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Resposta de *Herbaspirillum seropedicae* (estirpe HRC54) ao líquido apoplástico de cana-de-açúcar: transcristos e proteínas diferencialmente expressos e seu papel na interação planta-microrganismo

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"Aqui, no entanto, nós não olhamos para trás por muito tempo. Nós continuamos seguindo em frente, abrindo novas portas e fazendo coisas novas. Porque somos curiosos... e a curiosidade continua nos conduzindo por novos caminhos. Siga em frente."

Walt Disney

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um dia e possa entender porque não consegui te dar atenção direito.
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Resumo

Herbaspirillum seropedicae é uma bactéria endofítica diazotrófica recomendada como inoculante para gramíneas devido a sua capacidade de promover o crescimento vegetal. No caso da estirpe HRC54 de *H. seropedicae*, esta característica já foi observada em milho, sorgo, arroz e cana-de-açúcar. O uso de bactérias endofíticas diazotróficas como inoculante em gramíneas de interesse econômico pode ser uma alternativa sustentável ao uso de fertilizantes nitrogenados. Diversas pesquisas têm mostrado o efeito benéfico da inoculação, mas ainda existem algumas etapas dessa interação que necessitam ser mais bem esclarecido. O estudo do conjunto de trasncritos e proteínas expressos em resposta a diferentes estímulos pode ser a chave para o melhor entendimento desses processos, nos auxiliando a compreender quais mudanças e adaptações são necessárias para que o microrganismo possa infectar a planta e contribuir para o crescimento vegetal. A transcriptômica e a proteômica podem auxiliar na identificação dos padrões de expressão em diferentes condições, incluindo estudos de interação com bactérias promotoras do crescimento vegetal. Nesse sentido, essas duas abordagens foram utilizadas para a identificação dos transcritos e proteínas diferencialmente expressas na estirpe HRC54 de *H. seropedicae* em resposta à exposição de 2 horas ao líquido apoplástico de cana-de-açúcar da variedade RB867515. Os resultados observados nas análises *in silico* foram validados por RT-qPCR. Dentre os 89 transcritos e 123 proteínas diferencialmente expressos, a estirpe alterou a expressão do sistema de transporte e metabolismo de diferentes nutrientes, sugerindo que a bactéria possivelmente reconheceu o líquido do apoplastico como um componente do hospedeiro e modificou seus sistemas metabólico para adaptação ao crescimento em condições endofíticas.

Palavras chaves: *Herbaspirillum seropedicae*; cana-de-açúcar; transcriptoma; proteoma; interação planta-microrganismo.

Abstract

Herbaspirillum seropedicae is an endophytic diazotrophic bacteria recommended as inoculant for gramineous plant due to its characteristic as plant growth promotion bacteria. For strain HRC54 of *H. seropedicae*, its beneficial effects have already been observed in maize, rice, sorgum and sugarcane. The use of endophytic diazotrophic bacteria as inoculante for economic interest gramineuos can be a sustainable alternative against the use of nitrogen fertilizers. Researchs have showed the beneficial effects of these inoculants, but there are still some steps in the interaction process that need clarification. The study of transcripts and proteins differentially expressed as a microorganism's response to exposures to diferent situations could be the key for a better understanding of these process, helping clarify which changes and adaptations are necessary for the microrganism to infect the plant and contribute with its growth. Transcriptome and proteome can support the identification of expression patterns under different conditions, including interaction studies with plant growth promotion bacteria. Whit this, these two approaches were used to identify transcripts and proteims differentially expressed in *H. seropedicae* HRC54 respondign to a two hour exposure to sugarcane apoplastic fluid from the variety RB867515. The results observed on the *in silico* analyses were validated towards RT-qPCR. Among the 89 transcripts and 123 proteins differentially expressed, the strain altered the expression of systems related to transport and metabolism of different nutrients, susggesting that bacteria possibily recognized the apoplastic fluid as host component and modified its metabolic system in order to adapt a growth in endophytic conditions.

Keywords: *Herbaspirillum seropedicae*; sugarcane; transcriptome; proteome; plant microorganism interaction.

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Lista de Abreviaturas

BNF	*Biological Nitrogen Fixaton*
BPCV	Bactérias promotoras crescimento vegetal
cDNA	Ácido desoxirribonucleico complementar
CFU	*Colony-Forming Unit*
COG	Banco de dados *Clusters of Orthologous Groups* (COG)
Ct	*Cycle Threshold*
D.O./mL	Densidade ótica por mililitro
DEB	*Diazotrophic Endophytic Bacteria*
DEGs	*Differential Expressed Genes*
DNA	Ácido desoxirribonucleico
DUFFs	*Domains of Unkonwn Functions*
FBN	Fixação Biológica de Nitrogênio
FDR	*False Discovery Rate*
KEGG	Banco de dados *Kyoto Encyclopedia of Genes and Genomes*
Km	Antibiótico canamicina
mRNA	RNA mensageiro
ORFs	*Open Reading Frame*
PGPB	*Plant Growth-Promoting Bacteria*
RNA	Ácido ribonucleico
RNA-Seq	Sequenciamento de RNA
ROS	*Reative Oxigen Species*
RPKM	*Reads Per Kilobase of Transcript Per Million*
RT-qPCR	Reverse Transcriptase and Quantitative PCR
SSVI	Sistema secreção tipo VI
T6SS	*Type 6 Secretion System*
UFC/mL	Unidades formadora de colônia por mililitro

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1. Introdução

A cultura da cana-de-açúcar tem grande importância para o agronegócio, com a produção de açúcar e utilização para combustíveis como o etanol (Ryan et al., 2008). No entanto, os níveis de produtividade da cultura têm sido reduzidos devido a algumas condições abióticas, como o déficit hídrico, altas temperaturas e baixa fertilidades dos solos utilizados para o cultivo (Pereira et al., 2013). Neste cenário, a aplicação de fertilizantes na cultura da cana-de-açúcar tem se tornado uma prática recorrente, sendo mais frequente os adubos nitrogenados (Schultz et al., 2014). Porém, a utilização desses fertilizantes onera a produção e pode ocasionar problemas ambientais (Gomes et al., 2008a).

Uma alternativa para a redução do uso dos fertilizantes é a aplicação de inoculantes à base de bactérias endofíticas diazotróficas (BED), que podem atuar diretamente no suprimento de nitrogênio para a planta e promover o crescimento vegetal (Oliveira et al., 2002; Fischer et al., 2012). Apesar dos avanços já obtidos com o uso dessas bactéris, ainda existem diversas lacunas em relação a resposta à inoculação e a compreensão do processo de inoculação, sendo necessário um entendimento sobre mais detalhes dos mecanismos envolvidos na interação planta-bactéria (Monteiro et al., 2008).

Acredita-se que com ensaios *in vitro* de cultivo de um microrganismo endofítico em exposição de algum fluido ou tecido da planta, possam simular a interação do microrganismo com o tecido hospedeiro e alterar a expressão gênica de genes necessários para o nicho endofítico (*in vivo*) (Dong et al., 1994). Dessa forma, o cultivo de *Herbaspirillum seropedicae,* estirpe HRC54, na presença do líquido apoplástico (fluido presente nos espaços intercelulares das plantas que compreende à endosfera) de cana-de-açúcar é uma metodologia interessante para identificar os genes e proteínas da bactéria potencialmente relacionados com o processo de interação com a planta. Embora já existam relatos de estudos do transcriptoma e proteoma da *H. seropedicae* (Cordeiro et al., 2013; Pankievicz et al., 2016; Grillo-Puertas et al., 2021; Nunes et al., 2021), não há, até o momento, relatos do uso do líquido apoplástico de cana-de-açúcar em estudos de interação com esta espécie.

Deste modo, o presente estudo utilizou o líquido do apoplasto de cana-de-açúcar, aplicando a abordagem transcriptomica e proteômica, para avaliar os mecanismos envolvidos na interação planta-bactéria. Os resultados presentes nesse estudo trazem informações relevantes para o avanço no conhecimento do processo de interação da *H.*

seropedicae, estirpe HRC54, com a cana-de-açúcar, dando suporte para futuros bioinsumos.

1.1. Revisão de literatura

1.1.1. Cana-de-açúcar

A cana-de-açúcar pertence à classe Liliopsida; família *Poaceae*; tribo *Andropogoneae*, e gênero *Saccharum*. Há pelo menos seis espécies do gênero, sendo a cana-de-açúcar cultivada um híbrido multiespecífico, recebendo a designação *Saccharum* spp (Martins et al., 2020). A cultura canavieira foi inserida no Brasil no século XVI, tornando o país maior produtor dessa cultura desde 1980, seguido pela Índia e China (IBGE, 2017a). Seu cultivo possui grande importância para o agronegócio, em função do uso agronômico de sua matéria prima como melaço e rapadura, além da utilização do bagaço para produção de energia e seu potencial para produção de biocombustíveis (Ryan et al., 2008; IBGE, 2017b; Bordonal et al., 2018). Contudo, de acordo com a Companhia Nacional de Abastecimento - CONAB (2020), há uma estimativa de diminuição da produção de 4,3% em relação à safra 2020/21, resultando em 8.243,1 mil hectares para a produção de cana-de-açúcar, com destaque nas regiões Sul e Sudeste. Estima-se que nas regiões de maior área plantada, haja uma predominância da produção não-familiar de cana-de-açúcar (IBGE, 2017a). Essas reduções dos níveis de produtividade podem ser associados a solos de baixa fertilidade, condições edafoclimáticas e estresses abióticos (Schultz et al., 2015).

Diante deste cenário, diversas alternativas para aumentar a produção têm sido utilizadas, sendo mais frequente a aplicação de fertilizantes na cultura da cana-de-açúcar, com destaque para os adubos nitrogenados (Schultz et al., 2014). A utilização dos fertilizantes leva a aumentos do custo da produção, que podem não ser vantajosos para os pequenos produtores. A aplicação dos insumos também pode ocasionar problemas ambientais, como por exemplo, contaminação do solo e lençóis freáticos por lixiviação. Visando diminuir esses impactos na produção, alternativas sustentáveis tem sido buscadas para serem aplicadas por pequenos e médios produtores (Gomes et al., 2008a).

1.1.2. Fixação Biológica de Nitrogênio

Uma alternativa para a redução do uso de fertilizantes nitrogenados é o uso de inoculantes à base de bactérias diazotróficas, ou seja, capazes de tornar biodisponível para

as plantas o nitrogênio atmosférico (Boddey et al., 1995). A fixação biológica de nitrogênio (FBN) é um processo de ocorrência natural em alguns organismos procariotos que possuem a enzima nitrogenase, capazes de reduzir o nitrogênio atmosférico (N_2) para a forma inorgânica combinada NH_3 (Urquiaga et al., 1992; Pereira et al., 2020).

Devido à baixa disponibilidade de nitrogênio no solo, o processo de FBN se torna interessante, permitindo a obtenção desse nutriente que existe em abundância na atmosfera, porém em forma não assimilável pelas plantas (Pereira et al., 2013). Durante a associação entre plantas e microrganismos, as bactérias se beneficiam por ocuparem um ambiente protegido contra estresses bióticos e abióticos e com condições favoráveis para a sua manutenção nutricional, enquanto fornecem nutrientes (como o nitrogênio) e promovem o crescimento da planta hospedeira (Santi et al., 2013).

Além da FBN, o efeito da inoculação com bactérias diazotróficas na produção de cana-de-açúcar ocorre também através do crescimento do sistema radicular, o que proporciona maior absorção de nutrientes pelas plantas. Quando esses efeitos são identificados, pode ocorrer a seleção de genótipos mais suscetíveis à associação com bactérias diazotróficas, apresentando os efeitos da inoculação de forma mais intensa. A variedade comercial RB867515, por exemplo, é considerada responsiva à inoculação no que se refere ao aumento de produtividade, em testes sem a aplicação de fertilizantes nitrogenados (Reis et al., 2007). Além da FBN e a promoção do crescimento vegetal, a inoculação pode aumentar a tolerância a estresse abiótico, como apresentou Vargas et al., (2014), onde plantas de cana-de-açúcar inoculadas com *Gluconacetobacter diazotrophicus* foram menos afetadas pelo estresse hídrico.

1.1.3. Bactérias endofíticas diazotróficas

O termo “endofítico diazotrófico” foi inicialmente sugerido por Dobereiner (1992), para as bactérias fixadoras de nitrogênio com a capacidade de colonizar o interior das plantas. As bactérias endofíticas podem ser classificadas em obrigatórias, quando são dependentes da planta hospedeira para seu crescimento e sobrevivência durante todo seu ciclo; e facultativas, que possuem uma fase de seu ciclo dentro da planta hospedeira e outra na rizosfera (Baldani et al., 1997; Hardoim et al., 2008). Algumas destas bactérias são capazes de colonizar as plantas, da raiz até a parte aérea, o que pode trazer vantagens para o microrganismo e a planta hospedeira (Guo et al., 2008; Ryan et al., 2008).

A associação com o hospedeiro pode ocorrer incialmente pela a adesão da bactéria à superfície radicular, seguida de colonização de pontos de emergência de raízes

secundárias e penetração através de descontinuidades da epiderme, ocorrendo assim uma rápida ocupação dos espaços intercelulares, colonização do xilema e dispersão até a parte aérea (Hardoim et al., 2008; Balsanelli 2013). Bactérias com características associativas benéficas são também chamadas de promotoras do crescimento vegetal (BPCV) e podem se associar com diferentes plantas como gramíneas e leguminosas (Vejan et al., 2016).

Diversos estudos têm mostrado os efeitos positivos da inoculação de BPCV, não só através da FBN, mas também por meio de outros mecanismos, como a produção de fitohormônios, solubilização de fosfato e produção de sideróforos, na qual atuam diretamente na promoção do crescimento vegetal, podendo também auxiliar na tolerância a estresses abióticos e bióticos, como por exemplo proteção contra fitopatógenos (Figura 1) (Oliveira et al., 2002; Fischer et al., 2012; Vargas et al., 2014; de Souza et al., 2015; Pankievicz et al., 2021).

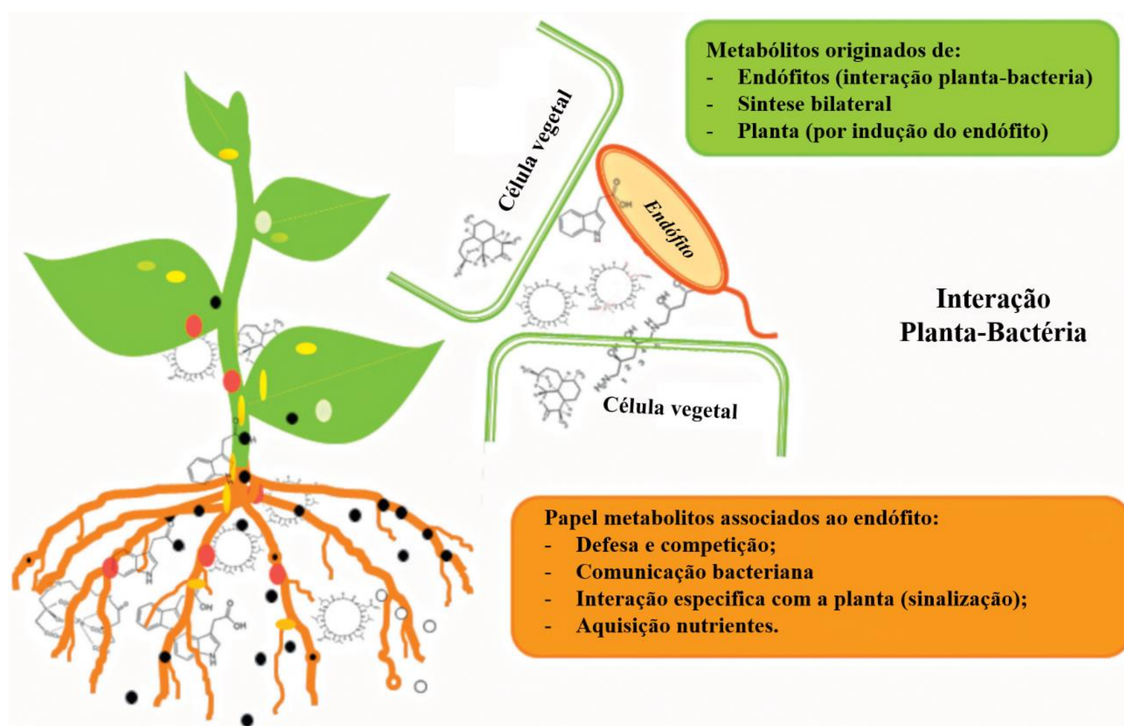

Figura 1: Destaque de algumas vantagens da interação planta-microrganismos, para ambos organismos envolvidos no processo. Figura adaptada de Brader et al., (2014).

Em 2007, a Embrapa propôs um inoculante para cana-de-açúcar composto por cinco estirpes de bactérias diazotróficas: *Gluconacetobacter diazotrophicus* PAl5, *Herbaspirillum rubrisulbalbicans* HCC103, *Herbaspirillum seropedicae* HRC54, *Nitrospirillum amazonense* CBAmC *e Paraburkholderia tropica* PPe8, relatadas como

sendo endófitos de cana-de-açúcar (Reis et al., 2007). A ação dessas bactérias já haviam sido avaliadas de forma individual em cana-de-açúcar e outras gramíneas, como milho e arroz (Olivares 1997). Também foram observados os efeitos satisfatórios quando uma ou mais estirpes foram misturadas, levando a sugestão de um consorcio ao invés de uma única espécie (Oliveira et al., 2006).

No entanto, a resposta à inoculação tem sido avaliada mais frequentemente em experimentos em escala menor, como em casas de vegetações, por exemplo. Quando avaliado em campos experimentais, ainda existem variabilidade relacionadas à eficácia do processo (Pereira et al., 2013). Ainda, os mecanismos bioquímicos e/ou moleculares envolvidos nesse processo de interação ainda não são totalmente conhecidos, tornando importante sua identificação e descrição (Sturz et al., 2000; Monteiro et al., 2008; Amaral 2014).

1.1.4. A espécie *Herbaspirillum seropedicae*

As bactérias do gênero *Herbaspirillum* são beta-proteobactérias aeróbicas Gram-negativas, vibrióides, com movimentação por flagelos. No total, existem 13 espécies descritas do gênero (Parte et al., 2020): *Herbaspirillum aquaticum* (Dobritsa et al., 2010), *H. autotrophicum* (Ding & Yokota, 2004), *H. camelliae* (Liu et al., 2020), *H. chlorophenolicum* (Im, 2004), *H. frisingense* (Kirchhof et al., 2001), *H. hiltneri* (Rothballer et al., 2006), *H. huttiensis* (Ding & Yokota, 2004), *H. lusitanum* (Valverde et al., 2003), *H. piri* (Xu et al. 2018), *H. rhizosphaerae* (Jung et al., 2007), *H. robiniae* (Fan et al., 2018), *H. rubrisubalbicans* (Baldani et al., 1996) e *H. seropedicae* (Baldani et al., 1986). As espécies *H. frisingense*, *seropedicae* e *rubrisulbalbicans* são classificadas como diazotróficas, sendo a última descrita em algumas espécies de plantas como patogênica, como por exemplo uma doença que acomete plantas de cana-de-açúcar, conhecida como estria mosqueada (Valdameri et al., 2017).

Algumas espécies desse gênero podem ser observadas em associação endofítica com plantas, com colonização endofítica na planta hospedeira por penetração passiva, no interior da raiz, no córtex e tecidos vasculares (Da Silva et al., 2003; Pedrosa et al., 2011; Monteiro et al., 2012), além de se destacarem por sua capacidade em fixar nitrogênio (Baldani et al., 1996; Pedrosa et al., 2011; Monteiro et al., 2012). Essas espécies também foram encontradas em tecidos de plantas superficialmente desinfestadas, sugerindo que se tratavam de bactérias endofíticas (James & Olivares, 1998; Hardoim et al., 2008).

A espécie *Herbaspirillum seropedicae* é uma bactéria endofítica fixadora de nitrogênio, descrita por Baldani et al. (1986) a partir de seu isolamento em plantas de arroz, milho e sorgo. Trabalhos posteriores mostraram que essa bactéria é capaz de colonizar também cana-de-açúcar, abacaxizeiro e bananeira (Olivares et al., 1996; James & Olivares, 1998; Cruz et al., 2001; James et al., 2002). Seus mecanismos de colonização são caracterizados, porém ainda pouco detalhados (Monteiro et al. 2012).

A estirpe HRC54 foi isolada de raízes de cana-de-açúcar por Dobereiner (1992), utilizando meios de culturas livres de nitrogênio, focados em buscar espécies fixadoras. A estirpe foi incluída no inoculante proposto pela Embrapa em 2007 (Oliveira et al., 2006; Reis et al., 2007), e já teve seus efeitos avaliados em algumas gramíneas, com o destaque para interação com cana-de-açúcar (Alves et al., 2014; dos Santos et al., 2020; Martins et al., 2020; Pereira et al., 2020). A estirpe SmR1, pertencente a espécie *H. seropedicae*, teve seu genoma sequenciado em 2011, destacando características interessantes como sistemas de secreção de proteínas diversificado, proteínas associadas à adesão celular e genes relacionados à FBN (Pedrosa et al., 2011). Contudo, ainda são necessários estudos mais detalhados para a identificação do padrão de expressão gênica associado ao processo de interação.

1.1.5. Apoplasto de plantas superiores

O apoplasto é a parede intercelular (não atravessa a membrana plasmática) e o líquido ou meio aquoso ali presente é chamado de liquido do apoplástico (Figura 2) (Haslam et al., 2003). Esse líquido pode se distribuir de forma contínua, vindo da raiz, passando pelo caule e chegando até as folhas, permitindo parte do transporte de água e nutrientes da rizosfera para os demais tecidos e órgãos da planta (Guo e Song 2009; Pechanova et al., 2010). Devido a características do simplasto que podem afetar a membrana plasmática dos microrganismos, como a presença de solutos e barreiras de íons, teoriza-se que as bactérias tenham preferência pelo apoplasto (Sattelmacher e Horst 2007).

O líquido apoplástico já foi caracterizado como um fluido composto por açúcares como sacarose, glicose, frutose; alguns ácidos orgânicos, como malato e citrato; e compostos nitrogenados como serina e ácido aspártico (Asis et al., 2003; Tejera et al., 2006). Esses nutrientes são similares aos aplicados em meios de cultura utilizados para bactérias, podendo estar relacionados com o controle do crescimento e da atividade

microbiana no líquido apoplástico, podendo auxiliar nos estudos sobre a promoção do crescimento vegetal (Sattelmacher & Horst, 2007).

