Unraveling the communication between maize roots and *Herbaspirillum seropedicae*

Luiz Eduardo Souza da Silva Irineu

UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO

CAMPOS DOS GOYTACAZES – RJ
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Tese apresentada ao Centro de Biociências e Biotecnologia da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como parte das exigências para obtenção do título de Doutor em Biotecnologia Vegetal.

Orientador: Prof. Dr. Fabio Lopes Olivares

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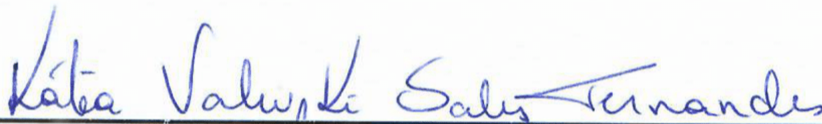

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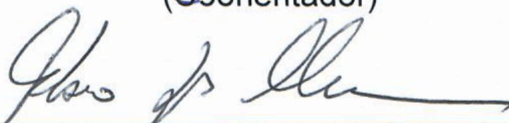

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“Levante-se e siga em Frente. Você é forte o suficiente para fazer seu próprio caminho.”

(Edward Elric)

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Resumo

O estabelecimento de *Herbaspirillum seropedicae* em raízes de plantas se inicia pela quimioatração por compostos exsudados, que atraem as bactérias até a raiz, onde ocorre a adesão apolar, seguida de recrutamento, proliferação e produção de biofilme. Após a colonização da superfície radicular, inicia-se a produção de auxinas, que induzem a formação de raízes laterais, que, ao romperem a epiderme da raiz, abrem portas para o estabelecimento endofítico. Apesar de muito bem elucidado esse mecanismo de infecção, os compostos dos exsudados responsáveis pela ativação dos sinais quimiotáticos em *H. seropedicae* ainda são indefinidos. Nesse sentido, essa tese apresenta um primeiro capítulo dedicado a revisar os conhecimentos obtidos sobre *H. seropedicae* e suas interações com plantas em 40 anos desde a identificação da espécie. Os outros dois capítulos têm como objetivos (i) entender como a inoculação de *H. seropedicae* induz mudanças no metabolismo das raízes do milho para se comunicar com a bactéria e como essas mudanças podem modular o bacterioma natural da planta; (ii) investigar a influência de ácidos orgânicos exsudados por plantas de milho sobre aspectos relacionados à colonização de *H. seropedicae* nas raízes, tais como quimioatração, biossíntese flagelar e formação de biofilme. Para isso, abordagens como a proteômica e análise da expressão gênica de raízes de milho inoculadas com *H. seropedicae* HRC54 foram empregadas. O fluxo de H^+ e o pH da superfície da raiz foram medidos e o bacterioma da raiz sequenciado. Para entender as respostas da bactéria quanto aos exsudados, realizou-se cinética de crescimento com ácidos orgânicos como única fonte de carbono, ensaio quimiotático, formação de biofilme e observação de flagelos, além de análise da expressão gênica. A inoculação de *H. seropedicae* alterou o ciclo do ácido tricarboxílico em nível transcricional e proteico, alterou o fluxo de H^+ e o pH na superfície da raiz. Essas mudanças modularam o bacterioma facilitando a interação com outros gêneros bacterianos como *Mesorhizobium*, *Novosphingobium*, *Rhizobium*, *Seratia* e *Stenotrophomonas*, comumente associados como promotores do crescimento vegetal. Todos os ácidos orgânicos testados induziram quimiotaxia, formação de flagelos e de biofilme, porém *H. seropedicae* usou somente os ácidos aconítico, cítrico, fumárico, málico e succínico como fontes de carbono. O perfil transcricional de HRC54 revelou que os compostos induziram genes receptores de quimiotaxia e biofilme e reprimiram os de formação de flagelo. Esse é o primeiro trabalho que analisa fenotipicamente o efeito quimiotático de exsudados de raízes de milho sobre *H. seropedicae*.

Palavras-chave: exsudados; quimiotaxia; ciclo do ácido tricarboxílico; interação bactéria-planta.

Abstract

The establishment of *Herbaspirillum seropedicae* in plant roots begins with chemoattraction by exudate compounds, which attract bacteria to the root, where nonpolar adhesion occurs, followed by recruitment, proliferation, and biofilm production. After the colonization of the root surface, the production of auxins begins, which induces the formation of lateral roots, which open doors for endophytic establishment by breaking the root epidermis. Although this infection mechanism has been well elucidated, the exudate compounds that activate chemotactic signals in *H. seropedicae* are still undefined. In this sense, this thesis presents a first chapter dedicated to reviewing the knowledge obtained about *H. seropedicae* and its interactions with plants in the 40 years since identifying the species. The other two chapters are aimed at (i) understanding how *H. seropedicae* inoculation induces changes in the metabolism of maize roots to communicate with the bacteria and how these changes can modulate the natural bacteriome of the plant; (ii) investigating the influence of organic acids exuded by maize plants on aspects related to the colonization of *H. seropedicae* in the roots, such as chemoattraction, flagellar biosynthesis and biofilm formation. For this, approaches such as proteomics and gene expression analysis of maize roots inoculated with *H. seropedicae* HRC54 were used. H^+ flux and root surface pH were measured, and the root bacteriome was sequenced. Growth kinetics were performed with organic acids as the only carbon source, chemotactic assay, biofilm formation and flagella observation, and gene expression analysis to understand the bacterial responses to the exudates. Inoculation of *H. seropedicae* altered the tricarboxylic acid cycle at transcriptional and protein levels and H^+ flux and pH on the root surface. These changes modulated the bacteriome, facilitating interaction with other bacterial genera such as *Mesorhizobium*, *Novosphingobium*, *Rhizobium*, *Serratia* and *Stenotrophomonas*, commonly associated with plant growth promoters. All organic acids tested induced chemotaxis, flagellar and biofilm formation; however, *H. seropedicae* used only aconitic, citric, fumaric, malic and succinic acids as carbon sources. Transcriptional profiling of HRC54 revealed that the compounds induced chemotaxis and biofilm receptor genes and repressed those for flagellum formation. This is the first work that phenotypically analyzes the chemotactic effect of maize root exudates on *H. seropedicae*.

Keywords: exudates; chemotaxis; tricarboxylic acid cycle; bacteria-plant interaction.

Introdução Geral

Herbaspirillum seropedicae é uma bactéria diazotrófica, endofítica, capaz de promover o crescimento vegetal por fornecer e modular a produção fitormônios, fixar nitrogênio biologicamente, solubilizar fosfato inorgânico e servir como controle biológico (MONTEIRO et al., 2012; MATTEOLI et al., 2020; IRINEU et al., 2023).

H. seropedicae se associa com espécies vegetais de grande importância econômica e alimentar como milho (*Zea mays*), arroz (*Oryza sativa*), cana-de-açucar (*Saccharum sp.*), trigo (*Triticum aestivum*), sorgo (*Sorghum bicolor*), abacaxi (*Ananas comosus*) e banana (*Musa sp.*) (BALDANI et al., 1986; OLIVARES et al., 1996; CRUZ et al., 2001; WEBER; FREIRE, 2003; BRASIL; BALDANI; BALDANI, 2005; SALA et al., 2005; BERGAMESHI et al., 2007).

O processo de colonização por *H. seropedicae* nas raízes de plantas se inicia pela atração da bactéria por exsudados liberados pelas raízes da planta hospedeira (quimiotaxia), levando-a a se movimentar em direção à rizosfera, seguido pela adesão apolar da bactéria à superfície das raízes e formação de complexas comunidades na forma de biofilmes. Subsequentemente, as bactérias aderidas secretam auxinas que induzem a atividade mitótica em células do periciclo e a formação de raízes laterais, que ao romper as camadas de células do córtex e epiderme, formam portas de entradas para a colonização endofítica da bactéria (OLIVARES et al., 1997; JAMES; OLIVARES, 1998).

O movimento quimiotático por influenciar o metabolismo e comportamento dos organismos direcionando a busca de nutrientes e a comunicação entre indivíduos (SINGH; KUMAR; AGRAWAL, 2014) e os componentes dos exsudados radiculares podem desempenhar importantes papéis como fonte de carbono e ou mediar sinais

para micro-organismos na rizosfera (SASSE; MARTINOIA; NORTHEN, 2018), podendo exercer atração quimiotática sobre grupos microbianos benéficos modulando o microbioma da rizosfera e a colonização da raiz (NARDI et al., 2000; BADRI; VIVANCO, 2009; ROLFE; GRIFFITHS; TON, 2019).

Raízes de milho podem exsudar ácidos orgânicos como os ácidos *cis* e *trans*-aconitico, benzóico, cítrico, isocítrico, fumárico, glutâmico, glioxílico, maleico, málico, malônico, oxálico, pirúvico, succínico e tartárico (KRAFFCZYK; TROLLDENIER; BERINGER, 1984; ERRO et al., 2009). A inoculação de *H. seropedicae* em milho induz alterações no padrão de exsudação das raízes das plantas (LIMA et al., 2014). Além disso, a inoculação foi capaz de alterar o metabolismo do ciclo dos ácidos tricarboxílico em folhas e raízes de plantas de milho (CANELLAS; OLIVARES; CANELLAS, 2019; IRINEU et al., 2023), sendo parte desses compostos os mesmos ácidos orgânicos definidos por Kraffczyk; Trolldenier; Beringer, (1984) e Erro et al. (2009).

Apesar de todo conhecimento sobre as propriedades de promoção do crescimento vegetal e da interação de *H. seropedicae* com plantas, ainda não existe nenhum estudo que descreva quais compostos exsudados exercem capacidade quimiotática sobre *H. seropedicae*. Entender quais compostos possuem tal atividade podem contribuir com melhoria da competência rizosférica e colonização de *H. seropedicae* em plantas inoculadas.

Esta tese investiga a interação de H. seropedicae e compostos dos exsudados radiculares e seus principais objetivos foram: (i) discutir os conhecimentos obtidos sobre *H. seropedicae* e suas interações com plantas em 40 anos desde a identificação da espécie; (ii) identificar alterações físico-químicas na rizosfera de plantas inoculadas com *H. seropedicae*; e (iii) avaliar os efeitos de ácidos orgânicos sobre a quimiotaxia

e aspectos da colonização de *H. seropedicae*. A tese está organizada em três capítulos idealizados como artigos independentes. Cada capítulo contém, introdução e discussão, além de detalhes metodológicos utilizados em cada um. Ao final são apresentadas as principais conclusões do trabalho.

REFERÊNCIAS

BADRI, D. V.; VIVANCO, J. M. Regulation and function of root exudates. **Plant, Cell and Environment**, v. 32, n. 6, p. 666–681, 2009.

BALDANI, J. I. et al. Characterization of *Herbaspirillum seropedicae* gen. nov. sp. nov. a Root- Associated Nitrogen-Fixing Bacterium. **INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY**, v. 36, n. 1, p. 86–93, 1986.

BERGAMESHI, C. et al. Occurrence of diazotrophic bacteria associated with forage sorghum cultivars Occurrence of diazotrophic bacteria associated with forage sorghum cultivars. **Ciência Rural**, v. 37, n. 3, p. 727–733, 2007.

BRASIL, M. DA S.; BALDANI, J.; BALDANI, V. L. D. Ocorrência e diversidade de bactérias diazotróficas associadas a gramíneas Forrageiras do pantanal sul Matogrossense. **Revista Brasileira de Ciencia do Solo**, v. 29, n. 2, p. 179–190, 2005.

CANELLAS, N. O. A.; OLIVARES, F. L.; CANELLAS, L. P. Metabolite fingerprints of maize and sugarcane seedlings: searching for markers after inoculation with plant growth - promoting bacteria in humic acids. **Chemical and Biological Technologies in Agriculture**, v. 6, n. 14, p. 1–10, 2019.

CRUZ, L. M. et al. 16S Ribosomal DNA Characterization of Nitrogen-Fixing Bacteria Isolated from Banana (. **APPLIED AND ENVIRONMENTAL MICROBIOLOGY**, v. 67, n. 5, p. 2375–2379, 2001.

ERRO, J. et al. Determination of organic acids in tissues and exudates of maize, lupin, and chickpea by high-performance liquid chromatography-tandem mass spectrometry. **Journal of Agricultural and Food Chemistry**, v. 57, n. 10, p. 4004–4010, 27 maio 2009.

IRINEU, L. E. S. DA S. et al. Multiomic Approaches Reveal Hormonal Modulation and Nitrogen Uptake and Assimilation in the Initial Growth of Maize Inoculated with *Herbaspirillum seropedicae*. **Plants**, v. 12, n. 1, 1 jan. 2023.

JAMES, E. K.; OLIVARES, F. L. Infection and Colonization of Sugar Cane and Other Graminaceous Plants by Endophytic Diazotrophs. **Critical Reviews in Plant Sciences**, v. 17, n. 1, p. 77--119, 1998.

KRAFFCZYK, I.; TROLLDENIER, G.; BERINGER, H. Soluble root exudates of maize: Influence of potassium supply and rhizosphere microorganisms. **Soil Biology and Biochemistry**, v. 16, n. 4, p. 315–322, 1984.

LIMA, L. DA S. et al. Root exudate profiling of maize seedlings inoculated with *Herbaspirillum seropedicae* and humic acids. **Chemical and Biological Technologies in Agriculture**, v. 1, n. 1, p. 1–18, 2014.

MATTEOLI, F. P. et al. *Herbaspirillum*. Em: AMARESAN, N. et al. (Eds.). **Beneficial Microbes in Agro-Ecology: Bacteria and Fungi**. 1. ed., Elsevier, 2020. p. 493–508.

MONTEIRO, R. A. et al. *Herbaspirillum*-plant interactions: Microscopical, histological and molecular aspects. **Plant and Soil**, v. 356, n. 1–2, p. 175–196, 2012.

NARDI, S. et al. Soil organic matter mobilization by root exudates. **Chemosphere**, v. 41, n. 5, p. 653–658, 2000.

OLIVARES, F. L. et al. Occurrence of the endophytic diazotrophs *Herbaspirillum spp.* in roots, stems, and leaves, predominantly of Gramineae. **Biology and Fertility of Soils**, v. 21, n. 3, p. 197–200, 1996.

OLIVARES, F. L. et al. Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirillum*. **New Phytologist**, v. 135, n. 4, p. 723–737, 1997.

ROLFE, S. A.; GRIFFITHS, J.; TON, J. Crying out for help with root exudates: adaptive mechanisms by which stressed plants assemble health-promoting soil microbiomes. **Current Opinion in Microbiology**, v. 49, p. 73-82, 2019.

SALA, V. M. R. et al. Ocorrência e efeito de bactérias diazotróficas em genótipos de trigo. **Revista Brasileira de Ciencia do Solo**, v. 29, n. 3, p. 345–352, 2005.

SASSE, J.; MARTINOIA, E.; NORTHEN, T. Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? **Trends in Plant Science**, v. 23, n. 1, p. 25–41, 2018.

SINGH, P.; KUMAR, V.; AGRAWAL, S. Evaluation of phytase producing bacteria for their plant growth promoting activities. **International Journal of Microbiology**, v. 2014, p. 7, 2014.

WEBER, O. B.; FREIRE, F. DAS C. O. Contribuição de bactérias diazotróficas na cultura da bananeira: perspectivas de utilização na produção integrada. **Boletim de Pesquisa e Desenvolvimento**, v. 16, p. 29p, 2003.

Chapter 1: Forty years of *Herbaspirillum seropedicae*, a review of agronomic discoveries and potential applications

Forty years of *Herbaspirillum seropedicae*, a review of agronomic discoveries and potential applications

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Abstract

Herbaspirillum seropedicae is the first described specie of the *Herbaspirillum* genus and has been identified in association with maize, sorghum, and sugarcane roots. Since then, several studies have characterized attributes that make it a plant-growth-promoting bacterium (PGPB), with its proven effect on crops of economic importance, such as maize, rice, forage, sugarcane, and wheat. This review highlights the physiological traits of *H. seropedicae* as PGPB, such as the production of phytohormones (e.g., auxin and gibberellin), nitrogen fixation, and phosphate solubilization. Furthermore, it will cover the ecological and structural interactions with plant roots and their colonization profile, including molecular cross-interactions. Moreover, will be revised research discoveries, biotechnological use of this specie as bioinoculants and its application in agroecosystems and other related products and patents in nearly 40 years.

Keywords: *Bioinoculant; biological nitrogen fixation; biostimulant; biofertilizer.*

2. Introduction

Herbaspirillum seropedicae was at the first time isolated from rhizosphere soil and roots of maize (*Zea mays*), sorghum (*Sorghum bicolor*), and rice (*Oryza sativa*) by Baldani et al. (1984) during an attempt to isolate the plant growth-promotion bacterium (PGPB) *Azospirillum amazonense* (reclassified as *Nitrospirillum amazonense*; LIN et al., 2014). The authors found different aspects from the typical colonies of *Azospirillum* that were smaller and with a greenish center when cultivated in a solid NFB medium, but when transferred to a semi-solid NFB medium, presented a typical *Azospirillum* pellicle demonstrating nitrogenase activity. This allowed the authors to presume this group as a new species, first named *Azospirillum seropedicae* (BALDANI et al., 1984).

Comparison analysis of DNA/RNA hybridization with *Azospirillum* species showed that the bacterium represented a new species (FALK et al., 1986), being designated as the first species of the genus *Herbaspirillum* (BALDANI et al., 1986).

Herbaspirillum seropedicae was described as an associative bacterium of seedy herbaceous plants (herb) that is small spiral-shaped (spirillum) and were characterized as Gram-negative cells, usually with vibrioid or sometimes helical cells; cells have 1-3 polar flagella with one or more flagella in both poles (figure 1); typical respiratory metabolism and unable to ferment sugars; able to fix atmospheric nitrogen under microaerobic conditions of developing well with N_2 as a sole nitrogen source; oxidase, catalase and urease positive; the organic acids such as malate, fumarate, succinate, pyruvate, citrate and trans-aconite are the preferred carbon sources for dependent growth of NH_4^+ and N_2; able to oxidizes sugars such as mannitol, sorbitol, glycerol, glucose, galactose and L-arabinose; does not hydrolyze starch and gelatin; the optimal pH range N_2-dependent growth is wider than the pH range for *Azospirillum* spp. When

limiting O2 conditions, it assimilates and disassembles nitrate, converting it to NO2(BALDANI et al., 1986).

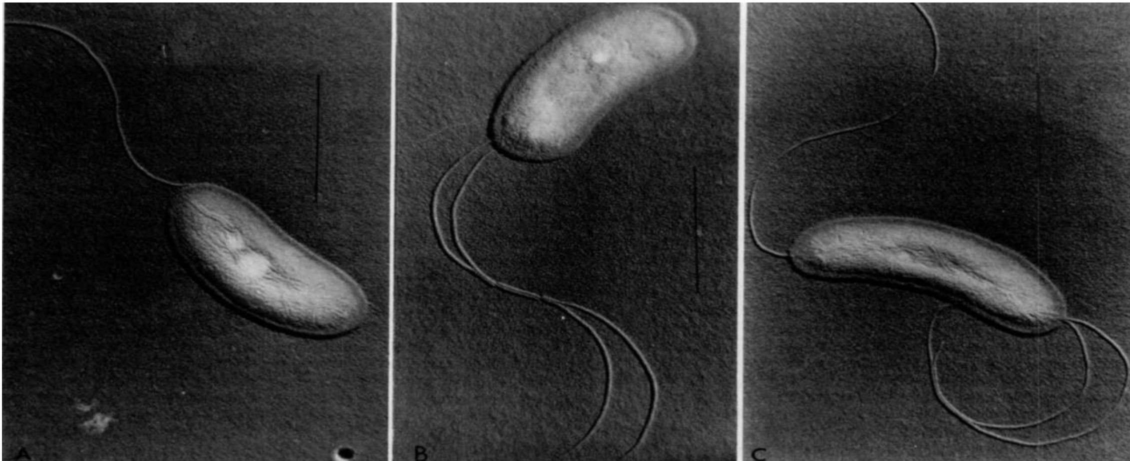

Figure 1: Transmission electron microscopy of *H. seropedicae* cells grown in nutrient agar. (A, B, and C) one, two, and three polar flagella on one or both poles, respectively. Bar = 1µm.Original photo by Baldani et al., (1986).

Herbaspirillum was classified as belonging to the phylum Proteobacteria, class Betaproteobacteria, order Burkholderiales, and family Oxalobacteraceae. The *Herbaspirillum* genus comprises 13 species (Table 1). Since the discovery, other *Herbaspirillum* species have been found in different environments, such as plant tissues, water bodies, soil, and sediments. T *Herbaspirillum* genus comprises 13 species (BALDANI et al., 2014; MATTEOLI et al., 2020; MONTEIRO et al., 2012).

Table 1: Representatives of the 13 species of genus *Herbaspirillum*, the sources of isolation and the country where it was first isolated.

Species	Country	Source	Reference
H. seropedicae	Brazil	Cereal roots	(BALDANI et al., 1986)
H. rubrisubalbicans	USA, Mauritius, others	*Saccharum officinarum*	(BALDANI et al., 1996)
H. frisingense	Germany and Brazil	C4-grasses	(KIRCHHOF et al., 2001)
H. lusitanum	Brazil	*Phaseolus vulgaris* root nodules	(VALVERDE et al., 2003)
H. autotrophicum	Switzerland	Eutrophic lake water	(DING; YOKOTA, 2004)

H. chlorophenolicum	South Korea	Soil	(IM et al., 2004)
H. hiltneri	Germany	Wheat root	(ROTHBALLER et al., 2006)
H. rhizosphaerae	South Korea	Rhizosphere soil	(JUNG et al., 2007)
H. huttiense subsp. huttiense	New Zealand	Distilled water	(DOBRITSA; REDDY; SAMADPOUR, 2010)
H. huttiense subsp. putei	Japan	Well water	(DOBRITSA; REDDY; SAMADPOUR, 2010)
H. aquaticum	USA	Water	(DOBRITSA; REDDY; SAMADPOUR, 2010)
H. robiniae	China	*Robinia pseudoacacia* root nodule	(FAN et al., 2018)
H. piri	China	Pear tree	(XU et al., 2018)

In addition to the biological nitrogen fixation (BNF) by nitrogenase activity, *H. seropedicae* can also produce plant growth regulators such as auxins and gibberellins (BASTIÁN et al., 1998; BOTTINI; CASSÁN; PICCOLI, 2004; LAMBRECHT et al., 2000). *H. seropedicae* presented auxin production six times higher when the culture medium was supplemented with tryptophan, a precursor of auxin synthesis (RADWAN; KAMEL; MASSENA, 2004).

Tests performed by Bergamaschi (2006), and Keyeo et al. (2011), verified the production of auxins by *H. seropedicae* and other diazotrophic bacteria, been several strains of *H. seropedicae* efficient in the production of this phytohormone when compared to the other microorganisms tested.

Also, inorganic phosphorus (Pi) solubilization was reported on *Herbaspirillum* strains with such ability, promoting increased rice production in the presence of phosphate tricalcium (ESTRADA et al. 2013). Figure 2 comprises the plant-promoted growth traits related above.

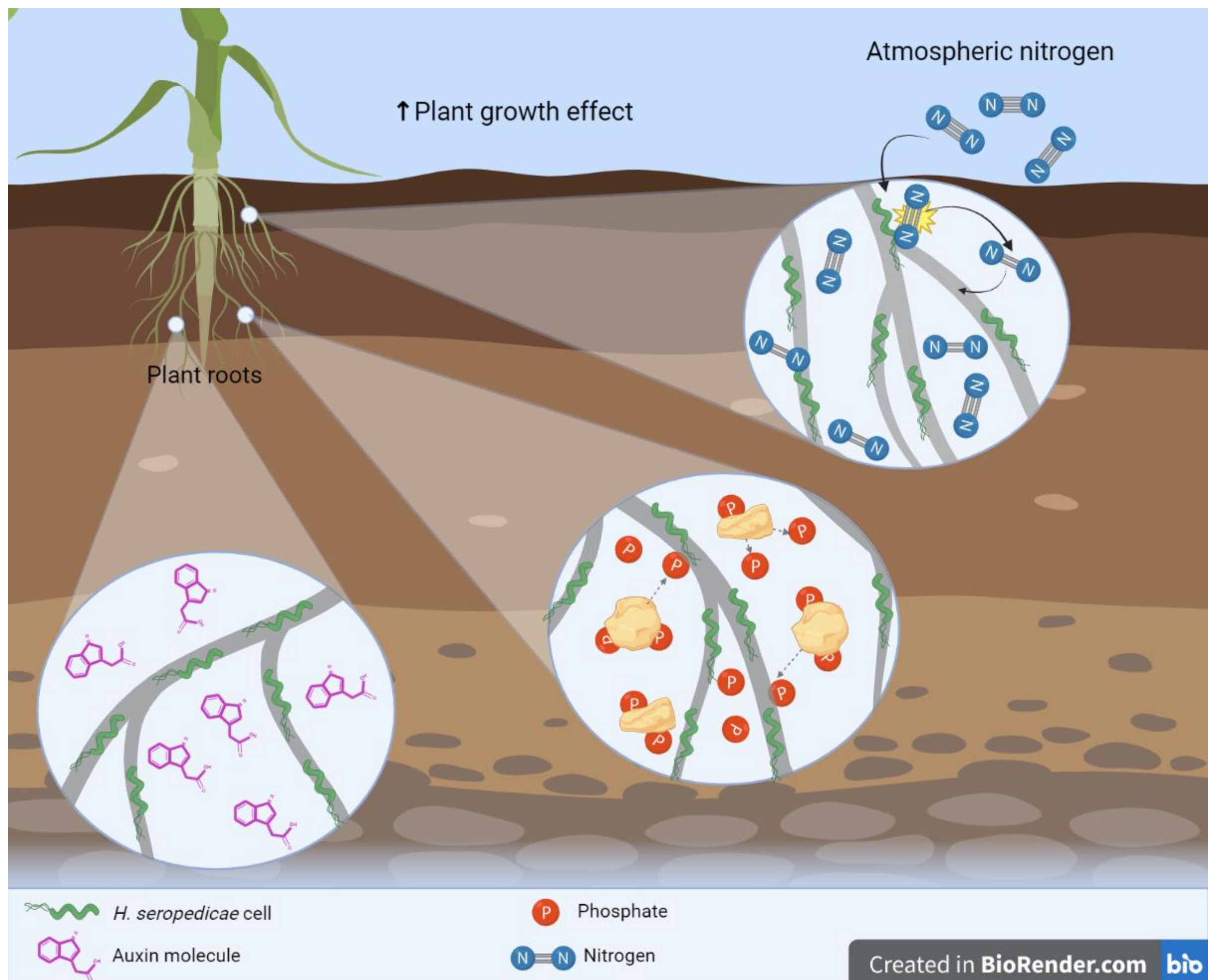

Figure 2: Schematic representation of the plant promotes growth traits. *H. seropedicae* can promote plant growth through auxin production, solubilization of inorganic phosphate and fixation of atmospheric nitrogen, making available these nutrients to plants. However, *H. seropedicae* and molecules are not to scale.

Later, the ecological survey of *H. seropedicae* was deeply investigated and detected in association with different plant species in different regions of Brazil, mainly members of the Poaceae family and different organs of the vegetative axis such as roots, stems and leaves in sugarcane and rice (OLIVARES et al., 1996; RODRIGUES et al., 2006), grassroots forage and wheat (BRASIL; BALDANI; BALDANI, 2005; SALA et al., 2005), and sorghum roots (BERGAMESHI et al., 2007). In addition, some isolates were associated with dicotyledonous plants such as pineapple and banana cultivated in Brazil (CRUZ et al., 2001; WEBER; FREIRE, 2003). ZHU et al. (2012) also detected *H. seropedicae* colonizing rice grown in China.

Döbereiner (1992) demonstrated that the diazotrophic species could colonize plant host tissues endophytically. This characteristic came to be proven by Olivares et al. (1997) and James; Olivares (1998), who showed the endophytic colonization of the xylem root vessels of sugarcane by strains of *H. seropedicae* and *H. rubrisubalbicans*. In the same way, has been shown the colonization of the intercellular spaces of roots and stems of two micropropagated varieties of Japanese sugarcane by (NJOLOMA et al., 2006) and the intercellular spaces of rice roots (JAMES et al., 2002).

Metagenomic studies of the diazotrophic bacterial community associated with the rhizosphere, roots and stem of maize cultivated in southern Brazil showed the presence of *Herbaspirillum* colonizing the interior of plant tissues but not in soil samples (ROESCH et al., 2008), which indicated that *H. seropedicae* could not survive on the soil without plants. Furthermore, studies conducted by Olivares et al. (1996) showed a rapid decline of bacteria introduced into the soil, suggesting it disappeared after four weeks. Interestingly, with the introduction of maize seeds in the soil, population levels of the bacteria were re-established, pointing to the importance of the presence of the host plant and possibly of root exudates on bacterial activation (OLIVARES et al., 1996).

Molecular approaches were pivotal to building solid scientific knowledge. For example, Chaves et al. (2007) constructed a reference map of *H. seropedicae* proteins using two-dimensional proteomics to contribute to the *H. seropedicae* genome annotation process and serve as a basis for comparative proteomic studies.

