of ABA induces stomata closure on stressed leaves, very important for the reduction of water loss by transpiration under water-stress conditions, such as saline stress (Taiz et al., 2015). Under saline conditions, inoculated plants also accumulated higher levels of SA (965 to 1,592 ng g^{-1} dry extract, considering both leaves and roots), but lower of JA (30 to 441 ng g^{-1} dry extract) (Fig. 3), than in non-stressing conditions (225 to 850 ng g^{-1} dry extract for SA and 142 to 2130 ng g^{-1} dry extract for JA) (Fig. 2). Further increases due to the inoculation of *A. brasilense* and *R. tropici*, alone or combined, were achieved for most treatments in SA in leaves and roots, but only two treatments, inoculated with Ab-V5 + Ab-V6 and with Ab-V5 + CIAT 899, increased JA in roots. Previously, the benefits of inoculation of *A. brasilense* Ab-V6 alone or co-inoculated with *R. tropici* CIAT 899 in mitigating saline stress have been reported (Fukami et al. 2017a). Now, our results indicate that the mechanisms related to this tolerance are mediated by SA and ABA.

In conclusion, we reported that the different methods of inoculation with *A. brasilense* strains Ab-V5 and Ab-V6, and also of their metabolites may confer protection to maize plants by simultaneous induction of JA and SA pathways. However, under salinity stress conditions, SA and ABA pathways are induced independently on the inoculation, but are further increased by inoculation with *A. brasilense* and *R. tropici*.

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8 CONSIDERAÇÕES FINAIS

Tendo em vista a necessidade de tecnologias mais sustentáveis para o agronegócio brasileiro, em um país em que genótipos mais produtivos, novos sistemas de cultivo e incremento na área sob cultivo de grãos resultam em demanda crescente de fertilizantes químicos, os inoculantes à base de *Azospirillum brasilense* representam uma tecnologia estratégica consesrvacionista e sustentável. Além da melhora pela substituição parcial de fertilizantes químicos, *A. brasilense* pode trazer outros benefícios às plantas, como a indução de mecanismos de tolerância a estresses abióticos.

Desde 2009 as estipres de *A. brasilense* Ab-V5 e Ab-V6 vêm sendo utilizadas de modo exponencial no Brasil para a inoculação de gramíneas e coinoculação de leguminosas partindo de 400 mil doses comercializadas naquele ano para cerca de 4,5 milhões na safra 2016/17. Contudo, estudos sobre estratégias de colonização e indução de tolerância a estresses com essas estirpes ainda não estavam disponíveis.

Neste estudo, foi demonstrado que as diferentes estirpes de uma mesma espécie apresentam mecanismos QS distintos durante o processo de colonização em raízes. Ademais, independente do método de inoculação, seja com células vivas ou com seus metabólitos (pulverização foliar) beneficiam o crescimentos das plantas de milho, e os mecanismos de resistência a estresses bióticos – *Azospirillum* – podem ser desencadeados pela indução simultânea via AJ e AS, ou de tolerância a estresses abióticos – estresse salino – via AS e ABA. Os efeitos benéficos da indução de tolerância do milho a estresses podem ser melhorados pela coinoculação com *Rhizobium tropici.*