Em cana-de-açúcar, Dong et al. (1994), realizaram análises no líquido apoplástico, caracterizando como um possível local para microorganismos dentro da planta. Dos-Santos et al. (2021) analisaram a microbiota do líquido apoplástico de cana-de-açúcar (variedade RB867515), detalhando os gêneros presentes neste fluido em plantas inoculadas com as cinco estirpes sugeridas pela Embrapa, e não inoculadas. Nas amostras não inoculadas, o gênero *Herbaspirillum* foi detectado em abundância.

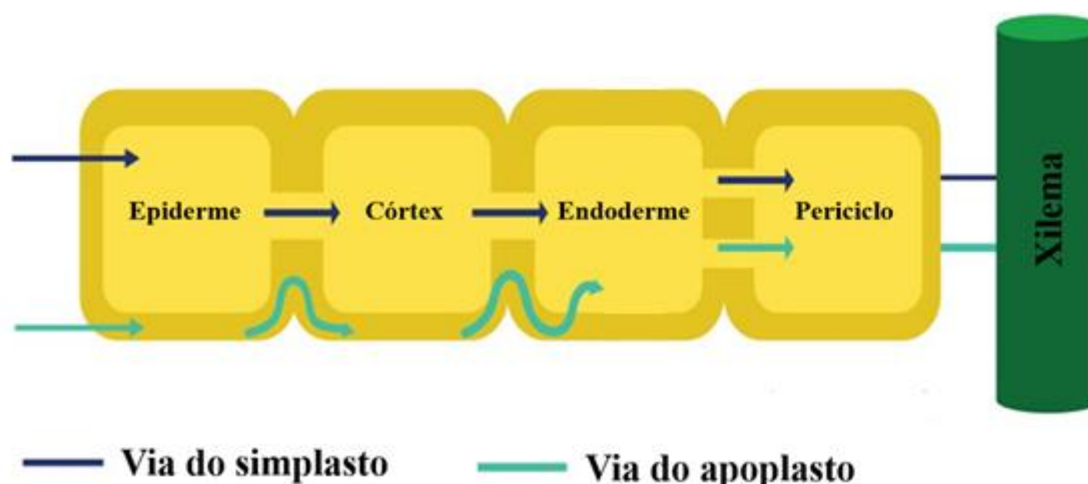

Figura 2: Representação gráfica do apoplasto. A via do simplasto (seta azul escura) permite o transporte intracelular, enquanto a via do apoplasto (seta azul piscina) ocorre de maneira extracelular. Ao atingirem a endoderme, as vias passam a ser extracelulares, chegando ao xilema. Acredita-se que nessas vias possam ser transportados nutrientes, e até mesmo microrganismos. Imagem adaptada, retirada do sítio https://www.vedantu.com/biology/apoplast.

1.1.6. Aspectos moleculares do processo da interação entre plantas e as bactérias promotoras crescimento vegetal

A efetividade do processo de interação entre as BPCV e as plantas está relacionado com diversos mecanismos que controlam a expressão gênica em ambos os organismos, de forma a proporcionar a associação. Entretanto, apesar de haver um conhecimento geral sobre a interação, ainda existe pouco conhecimento acerca das etapas desse processo (Bruto et al., 2014). A necessidade de melhor compreensão desse processo é fundamental

para aumentar a consistência de resposta à inoculação com bactérias associativas (Santoyo et al., 2016).

A expressão gênica envolvida no processo de interação vem se tornando o principal foco dos estudos. Sabe-se que para alcançar a simbiose, planta e microrganismo podem ativar ou reprimir sistemas e metabolismos que estão relacionados ao processo de interação (Pankievicz et al., 2021). (Baldani and Baldani 2005; Orozco-Mosqueda et al. 2021; Sheoran et al. 2021). Por exemplo, De Matos et al., 2021, apresentaram uma análise dos genes *nif* em bactérias do gênero *Bradyrhizobium* quando em associação com cana-de-açúcar, sugeriram a participação desses genes durante a interação. Porém, o estudo também demonstrou que pode ocorrer uma expressão diferenciada em plantas leguminosas e não-leguminosas, bem como entre as espécies do gênero, ressaltando a necessidade de uma avaliação específica do microrganismo com cada planta.

Os genes relacionados à produção de lipopolissacarídeos e exopolisacarídeos também já foram relatados como importantes para o processo de colonização das plantas. A estirpe SMR1 de *H. seropedicae* requer lipopolissacarídeos para adesão nas raízes e colonização interna em milho, como mostrado por Balsanelli et al. (2010). Em arroz, Meneses et al. (2011) mostraram que a produção de exopolissacarídeo é necessária para formação de biofilme e colonização das plantas pela bactéria endofítica fixadora de nitrogênio *Gluconacetobacter diazotrophicus*.

Diversos estudos têm mostrado que quanto maior a capacidade do microrganismo competir por sítios de infecção, maiores as chances de uma interação efetiva. Um exemplo é o uso dos sistemas de secreção por várias bactérias para aumentar seu poder competitivo. Em bactérias endofóticas, esse sistema também tem se mostrado importante, por auxiliar na capacidade de "infectar" a planta (Bernal et al. 2018; Speare et al., 2018; Goyal et al., 2021). O sistema de secreção do tipo VI, que até há pouco tempo havia sido identificado apenas em microrganismos patogênicos, tem sido determinante em bactérias com alta capacidade de invasão (Jani & Cotter, 2010; Speare et al., 2018). A espécie *H. seropedicae* possui os sistemas do tipo III e VI, podendo esse ser um dos motivos de sua alta capacidade de infectar a planta e colonizar os espaços intercelulares (Pedrosa et al., 2011; Monteiro et al., 2012).

Através do sequenciamento de mutantes gerados por transposon Do Amaral et al. (2020) identificaram vários genes necessários para o processo de colonização de *Setaria viridis* pelas bactérias *Azoarcus olearius* (DQS4T) e *H. seropedicae* (SmR1). Entre os genes descritos destacam-se genes da pirroloquinolina quinona (PQQ)-dependente alcool

dehidrogenase na estirpe DQS4T, e poli-3-hidroxialkanoato depolimerase (PhaZ1) na estirpe SMR1, que possuem um papel relevante no processo de competição e colonização da superfície das raízes.

Especificamente para a estirpe SMR1, genes relacionados à motilidade, transporte de aminoácidos e produção de energia são diferencialmente expressos durante as etapas iniciais da infecção e podem ter uma função importante na interação. A modificação em genes de motilidade é geralmente reportada em estudos de interação entre plantas e microrganismos, seja através de experimentos de inoculação e/ou ensaios *in vitro* (Taulé et al. 2021). Em *H. seropedicae* SMR1, a repressão dos genes de motilidade foram reportados em experimentos com cana-de-açúcar e milho, e em reposta a exposição ao caldo de cana ou flavonóides (Alberton et al., 2013; Cordeiro et al., 2013; Tadra-Sfeir et al., 2015; Balsanelli et al., 2016; Pankievicz et al., 2016). O uso do líquido apoplástico de cana-de-açúcar, também mostrou modificação nos padrões de expressão de genes relacionados com a motilidade em outras espécies de fixadoras como *P. tropica* PPe8 e *N. amazonense* CBAmC (Silva et al., 2018; Terra et al., 2020).

Apesar de alguns estudos já terem identificado genes potencialmente relevantes para algumas das etapas importantes da interação entre bactérias endofíticas diazotróficas e plantas, a maioria dos autores concorda que falta uma anotação funcional mais detalhada, reforçando a necessidade de pesquisas que possam avaliar as vias metabólicas envolvidas nas etapas do processo de interação (Levy et al., 2018; Do Amaral et al., 2020). Esses resultados mostram que há necessidade de ampliar o número de genes e proteínas estudados, possibilitando a identificação de mecanismos que sejam específicos de cada microrganismo, havendo assim a necessidade de avaliar cada espécie ou grupo de BPCV. Os conhecimentos obtidos nesses experimentos podem ser utilizados na seleção de estirpes ou espécies com maior capacidade de interação, auxiliando na busca de melhores inoculantes a base de BPCV (Backer et al. 2018).

1.1.7. Ciências "ômicas" para avanços de conhecimento sobre a interação planta-bactéria

As análises de transcriptoma e proteoma podem ser utilizadas nos estudos de expressão gênica graças aos avanços tecnológicos na área de biologia molecular (Liu et al., 2012). Para o transcriptoma, o sequenciamento de RNA em larga escala (RNA-Seq) tem mostrado ser uma tecnologia de alta eficiência, capaz de fornecer uma grande quantidade de dados em pouco tempo (Sharma and Vogel 2014). As sequências que

resultam do RNA-Seq podem ser alinhadas a um genoma de referência, a fim de identificar os genes diferencialmente expressos, permitindo, por exemplo, mostrar mudanças nos níveis de expressão gênica que possam indicar quais genes estão envolvidos com a relação entre bactérias e plantas (Wang et al., 2009; Sharma & Vogel, 2014; Souza et al., 2016).

As análises de proteômica, por outro lado, vem sendo potencializadas com o surgimento de metodologias livres de gel (*gel-free*) e livres de marcação (*label-free*), que utilizando espectrometria de massas, tornam possível obter um maior número de proteínas identificadas, quando comparadas com a eletroforese bidimensional (análise de *spots*) (Baggerman et al. 2005; Roe & Griffin, 2006; Armengaud, 2013).

A utilização dessas técnicas em estudos da simbiose entre microrganismo:hospedeiro, que simulem condições ambientais, podem ser a chave para aumentar o entendimento dos genes e proteínas expressas pelo microrganismo ao iniciar o processo de interação. Contudo, esses tipos de experimentos, quando aplicados em plantas inoculadas, podem trazer uma grande quantidade de dados, podendo tornar a interpretação dos resultados confusa. Abordagens que foquem em avaliar o padrão de expressão da bactéria frente a presença ou exposição da planta, podem tornar os experimentos menores e permitindo avaliar o processo de interação (Zhang et al. 2017).

Para a espécie *H. seropedicae*, a identificação de genes e proteínas diferencialmente expressas já vem sendo utilizada para um maior entendimento dos processos metabólicos envolvidos na interação. Balsanelli et al. (2016) e Pankievicz et al. (2016) observaram, através de análises de RNA-Seq, que a estirpe SMR1 induziu genes relacionados a colonização de milho e trigo, respectivamente. Cordeiro et al. (2013) identificaram a expressão diferenciada de proteínas relacionadas ao estabelecimento da parede celular quando a *H. seropedicae* estirpe SMR1 foi cultivada com 5% de caldo de cana-de-açúcar. Tadra-Sfeir et al. (2015) mostrou que a resposta da estirpe SMR1 a presença do flavonoide naringenina resultou na repressão de genes relacionados a formação de flagelos e quimiotaxia. Ambos resultados apresentados anteriormente sugerem a preparação da estirpe SMR1 para interação/colonização na superfície da raiz da planta.

Dessa forma, o cultivo de *H. seropedicae* HRC54 na presença do líquido apoplástico de cana-de-açúcar é uma alternativa interessante para identificar os genes e proteínas da bactéria potencialmente relacionados com o processo de interação com a planta. Embora já existam relatos de estudos do transcriptoma e proteoma da espécie *H. seropedicae* (Cordeiro et al. 2013; Pankievicz et al., 2016; Grillo-Puertas et al., 2021;

Nunes et al., 2021) não há, até o momento, relatos do uso do líquido apoplástico e a estirpe HCR54 de cana-de-açúcar em estudos de interação.

A utilização do líquido apoplástico de cana-de-açúcar como estudo de interação se mostrou viável na avaliação dos níveis de expressão diferencial das bactérias *P.tropica* estirpe Ppe8 e *N. amazonense* estirpe CBAmC quando expostas durante duas horas ao líquido apoplástico de cana-de-açúcar (variedade RB867515). Ambos os estudos destacaram a repressão de genes relacionados com motilidade, quimiotaxia, metabolismo e transporte de carboidratos e aminoácidos. Essas alterações indicam uma tentativa de adaptação das estirpes à presença do fluido e busca por uma mudança de vida livre para endofítica (Silva et al., 2018; Terra et al., 2020).

Com isso, no presente trabalho, a estirpe HRC54 de *H. seropedicae* foi exposta ao líquido apoplástico da variedade RB867515 de cana-de-açúcar para análises de transcriptoma e proteoma diferencial, utilizando as abordagens de RNA-Seq e livre de gel, respectivamente. A variedade RB867515, utilizada neste estudo, é considerada responsiva à inoculação no que se refere ao aumento de produtividade, em testes sem a aplicação de fertilizantes nitrogenados (Reis et al., 2007; dos Santos et al., 2020).

1.2. Justificativa

A fase inicial de uma colonização endofídica pode ser crucial para uma associação bem sucedida com o hospedeiro (Rosconi et al., 2016). Compreender as etapas envolvidas neste processo pode ajudar a esclarecer e melhorar sua performance. Ferramentas de análise do padrão de expressão gênica em diferente níveis, como mRNA e proteínas, podem auxiliar no entendimento dos mecanismos envolvidos na interação (Kaul et al., 2016). Espécies de BPCV vem sendo usadas como inoculante visando o aumento da produção e reduzindo o impacto ambiental do uso de fertilizantes, mostrando ser uma aplicação viável e sustentável (dos Santos et al., 2020). Contudo, ainda existem algumas inconsistências na reposta à inoculação que podem ser elucidadas por meio da avaliação dos mecanismos moleculares utilizados por essas espécies de bactérias durante a interação com a planta (Lardi and Pessi 2018).

A avaliação do conjunto de genes e proteínas expressos em diferente condições de crescimento e/ou exposição de nutrientes pode auxiliar na obtenção de respostas relacionadas a questões de qual(is) sistema(s) pode(m) ser chave(s) para o processo de interação (Kaul et al., 2016; Zhang et al., 2017). Acredita-se que com ensaio *in vitro* de microrganismos na presença de algum fluido ou tecido da planta, seja possível simular o

que ocorre com estes organismos quando associado com o hospedeiro (Dong et al., 1994). O fato de algumas BPCV serem capazes de ocupar os espaços intercelulares, torna o líquido apoplástico de cana-de-açúcar um material candidato para estudos com objetivo de simular o nicho endofítico (James e Olivares, 1998).

Diversos autores já mostraram a ocorrência de alterações moleculares durante a interação planta-bactéria, como por exemplo, um padrão de expressão gênica específico; modificações nas respostas de defesa a possíveis patógenos e sinalizações através de fitohormônios (Brusamarello-Santos et al. 2012; Tadra-Sfeir et al., 2015; Balsanelli et al., 2016). Deste modo, é interessante avaliar as respostas iniciais da bactéria *H. seropedicae* quando exposta ao líquido apoplástico e estudar as modificações de expressão gênica e/ou proteica induzidas.

O presente estudo pode revelar os sistemas e metabolismos diretamente envolvidos na interação planta-microrganismo, ampliando o entendimento sobre este processo. Especificamente, no caso da cana-de-açúcar, o uso do líquido apoplástico tem se mostrado promissor em estudos de interação bactéria-planta (Klonowska et al., 2018; Dos Santos et al., 2020; Leandro et al., 2021; Nunes et al., 2021), permitindo identificar quais vias metabólicas são diretamente influenciadas e importantes para este processo de interação (Silva et al., 2018; Terra et al., 2020).

1.3. **Hipótese**

O uso do líquido apoplástico em ensaios *in vitro* com BPCV, podem ajudar a simular o nicho endofítico e auxiliar nos estudos que visam a compreensão do processo de interação bactéria-planta, uma vez que, a bactéria pode reconhecer essa substância como a presença do hospedeiro, e modificar sua expressão gênica para as condições de associação e colonização endofítica.

2. **Objetivos**

Identificar genes e proteínas diferencialmente expressos em *H. seropedicae*, estirpe HRC54, em resposta ao líquido apoplástico de cana-de-açúcar.

2.1. Objetivos específicos

- Identificar genes e proteínas diferencialmente expressos na estirpe HRC54 após a exposição por 2 horas ao líquido apoplástico de cana-de-açúcar variedade RB867515;

- Validar do perfil de expressão identificado nas análises de genes e proteínas diferencialmente expressos, através da técnica de RT-qPCR;

- Comparar sistemas e metabolismos diferencialmente expressos na estirpe HRC54 em resposta ao líquido apoplástico de cana-de-açúcar.

3. Material e Métodos

3.1. Coleta do líquido apoplástico de cana-de-açúcar

Plantas de cana-de-açúcar da variedade RB867515 foram coletadas plantas com 1 ano de idade (soca) na área conhecida como "Campo Novíssimo" (22°44'50.3''S/43°40'15.7''W), no campo experimental da Embrapa Agrobiologia (Dos Santos et al., 2020). Após coleta no campo, as plantas foram imediatamente descascadas e seus nós foram retirados e entrenós cortados em pedaços menores, de forma que coubessem em tubos de fundo cônico do tipo "Falcon" com capacidade de 50 mL. Os entrenós foram flambados com álcool a 70% (v/v), e ponteiras estéreis de 300 uL foram inseridas na parte inferior do entrenó (de forma que a ponteira impedisse o contato do entrenó com o fundo do tubo), colocados nos tubos e mantidos fechados em gelo. Para a obtenção do líquido apoplástico, foi realizada uma centrifugação a 3.000 x g por 20 minutos a 4°C, a fim de proporcionar a manutenção das propriedades do líquido, de acordo com a metodologia de Dong et al. (1994).

3.2. Cultivo da estirpe HRC54 de *H. seropedicae*

A bactéria *H. seropedicae*, estirpe HRC54 (Baldani et al., 2014), foi obtida do Centro de Recursos Biológicos Johanna Döbereiner da Embrapa Agrobiologia (Zilli e Soares, 2016). A partir de uma colônia isolada, a bactéria foi repicada nos meios rico e semi-seletivo DYGS e JNFB (Rodrigues et al., 1986), respectivamente. O tempo de crescimento para culturas em meio líquido é de 16 a 24 horas a 30°C; e para culturas em placas de Petri com meio JNFB sólido, 5 dias a 30°C.

3.3. Experimento da estirpe HRC54 com líquido apoplástico de cana-de-açúcar

Para a realização do experimento de crescimento de *H. seropedicae*, na presença e ausência do líquido apoplástico de cana-de-açúcar, a estirpe HRC54 foi incubada durante 16 horas em 60 mL de JNFB líquido até atingir a densidade ótica a 600 nm ($D.O._{600}$) de 1,1, representando o início da fase estacionária (Pessoa, 2016). Com isso, a cultura foi separada em seis novos Erlenmeyers (capacidade 125 mL), onde para cada 15 mL de cultura, foram adicionados 15 mL do líquido apoplástico; e para o controle, 15 mL de água destilada esterilizada. As amostras do ensaio foram mantidas a 30ºC sob agitação a 100 rpm em um agitador orbital por mais 2 horas. Esse tempo foi estipulado com o

objetivo de obter o padrão de expressão que corresponda a uma resposta inicial da bactéria na presença ou ausência do líquido apoplástico no meio de cultura. Os ensaios foram feitos com 3 repetições biológicas. Após o período de 2 horas, foram coletados materiais para extração de RNA e proteínas totais, através de centrifugações a 8.300 x g por 10 minutos a 4ºC.

3.4. Avaliação do transcriptoma e proteoma da estirpe HRC54 em resposta a presença do líquido apoplástico

3.4.1. Identificação dos genes diferencialmente expressos estirpe HRC54 em resposta ao líquido apoplástico de cana-de-açúcar

O RNA total foi extraído utilizando o reagente TRIZOL™ (Invitrogen), de acordo com as especificações do fabricante. Em sequência, as amostras de RNA foram submetidas a um tratamento com DNAse I (Epicentre®, Cat. No. D9902K) para a completa remoção de DNA genômico. Para o enriquecimento do RNA mensageiro foi utilizado o kit MICROBExpress™ Bacterial mRNA Enrichment (Ambion), de acordo com as especificações do fabricante. No total, seis bibliotecas de cDNA foram construídas utilizando o kit Ion PITM Sequencing 200 e sequenciadas através do sequenciador do tipo Ion Proton System (Thermo Fisher Scientific). A qualidade dos dados brutos gerados no sequenciamento foi analisada através do software FastQC (Andrews, 2010). As sequências oriundas desta análise foram trimadas usando FASTX-Toolkit (http:// hanno nlab. cshl. edu/ fastx_ toolk it) e mapeadas contra o genoma de *H. seropedicae* HRC54 utilizando o programa EDGE-pro (Magoc et al., 2013). Apenas as *reads* com qualidade 20 e tamanho de 50 pares de bases foram utilizadas para o mapeamento. As leituras de sequencimanto (*reads*) mapeadas foram utilizadas para o cálculo de RPKM (do inglês *Reads Per Kilobase Of Transcript Per Million*) no programa EDGE-pro (Magoc et al., 2013). Apenas sequências alinhadas uma vez com o genoma de referência foram utilizadas nas análises seguintes. A identificação dos genes diferencialmente expressos e seus valores de *fold-change* e significância foram estimados através do pacotes do R DESeq2 (Love et al., 2014b) e edgeR (Robinson et al., 2010), com um valor *p* de 0,05 e log2fold-change de 1,5. Neste valor de *fold-change,* os maiores que 1,5 foram considerados induzidos (*up-regulated*) e aqueles menores que -1,5 foram considerados reprimidos (*down-regulated*). Foram considerados como genes difenrenfialmente expressos e subsequentes análises apenas os genes identificados tanto no DESeq2 como

no edgeR, com objetivo de garantir segurança aos dados obtidos *in silico*. A anotação funcional desses genes foi feita através do programa BLAST2GO (Conesa et al., 2005) e dos bancos de dados do *Kyoto Encyclopedia of Genes and Genomes* (KEGG) e *Clusters of Orthologous Groups* (COG) (Kanehisa and Goto 2000; Tatusov et al., 2000).