Pedrosa et al. (2011) completely sequenced the genome of the *Herbaspirillum seropedicae* strain SmR1, which consists of a circular chromosome of 5,513,887 base pairs with 4,804 genes, which encode 3,108 proteins with known functions, 497 with a general prediction of their functions and 1,130 with unknown functions. *H. seropedicae*

has genes that encode multiple secretion systems, including type I, II, III, V, VI and type IV pili. In addition, it also has genes for four metabolic pathways for indole acetic acid biosynthesis, for hemagglutinins, hemolysins and adhesins and a gene coding for 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Such phenological traits allowed a better understanding of its physiology through new studies on the basis's molecular aspects of its interaction with plants.

Studies using strains of genetically modified *H. seropedicae* contributed to understanding and unraveling the function of some genes and their interaction with plants (Table 2). For example, Monteiro et al. (2008) constructed an *H. seropedicae* mutant capable of expressing the ds-RED protein and thus constitutively emitting red fluorescence, which contributed to the understanding of how the *Herbaspirillum*-plant interaction occurs, as described by Olivares et al. (1997) and James & Olivares (1998).

H. seropedicae strains defective in the production of lipopolysaccharides (LPS) and exopolysaccharides (EPS) (BALSANELLI, 2013) helped to elucidate how these molecules are essential for anchoring to root lectins and serving as a matrix for biofilm formation. Balsanelli et al. (2016), using the Hsero_3720ΩnptI mutant strain, defective for a gene that encodes a Methyl-accepting Chemotaxis Protein (MCP), reported that these receptors are essential for mediating signals in the rhizosphere, allowing colonization.

Table 2: Genetically modified strains of *H. seropedicae.*

Strain	Features	Reference
***H. seropedicae* SmR1**	Strain spontaneous variant Z78 SM^r, *nif*$^+$, isolated from sorghum roots	(PEDROSA et al., 1997)
***H. seropedicae* RAM4**	*H. seropedicae* SmR1 expressing Ds-RED, SMr, KM^r.	(MONTEIRO et al., 2008)
***H. seropedicae* MHS1**	*H. seropedicae* SmR1 *epsG*::lacZ, Sm^r, Km^r, Cm^r	(TADRA-SFEIR et al., 2011)
***H. seropedicae* LPSEB**	*H. seropedicae* SmR1 *waaL*$^-$, Sm^r, Km^r	(BALSANELLI, 2013)

***H. seropedicae* EPSB**	*H. seropedicae* SmR1 *epsB*⁻, Sm^r, Km^r	(BALSANELLI, 2013)
***H. seropedicae* ΔphbC1**	*H. seropedicae* SmR1 *ΔphbC1*⁻, Sm^r	(TIRAPELLE et al., 2013)
***H. seropedicae* IM40**	*H. seropedicae* SmR1 containing chromosomal fusion *nifH*::lacZ, Sm^r, Km^r	(MACHADO et al., 1996)
***H. seropedicae* pEMS120**	*H. seropedicae* SmR1 containing plasmid fusion *nifA*::lacZ, Sm^r, Tc^r	(SOUZA et al., 2000)
***H. seropedicae* pEMS140**	*H. seropedicae* SmR1 containing plasmid fusion nifB::lacZ, Sm^r, Tc^r	(REGO et al., 2006)
***H. seropedicae* RAMEBB**	*H. seropedicae* SmR1 rfbB$^-$ mutant, Sm^r, Tc^r	(BALSANELLI et al., 2010)
***H. seropedicae* RAMEBC**	*H. seropedicae* rfbC$^-$ mutant, Sm^r, Tc^r	(BALSANELLI et al., 2010)
***H. seropedicae* SmR1+pHC60**	*H. seropedicae* SmR1 constitutively expressing GFP from pHC60, Sm^r, Tc^r	(BALSANELLI et al., 2014)
***H. seropedicae* EPSB+pHC60**	*H. seropedicae epsB* constitutively expressing GFP de pHC60, Smr, Km^r, Tc^r	(BALSANELLI et al., 2014)
Hsero_4782ΩnptI	*H. seropedicae* putative ABC-Type multi-drug transporter$^-$	(BALSANELLI et al., 2016)
Hsero_3720ΩnptI	*H. seropedicae* methyl-accepting chemotaxis protein$^-$	(BALSANELLI et al., 2016)
Hsero_fliCΩnptI	*H. seropedicae* flagenina$^-$	BALSANELLI et al., 2016

Sm = streptomycin; Km = kanamycin; Cm = chloramphenicol; Tc = tetracycline; superscript r = resistant, and - superscript = defective.

About to celebrate 40 years since its discovery, *H. seropedicae* has accumulated several publications about it (Fig. 3), revealing its potential to promote plant growth in different cultures. This review will cover highlights of discoveries about this bacterium and its functionalities, interaction with plants and the potential for biotechnological products.

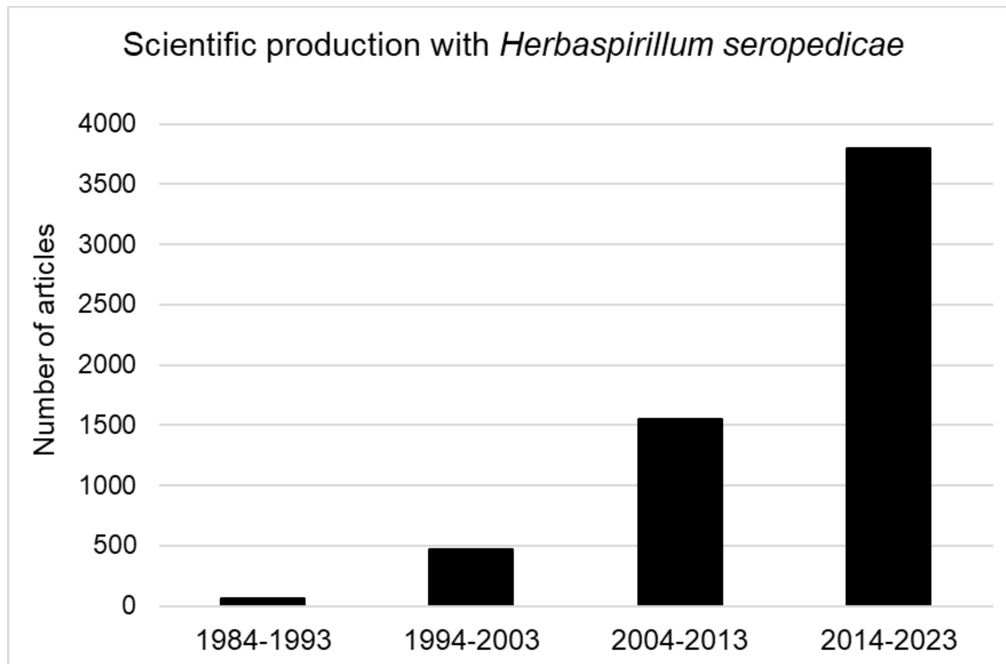

Figure 3: Increase in decades of publication about *H. seropedicae* since the first isolation. Source: Academic google.

3. Interaction between *Herbaspirillum seropedicae* and plants

2.1. Molecular aspects of *Herbaspirillum-plant* interactions

2.1.1. Chemotaxis

The first step towards the initiation of rhizospheric colonization by *H. seropedicae* is chemotaxis, where the different chemical compounds exuded by the root of the plant can serve as a chemical attractant for microorganisms present in that environment (MONTEIRO et al., 2012). These compounds bind to proteins that serve as membrane chemoreceptors known as “Methyl-accepting Chemotaxis Proteins” (MCPs) (GREBE; STOCK, 1998). *H. seropedicae* has 66 genes encoding *mcp*s and 46 genes involving biosynthesis, assembly, and flagella structure (PEDROSA et al., 2011).

Maize roots inoculated with *H. seropedicae* showed changes in the exudation profile, and several exuded compounds were identified in the presence of bacteria,

indicating a communication that mediated the interaction between organisms (LIMA et al., 2014).

Using the RNA sequencing approach (RNA-seq), PANKIEVICZ et al. (2016) analyzed the transcriptome of *H. seropedicae* colonizing wheat roots, revealing that the genes *Hsero_2914*, *Hsero_2915*, *Hsero_2723*, which encode MCPs were highly induced when bacterial cells colonized the wheat roots epiphytically. Similar results were found by Balsanelli et al. (2016), where genes encoding MCPs (*Hsero_2723* and *Hsero_3720*) were up-regulated in the presence of maize root. Furthermore, Balsanelli et al. (2016) analyzed a mutant of *H. seropedicae* strain SmR1 for the gene *Hsero_3720*, showing that these bacteria decreased the ability to adhesion of the bacteria to the roots, suggesting that the expression of this gene is necessary for the perception of signals from the rhizospheric environment and adhesion of *H. seropedicae* to the root surface.

Tadra-Sfeir et al. (2015) also demonstrated that genes related to MCPs and flagella were upregulated in the presence of the flavonoid naringenin, a potential chemoattractant related to the endophytic interaction.

2.1.2. *Defense*

The expression pattern of the fde operon ("flavonoid degradation") in *H. seropedicae* strain SmR1 was evaluated through assays of β-galactosidase activity. The results showed that flavonoid naringenin increased β-galactosidase activity compared to the control condition. Furthermore, this strain was able to degrade the flavonoid naringenin up to undetectable levels after 9 hours of growing. However, a mutant strain in the *fdeA* gene could not degrade the flavonoid, indicating that the operon is responsible for the degradation of flavonoids in *H. seropedicae* (MARIN et al., 2013).

AMARAL et al. (2014) analyzed the gene chalcone synthase (Zmchs) expression in maize roots inoculated with *H. seropedicae* SmR1 after 1, 4, 7 and 10 days of inoculation. This gene synthesizes defense-related flavonoids in plants, and they visualized its repression with a significant change only on the seventh day.

H. seropedicae can control the production of peptidoglycan in response to signals from the host plant (TADRA-SFEIR et al., 2015). Peptidoglycans can be recognized as an elicitor of innate immunity of the plant, known as a "microbe-associated molecular pattern" (MAMP) (ERBS; NEWMAN, 2012). The *mur* genes, responsible for peptidoglycan biosynthesis, were less expressed in *H. seropedicae* in initiating epiphytic colonization when compared to *H. seropedicae* under planktonic conditions. In addition, murein lytic transglycosylase genes (Hsero_3419 and Hsero_3582) were six times more expressed when epiphytically colonizing maize roots, which suggests loss of layers of peptidoglycan during this condition (BALSANELLI et al. 2016). In the presence of the flavonoid naringenin, genes encoding Mur ligase enzymes responsible for assembling the peptidoglycan (*murC*, *murD*, *murE* and *murF*) were repressed.

The gene expression profile in rice roots inoculated with *H. seropedicae* showed a decrease in transcripts related to defense, probenazole-inducible protein (PBZ1), and thionins, suggesting that bacterial inoculation can modulate the plant defense to allow the establishment of efficient cooperation between host bacteria (Brusamarello-Santos et al., 2012).

2.1.3. Adhesion

Balsanelli et al. (2010) verify that lipopolysaccharides (LPS) of *H. seropedicae* participate in bacterial adhesion to maize roots, possibly anchoring the N-acetyl glucosamine moieties to host plant receptors. The adhesion of *H. seropedicae* strain

SmR1 to the surface of maize roots was shown to be mediated by the binding of LPS to root lectins. This mechanism occurs as also in wheat and rice, and changes in the structure of LPS reduce bacterial adhesion capacity by 90% (BALSANELLI et al., 2013). Furthermore, Pankievicz et al. (2016) demonstrated that the genes that encode the filamentous hemagglutinin (FHA) (*Hsero_1294* and *Hsero_3251*) responsible for cell adhesion were expressed 2.8 and 2.3 times more, respectively when adhered to wheat roots compared to cells of bacteria under planktonic conditions.

A mutation in the *epsB* gene, responsible for the biosynthesis of exopolysaccharides (EPS) and biofilm formation, decreased EPS production and, consequently, biofilm production. However, *H. seropedicae* mutants could colonize maize roots similarly to the wild strain, suggesting that epiphytic and endophytic colonization does not depend on biofilm formation (BALSANELLI et al. 2014). Furthermore, coding genes were not found when analyzing the transcripts of free-living *H. seropedicae* and adhered to wheat roots, corroborating the data found by Balsanelli et al. (2014) (PANKIEVICZ et al. 2016).

Genes involved in flagella biosynthesis had their expression levels repressed in *H. seropedicae* living epiphytically, suggesting a free lifestyle change to a sessile state, where *H. seropedicae* can utilize twitching motility through the use of type IV pili since the expression of a gene encoding for spasmodic motility (*pilI)* increased its expression in adhered bacterial cells (PANKIEVICZ et al., 2016). Moreover, Balsanelli et al. (2016) detected high levels of expression of pilOPQRSM genes in bacterial cells before adhesion to the source.

2.1.4. Nitrogen metabolism

Baldani et al. (1986) described that *H. seropedicae* could grow aerobically using nitrate as a nitrogen source. Nevertheless, this capacity is lost under anaerobic

conditions, also losing the capacity for denitrification. Bonato et al. (2016) studied the importance of genes encoding the respiratory nitrate reductase (NAR) in *H. seropedicae*. They found that the enzyme is not necessary to assimilate nitrate but is involved in producing nitric oxide, an essential molecule during cell signaling bacteria with the host plant.

Pankievicz et al. (2016) demonstrated that the operon containing *nif* genes was expressed during association with host plant roots, strongly indicating nitrogen fixation activity, being more expressed in epiphytically adherent bacterial cells than free-living bacterial cells. Furthermore, during colonization, the nitrogen regulation system (NTR) was induced, activating genes related to the metabolism of nitrogen, which includes nitrate, nitrite, ammonia, transporters of urea and the transcriptional activator *nifA*, thereby activating the *nif* genes (BALSANELLI et al. 2016).

These authors also verified that cells of free-living bacteria had an increase in the expression of *nif* genes three days after inoculation compared to those epiphytically attached bacterial cells that had a decrease in the expression of these genes, perhaps as a result of nitrogen sources provided by the host plant. Lima et al. (2014) identified an increase in the content of nitrogenous compounds exuded by maize roots inoculated with *H. seropedicae*.

Bonato et al. (2016) identified that the inoculation of an *H. seropedicae* mutant in the catalytic subunit of the *narG* (nitrate reductase) gene resulted in lower accumulation rates of the dry mass of the root and shoot of wheat plants when compared with the inoculated wild strain.

KUANG et al. (2022) demonstrated thaH. seropedicae alters N-dependent root growth dynamics and nitrate and ammonium uptak*e* in the cereal model *Brachypodium distachyon*. Multiomic approaches suggested that growth promotion in the early stages

of maize inoculated with *H. seropedicae* arose from modulation of N uptake and assimilation induced by the inoculation (IRINEU et al. 2023).

2.1.5. Hormone metabolism

Herbaspirillum seropedicae has genes for four possible production routes of indole acetic acid (IAA), also known as the phytohormone auxin (PEDROSA et al. 2011). Genes involved in IAA biosynthesis (*Hsero_4278* and *Hsero_1422*) were up-regulated in *H. seropedicae* when attached to wheat roots, which suggests that IAA production is stimulated by contact with the host plant (PANKIEVICZ et al., 2016). Furthermore, (BRUSAMARELLO-SANTOS et al., 2012a) analyzed the transcripts during roots of rice-*H. seropedicae* interaction and identified two auxin-responsive repressors (*IAA18-like* and *IAA11-like*) negatively regulated, which can be explained by the production of auxin by the microorganism.

On the other hand, the presence of IAA of bacterial origin can stimulate the presence of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, negatively affect ethylene production, and allow plant root growth under stressful abiotic conditions (PEDROSA et al., 2011). Therefore, Alberton et al. (2013) show that rice roots inoculated with *H. seropedicae* SmR1 had reduced levels of ethylene, and a proteomics approach demonstrated that *H. seropedicae* stimulates the recycling of methionine, the precursor of biosynthesis of ethylene.

The analyzes made by Brusamarello-Santos et al. (2012) investigated rice roots inoculated with *H. seropedicae* and demonstrated that the transcription factor involved in response to ethylene (ERF2-like) was repressed when in the presence of the microorganism. The genes “ent-copalyl diphosphate synthase” (*Zmcps1*), “ent-kaurene oxidase” (*Zmko1*) and gibberellin 20 oxidase 4 (*Zmga20ox4*), involved in

gibberellin biosynthesis in plants, were differentially expressed in maize plants inoculated with *H. seropedicae* SmR1.

2.1.6. General metabolism

In several organisms, Fnr proteins act as a regulator of genes required for metabolic change in response to O_2 levels. For example, in *H. seropedicae*, this protein regulates the configuration of chain electron carriers to explore respiratory flexibility and optimize the energy coupling in response to oxygen availability (BATISTA et al. 2013). Transcripts analyzes of *H. seropedicae* colonizing wheat roots revealed that the *Fnr1* (*Hsero_3197*) and *Fnr2* (*Hsero_2381*) genes had their levels of expression increased by 16.8 and 38.9 times, respectively (PANKIEVICZ et al. 2016).

According to Kadouri; Jurkevitch; Okon (2003), and Ratcliff & Denison (2010), the ability to produce and store polyhydroxybutyrate (PHB) positively influences survival and/or stress in competitive environments. According to Pankievicz et al. (2016), PHB genes were induced in adhered cells, such as *phaA2* (*Hsero_0239*), *phaB* (*Hsero_2998*), and *phaC1*. Balsanelli et al. (2016) also reported increased expression levels of *phb* genes (ABC) in adherent bacteria compared to free-living ones. The expression of genes involved in PHB metabolism in different stages suggests that PHB biosynthesis and degradation are required in the early stages of bacterial colonization on the host plant.

For inorganic phosphorus (Pi) solubilization, Estrada et al. (2013) were the first to report on *Herbaspirillum* strains with such ability, which was reflected by the promotion of an increase in rice production in the presence of tricalcium phosphate. Irineu et al. (2023) demonstrated that *H. seropedicae* inoculation increased the expression of phosphate transporters genes in maize roots in the early stages of development.

Investigations into the effect of phosphate concentrations show that *H. seropedicae* growing on high Pi conditions demonstrates better overall fitness, increasing Pi metabolism, bacterial flagella biosynthesis, chemotaxis, energy production processes, and PHB metabolism, which could be an essential feature to consider when the bacterium is used as plant inoculant (GRILLO-PUERTAS et al. 2021).

2.2. Applications and growth promotion in plants inoculated with H. seropedicae.

Beneficial bacteria offer advantages to the host plant, primarily in promoting plant growth (ALI; CHARLES; GLICK, 2012), known as *plant growth-promoting bacteria* (PGPB). Among the various mechanisms of plant growth promotion mediated by bacteria, we can mention biological nitrogen fixation, hormone production and phosphorus and iron acquisition (GLICK; PENROSE; LI, 1998; IRINEU et al. 2023). The understanding of the plant growth promotion mechanisms by *H. seropedicae* was leveraged in the last decade (2010 to 2020s).

Several studies demonstrated the association of *Herbaspirillum* bacteria with a wide variety of plants belonging to the Poaceae family, such as rice, maize, sorghum, and sugarcane (BALDANI et al., 1986; PIMENTEL et al., 1991; OLIVARES et al., 1996), as well as beans (*Phaseolus vulgaris*) nodules (VALVERDE et al., 2003) and soybean (*Glycine max*) surface-sterilized roots (KUKLINSKY-SOBRAL et al., 2005). Other studies also found an association with banana plants (*Musa* spp.) and pineapple plants (*Ananas comosus* (L.) Merril) plants (CRUZ et al., 2001; WEBER; FREIRE, 2003).

In addition to the biological nitrogen fixation, phytohormones production, ACC deaminase and siderophores production (MONTEIRO et al. 2012), *H.* seropedicae has been recognized as an efficient plant-colonizing bacterium, arising technological interest as a microbial inoculant in agroecosystems to improve agricultural productivity.

H. seropedicae plant-colonization initiates with attraction to root exudates (chemotaxis). The chemical signaling leads to the expression of the machinery involved in moving towards the rhizosphere compartment (Fig 4A), with an increase in the bacterial population in this region, followed by the apolar adhesion of the bacteria to the surface of the roots (Fig. 4B). The preferential sites of adhesion to the root axis are the elongation/differentiation and the root hairs zones. Bacterial cells adhered to the root surface form aggregates with different dimensions by cell division and recruitment of non-adherent bacteria, which can produce complex microbial communities as biofilms (Fig 4C). Subsequently, these adhered bacteria secrete bioactive compounds and other molecules that modulate the interaction (i.e., auxins and other bioactive compounds), which induce mitotic activity in pericycle cells and the formation of lateral roots. Infection occurs through the emergence points of the lateral roots that break through the layers of cortex and epidermis cells, which serve as a gateway for endophytic colonization (Fig 4D) (JAMES; OLIVARES, 1998; MONTEIRO et al., 2012; OLIVARES et al., 1997).

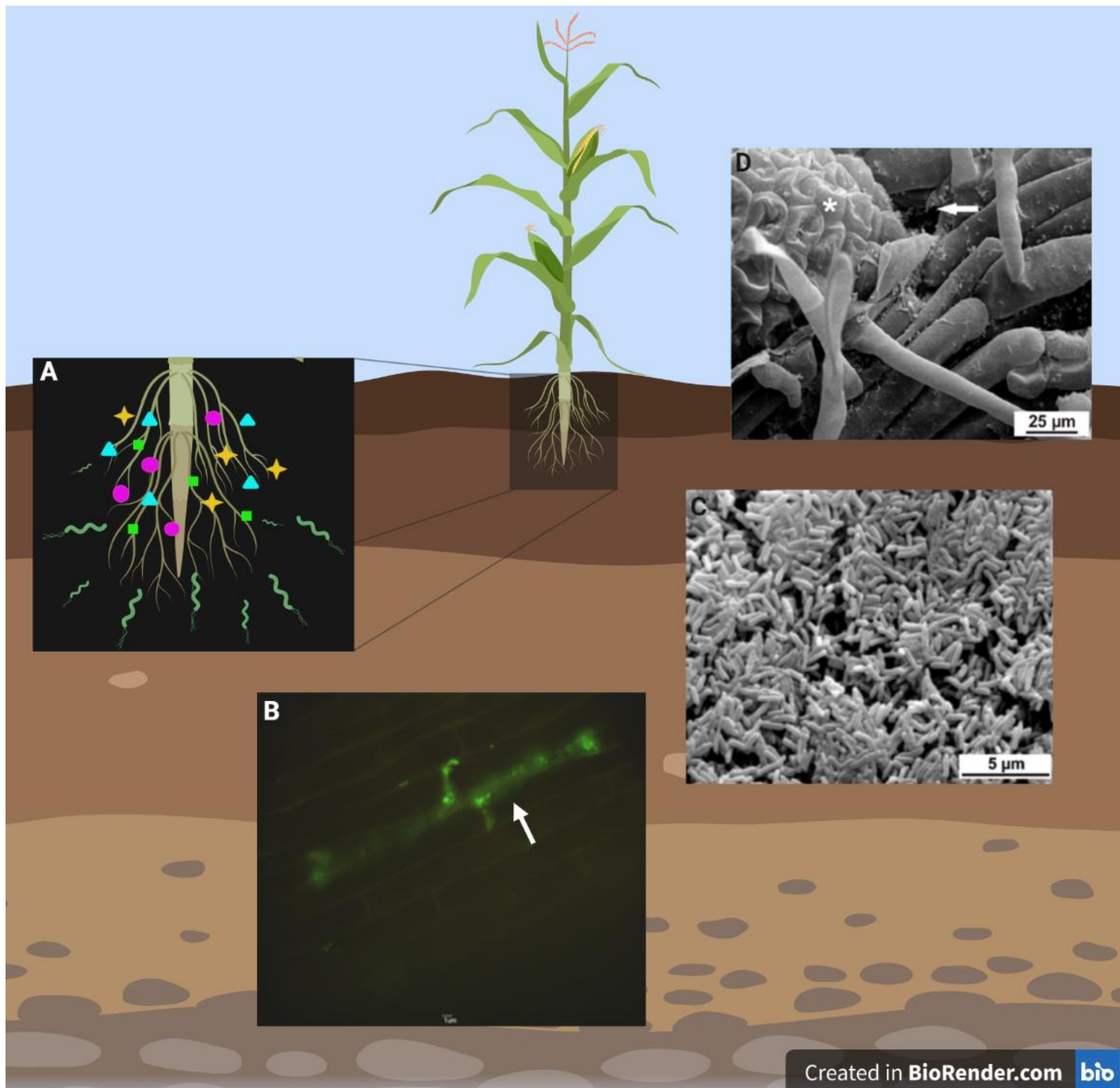

Figure 4: Schematic graphical demonstrating the colonization pathway of *H. seropedicae*. (**A**) Rhizosphere exudation attracting *H. seropedicae*. (**B**) Fluorescence microscopy showing epiphytic colonization of *H. seropedicae* RAM10 of the epidermal cell wall (arrow) of maize root after 7 days of inoculation (personal image). (**C**) Scanning electron microscopy shows bacterial cells form biofilm in pineapple plantlets' roots 21 days after inoculation (BALDOTTO; OLIVARES; BRESSAN-SMITH, 2011). (**D**) Scanning electron microscopy showing bacterial infection through the cavity (arrow) resulting from the rupture of epidermal cells during the emergence of lateral roots (asterisk) (BALDOTTO; OLIVARES; BRESSAN-SMITH, 2011).

The growing use of PGPBs stands against the excessive and harmful aspect of pesticides and fertilizers, especially in the face of the urgent demand to increase food production. Thus, sustainable and ecologically correct practices are necessary to guarantee an integrated agricultural and livestock production system in the long term (AJIJAH et al., 2023). In this context, *Herbaspirillum* has been studied in biofertilizer

formulations and providing increased agricultural productivity of different crops (Table 3).

Table 3: *H. seropedicae* use in different crops and its growth-promoting function.

Strain	Crop systems	Function in growth	References
***Herbaspirillum seropedicae* Z67**	Rice seedling cv. IR42 and IR72	Increase in N, and it also incorporated a significant amount of $15N_2$.	(JAMES et al., 2002)
***Herbaspirillum seropedicae* Z152**	*Oryza sativa* L. *cv "El Paso 144"*	Increase ethylene production and ACS activity	(CURZI et al., 2008)
Herbaspirillum seropedicae	*Oryza sativa* ssp. *japonica*	Alteration in the expression of genes involved in the synthesis of auxin and ethylene.	(BRUSAMARELLO-SANTOS et al., 2012a)
***Herbaspirillum seropedicae* SmR1**	*Triticum aestivum* L.	Increase in plant biomass in the early stages of culture and grain yield, the capacity of providing the source of nutrients to plants and pointed out to a responsive wheat cultivar.	(NEIVERTH et al., 2014)
***Herbaspirillum seropedicae* SmR1**	*Zea mays* L. cv. DKB 240	Increase in the expression of one gene involved in the gibberellin biosynthesis pathway (*Zmko1*) and one gene of NADPH oxidase (*ZmrbohC*)	(AMARAL et al., 2014)
***Herbaspirillum seropedicae* SmR2**	Maize seeds (cv. Pioneer 30F53)	Increase in leaf area.	(DALL'ASTA et al., 2019)
***Herbaspirillum seropedicae* SmR1**	*Setaria viridis* A10.1	Synthesis and accumulation of Poli-3-hydroxybutyrate (PHB)	(ALVES et al., 2019)
***Herbaspirillum seropedicae* HRC54**	*Oryza sativa*	The stimulus of root vacuolar H^+-pumps (vacuolar H^+-ATPase and vacuolar H^+-PPase) increased plant growth, nutrient contents and photosynthetic efficiency.	(RAMOS et al., 2020)
***Herbaspirillum seropedicae* Z67**	*Brachypodium distachyon*	Promoting uptake of N from the root.	(KUANG et al., 2022)
***Herbaspirillum seropedicae* SmR1**	Maize variety DKB 390	Increase of plant shoot length and nitrogen content of shoot.	(Cunha et al., 2022)

3. *Herbaspirillum seropedicae* products and patents

With the development of microbial bioinoculants technology, many products have been produced based on this technology, increasing innovations and the number of patents. *H. seropedicae* is part of a select group of microorganisms capable of

promoting plant growth and development, essential for bioinoculant microbial preparations.

However, the number of market products with this bacterium is still scarce. Therefore, bioinoculants were developed by Empresa Brasileira de Pesquisa Agriculture (EMBRAPA), one with *H. seropedicae* strain BR11417 recommended for the cultivation of maize (REIS et al. 2009), and the other one based on a bacterial consortium of the strains *Gluconacetobacter diazotrophicus* (BR11281), *H. seropedicae* (BR11335), *H. rubrisubalbicans* (BR11504), *Nitrospirillum amazonense* (BR11145) and *Paraburkholderia tropica* (BR11366) for sugarcane cultivation (REIS; BALDANI; URQUIAGA, 2009), both.

Combining *H. seropedicae* with other microorganisms represents a technological alternative for better inoculation results. This approach can be observed in the products and patents containing *H. seropedicae* (Table 4).