3.4.2. Identificação das proteínas diferencialmente expressas de HRC54 em resposta ao líquido apoplástico de cana-de-açúcar

Foi realizada uma avaliação das proteínas diferencialmente expressas utilizando uma abordagem chamada *gel-free*. O sedimento celular obtido pelo cultivo da estirpe HRC54 na presença e ausência do líquido apoplástico de cana-de-açúcar, foi ressuspenso em 1 mL de solução de ressuspensão (10 mM de Tris-HCl, 1,5 mM de MgCl2 e 10 mM de KCl, pH 8,0) e, novamente sedimentado por meio de centrifugação por 10 minutos a 12.000xg a 4°C. Ao sedimento bacteriano foram adicionados 2 mL de solução de ressuspensão, para transferir o conteúdo para um tubo de microcentrífuga com capacidade de 2 mL. O sedimento celular foi recuperado por meio de centrifugação por 10 minutos a 12.000xg a 4°C e congelado até dar início ao processo de extração. Em seguida, foram adicionados ao sedimento celular 0,5 mL de solução de solubilização (7 M de uréia, 2 M de tiouréia, 2% de Triton X-100, 1 mM de PMSF e 20 mM de DTT), e a mistura agitada vigorosamente por 30 segundos com auxílio de agitador do tipo vórtex. Em seguida, o sedimento foi mantido por 30 minutos à temperatura ambiente sob agitação de 500 rpm (Eppendorf ThermoMixer). Em banho de gelo, foi feito o processo de lise por sonicação: 10 ciclos de 20 segundos de pulso a 10% de amplitude intercalados por um período de descanso de 1 minuto. Após, as amostras foram centrifugadas por 90 minutos a 12.000xg a 4°C. Em seguida, o sobrenadante foi coletado e transferido para um novo tubo de microcentrífuga com capacidade de 2 mL, e mantido a -20°C até novas análises.

O sobrenadante foi utilizado para análise de espectrometria de massa do tipo ESI-LC-MS/MS, com coluna nanoacquity UPLC conectado a um espectrômetro de massa Synapt G2-Si HMDS (Waters, Manchester, UK). As amostras foram carregadas na coluna de afinidade C18 nanoAcquity UPLC 5 µm a 5 µL/min durante 3 min e depois na coluna de fase reversa analítica nanoAcquity HSS T3 de 1,8 µm a 400 nL/min, com temperatura de 45°C. Para a eluição peptídica, utilizou-se um gradiente binário, com fases móveis de ácido fórmico (Sigma-Aldrich) 0,1% em água (Tedia, Fairfield, Ohio, EUA) e ácido fórmico 0,1% em acetonitrila (Sigma-Aldrich).

A espectrometria de massa foi realizada em modo positivo e de resolução (modo V-35,000 FWHM) com mobilidade iónica; no modo de aquisição independente de dados (DIA), e separação da mobilidade iónica (HDMS-E). Nos parâmetros TOF, o tempo de varredura foi ajustado para 0,5 s no modo contínuo com uma faixa de massa de 50 a 2000 Da. Foi utilizado como calibração externa o Glu1-fibrinopeptídeo B (Sigma-Aldrich) a 100 fmol/µL e a aquisição de massa de bloqueio foi realizada a cada 30 segundos. A aquisição do espectro de massa foi realizada pelo programa MassLynx v4.0.

A análise foi feita com triplicata técnica de cada réplica biológica. O processamento de espectros e as condições de busca de banco de dados foram realizadas pela *Progenesis QI para Proteomics Software* V.2.0 (Nonlinear Dynamics, Newcastle, Reino Unido). Para análise foi utilizado o banco de dados de proteína de *H. seropedicae*, estirpe HRC54. As análises quantitativas relativas livres foram realizadas com base na proporção de contagens de íons proteicos entre as amostras contrastantes. Após o processamento de dados e para garantir a qualidade, apenas foram aceitas proteínas únicas ou presentes em 3 das 3 corridas. Além disso, as proteínas diferencialmente abundantes foram selecionadas com base em um *fold change* de maior que 1,5 e menor que 0,65 e ANOVA P <0,05 consideradas induzidas e reprimidas, respectivamente. As que ficaram no intervalo de 1,5 a 0,65 foram consideradas *Unchanged* (não alteradas). A anotação funcional dessas proteínas também foi feita através do programa BLAST2GO (Conesa et al., 2005) e dos bancos de dados do KEGG e COG (Kanehisa e Goto, 2000; Tatusov et al., 2000).

3.5. Validação dos dados do transcriptoma e proteoma através da técnica de RT-qPCR

Vinte e quatro genes e proteínas diferencialmente expressas identificados no transcriptoma e proteoma foram selecionados para a técnica RT-qPCR, a fim de validar os resultados obtidos (Tabela 1). Para o desenho dos iniciadores para o genes identificados nas análises de proteômica, foram utilizadas as sequências de amino ácidos das *open reading frames* (ORFs) correspondente a cada proteínas. O programa Primer 3 Plus (Untergasser et al., 2007) foi utilizado para o desenho dos iniciadores de acordo com os seguintes critérios: 19-22 nucleotídeos de tamanho; temperatura de anelamento entre 58-62°C; conteúdo GC de 50-80% e seus produtos na faixa de 100 a 180 pares de bases (pb). Em seguida, os iniciadores foram também avaliados no programa Oligo Explorer v.1.1.2 (http://www.genelink.com/tools/OEreg.asp).

O RNA total foi extraído (vide tópico 5.4.1.) e o cDNA sintetizado através da enzima Superscript™ III Reverse Transcriptase (Invitrogen), utilizando primers randômicos presentes no kit, seguindo as orientações do fabricante. O cDNA utilizado para os ensaios são oriundos de um novo experimento, seguindo as mesmas condições descritas no tópico 5.3. Para a reação de transcrição reversa seguida de PCR em tempo real (RT-qPCR) foi utilizado o equipamento 7500 Fast Real-Time PCR System (Applied Biosystems Cat. No. 275013373). Os experimentos foram realizados em triplicata técnica para 3 repetições biológicas, com 7,5 uL de GoTaq® qPCR Master Mix e 6,5 uL de cDNA, utilizando os primers da Tabela 1. Os dados brutos produzidos pelo ensaio de RT-qPCR foram inseridos no programa Miner (Zhao e Fernald, 2005), para calcular os valores de Ct (do inglês *Cycle Threshold*). Os níveis de expressão relativa foram obtidos com o programa qBASE v.1.3.5 (Hellemans et al., 2007). Como genes de referência, foram utilizados *rpoA* e *groEL*, como descrito por Pessoa et al. (2016).

Tabela 1: Sequências dos iniciadores utilizados para as reações de RT-qPCR e validação dos dados do transcriptoma e proteoma de estirpe HRC54 de *H. seropedicae* em resposta ao líquido apoplástico de cana-de-açúcar.

Gene	Anotação Funcional	Resultado que levou à síntese do iniciador	Iniciador (Foward 5´-3´)	Iniciador (Reverse 5´-3´)	Fragmento (pb)
Hs4167	Translocação de proteínas	UP - Transcriptoma	CACCACCCCCGAGATGTTTG	ACAGGTTCCAGATTTCGGCG	209
Hs4308	desconhecida	UP - Transcriptoma	GTGGAAGAACAACCGCCGCC	GTTCGGGATTGAGCCAGACG	227
pal	divisão celular	UP - Transcriptoma	CGACTACGACAGCTACACCG	CCCTGGATGATGACCTTGCG	102
Hs4729	transporte transmembranar	UP - Transcriptoma	GCCTGTCCGCAGTGTTCAAC	GTGTCAGCGAAGTCGGTCTG	163
hfq	ligação	UP - Transcriptoma	CCTGGTCAACGGCATCAAGC	GCACGACGGTAGAGATGGCG	116
Hs799	transferase	UP - Transcriptoma	ATGGGGGACACCGACAAACC	TGTGGGCGATGACTTCCTGC	214
Hs2067	hemaglutinina	DOWN - Transcriptoma	CCGTTCAACTTCCAGAGCAC	CGGAGGCATAGAAGGTCGTG	160
thiD	transferase	DOWN - Transcriptoma	CGTCAGCGTCTTTGCCGTGC	CTGGTCTCCCTTGCCATTGG	220
smoG	transporte	DOWN - Transcriptoma	CGCCATCGTGCTGTTCTTCC	ATACCCCAGATAGTGACTGC	145
ugpC	transporte	DOWN - Transcriptoma	ATCGTAGTGCTCTCCGAGGG	TGGGCGAACCGATGAAACCG	103
Hs245	dioxigenase	DOWN - Transcriptoma	GCAGCAAGGCGTTTTACGGC	ATGTCTTCCAGGTCGTCCGC	166
Hs707	transdução sinal	DOWN - Transcriptoma	TCATTCCCTTTGCCGAGACC	TGGAGAGGTTGACCGAGAAC	129
rpoA	RNA polimerase	Normalizador	GCACGAATACTCGTCCCTGG	CCTTCGCCTTCCTTCTTCAG	129
groEL	chaperona molecular	Normalizador	GCTACCTGTCGCCGTACTTC	GGTCACGGATGTTGGAGATC	105
PdxI	NADP(H)-dependente aldo-keto redutase	UP - Proteoma	CAGCATGTGGAGGACGAGAG	TACGGGTTCTGGATGGTGAC	185
HtpG	Chaperona molecular HtpG	UP - Proteoma	GCGTGGACGAATGGATGCTG	GGGTCTCTTCGTGCTGCTTC	129
Hs.1741	Glutamato/leucina dehidrogenase	UP - Proteoma	GCCGTGAACGTGCCCTATGG	AAGAGGTGCTGCCCTGGTTC	220
Hs.2721	keto-deoxi-fosfogluconato aldolase	UP - Proteoma	GGCATCACGCAAGAGACCGC	ATCTTGTCCCAGTCACCCGC	113
Hs.3070	proteína Sistema secreção tipo VI Hcp	UP - Proteoma	ATCAGCAGCGTCAAGCCCAG	GCCGCCATCCTTCTTCTGCT	111
Hs.4729	transporte transmembranar	UP - Proteoma	GCCTGTCCGCAGTGTTCAAC	GTGTCAGCGAAGTCGGTCTG	163
DppD	transportador ABC	DOWN - Proteoma	ATCGTCTTCGTCACCCACAG	CGTTGCTGCTCTTCCATCAC	191
DctP	transportador ABC C4-dicarboxylate	DOWN - Proteoma	GAGGTCTATCCCAACAGCAC	TACAGCACTTCCTTGGTCGG	170
PstS	transportador ABC	DOWN - Proteoma	AAGTGCTGGGCGACATCTAC	TGGACAGGTAGGAGGTGAAG	159
Hs.1210	transportador MFS	DOWN - Proteoma	CATCGGCAATGGCTGGTTCG	ACGCACGGTTTCCTTGACGA	154
Hs.1485	Proteína de quimiotaxia CheV	DOWN - Proteoma	TCTCCTTCGTCCTGACCGAT	CGAACTTGGCGACATAGGCG	180
Hs.3916	transportador ABC	DOWN - Proteoma	TGGGGGTGAACTTCTACGAC	AATTCAGGCGAGAGCGTCAC	166

4. Resultados e Discussão

4.1. Artigo "*Herbaspirillum seropedicae* strain HRC54 expression profile in response to sugarcane apoplastic fluid"

Os resultados da análise de transcriptoma da estirpe HRC54 em resposta ao líquido apoplástico de cana-de-açúcar foram publicados na revista 3Biotech em julho de 2021. A versão online pode ser conferida através do DOI https://doi.org/10.1007/s13205-021-02848-y. A versão completa pode ser conferida abaixo, com material suplementar no anexo (6.1).

Herbaspirillum seropedicae strain HRC54 expression profile in response to sugarcane apoplastic fluid

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Abstract

Bacterial transcriptome profiling in the presence of plant fluids or extracts during microbial growth may provide relevant information on plant–bacteria interactions. Here, RNA sequencing (RNA-Seq) was used to determine the transcriptomic profile of *Herbaspirillum seropedicae* strain HRC54 at the early stages of response to sugarcane apoplastic fluid. Differentially expressed gene (DEG) analysis was performed using the DESeq2 and edgeR packages, followed by functional annotation using Blast2GO and gene ontology enrichment analysis using the COG and KEGG databases. After 2 h of sugarcane apoplastic fluid addition to the *H. seropedicae* HRC54 culture, respectively 44 and 45 genes were upregulated and downregulated. These genes were enriched in bacterial metabolism (e.g., oxidoreductase and transferase), ABC transporters, motility,

secretion systems, and signal transduction. RNA-Seq expression profiles of 12 genes identified in data analyses were verified by RT-qPCR. The results suggested that *H. seropedicae* HRC54 recognized sugarcane apoplastic fluid as the host signal, and some DEGs were closely involved at the early stages of the establishment of plant–bacteria interactions.
Keywords: endophytic diazotrophic bacteria; plant growth promotion; apoplastic fluid; RNA-Seq; differential gene expression; plant–bacteria interaction.

Introduction

Sugarcane is an important crop in Brazilian agribusiness because of the high value of its products in various industries, including bioenergy production (Bordonal et al. 2018). Over the last decades, sugarcane yield has decreased slightly but significantly, which is typically attributed to low soil fertility and climate change (Pereira et al. 2013; CONAB 2020). In this light, need for the application of fertilizers, particularly nitrogen, has increased (Schultz et al. 2015), which can be harmful to the environment and increase agricultural costs (Gomes et al. 2008b). An alternative to reduce fertilizer application is the use of inoculants with diazotrophic endophytic bacteria (DEB). DEB can convert N_2 to NH_3 through biological nitrogen fixation (BNF) and can act as plant growth-promoting bacteria (PGPB) (Baldani and Baldani 2005; Oliveira et al. 2006; de Souza et al. 2015; da Fonseca Breda et al. 2019; dos Santos et al. 2020).

Previous studies have shown the positive effects of DEB inoculation on plant growth via BNF, hormone production, defense response, nutrient acquisition, and abiotic and biotic stress tolerance (Oliveira et al. 2002, 2006, 2009; de Souza et al. 2015; Kandel et al. 2017). In sugarcane, the positive effects of inoculating five DEB strains, including *Gluconacetobacter diazotrophicus* PAL5^T (BR11281), *Herbaspirillum rubrisubalbicans* HCC103 (BR11504), *H. seropedicae* HRC54 (BR11335), *Nitrospirillum* (formerly *Azospirillum*) *amazonense* CBAmC (BR11145), and *Paraburkholderia* (formerly *Burkholderia*) *tropica* PPe8^T (BR11366), have been previously reported (Oliveira et al. 2006; Reis et al. 2009; Renan et al. 2016; dos Santos et al. 2020; Martins et al. 2020; Ramos et al. 2020). However, the beneficial effects attributed to inoculation are highly variable, and their underlying mechanisms remain unclear. Therefore, additional information on the fundamental aspects of plant–microorganism interaction is imperative (Monteiro et al. 2008).

H. seropedicae is an diazotrophic endophytic β-proteobacteria, isolated from crops such as rice, maize, and sugarcane (Baldani et al. 1986; Olivares et al. 1996), and it has also been used as a sugarcane inoculant. This bacterium has been shown to increase plant biomass (Guimarães et al. 2003) through acting on phytohormone signaling (Amaral et al. 2014; Tadra-Sfeir et al. 2015), plant defense responses (Brusamarello-Santos et al. 2012), nitrogen metabolism (Breda et al. 2018), siderophore production, and polyhydroxybutyrate synthesis (Tirapelle et al. 2013; Brader et al. 2014). Furthermore, diverse protein secretion systems help bacteria to successfully interact with plants (Monteiro et al. 2008; Pedrosa et al. 2011). *H. seropedicae* strain HRC54 is a diazotrophic entophyte isolated from sugarcane roots (Baldani et al. 1996), with potential to serve as a PGPB, particularly for gramineuos plants (Junior et al. 2008; Alves et al. 2014; Martins et al. 2020). However, no study has reported the molecular overview of the interaction between HRC54 and sugarcane.

In 2011, the complete genome of *H. seropedicae* strain SmR1 was compiled, which enabled the identification of genes involved in several pathways such as auxin biosynthesis, BNF, siderophore production, protein secretion, and plant–bacteria interactions (e.g., chemotaxis proteins, secretion systems, and flagellar biosynthesis) (Pedrosa et al. 2011). These genes allow the bacteria to recognize plant signals and modulate plant gene expression for endophytic colonization and plant growth promotion (Monteiro et al. 2012). However, the molecular pathways involved at the initial stages of the bacterial endophytic lifestyle have not been well studied.

The aboveground parts of host plants, specifically intercellular spaces, are frequently occupied by endophytic bacteria, and the apoplastic space is a niche for bacterial development, providing nutrients such as sugar, ammonium, nitrite, nitrate, amino acids, and proteins, which are essential for bacterial growth (Asis et al. 2003; Haslam et al. 2003; Tejera et al. 2006; Sattelmacher and Horst 2007; Pechanova et al. 2010). An *in vitro* system to grow microorganisms in the presence of specific plant fluids or tissues can mimic the endophytic environment and help study the initial stages of plant–microbe interactions and provide information on the expression profiles of genes involved in these processes (Dong et al. 1994). Therefore, the exposure of a bacterial culture to apoplastic fluid during a certain period can provide insight into the signaling cascades that activate the bacterial genes necessary for metabolic adaptations to live within the plant.

Transcriptomics can be applied in such experiments, allowing for the evaluation of microbial gene expression profiles under different scenarios, such as abiotic stress, or in

specific pathways and interactions (Vacheron et al. 2013; Chandra et al. 2019; Raju et al. 2020). However, in most plant-associated bacterial transcriptomic studies conducted thus far, bacteria were cultured separately from the host plant to obtain sufficient bacterial mRNA transcripts (Levy et al. 2018). We previously used sugarcane apoplastic fluid in *in vitro* experiments on *P. tropica* PPe8^T and *N. amazonense* CBAmC and found this approach to be appropriate for evaluating bacterial responses to plant signals (Silva et al. 2018; Terra et al. 2020). The short exposure time to apoplastic fluid set in those experiments allowed us to elucidate initial gene expression changes and their importance at the early stages of plant–bacteria interactions (Pinski et al. 2019; Taulé et al. 2021). To this end, in the present study, we sought to identify the differentially expressed genes (DEGs) of *H. seropedicae* HRC54 in response to the short-term exposure of its culture to sugarcane (RB867515 variety) apoplastic fluid.

Materials and Methods

Sugarcane apoplastic fluid collection

The sugarcane variety RB867515 was sampled from the Embrapa Agrobiologia Experimental Field Station. This commercial variety is characterized by its ability to adapt to low-fertility soils, and it is responsive to PGPB inoculation (Schultz et al. 2014; dos Santos et al. 2020). After superficial washing, the stems were peeled, and the internodes were removed and disinfected by flaming (Silva et al. 2018). Sugarcane apoplastic fluid was obtained by centrifuging the stems in 50 mL tubes at 3,000 ×*g* for 20 min at 4°C (Dong et al. 1994). The extracted apoplastic fluid was passed through a 0.22 µm filter (diameter, 47 mm) and stored at −70°C until use.

H. seropedicae HRC54 growth in the presence of sugarcane apoplastic fluid

A pre-inoculum of *H. seropedicae* HRC54 was grown in 5 mL of DYGS medium (Baldani et al. 2014) for 12 h at 30°C while shaking at 100 rpm. Next, the bacteria (10^5 cells mL^{-1}) were inoculated in 100 mL of semi-selective JNFB liquid medium containing nitrogen (Baldani et al. 2014). After 16 h of growth under the same conditions mentioned above, the culture was divided in half and subjected to the following treatment: addition of sugarcane apoplastic fluid or water to the bacterial culture (at 1:1 proportion). The experiment was performed with three biological replicates for each treatment, resulting in six samples. The treatment lasted for 2 h at 30°C while shaking at 100 rpm. From each

replicate of both treatments, 5 mL of the culture was collected and centrifuged at 2,300 ×*g* for 10 min (Cordeiro et al. 2013), and the residual medium was discarded.

Cellular viability of H. seropedicae HRC54 in the presence of apoplastic fluid

To evaluate the effects of sugarcane apoplastic fluid on *H. seropedicae* HRC54, a *LIVE/DEAD* cell viability assay was performed. At the time of the start of the treatment (T0) and after 2 h (T2), samples (1 mL) were collected and centrifuged at 10,000 ×*g* for 10 min; the pellet was resuspended in 1 mL of 0.85% saline solution (NaCl, w/v). Then, 1.5 µL each of reagents A and B from the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen™) was added, and the samples were incubated in the dark for 15 min. An aliquot of 10 µL of the incubated sample was used for microscopy using LSM700 AxioObserver (Zeiss) equipped with the Plan-Apochromat 40x/1.3 Oil DIC M27 and 63x/1.4 Oil DIC M27 objective lenses. For SYTO 9 staining, the excitation wavelength was 555 nm and detection wavelength was 578-800 nm (green fluorescence, live cells). For propidium iodide staining, the excitation wavelength was 488 nm and detection wavelength was 300-578 nm (red fluorescence, dead cells). Transmitted light was used with a T-PMT detector. To confirm the results of microscopy, bacterial colony-forming units were counted ($CFU \cdot mL^{-1}$) using selective media for *Herbaspirillum* sp. JNFb (Baldani et al. 2014). Briefly, 1 mL of sample from each biological replicate was centrifuged at 10,000 ×*g* for 10 min, and the resulting pellet was resuspended in 1 mL of 0.9% saline solution (NaCl, w/v). The suspensions were serially diluted to a density of 10^{-10} $cells \cdot mL^{-1}$; then, 10 µL of the diluted suspension was spread on JNFb solid medium containing a nitrogen source and cultured for 24 h at 30°C. Statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/). The results were compared using Student's t-test, considering 0.05 as the level of significance (p).

Total RNA extraction, mRNA enrichment, and RNA-Sequencing (RNA-Seq)

Total RNA was extracted using the TRIZOL™ reagent (Invitrogen), followed by DNase I (Epicenter) treatment to completely remove genomic DNA. Both procedures were performed according to the manufacturer's protocols. mRNA enrichment was performed using the MICROBExpress™ Bacterial mRNA Enrichment Kit (Ambion), following the manufacturer's instructions. Six cDNA libraries were constructed using the Ion PITM Sequencing 200 Kit and sequenced using an ion proton semiconductor sequencer (Life).

Transcriptomic data analysis

The quality of raw reads generated from RNA-Seq was analyzed using FastQC (Andrews 2010). The reads were trimmed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and mapped to the genome of *H. seropedicae* HRC54 (unpublished). Read mapping data were used to calculate gene expression levels as reads per kilobase of transcript per million mapped reads (RPKM) using EDGE-pro (Magoc et al. 2013). Only reads aligned at least once with the reference genome of *H. seropedicae* HRC54 were used for the subsequent analyses. The list of DEGs with associated fold changes and significance estimates was generated using the R packages DESeq2 (Love et al. 2014a) and edgeR (Robinson et al. 2010), with a cut-off p-value of 0.05 and a log2 fold change of 1.5 (fold change of ≤1.5 indicated downregulated genes, and fold change of ≥1.5 indicates upregulated genes). The results from both packages were used for DEGS analyses to ensure the reliability of our final data. In the edgeR package, the false discovery rate (FDR) was analyzed at a cut-off of ≤0.05. The fold-change was calculated as the difference between the strain+apoplast (apoplast) and strain+water (water) treatments. Only genes that were differentially expressed in both packages were selected for functional annotation with Blast2GO (Conesa et al. 2005) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups (COG) databases (Kanehisa and Goto 2000; Tatusov et al. 2000).

RT-qPCR

To validate the results of RNA-Seq analysis, 12 DEGs were randomly selected for RT-qPCR. Primers were designed using Primer3Plus (Untergasser et al. 2007) according to the following criteria: sequence length of 19–22 nucleotides; annealing temperature of 58–62°C; GC content of 50%–80%; and amplicon size of 100–180 base pairs. The primers designed were also checked for dimer and hairpin formation using Oligo Explorer (http://www.uku.fi/~kuulasma/OligoSoftware/) (Table S2). For this assay, a new experiment was designed using the same conditions as above and the same total RNA extraction and DNAse I treatment protocols, followed by cDNA synthesis using SuperScript™ III Reverse Transcriptase (Invitrogen). RT-qPCR was performed using the 7500 Fast Real-Time PCR System and QuantiTect SYBR® Green PCR (Qiagen). The raw fluorescence data were converted to cycle threshold (Ct) using Miner (Zhao and Fernald 2005), and relative expression was calculated using the delta-delta Ct method ($2^{-\Delta\Delta CT}$) and

qBase v1.3.5 (Livak and Schmittgen 2001; Hellemans et al. 2007). *rpoA* and *groEL* were used as the reference genes (Pessoa et al. 2016). Statistical analyses were performed as described above (see section *Cellular viability of H. seropedicae HRC54 in the presence of apoplastic fluid*).

Results and Discussion

Apoplastic fluid does not negatively affect the viability of H. seropedicae HRC54 cells

Confocal microscopy showed that the addition of sugarcane apoplastic fluid to the culture did not negatively affect the viability of *H. seropedicae* HRC54 cells (Figure 1). However, the result observed in CFU counts was different; as such, the number of cells was not higher following the addition of apoplastic fluid (Figure 2). Indeed, bacterial growth following the addition of water was higher than that after the addition of apoplastic fluid; however, this analysis was focused on the viability of HRC54 cell following exposure to sugarcane apoplastic fluid. In micrographs D, H, M, and Q, the bacterial morphology was not altered following the addition of sugarcane apoplastic fluid.

Transcriptomic analysis revealed 89 DEGs in H. seropedicae HRC54 in response to sugarcane apoplastic fluid

After raw data quality analysis, the trimmed reads were mapped against the *H. seropedicae* HRC54 genome, and the RPKM values were calculated (Table 1). Analyses using DESeq2 and edgeR with a p-value of 0.05 revealed that the expression patterns of 1,009 genes were altered. Using a cut-off of log2 fold change of ±1.5, 89 genes were identified as differentially expressed under the experimental conditions, of which respectively 44 and 45 were upregulated and downregulated (Table 2). Of these DEGs, 18% encoded proteins or domains with unknown functions (DUFFs), 16.9% were related to oxidoreductase activity, and 13.5% were involved in transport and transmembrane transport (Tables S4 and S5). In addition, some DEGs encoded proteins related to signal transduction, transferase activity, secretion systems, cell division, and motility (Figure 3). Finally, few DEGs encoded proteins related to chaperone, dioxygenase, isomerase, ligase, and translation activities (“Others” in Table 2).

Expression of H. seropedicae HRC54 genes involved in specific metabolic subsystems was altered in response to sugarcane apoplastic fluid

Oxidoreductase activity

Six downregulated and nine upregulated genes were involved in oxidoreductase activity at the early stages of plant–bacteria interactions, which is associated with response to oxidative stress (Knief et al. 2011; Imlay 2014). Among the six downregulated genes, four were involved in the cytochrome C enzymatic chain (*ccoP*, *coxB*, *coxA*, and *cox11*), which are likely required for bacterial adaptation to different oxygen concentrations (Kulajta et al. 2006). Similarly, in *H. seropedicae* SmR1 inoculated on wheat plants, genes from the cytochrome C enzymatic chain were suppressed after 3 days (Pankievicz et al. 2016). The remaining two downregulated genes encoded glutamate synthase subunits alpha and beta (*gltB* and *gltD*). Six of the nine upregulated genes were related to NADP or NADPH redox activity (*nfnB*, *yahK*, *Hs2057*, *Hs2266*, *Hs3119*, and *Hs3748*), and the remaining three encoded peroxiredoxin (*PRDX2I)*, an amino acid dehydrogenase (*glud1_2*), and an aldo/keto reductase (*Hs2082*). *nfnB* encodes NAD(P^+)H nitroreductase, which is involved in the metabolism of nitrogen-containing compounds. *yahK* encodes NAD(P^+)-dependent alcohol dehydrogenase required for glycolysis. In addition, *Hs3748* encodes NADH dehydrogenase, which is involved in metabolic processes that generally require oxidoreductase activity. The *Hs3119* open reading frame (ORF) was annotated as a transhydrogenase and the *Hs2057* and *Hs2266* ORFs as NADP-dependent oxidoreductases, but no additional functional information was found. Previous experiments with *Paraburkholderia tropica* PPe8^T revealed the augmentation of genes involved in the oxidoreductase pathway and suppression of genes in the cytochrome C enzymatic chain in response to sugarcane apoplastic fluid (Silva et al. 2018). The differential modulation of these genes could be related to the initial bacterial attempt of adaptation to the presence of sugarcane apoplastic fluid.

Transferases and hydrolases

In *H. seropedicae* HRC54, the expression patterns of nine genes related to transferase activity were altered in response to sugarcane apoplastic fluid (Figure 3). This modulation of transferase/hydrolase activity may be required for the metabolic adaption and survival of bacteria under stress and may involve compounds necessary for beneficial plant–microbe interactions (Orellana et al. 2017). The *gst*, *puuE*, *tyrB*, *ndk*, *mdoH*, and *Hs799* ORFs were upregulated. *tyrB* encodes an aromatic amino acid transaminase, and *puuE* encodes 4-aminobutyrate aminotransferase, both related to amino acid metabolism and nitrogen group transfer. *mdoH* encodes a membrane glycosyltransferase involved in the

transfer and metabolism of carbohydrates. *ndK* encodes a nucleoside-diphosphate kinase, a transferase related to ATP binding, and *gst* encodes a protein similar to glutathione S-transferase, a transferase involved in the transfer of aryl and acyl groups in glutathione metabolism. Glutathione S-transferase overexpression has been observed during *Enterobacter lignolyticus* growth in the presence of lignin (Orellana et al. 2017). The *Hs799* ORF encoded a protein similar to glutamine amidotransferase, but no additional functional information was found. The downregulated genes *thiD*, *selU*, *fadA*, and *tktA* were annotated as hydroxymethylpyrimidine/phosphomethylpyrimidine, tRNA 2-selenouridine synthase, acetyl-CoA C-acyltransferase, and transketolase, respectively. Acetyl-CoA C-acyltransferase suppression was observed in *Burkholderia kururiensis* exposed to rice plant extract (Coutinho et al. 2015). *thiD* is involved in thiamine metabolism, and *fadA* and *tktA* are involved in carbohydrate, specifically fatty acid and pentose phosphate, metabolism. *selU* is involved in transfers during RNA biogenesis. Three genes related to hydrolase activity were differentially expressed, and only *cbiG*, which encodes a cobalamin biosynthesis protein, was downregulated. *cbiG* acts on the carbon–carbon bonds and is involved in porphyrin and chlorophyll metabolism. The *ostB* and *Hs4740* ORFs were upregulated. *ostB* encodes trehalose 6-phosphate phosphatase, which is involved in starch and sucrose metabolism. The *Hs4740* ORF encoded a protein similar to glycoside hydrolase, which is also involved in carbohydrate metabolism, but no additional functional information was found. *H. seropedicae* SmR1 associated with maize has been reported to exhibit differential expression patterns of hydrolases (Balsanelli et al. 2016). Overall, the overexpression of genes related to transferase and hydrolase activities suggests that the bacteria altered their gene expression pattern to adapt to and metabolize nutrients in sugarcane apoplastic fluid.

Signal transduction

Nine genes involved in signal transduction were differentially expressed, of which eight were downregulated and one was upregulated (Figure 3). During plant–bacteria interactions, chemotaxis and signal transduction systems are key to other processes such as host adhesion and recognition (Falke et al. 1997; Batista et al. 2018). The upregulated gene *glrR* is a response regulator that enables bacteria to adapt and respond to the environment. The signal transduction genes *aer*; *mcp*; and the *Hs2330*, *Hs3454*, *Hs3539*, *Hs4087*, *Hs505*, and *Hs707* ORFs were downregulated. *mcp* encodes a methyl-accepting chemotaxis protein, which serves as an aerotaxis receptor. Similar expression patterns

have been reported during the growth of *H. seropedicae* SmR1 associated with maize and in the presence of the flavonoid naringenin (Tadra-Sfeir et al. 2015; Balsanelli et al. 2016) as well as in *Nitrospirillum amazonense* CBAmC in response to sugarcane apoplastic fluid (Terra et al. 2020). The *Hs3454*, *Hs3539*, *Hs2330*, and *Hs505* ORFs were also annotated as chemotaxis proteins, but no additional functional information was found. These DEGs involved in signal transduction may be related to bacterial adaptation, signaling, and recognition. However, the annotation of these genes was automatic and no specific function was found; therefore, additional experiments are warranted to elucidate their biological function and involvement in the interaction process.

Transport system

Several genes encoding sorbitol, mannitol, and branched-chain amino acids of the ABC transport system and saccharide transporters were suppressed. ABC transporters are extremely important for bacterial metabolism because of their function in nutrient uptake and exchange (Fatht and Kolter 1993). Several genes related to ABC transporters (Figure 3), such as *smog* and *ugpC* involved in saccharide transport as well as *livH* and *livM* involved in amino acid transport, were suppressed. Similarity analysis did not identify the orthologous of the *Hs1900* OFR, but its location in the *H. seropedicae* HRC54 genome was very close to that of the *Hs1901* and *Hs1902* ORFs (*livH* and *livM*), suggesting that *Hs1900* is related to the *liv* enzymatic chain. However, additional experiments are required to confirm this hypothesis. Similar expression profiles of genes related to transport systems were found in *H. seropedicae* SmR1 associated with wheat plants (Pankievicz et al. 2016) as well as in *P. tropica* PPe8^T and *N. amazonense* CBAmC in response to sugarcane apoplastic fluid (Silva et al. 2018; Terra et al. 2020). The *Hs80* ORF, annotated as a tripartite tricarboxylate transporter, was downregulated, but its function remains unknown. Only two genes involved in this pathway were upregulated in the presence of sugarcane apoplastic fluid. *ctpV* (copper transport) and the *Hs2747* ORF were annotated as ABC transporter substrate-binding proteins. Copper is involved in homeostatic processes that regulate plant defense mechanisms, allowing infection and colonization during interaction (Thiebaut et al. 2014). Suppression of these genes suggests that the bacteria specifically modulated carbohydrate metabolism, probably in response to sugars present in the apoplastic fluid.

Four genes related to transmembrane transport, a more specific pathway within the transport system, were differentially expressed in *H. seropedicae* HRC54 in the presence

of sugarcane apoplastic fluid. *acrA* and *acrB*, which are part of the multi-drug efflux system in gram-negative bacteria, were upregulated. This family participates in the antimicrobial resistance pathway, specifically the surface adhesion chain. The resistance nodulation and cell division family (RND) proteins are responsible for bacterial surface adhesion. Genes encoding these proteins were are also upregulated in *Azospirillum* sp. associated with wheat plants (Camilios-neto et al. 2014) and *Burkholderia kururiensis* exposed to rice plant extract (Coutinho et al. 2015). During competition with other bacteria in the presence of *Mimosa* plant extract, genes of the RND family were induced in *Burkholderia phymatum* (Klonowska et al. 2018). The upregulated expression of the RND family genes in different plant–bacteria interaction experiments highlights the importance of this family in the interaction process. The *Hs4729* ORF, a putative porin, was also upregulated. This protein is part of small exchange channels in the membrane and is involved in the membrane exchange and metabolism of poly-beta-hydroxybutyrate, an important compound in interaction (Tirapelle et al. 2013). The upregulation of this gene has also been reported in *H. seropedicae* SmR1 associated with wheat and maize (Balsanelli et al. 2016; Pankievicz et al. 2016). The bile acid gene *Hs3295*, a sodium transporter, was the only downregulated gene among those belonging to the transmembrane transport system. This gene may be related to photorespiratory metabolism in plants, but its function in bacteria remains unknown. Changes in the expression patterns of these genes highlight the importance of transport systems, which can help the bacteria to metabolize host-derived nutrients. However, further experiments are required to confirm this hypothesis.

Binding and cell division

Four genes related to binding were differentially expressed (Figure 3). Meanwhile, *polB* and the *Hs2304* and *Hs2300* ORFs were downregulated. *polB* encodes a type II DNA polymerase responsible for DNA repair during DNA synthesis. The *Hs2304* and *Hs2300* ORFs were annotated as a DNA-binding response regulator and tetratricopeptide repeat protein, respectively. The DNA-binding response regulator is a part of the output domain of response regulators related to cellular processes, while the tetratricopeptide repeat protein is a motif with no specific function reported to date. *hfq*, an RNA-binding protein (RBP), was upregulated. RBPs are responsible for RNA regulation and metabolism and play crucial roles in cellular function, transport, and localization.

Regarding cell division, only one gene was upregulated and the others were downregulated. *pal*, a peptidoglycan-associated lipoprotein involved in bacterial survival under endophytic conditions (Godlewska et al. 2009), was upregulated. Although its role in virulence or interaction remains unclear, *pal* upregulation indicates the attempt of *H. seropedicae* HRC54 to identify sugarcane apoplastic fluid as a possible host plant signal. Cell division proteins are important during the initial stages of plant–bacteria interactions (dos Santos et al. 2010; Knief et al. 2011). *ftsZ*, required for the formation of a contractile ring structure (Z ring) at the future cell division and bacterial multiplication site, was downregulated. The ring assembly regulates the timing and site of cell division. In contrast, upregulation of this protein was reported in *Gluconactobacter diazotrophicus* associated with sugarcane (Lery et al. 2011) and *Rhizobium tropici* in response to heat stress (Gomes et al. 2012). Additional experiments with prolonged exposure to sugarcane apoplastic fluid may provide more details of genes related to cell division and their roles in plant–bacteria interaction.

Motility and secretion system

The flagelin and flagellar transcription genes *fliC* and *flhC*, which are important primary genes in bacterial flagellar assembly, were downregulated. This suppression of the motility system can be related to the bacterial switch from free-swimming to motile habit for attaching to the host surface. Regarding the secretion system, three genes were upregulated at only 2 h after the addition of apoplastic fluid. *Lip*, *dotU*, and *vgrG* are members of the type VI secretion system (T6SS), involved in bacterial symbiosis and other processes of the plant–bacteria interaction. T6SS comprises nine genes, of which one-third were upregulated in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid. The motility system is important for bacterial interaction and is typically related to chemotaxis (Pedrosa et al. 2011; Monteiro et al. 2012), which is an initial step during interaction (Pankievicz et al. 2016).

Suppression of the motility system-related genes has also been reported in *H. seropedicae* SmR1 associated with maize and wheat plants as well as in response to naringenin and sugarcane extract (Cordeiro et al. 2013; Tadra-Sfeir et al. 2015; Balsanelli et al. 2016; Pankievicz et al. 2016). Similar suppression of these genes has also been reported in other species such as *P. tropica* PPe8^{T} and *N. amazonense* CBAmC in response to sugarcane apoplastic fluid (Silva et al. 2018; Terra et al. 2020). In the present experiment, *H. seropedicae* HRC54 genes related to flagelin and flagellar transcription were suppressed

in response to sugarcane apoplastic fluid, suggesting that the bacteria recognized the fluid as part of the host plant and initiated changes in the motility system essential to the endophytic life.

T6SS is believed to be exclusive to pathogenic bacteria (Jani and Cotter 2010). Nevertheless, recent studies have shown the importance of this secretion system in competition with other bacteria and further successful interaction with plants (Filloux 2009; Jani and Cotter 2010). Interestingly, three members of the T6SS (*Lip*, *dotU*, and *vgrG*) were overexpressed in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid. T6SS was also upregulated in *B. kururiensis* in the presence of rice plant extract as well as in *P. tropica* in response to sugarcane apoplastic fluid (Coutinho et al. 2015; Silva et al. 2018). These results highlight the importance of T6SS at the initial stages of beneficial plant–microbe interactions and the need for the comprehensive investigation of this system.

Hemagglutinins and chaperones

fhaB and the *Hs2067* ORF, which encode filamentous hemagglutinin N-terminal domain proteins, were downregulated. Hemagglutinins may be related to bacterial virulence, but more recent data suggest that in some gram-negative bacteria, these proteins are involved in adhesion and surface attachment as well as biofilm formation - aspects that are often related to bacterial invasion; endophytic bacteria may use similar mechanisms for interaction with plants (Bernal et al. 2018). Genes related to the chaperone activity, including *trxA* (thioredoxin) and *hslU* (ATP-dependent protease), were upregulated in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid (Figure 3). ATP-dependent proteases are required for the degradation of specific intracellular molecules. Thioredoxin is part of the cellular antioxidant system, which is important for the protection of bacteria from oxidative damage caused by reactive oxygen species (ROS). With the addition of sugarcane apoplastic fluid, the bacteria were exposed to a “new” environment, with a high probability of the presence of ROS. However, identification of only one gene is not sufficient to support this hypothesis.

Genes encoding hemagglutinin are relevant to plant–microbe interactions. Such genes have been identified in the genome of *H. seropedicae* SmR1 (Pedrosa et al. 2011). In the present experiment, two genes encoding hemagglutinin (*fhaB* and *Hs2067*) were suppressed in *H. seropedicae* HRC54 in response to apoplastic fluid. Although hemagglutinin has generally been believed to be related to the virulence of pathogenic

bacteria, more recent data have shown its importance in surface attachment and biofilm formation (Ariyakumar and Nishiguchi 2009; Gottig et al. 2009; Bernal et al. 2018; Taulé et al. 2021). Interestingly, in the present experiment, genes related to another system believed to be exclusive to pathogenic bacteria (T6SS) were also differently expressed in HRC54 in response to sugarcane apoplastic fluid. Perhaps, these systems are related to plant–bacteria interactions and warrant further exploration.

Several ORFs annotated with general functions such as lyase activity, transcription, and protein activation in bacterial metabolism, including *Hs245*, *Hs947*, and *Hs430*, were downregulated. *trpS*, *bpeT*, *pmm-pgm*, *RP-L31*, and the *Hs4167* ORF were upregulated in response to sugarcane apoplastic fluid. *trpS* and *RP-L31* are related to the translation and metabolism of tryptophan, which is an important amino acid for bacterial growth (Glick 2015). *pmm-pgm* is a phosphomannomutase/phosphoglucomutase, which is related to glycolysis and involved in virulence factor synthesis in *Pseudomonas aeruginosa* (Regni et al. 2006). The *Hs4167* ORF was annotated as a Tim44 domain-containing protein, which is responsible for the translocation of nuclear-encoded proteins across the mitochondrial inner membrane. These processes could be relevant to plant–bacteria interaction process and warrant further research.

Unknown and other functions

Several metabolic functions were affected by at least one of the altered genes (Figure 3). *Hs38*, a putative membrane protein; *Hs245*, a glyoxalase/bleomycin resistance/extradiol dioxygenase; *Hs430*, a class I SAM-dependent methyltransferase; and *Hs947*, related to the TetR family transcriptional regulators, were downregulated. Meanwhile, *trpS*, *bpeT, pmm-pgm*, *RP-L31*, and the *Hs4167* ORF were upregulated. *TrpS* is a tryptophan–tRNA ligase and *RP-L31* is a type B 50S ribosomal protein, both related to translation. They are also members of the LysR transcriptional regulator family, a diverse family of genes related to virulence, motility, and quorum sensing. Similarity analysis showed that some DEGs were annotated as unknown functions in the COG and KEGG databases (Table S1). However, based on the position of their ORFs in the *H. seropedicae* HRC54 genome, it is reasonable to assume that they serve some specific functions hitherto unknown. For instance, the *Hs3922* and *Hs3926* ORFs were identified as proteins with domains of unknown function (DUFFs), whereas *Hs3924* and *Hs3925* are related to cytochrome C oxidase. Nevertheless, more detailed studies are warranted to confirm this hypothesis. Such domains or putative proteins in *H. seropedicae* strains have not been previously

reported; therefore, future comprehensive studies of some of these differentially expressed proteins are essential to identify their functions at the initial stages of plant–bacteria interactions.

Expression patterns of 12 genes were validated with RT-qPCR

To confirm the expression patterns of *H. seropedicae* HRC54 genes in response to sugarcane apoplastic fluid, 12 genes were randomly selected for validation using RT-qPCR, of which six each were upregulated and downregulated (Table S3). The upregulated genes showed higher relative expression levels and the downregulated genes showed lower relative expression levels in samples exposed to sugarcane apoplastic fluid. The expression patterns of 9 of the 12 genes were confirmed by the assay (Figure 4). The *Hs4308* ORF showed a lower relative expression level in samples exposed to sugarcane apoplastic fluid, which was contradictory to the results of RNA-Seq analysis, in which this gene was upregulated. Similarly, *smoG* and *ugpC* showed higher relative expression levels in samples exposed to sugarcane apoplastic fluid, which was contradictory to the results of transcriptomic analysis (Figure 4). This small variation in the results can be attributed to the fact that RT-qPCR is a more sensitive method. However, given that the expression patterns of majority of the genes were confirmed by the RT-qPCR assay, our transcriptomic data derived from *in silico* analyses can be considered reliable.

Conclusions

DEGs explored in the present study constitute the early response of *H. seropedicae* HRC54 to compounds present in the apoplastic fluid of the sugarcane variety RB867515. The transcriptomic profile of *H. seropedicae* HRC54 during 2 h in the presence of sugarcane apoplast fluid showed the modulation of genes related to carbohydrate and amino acid metabolism, motility, secretion, oxidoreductase activity, and signal transduction. This expression pattern is consistent with bacterial response to host plant signals and adaptation to and survival under endophytic life conditions, suggesting that the bacteria recognized sugarcane apoplast fluid as the host plant environment. Additionally, in *H. seropedicae* HRC54, metabolism was altered to use compounds in the apoplastic fluid as a nutrient source and genes related to motility, secretion, transferase activity, and signal transduction necessary to establish interaction with the host plant were modulated. Furthermore, many putative proteins or proteins with DUFFs were differentially expressed in the transcriptomic data. Therefore, additional experiments are

warranted to determine the importance of such proteins in the sugarcane–*H. seropedicae* interaction.