TRIPLETT; KAEPPLER; CHELIUS (2008) designed an inoculant containing bacteria capable of increasing plant growth. This formulation includes *H. seropedicae* 2A, *H. seropedicae* Z152, *Pantoea agglomerans* P101, *Pantoea agglomerans* P102, *Klebsiella pneumoniae* 342, *Klebsiella pneumoniae* Zmvsy, and *Gluconacetobacter diazotrophicus* PAL 5. Another combination of *H. seropedicae* strains is the invention of a kit and method for enhancing plant growth utilizing *H. seropedicae* combined with *Azospirillum lipoferum* or *Bacillus subtilis* (MERRITT, 2013)

H. seropedicae can be used in developing subproducts derived from its metabolism. Koppisch et al. (2013) patented compound production methods (3,4-dihydroxybenzoate, catechol, cis,cis-muconate, or 3-carboxy-cis,cis-muconic acid) utilizing biosynthetic pathways in prokaryotic organisms expressing one or more heterologous genes.

Table 4: The claims contain patents involving *H. seropedicae* or its metabolic subproducts.

Patent nº	Title	Applicants	Inventors	Publication date
US007393678B2	*Klebsiella pneumoniae* inoculants for enhancing plant growth	Wisconsin Alumni Research Foundation	(TRIPLETT; KAEPPLER; CHELIUS, 2008)	01/07/2008
WO2012/140177 A1	Method for cultivating sugar cane	BASF SE	(DEGASPARI et al., 2012)	18/10/2012
WO2013029112 A1	Microbial composition, method, and kit for enhancing plant growth	ThinkBio Pyt Ltd	(MERRITT, 2013)	03/07/2013
US 2013/0252294 A1	Production of industrially relevant compounds in prokaryotic organisms	The National University of Singapore, Singapore (SG); Los Alamos National Laboratory, LC/IP, Los Alamos, NM (US)	(KOPPISCH et al., 2013)	29/09/2013
EP 2 778 228 B1	Method for producing 2-ketoglutaric acid and derivatives thereof by using a bacterium from the genus *Pantoea* or the genus *Corynebacterium*	Ajinomoto Co., Inc. Tokyo	(NISHIO et al., 2017)	17/05/2017

4. Final considerations

The use of microbial inoculants with biofertilizer and biostimulant properties is an alternative to promote plant growth and protection and reduce the environmental impacts of industrial agriculture practices by replacing or complementing the complex synthetic fertilizers, that increase costs every year and contribute to contamination of soil, water bodies and negative impact by the atmospheric emission of nitrous oxide.

Forty years of research with *Herbaspirillum seropedicae* has proven its ability to promote plant growth and increase yields in some crops of great economic importance. However, no products on the market or even a few patents exploring these bacteria as a biotechnological source exist.

There are still challenges to be overcome so that biological-based agricultural inputs and technologies containing *H. seropedicae* reach more producers and contribute to reducing the use of chemical fertilizers.

REFERENCES

AJIJAH, N. et al. Plant Growth-Promoting Bacteria (PGPB) with Biofilm-Forming Ability: A Multifaceted Agent for Sustainable Agriculture. **Diversity**, v. 15, n. 1, p. 112, 2023.

ALBERTON, D. et al. Comparative proteomics analysis of the rice roots colonized by *Herbaspirillum seropedicae* strain SmR1 reveals induction of the methionine recycling in the plant host. **Journal of Proteome Research**, v. 12, n. 11, p. 4757–4768, 2013.

ALI, S.; CHARLES, T. C.; GLICK, B. R. Delay of flower senescence by bacterial endophytes expressing 1-aminocyclopropane-1-carboxylate deaminase. **Journal of Applied Microbiology**, v. 113, n. 5, p. 1139–1144, 2012.

ALVES, L. P. S. et al. Importance of poly- 3-hydroxybutyrate metabolism to the ability of *Herbaspirillum seropedicae* to promote plant growth. **Applied and Environmental Microbiology**, v. 85, n. 6, 2019.

AMARAL, F. P. et al. Gene expression analysis of maize seedlings (DKB240 variety) inoculated with plant growth-promoting bacterium *Herbaspirillum seropedicae*. **Symbiosis**, v. 62, n. 1, p. 41–50, 2014.

BALDANI, J. I. et al. A fourth *Azospirillum* species from cereal roots. **An. Acad. Bras. Cienc.**, v. 56, p. 365, 1984.

BALDANI, J. I. et al. Characterization of *Herbaspirillum seropedicae* gen. nov. sp. nov. a Root- Associated Nitrogen-Fixing Bacterium. **INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY**, v. 36, n. 1, p. 86–93, 1986.

BALDANI, J. I. et al. Emended Description of *Herbaspirillum*; Inclusion of [*Pseudomonas*] *rubrisubalbicans*, a Mild Plant Pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and Classification of a Group of Clinical Isolates (EF Group 1) as *Herbaspirillum* Species 3. **INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY**, v. 46, n. 3, p. 802–810, 1996.

BALDANI, J. I. et al. The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. **Plant and Soil**, v. 384, n. 1–2, p. 413–431, 2014.

BALDOTTO, L. E. B.; OLIVARES, F. L.; BRESSAN-SMITH, R. Structural interaction between gfp-labeled diazotrophic endophytic bacterium *Herbaspirillum seropedicae* ram10 and pineapple plantlets “vitória”. **Brazilian Journal of Microbiology**, v. 42, p. 114–125, 2011.

BALSANELLI, E. et al. *Herbaspirillum seropedicae rfbB* and *rfbC* genes are required for maize colonization. **Environmental Microbiology**, v. 12, n. 8, p. 2233–2244, 2010.

BALSANELLI, E. **Caracterização dos fatores moleculares envolvidos na interação de *Herbaspirillum seropedicae* com gramíneas.** Tese de Doutorado—Curitiba: Universidade Federal do Paraná, 13 dez. 2013.

BALSANELLI, E. et al. Exopolysaccharide biosynthesis enables mature biofilm formation on abiotic surfaces by *Herbaspirillum seropedicae*. **PLoS ONE**, v. 9, n. 10, 2014.

BALSANELLI, E. et al. Molecular adaptations of *Herbaspirillum seropedicae* during colonization of the maize rhizosphere. **Environmental microbiology**, v. 18, n. 8, p. 2343–2356, 2016.

BASTIÁN, F. et al. Production of indole-3-acetic acid and gibberellins A 1 and A 3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically-defined culture media. **Plant Growth Regulation**, v. 24, p. 7–11, 1998.

BATISTA, M. B. et al. The *Herbaspirillum seropedicae* SmR1 *Fnr* orthologs controls the cytochrome composition of the electron transport chain. **Scientific Reports**, v. 3, p. 1–11, 2013.

BERGAMASCHI, C. **Ocorrência De Bactérias Diazotróficas Associadas a Raízes E Colmos De Cultivares De Sorgo**. Dissertação de Mestrado—Porto Alegre: Universidade Federal do Rio Grande do Sul, fev. 2006.

BERGAMESHI, C. et al. Occurrence of diazotrophic bacteria associated with forage sorghum cultivars. **Ciência Rural**, v. 37, n. 3, p. 727–733, 2007.

BONATO, P. et al. RNA-seq analyses reveal insights into the function of respiratory nitrate reductase of the diazotroph *Herbaspirillum seropedicae*. **Environmental microbiology**, v. 18, n. 8, p. 2677–2688, 2016.

BOTTINI, R.; CASSÁN, F.; PICCOLI, P. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. **Applied Microbiology and Biotechnology**, v. 65, n. 5, p. 497–503, 2004.

BRASIL, M. DA S.; BALDANI, J.; BALDANI, V. L. D. Ocorrência e diversidade de bactérias diazotróficas associadas a gramíneas Forrageiras do pantanal sul Matogrossense. **Revista Brasileira de Ciencia do Solo**, v. 29, n. 2, p. 179–190, 2005.

BRUSAMARELLO-SANTOS, L. C. C. et al. Differential gene expression of rice roots inoculated with the diazotroph *Herbaspirillum seropedicae*. **Plant and Soil**, v. 356, n. 1–2, p. 113–125, 2012.

CRUZ, L. M. et al. 16S Ribosomal DNA Characterization of Nitrogen-Fixing Bacteria Isolated from Banana. **APPLIED AND ENVIRONMENTAL MICROBIOLOGY**, v. 67, n. 5, p. 2375–2379, 2001.

CURZI, M. J. et al. Changes in the content of organic and amino acids and ethylene production of rice plants in response to the inoculation with *Herbaspirillum seropedicae*. **Journal of Plant Interactions**, v. 3, n. 3, p. 163–173, 2008.

DA CUNHA, E. T. et al. Inoculation of *Herbaspirillum seropedicae* strain SmR1 increases biomass in maize roots DKB 390 variety in the early stages of plant development. **Archives of Microbiology**, v. 204, n. 7, 2022.

DALL'ASTA, P. et al. *Herbaspirillum seropedicae* promotes maize growth but fails to control the maize leaf anthracnose. **Physiology and Molecular Biology of Plants**, v. 25, n. 1, p. 167–176, 2019.

DEGASPARI, N. et al. **Method for cultivating sugar cane**. Australia, oct 18. 2012.

DING, L.; YOKOTA, A. Proposals of *Curvibacter gracilis* gen. nov., sp. nov. and *Herbaspirillum putei* sp. nov. for bacterial strains isolated from well water and reclassification of [*Pseudomonas*] *huttiensis*, [*Pseudomonas*] *lanceolata*, [*Aquaspirillum*] *delicatum* and [*Aquaspirillum*] *autotrophicum* as *Herbaspirillum huttiense* comb. nov., *Curvibacter lansceolatus* comb. nov., *Curvibacter delicatus* comb. nov. **International Journal of Systematic and Evolutionary Microbiology**, v. 54, n. 6, p. 2223–2230, 2004.

DÖBEREINER, J. Recent changes in concepts of plant-bacteria interactions: endophytic N2 fixing bacteria. **Ciência e Cultura**, v. 44, n. 5, p. 310–313, 1992.

DOBRITSA, A. P.; REDDY, M. C. S.; SAMADPOUR, M. Reclassification of *Herbaspirillum putei* as a later heterotypic synonym of *Herbaspirillum huttiense*, with the description of *H. huttiense* subsp. *huttiense* subsp. nov. and *H. huttiense* subsp. *putei* subsp. nov., comb. nov., and description of *Herbaspirillum aquaticum* sp. nov. **International Journal of Systematic and Evolutionary Microbiology**, v. 60, n. 6, p. 1418–1426, 2010.

ERBS, G.; NEWMAN, M. A. The role of lipopolysaccharide and peptidoglycan, two glycosylated bacterial microbe-associated molecular patterns (MAMPs), in plant innate immunity. **Molecular Plant Pathology.** v. 13, n.1, p. 95-104, 2011.

ESTRADA, G. A. et al. Selection of phosphate-solubilizing diazotrophic *Herbaspirillum* and *Burkholderia* strains and their effect on rice crop yield and nutrient uptake. **Plant and Soil**, v. 369, n. 1–2, p. 115–129, 2013.

FALK, E. C. et al. Deoxyribonucleic and Ribonucleic Acid Homology Studies of the Genera *Azospirillum* and *Conglomeromonas*. **International Journal of Systematic Bacteriology**, v. 36, n. 1, p. 80–85, 1986.

FAN, M. C. et al. *Herbaspirillum robiniae* sp. nov., isolated from root nodules of *Robinia pseudoacacia* in a lead-zinc mine. **International Journal of Systematic and Evolutionary Microbiology**, v. 68, n. 4, p. 1300–1306, 2018.

GLICK, B. R.; PENROSE, D. M.; LI, J. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. **J. Theor. Biol**. v. 190, p.63-68, 1998.

GREBE, T. W.; STOCK, J. Bacterial chemotaxis: The five sensors of a bacterium. **Current Biology**, v. 8, p. 154–157, 1998.

GRILLO-PUERTAS, M. et al. Transcriptional Responses of *Herbaspirillum seropedicae* to Environmental Phosphate Concentration. **Frontiers in Microbiology**, v. 12, 2021.

IM, W. T. et al. *Herbaspirillum chlorophenolicum* sp. nov., a 4-chlorophenol-degrading bacterium. **International Journal of Systematic and Evolutionary Microbiology**, v. 54, n. 3, p. 851–855, 2004.

IRINEU, L. E. S. DA S. et al. Multiomic Approaches Reveal Hormonal Modulation and Nitrogen Uptake and Assimilation in the Initial Growth of Maize Inoculated with *Herbaspirillum seropedicae*. **Plants**, v. 12, n. 1, 2023.

JAMES, E. K. et al. Infection and Colonization of Rice Seedlings by the Plant Growth-Promoting Bacterium *Herbaspirillum seropedicae* Z67. **Molecular Plant-Microbe Interactions**, v. 15, n. 9, p. 894–906, 2002.

JAMES, E. K.; OLIVARES, F. L. Infection and Colonization of Sugar Cane and Other Graminaceous Plants by Endophytic Diazotrophs. **Critical Reviews in Plant Sciences**, v. 17, n. 1, p. 77-119, 1998.

JUNG, S. Y. et al. *Herbaspirillum rhizosphaerae* sp. nov., isolated from rhizosphere soil of *Allium victorialis* var. platyphyllum. **International Journal of Systematic and Evolutionary Microbiology**, v. 57, n. 10, p. 2284–2288, 2007.

KADOURI, D.; JURKEVITCH, E.; OKON, Y. Involvement of the reserve material poly-β-hydroxybutyrate in *Azospirillum brasilense* stress endurance and root colonization. **Applied and Environmental Microbiology**, v. 69, n. 6, p. 3244–3250, 2003.

KIRCHHOF, G. et al. *Herbaspirillum frisingense* sp. nov., a new nitrogen-fixing bacterial species that occurs in C4-fibre plants. **International Journal of Systematic and Evolutionary Microbiology**, v. 51, p.157-168, 2001.

KOPPISCH, A. T. et al. **PRODUCTION OF INDUSTRIALLY RELEVANT COMPOUNDS IN PROKARYOTC ORGANISMS**. United States, sept 26. 2013.

KUANG, W. et al. N-dependent dynamics of root growth and nitrate and ammonium uptake are altered by the bacterium *Herbaspirillum seropedicae* in the cereal model *Brachypodium distachyon*. **Journal of Experimental Botany**, v. 73, n. 15, p. 5306–5321, 2022.

KUKLINSKY-SOBRAL, J. et al. Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. **Plant and Soil**, v. 273, n. 1–2, p. 91–99, 2005.

LAMBRECHT, M. et al. Indole-3-acetic acid: a reciprocal signaling molecule in bacteria-plant interactions. **Trends in microbiology**, v. 8, n. 7, p. 298–300, 2000.

LIMA, L. DA S. et al. Root exudate profiling of maize seedlings inoculated with *Herbaspirillum seropedicae* and humic acids. **Chemical and Biological Technologies in Agriculture**, v. 1, n. 1, p. 1–18, 2014.

MACHADO, I. M. et al. Cloning and sequencing of the nitrogenase structural genes *nifHDK* of *Herbaspirillum seropedicae*. **Brazilian journal of medical and biological research**, v. 29, n. 12, p. 1599—1602, 1996.

MARIN, A. M. et al. Naringenin degradation by the endophytic diazotroph *Herbaspirillum seropedicae* SmR1. **Microbiology (United Kingdom)**, v. 159, n. 1, p. 167–175, 2013.

MATTEOLI, F. P. et al. *Herbaspirillum*. Em: AMARESAN, N. et al. (Eds.). **Beneficial Microbes in Agro-Ecology: Bacteria and Fungi**. 1. ed. Elsevier, 2020. p. 493–508.

MERRITT, K. **Microbial composition, method, and kit for enhancing plant growth**. Australia, 7 mar. 2013.

MONTEIRO, R. A. et al. Early colonization pattern of maize (*Zea mays* L. Poales, Poaceae) roots by *Herbaspirillum seropedicae* (*Burkholderiales*, *Oxalobacteraceae*). **Genetics and Molecular Biology**, v. 31, n. 4, p. 932–937, 2008.

MONTEIRO, R. A. et al. *Herbaspirillum*-plant interactions: Microscopical, histological and molecular aspects. **Plant and Soil**, v. 356, n. 1–2, p. 175–196, 2012.

NEIVERTH, A. et al. Performance of different wheat genotypes inoculated with the plant growth-promoting bacterium *Herbaspirillum seropedicae*. **European Journal of Soil Biology**, v. 64, p. 1–5, 2014.

NISHIO, Y. et al. **METHOD FOR PRODUCING 2-KETOGLUTARIC ACID AND DERIVATIVES THEREOF BY USING A BACTERIUM FROM THE GENUS PANTOEA OR THE GENUS CORYNEBACTERIUM**, May 17, 2017.

NJOLOMA, J. et al. Infection and colonization of aseptically micropropagated sugarcane seedlings by nitrogen-fixing endophytic bacterium, *Herbaspirillum sp.* B501gfp1. **Biology and Fertility of Soils**, v. 43, n. 2, p. 137–143, 2006.

OLIVARES, F. L. et al. Occurrence of the endophytic diazotrophs *Herbaspirillum spp.* in roots, stems, and leaves, predominantly of Gramineae. **Biology and Fertility of Soils**, v. 21, n. 3, p. 197–200, 1996.

OLIVARES, F. L. et al. Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirillum*. **New Phytologist**, v. 135, n. 4, p. 723–737, 1997.

PANKIEVICZ, V. C. S. et al. RNA-seq transcriptional profiling of *Herbaspirillum seropedicae* colonizing wheat (*Triticum aestivum*) roots. **Plant Molecular Biology**, v. 90, n. 6, p. 589–603, 2016.

PEDROSA, F. O. et al. Structural organization and regulation of the *nif* genes of *Herbaspirillum seropedicae*. **Soil Biology and Biochemistry**, v. 29, n. 5–6, p. 843–846, 1997.

PEDROSA, F. O. et al. Genome of *Herbaspirillum seropedicae* strain SmR1, a specialized diazotrophic endophyte of tropical grasses. **PLoS Genetics**, v. 7, n. 5, 2011.

PIMENTEL, J. et al. Dinitrogen fixation and infection of grass leaves by *Pseudomonas rubrisubalbicans* and *Herbaspirillum seropedicae*. **Plant and soil**, v. 137, p. 61-65, 1991.

RADWAN, T. E. E.; KAMEL, Z.; MASSENA, V. Efeito da inoculação de *Azospirillum* e *Herbaspirillum* na produção de compostos indólicos em plântulas de milho e arroz. **Pesquisa Agropecuaria Brasileira**, v. 39, n. 10, p. 987–994, 2004.

RAMOS, A. C. et al. Inoculation with the endophytic bacterium *Herbaspirillum seropedicae* promotes growth, nutrient uptake and photosynthetic efficiency in rice. **Planta**, v. 252, n. 5, p. 1–8, 2020.

RATCLIFF, W. C.; DENISON, R. F. Individual-level bet hedging in the bacterium *Sinorhizobium meliloti*. **Current Biology**, v. 20, n. 19, p. 1740–1744, 2010.

REGO, F. G. M. et al. The expression of *nifB* gene from *Herbaspirillum seropedicae* is dependent upon the *NifA* and *RpoN* proteins. **Canadian Journal of Microbiology**, v. 52, n. 12, p. 1199–1207, 2006.

REIS, V. M. et al. Recomendação de inoculante para cultura de milho utilizando a bactéria *Herbaspirillum seropedicae* estirpe BR 11417. **Comunicado técnico 119**, p. 2, 2009.

REIS, V. M.; BALDANI, J. I.; URQUIAGA, S. Recomendação de uma mistura de estirpes de cinco bactérias fixadoras de nitrogênio para inoculação de cana-de-açúcar: *Gluconacetobacter diazotrophicus* (BR 11281), *Herbaspirillum seropedicae* (BR 11335), *Herbaspirillum rubrisubalbicans*. **Circular Técnica - Embrapa Agrobiologia**, v. 30, p. 1–4, 2009.

RODRIGUES, L. S. et al. Diversidade de bactérias diazotróficas endofíticas dos gêneros *Herbaspirillum* e *Burkholderia* na cultura do arroz inundado. **Pesquisa Agropecuaria Brasileira**, v. 41, n. 2, p. 275–284, 2006.

ROESCH, L. F. W. et al. Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. **Plant and Soil**, v. 302, n. 1–2, p. 91–104, 2008.

ROTHBALLER, M. et al. *Herbaspirillum hiltneri* sp. nov., isolated from surface-sterilized wheat roots. **International Journal of Systematic and Evolutionary Microbiology**, v. 56, n. 6, p. 1341–1348, 2006.

SALA, V. M. R. et al. Ocorrência e efeito de bactérias diazotróficas em genótipos de trigo. **Revista Brasileira de Ciencia do Solo**, v. 29, n. 3, p. 345–352, 2005.

SOUZA, E. M. et al. Expression of the *nifA* gene of *Herbaspirillum seropedicae*: Role of the *NtrC* and *NifA* binding sites and of the -24/-12 promoter element. **Microbiology**, v. 146, n. 6, p. 1407–1418, 2000.

TADRA-SFEIR, M. Z. et al. Naringenin regulates expression of genes involved in cell wall synthesis in *Herbaspirillum seropedicae*. **Applied and Environmental Microbiology**, v. 77, n. 6, p. 2180–2183, 2011.

TADRA-SFEIR, M. Z. et al. Genome wide transcriptional profiling of *Herbaspirillum seropedicae* SmR1 grown in the presence of naringenin. **Frontiers in Microbiology**, v. 6, 2015.

TIRAPELLE, E. F. et al. Identification of Proteins Associated with Polyhydroxybutyrate Granules from *Herbaspirillum seropedicae* SmR1 - Old Partners, New Players. **PLoS ONE**, v. 8, n. 9, 2013.

TRIPLETT, E. W.; KAEPPLER, S. M.; CHELIUS, M. K. ***KLEBSIELLA PNEUMONIAE* INOCULANTS FOR ENHANCING PLANT GROWTH**. United States, 1 jul. 2008.

VALVERDE, A. et al. *Herbaspirillium lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of Phaseolus vulgaris. **International Journal of Systematic and Evolutionary Microbiology**, v. 53, n. 6, p. 1979–1983, 2003.

WEBER, O. B.; FREIRE, F. DAS C. O. Contribuição de bactérias diazotróficas na cultura da bananeira: perspectivas de utilização na produção integrada. **Boletim de Pesquisa e Desenvolvimento**, v. 16, p. 29p, 2003.

XU, G. et al. *Herbaspirillum piri* sp. nov., isolated from bark of a pear tree. **International Journal of Systematic and Evolutionary Microbiology**, v. 68, n. 11, p. 3652–3656, 2018.

ZHU, B. et al. Genome sequence of the pathogenic *Herbaspirillum seropedicae* strain Os45, isolated from rice roots. **Journal of Bacteriology**, v. 194, n. 24, p. 6995–6996, 2012.

Chapter 2: Changes in the rhizosphere and the seed-borne bacteriome associated with maize roots by *Herbaspirillum seropedicae* inoculation.

Changes in the rhizosphere and the seed-borne bacteriome associated with maize roots by *Herbaspirillum seropedicae* inoculation.

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Abstract

Plants can react to a biotic and abiotic stimuli for survival and development. Several studies have proven that inoculation of *Herbaspirillum seropedicae*, a plant-growth promoter bacteria (PGPB), alters the metabolism of plants favoring the colonization of bacteria in the host plant. This work aims to understand how inoculation of *H. seropedicae* induces changes in maize root metabolism to communicate with the bacteria and how these changes can modulate the natural plant bacteriome. For this, proteomics and gene expression approaches of maize roots inoculated or not with *H. seropedicae* strain HRC54 were used to assess the metabolic changes. The roots' H+ flux and surface pH were measured by the non-invasive scanning ion-selective electrode technique (SIET). Illumina MiSeq made sequencing of the root bacteriome. Inoculation of *H. seropedicae* changed the tricarboxylic acid cycle (TCA) on transcriptional and post-transcriptional levels, altered the H^+ flux and pH on the root surface, and regulated the H^+-ATPase on the protein level. These changes modulated the bacteriome of the roots, increasing bacterial genera *Mesorhizobium*, *Novosphingobium*, *Rhizobium*, *Serratia*, and *Stenotrophomonas*, commonly associated with PGPB. These results suggest that maize roots inoculated with *H. seropedicae* can respond to the inoculation modulating their metabolism to exude compounds of the TCA and change the pH of the root surface to favor the inoculated bacteria and select the bests candidates of the root bacteriome.

Keywords: *organic acids; exudates; chemotaxis; seed-borne bacteria; bioinoculant*.

1. Introduction

Using microbial inoculants with biofertilizer and biostimulant properties emerges as an alternative to replace or complement synthetic fertilizers, to promote plant growth and protection, and to reduce the environmental impacts of industrial agriculture practices (TOYOTA; WATANABE, 2013).

Herbaspirillum seropedicae is a nitrogen-fixing bacterium of the β-Proteobacteria subclass, preferably associated with important agricultural crops, such as wheat (*Triticum aestivum*), maize (*Zea mays*), sugar cane (*Saccharum officinarum*), sorghum (*Sorghum bicolor*) and rice (*Oryza sativa*) (BALDANI et al., 1996; MONTEIRO et al., 2012; OLIVARES et al., 1996).

The bacteria survive poorly in soil but can be found endophytically associated with roots, stems and leaves of different plant hosts (BALDANI et al., 1996; ESTRELA BORGES BALDOTTO; LOPES OLIVARES; BRESSAN-SMITH, 2011; JAMES; OLIVARES, 1998; MONTEIRO et al., 2012; OLIVARES et al., 1996). *H. seropedicae* adheres to plant surfaces as epiphytes or migrates to apoplastic compartments of the plant body, colonizing intercellular spaces and xylem lumen as endophytes (JAMES; OLIVARES, 1998; MONTEIRO et al., 2012) helps to supply the N demand of plants(JAMES et al., 2002; RAMOS et al., 2020; RONCATO-MACCARI et al., 2003; SCHMIDT et al., 2011), produces growth regulators (BASTIÁN et al., 1998; BERGAMESHI et al., 2007; BOTTINI; CASSÁN; PICCOLI, 2004; KEYEO; AI'SHAH; AMIR, 2011; LAMBRECHT et al., 2000; RADWAN; KAMEL; MASSENA, 2004), and solubilizes inorganic phosphorus (ESTRADA et al., 2013).

Plants can use root exudates to mediate interactions in the rhizosphere positively and/or negatively. Positive interactions include symbiotic and non-symbiotic associations with microorganisms such as mycorrhiza, rhizobia and plant growth-

promoting bacteria (PGPB). They can also exert chemotactic actions for certain groups of microorganisms or act as repellents for other groups, thus modulating the rhizosphere microbiota and symbiosis (BADRI; VIVANCO, 2009; NARDI et al., 2000; SASSE; MARTINOIA; NORTHEN, 2018).

Plant root exudates are one of the leading forces of the rhizosphere processes, which include low molecular weight (i.e., organic acids, amino acids, sugars, phenolic acids, flavonoids) and high molecular weight (i.e., carbohydrates, enzymes) organic compounds, depending on plant species and/or environmental conditions (MIMMO et al., 2011). Exudates could represent an easily accessible C source for microorganisms in the rhizosphere, where the concentration of these compounds is higher than in the bulk soil (HINSINGER et al., 2009)

Maize roots can exudate organic acids, such as benzoic, *cis* and *trans*-aconitic, citric, isocitric, fumaric, glutamic, glyoxylic, maleic, malic, malonic, oxalic, pyruvic, succinic, tartaric acids (KRAFFCZYK; TROLLDENIER; BERINGER, 1984; ERRO et al., 2009).

Also, roots can change their rhizosphere pH to counterbalance the release of organic anions (i.e., malate, citrate, oxalate) by an equivalent influx of OH^- or efflux of H^+. The transmembrane electrochemical gradient is maintained by the plasma membrane H^+-ATPase, and the activity of this enzyme is essential for the movement of solutes and, considering nutrient-acquisition processes in plants (PII et al., 2015). Then, plant systems can benefit from H^+ efflux and organic acids exudation (HINSINGER et al., 2003; BALASUBRAMANIAN et al., 2021).

Lima et al. (2014) demonstrated that co-inoculation of *H. seropedicae* and humic acids in maize plants changes the exudation profile 14 and 21 days after inoculation (DAI). Canellas; Olivares; Canellas (2019) reported an increase in the production of

organic acids of the tricarboxylic acid cycle (TCA) in maize and sugarcane co-inoculated with *H. seropedicae*, *Gluconacetobacter diazotrophicus* and humic acids. Also, seedlings treated with *H. seropedicae* and humic substances caused stimulation of plasma membrane H^+-ATPase in maize roots (CANELLAS et al., 2013).

Research on the processes that involve maize root and rhizosphere changes in the presence of H*. seropedicae* could support the knowledge of how plants communicate with microorganisms in the soil. Furthermore, the results obtained by the inoculation of *H. seropedicae* with other plant-growth promoter bacteria (PGPB) and humic substances open precedents to investigate the isolated effect of the inoculation of *H. seropedicae* on the TCA and the H^+-ATPase of maize roots and understand how the activity of these modulate the maize rhizosphere bacteriome.