Table 1: Reads mapped against the genome of *Herbaspirillum seropedicae* strain HRC54 using EDGE-pro software.

Sample	Raw data reads	Total reads after quality analysis	Total reads mapped against HRC54 genome	% reads mapped against genome	% unique reads mapped against genome
Apoplast+HRC54 1	4,660,273	3,201,458	2,354,893	79.07	5.51
Apoplast+HRC54 2	4,370,557	3,007,949	2,407,667	83.28	3.24
Apoplast+HRC54 3	7,181,611	6,032,121	5,261,784	88.72	1.49
Water+HRC54 1	2,801,113	1,339,224	1,085,216	86.98	5.94
Water+HRC54 2	3,618,072	1,895,847	1,390,060	77.66	4.34
Water+HRC54 3	4,364,421	3,128,588	2,676,803	87.78	2.23

Table 2: Total number of differentially expressed genes from *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid identified using DESeq2 and edgeR packages (with cut off p-value of 0.05 and log2 fold change of 1.5, in both packages) and their main functions.

Function	Up-regulated	Down-regulated	Total genes
oxireductase	9	6	15
transferase	6	4	10
hydrolase	2	1	3
signal transduction	1	8	9
transport	2	6	8
transmenbranar transport	3	1	4
binding	1	3	4
cell division	1	1	2
motility	-	2	2
secretion system	3	-	3
chaperone	2	-	2
hemagglutinin	-	2	2
others	5	4	9
unknown	9	7	16
Differentially expressed	44	45	89

Figures

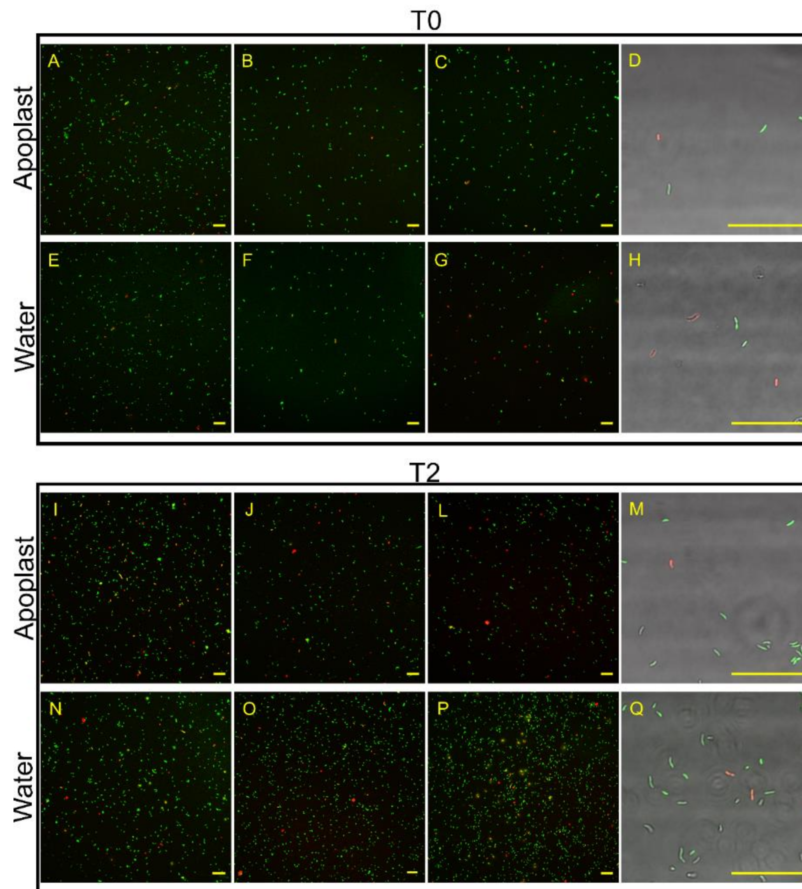

Figure 1: Confocal microscopy of *H. seropedicae* HRC54 in the presence of sugarcane apoplastic fluid using LIVE/DEAD BacLight Bacterial Viability Kits (Invitrogen™). Micrographs A, B, C, and D are from sample Apoplast; and micrographs E, F, G and H are from sample Water in the moment of the addition of the treatments (T0). Micrographs I, J, L, and M are from sample Apoplast; and micrographs N, O, P and Q are from sample Water after the two hours exposure of the treatments (T0). Red fluorescence represents dead cells and green fluorescence represents the live cells. Micrographs D, H, M and Q are using the 63x lent, presenting the cell morphology. Scale bars represents 20 µm.

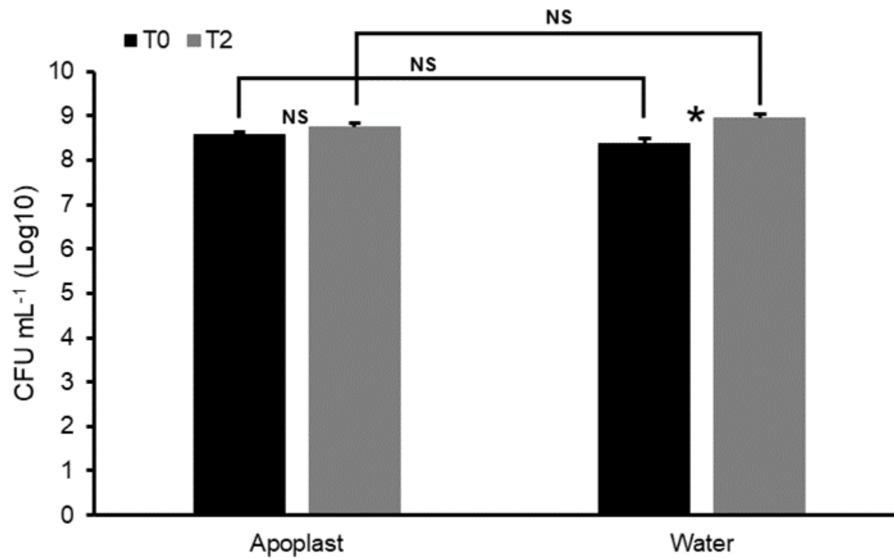

Figure 2: Count of the UFC/mL from *H. seropedicae* HRC54 from the experiment with apoplastic fluid from sugarcane. Were evaluate for this assay, the moment from addition of the treatments (T0) and after two hours of exposure (T2). The asterisks represent a significant difference in t-test ($p < 0.05$), and NS represents a non-significant difference in t-test ($p > 0.05$).

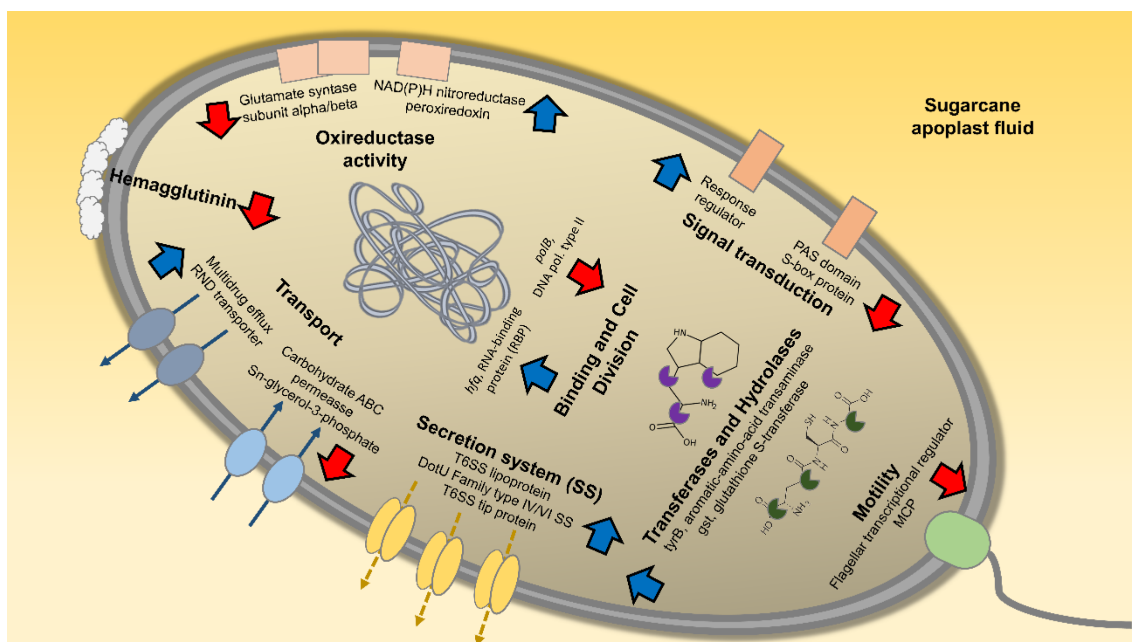

Figure 3: Overview of differential expressed genes (DEGs) from *H. seropedicae* HRC54 in the presence of sugarcane apoplastic fluid, highlighting predominant functions with up and down-regulation such as transport, oxidoreductase activity and signal transduction; and unique regulations as motility, secretion system and hemagglutinin. The blue arrows represent up-regulation and red arrows represent down-regulation.

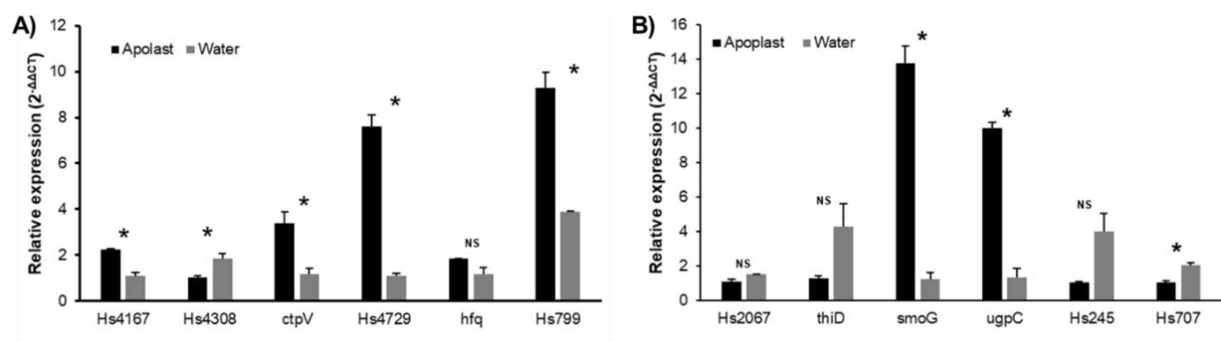

Figure 4: Relative expression of genes from *H. seropedicae* HRC54 in the presence of sugarcane apoplastic fluid using RT-qPCR. Validation was performed using up (A) and down-regulated genes (B). The expression level was calculated by qBASE software using Delta CT method ($2^{-\Delta\Delta CT}$). The symbols represents a significant difference in t-test ($p < 0.05$), and NS represents a non-significant difference in t-test ($p > 0.05$).

4.1.1. Material suplementar artigo "*Herbaspirillum seropedicae* strain HRC54 expression profile in response to sugarcane apoplastic fluid"

Table S1: Log2 fold change values from the differential expressed genes identified in strain HRC54 of *H. seropedicae* in response to sugarcane apoplastic fluid, annotated as hypothetical proteins or domains of unknown function (DUFF).

ORF	Function	Log2 Fold Change
Hs2123	hypothetical protein	-2.209
Hs3303	attG domain containing protein	-2.235
Hs3922	DUF2244 domain-containing protein	-3.135
Hs3926	DUF2970 domain-containing protein	-4.084
Hs708	DUF3034 domain-containing protein	-2.546
Hs244	DUF2026 domain-containing protein	-2.360
Hs1727	DUF3047 domain-containing protein	-2.439
Hs1697	hypothetical protein	3.639
Hs1843	DUF4148 domain-containing protein	3.128
Hs1844	DUF4148 domain-containing protein	4.432
Hs257	MULTISPECIES: hypothetical protein	2.328
Hs258	hypothetical protein Hsero_3161	3.350
Hs3054	hypothetical protein Hsero_0762	2.163
Hs1821	DUF3567 domain-containing protein	2.239
Hs1297	DUF4148 domain-containing protein	4.711
Hs4308	VWA domain-containing protein	2.088

Table S2: Primers RT-qPCR assays from DEGs identified in *H. seropedicae* strain HRC54 in the presence of the sugarcane apoplastic fluid.

Gene	Function*		Forward primer (5´-3´)	Reverse primer (5´-3´)	Amplicon (bp)
Hs4167	translocation of proteins	UP	CACCACCCCCGAGATGTTTG	ACAGGTTCCAGATTTCGGCG	209
Hs4308	unknown	UP	GTGGAAGAACAACCGCCGCC	GTTCGGGATTGAGCCAGACG	227
pal	cell division	UP	CGACTACGACAGCTACACCG	CCCTGGATGATGACCTTGCG	102
Hs4729	transmembrane transport	UP	GCCTGTCCGCAGTGTTCAAC	GTGTCAGCGAAGTCGGTCTG	163
hfq	binding	UP	CCTGGTCAACGGCATCAAGC	GCACGACGGTAGAGATGGCG	116
Hs799	transferase	UP	ATGGGGGACACCGACAAACC	TGTGGGCGATGACTTCCTGC	214
Hs2067	hemagglutinin	DOWN	CCGTTCAACTTCCAGAGCAC	CGGAGGCATAGAAGGTCGTG	160
thiD	transferase	DOWN	CGTCAGCGTCTTTGCCGTGC	CTGGTCTCCCTTGCCATTGG	220
smoG	transport	DOWN	CGCCATCGTGCTGTTCTTCC	ATACCCCAGATAGTGACTGC	145
ugpC	transport	DOWN	ATCGTAGTGCTCTCCGAGGG	TGGGCGAACCGATGAAACCG	103
Hs245	dioxygenase	DOWN	GCAGCAAGGCGTTTTACGGC	ATGTCTTCCAGGTCGTCCGC	166
Hs707	signal transduction	DOWN	TCATTCCCTTTGCCGAGACC	TGGAGAGGTTGACCGAGAAC	129

Table S3: Genes evaluated by RT-qPCR to validate the DEGs identified in *H. seropedicae* strain HRC54 in the presence of sugarcane apoplastic fluid using RNA-Seq.

Gene	Description	Function	RNA-Seq		RT-qPCR Relative Expression	
			Log2 Fold-change	Expression	Apoplast+HCR54	Water+HRC54
Hs4167	Tim44 domain-containing protein	translocation	3.188	UP	2.541	1.879
Hs4308	VWA domain-containing protein	unknown	2.088	UP	1.074	3.657
pal	peptidoglycan-associated lipoprotein	cell division	1.664	UP	3.742	2.161
Hs4729	porin	transport	4.475	UP	8.209	2.022
hfq	RNA-binding protein	binding	1.601	UP	1.652	1.498
Hs799	type 1 glutamine amidotransferase	transferase	3.442	UP	7.253	2.933
Hs2067	filamentous hemagglutinin N-terminal domain	hemagglutinin	-4.037	DOWN	1.332	2.839
thiD	hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	transferase	-3.186	DOWN	1.198	6.930
smoG	carbohydrate ABC transporter permease	transport	-3.123	DOWN	12.815	2.574
ugpC	sn-glycerol-3-phosphate ABC transporter ATP-binding protein	transport	-2.942	DOWN	11.053	2.951
Hs245	glyoxalase/bleomycin resistance/extradiol dioxygenase family	dioxygenase	-3.769	DOWN	1.217	6.679
Hs707	GGDEF domain-containing protein	signal transduction	-1.928	DOWN	1.189	3.346

Table S4: Down-regulated genes from *H. seropedicae* HRC54 response to sugarcane apoplastic fluid. Genes identified using DESeq2 and edgeR package (with cut off p-value of 0.05 and log2 fold change of 1.5).

ORF ID	log2FoldChange	p-value	FDR	Gene or ORF	Blast2GO	KEEG	COG
Hs.peg.1369	-2.301795146	2.71E-06	2.77E-57	*fliC*	flagellin	fliC; flagellin	Flagellin and related hook-associated proteins
Hs.peg.169	-2.077113804	0.022560026	1.01E-97	*fhaB*	filamentous hemagglutinin N-terminal domain-containing protein		
Hs.peg.1727	-2.43907054	0.025974368	1.18E-81	Hs1727	DUF3047 domain-containing protein		
Hs.peg.1900	-2.663611722	0.00044878	6.58E-123	Hs1900	ABC transporter permease		
Hs.peg.1901	-4.407070284	0.001111097	6.43E-86	*livH*	branched-chain amino acid ABC transporter permease	livH; branched-chain amino acid transport system permease protein	Branched-chain amino acid ABC-type transport system, permease components
Hs.peg.1902	-1.653392754	0.036996137	8.97E-108	*livM*	branched-chain amino acid ABC transporter permease	livM; branched-chain amino acid transport system permease protein	ABC-type branched-chain amino acid transport system, permease component
Hs.peg.2062	-1.991377377	0.04484644	9.65E-93	*tktA*	transketolase	E2.2.1.1; transketolase [EC:2.2.1.1]	Transketolase
Hs.peg.2067	-4.037472453	0.002941638	6.90E-82	Hs2067	filamentous hemagglutinin N-terminal domain-containing protein		
Hs.peg.2104	-2.148177429	0.009735745	2.39E-107	*ftsZ*	cell division protein FtsZ	ftsZ; cell division protein FtsZ	Cell division GTPase
Hs.peg.2123	-2.209066952	0.018824987	8.98E-99	Hs2123	hypothetical protein		
Hs.peg.218	-1.891784454	0.002078847	1.49E-118	*ccoP*	cytochrome-c oxidase, cbb3-type subunit III		
Hs.peg.2193	-3.18689174	0.011013301	2.22E-78	*thiD*	hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	thiD; hydroxymethylpyrimidine/phosphomethylpyrimidine kinase [EC:2.7.1.49 2.7.4.7]	Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase
Hs.peg.2206	-3.123901502	0.039353907	2.23E-91	*smoG*	carbohydrate ABC transporter permease	smoG; sorbitol/mannitol transport system permease protein	ABC-type sugar transport system, permease component
Hs.peg.2208	-2.942336272	0.027246245	4.62E-77	*ugpC*	sn-glycerol-3-phosphate ABC transporter ATP-binding protein UgpC	malK; multiple sugar transport system ATP-binding protein [EC:3.6.3.-]	ABC-type branched-chain amino acid transport systems, ATPase component
Hs.peg.2219	-2.66009306	0.001438531	2.17E-127	*mcp*	methyl-accepting chemotaxis protein	mcp; methyl-accepting chemotaxis protein	Methyl-accepting chemotaxis protein
Hs.peg.2300	-1.638765722	0.031132763	3.99E-127	Hs2300	DNA-binding response regulator		
Hs.peg.2304	-3.569995762	0.019367005	5.80E-91	Hs2304	tetratricopeptide repeat protein		
Hs.peg.2330	-1.879466397	0.000341278	1.20E-95	Hs2330	chemotaxis protein		
Hs.peg.244	-2.360812172	0.028083037	4.87E-83	Hs244	DUF2026 domain-containing protein		
Hs.peg.245	-3.769803901	0.040948234	5.46E-72	Hs245	glyoxalase/bleomycin resistance/extradiol dioxygenase protein	uncharacterized protein	Lactoylglutathione lyase and related lyases
Hs.peg.264	-5.209581489	0.002183082	3.09E-84	*selU*	tRNA 2-selenouridine(34) synthase MnmH	selU; tRNA 2-selenouridine synthase [EC:2.9.1.-]	Predicted ATPase

Continuation table S4: Down- regulated genes from *H. seropedicae* HRC54 response to sugarcane apoplastic fluid. Genes identified using DESeq2 and edgeR package (with cut off p-value of 0.05 and log2 fold change of 1.5).

ORF ID	log2FoldChange	p-value	FDR	Gene or ORF	Blast2GO	KEEG	COG
Hs.peg.3295	-3.110577069	0.006402762	3.65E-83	Hs3295	bile acid:sodium symporter		
Hs.peg.3303	-2.235134487	0.00187872	2.96E-130	Hs3303	attG domain containing protein		
Hs.peg.3304	-3.25618821	0.00032339	1.47E-114	*aer**	PAS domain S-box protein	aer; aerotaxis receptor	Methyl-accepting chemotaxis protein
Hs.peg.3454	-4.215450289	0.01046757	4.69E-67	Hs3454	methyl-accepting chemotaxis protein		
Hs.peg.3539	-1.90363504	0.011645227	3.53E-130	Hs3539	methyl-accepting chemotaxis protein	mcp; methyl-accepting chemotaxis protein	Methyl-accepting chemotaxis protein
Hs.peg.38	-5.446493285	0.000789012	2.09E-92	Hs38	putative membrane protein		
Hs.peg.3922	-3.13577526	0.000790142	4.16E-106	Hs3922	DUF2244 domain-containing protein		
Hs.peg.3923	-1.858622907	0.039379141	5.88E-107	*coxB*	cytochrome c oxidase subunit II		
Hs.peg.3924	-4.127460425	3.41E-06	2.62E-131	*coxA*	cytochrome c oxidase subunit I	coxA; cytochrome c oxidase subunit I [EC:1.9.3.1]	Heme/copper-type cytochrome/quinol oxidases, subunit 1
Hs.peg.3925	-3.088155764	0.000192691	6.51E-131	*cox11*	cytochrome c oxidase assembly protein		
Hs.peg.3926	-4.084084338	0.003867877	1.39E-78	Hs3926	DUF2970 domain-containing protein		
Hs.peg.4087	-1.956972334	0.006457509	7.55E-127	Hs4087	response regulator		
Hs.peg.4144	-2.121304292	0.003377188	2.36E-122	*gltB*	glutamate synthase subunit alpha	gltB; glutamate synthase (NADPH/NADH) large chain [EC:1.4.1.13 1.4.1.14]	Glutamate synthase domain 2
Hs.peg.4145	-3.860688694	0.000121641	1.10E-117	*gltD*	glutamate synthase subunit beta	gltD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13 1.4.1.14]	NADPH-dependent glutamate synthase beta chain and related oxidoreductases
Hs.peg.429	-1.993291476	0.004152049	4.43E-130	*flhC*	flagellar transcriptional regulator FlhC		
Hs.peg.430	-2.990647008	0.007817012	4.46E-86	Hs430	class I SAM-dependent methyltransferase		
Hs.peg.4798	-3.213927601	0.002080448	2.33E-93	*fadA*	acetyl-CoA C-acyltransferase	fadA; acetyl-CoA acyltransferase [EC:2.3.1.16]	Acetyl-CoA acetyltransferase
Hs.peg.505	-2.304148521	0.017969055	9.60E-92	Hs505	methyl-accepting chemotaxis protein		
Hs.peg.707	-1.928729643	0.040990366	3.38E-95	Hs707	GGDEF domain-containing protein		
Hs.peg.708	-2.546363916	0.037102714	1.35E-73	Hs708	DUF3034 domain-containing protein		
Hs.peg.761	-4.139173317	0.014756285	3.72E-83	*cbiG*	cobalamin biosynthesis protein CbiG		
Hs.peg.80	-5.017728716	0.004143032	1.55E-74	Hs80	tripartite tricarboxylate transporter substrate binding protein		
Hs.peg.947	-4.829360504	0.006646757	4.42E-70	Hs947	TetR family transcriptional regulator		
Hs.peg.987	-2.434413487	0.047917403	9.52E-74	*polB*	DNA polymerase II	polB; DNA polymerase II [EC:2.7.7.7]	DNA polymerase elongation subunit (family B)

Table S5: Up-regulated genes from *H. seropedicae* HRC54 response to sugarcane apoplastic fluid. Genes identified using DESeq2 and edgeR package (with cut off p-value of 0.05 and log2 fold change of 1.5).

ORF ID	log2FoldChange	p-value	FDR	Gene or ORF	Blast2GO	KEEG	COG
Hs.peg.1297	4.711401344	0.001288105	3.26E-94	Hs1297	DUF4148 domain-containing protein		
Hs.peg.141	1.797180179	0.035555429	9.12E-117	*trpS*	tryptophan--tRNA ligase	WARS; tryptophanyl-tRNA synthetase [EC:6.1.1.2]	Tryptophanyl-tRNA synthetase
Hs.peg.1697	3.639796189	0.001313961	4.01E-108	Hs1697	hypothetical protein		
Hs.peg.1741	2.375719103	1.03E-05	2.78E-92	*glud1_2*	Glu/Leu/Phe/Val dehydrogenase	GLUD1_2; glutamate dehydrogenase (NAD(P)+) [EC:1.4.1.3]	Glutamate dehydrogenase/leucine dehydrogenase
Hs.peg.1821	2.239403418	0.00839604	4.44E-129	Hs1821	DUF3567 domain-containing protein		
Hs.peg.1843	3.128686722	0.003007496	6.70E-112	Hs1843	DUF4148 domain-containing protein		
Hs.peg.1844	4.432370961	0.004069703	3.71E-84	Hs1844	DUF4148 domain-containing protein		
Hs.peg.1956	3.405587402	0.040983043	1.09E-68	*glrR*	response regulator	glrR; two-component system, NtrC family, response regulator GlrR	Response regulator containing CheY-like receiver, AAA-type ATPase, and DNA-binding domains
Hs.peg.2057	3.326036883	0.016135616	8.60E-83	Hs2057	SDR family NAD(P)-dependent oxidoreductase		
Hs.peg.2079	3.954531392	0.002995654	1.14E-96	*bpeT*	LysR family transcriptional regulator		
Hs.peg.2082	2.387689752	0.044104004	1.16E-86	Hs2082	aldo/keto reductase		
Hs.peg.2235	2.802119254	0.028406194	3.93E-83	*pmm-pgm*	phosphomannomutase/phosphoglucomutase	pmm-pgm; phosphomannomutase / phosphoglucomutase [EC:5.4.2.8 5.4.2.2]	Phosphomannomutase
Hs.peg.2266	4.90615883	6.33E-05	3.46E-129	Hs2266	NADP-dependent oxidoreductase	uncharacterized protein	Putative NADP-dependent oxidoreductases
Hs.peg.2283	3.190488467	0.006718108	5.52E-97	*nfnB*	NAD(P)H nitroreductase		
Hs.peg.257	2.32804183	2.86E-05	1.03E-94	Hs257	MULTISPECIES: hypothetical protein		
Hs.peg.258	3.350033037	0.015804109	1.17E-80	Hs258	hypothetical protein Hsero_3161		
Hs.peg.2589	2.260675441	0.007761235	1.33E-114	*mdoH*	membrane glycosyltransferase		
Hs.peg.2744	3.409212812	0.04342148	2.07E-69	*puuE*	4-aminobutyrate--2-oxoglutarate transaminase	puuE; 4-aminobutyrate aminotransferase [EC:2.6.1.19]	4-aminobutyrate aminotransferase and related aminotransferases
Hs.peg.2747	4.097788237	0.028528932	2.55E-62	Hs2747	ABC transporter substrate-binding protein	ABC.SP.S; putative spermidine/putrescine transport system substrate-binding protein	Spermidine/putrescine-binding periplasmic protein
Hs.peg.2949	1.660629562	0.034422222	1.24E-126	*trxA*	thioredoxin TrxA		
Hs.peg.2951	1.733256988	0.000851208	1.22E-96	*RP-L31*	type B 50S ribosomal protein L31	RP-L31; large subunit ribosomal protein L31	Ribosomal protein L31
Hs.peg.3054	2.163768947	0.016455143	1.76E-110	Hs3054	hypothetical protein Hsero_0762		
Hs.peg.3074	4.304208812	0.013431377	2.73E-66	*Lip*	type VI secretion system lipoprotein TssJ		

Continuation table S5: Up- regulated genes from *H. seropedicae* HRC54 response to sugarcane apoplastic fluid. Genes identified using DESeq2 and edgeR package (with cut off p-value of 0.05 and log2 fold change of 1.5).

ORF ID	log2FoldChange	p-value	FDR	Gene or ORF	Blast2GO	KEEG	COG
Hs.peg.3076	3.221787215	0.023695916	6.33E-82	*dotU*	DotU family type IV/VI secretion system protein	impK; type VI secretion system protein ImpK	Uncharacterized protein conserved in bacteria
Hs.peg.3077	3.521596192	0.000904985	4.74E-113	*vgrG*	type VI secretion system tip protein VgrG		
Hs.peg.3119	3.529924053	0.035018422	3.96E-69	Hs3119	Re/Si-specific NAD(P)(+) transhydrogenase subunit alpha		
Hs.peg.3329	1.736472319	0.049333925	1.31E-112	*acrA*	MexE family multidrug efflux RND transporter periplasmic adaptor subunit	acrA; membrane fusion protein, multidrug efflux system	Membrane-fusion protein
Hs.peg.3459	3.861129767	0.040928249	1.64E-48	*acrB*	hydrophobe/amphiphile efflux-1 family RND transporter	acrB; multidrug efflux pump	Cation/multidrug efflux pump
Hs.peg.3504	3.258485466	0.018812717	2.37E-83	*gst*	glutathione S-transferase		
Hs.peg.3593	5.187961112	0.000303934	6.84E-106	*yahK*	NAD(P)-dependent alcohol dehydrogenase	yahK; uncharacterized zinc-type alcohol dehydrogenase-like protein [EC:1.-.-.-]	Zn-dependent alcohol dehydrogenases
Hs.peg.3677	3.330955259	0.048748525	1.33E-66	*tyrB*	aspartate/tyrosine/aromatic aminotransferase	tyrB; aromatic-amino-acid transaminase [EC:2.6.1.57]	Aspartate/tyrosine/aromatic aminotransferase
Hs.peg.3748	3.941866736	0.036944795	5.07E-56	Hs3748	NADH dehydrogenase		
Hs.peg.3775	2.462847158	0.00759008	1.10E-120	*PRDX2*	peroxiredoxin	PRDX2_4; peroxiredoxin (alkyl hydroperoxide reductase subunit C) [EC:1.11.1.15]	Peroxiredoxin
Hs.peg.4167	3.188533336	0.032725358	3.66E-74	Hs4167	Tim44 domain-containing protein		
Hs.peg.4308	2.08800868	0.016982227	7.58E-109	Hs4308	VWA domain-containing protein	uncharacterized protein	Uncharacterized protein conserved in bacteria
Hs.peg.4310	2.652464954	0.008710213	2.02E-101	*ctpV*	copper-translocating P-type ATPase	copA; Cu+-exporting ATPase [EC:3.6.3.54]	Cation transport ATPase
Hs.peg.464	1.537104759	0.045985804	4.20E-119	*ndK*	nucleoside-diphosphate kinase	ndk; nucleoside-diphosphate kinase [EC:2.7.4.6]	Nucleoside diphosphate kinase
Hs.peg.4696	1.664821676	0.023508729	9.14E-130	*pal*	peptidoglycan-associated lipoprotein Pal		
Hs.peg.4726	1.949565993	0.017063314	1.54E-126	*hsiU*	ATP-dependent protease ATPase subunit HslU	hslU; ATP-dependent HslUV protease ATP-binding subunit HslU	ATP-dependent protease HslVU (ClpYQ), ATPase subunit
Hs.peg.4729	4.475741181	3.69E-06	3.86E-134	Hs4729	porin		
Hs.peg.473	1.601847657	0.00733939	5.56E-119	*hfq*	RNA-binding protein Hfq		
Hs.peg.4739	2.796939826	0.02143491	6.38E-85	*otsB*	trehalose-phosphatase	otsB; trehalose 6-phosphate phosphatase [EC:3.1.3.12]	Trehalose-6-phosphatase
Hs.peg.4740	3.21988272	0.024339244	3.73E-82	Hs4740	glycoside hydrolase family 15 protein		
Hs.peg.799	3.442515413	0.040545294	1.24E-67	Hs799	type 1 glutamine amidotransferase domain-containing protein		

4.2. Artigo "Protein expression profile of *Herbaspirillum seropedicae* HRC54 in response to sugarcane apoplastic fluid provides insights into the initial steps of plant-bacteria interaction"

Manuscrito com os dados do experimento de proteoma de *Herbaspirillum seropedicae* HRC54 em resposta ao líquido apoplástico de cana-de-açúcar submetido na revista *Biochimica et Biophysica Acta - Proteins and Proteomics*. A versão completa pode ser lida abaixo, com material suplementar no tópico 6.2.

Protein expression profile of *Herbaspirillum seropedicae* HRC54 in response to sugarcane apoplastic fluid provides insights into the initial steps of plant-bacteria interaction

Highlights

- A total of 123 HRC54 proteins were dysregulated in response to apoplastic fluid
- A type VI secretion system is important to initiate the interaction process
- Host recognition changes metabolism and transport processes in HRC54

Abstract

Differential protein expression profiling can provide important insights into the mechanisms that drive metabolic changes to adapt to different environmental conditions. Analyses using endophytic diazotrophic bacterial species exposed to plant fluids are an interesting approach to identify and understand these changes. Therefore, this study evaluated the protein profile of *Herbaspirillum seropedicae* strain HRC54 exposed to sugarcane apoplastic fluid for two hours. The HRC54 protein expression pattern highlighted the importance of the transport and metabolism of several nutrients and the bacterial secretion system in the interaction process. These expression patterns allow bacteria to adapt to changing conditions during the early stages of the plant-bacteria interaction process, suggesting that HRC54 recognized the plant apoplastic fluid as a host site.

Keywords: *Herbaspirillum seropedicae*, sugarcane, diazotrophic bacteria, proteome, gel-free approach

1. Introduction

Climate change and global warming have resulted in significant losses in soil quality, thus increasing the need for fertilizers for large crops (Bordonal et al. 2018). However, this practice can be harmful and expensive and therefore, alternative strategies have been developed to increase crop productivity (Schultz et al. 2017). Among these strategies, recent studies have identified plant growth-promoting bacteria (PGPB) that promote symbiosis with several plant species, thus benefiting both organisms (Reis 2005). An example of a successful PGPB is the endophytic diazotrophic bacterium *Herbaspirillum seropedicae*, which can associate with grasses as maize, sugarcane and wheat (James et al. 1997). To date, its use as an inoculant in sugarcane has been explored as an alternative to high concentrations of nitrogen fertilizers (Alves et al. 2014; Schultz et al. 2017; dos Santos et al. 2020). Nevertheless, the mechanisms that drive plant-bacteria interactions remain poorly understood, and the failure of critical steps during the interaction can decrease the inoculation effectiveness (Monteiro et al. 2008).

Herbaspirillum seropedicae strain HRC54 (BR11335) was isolated from sugarcane roots in Brazil using a nitrogen-free culture (Dobereiner 1992) and its potential as an inoculant has been evaluated in grass plants such as sugarcane (Alves et al. 2014; dos Santos et al. 2020; Martins et al. 2020). The *H. seropedicae* genome (strain SMR1) was sequenced in 2011 and this bacterium is known to colonize its host through passive penetration, in addition to being capable of fixing nitrogen, producing phytohormones, and inhabiting plant tissues and organs without adversely affecting the host. These characteristics enable this bacterium to establish symbiotic interactions with plant hosts (Pedrosa et al. 2011).

The study of gene and protein expression patterns (i.e., transcriptomes and proteomes, respectively) has been widely applied to understand the molecular processes that mediate the interactions between organisms (Trapnell et al. 2013; Van Dijk et al. 2014; Taulé et al. 2021). Plant tissues or extracellular fluids can be used to induce changes in the gene/protein expression patterns of microorganisms, thus providing a useful approach to evaluate the mechanisms that drive the plant-microbe interactions of different PGPB species (Silva et al. 2018; Dos Santos et al. 2020; Terra et al. 2020; Pessoa et al. 2021). Proteome analyses can be uniquely challenging due to their reliance on complex

methodologies and specialized equipment, which highlights the need for alternative label-free and gel-free approaches (Baggerman et al. 2005; Porteus et al. 2011; Armengaud 2016). These technologies enable researchers to easily evaluate and compare different proteomes, their compositions, and the potential factors that drive gene/protein dysregulation (Gevaert et al. 2007). For example, combined studies integrating transcriptome and proteome analyses can elucidate the molecular mechanism involved in complex processes such as plant-microorganism interactions (Zhang et al. 2017; Lardi and Pessi 2018).

Transcriptomic analyses of *H. seropedicae* (HRC54) exposed to sugarcane apoplastic fluid for 2 h indicated that 89 genes were differentially expressed, including genes related to the regulation of bacterial metabolism, nutrient transport, secretion system, and signal transduction (Pessoa et al. 2021). In the past, the type VI secretion system (T6SS) was believed to be present only in pathogenic bacteria (Filloux et al. 2008; Jani and Cotter 2010). However, the genome of *H. seropedicae* SMR1 exhibits the T6SS, which likely promotes the ability of this bacterium to invade its host and become established in its tissues during the interaction process (Pedrosa et al. 2011; Bernal et al. 2018; Drebes Dörr and Blokesch 2018). Interestingly, 33% of T6SS genes in HRC54 were upregulated upon plant fluid exposure (Pessoa et al. 2021), suggesting that this system plays an important role during the initial steps of the interaction process. To better understand the response of HRC54 to sugarcane apoplastic fluid, a gel-free proteomic approach was used to identify differentially expressed proteins. Our study focused on the initial response of the bacteria to fluid exposure, as this would provide insights into the mechanisms that mediate the early establishment of plant-microbe interactions. To the best of our knowledge, our study is the first to evaluate the protein-level effects of sugarcane apoplastic fluid on strain HRC54 to elucidate the mechanisms that drive plant-microbe symbiosis.

2. Materials and Methods

2.1 Harvesting of apoplastic fluid from sugarcane RB867515

Sugarcane plants (*Saccharum officinarum*, variety RB867515) were collected from an Embrapa Agrobiologia field at approximately 1 year of age. This commercial variety is responsive to inoculation with endophytic diazotrophic bacteria (Schultz et al. 2015; dos Santos et al. 2020). To extract the liquid, the plants were harvested and immediately

peeled, after which the node was removed and cut into smaller pieces to fit in 50 mL conical-bottom tubes. These pieces were then flamed with 70% alcohol to obtain the apoplastic liquid, which was then centrifuged at 3,000 × g for 20 min at 4 °C to maintain the liquid properties, as described by Dong et al. (1994) (Dong et al. 1994).

2.2 *H. seropedicae* HRC54 growth in the presence of sugarcane apoplastic fluid

H. seropedicae strain HRC54 (BR11335) was obtained from the Biological Resource Center, Johanna Döbereiner Embrapa Agrobiologia, Seropédica, Brazil (Zilli and Soares 2016). To evaluate differential protein expression, strain HRC54 was grown in 60 mL of JNFB medium for 16 h at 100 rpm. Afterward, the culture was equally separated into two vessels, each containing the same proportion of apoplastic fluid and water. The samples were then labeled as HRC54+apoplast (APOPLAST) and HRC54+water (WATER). The samples were maintained at 100 rpm for 2 h. This collection time was selected to characterize the early bacterial response to the presence of sugarcane apoplastic fluid. For this, 5 mL of each sample was collected and centrifuged for 10 min at 8.300 × g at 4 °C (Pessoa et al. 2021).

2.3 Identification of differentially expressed proteins

2.3.1 Protein extraction

The cellular sediment obtained from the HRC54 culture and apoplastic fluid experiment mentioned above was resuspended in a resuspension solution (10 mM Tris-HCl, 1,5 mM $MgCl_2$, and 10 mM KCl, pH 8,0) and the sediment was centrifuged again for 10 min at 12,000 × g and 4 °C. Afterward, the supernatant was discarded, and the sediment was once again resuspended in another 2 mL of resuspension solution. The samples were then centrifuged again for 10 min at 12.000 × g at 4 °C. Next, 0.5 mL of solubilization solution (7 M urea, 2 M urea, 2% Triton X-100, 1 mM PMSF, and 20 mM DTT) was added to the samples, after which they were agitated for 30 min. Next, the samples were sonicated in an ice bath (10 cycles of 20 s with an amplitude of 10%), then centrifuged for 90 min at 12,000 × g at 4 °C. Supernatants were collected and transferred to new microtubes, and the protein extract obtained from each biological sample was quantified using the 2-D Quant Kit (GE Healthcare).

2.3.2 Mass spectrometry analyses

Mass spectrometry ESI-LC-MS/MS was performed using a nanoACQUITY ultra-performance liquid chromatograph (UPLC) coupled with a Synapt G2-Si HMDS instrument (Waters, Manchester, UK). The protein concentrations were normalized to 500 ng/mL per sample. To ensure standardized molar values for all conditions, normalization was conducted between the samples based on stoichiometric measurements of the total ion counts of scouting runs prior to the analyses using the ProteinLynx Global SERVER v. 3.0 program (PLGS; Waters). During separation, the samples were loaded onto a nanoACQUITY UPLC 5 µm C18 trap column (180 µm × 20 mm; Waters) at 5 µL min^{-1} for 3 min, followed by a nanoACQUITY HSS T3 1.8 µm analytical reversed-phase column (75 µm × 150 mm; Waters) at 400 nL min^{-1} with a column temperature of 45 °C. A binary gradient was used for peptide elution. Mobile phase A consisted of water and 0.1% formic acid, whereas mobile phase B consisted of acetonitrile and 0.1% formic acid. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 FWHM, with ion mobility (IMS) (HDMSE), and in data-independent acquisition (DIA) mode. For the time-of-flight (TOF) parameters, the scan time was set to 0.5 s in continuous mode, with a mass range of 50 to 2000 Da. Human [Glu1]-fibrinopeptide B (100 fmol μL^{-1}) was used as an external calibrant, and lock mass acquisition was performed every 30 s. Mass spectra were subsequently acquired using the MassLynx v4.0 software.

2.3.3 Data processing protocol

Spectra processing and database searches were performed using the Progenesis QI for Proteomics software V.2.0 (Nonlinear Dynamics, Newcastle, UK). The analyses were conducted using the following parameters: Apex3D of 150 counts for low-energy threshold, 50 counts for elevated energy threshold, and 750 counts for intense threshold; one missed cleavage; minimum fragment ions per peptide equal to two; minimum fragment ions per protein equal to five; minimum peptides per protein equal to two; fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY); maximum default false discovery rate of 1%; peptide score greater than four; and maximum mass error of 10 ppm. Label-free relative quantitative analyses were performed based on the ratio of protein ion counts between the samples. For protein identification, MS data were processed against the *H. seropedicae* HRC54 protein database (UniProt). Only the proteins present in all three runs were accepted and subjected to differential abundance analysis. The WATER sample was used as the control

for all downstream comparisons. Proteins in the APOPLAST samples with a fold change (FC) greater than 1.5 were considered upregulated, whereas downregulated proteins were defined as those with an FC below 0.67. In both cases, the protein expression levels had to be significantly different to the control (ANOVA; $P < 0.05$) for the proteins to be considered either up- or downregulated. Functional annotation of these proteins was performed using BLAST2GO (Conesa et al. 2005) and gene ontology was characterized using the KEGG and COG databases (Kanehisa and Goto 2000; Tatusov et al. 2000).

2.4. Data validation with RT-qPCR

To validate the differentially expressed proteins identified in HRC54 in response to the presence of sugarcane apoplastic fluid, six upregulated and downregulated proteins were randomly selected for RT-qPCR analyses. Primers were designed using the coding sequences of the selected proteins. Twelve genes were evaluated and two genes (*groEL* and *rpoA*) were used as a reference, as recommended by Pessoa et al. (Pessoa et al. 2016, 2021). A new RT-qPCR experiment was performed as described by Pessoa et al. (2021) (Pessoa et al. 2021). Statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/). $P < 0.05$ was considered statistically significant according to the Student's t-test. The primer sequences employed for our RT-qPCR experiments are summarized in Supplementary Table S3.

3. Results and Discussion

3.1. Identification of differentially expressed proteins

A total of 657 proteins were identified in *H. seropedicae* HRC54 exposed to sugarcane apoplastic fluid. Among them, the expression of 527 proteins remained unaltered, whereas 123 were differentially expressed. Among these dysregulated 123 proteins, 76 were upregulated (fold change >1.5) and 47 were downregulated (fold change <0.65) (Table 1). The 123 proteins were submitted for functional annotation analyses using the KEGG and COG databases (Supplementary Tables S1 and S2). The information found in the databases was used to classify the dysregulated proteins and group them according to these categories. The most dysregulated proteins, both up- and downregulated, served to elucidate the metabolic pathways that were most affected in HRC54 in response to sugarcane apoplastic fluid (Table 2). Eleven proteins identified in this study were also found in RNA-Seq analyses of the HRC54 response to apoplastic fluid (Supplementary

Table S4). HRC54 differentially expressed proteins were generally associated with a diverse set of functions (Figure 1), which is detailed in the next section.