2. Materials and Methods

2.1. Preparation of inoculum

The microorganism used in this study was the *H. seropedicae* strain HRC54 (SisGen nº AFD1CAD), originally isolated from sugarcane roots. The pre-inoculum was obtained from a pure plate colony and after growth in DYGS (RODRIGUES-NETO; MALAVOLTA JR.; VICTOR, 1986) liquid medium for 24h at 30°C in an orbital shaker at 150rpm. After growth, a 20 µL aliquot of the bacterial suspension was transferred to JNFB (DÖBEREINER; BALDANI; BALDINI, 1995) liquid medium supplemented with NH_4Cl (1g.L^{-1}) for 48h under the same conditions described above after the bacterial cells were sedimented by centrifugation (5.000 × g for 15 min) and resuspended in sterile distilled water at a cell density of $4x10^9$ CFU.mL^{-1}.

2.2. Plant inoculation

Maize seeds (*Zea mays* L., var. Dekalb 7815) were surface disinfected by immersion in 0.5% NaClO for 5 min, followed by rinsing and immersion in distilled water for 6h. The seeds were then placed to germinate on Germitex paper for 72h. Seeds with approximately 2.5 cm long radicles were selected and placed in 2 L pots filled with 0.2 M CaCl2 solution, consisting of 40 seeds per pot. Maize seedlings were inoculated with a 20 mL aliquot of the bacterial inoculum. Tests were carried out under the following conditions: uninoculated maize plants (Control) and inoculated maize plants, with $2x10^7$ cells mL^{-1} *H. seropedicae* strain HRC54 at 28ºC, photoperiod of 16h/8h with constant aeration.

After five days of biostimulation, the plants were collected, and the roots were separated for the extraction of RNA and DNA and proteins and stored in a -70 ºC freezer.

2.3. Proteomic procedures

2.3.1. Protein extraction

Three biological replicates (300 mg of root material for each replicate) of the treatment control and inoculated after five days of growth were used for protein extraction. Proteins were extracted using the TCA/acetone precipitation method developed by Damerval et al. (1986). Each sample was resuspended in 1 mL of chilled extraction buffer containing 10% (w/v) trichloroacetic acid (TCA; Sigma Chemical Co., St. Louis, MO) in acetone with 20 mM dithiothreitol (DTT; GE Healthcare, Little Chalfont, UK) vortexed for 30min at 8 ºC and left at -20 °C for 1h for protein precipitation. The mixture was centrifuged at 16,000×g for 30min at 4 °C. The resulting pellets were washed three times with cold acetone plus 20 mM DTT, vortexed for 30 s and centrifuged for 5min at 4 °C for each wash. Finally, pellets were air-dried and

resuspended in 1 mL of buffer containing 7 M urea (GE Healthcare, Little Chalfont, UK), 2 M thiourea (GE Healthcare), 2% Triton X-100 (GE Healthcare), 1% DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and incubated for 30min on ice. Samples were then vortexed at 8 ºC for 30 min and centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant was collected, and the protein concentrations were determined using the 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

2.3.2. Protein digestion

For protein digestion, 100 µg of extracted proteins from each biological replicate were precipitated with methanol/chloroform, according to Nanjo et al. (2012), to remove any interference from the samples. After the protein precipitation, the samples were resuspended in 7 M urea/2 M thiourea solution. Tryptic protein digestion (1:100 enzyme: protein, V5111, Promega, Madison, USA) was subsequently performed using the modified filter-aided sample preparation (FASP) method as described by Burrieza et al. (2019). The resulting peptides were quantified according to the $A_{205\ nm}$ protein and peptide method using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

2.3.3. Mass spectrometry analyses

Nano-LC-electrospray ionization (ESI)-MS/MS analysis was carried out using a nanoAcquity UPLC (Waters) coupled to a Synapt G2-Si mass spectrometer (Waters). The peptide mixtures were separated by liquid chromatography by loading 1 µg of digested peptides onto a nanoAcquity UPLC 5-µm C18 trap column (180 µm by 20 mm; Waters), followed by loading onto a nanoAcquity HSS T3 1.8-µm analytical column (75 µm by 150 mm; Waters) at a rate of 400 $nL.min^{-1}$ at 45 °C. The

chromatographic method, mass spectrometer parameters, and run conditions were performed according to methods described by Almeida et al. (2019).

2.3.4. Proteomics data analysis

Spectral processing and database searching was performed using ProteinLynx Global Server (PLGS; v3.0.2; Waters) and ISOQuant workflow software (DISTLER et al., 2014, 2016). The *Z. mays* protein database from Uniprot (Proteome ID: UP000007305) was used for protein identification. Label-free relative quantitative analyses were performed based on the normalized protein ion counts. The spectra processing in PLGS and comparative label-free quantification analysis parameters in ISOQuant were described by Burrieza et al. (2019). After ISOQuant data analyses, only the present or absent proteins (for unique proteins) in all three biological replicates were considered for differential abundance analysis. Data were analyzed using Student's t-test (two-tailed). Proteins with $P \leq 0.05$ were considered up-accumulated if the $\log_2$ of the fold change was more significant than 0.5 and downregulated if the $\log_2$ of the fold change was lower than -0.5.

Finally, proteins were blasted against the non-redundant (nr) Plants/ Viridiplantae_Protein_Sequences database by using the Blast2GO software (www.blast2go.com) (CONESA et al., 2005) for protein ontology and cellular component term annotation and the metabolic classification executed with MapMan version 3.6.0RC1 (THIMM et al., 2004; USADEL et al., 2009) (https://mapman.gabipd.org/).

2.4. Gene expression analysis and *H. seropedicae* presence confirmation.

A total of 100 mg of control and inoculated roots were macerated in liquid nitrogen for RNA extraction with Trizol® reagent (Thermo Fischer), quantified in

NanoDrop 2000® spectrophotometer (Thermo Scientific). The cDNA synthesis was carried out using the SuperScript™ III Reverse Transcriptase (Thermo Fischer) as described in the manufacturer's protocol using the 1µg from RNA extracted from the inoculated and uninoculated samples.

Real-time PCR quantification validated the differentially expressed genes previously identified through proteomic analyses. Specific primers for each gene to be analyzed (Table 1) were designed using Oligo Explorer™ software (Teemu Kuulasmaa, Finland) and confirmed in Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (YE et al., 2012). Genes for *α*-Tubulin (*α-Tub*) and Tubulin *β*-chain (*β-Tub*) were used as normalizing genes, according to Lin et al. (2014).

Table 1: Genes selected, primers designed, and concentration used for RT-qPCR.

Gene	Gene product	Primers sequences (5'-3')	Amplicon size
Aco	Aconitate hydratase	F: GTTTGGGTTCTTGAAATGGG	143bp
		R: CGCTGTCAGGGTAAAGGATC	
Cit	Citrate synthase	F: TCCTGTGCTGTTTGCTATTC	143bp
		R: GTTCTCTGACGGGGGTATAG	
Fum	Fumarase	F: TGGCTGAGGAAACAAACC	145bp
		R: GCCCTTCCAACTGCTAAAC	
Iso	Isocitrate dehydrogenase	F: TCAGGACCATGCTATCTTCG	131bp
		R: ATTGCAAGTGCCTCAACATC	
Mal	Malate dehydrogenase	F: GGGCGTCTACAATCTCAAG	117bp
		R: ACAACTGGGACATCAACATC	
Suc	Succinate--CoA ligase	F: TGCACTGCGTGATACAACTC	100bp
		R: CACCATGCAACCAATCTCTC	
Tub	Tubulin alpha-3 chain	F: GCGCACCATCCAGTTCGT	61bp
		R: CTGGTAGTTGATTCCGCACTTG	
β-tub	Tubulin beta-4 chain	F: CTACCTCACGGCATCTGCTATGT	139bp
		R: GTCACACACACTCGACTTCACG	

Legend: F- forward; R: reverse; bp: base pairs.

Primers were used in RT-qPCR reactions at concentrations of 500 nM, using 7.5 µL of FAST SYBR Green PCR Master Mix (Applied Biosystems) in Step One Plus Real-Time PCR machine (Applied Biosystems) under the following conditions: 40 cycles 95° C for 1min, 60°C for 1min and 72°C for 1min. All qPCR runs were conducted

in triplicate. The relative quantification was determined according to the $2^{-\Delta\Delta Ct}$ method (LIVAK; SCHMITTGEN, 2001).

For confirmation, *H. seropedicae* stabilized in the inoculated and non-inoculated maize roots, and total DNA was isolated using Cetyltrimethylammonium bromide (CTAB) (CHEN; RONALD, 1999; DOYLE; DOYLE, 1987). Total DNA samples were quantified using a NanoDrop 2000® spectrophotometer (Thermo Scientific). Specific primers for *H. seropedicae* ribosomal DNA 16S (16S rDNA) were used (5′-CTAATACCGCATACGATCTAC-3' and 5′-TTCTGGATATTAGCCAAAACC-3') for PCR amplification. The protocol consisted of an initial incubation at 50 °C for 2min, 95 °C incubation for 10min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1min (PEREIRA et al., 2014). This PCR aimed to detect the presence of the bacteria in the extracted material from three control and three inoculated plants.

2.5. Sequencing of maize root bacteriome

200 mg of root samples from each treatment were macerated in liquid nitrogen for total DNA extraction with CTAB (Cetyltrimethylammonium bromide) (Chen and Ronald, 1999; Doyle and Doyle, 1987). The extracted DNA was quantified in NanoDrop 2000® spectrophotometer (Thermo Scientific), and the quality was confirmed in agarose gel (0.8%) electrophoresis (80 V, for 70 min). The total DNA was sent to the company “NGS Soluções Genômicas” (Piracicaba – SP, Brazil) for sequencing the 16S rRNA gene in Illumina MiSeq, with three repetitions per treatment. The samples were amplified with primers 515FB (GTGYCAGCMGCCGCGGTAA) and 806RB (GGACTACNVGGGTWTCTAAT) (Caporaso et al. 2011; 2012), with modifications to primer degeneracy done by the labs of Jed Furhman (PARADA; NEEDHAM; FUHRMAN, 2016) and Amy Apprill (APPRILL et al., 2015). For PCR, 10 µL of PCR

master mix, 0.5 µL of each primer (10 µM), 1 µL of DNA template, and 13 µL of PCR-grade water were used, totaling 25 µL of reaction. The PCR conditions were: initial denaturation at 94 ºC for 3min, followed by 30 cycles of 94 ºC for 30 s, 50 ºC for 30s and 72 ºC for 30s and a final extension of 72 ºC for 10min. The sequencing was performed on the Illumina MiSeq platform.

2.6. 16S profiling data analysis

The raw sequences of the Illumina system were analyzed following the recommendations of the Brazilian Microbiome Project (PYLRO et al., 2014) and using the BMP Operating System (BMPOS) (PYLRO et al., 2016). Briefly, the sequences were filtered, grouped into Operational Taxonomic Units (OTUs) with a 97% similarity cut (EDGAR et al., 2011) and taxonomically classified with the QIIME software (CAPORASO et al., 2010) using Greengenes reference sequences (MCDONALD et al., 2012)

The BIOM file was imported into the R environment using the phyloseq package (MCMURDIE; HOLMES, 2013), and the sampling quality was estimated from Good's coverage (Good, 1953). Taxonomy up to gender level was estimated in centered log-ratio (clr) transformed abundance (GLOOR; REID, 2016). Beta diversity (bacterial diversity between different samples) was compared by principal coordinates analysis (PCoA) using the phyloseq package, and the significance between groups was visualized by Permutational multivariate analysis of variance (Permanova) (ANDERSON, 2017) with the adonis function available in vegan package (OKSANEN et al., 2015). Alpha diversity (bacterial diversity within each sample) was calculated (estimate_richness function in the phyloseq package) by the species observed and by the Shannon diversity index (considering the number and abundance of species). The

diversity indices were submitted to analysis of variance (ANOVA) and normality confirmed by the Shapiro-Wilk W test ($p < 0.05$).

2.7. Rhizospheric H^+ fluxes and microenvironmental pH measurements

Proton fluxes and pH were measured across the roots through the non-invasive Scanning Ion-selective Electrode Technique (SIET). Ion-specific microelectrodes were produced from 1.5 mm borosilicate glass capillaries and backfilled with an electrolyte (15 mM KCl and 40 mM KH2PO4, pH 6.0) and front-loaded with the ion-selective cocktail (Sigma-Aldrich, hydrogen ionophore I, Cocktail B, Cat. No. 95293). The microelectrodes were calibrated using standard pH solutions to obtain a calibration line. The slope and intercept of the calibration line were used to calculate the H^+ concentration from the mV values measured during the experiments.

Maize seeds (*Zea mays* cv. DKB 177) were surface disinfected with 0.5% NaClO for 5 min, followed by rinsing and immersion in distilled water for 6 h. Afterward, the seeds were placed to germinate on Germitex paper for 72 h. Seeds with radicles approximately 5 cm long were selected and placed in 1/10 strength Murashige and Skoog (MS) medium for acclimatization for 24 hours.

Vibrating microelectrodes were positioned near the root bathed in fresh 1/10 strength MS, and the net H^+ fluxes and root microenvironment pH were measured as voltage–current (µV) differences over an excursion distance of 15 µm of root surface near the root cap, elongation zone and root hair zone for 5–10 min. Control background measurements were performed at ~700 µm from the root surface and subtracted from measurements performed near (15 µm). After the measurements, an aliquot of 5×10^7 cells.mL^{-1} of *H. seropedicae* previously prepared was added to the solution, and once again, the measurements were performed in the areas mentioned before.

2.8. Rhizoplane colonization dynamics of the root axis

Seeds of maize plants (*Zea mays* cv. DKB 177) were surface disinfected for 1 min in ethanol (70%), followed by sodium hypochlorite (NaOCl, 2.5%) for 5min and four washes in sterilized water (5min per wash). Seeds were germinated in wet filter paper in the dark at 28 °C for 3-d and transferred with an approximate 4 cm root length to a Petri plate containing 20 mL of Murashige and Skoog (MS-salt) medium at $1/10^{th}$ ionic strength (Murashige and Skoog, 1962) as visualized in Figure 1A.

The bacterium inoculum of *H. seropedicae* strain HRC54 was obtained according to Baldani et al. (2014) using a liquid Digys medium to produce the cell biomass at 30 °C and 180 rpm in a rotatory shake for 36h. After that, 1 mL of the culture (DO_{595nm}=1.3; equivalent to 5 x 10^8 cells. mL^{-1}) was centrifugated for 10 min at 10,000 rpm and resuspended in 1mL of MS-salt ($1/10^{th}$ ionic strength). Finally, 200 µL of the bacterial suspension was applied to the liquid medium at 4 cm from the germinated maize root plantlet (Figure 1A).

Root segments of 1 cm in length containing root tip zone (i), elongation and differentiation zone (ii) and root hair zone (iii) - see Fig 1B; were collected and prepared for scanning electron microscopy (SEM) at 30min, 2h, 4h and 24h after bacteria application to evaluate the bacteria interaction pattern with distinct root axis zone over the time. SEM observations were done using an EVO 40 Zeiss at 10-20 Kv in mode high deep focus for low magnification (less than 500 X magnification - Fig 1C) and the high mode resolution (highest magnification - Figure 1D). To evaluate the root attachment frequency in different anatomical zones and times, a digital square of 10 x 10 µm (area of 100 μm^2) was generated to randomly evaluate the presence of bacteria attached to the root surface (Figure 1E). The results were expressed as the average frequency $\pm$ standard deviation of the 30 squares evaluation (n = 30).

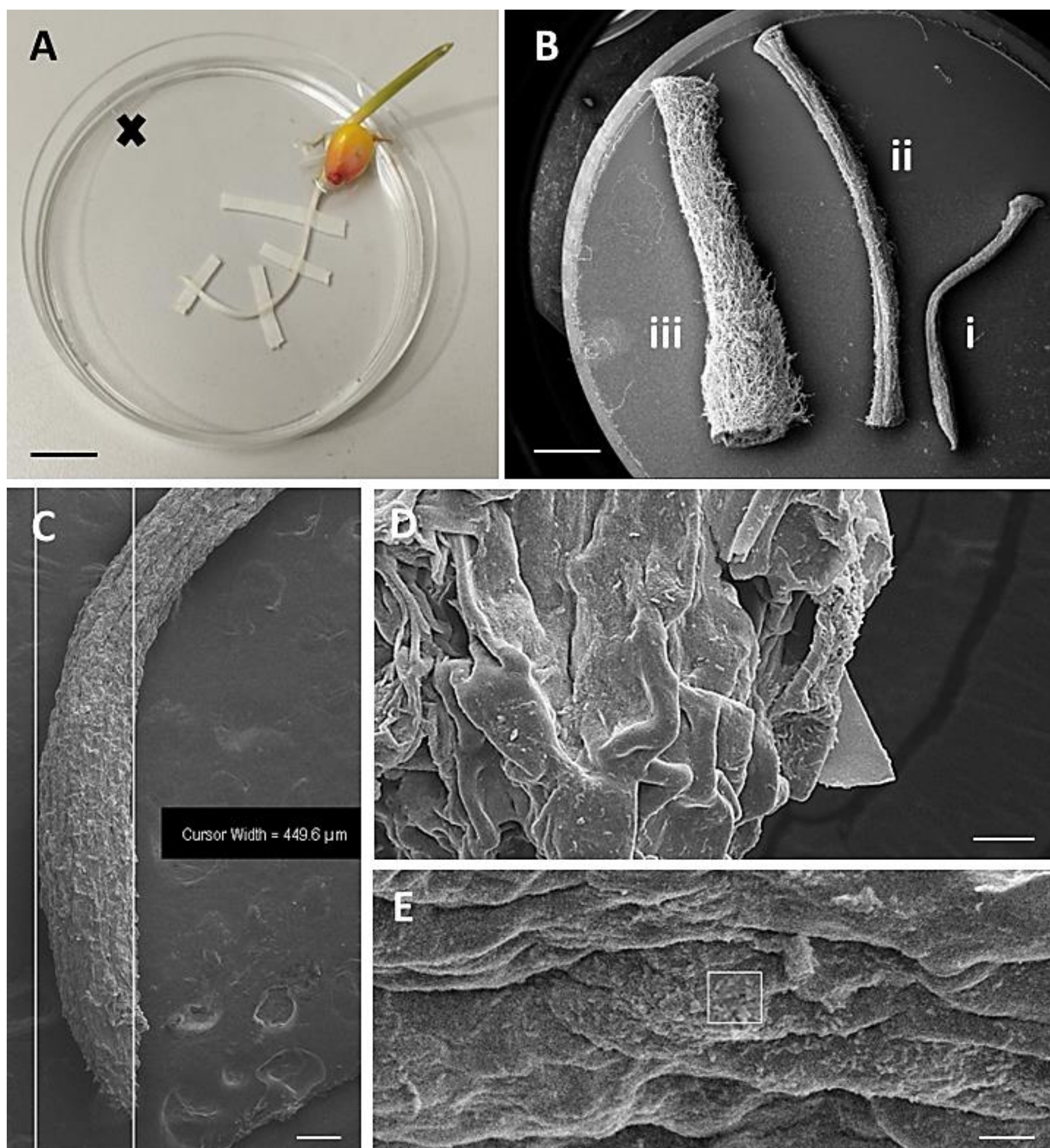

Figure 1: (A) Experimental setup designed to evaluate *H. seropedicae*-maize root interaction by SEM. X means inoculation point, bar = 1 cm; (**B**) SEM preparation in aluminum stub containing 1-cm root segment of (i) root cap region, (ii) elongation-differentiation region and (iii) root hair zone, bar = 2 mm; (**C**) SEM view for high deep focus study of bacterium colonization, bar = 200 µm; (**D**) SEM view for high-resolution study of cell-attachment pattern, bar = 10 µm; (**E**) SEM view of the digital square area (10 x 10 µm) used to evaluate the presence frequency of the bacteria in time-space root segments, bar = 10 µm.

3. Results

3.1. Confirmation of maize roots inoculation.

The confirmation of *H. seropedicae* in the roots was assessed by conventional PCR amplification using specific primers for the 16S rDNA of *H. seropedicae.*

Electrophoretic analyses showed band formation only in the inoculated samples, confirming the presence of *H. seropedicae* in the root tissue (Figure 2).

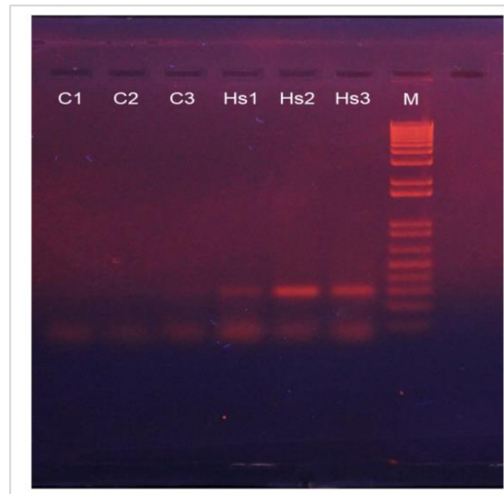

Figure 2: Electrophorese gel demonstrating the presence of *H. seropedicae* in the maize roots of inoculated plants. Legend: **C1**, **C2** and **C3** – uninoculated plants (control); **Hs1**, **Hs2** and **Hs3** – inoculated plants (*H. seropedicae*); **M** - molecular weight marker.

3.2. Proteomic profile in response to inoculation with *H. seropedicae*

Proteomic analysis performed using three independent biological replicates generated a total of 240 proteins in each experimental condition (control and inoculated with *H. seropedicae*), with statistical significance ($p<0.05$). The biological replicates demonstrated a high level of correlation ($r^2>0.9311$).

In summary, 91 proteins were down-accumulated, and 132 were up-accumulated. Eleven proteins appeared exclusive in control plants, while six others appeared only in plants treated with *H. seropedicae*.

Functional classification demonstrated a total of 208 proteins matched. In addition, 23 proteins with unknown functions were matched. According to the objective of this work, the analyses were focused on proteins of the TCA cycle and transporters (Figure 2).

Proteins of TCA like Aconitate hydratase (A0A1D6PJL0), Acetyltransferase component of pyruvate dehydrogenase complex (A0A1D6QQE1), Fumarate

hydratase (C0PDA6), Isocitrate dehydrogenase (A0A1D6HC33), and Succinate-CoA ligase (A0A1D6HDG4) were up-accumulated (Figure 3A).

Some proteins with transport activity show to be regulated by inoculation. For example, eight aquaporins (A0A1D6EMF1, A0A1D6HYQ4, A0A1R3N4Y1, Q41870, Q9AQU5, Q9AR14, Q9ATM6 and Q9ATM7) two sugar transporters (A0A1D6K7N5 and C4IYM7) one amino acids transporter (A0A1D6P3W8) appears down-accumulated, and two ATPases appear down-accumulated (A0A1D6KZT0 and A0A1D6MV35) and two up-accumulated (A0A1D6JJK6 and A0A1D6NQK5) (Figure 3B).

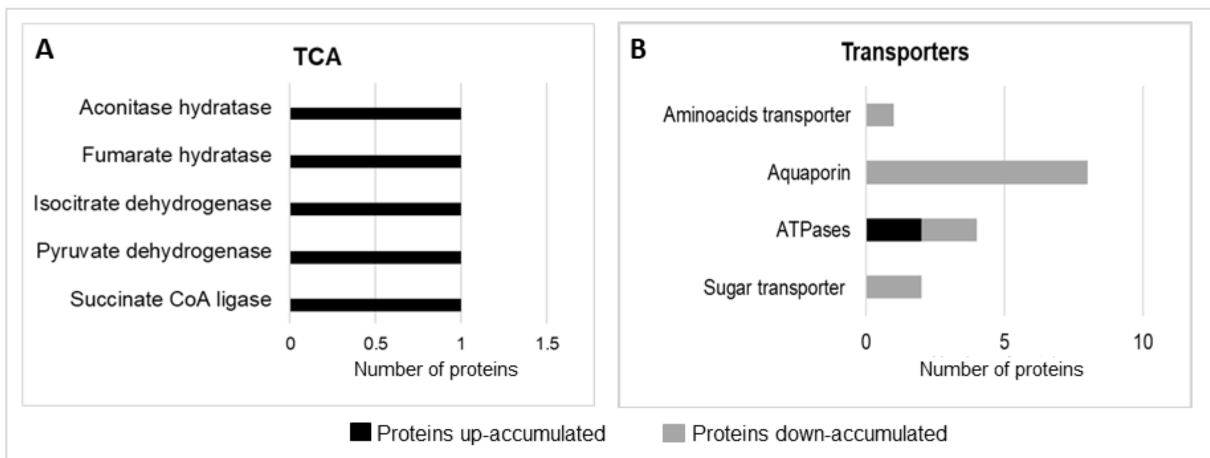

Figure 3: Mapman visualization of differentially accumulated proteins (DAPs). (**A**) Tricarboxylic acid pathway; (**B**) Transporters.

3.3. Transcriptional profile of maize roots

Gene expression tracked by RT-PCR of the enzymes of TCA in maize roots was investigated in inoculated and control plants. The genes encoding the enzymes Aconitase hydratase, Citrate synthase, Isocitrate dehydrogenase and Succinate CoA-ligase were more expressed at 0.58, 1.36, 2.18 and 1.49-fold, respectively, and Fumarate hydratase and Malate dehydrogenase have the expression repressed 0.34 and 0.11-fold when compared with the plant controls (Figure 4).

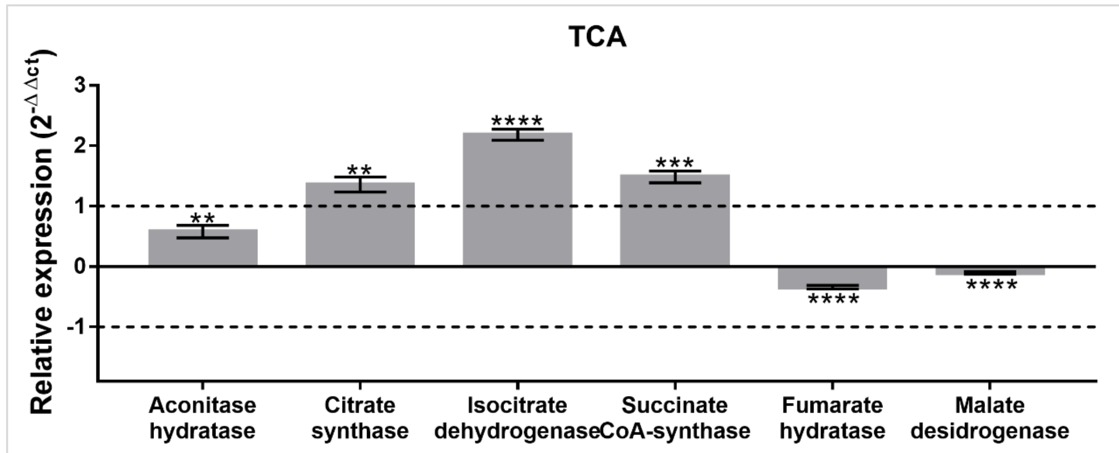

Figure 4: Transcriptional profile of tricarboxylic acid cycle of maize roots inoculated with *H. seropedicae*. The dotted line represents the expression value of the control sample. Means marked by asterisk differ from control condition (* p value < 0.01; ** p value < 0.05; *** p value < 0.005; **** p value < 0.0001). Bars represent the standard error (n = 3).

3.4. Changes in rhizospheric H^+ fluxes and pH

Inoculation of *H. seropedicae* influenced the H^+ flux and pH on maize root rhizosphere. The superficial flux of H^+ on the root cap and root hair zone without inoculation demonstrated an influx of H^+ that decreased with *H. seropedicae*. In the elongation zone, a slight efflux of H^+ could be observed without the effect of the inoculation, which changed to a higher efflux after the inoculation (Figures 5A and B).

These changes in H^+ flux in the maize roots by the *H. seropedicae* inoculation altered the pH across the root surface. In the root cap and root hair zone, where there was a decrease in influx, the pH in these zones became less acidic by the presence of *H. seropedicae*. However, the opposite also applied to the elongation zone, where the efflux of protons increased, making this zone more acidic (Figure 5C).

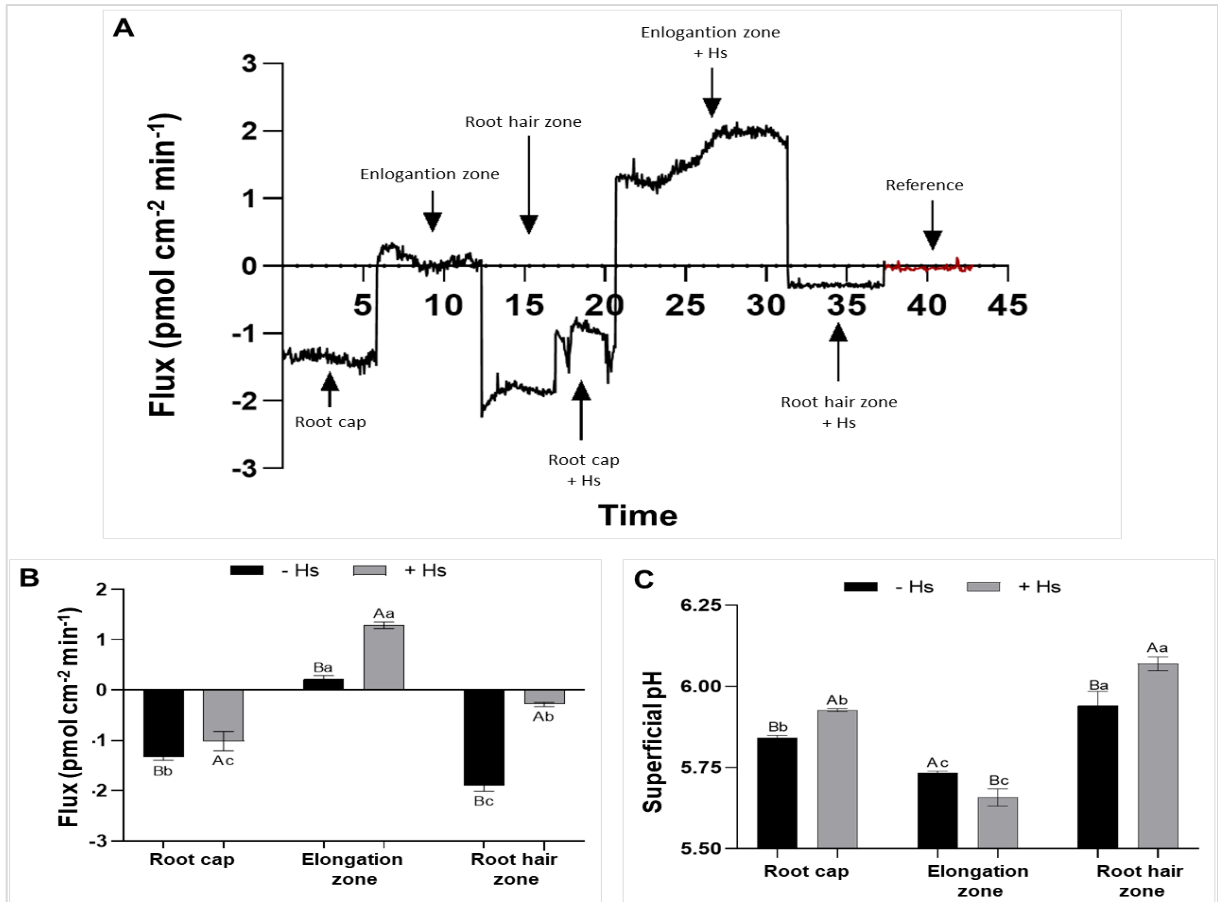

Figure 5: Rhizospheric changes in the regions of the root cap, elongation zone and root hairs of maize plants before and after *H. seropedicae* inoculation. **A**) Profile of extracellular H^+ flux. (**B**) Analysis of extracellular H^+ flux (**C**) Surface pH analysis. Bars represent mean ± SD values from three independent experiments. For the conditions of inoculation with the bacterium H. seropedicae, the bars followed by the same capital letter are not significantly different by the Tukey test at $p<0.05$. For each measurement region, bars followed by the same lowercase letter are not significantly different by Tukey's test at $p<0.05$ (n=50).

3.5. Rhizoplane colonization dynamics of the root axis

Scanning electron microscopy allows the visualization of the colonization profile of maize root rhizoplane over time, with the characteristic curved shape of *H. seropedicae* could being observed. After 30min of inoculation, the bacteria were observed along all the root zones, in apolar adhesion, arranged individually. In the root cap, after 30 min, a few bacteria could be observed adhered to the surface (Figure 6A). An increase in bacterial number adhered to the rot cap could be observed within 2 and 4h culminating in bacterial aggregates in biofilms after 24 hours of inoculation (Figure 6B-D).

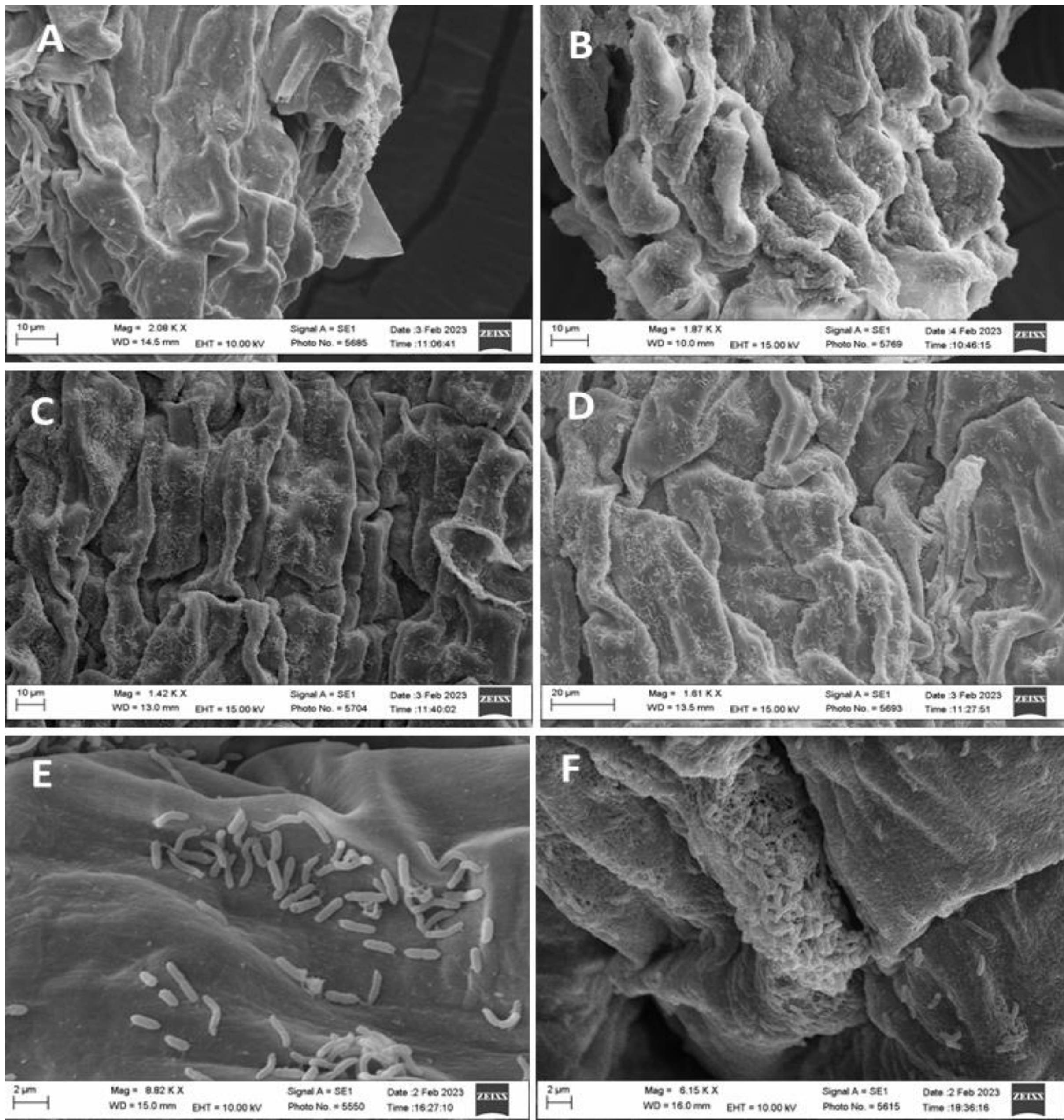

Figure 6: SEM view of maize root cap segments inoculated with *H. seropedicae* strain HRC54. (**A**) General view showing very few single bacteria cells anchored by apolar attachment to root cap surface at 30 minutes after inoculation; (**B-D**) General view showing an increased number of bacteria cells anchored by apolar attachment to root cap surface until 24-h after inoculation; (**E**) Detailed view of the pattern of cell distribution in the earlier time (30 min) and (**F**) Detailed view of the pattern of cell distribution in the later time (24-h). Note an increase in bacteria cell density and cell aggregation with time.

The elongation and differentiation zone after 30min of inoculation was highly colonized by single cells of *H. seropedicae* (Figure 7A-C). Over the hours, an increase in the bacterial population in the monolayer was visible (Figure 7b-D). After 24 hours of inoculation, high bacterial density in aggregates could be observed colonizing the elongation zone (Figure 7F).

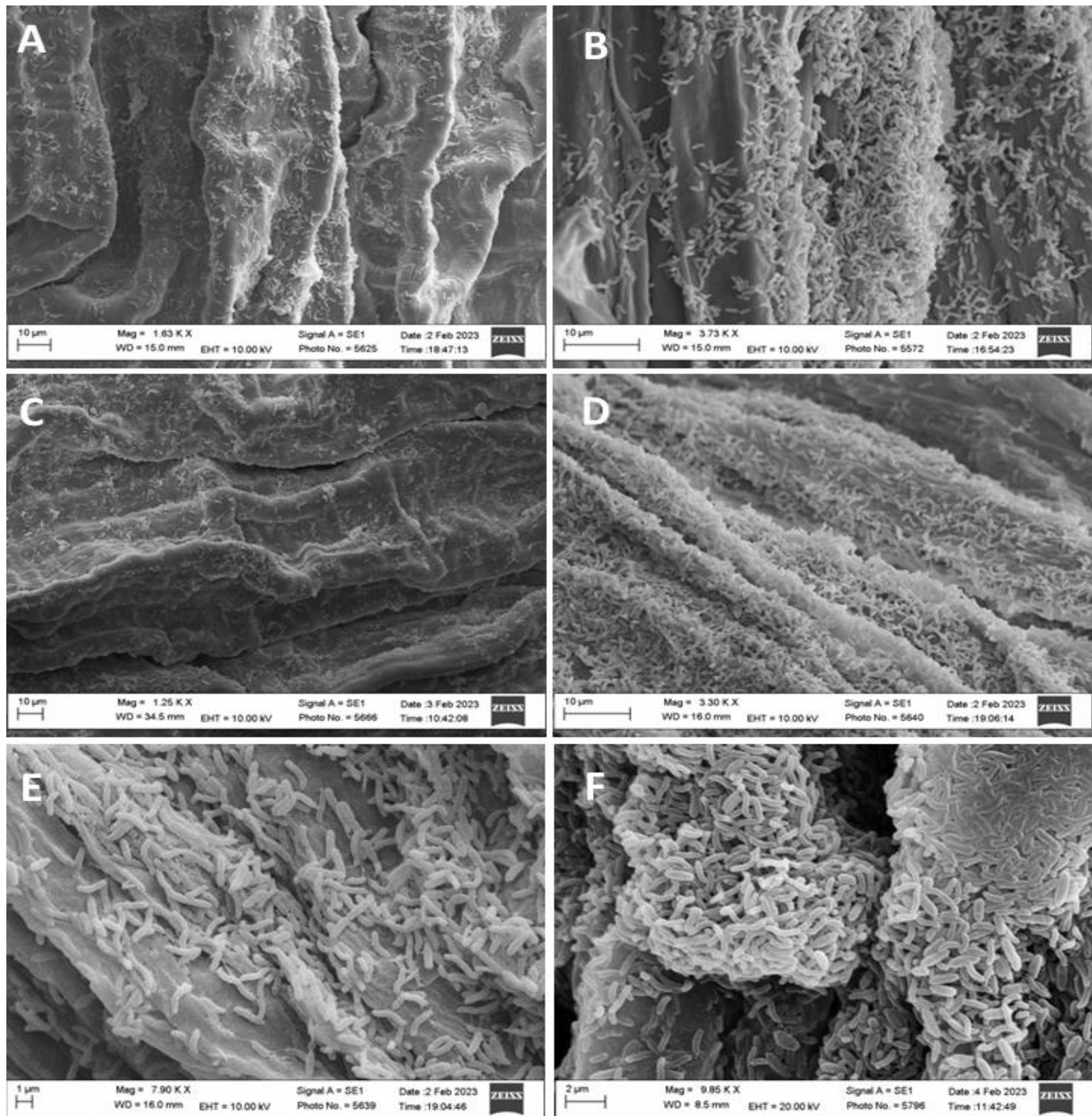

Figure 7: SEM view of maize root elongation-differentiation region segments inoculated with *H.seropedicae* strains HRC54. (**A-C**) General view showing high frequency of single bacteria cells anchored by apolar attachment to root elongation zone at 30 minutes after inoculation; (**B-D**) General view showing highest frequency of aggregated bacteria cells (compared to B-D) anchored by apolar attachment to root elongation zone at 24 hours after inoculation; (**E**) Detailed view of the pattern of cell distribution in the earlier time (4 hours) and (**F**) Detailed view of the pattern of cell distribution in the later time (24-h). Note an increase in bacteria cell density and cell aggregation with time.

The root-hair zone did not present bacterial cells adhered to the hoot hairs, only in the pilus base after 30min of inoculation (Figure 8A-B). However, the number of bacterial cells of *H. seropedicae* adhered to the root-hair zone increased after 4h of

inoculation (Figure 8C) and after 24 hours, it was possible to observe bacterial aggregates (Figures 8E-F).

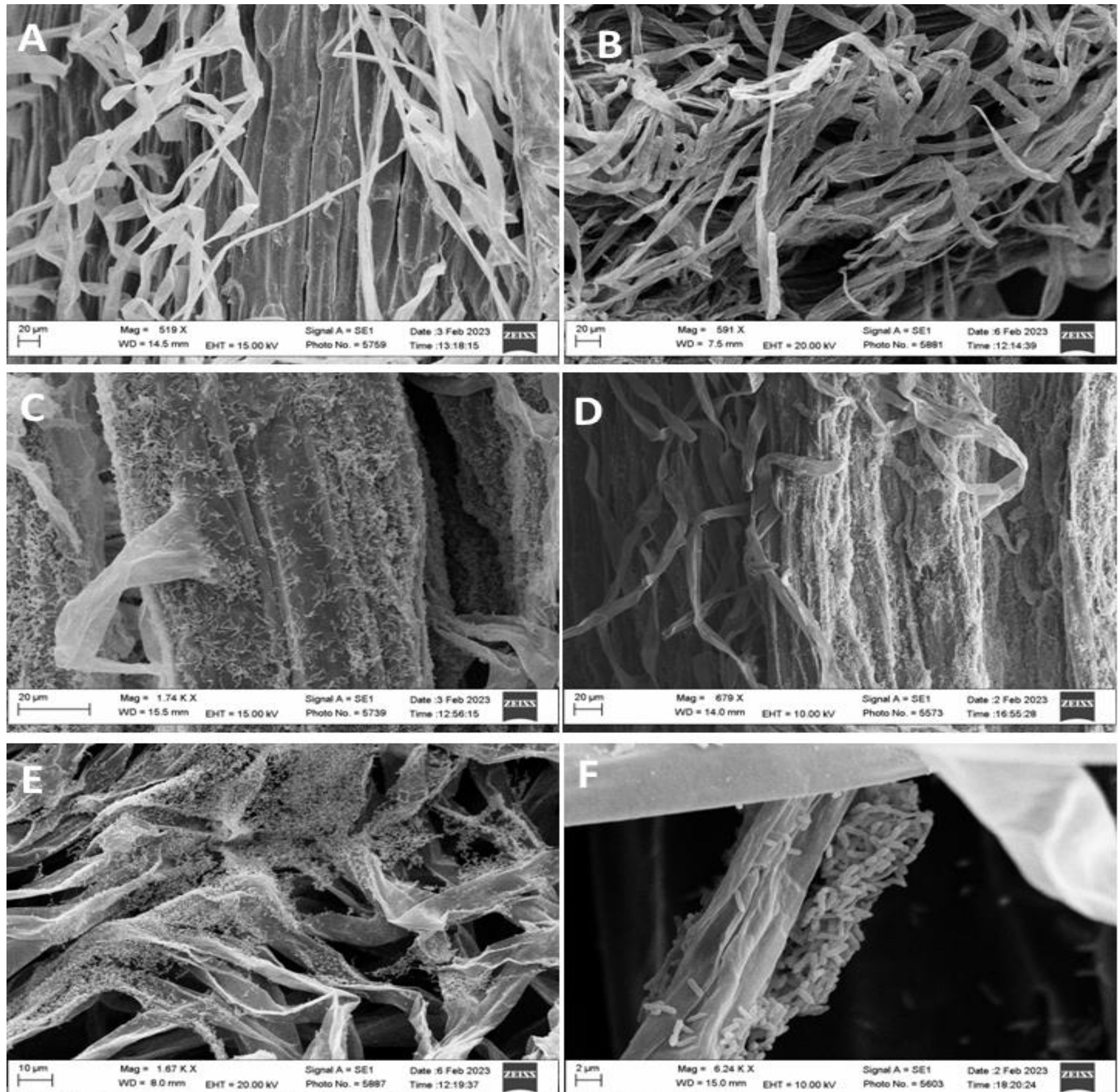

Figure 8: SEM view of maize root-hair region segments inoculated with *H. seropedicae* strains HRC54. (**A**) General view showing very few bacteria cells attached to the ordinary epidermal cells anchored by apolar attachment to root elongation zone at 30 minutes after inoculation; (**B**) General view showing no bacteria cells attached to the root hair surface at 30 minutes after inoculation; (**C**) Increased bacteria cell density on epidermal plant cell at the bottom of the root hair (4-h after inoculation; (**D**) Increased bacteria cell density on epidermal plant cell at the bottom of the root hair (24-h after inoculation and (**E**) in the surface of mature root hairs (24-h after inoculation); (**F**) Detailed view of the pattern of cell distribution in the later time (24-h) on the surface of a root hair.

The frequency of bacterial colonization followed a pattern where *H. seropedicae* preferred to colonize the elongation and differentiation zone compared to the other regions (Figure 9).

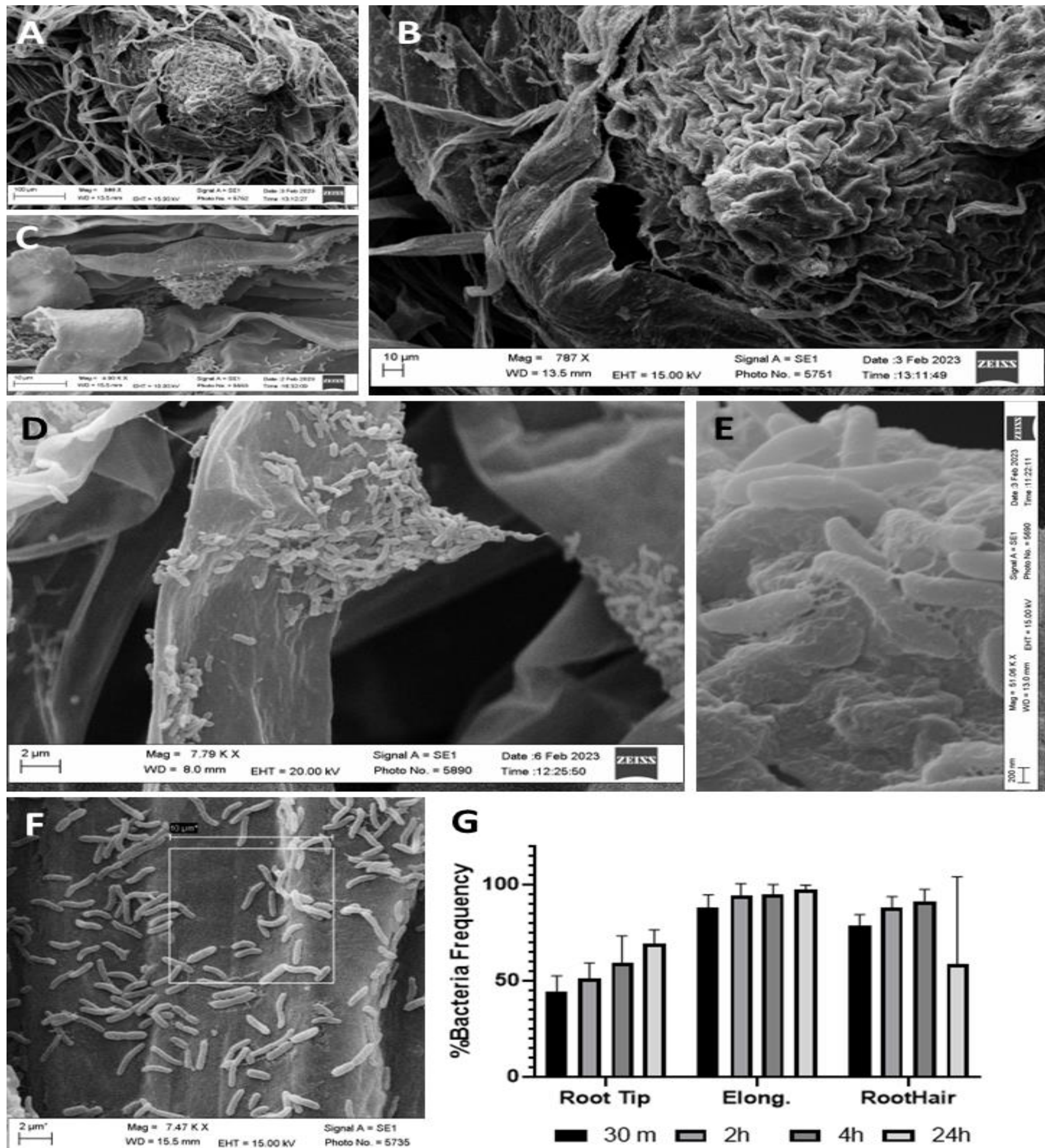

Figure 9: SEM view of maize root inoculated with *H. seropedicae* strains HRC 54 (**A**) General view showing lateral root emission; (**B**) Detail of (**A**) showing bacteria aggregates close to root tissue rupture and root tip of the new emerging root; (**C-D**) Bacteria aggregates to biofilms in root hairs; (**E**) bacteria cell fibrils connection; (**F**) 100 µm^2 square used to estimate the frequency of bacteria present in different regions of the root axis and (**G**) percentage of positive observation per region and time (n=30).

3.6. Maize root bacteriome modulation

The influence of *H. seropedicae* inoculation on the maize root bacterial community was analyzed by Illumina Miseq sequencing. A total of 104.332 reads were obtained from six samples.

Differences in the beta diversity (bacterial diversity between different samples) of maize root after the *H. seropedicae* inoculation were obtained by non-metric multidimensional scaling (NMDS) and visualized by principal coordinate analysis (PCoA) (Figure 10A). Inoculated samples were grouped without the influence of control samples and indicated changes in the bacteriome of the roots at the genus level (Figure 10B).

Alpha diversity (bacterial diversity within each sample) revealed that the species observed, and Shannon diversity of inoculated maize roots was more significant than the control roots but not significantly (Figure 10C).

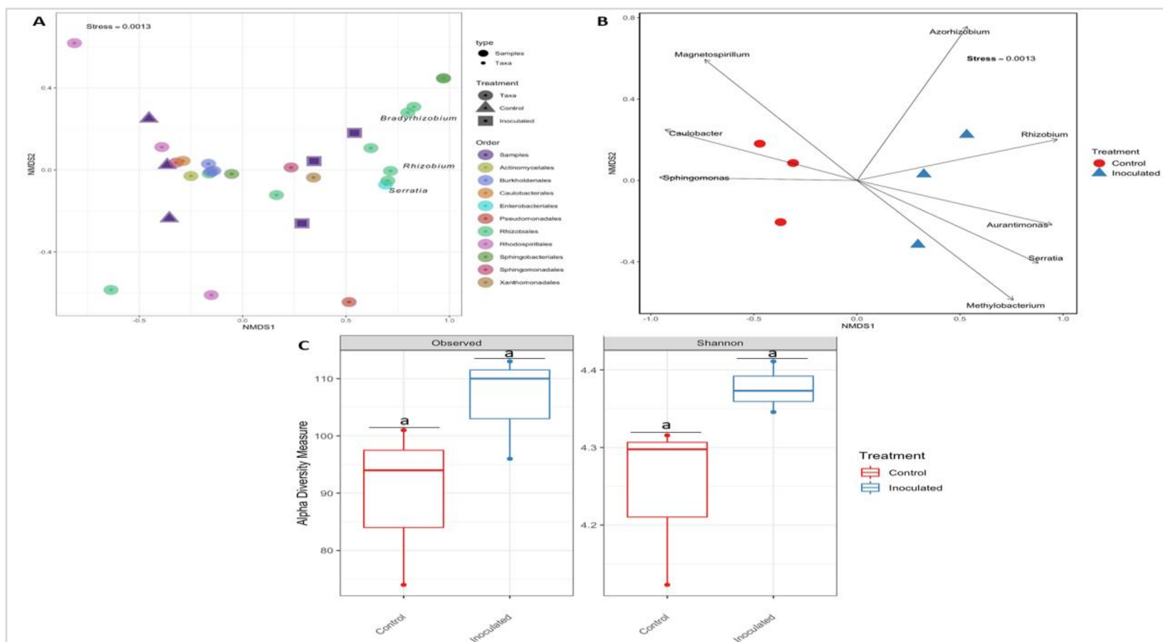

Figure 10: Beta and Alfa diversity. (**A**). Non-metric multidimensional scaling (NMDS) of the Bray–Curtis distance presents two distinct clusters of the root's bacteriome. Triangles and squares represent a microbial community from Control and Inoculated samples. Each circle represents a microbial genus color-coded by microbial order. Important genera dislocated by the inoculation are annotated in the figure. (**B**) The fitted vectors are arrows with the following interpretation: The arrow points to the direction of the most rapid change in the genus. Often this is called the direction of the gradient. The length of the arrow is proportional to the correlation between ordination and environmental variable. Only a subset of significant (p-value 0.05) variables are shown. (**C**) Alpha diversity measure. Shannon index and observed number of species. The bars followed by the same capital letter are not significantly different by the Kruskal-Wallis followed by Pairwise comparisons using the Wilcoxon test.

Inoculation of *H. seropedicae* in maize seedlings altered the root bacteriome at different taxonomic levels. These modulations were presented in a box plot with

centered log ratio (clr) transformed abundance values. The inoculation reduced the abundance of the Proteobacteria phylum and slightly stimulated bacteria of the phylum Actinobacteria and Bacteroidetes (Figure 11).

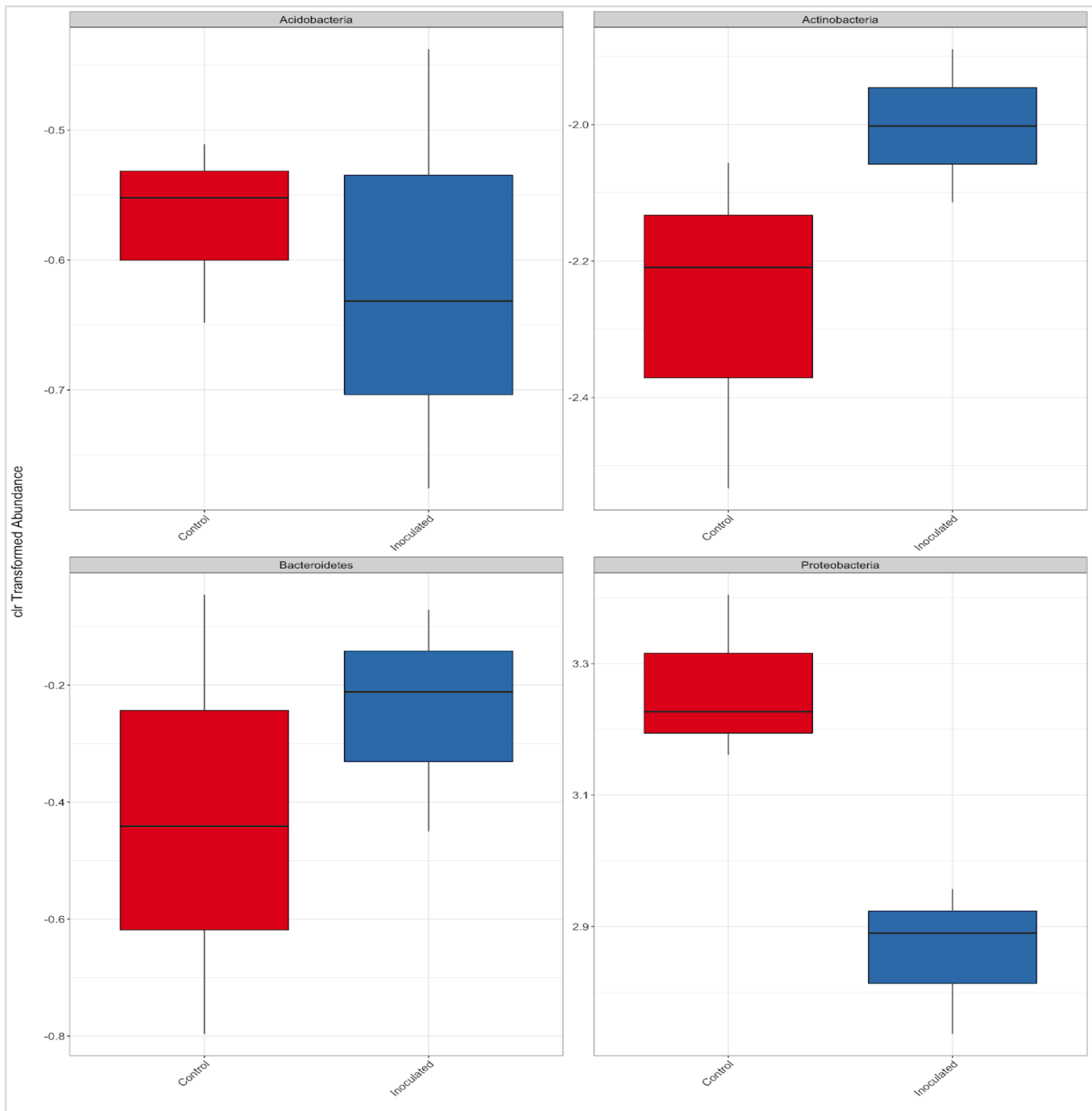

Figure 11: Modulation of phylum abundance of maize roots bacteriome by the inoculation of *H. seropedicae*. Box plots are organized according to treatment and facet by microbial phylum using the centered log-ratio transformation of the rarefied raw sequence counts. Boxes denote the interquartile range; the horizontal line inside the boxes represents the median.