3.2. Functional annotation and gene ontology

Among the 123 differentially expressed proteins elucidated herein, the two most abundant groups were 49 proteins that were not identified, followed by 10 proteins from multiple class categories. The next groups, with more than five proteins each, were associated with carbohydrate metabolism and transport, signal transduction, amino acid metabolism and transport, and cell wall/membrane/envelope biogenesis. Other groups, such as membrane transport, bacterial secretion system, and energy production and conversion, were also linked to 3-4 proteins.

3.2.1. Amino acid and carbohydrate transport, metabolism, and membrane transport

The transport and metabolism of carbohydrates and amino acids is required for *in vitro* bacterial growth, including that of *H. seropedicae* (Rosconi et al. 2016). Twenty-one proteins were differentially expressed in HRC54 in response to apoplastic fluid from sugarcane (Table 1). Among the upregulated proteins, AfuA and AfuC (iron transport) and MlaC and RbsB (phospholipid and ribose transport) are related to membrane transport, whereas AdhP [NAD(P)-dependent alcohol dehydrogenase] and LhgO (hydroxyglutarate oxidase) are related to carbohydrate transport and metabolism. In contrast, *phbB* (acetoacetyl-CoA reductase), which is related to carbohydrate metabolism, was downregulated in HRC54 as observed in *Gluconacetobacter diazotrophicus* when co-cultivated with sugarcane and *H. seropedicae* SMR1 was cultivated in sugarcane extract (dos Santos et al. 2010; Cordeiro et al. 2013). PtsA (phosphoenolpyruvate-protein phosphotransferase), PpsA (phosphoenolpyruvate synthase), DctP (open reading frames *Hs721* and *Hs2964* C4-dicarboxylate ABC transporter), and RbsB (ABC transporter substrate-binding protein), which are also related to carbohydrate transport and metabolism, were all downregulated (Table S2). This category was also repressed in *Paraburkholderia tropica* Ppe8 in response to sugarcane apoplastic fluid, as well as in *H. seropedicae* SMR1, in response to naringenin and environmental phosphate, and *G. diazotrophicus* exposed to polyethylene glycol (Tadra-Sfeir et al. 2015; Silva et al. 2018; Grillo-Puertas et al. 2021; Leandro et al. 2021). This pattern was also observed in AroG1, LivK, LivG, GsiB, and TyrA, which encode proteins involved in amino acid transport and metabolism. These types of transporters were also downregulated in the

transcriptomic analyses of HRC54, *P. tropica* Ppe8, and *Nitrospirillum amazonense* CBAmC in response to sugarcane apoplastic fluid (Silva et al. 2018; Terra et al. 2020; Pessoa et al. 2021). These results highlight the importance of the ABC transporter as a mediator of bacterial adaptation to its habitat and differential expression of the ABC transporter in bacteria can be related to the nutrients present in the fluid or exudates, possibly during the recognition of the host or the initiation of microbe-plant interaction.

3.2.2. Signal Transduction

The signal transduction system regulates the changes in bacterial gene expression in response to external signals and can be present at all expression levels (Galperin 2004, 2010; Olanrewaju et al. 2017; Khatabi et al. 2019). A total of eight proteins associated with signal transduction were differentially expressed in HRC54 exposed to sugarcane apoplastic fluid (Table 1), among which seven were downregulated and one was upregulated. Six open reading frames (ORFs) were annotated as Tar (methyl-accepting chemotaxis). Based on these analyses, the Med (BMP family), CheV (chemotaxis protein), and Tar proteins were found to be downregulated and one Tar protein (OFR *Hs685*) was upregulated. Similar changes in expression patterns were observed in transcriptomic analyses of *H. seropedicae* HRC54 and *P. tropica* Ppe8, as well as a proteomic evaluation of *N. amazonense* CBAmC after exposure to sugarcane apoplastic fluid (Silva et al. 2018; Terra et al. 2020; Pessoa et al. 2021). However, a different regulation of these proteins was observed in *Azospirillum lipoferum* 4 B in association with rice and *H. seropedicae* in response to iron availability (Drogue et al. 2014; Trovero et al. 2018). This difference between the expression patterns appeared to be largely caused by the strategy used to evaluate the plant-microorganism interaction. However, the role of signal transduction cannot be overlooked, particularly as genes associated with this process were dysregulated in strain HRC54 in response to sugarcane apoplastic fluid, as demonstrated by transcriptome- and protein-level analyses (Pessoa et al. 2021). Nevertheless, further investigation is required to reaffirm this system’s importance in the interaction process.

3.2.3. Cell wall, membrane, and envelop biogenesis

Six proteins involved in membrane and cell wall maintenance were differentially expressed in HRC54 in response to sugarcane apoplastic fluid (Table 1). This protein category is the second most abundantly required for *in vitro* bacterial growth (Rosconi et

al. 2016). OmpC (ORF *Hs4729*, porin), OsmY (lipoprotein), NlpD (peptidoglycan-binding), and GalU (glucose-1-phosphate uridylyltransferase) were upregulated and two ORFs (*Hs1194* and *Hs2786*), annotated as OmpC, were downregulated. These proteins encode porins, which are involved in nutrient exchange. Overexpression of these porins was reported in *P. tropica Ppe8* in response to sugarcane apoplastic fluid, as well as in *H. seropedicae* Z67 in response to iron availability, and during the interaction of *Burkholderia kururiensis* M130 and *H. seropedicae* SMR1 with rice (Coutinho et al. 2015; Brusamarello-Santos et al. 2018; Silva et al. 2018; Trovero et al. 2018). The induction of these proteins suggests that HR54 was able to recognize the fluid as a host.

3.2.4. Post-translational modification, protein turnover, and chaperone functions

Post-translational modifications are expected in comparative proteomic studies using plant extracts (Baggerman et al. 2005; Knief et al. 2011). This category appeared to be more active at the protein level than the transcript level; however, one level is directly related to the other (Vogel and Marcotte 2013). The proteins Gst (glutathione S-transferase), Dcp (peptidase), DegQ (periplasmic serine protease), and HslU and HflC (proteases) were upregulated when HRC54 was exposed for 2 h to sugarcane apoplastic fluid (Table 1), which was also observed in *P. tropica* Ppe8 and *N. amazonense* CBAmC (Silva et al. 2018; Terra et al. 2020). The induction of these groups during the response to the fluid can be related to the establishment of bacteria-plant interaction.

3.2.5. Type VI secretion system

T6SS in non-pathogenic bacteria appears to be involved in recognition and signaling, thus acting as a successful apparatus for competition against other microorganisms and during plant invasion (Pedrosa et al. 2011; Bernal et al. 2018). The upregulation of T6SS was also observed in RNA-Seq analyses of strain HRC54 in response to sugarcane apoplastic fluid (Pessoa et al. 2021). Proteins Hcp (membrane subunit), ImpC, ImpB (contractile sheath), and ImpJ (baseplate) from T6SS were upregulated in HRC54 proteomic analyses. Upregulation of this system was also observed in RNA-Seq analyses of *H. seropedicae* SMR1 in response to phosphate concentration, as well as in *N. amazonense* CBAmC n response to apoplastic fluid, and in *Burkholderia phymatum* STM815 in response to *Mimosa pudica* root exudates, suggesting that this system is required in all types of symbiosis (Klonowska et al. 2018; Terra et al. 2020; Grillo-Puertas et al. 2021). Of the

nine gene components of T6SS, eight were differentially expressed in HRC54 in response to apoplastic fluid. One was identified as a “hypothetical protein.” However, based on the gene locus and its position in the genome of HRC54, we speculated that this gene belonged to the T6SS operon (Table 3). Genes *hcp* and *vgrG* are essential for T6SS functioning (Cascales and Cambillau 2012), and both were identified in strain HRC54 (Table 3). The differential expression of the genes related to this system at both the transcript and protein levels highlight the importance of the type VI secretion system in the initial steps of the interaction process. However, the species-specific variations of this response and the abundance of its components remain largely uncharacterized and thus warrant additional research (Lin et al. 2019).

3.2.6. Energy production and conversion, mineral and organic ion transporters, and replication and repair

Three proteins related to COG S category C were differentially expressed in HRC54 (Table 1). Specifically, one protein was downregulated (TctC-tricarboxylate transporter substrate) and the other two were upregulated (FumC-class II fumarate hydratase and GpsA-3-hydroxyacyl-CoA dehydrogenase). The proteins OpuA, OpuBD, and OpuC (osmoprotectant transport system), associated with mineral and organic ion transport, were upregulated. The expression of three proteins involved in replication and repair was altered, among which two were upregulated (RecA - recombinase A protein and UvrA - excinuclease ABC) and one was downregulated (BRR2 - replicative superfamily II helicase). An upregulation of energy production and gene conversion was observed in a proteomic evaluation of the exponential phase of *G. diazotrophicus* and *N. amazonense* CBAmC proteomes in response to sugarcane apoplastic fluid (Lery et al. 2008; Terra et al. 2020). It is also worth noting that our HRC54 proteome analyses were also conducted at the exponential phase. The aforementioned protein classifications were likely associated with the adaptation to the presence of apoplastic fluid. However, further experiments are required to confirm this hypothesis.

3.2.7. Other functions

Six proteins from categories P, I, and J were differentially expressed in HRC54 exposed to sugarcane apoplastic fluid (Table 1). The proteins *Bfr* (bacterioferritin) and *PstS* (phosphate ABC transporter) involved in inorganic ion transport and metabolism were upregulated and downregulated, respectively. Regarding lipid metabolism, the protein

AdhE (acyl-CoA reductase) was upregulated and *CphA* (cyanophycin synthetase) was downregulated. *Rph* (ribonuclease PH) and *RraA*, which are related to translation, were downregulated. Inorganic ion transport and metabolism proteins were also differentially expressed during the proteomic profiling of *R. tropici* PRF 81 responses to heat stress and *Chromobacterium violaceum* exposed to cyanate (Baraúna et al. 2011; Gomes et al. 2012). Proteins related to lipid metabolism were also identified during proteome analysis of *Pseudomonas putida* F1 genes in different soil environments [57], and one set of samples in *G. diazotrophicus* co-cultivated with sugarcane plantlets also exhibited upregulation of proteins associated with lipid metabolism (dos Santos et al. 2010). The repression of translation proteins was also observed in the *C. violaceum* and *G. diazotrophicus* studies mentioned above (dos Santos et al. 2010; Baraúna et al. 2011).
A diverse set of functions was identified with at least one representative (Table 1). Proteins involved in cell motility (CheZ - protein phosphatase), coenzyme metabolism (ThiC-phosphomethylpyrimidine synthase), and transcription (RpoD - RNA polymerase) were downregulated. Cell motility repression is a common feature in studies of interaction processes (Tadra-Sfeir et al. 2015; Balsanelli et al. 2016; Silva et al. 2018; Terra et al. 2020; Pessoa et al. 2021). The upregulation occurred in proteins related to defense mechanisms (AhpF - alkyl hydroperoxide reductase subunit F), intracellular trafficking and secretion (TolB-Tol-Pal system protein), lipopolysaccharide biosynthesis (PagP lipid IVA palmitoyltransferase), and secondary structure (YcgM - 2-keto-4-pentenoate hydratase). Although these categories represented a low number of genes, their roles in the initial interaction process should be further characterized.

3.2.8. Multiple classes

Ten proteins were identified in COG categories as multiple classes (Table 1). This means that these proteins were associated with not only a single function but also two or more, indicating that these proteins likely play diverse roles (Galperin et al. 2021). LeuB and CstA, associated with categories C and E (energy production and conversion and amino acid metabolism and transport), were downregulated. This pattern was also identified for the proteins PabA and IlvC from categories E and H (amino acid metabolism and transport and coenzyme metabolism). Two proteins from categories E and R (amino acid metabolism and transport and general function, prediction only) were also differentially expressed. HppD and GltD were upregulated and downregulated, respectively. The proteins AcrA and AcrB associated with categories V and M (defense mechanisms and

cell wall/membrane/envelope biogenesis) were upregulated. PdxI from categories H and R (coenzyme metabolism and general function, prediction only) was upregulated, whereas *dppD* from the E and P categories (amino acid metabolism and transport and inorganic ion transport and metabolism) was downregulated. The differential expression of some of the functions mentioned here has already been discussed in the previous sections.

3.2.9. Non-identified categories or functions

A group of 49 proteins were identified by BLAST2GO, but no further information about their functional annotation was obtained (Table 1). In this group, 39 proteins were upregulated and 10 were downregulated. In addition to the presence of three proteins described as DUF domains (Goodacre et al. 2014), two for BON domains [61], and one hypothetical protein, the other proteins did not present matches in other databases such as KEGG and COG. However, the presence of proteins described for T6SS (ORF *Hs3058*), glutamate, and glutathione (*Hs1741*, *Hs1800*, *Hs1801*, and *Hs2247*), and a high number of proteins annotated as ABC transporter substrate-binding proteins (*Hs74*, *Hs1783*, *Hs2374*, and *Hs3916*) should not be ignored. For instance, ABC transporters were differentially expressed in *G. diazotrophicus* when exposed to *Arabidopsis thaliana* exudates and exposed to different PEG concentrations (Dos Santos et al. 2020; Leandro et al. 2021). Even with insufficient annotation, these proteins appear to be relevant for the initial steps of the plant-bacteria interaction process.

3.3. Differential protein expression pattern validation with RT-qPCR

The validation of 12 differentially expressed proteins was performed using RT-qPCR (Figure 2). These transcript-level analyses were performed based on our proteomic analyses, which is considered an important bottleneck in proteomic validations (Armengaud 2013). Among the upregulated proteins (Figure 2A), only the expression pattern of the *Hs4729* gene was confirmed, with a high relative expression value in the APOPLAST sample. The same occurred in the case of *Hs1741* and *Hs3070*; however, the statistical analyses indicated that there was a non-significant difference between the relative expression levels. The *pdxI*, *htpG*, and *Hs2721* genes had higher expression levels in the WATER samples, which did not match our proteomic data. The downregulated proteins (Figure 2B) presented more consistent results, with four out of six genes confirmed. The relative expression of the genes *dppD*, *dctP*, *Hs1210*, and *Hs1485* was

higher in the WATER samples, thus confirming the data obtained from the proteomic analyses. The same was true for genes *pstS* and *Hs3916*; however, both results were considered statistically non-significant. The divergence in these results could be due to the high sensitivity of the RT-qPCR technique, as well as post-translational modifications (Zhang et al. 2017). Overall, although several of the APOPLAST RT-qPCR analyses could not statistically confirm the protein-level expression results, there was still an acceptable level of consistency with our *in silico* data.

4. Conclusions

Our study evaluated the protein expression patterns of *H. seropedicae* strain HRC54 in response to sugarcane apoplastic fluid. In most cases, the differentially expressed proteins had functions associated with transport and metabolism (presumably because the apoplastic fluid was detected by the bacteria as a new nutrient source), thus highlighting the importance of these functions in the initial steps of the plant-microbe interaction process. Additionally, T6SS proteins were also differentially expressed, suggesting that this system is also necessary for the initial steps of the interaction process. Taken together, our findings confirm that exposing bacteria to plant fluids is a useful tool for the characterization of plant-microbe interactions, which renders diverse but consistent results that provide useful insights into the mechanisms that drive plant-microbe symbioses.

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Table 1: Functional annotation distribution of differentially expressed proteins identified in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid using a gel-free approach.

Class or Group Description	UP	DOWN	Total
Non-identified	39	10	49
Multiple classes	4	6	10
Carbohydrate metabolism and transport	2	6	8
Signal Transduction	1	7	8
Amino Acid metabolism and transport	-	7	7
Cell wall/membrane/envelop biogenesis	4	2	6
Post-translational modification, protein turnover, chaperone functions	5	-	5
Bacterial secretion system	4	-	4
Membrane transport	4	-	4
Energy production and conversion	2	1	3
Mineral and organic ion transporters	3	-	3
Replication and repair	2	1	3
Inorganic ion transport and metabolism	1	1	2
Lipid metabolism	1	1	2
Translation	-	2	2
Cell motility	-	1	1
Coenzyme metabolism	-	1	1
Defense mechanisms	1	-	1
Intracellular trafficking and secretion	1	-	1
Lipopolysaccharide biosynthesis	1	-	1
Secondary Structure	1	-	1
Transcription	-	1	1
Differential Expressed Protein		123	

Table 2: Highest fold change values of differentially expressed proteins identified in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid using a gel-free approach.

Locus	Protein	Product	Class/KO*	Description	Fold Change	Expression pattern
Hs.peg.685	Tar	HAMP domain-containing protein	T	Signal Transduction	19.92	Up regulated
Hs.peg.741	GalU	UTP--glucose-1-phosphate uridylyltransferase	M	Cell wall/membrane/envelop biogenesis	9.52	Up regulated
Hs.peg.4245	HppD	4-hydroxyphenylpyruvate dioxygenase	ER	Multiple classes	7.51	Up regulated
Hs.peg.3593	AdhP	NAD(P)-dependent alcohol dehydrogenase	G	Carbohydrate metabolism and transport	6.61	Up regulated
Hs.peg.4714	OsmY	periplasmic or secreted lipoprotein	M	Cell wall/membrane/envelop biogenesis	5.49	Up regulated
Hs.peg.4201	OpuA	osmoprotectant transport system ATP-binding	K05847	Mineral and organic ion transporters	5.09	Up regulated
Hs.peg.4729	OmpC	porin	M	Cell wall/membrane/envelop biogenesis	4.25	Up regulated
Hs.peg.4202	OpubD	osmoprotectant transport system permease	K05846	Mineral and organic ion transporters	3.86	Up regulated
Hs.peg.3546	AfuC	iron(III) transport system ATP-binding	K02010	Membrane transport	3.63	Up regulated
Hs.peg.3544	AfuA	iron(III) transport system substrate-binding	K02012	Membrane transport	2.81	Up regulated
Hs.peg.1305	RpH	ribonuclease PH	J	Translation	0.67	Down regulated
Hs.peg.4482	Arog1	3-deoxy-7-phosphoheptulonate synthase	E	Amino Acid metabolism and transport	0.66	Down regulated
Hs.peg.4437	LeuB	Isocitrate/isopropylmalate dehydrogenase	CE	Multiple classes	0.66	Down regulated
Hs.peg.3654	BRR2	Replicative superfamily II helicase	L	Replication and repair	0.66	Down regulated
Hs.peg.419	PhbB	acetoacetyl-CoA reductase	K00023	Carbohydrate metabolism	0.65	Down regulated
Hs.peg.3819	Med	BMP family ABC transporter substrate-binding	T	Signal Transduction	0.64	Down regulated
Hs.peg.1797	PtsA	phosphoenolpyruvate-protein phosphotransferase	G	Carbohydrate metabolism and transport	0.63	Down regulated
Hs.peg.4145	GltD	glutamate synthase subunit beta	ER	Multiple classes	0.62	Down regulated
Hs.peg.4660	Tar	methyl-accepting chemotaxis	T	Signal Transduction	0.61	Down regulated
Hs.peg.186	Tar	chemotaxis protein	T	Signal Transduction	0.61	Down regulated

***KO:** KEGG Ontology

Table 3: Differentially expressed type VI secretion system proteins identified in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid.

Locus	**Gene**	**Product**	**Fold Change**	**Approach**	**Reference**
Hs.peg.3058	Hs3058	type VI secretion system membrane subunit	2.24	Proteome	
Hs.peg.3070	Hcp	type VI secretion system tube protein	2.20	Proteome	
Hs.peg.3071	ImpC	type VI secretion system contractile sheath large subunit	2.09	Proteome	This work
Hs.peg.3072	ImpB	type VI secretion system contractile sheath small subunit	1.56	Proteome	
Hs.peg.3073	-	hypothetical protein	2.21	Proteome	
Hs.peg.3074	*Hs3074*	type VI secretion system lipoprotein	4.30	Transcriptome	Pessoa et. al., 2021
Hs.peg.3075	ImpJ	type VI secretion system baseplate subunit	1.85	Proteome	This work
Hs.peg.3076	*dotU*	family type IV/VI secretion system protein	3.22	Transcriptome	Pessoa et. al., 2021
Hs.peg.3077	*vgrG*	type VI secretion system tip protein	3.52	Transcriptome	Pessoa et. al., 2021

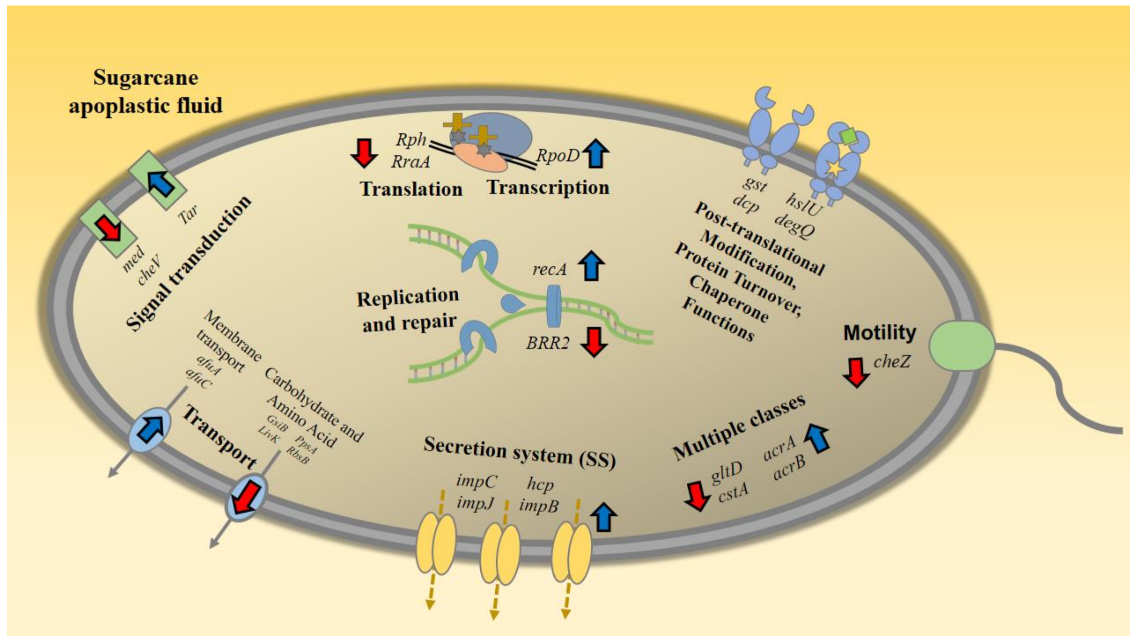

Figure 1: Differentially expressed proteins identified in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid using a gel-free approach, including the most abundant systems identified herein. Blue arrows: upregulation; red arrows: downregulation.