At the class level, the abundance of Alphaproteobacteria and Betaproteobacteria was reduced by the inoculation of *H. seropedicae*, and the class of Actinobacteria and Gammaproteobacteria were induced by the inoculation (Figure 12).

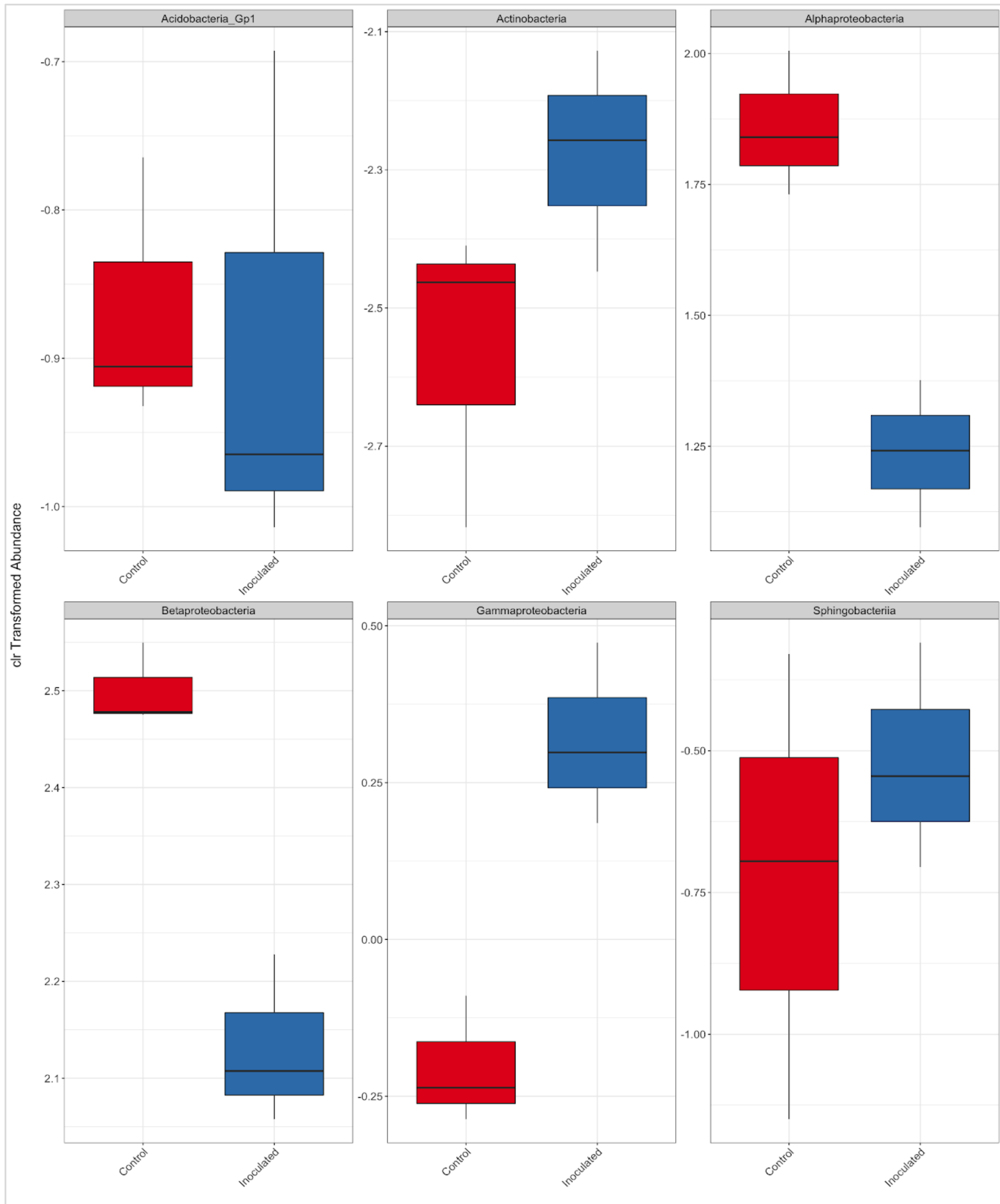

Figure 12: Modulation of class abundance of maize roots bacteriome by the inoculation of *H. seropedicae*. Box plots are organized according to treatment and facet by microbial Class using the centered log-ratio transformation of the rarefied raw sequence counts. Taxa not seen more than 3 times in at least 20% of the samples were removed to protect against an OTU with small mean and trivially large C.V. Boxes denote the interquartile range; the horizontal line inside the boxes represents the median.

The structure of the bacteriome at the order level demonstrated that inoculation reduced the abundance of Burkholderiales, Caulobacteriales, Rhodociales, Rhodospirillales, and Sphingomonadales order but increased the abundance of

Actinomycetales, Enterobacteriales, Pseudomonadales, Rhizobiales, and Xanthomonadales order (Figure 13).

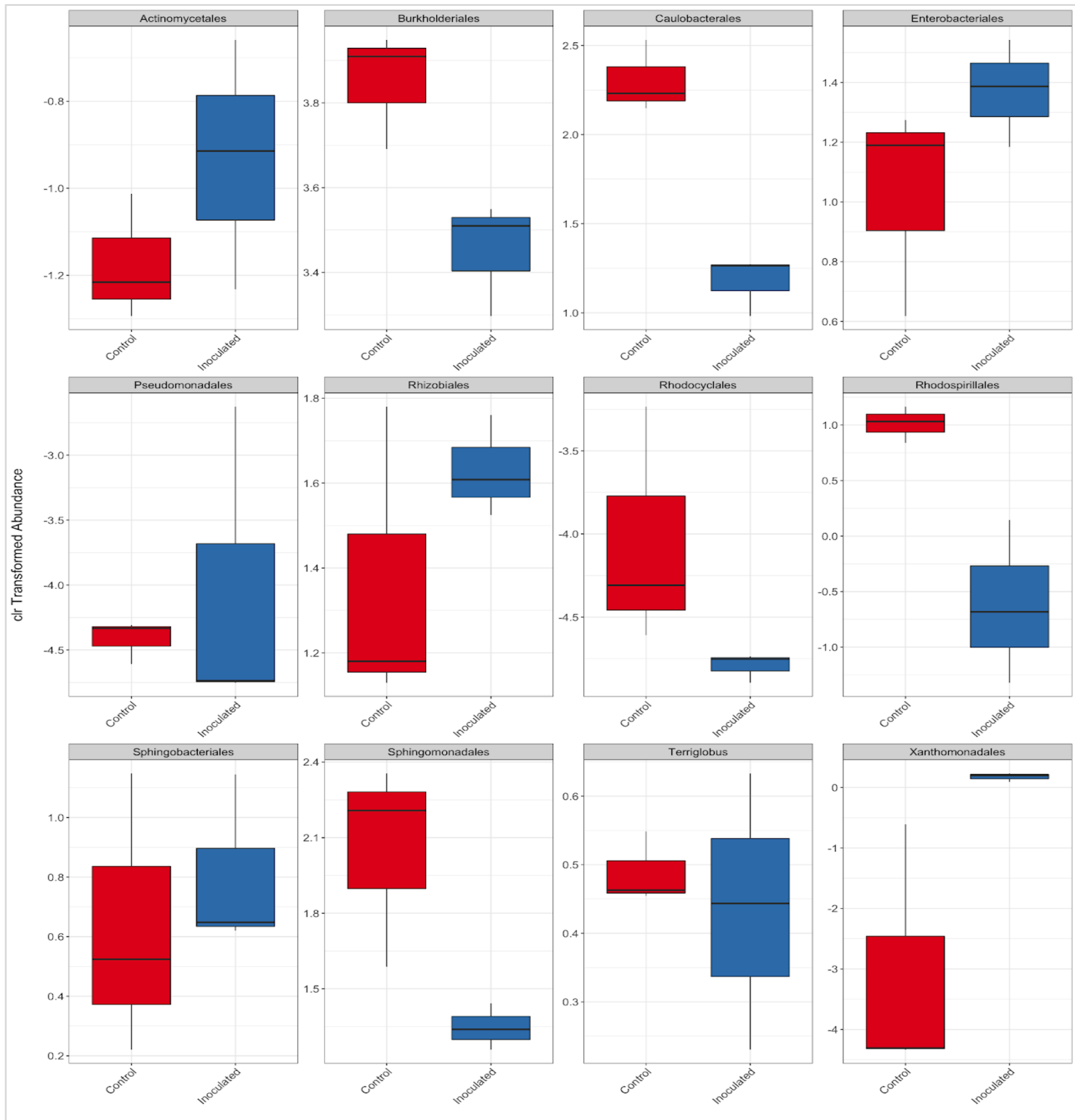

Figure 13: Modulation of order abundance of maize roots bacteriome by the inoculation of *H. seropedicae.* Box plots are organized according to treatment and facet by microbial Order using the centered log-ratio transformation of the rarefied raw sequence counts. Taxa not seen more than 3 times in at least 20% of the samples were removed to protect against an OTU with small mean and trivially large C.V. Boxes denote the interquartile range; the horizontal line inside the boxes represents the median.

The inoculation modulated the bacteriome composition at the family level with a reduction of Acetobacteriaceae, Bradyrhizobiaceae, Caulobacteriaceae, Comamonadaceae, Enterobacteriaceae, Microbacteriaceae, Oxalobacteriaceae,

Rhodocyclaceae, Rhodospirillaceae, Sphingobacteriaceae and, Sphingomonodaceae families, and by the increase of Aurantimonadaceae, Methylobacteriaceae, Phyllobacteriaceae, Rhizobiaceae, Xanthobacteriaceae, and Xanthomonadaceae families (Figure 14).

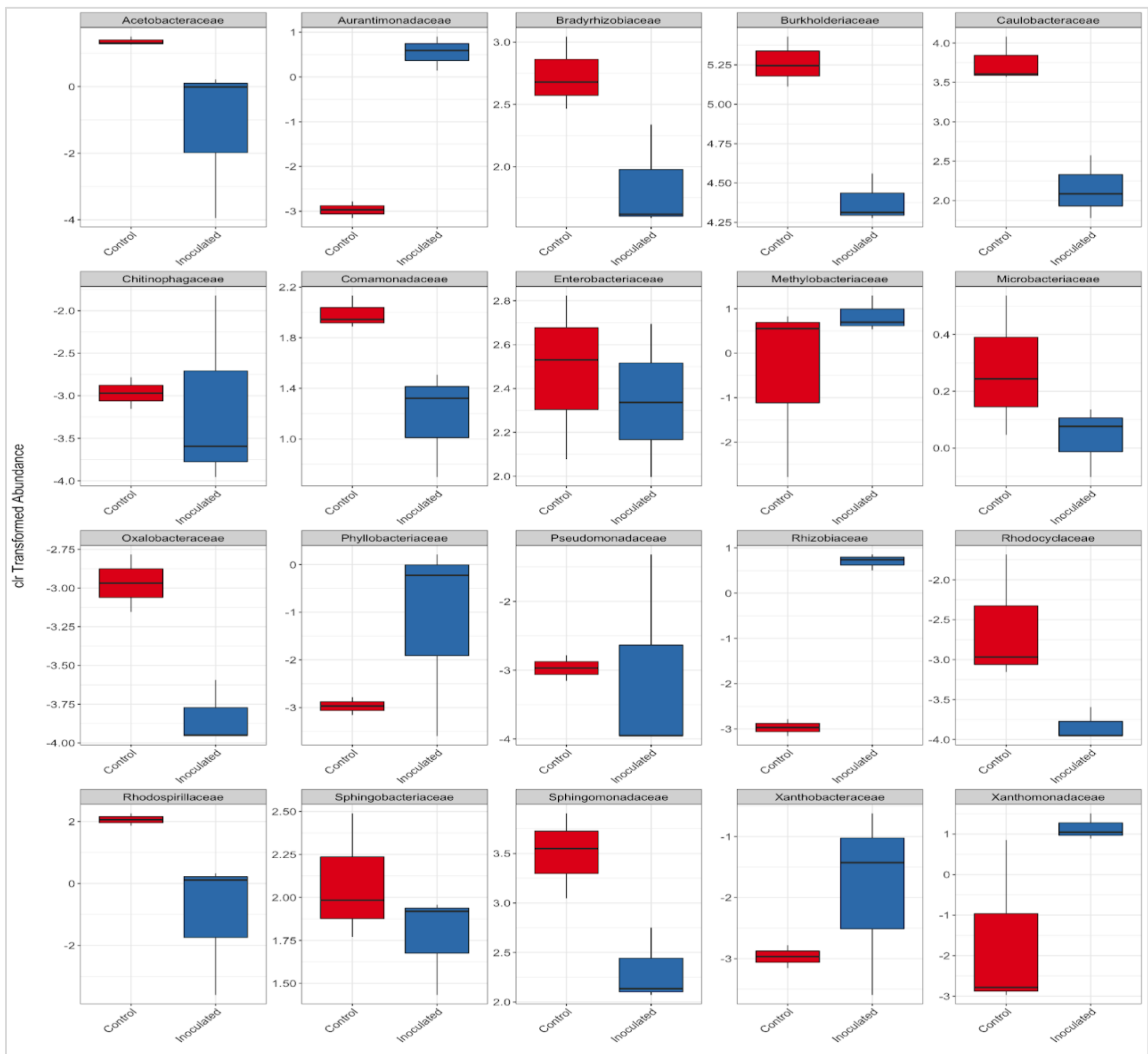

Figure 14: Modulation of family abundance of maize roots bacteriome by the inoculation of *H. seropedicae.* Box plots are organized according to treatment and facet by microbial Family using the centered log-ratio transformation of the rarefied raw sequence counts. Taxa not seen more than 3 times in at least 20% of the samples were removed to protect against an OTU with small mean and trivially large C.V. Boxes denote the interquartile range; the horizontal line inside the boxes represents the median.

Among the genera identified by sequencing, the abundance of *Aurantimonas*, *Mesorhizobium*, *Novosphingobium*, *Rhizobium*, *Serratia*, and *Stenotrophomonas* was increased by the inoculation of *H. seropedicae*. On the other hand, other genera, such

as *Bosea*, *Bradirhizobium*, *Burkholderia*, *Caulobacter*, *Enterobacter*, *Gluconacetobacter*, *Magnetospirillum*, *Pelomonas*, *Ralstonia*, *Sphingomonas* had their abundance reduced by the inoculation (Figure 15). All genera shared among treatments are described in Table 2. Table 3 contains the Genus differential abundance between the treatments.

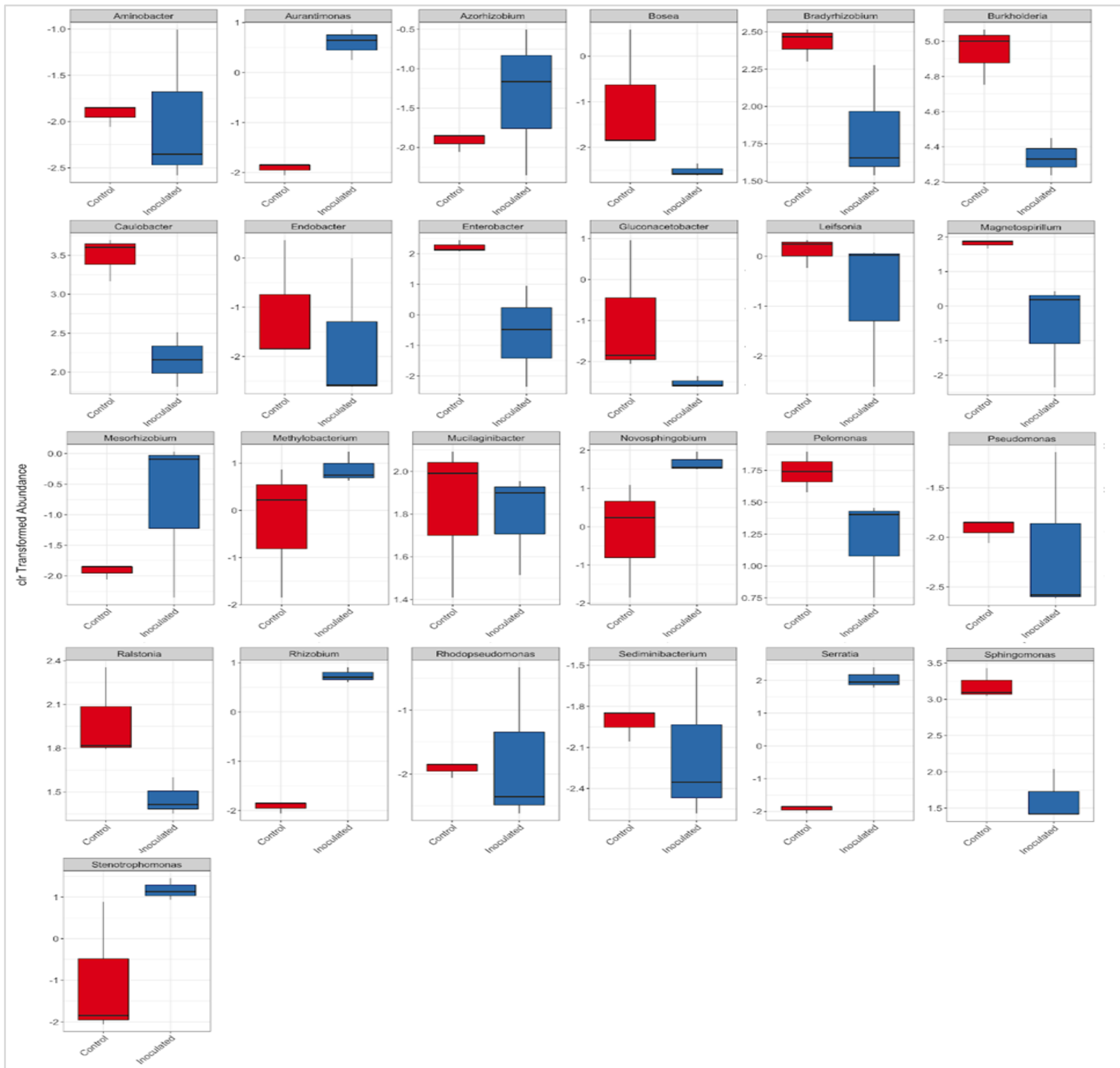

Figure 15: Modulation of genera abundance of maize roots bacteriome by the inoculation of *H. seropedicae*. Box plots are organized according to treatment and facet by microbial genus using the centered log-ratio transformation of the rarefied raw sequence counts. Taxa not seen more than 3 times in at least 20% of the samples were removed to protect against an OTU with small mean and trivially large C.V. Boxes denote the interquartile range; the horizontal line inside the boxes represents the median.

Table 2: List of Genera shared among treatments.

Phylum	**Class**	**Order**	**Family**	**Genus**	**Control**	**Inoculated**
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*	15960	15606
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Caulobacter*	3751	1781
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	*Magnetospirillum*	681	190
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	2794	1034
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Pelomonas*	641	684
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Serratia*	0	1572
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	80	646
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	151	1073
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Ralstonia*	876	876
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	0	364
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	1297	1277
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Mucilaginibacter*	742	1228
Acidobacteria	Acidobacteria_Gp1	Terriglobus	NA	NA	606	841
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*	1048	228
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*	0	418

Proteobacteria	Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	*Aurantimonas*	0	357
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	NA	194	94
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	*Methylobacterium*	127	479
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	NA	0	87
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Leifsonia*	112	125
Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium*	0	132
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Gluconacetobacter*	88	0
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bosea*	73	0
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Endobacter*	57	52
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Rhodopseudomonas*	0	46
Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	*Azorhizobium*	0	57
Proteobacteria	Alphaproteobacteria	NA	NA	NA	0	29
Proteobacteria	Gammaproteobacteria	NA	NA	NA	41	0
Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Aminobacter*	0	22
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Sediminibacterium*	0	11
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	0	13

Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	NA	3	0

Table 3: Genus differential abundance

Genus	rab.all [a]	rab.win.Control [b]	rab.win.Inoculated [c]	diff.btw [d]	diff.win [e]	Effect [f]	Overlap [g]	we.ep [h]
Sphingomonas	6.0032615	7.111431	3.331728	-3.610247	0.9902360	-3.376087	0.0003653998	0.01576662
Caulobacter	6.1790496	7.617294	4.269644	-3.170432	1.1154855	-2.843619	0.0052331958	0.01892268
Serratia	1.4302729	-5.107492	4.029571	9.610914	2.6921318	3.628195	0.0003653998	0.02200195
Ralstonia	4.4022238	5.396473	3.408144	-1.956094	0.6855116	-2.968396	0.0052331958	0.02228642
Burkholderia	8.6239344	9.675232	7.496809	-2.097447	0.8108816	-2.412213	0.0104432569	0.02982281
Aurantimonas	0.4429797	-4.534893	2.044028	6.662575	2.3116269	2.900641	0.0003653998	0.03338331
Rhizobium	0.8084178	-4.856286	2.373071	7.477315	2.5438001	2.989902	0.0003653998	0.03558587

Legend: [a] median clr value for all samples in the feature; [b] rab.win.x - median clr value for the group x; [c] rab.win.y - median clr value for the group y; [d] dif.btw - the median difference in clr values between x and y groups; [e] dif.win - the median of the largest difference in clr values within x and y groups; [f] effect - median effect size: dif.btw / max(dif.win) for all instances; [g] overlap - proportion of effect size that overlaps 0 (i.e., no effect); [h] we.ep - Expected P value of Welch's t-test.

4. Discussion

In this study, we investigated the effects of *H. seropedicae* inoculation on maize plants, the changes made by the plant roots to establish an interaction with the inoculated bacteria, and how this interaction can modulate the entire natural bacteriome of the seed.

The presence of *H. seropedicae* in the root tissue five days after the inoculation could be confirmed by PCR. This interaction altered transcriptional and proteomic profiles in TCA metabolism, corroborating the findings of Canellas; Olivares; Canellas (2019), where the inoculation increased the regulation of proteins involved in this pathway. The transcriptional profile of rice roots inoculated with *H. seropedicae* also stimulated TCA enzymes (BRUSAMARELLO-SANTOS et al., 2019).

As it is known, *H. seropedicae* is a diazotrophic bacteria that can provide N for plants by biological nitrogen fixation (PIMENTEL et al., 1991; MATTEOLI et al., 2020). Maize plants inoculated with *H. seropedicae* demonstrated an increase in the transcription of nitrate transporters (AZEVEDO et al., 2019; HARDOIM et al., 2020; IRINEU et al., 2023), improving the uptake of this nutrient by the plant, resulting in increased N content, as described by Ramos et al. (2020) in rice plants.

In our previous work (IRINEU et al., 2023), we show that the balance in the C/N ratio through the crosstalk of N metabolism and the TCA, suggesting that N coming from the N fixation by the inoculation of *H. seropedicae*, and also assimilated in the form of glutamate, was incorporated into other amino acids, and these were able to supply carbon to TCA, integrating the metabolic pathways.

Here, we show that inoculation of *H. seropedicae* influenced the superficial H^+ flux, pH on maize root, and changes in the H^+-ATPase accumulation on protein level. Furthermore, data obtained by inoculating *H. seropedicae* in maize and rice proved

that the plant-*Herbaspirillum* interaction stimulated root H^+-ATPase (AZEVEDO et al., 2019; RAMOS et al., 2020).

It has been demonstrated that the influx of H^+ is coupled with the transport of NO_3^- and that the hyperpolarization of the transmembrane electrochemical gradient favors nutrients' movement across the membranes. For these reasons, the enhanced H^+ extrusion by PGPB inoculation might be crucial in the nutritional process (PII et al., 2015).

As Erro et al. (2009) described, maize roots can exudate organic acids provided by TCA, and according to Balasubramanian et al. (2021), roots can significantly change the rhizosphere pH by the release of H^+ for compensate organic acids exudation, that is exuded in anion form. Our data suggest the regulation of proteins and genes related to TCA, possibly indicating the exudation of organic acids in the rhizosphere. Interestingly, the bacterial frequency in the root regions was higher in the elongation zone, where the H^+ efflux was increased, decreasing the pH.

These effects on the root surface and rhizosphere can promote changes in the root bacteriome. Here, root sequencing by Illumina MiSeq revealed that inoculation modulated the bacteriome since the roots inoculated showed high bacterial diversity by Shannon index and Observed index, although statistically insignificant values.

Among the bacteria, groups identified by sequencing are from the genera *Bosea*, *Bradyrhizobium*, *Burkholderia*, *Caulobacter*, *Enterobacter*, *Gluconacetobacter*, *Magnetospirillum*, *Pelomonas*, *Ralstonia*, and *Sphingomonas* had their abundance reduced by the inoculation. This reduction could be explained by the niche theory, which predicts that the microbial diversity of plants is determined by the number of available niches (HARDOIM et al., 2012).

Here, the high number of *H. seropedicae* cells inoculated in the plants may have occupied the possible niches that these genera would have occupied, suppressing them. Also, the modifications that happened on the maize root surface and rhizosphere, due to changes in pH and the possible exudation of organic acids, may favor the increase of the *H. seropedicae* population, suppressing other genera.

Interestingly, the inoculation of *H. seropedicae* increased the abundance of bacteria from the Proteobacteria phylum. Some plant species are evidence for different vertical transmission of seed-borne endophytes, mainly belonging to Proteobacteria (HACQUARD et al., 2015). Among the genera identified by sequencing and increased by the inoculation, we can observe the presence of *Mesorhizobium, Novosphingobium, Serratia,* and *Stenotrophomonas,* all these genera possessing endophytic species with characteristics of PGPB (FILIPINI et al., 2021; LARANJEIRA et al., 2021; MARTÍNEZ-CANO et al., 2022; MATSUMURA et al., 2015; REIS et al., 2021; WANG et al., 2021; WOŹNIAK et al., 2019).

5. Conclusion

Overall results indicate that maize plants inoculated with *H. seropedicae* induce root metabolic changes on the TCA cycle that probably influence the root exudation combined with the regulation of H^+-ATPases altering the H^+ flux and pH of the root surface and rhizosphere, which contribute to *H. seropedicae* adhesion and colonization. Together, these changes contribute to alterations in the natural bacteriome of the seeds, selecting beneficial PGPB that can collaborate with the growth and development of the plants.

These findings allow the understanding of host-bacteria communication and how the plant changes its metabolism to select the microbiota associated, seeking the best collaborators for its development.

REFERENCES

ALMEIDA, F. A. et al. LED lamps enhance somatic embryo maturation in association with the differential accumulation of proteins in the *Carica papaya* L. 'Golden' embryogenic callus. **Plant Physiology and Biochemistry**, v. 143, n. February, p. 109–118, 2019.

Anderson, M.J. (2017). Permutational Multivariate Analysis of Variance (PERMANOVA). In **Wiley StatsRef: Statistics Reference Online** (eds N. Balakrishnan, T. Colton, B. Everitt, W. Piegorsch, F. Ruggeri and J.L. Teugels). 2017. p. 1–15. Disponível em: https://doi.org/10.1002/9781118445112.stat07841

APPRILL, A. et al. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. **Aquatic Microbial Ecology**, v. 75, n. 2, p. 129–137, 2015.

AZEVEDO, I. G. DE et al. Humic acids and *Herbaspirillum seropedicae* change the extracellular H^+ flux and gene expression in maize roots seedlings. **Chemical and Biological Technologies in Agriculture**, v. 6, n. 1, 2019.

BADRI, D. V.; VIVANCO, J. M. Regulation and function of root exudates. **Plant, Cell and Environment**, v. 32, n. 6, p. 666–681, 2009.

BALASUBRAMANIAN, V. K. et al. Molecular Mechanisms of Plant–Microbe Interactions in the Rhizosphere as Targets for Improving Plant Productivity. Em: VADAKATTU, V. S. R. G.; SHARMA, A. K. (Eds.). **Rhizosphere Biology: Interactions Between Microbes and Plants**. Singapore: Springer Nature, 2021. p. 295–335.

BALDANI, J. I. et al. Emended Description of *Herbaspirillum*; Inclusion of [*Pseudomonas*]*rubrisubalbicans*, a Mild Plant Pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and Classification of a Group of Clinical Isolates (EF Group 1) as *Herbaspirillum* Species 3. **INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY**, v. 46, n. 3, p. 802–810, 1996.

BALDANI, J. I. et al. The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. **Plant and Soil**, v. 384, n. 1–2, p. 413–431, 2014.