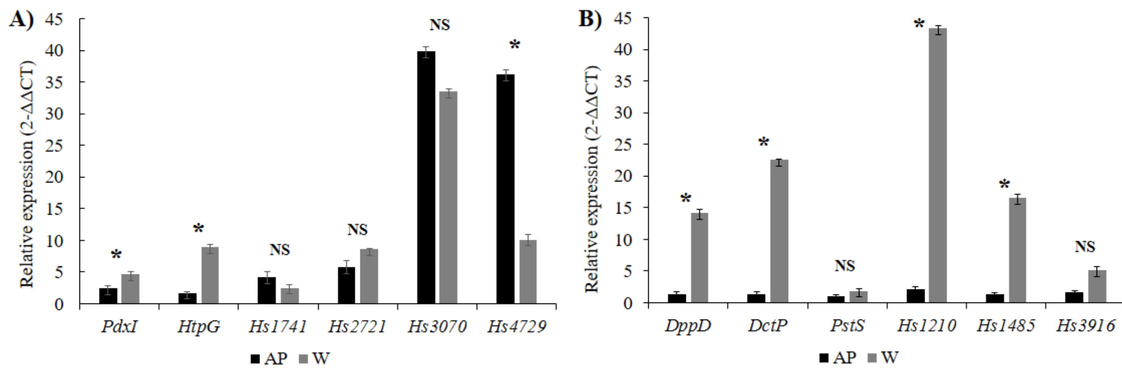

Figure 2: RT-qPCR validation of 12 upregulated (A) and downregulated (B) proteins identified in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid using a gel-free approach. APOPLAST (AP) and WATER (W) samples were evaluated via RT-qPCR to validate the proteomics results. Relative expression was calculated using the Delta CT method (2−ΔΔCT) using *groEL* and *rpoA* as reference genes. All pairwise comparisons were conducted using Student's t-test ($p < 0.05$). The asterisks (*) indicate significant differences. NS: non-significant difference.

4.2.1. Material suplementar artigo “Differential protein expression of *Herbaspirillum seropedicae* HRC54 in response to sugarcane apoplastic fluid elucidates the initial steps of plant bacteria interaction process”

Table S1: Differential expressed proteins identified on *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid using a gel-free approach, with expression patterned up regulated (fold change > 1.5).

Locus	Protein	Product	Class/KO	Description	Fold Change	Expression pattern
Hs.peg.218		cytochrome-c oxidase, cbb3-type subunit III			1.55	Up regulated
Hs.peg.271		5-dehydro-4-deoxyglucarate dehydratase			22.57	Up regulated
Hs.peg.293		methylisocitrate lyase			2.11	Up regulated
Hs.peg.304		alkene reductase			2.09	Up regulated
Hs.peg.402	Gst	glutathione S-transferase	O	Post-translational modification, protein turnover, chaperone functions	1.93	Up regulated
Hs.peg.459	NlpD	LysM peptidoglycan-binding domain-containing protein	M	Cell wall/membrane/envelop biogenesis	1.74	Up regulated
Hs.peg.476	HflC	HflC protein	O	Post-translational modification, protein turnover, chaperone functions	1.74	Up regulated
Hs.peg.685	Tar	HAMP domain-containing protein	T	Signal Transduction	19.92	Up regulated
Hs.peg.692	PdxI	Pyridoxal reductase related oxidoreductase	HR	Multiple classes	1.66	Up regulated
Hs.peg.741	GalU	UTP--glucose-1-phosphate uridylyltransferase	M	Cell wall/membrane/envelop biogenesis	9.52	Up regulated
Hs.peg.1284		oxidative stress resistance two component response regulator			1.72	Up regulated
Hs.peg.1357		alkyl hydroperoxide reductase			1.64	Up regulated
Hs.peg.1506		DegQ family serine endoprotease			1.73	Up regulated
Hs.peg.1617		heat-shock protein			1.82	Up regulated
Hs.peg.1639		molecular chaperone HtpG			1.58	Up regulated
Hs.peg.1704		SIMPL domain-containing protein			2.22	Up regulated
Hs.peg.1712		murein transglycosylase			1.63	Up regulated
Hs.peg.1717		NAD(P)-dependent oxidoreductase			2.17	Up regulated
Hs.peg.1718		fumarylacetoacetate hydrolase			7.65	Up regulated

Continuation table S1

Locus	Protein	Product	Class/KO	Description	Fold Change	Expression pattern
Hs.peg.1720		amino acid ABC transporter			7.23	Up regulated
Hs.peg.1741		glutamate dehydrogenase			4.95	Up regulated
Hs.peg.1800		glutathione synthase			1.82	Up regulated
Hs.peg.1801		glutamate-cysteine ligase			2.57	Up regulated
Hs.peg.1826		basic amino acid ABC transporter substrate-binding protein			2.02	Up regulated
Hs.peg.1935		Phasin (PHA-granule associated protein)			2.17	Up regulated
Hs.peg.2074		DUF1993 domain-containing protein			7.75	Up regulated
Hs.peg.2082		aldo/keto reductase			2.49	Up regulated
Hs.peg.2109		preprotein translocase subunit SecA			1.60	Up regulated
Hs.peg.2135		MULTISPECIES: signal recognition particle protein			1.50	Up regulated
Hs.peg.2217		Rossmann fold nucleotide-binding protein			1.58	Up regulated
Hs.peg.2235		phosphomannomutase/phosphoglucomutase			1.87	Up regulated
Hs.peg.2247		glutathione peroxidase			2.52	Up regulated
Hs.peg.2259		LysR family transcriptional regulator			2.08	Up regulated
Hs.peg.2578		amino-acid N-acetyltransferase			1.89	Up regulated
Hs.peg.2593		thymidylate synthase			1.50	Up regulated
Hs.peg.2721		keto-deoxy-phosphogluconate aldolase			1.66	Up regulated
Hs.peg.2722		phosphogluconate dehydratase			1.95	Up regulated
Hs.peg.2724		glucose-6-phosphate dehydrogenase			1.83	Up regulated
Hs.peg.2942		GatB/YqeY domain-containing protein			2.51	Up regulated
Hs.peg.3058		type VI secretion system membrane subunit TssM			2.24	Up regulated
Hs.peg.3060		BON domain-containing protein			2.71	Up regulated

Continuation table S1

Locus	Protein	Product	Class/KO	Description	Fold Change	Expression pattern
Hs.peg.3070	Hcp	type VI secretion system tube protein Hcp	K11903	Bacterial secretion system	2.20	Up regulated
Hs.peg.3071	ImpC	type VI secretion system contractile sheath large subunit	K11900	Bacterial secretion system	2.09	Up regulated
Hs.peg.3072	ImpB	type VI secretion system contractile sheath small subunit	K11901	Bacterial secretion system	1.56	Up regulated
Hs.peg.3073		hypothetical protein			2.21	Up regulated
Hs.peg.3075	ImpJ	type VI secretion system baseplate subunit	K11893	Bacterial secretion system	1.85	Up regulated
Hs.peg.3128	Bfr	bacterioferritin	P	Inorganic ion transport and metabolism	2.24	Up regulated
Hs.peg.3160	RecA	recombinase A protein	L	Replication and repair	1.55	Up regulated
Hs.peg.3329	AcrA	efflux RND transporter periplasmic adaptor subunit	VM	Multiple classes	1.85	Up regulated
Hs.peg.3330	AcrB	multidrug efflux RND transporter permease subunit	VM	Multiple classes	2.09	Up regulated
Hs.peg.3394		BON domain-containing protein			1.79	Up regulated
Hs.peg.3544	AfuA	iron(III) transport system substrate-binding protein	K02012	Membrane transport	2.81	Up regulated
Hs.peg.3546	AfuC	iron(III) transport system ATP-binding protein	K02010	Membrane transport	3.63	Up regulated
Hs.peg.3593	AdhP	NAD(P)-dependent alcohol dehydrogenase	G	Carbohydrate metabolism and transport	6.61	Up regulated
Hs.peg.3695		OmpA family protein			2.25	Up regulated
Hs.peg.3776	AhpF	alkyl hydroperoxide reductase subunit F	V	Defense mechanisms	1.94	Up regulated
Hs.peg.3860	LhgO	L-2-hydroxyglutarate oxidase	G	Carbohydrate metabolism and transport	1.54	Up regulated
Hs.peg.3993	MlaC	phospholipid transport system substrate-binding protein	K07323	Membrane transport	1.56	Up regulated
Hs.peg.4043	RbsB	ribose transport system substrate-binding protein	K10439	Membrane transport	1.89	Up regulated
Hs.peg.4102	UvrA	excinuclease ABC subunit UvrA	L	Replication and repair	1.56	Up regulated
Hs.peg.4158	PagP	lipid IVA palmitoyltransferase [EC:2.3.1.251]	K12973	Lipopolysaccharide biosynthesis	1.74	Up regulated
Hs.peg.4201	OpuA	osmoprotectant transport system ATP-binding protein	K05847	Mineral and organic ion transporters	5.09	Up regulated
Hs.peg.4202	OpuBD	osmoprotectant transport system permease protein	K05846	Mineral and organic ion transporters	3.86	Up regulated
Hs.peg.4203	OpuC	osmoprotectant transport system substrate-binding protein	K05845	Mineral and organic ion transporters	2.31	Up regulated

Continuation table S1

Locus	Protein	Product	Class/KO	Description	Fold Change	Expression pattern
Hs.peg.4245	HppD	4-hydroxyphenylpyruvate dioxygenase	ER	Multiple classes	7.51	Up regulated
Hs.peg.4392	FumC	class II fumarate hydratase	C	Energy production and conversion	1.82	Up regulated
Hs.peg.4411		LacI family transcriptional regulator			2.99	Up regulated
Hs.peg.4453	Dcp	M3 family peptidase	O	Post-translational modification, protein turnover, chaperone functions	1.98	Up regulated
Hs.peg.4466	AdhE	Acyl-CoA reductase or other NAD-dependent aldehyde dehydrogenase	I	Lipid metabolism	1.64	Up regulated
Hs.peg.4507	DegQ	Periplasmic serine protease, S1-C subfamily, contain C-terminal PDZ domain	O	Post-translational modification, protein turnover, chaperone functions	2.24	Up regulated
Hs.peg.4646	YcgM	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase	Q	Secondary Structure	2.79	Up regulated
Hs.peg.4695	TolB	Tol-Pal system protein TolB	U	Intracellular trafficing and secretion	1.57	Up regulated
Hs.peg.4714	OsmY	periplasmic or secreted lipoprotein	M	Cell wall/membrane/envelop biogenesis	5.49	Up regulated
Hs.peg.4726	HslU	ATP-dependent protease ATPase subunit HslU	O	Post-translational modification, protein turnover, chaperone functions	1.56	Up regulated
Hs.peg.4729	OmpC	porin	M	Cell wall/membrane/envelop biogenesis	4.25	Up regulated
Hs.peg.4797	GpsA	3-hydroxyacyl-CoA dehydrogenase	C	Energy production and conversion	1.53	Up regulated

Table S2: Differential expressed proteins identified on *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid using a gel-free approach, with expression patterned down regulated (fold change < 0.65).

Locus	Protein	Product	Class/KO	Description	Fold Change	Expression pattern
Hs.peg.74		ABC transporter substrate-binding protein			0.66	Down regulated
Hs.peg.80	TctC	tripartite tricarboxylate transporter substrate binding protein	C	Energy production and conversion	0.51	Down regulated
Hs.peg.186	Tar	chemotaxis protein	T	Signal Transduction	0.61	Down regulated
Hs.peg.320	CheZ	protein phosphatase CheZ	K03414	Cell motility	0.60	Down regulated
Hs.peg.343	LivK	ABC transporter permease	E	Amino Acid metabolis and transport	0.59	Down regulated
Hs.peg.419	PhbB	acetoacetyl-CoA reductase [EC:1.1.1.36]	K00023	Carbohydrate metabolism	0.65	Down regulated
Hs.peg.507		DUF4440 domain-containing protein			0.64	Down regulated
Hs.peg.721	DctP	C4-dicarboxylate ABC transporter	G	Carbohydrate metabolism and transport	0.15	Down regulated
Hs.peg.1194	OmpC	outer membrane porin protein	M	Cell wall/membrane/envelop biogenesis	0.45	Down regulated
Hs.peg.1210		MFS transporter			0.31	Down regulated
Hs.peg.1242	PpsA	phosphoenolpyruvate synthase	G	Carbohydrate metabolism and transport	0.47	Down regulated
Hs.peg.1267	CphA	cyanophycin synthetase	I	Lipid metabolism	0.51	Down regulated
Hs.peg.1305	Rph	ribonuclease PH	J	Tranlsation	0.67	Down regulated
Hs.peg.1479	CstA	carbon starvation protein A	CE	Multiple classes	0.44	Down regulated
Hs.peg.1485	CheV	chemotaxis protein CheV	K03415	Signal Transduction	0.50	Down regulated
Hs.peg.1572		peptidyl-prolyl cis-trans isomerase			0.54	Down regulated
Hs.peg.1596	GsiB	glutathione ABC transporter substrate-binding protein	E	Amino Acid metabolis and transport	0.44	Down regulated
Hs.peg.1783		ABC transporter substrate-binding protein			0.15	Down regulated
Hs.peg.1790	IlvC	ketol-acid reductoisomerase	EH	Multiple classes	0.45	Down regulated
Hs.peg.1797	PtsA	phosphoenolpyruvate--protein phosphotransferase	G	Carbohydrate metabolism and transport	0.63	Down regulated
Hs.peg.1898	LivG	ABC-type branched-chain amino acid transport system	E	Amino Acid metabolis and transport	0.61	Down regulated
Hs.peg.1900	LivK	ABC transporter permease	E	Amino Acid metabolis and transport	0.61	Down regulated
Hs.peg.2139	DppD	ABC transporter ATP-binding protein	EP	Multiple classes	0.57	Down regulated

Continuation table S2

Locus	Protein	Product	Class/KO	Description	Fold Change	Expression pattern
Hs.peg.2190	ThiC	phosphomethylpyrimidine synthase ThiC	H	Coenzyme metabolis	0.35	Down regulated
Hs.peg.2219	Tar	methyl-accepting chemotaxis protein	T	Signal Transduction	0.42	Down regulated
Hs.peg.2374		ABC transporter substrate-binding protein			0.58	Down regulated
Hs.peg.2389	LivK	ABC transporter substrate-binding protein	E	Amino Acid metabolis and transport	0.08	Down regulated
Hs.peg.2603	Tar	methyl-accepting chemotaxis protein	T	Signal Transduction	0.25	Down regulated
Hs.peg.2786	OmpC	porin	M	Cell wall/membrane/envelop biogenesis	0.20	Down regulated
Hs.peg.2812	RbsB	autoinducer 2 ABC transporter substrate-binding protein	G	Carbohydrate metabolism and transport	0.24	Down regulated
Hs.peg.2939	RpoD	RNA polymerase sigma factor RpoD	K	Transcription	0.19	Down regulated
Hs.peg.2964	DctP	C4-dicarboxylate ABC transporter	G	Carbohydrate metabolism and transport	0.54	Down regulated
Hs.peg.3253		DUF1852 domain-containing protein			0.25	Down regulated
Hs.peg.3278	Tar	chemotaxis protein	T	Signal Transduction	0.39	Down regulated
Hs.peg.3623	RraA	RraA family protein	J	Tranlsation	0.38	Down regulated
Hs.peg.3624	TyrA	Prephenate dehydrogenase	E	Amino Acid metabolis and transport	0.27	Down regulated
Hs.peg.3654	BRR2	Replicative superfamily II helicase	L	Replication and repair	0.66	Down regulated
Hs.peg.3819	Med	BMP family ABC transporter substrate-binding protein	T	Signal Transduction	0.64	Down regulated
Hs.peg.3916		ABC transporter substrate-binding protein			0.59	Down regulated
Hs.peg.3923	CoxB	cytochrome c oxidase subunit II			0.50	Down regulated
Hs.peg.4145	GltD	glutamate synthase subunit beta	ER	Multiple classes	0.62	Down regulated
Hs.peg.4175	PabA	aminodeoxychorismate/anthranilate synthase component II	EH	Multiple classes	0.59	Down regulated
Hs.peg.4279		SAM-dependent methyltransferase			0.16	Down regulated
Hs.peg.4437	LeuB	Isocitrate/isopropylmalate dehydrogenase	CE	Multiple classes	0.66	Down regulated
Hs.peg.4482	AroG1	3-deoxy-7-phosphoheptulonate synthase	E	Amino Acid metabolis and transport	0.66	Down regulated
Hs.peg.4655	PstS	phosphate ABC transporter substrate-binding protein PstS	P	Inorganic ion transport and metabolism	0.45	Down regulated
Hs.peg.4660	Tar	methyl-accepting chemotaxis protein	T	Signal Transduction	0.61	Down regulated

Table S3: Primers designed for RT-qPCR validation differential expressed proteins identified on *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid using a gel-free approach.

Gene ID	Expression pattern	Function	Sequence	Amplicon
PdxI.F *PdxI.R*	Up regulated	NADP(H)-dependent aldo-keto reductase	CAGCATGTGGAGGACGAGAG TACGGGTTCTGGATGGTGAC	185 bp
HtpG.F *HtpG.R*	Up regulated	molecular chaperone HtpG	GCGTGGACGAATGGATGCTG GGGTCTCTTCGTGCTGCTTC	129 bp
Hs.1741.F *Hs.1741.R*	Up regulated	Glutamate/leucine dehydrogenase	GCCGTGAACGTGCCCTATGG AAGAGGTGCTGCCCTGGTTC	220 bp
Hs.2721.F *Hs.2721.R*	Up regulated	keto-deoxy-phosphogluconate aldolase	GGCATCACGCAAGAGACCGC ATCTTGTCCCAGTCACCCGC	113 bp
Hcp.F *Hcp.R*	Up regulated	type VI secretion system tube protein Hcp	ATCAGCAGCGTCAAGCCCAG GCCGCCATCCTTCTTCTGCT	111 bp
Hs.4729.F *Hs.4729.R*	Up regulated	transmembrane transport	GCCTGTCCGCAGTGTTCAAC GTGTCAGCGAAGTCGGTCTG	163 bp
DppD.F *DppD.R*	Down regulated	ABC transporter ATP-binding protein	ATCGTCTTCGTCACCCACAG CGTTGCTGCTCTTCCATCAC	191 bp
DctP.F *DctP.R*	Down regulated	C4-dicarboxylate ABC transporter	GAGGTCTATCCCAACAGCAC TACAGCACTTCCTTGGTCGG	170 bp
PstS.F *PstS.R*	Down regulated	phosphate ABC transporter substrate-binding protein	AAGTGCTGGGCGACATCTAC TGGACAGGTAGGAGGTGAAG	159 bp
Hs.1210.F *Hs.1210.R*	Down regulated	MFS transporter	CATCGGCAATGGCTGGTTCG ACGCACGGTTTCCTTGACGA	154 bp
Hs.1485.F *Hs.1485.R*	Down regulated	chemotaxis protein CheV	TCTCCTTCGTCCTGACCGAT CGAACTTGGCGACATAGGCG	180 bp
Hs.3916.F *Hs.3916.R*	Down regulated	ABC transporter substrate-binding protein	TGGGGGTGAACTTCTACGAC AATTCAGGCGAGAGCGTCAC	166 bp

Table S4: Differential expressed genes and proteins identified on *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid common for transcriptome and proteome analyses.

Locus	Gene/Protein	Product	Expression pattern	Fold Change	
				Transcriptome	Proteome
Hs.peg.80	*tctC*	tripartite tricarboxylate transporter substrate binding protein	Down regulated	-5.02	0.51
Hs.peg.1741	*Hs1741*	glutamate dehydrogenase	Up regulated	2.37	4.95
Hs.peg.1900	*livK*	ABC transporter permease	Down regulated	-2.66	0.61
Hs.peg.2082	*Hs2082*	aldo/keto reductase	Up regulated	2.38	2.49
Hs.peg.2219	*tar*	methyl-accepting chemotaxis protein	Down regulated	-2.66	0.42
Hs.peg.2235	*Hs2235*	phosphomannomutase/phosphoglucomutase	Up regulated	2.80	1.87
Hs.peg.3329	*acrA*	efflux RND transporter periplasmic adaptor subunit	Up regulated	1.74	1.85
Hs.peg.3923	*Hs3923*	cytochrome c oxidase subunit II	Down regulated	-1.86	0.50
Hs.peg.4145	*gltD*	glutamate synthase subunit beta	Down regulated	-3.86	0.62
Hs.peg.4726	*hslU*	ATP-dependent protease ATPase subunit HslU	Up regulated	1.95	1.56
Hs.peg.4729	*ompC*	porin	Up regulated	4.48	4.25

5. Conclusão

Durante a exposição de duas horas ao líquido apoplástico de cana-de-açúcar da variedade RB867515, a estirpe HRC54 alterou a expressão de 212 moleculas, sendo 89 transcritos e 123 proteínas. Comparando as duas abordagens utilizadas neste trabalho, genes e proteínas relacionados a metabolismo e transporte de carboidratos e aminoácidos; transdução de sinal e sistema de secreção, foram detectados em ambas as análises. Para além destas funções, o transcriptoma também nos apresentou genes relacionados a motilidade e o proteoma mostrou a alteração no padrão de expressão de proteínas relacionadas ao transporte membranar, parede celular e modificações pós-transducionais. A modulação do metabolismo e transporte de carboidratos e aminoácidos pode estar relacionado à adaptação da estirpe a presença do líquido apoplástico, mostrando que a bactéria metabolizou esses nutrientes que se encontram presentes no fluido. O sistema de secreção do tipo 6 é o grande destaque deste trabalho, sendo identificado como induzido em ambas as abordagens, sugerindo que pode ter uma função de grande importância para as etapas iniciais do processo de interação.

Esses resultados mostram que o uso do líquido apoplástico como meio para simular as condições presentes neste nicho endofítico, permitiu avaliar o comportamento da estírpe HRC54 da espécie *H. seropedicae* na etapa inicial de sua adaptação ao habitat interno da planta, visto que os transcritos e proteínas aqui identificados possuem funções relacionadas as modificações necessárias para a mudança de "estilo de vida" da bactéria. Essas modificações sugerem que a estirpe possivelmente identificou o líquido apoplástico como parte do hospedeiro, o que levou à indução da expressão de várias proteínas com intuito de iniciar o processo de interação. Adicionalmente, em ambas as análises, foram identificados genes e proteínas paras os quais até o presente trabalho, não possuíam anotação funcional, não permitindo assim associá-las a um grupo específico. Deste modo, é sugerido que futuros trabalhos investiguem estes genes, pois ainda há funções desconhecidas que poderiam desempenhar um importante papel no processo de interação bactéria-planta.

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