BALDOTTO, L. E. B.; OLIVARES, F. L.; BRESSAN-SMITH, R. Structural interaction between gfp-labeled diazotrophic endophytic bacterium *Herbaspirillum seropedicae* RAM10 and pineapple plantlets "vitória". **Brazilian Journal of Microbiology**, v. 42, p. 114–125, 2011

BASTIÁN, F. et al. Production of indole-3-acetic acid and gibberellins A 1 and A 3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically-defined culture media. **Plant Growth Regulation**, v. 24, p. 7–11, 1998.

BERGAMESHI, C. et al. Occurrence of diazotrophic bacteria associated with forage sorghum cultivars. **Ciência Rural**, v. 37, n. 3, p. 727–733, 2007.

BOTTINI, R.; CASSÁN, F.; PICCOLI, P. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. **Applied Microbiology and Biotechnology**, v. 65, n. 5, p. 497–503, 2004.

BRUSAMARELLO-SANTOS, L. C. C. et al. Modulation of defence and iron homeostasis genes in rice roots by the diazotrophic endophyte *Herbaspirillum seropedicae*. **Scientific Reports**, v. 9, n. 1, 2019.

BURRIEZA, H. P. et al. Shotgun proteomic analysis of quinoa seeds reveals novel lysine-rich seed storage globulins. **Food Chemistry**, v. 293, p. 299–306, 2019.

CANELLAS, L. P. et al. A combination of humic substances and *Herbaspirillum seropedicae* inoculation enhances the growth of maize (*Zea mays* L.). **Plant and Soil**, v. 366, n. 1–2, p. 119–132, 2013.

CANELLAS, N. O. A.; OLIVARES, F. L.; CANELLAS, L. P. Metabolite fingerprints of maize and sugarcane seedlings: searching for markers after inoculation with plant growth - promoting bacteria in humic acids. **Chemical and Biological Technologies in Agriculture**, v. 6, n. 14, p. 1–10, 2019.

CHEN, D.-H.; RONALD, P. C. A Rapid DNA Minipreparation Method Suitable for AFLP and Other PCR Applications. **Plant Molecular Biology Reporter**, v. 17, p. 53–57, 1999.

CONESA, A. et al. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. **Bioinformatics**, v. 21, n. 18, p. 3674–3676, 2005.

DAMERVAL, C. et al. Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. **Electrophoresis**, v. 7, n. 1, p. 52–54, 1986.

DISTLER, U. et al. Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. **Nature Methods**, v. 11, n. 2, p. 167–170, 2014.

DISTLER, U. et al. Label-free quantification in ion mobility-enhanced data-independent acquisition proteomics. **Nature Protocols**, v. 11, n. 4, p. 795–812, 2016.

DÖBEREINER, J.; BALDANI, V. L. D.; BALDINI, J. I. **Como Isolar e identificar bactérias diazotróficas de plantas não-leguminosas**. EMBRAPA-SPI, 1995. v. 136

DOYLE, J. J.; DOYLE, J. L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. **Phytochemical Bulletin**, v. 19, n. 1, p. 11–15, 1987.

EDGAR, R. C. et al. UCHIME improves sensitivity and speed of chimera detection. **Bioinformatics**, v. 27, n. 16, p. 2194–2200, 2011.

ERRO, J. et al. Determination of organic acids in tissues and exudates of maize, lupin, and chickpea by high-performance liquid chromatography-tandem mass spectrometry. **Journal of Agricultural and Food Chemistry**, v. 57, n. 10, p. 4004–4010, 2009.

ESTRADA, G. A. et al. Selection of phosphate-solubilizing diazotrophic *Herbaspirillum* and *Burkholderia* strains and their effect on rice crop yield and nutrient uptake. **Plant and Soil**, v. 369, n. 1–2, p. 115–129, 2013.

FILIPINI, L. D. et al. Application of *Azospirillum* on seeds and leaves, associated with *Rhizobium* inoculation, increases growth and yield of common bean. **Archives of Microbiology**, v. 203, n. 3, p. 1033–1038, 2021.

GLOOR, G. B.; REID, G. Compositional analysis: A valid approach to analyze microbiome high-throughput sequencing data. **Canadian Journal of Microbiology**, v. 62, n. 8, p. 692–703, 2016.

HACQUARD, S. et al. Microbiota and host nutrition across plant and animal kingdoms. **Cell Host and Microbe,** v. 17, p. 603-616, 2015.

HARDOIM, P. R. et al. Dynamics of seed-borne rice endophytes on early plant growth stages. **PLoS ONE**, v. 7, n. 2, 2012.

HARDOIM, P. R. et al. Genome-wide transcriptome profiling provides insights into the responses of maize (*Zea mays* L.) to diazotrophic bacteria. **Plant and Soil**, v. 451, n. 1–2, p. 121–143, 2020.

HINSINGER, P. et al. Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: A review. **Plant and Soil**, v. 248, p. 43-59, 2003.

HINSINGER, P. et al. Rhizosphere: Biophysics, biogeochemistry and ecological relevance. **Plant and Soil**, v. 321, n. 1–2, p. 117–152, 2009.

IRINEU, L. E. S. DA S. et al. Multiomic approaches reveal hormonal modulation and nitrogen uptake and assimilation in the initial growth of maize inoculated with *Herbaspirillum seropedicae*. **Plants**, v. 12, n. 1, 2023.

JAMES, E. K. et al. Infection and Colonization of Rice Seedlings by the Plant Growth-Promoting Bacterium *Herbaspirillum seropedicae* Z67. **Molecular Plant-Microbe Interactions**, v. 15, n. 9, p. 894–906, 2002.

JAMES, E. K.; OLIVARES, F. L. Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. **Critical Reviews in Plant Sciences**, v. 17, n. 1, p. 77-119, 1998.

KEYEO, F.; AI'SHAH, O. N.; AMIR, H. G. The effectes of nitrogen fixation activity and phytohormone production of diazotroph in promoting growth of rice seedlings. **Biotechnology**, v. 10, n. 3, p. 267–273, 2011.

KRAFFCZYK, I.; TROLLDENIER, G.; BERINGER, H. Soluble root exudates of maize: Influence of potassium supply and rhizosphere microorganisms. **Soil Biology and Biochemistry**, v. 16, n. 4, p. 315–322, 1984.

LAMBRECHT, M. et al. Indole-3-acetic acid: a reciprocal signaling molecule in bacteria-plant interactions. **Trends in microbiology**, v. 8, n. 7, p. 298–300, 2000.

LARANJEIRA, S. et al. Inoculation of plant growth-promoting bacteria and arbuscular mycorrhizal fungi improve chickpea performance under water deficit conditions. **Applied Soil Ecology**, v. 164, 2021.

LIMA, L. DA S. et al. Root exudate profiling of maize seedlings inoculated with *Herbaspirillum seropedicae* and humic acids. **Chemical and Biological Technologies in Agriculture**, v. 1, n. 1, p. 1–18, 2014.

LIN, Y. et al. Validation of potential reference genes for qPCR in maize across abiotic stresses, hormone treatments, and tissue types. **PLoS ONE**, v. 9, n. 5, 2014.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-$^{\Delta\Delta}$CT method. **Methods**, v. 25, p. 402–408, 2001.

MARTÍNEZ-CANO, B. et al. Effect of Two Strains of *Stenotrophomonas rhizophila* Isolated from Compost on the Growth of Tomato, Lettuce, and Jalapeno Pepper. **International Journal of Agriculture and Biology**, v. 27, n. 6, p. 423–430, 2022.

MATSUMURA, E. E. et al. Composition and activity of endophytic bacterial communities in field-grown maize plants inoculated with *Azospirillum brasilense*. **Annals of Microbiology**, v. 65, n. 4, p. 2187–2200, 2015.

MATTEOLI, F. P. et al. *Herbaspirillum*. Em: AMARESAN, N. et al. (Eds.). **Beneficial Microbes in Agro-Ecology: Bacteria and Fungi**. 1. ed. Elsevier, 2020. p. 493–508.

MCDONALD, D. et al. An improved green genes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. **ISME Journal**, v. 6, n. 3, p. 610–618, 2012.

MCMURDIE, P. J.; HOLMES, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. **PLoS ONE**, v. 8, n. 4, p. 1–11, 2013.

MIMMO, T. et al. Time and substrate-dependent exudation of carboxylates by *Lupinus albus* L. and *Brassica napus* L. **Plant Physiology and Biochemistry**, v. 49, n. 11, p. 1272–1278, 2011.

MONTEIRO, R. A. et al. *Herbaspirillum*-plant interactions: Microscopical, histological and molecular aspects. **Plant and Soil**, v. 356, n. 1–2, p. 175–196, 2012.

NANJO, Y. et al. Mass spectrometry-based analysis of proteomic changes in the root tips of flooded soybean seedlings. **Journal of Proteome Research**, v. 11, n. 1, p. 372–385, 2012.

NARDI, S. et al. Soil organic matter mobilization by root exudates. **Chemosphere**, v. 41, n. 5, p. 653–658, 2000.

OKSANEN, J. et al. **Package “vegan” Title Community Ecology Package**, 2015.

OLIVARES, F. L. et al. Occurrence of the endophytic diazotrophs *Herbaspirillum spp.* in roots, stems, and leaves, predominantly of Gramineae. **Biology and Fertility of Soils**, v. 21, n. 3, p. 197–200, 1996.

PARADA, A. E.; NEEDHAM, D. M.; FUHRMAN, J. A. Every base matter: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. **Environmental Microbiology**, v. 18, n. 5, p. 1403–1414, 2016.

PEREIRA, T. P. et al. Real-time PCR quantification of the plant growth-promoting bacteria *Herbaspirillum seropedicae* strain SmR1 in maize roots. **Molecular Biotechnology**, v. 56, n. 7, p. 660–670, 2014.

PII, Y. et al. Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. **Biology and Fertility of Soils**, v. 51, n. 4, p. 403–415, 2015.

PIMENTEL, J. et al. Dinitrogen fixation and infection of grass leaves by *Pseudomonas rubrisubalbicans* and *Herbaspirillum seropedicae*. **Plant and soil**, v. 137, p. 61-65, 1991.

PYLRO, V. S. et al. Data analysis for 16S microbial profiling from different benchtop sequencing platforms. **Journal of Microbiological Methods**, v. 107, p. 30–37, 2014.

PYLRO, V. S. et al. BMPOS: a Flexible and User-Friendly Tool Sets for Microbiome Studies. **Microbial Ecology**, v. 72, n. 2, p. 443–447, 1 ago. 2016.

RADWAN, T. E. E.; KAMEL, Z.; MASSENA, V. Efeito da inoculação de *Azospirillum* e *Herbaspirillum* na produção de compostos indólicos em plântulas de milho e arroz. **Pesquisa Agropecuaria Brasileira**, v. 39, n. 10, p. 987–994, 2004.
RAMOS, A. C. et al. Inoculation with the endophytic bacterium *Herbaspirillum seropedicae* promotes growth, nutrient uptake and photosynthetic efficiency in rice. **Planta**, v. 252, n. 5, p. 1–8, 2020.

REIS, R. J. DE A. et al. Mutualistic interaction of native *Serratia marcescens* UENF-22GI with *Trichoderma longibrachiatum* UENF-F476 boosting seedling growth of tomato and papaya. **World Journal of Microbiology and Biotechnology**, v. 37, n. 12, 2021.

RODRIGUES-NETO, J.; MALAVOLTA JR., V.; VICTOR, O. Meio simples para o isolamento e cultivo de *Xanthomonas campestris* pv. *citri* tipo B. **Summa Phytopathol**, v. 12, p. 16, 1986.

RONCATO-MACCARI, L. D. B. et al. Endophytic *Herbaspirillum seropedicae* expresses nif genes in gramineous plants. **FEMS Microbiology Ecology**, v. 45, n. 1, p. 39–47, 2003.

SASSE, J.; MARTINOIA, E.; NORTHEN, T. Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? **Trends in Plant Science**, v. 23, n. 1, p. 25–41, 2018.

SCHMIDT, M. A. et al. Evidence for the endophytic colonization of Phaseolus vulgaris (common bean) roots by the diazotroph *Herbaspirillum seropedicae*. **Brazilian Journal of Medical and Biological Research**, v. 44, n. 3, p. 182–185, 2011.

THIMM, O. et al. MAPMAN: A user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. **Plant Journal**, v. 37, n. 6, p. 914–939, 2004.

USADEL, B. et al. A guide to using MapMan to visualize and compare Omics data in plants: A case study in the crop species, Maize. **Plant, Cell and Environment**, v. 32, n. 9, p. 1211–1229, 2009.

WANG, Z. et al. High-throughput sequencing-based analysis of the composition and diversity of endophytic bacterial community in seeds of upland rice. **Archives of Microbiology**, v. 203, n. 2, p. 609–620, 2021.

WOŹNIAK, M. et al. Endophytic bacteria potentially promote plant growth by synthesizing different metabolites and their phenotypic/physiological profiles in the biolog gen iii microplate™ test. **International Journal of Molecular Sciences**, v. 20, n. 21, 2019.

Chapter 3: Influence of organic acids of maize roots exudates in initial aspects of root colonization by *Herbaspirillum seropedicae*

Influence of organic acids of maize roots exudates in initial aspects of root colonization by *Herbaspirillum seropedicae*

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Abstract

Herbaspirillum seropedicae is a plant growth-promoting bacterium. Its interaction with the host plant is mediated by chemical compounds exuded by the roots that modulate recognition and chemoattraction events towards the roots, which are essential for the endophytic establishment of the bacterium. This work investigates the influence of organic acids exuded by maize plants on chemotaxis, flagellar biosynthesis, and biofilm formation on *H. seropedicae*. For this, the kinetics of the use of organic acids (aconitic, citric, fumaric, maleic, malic, malonic, oxalic, and succinic acids) as sole carbon source, chemotactic assay, biofilm formation and flagella observation were performed combined with gene expression of related genes (chemotaxis: *tar*, *tsr* and *Hsero_3720*, and *cheABC*; flagella biosynthesis: *fliOPQ*; and biofilm formation: *EpsB*). All organic acids tested induced chemotaxis and flagella on electron transmission microscopy. The results obtained with 72 hours of growth demonstrated that *H. seropedicae* can use aconitic, citric, fumaric, malic, and succinic acids as carbons sources: the organic acids, the aconitic acid, the most inductor of biofilm formation. The transcriptional profile reveals that *tar*, *tsr*, *Hsero_3720, cheA e cheD* are repressed and up-regulated by aconitic and other organic acids, respectively, indicating that the organic acids activated *tar*, *tsr* and *Hsero_3720* and transmitted the signal to *cheA* and the mcps are deactivated by *cheD*. *cheB* was induced only by oxalic acid, inducing demethylation of *tar*, *tsr* and *Hsero_3720*. The influence of organic acids differentially expressed genes involved in biofilm formation, and flagella biosynthesis was repressed. These results show for the first time that the phenotypic effects of maize root exudates on *H. seropedicae* chemotaxis e can contribute to understanding the rhizospheric communication between plant host and *H. seropedicae*.

Keywords: *chemotaxis; flagellum; adhesion; rhizosphere; colonization*.

1. Introduction

Plant root exudates are substances released into the rhizosphere by healthy and intact plant roots (ROVIRA, 1969). Plants can exude up to 20% of carbon and 15% of fixed nitrogen, which includes sugars, organic acids, amino acids, and secondary metabolites (SASSE; MARTINOIA; NORTHEN, 2018), and there may be variations in the composition of these exudates according to plant species, their stage of development and biotic and abiotic stimuli (NARDI et al., 2000).

Plant root exudates can be critical in mediating interactions in the rhizosphere with symbiotic and non-symbiotic microorganisms, mycorrhiza, rhizobia (NARDI et al., 2000) and plant growth-promoting bacterium (BADRI; VIVANCO, 2009). They also can exert chemotactic attractions over certain groups of microorganisms, or they can act as repellents for other groups, thus modulating the rhizosphere microbiota and symbiosis, like a "cry-for-help" (NARDI et al., 2000; ROLFE; GRIFFITHS; TON, 2019; SASSE; MARTINOIA; NORTHEN, 2018).

Rice root exudates have been shown to induce high chemotactic capacity over the natural endophytes of rice plants *Corynebacterium flavescens* and *Bacillus pumilis* (BACILIO-JIMÉNEZ et al., 2003). Furthermore, maize root exudates increased the recruitment of *Bacillus amyloliquefaciens* to inhibit *Fusarium graminearum* infection (XIE et al., 2022). In addition, maize root exudates promoted the colonization of *Bacillus velezensis* in root tissue (JIN et al., 2019).

The importance of orientation and active movement for the survival of many living organisms led to several motility mechanisms. Bacteria can respond to a broad spectrum of stimuli, such as concentrations of chemical substances (chemotaxis), light (phototaxis), electric fields (galvanotaxis), magnetic fields (magnetotaxis), pH (pH-taxis) and temperature (thermotaxis) (WONG-NG; CELANI; VERGASSOLA, 2018).

The chemotactic movement and the chemical substances involved influence the metabolism and behavior of some unicellular organisms, such as directing the search for nutrients and communication between individuals during the formation of colonies or groups (SINGH; KUMAR; AGRAWAL, 2014).

The process of colonization by *Herbaspirillum seropedicae* in Poaceae roots begins with chemotaxis induced by root exudates of the host plant, which leads to the expression of the machinery involved in the movement towards the rhizosphere, followed by non-polar adhesion of the bacteria to the zone of elongation/differentiation and the zone of root hair formation, where they will form complex communities in the form of biofilms (JAMES; OLIVARES, 1998; OLIVARES et al., 1997). *Herbaspirillum seropedicae* is a diazotrophic endophytic bacterium that supplies the plant host with phytohormones (BASTIÁN et al., 1998; BOTTINI; CASSÁN; PICCOLI, 2004; LAMBRECHT et al., 2000), biological nitrogen fixation (Alves, 2007, Pimentel), phosphate solubilization (ESTRADA et al., 2013) and biological control (CRUZ et al., 2001; WEBER; FREIRE, 2003).

Previous works with *H. seropedicae* inoculation in maize demonstrated that inoculated plants had their exudation profile altered in the presence of the microorganism (LIMA et al., 2014). Furthermore, maize plants inoculated with *H. seropedicae* plus *Gluconacetobacter diazotrophicus* and humic acids had the content of their metabolites modulated, showing an increase of organic acids derived from the Tricarboxylic acid cycle (TCA) (CANELLAS; OLIVARES; CANELLAS, 2019).

Data presented in the previous chapter demonstrated that *H. seropedicae* influence maize root enzymes of TCA at transcriptional and proteins level, indicating that organic acids from this cycle can modulate the chemotaxis movement of *H. seropedicae* towards the roots. This work investigates the influence of organic acids

exuded by maize roots on chemotaxis, flagellar biosynthesis, and biofilm formation by *H. seropedicae*.

2. Materials and methods

2.1. Preparation of Inoculum

The microorganism used was *H. seropedicae* strain HRC54 (SisGen nº AFD1CAD), originally isolated from sugarcane roots. The pre-inoculum was obtained from a pure plate colony and after growth in DYGS (RODRIGUES-NETO; MALAVOLTA JR.; VICTOR, 1986) liquid medium for 24h at 30°C in an orbital shaker at 150rpm. After growth, a 20 µL aliquot of the bacterial suspension was transferred to JNFB (DÖBEREINER; BALDANI; BALDINI, 1995) liquid medium supplemented with NH_4Cl (1g.L^{-1}) for 48 hours under the same conditions described above after the bacterial cells were sedimented by centrifugation (5.000 × g for 15 min) and resuspended in sterile distilled water at a cell density of 4x10^{9} CFU.ml^{-1}.

2.2. Growth curve of *H. seropedicae*

To analyze the growth rate of *H. seropedicae* using the organic acids of maize roots exudate, according to (CANELLAS; OLIVARES; CANELLAS, 2019), the JNFB medium (DÖBEREINER; BALDANI; BALDINI, 1995) liquid supplemented with NH4CL (1g.L^{-1}), replacing the original carbon source (malic acid) of the medium by the compounds of interest found in the exudate (aconitic, citric, fumaric, maleic, malonic, oxalic and succinic acids) and disposed of in 200 µL in 96-well plates in triplicates. An aliquot of 5µL of bacterial suspension obtained from the pre-inoculum (5x10^{2} cells/µL) was inoculated in the medium and cultivated at 30 ºC in orbital shake at 150rpm. To

build the growth curve, the plates were read every 4h in a spectrophotometer from the Versa Max Microplate Reader brand at a wavelength of 600nm.

2.3. Evaluation of the chemotactic activity and flagella of *H. seropedicae*

A 1mL aliquot of *H. seropedicae* HRC54 inoculum was centrifuged for chemotaxis assays, and the supernatant was discarded. The bacterial pellet was diluted in sterilized distilled water and normalized to a population of $1x10^7$ cells.mL^{-1}. Chemotaxis chambers were prepared according to Mandimba et al. (1986), adapted by (BARBOSA et al. 2020).

The chemotactic chamber was assembled from a sterilized Petri dish (2 cm high and 14.5 cm in diameter), where a sheet of absorbent paper saturated with sterilized distilled water, a capillary tube with a total volume of 5µL loaded with organic acids (aconitic, citric, fumaric, maleic, malic, malonic, oxalic, succinic and sterilized distilled water for control) solution, separately, at a concentration of 9,2mM according to (BAUDOIN; BENIZRI; GUCKERT, 2003), with one end in contact with the bacterial cell suspension (50µL at a concentration of $1x10^7$ cells/mL) and the other end on support, forming an angle of approximately 30º with the Petri dish, according to figure 1.

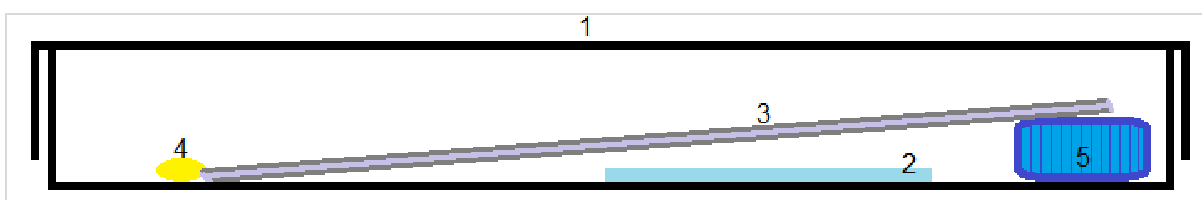

Figure 1: Chemotaxis chamber - 1: Petri dish; 2: paper saturated with water; 3: capillary tube with an organic acid solution; 4: bacterial suspension; 5: support.

After assembling the chemotactic chambers for the different compounds tested, the plates were incubated in the dark at 30 ºC for 4h. To determine the attraction of *H. seropedicae* into the capillary, 2 µL from the top was obtained, diluted in 50µL for serial dilution and plated from 10^{-3} to 10^{-5} for count using the technique of Colony Forming

Unit (CFU) count. Different chambers were used for each compound evaluated in triplicate. Data were analyzed by two-way statistical analysis of variance (ANOVA) followed by a Tukey's test. Data analyses were carried out using GraphPad Prism 7.00.

For valuation of the gene expression related to chemotaxis and flagella, 2mL of the pre-inoculum was transferred to sterile Eppendorf tubes, centrifuged and the medium was discarded. The precipitated sample was washed with sterile distilled water, centrifuged, and discarded supernatant. A solution containing organic acids (aconitic, citric, fumaric, maleic, malic, malonic, oxalic, succinic, and sterilized distilled water for control) at a concentration of 9.2 mM was gently added to each tube separately and incubated for 1h at 30ºC. After the incubation, the tubes were vortexed and centrifugated, the supernatant was discarded, and the precipitated material was followed for RNA extraction.

For visualization of flagella of *H. seropedicae,* the experiment of chemotaxis was repeated, and the final 2µL from the top of the capillary was collected and fixed on Cooper 300 Hex Mesh grids coated with Formvar and stained with 5% uranyl acetate solution and observed under transmission electron microscopy (TEM) ZEISS M-900 operated at 80 kW (OLIVARES, 1997).

2.4. Quantification and visualization of *H. seropedicae* biofilm

For valuation of the *H. seropedicae* production of biofilm using the organic acids from maize roots exudates, an aliquot of $1x10^7$ cells/mL of HRC54 strain pre-inoculum was inoculated in test tubes containing JNFB medium with the carbon source modified for organic acids (aconitic, citric, fumaric, maleic, malic, malonic, oxalic, and succinic) and 0.1g of glass wool (OOSTHUIZEN et al., 2001). The cultures were incubated for 48h at 30º with agitation at 150 rpm. After the cultivation, the broth was discarded,

maintaining the glass wools. The JNFB medium with glass wool without HRC54 was used as a control.

The glass wools were transferred to Petri dishes and add 1ml of crystal violet solution 1% (v/v) and incubated at room temperature for 30min. After the incubation, the glass wools were washed twice with phosphate buffer 0.5 M by the agitation of 150rpm for 15min. Finally, the biofilm stained with crystal violet that remained adhered to the glass wool was extracted with 2 washes with 5ml of absolute ethanol.

A 200µL of the ethanol stained was transferred to a 96-well plate and measured in a spectrophotometer from the Versa Max Microplate Reader brand at a wavelength of 560nm.

The experiment was repeated to evaluate the gene expression related to biofilm. The glass wools were transferred to falcon tubes of 15 mL with 2mL of sterile distilled water and vortexed for 2min to liberate the cells adhered to the glass wools. For the control samples, JNFB modified without glass wool was used. The contents of each tube were transferred to Eppendorf tubes, centrifuged, and the supernatant discarded and sent to RNA extraction.

To visualize the biofilm formed for *H. seropedicae* in the glass wool were followed the procedures cited above and the glass wools were stained with safranin solution 1% (v/v) and incubated at room temperature for 30 minutes. After the incubation, the glass wools were washed twice with distilled water and transferred to glass slides for visualization in Zeiss Axioplan optical microscope with an attached ToupCam™ camera.

Data were analyzed by two-way statistical analysis of variance (ANOVA) followed by a Tukey's test. Data analyses were carried out using GraphPad Prism 7.00.

2.5. Gene expression related to chemotaxis and biofilm formation.

Real-time PCR quantification was used to investigate the differentially expressed genes in the phenotypes of chemotaxis and biofilm. Specific primers for each gene to be analyzed (Table 1) were designed based on the genome of the *H. seropedicae* SmR1, using Oligo Explorer™ software (Teemu Kuulasmaa, Finland) and confirmed in Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (YE et al., 2012). In addition, *groEL* for the molecular chaperone(PESSOA et al., 2016) and 16s rDNA (PEREIRA et al., 2014) were used as normalizing genes.

Table 1: Primers designed for RT-PCR based on the *H. seropedicae* SmR1 genome.

Gene	Gene product	Primers sequences (5'-3')	Amplicon size
		Chemotaxis	
tar	Methyl-accepting chemotaxis transducer transmembrane protein	F: ATTTCCCCACCCACAACAAG R: GGTGCGATCACTGAAGATGC	101bp
tsr	Methyl-accepting chemotaxis protein I, serine sensor receptor protein	F: GAGCGCTTCGATTTCCTTG R: GCCTTCCAGACCAATATCCTG	147bp
Hsero _3720	Methyl-accepting chemotaxis transducer transmembrane protein	F: GGCATTGTCGGCATTCTG R: GGCAACATGGACCTGTCTTC	111bp
CheA	Signal-transducing histidine kinase *CheA* protein	F: TCCATACGCTCAAGGGAAA R: CAGCACCTCCGAATAGGC	122 bp
CheB	Chemotaxis-specific methylesterase protein	F: CTCACGCTGGACGTGGAAAT R: GCAAGGTGATTTCCGAACCG	127bp
CheD	Chemoreceptor glutamine deamidase protein	F: GATTGCCTCCAATCTCTACTAC R: CTTGGTGGTGAAGTAATACTCC	85bp
		Flagellum	
fliO		F: CCGAGTTCGTGTGGTACCTA	

	flagellar biogenesis *FliO* protein	R: TAGGTAACGAGTGGGTGGAG	
fliP	Flagellar biosynthetic protein *FliP* precursor protein	F: GTGAAGCAGGTCATCATCAG R: GTATTCCCGCACTCAACAG	130bp
fliQ	flagellar biosynthetic *FliQ* protein	F: AATCCACGATCACCGTCAG R: GACCCTGGCCTTCATTCC	86bp
	Biofilm formation		
EpsB	Glucosyltransferase involved in lipopolysaccharide synthesis protein	F: GATCGGCTATGTCTGCGAGT R: ACTGTGCATACTGCGACACC	104bp

Legend: F- forward; R- reverse; bp- base pairs.

The RNA of chemotaxis and biofilm samples were extracted with Trizol® reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the cDNA synthesis was carried out using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) as described in the manufacturer's protocol using 1µg from RNA extracted from the samples.

Primers were used in RT-qPCR reactions at concentrations of 100 nM, using 7.5 µL of FAST SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) in Step One Plus Real-Time PCR machine (Applied Biosystems) under the following conditions: 40 cycles 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. All qPCR runs were conducted in triplicate. The relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method (LIVAK; SCHMITTGEN, 2001). Student's t-test analyzed data using GraphPad Prism (version 7.00).

3. Results

3.1. Dynamics of the use of organic acids like carbon sources

The growth rate of *H. seropedicae* using the organic acids derived from TCA as a source of carbon, according to Canellas, Olivares and Canellas (2019) and our previous results, show us that *H. seropedicae* can use the aconitic, citric, fumaric, malic, and succinic acids as a sole source of carbon. Furthermore, our results demonstrated that *H. seropedicae* preferentially uses malic acid as the sole carbon source, which has been shown to initiate growth within the first 8 hours of cultivation. On the other hand, *H. seropedicae* did not use the organic acids, maleic, malonic, and oxalic soles as carbon sources, resulting in no growth after 72h (Figure 2).

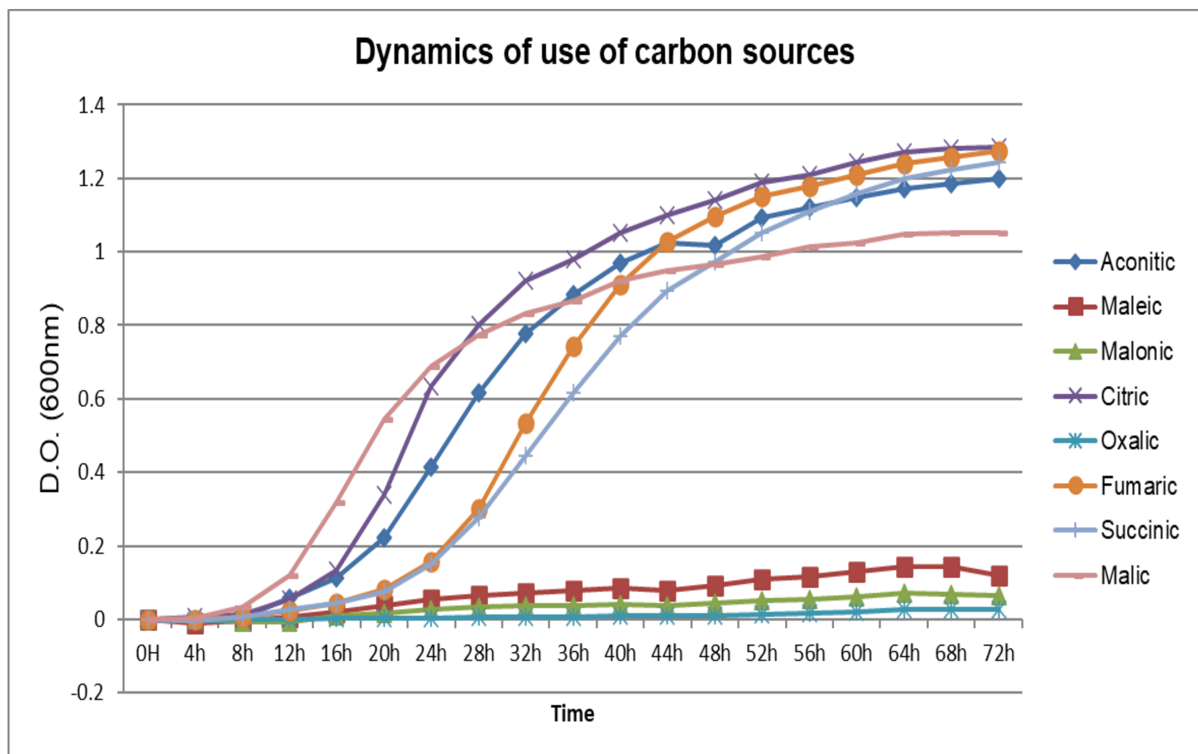

Figure 2: Dynamic growth of *H. seropedicae* using organic acids of maize exudates as carbon source.

3.2. *H. seropedicae* chemotaxis activity

The chemotaxis assay demonstrated that *H. seropedicae* could use all the organic acids derived from maize root exudates as a signal to move toward the carbon source. The best chemoattractants were malonic, succinic, malic, and oxalic acids.

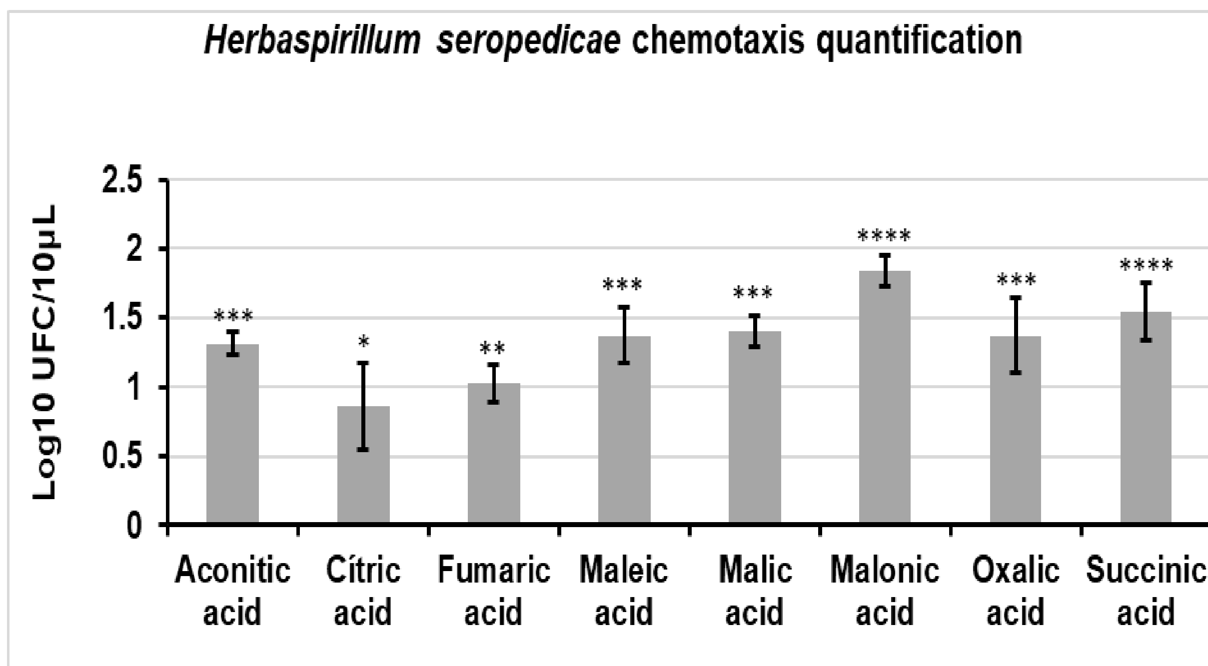

Figure 3: Chemotactic quantification of *H. seropedicae* using organic acids of maize exudates as chemoattractant. Means marked by asterisk differ from control condition (* p value < 0,01; ** p value < 0.05; *** p value < 0,005; **** p value < 0,0001). Bars represent the standard error (n = 3).

The transmission electron microscopy visualization of flagella formation demonstrated that *H. seropedicae* is influenced by organic acids to form flagella for chemotaxis activity. *H. seropedicae* in contact only with water did not show flagellar formation (Figure 4A). Fumaric, maleic and oxalic acids formed double flagella in one cell pole (Figure 4D, E and H). Citric and oxalic acids induced a unique flagellum (Figures 4C and G). Malic and succinic acids induced multiple flagella in one pole (Figures F and J). Aconitic acid induced the formation of double flagella in both poles of the cell of *H. seropedicae* (Figure B).

In addition to the flagella, it was possible to observe the formation of polyhydroxybutyrate (PHB) granules in cells chemoattracted by malic acid (Figure 4F).

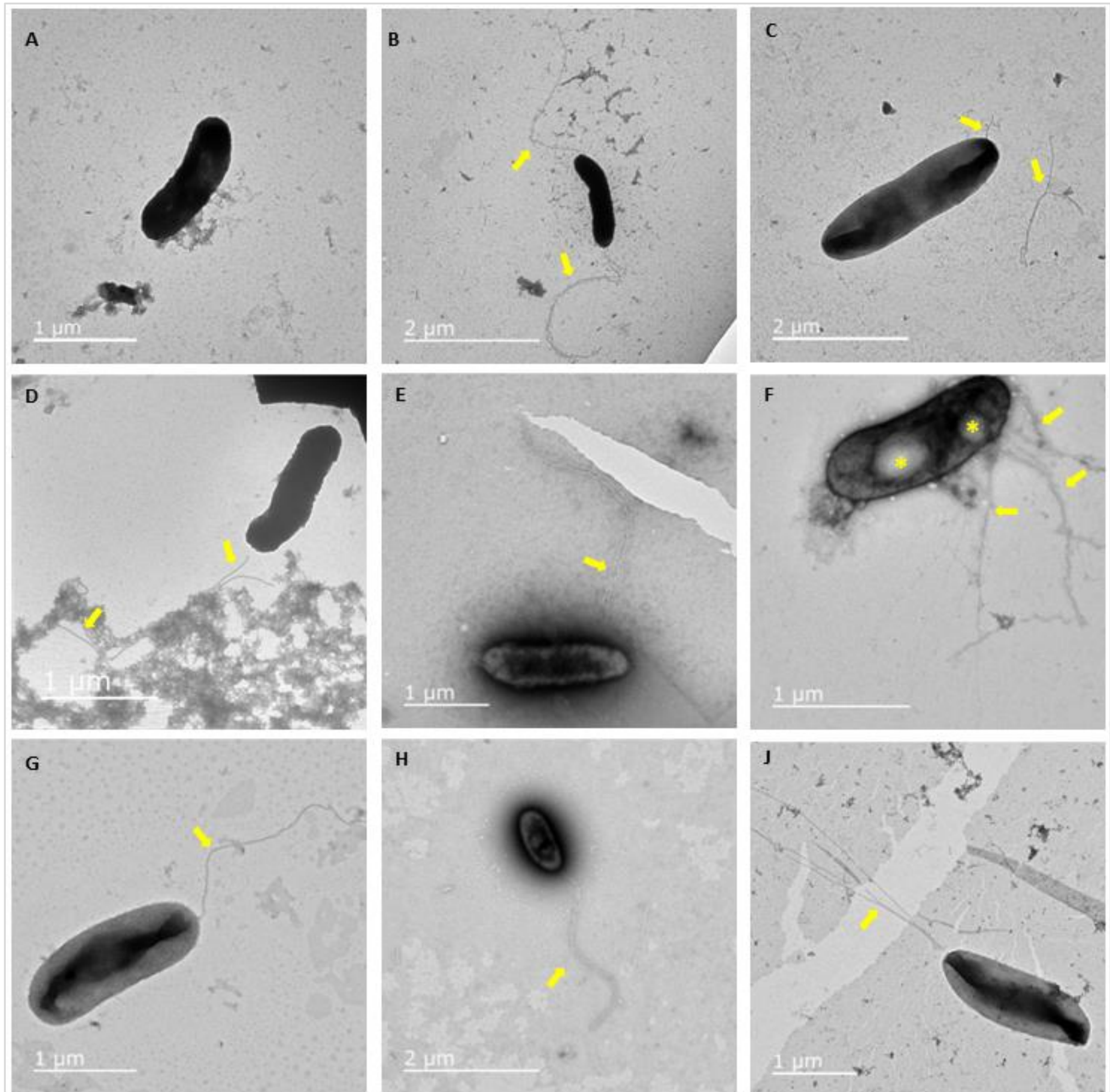

Figure 4: Transmission electron microscopy of flagella of *H. seropedicae* chemoattract by organic acids from maize roots exudates. (**A**) Control, (**B**) Aconitic acid, (**C**) Citric acid, (**D**) Fumaric acid, © Maleic acid, (**F**) Malic acid, (**G**) Malonic acid and (**H**) Oxalic acid, and (**I**) Succinic acid. (Yellow arrows indicate the flagella) (Yellow asterisk shows PHB granules).

3.3. Biofilm formation in response to organic acids

To identify the influence of the organic acids exudated by maize roots in the formation of *H. seropedicae*, biofilm was quantified after 48h of growth in JNFB, modifying the carbon source for the organic acids and glass wools. The controls were the same modified JNFB medium without the glass wools.

According to the quantification of biofilm formation, *H. seropedicae* was strongly induced to produce biofilm in the presence of aconitic, maleic, fumaric and citric acid. On the other hand, in the presence of malic, malonic, and oxalic acid, there was not as much biofilm formation, and with succinic acid, biofilm formation was minimal, not showing a difference from the control (Figure 5).

The visualization of the biofilm in glass wools (Figure 6) confirms the results of quantification, where *H. seropedicae* in the presence of aconitic and maleic acids formed significant structures of biofilm (figure 6B and D) and in the presence of succinic acid, a minimal biofilm structure formation (Figure 6H).

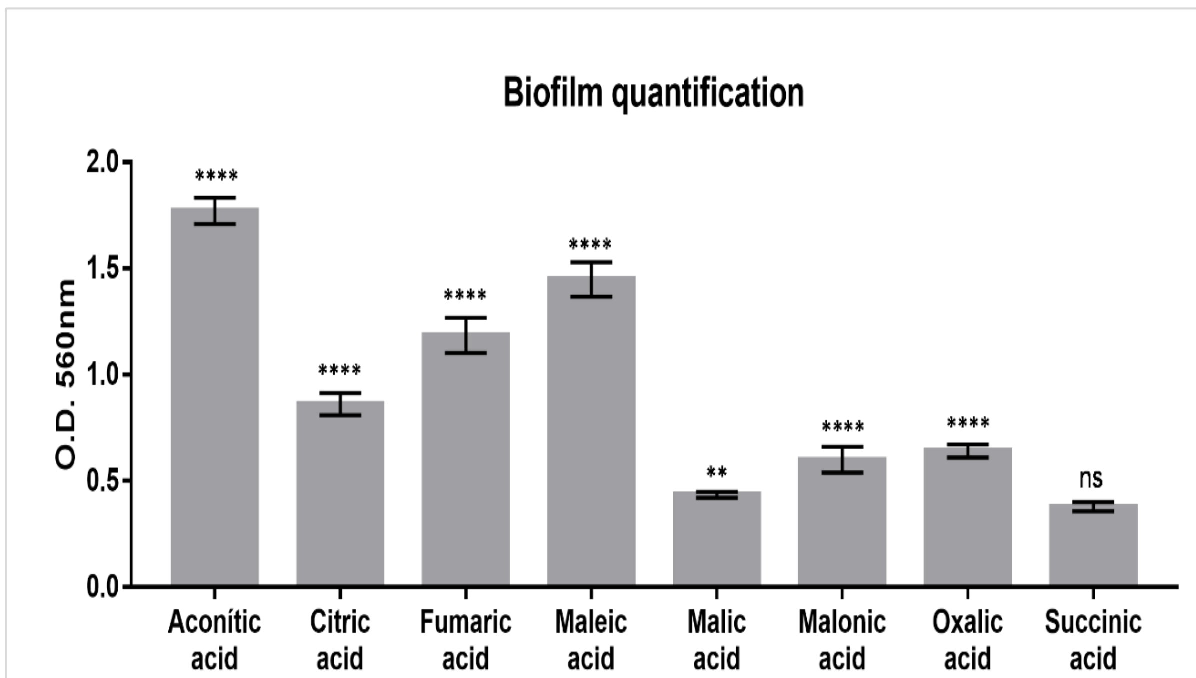

Figure 5: Biofilm quantification of *H. seropedicae* in the presence of organic acids from the maize root exudates. Means marked by asterisk differ from the control condition (** p value < 0.005; **** p value < 0,0001; ns = non-significant). Bars represent the standard error (n = 3).

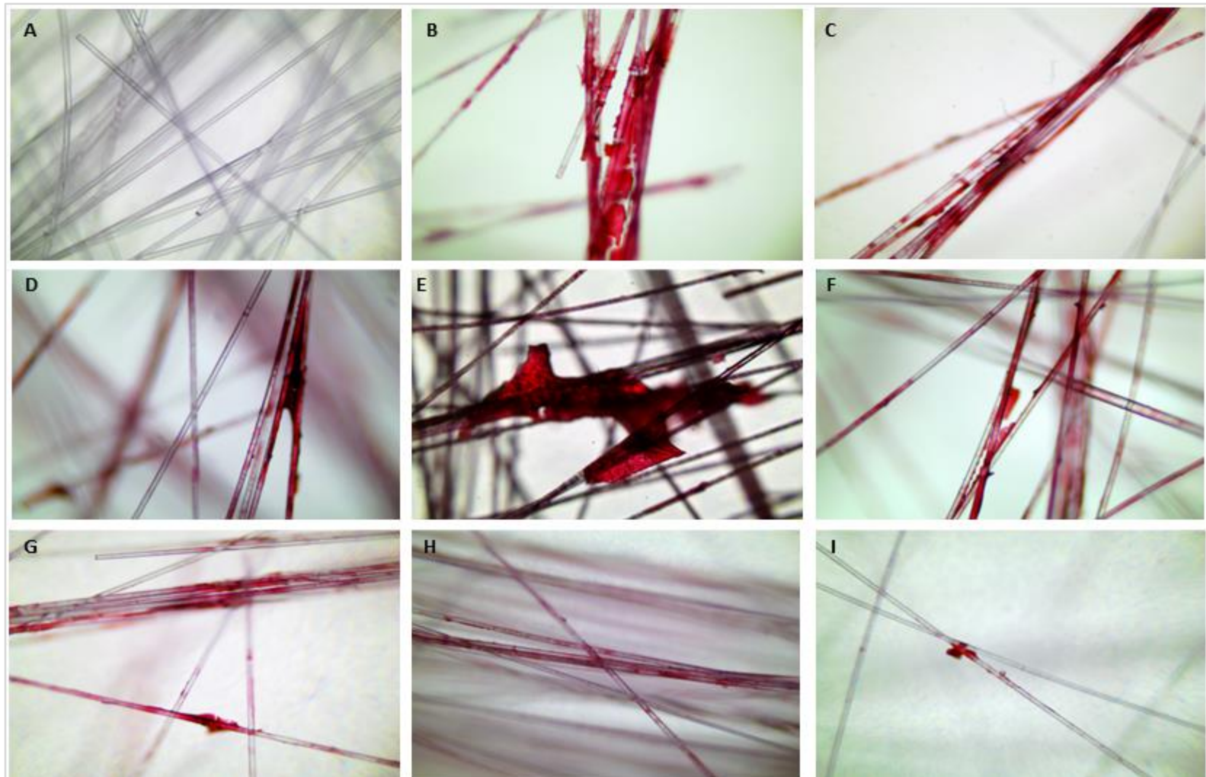

Figure 6: Bright field microscopy of biofilm of *H. seropedicae* grown in the presence of organic acids from the maize roots and glass wool for 48h. (**A**) Control, (**B**) Aconitic acid, (**C**) Citric acid, (**D**) Fumaric acid, (**E**) Maleic acid, (**F**) Malic acid, (**G**) Malonic acid and (**H**) Oxalic acid, and (**I**) Succinic acid.

3.4. Transcriptional profile in response to organic acids

Gene expression related to chemotactic signal receptors was analyzed to understand *H. seropedicae* response after 1h of exposure to the organic acids of maize exudates. The genes encoding signal receptors of chemotaxis, *tar*, *tsr* and *Hsero_3720* were more expressed in the presence of all organic acids but were repressed by aconitic acid (figure 7).

The *tar* gene was repressed -3.55 fold by the aconitic acid and expressed 2.66, 1.87, 2.27, 1.86, 2.76, 3.82 and 2.55-fold by the citric, fumaric, maleic, malic, malonic, oxalic, and succinic acids, respectively, when compared to control (figure 7a). The *tsr* gene was repressed -0.33-fold by the aconitic acid and expressed 5.47, 7.94, 3.95, 6.90, 3.33, 8.22 and 1.55-fold by the citric, fumaric, maleic, malic, malonic, oxalic, and succinic acids, respectively (figure 7b). The *Hsero_3720* gene was repressed -0.33

and -0.46-fold by the aconitic and succinic acids and expressed 4.48, 4.18, 1.47, 4.85, 1.49 and 6.12-fold by the citric, fumaric, maleic, malic, malonic, and oxalic acids, respectively (figure 7c).

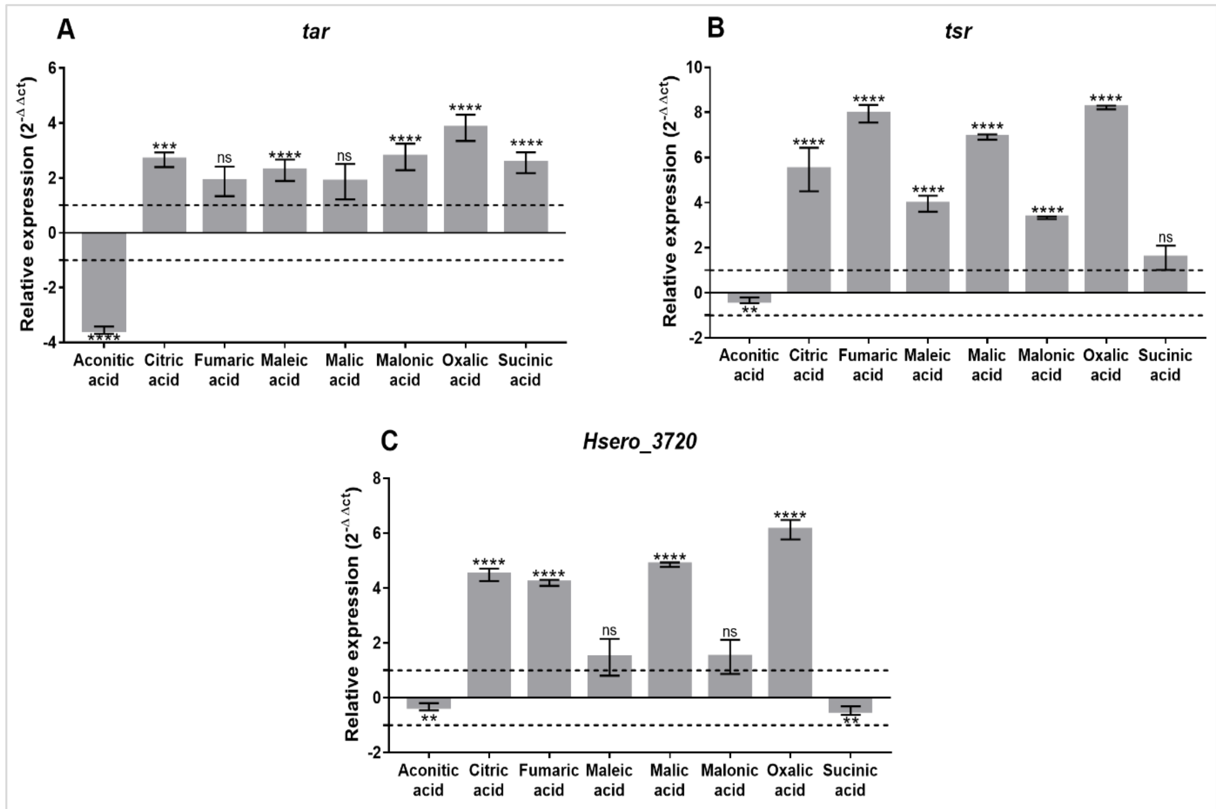

Figure7: Gene expression of chemotactic receptors of *H. seropedicae* in contact with organic acids of maize exudates as chemoattractant. (**A**) Methyl-accepting chemotaxis transducer transmembrane protein. (**B**) Chemotaxis-specific methylesterase protein. (**C**) Methyl-accepting chemotaxis transducer transmembrane protein. The dotted line represents the expression value of the control sample. Means marked by asterisk differ from control condition (* p value < 0,01; ** p value < 0.005; *** p value < 0,005; **** p value < 0,0001). Bars represent the standard error (n = 3).

Genes encoding signal the cytoplasmic receptors of chemotaxis *cheA* and *cheD* were more expressed in contact with all organic acids but were repressed when in contact with aconitic acid (figure 8). On the other hand, the gene encoding the *cheD* was repressed by all the organic acids and expressed only by the oxalic acid.

The *cheA* gene was repressed -4.73-fold by the aconitic acid and expressed 7.99, 7.03, 7.94, 5.56, 6.55, 7.88 and 7.83-fold by the citric, fumaric, maleic, malic, malonic, oxalic, and succinic acids, respectively (figure 8a).

The *cheB* gene was repressed -5.81, -0.09, -0.64, -0.03, -2.01, -2.03, and -3.06-fold by the citric, fumaric, maleic, malic, malonic, and succinic acids, respectively and slightly expressed 0.76-fold by the oxalic acid (figure 8b).

The *cheD* gene was repressed -4.90-fold by the aconitic acid and expressed 4.91, 3.73, 4.48, 3.19, 2.80, 4.75, and 1.75-fold by the citric, fumaric, maleic, malic, malonic, oxalic, and succinic acids, respectively (figure 8c).

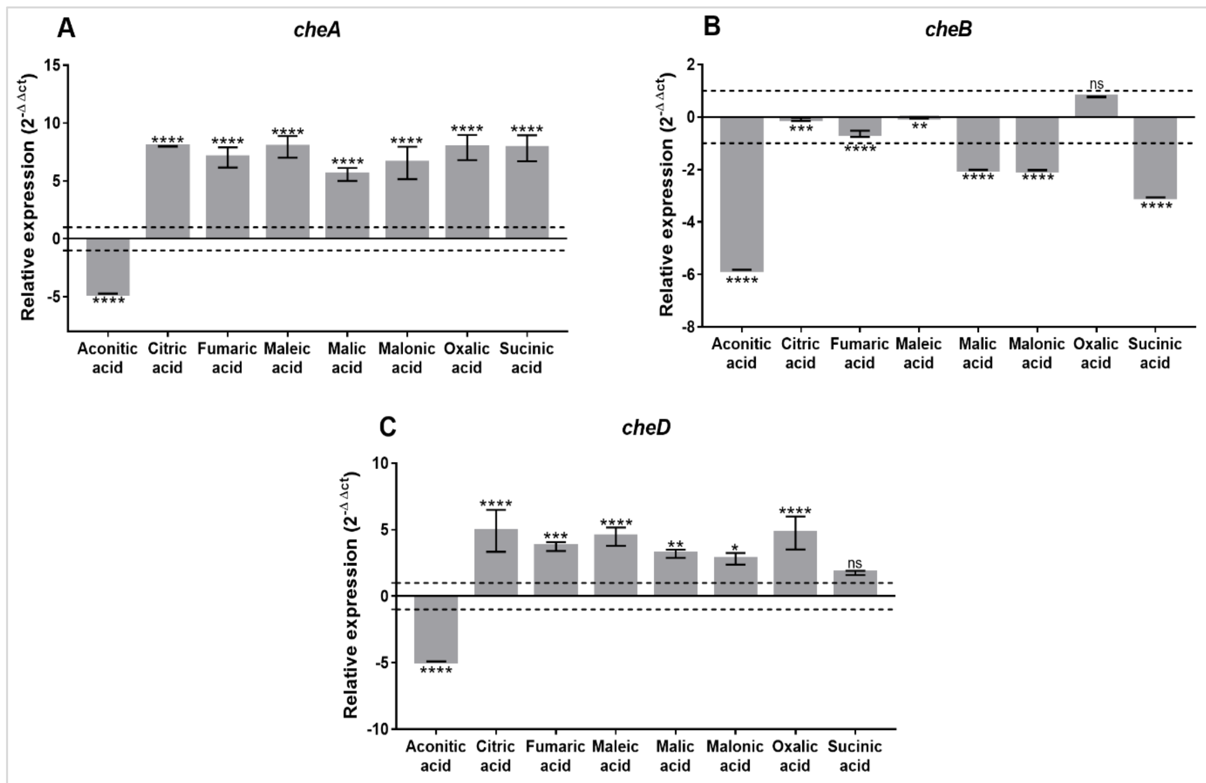

Figure 8: Gene expression of chemotactic transducer signals of *H. seropedicae* in contact with organic acids of maize exudates as chemoattractant. (**A**) Signal-transducing histidine kinase CheA protein. (**B**) Methyl-accepting chemotaxis protein I, serine sensor receptor protein. (**C**) Chemoreceptor glutamine deamidase protein. The dotted line represents the expression value of the control sample. Means marked by asterisk differ from control condition (* p value < 0,01; ** p value < 0.005; *** p value < 0,005; **** p value < 0,0001). Bars represent the standard error (n = 3).

Gene expression related to flagella biosynthesis was analyzed to understand *H. seropedicae* response in contact after 1h of exposure to the organic acids of maize exudates. The expression of *fliO* was slightly induced by fumaric acid 0.10-fold and repressed -3.47, -0.17, -1.28, -0.82, -0.30, -0.03, and -2.05-fold by the aconitic, citric, maleic, malic, malonic, oxalic, and succinic acid, respectively (Figure 9A).

The gene expression *fliP* was repressed -11.41, -5.37, -6.02, -2.45, -3.66, -2.75, -0.71, and -1.84-fold by the aconitic, citric, fumaric, maleic, malic, malonic, oxalic, and succinic acid, respectively (Figure 9B). Also, the gene expression of *fliQ* was repressed -5.95, -2.00, -3.14, -1.73, -3.46, -3.37, -1.32, and -3.38-fold by the aconitic, citric, fumaric, maleic, malic, malonic, oxalic, and succinic acid, respectively (Figure 9C).

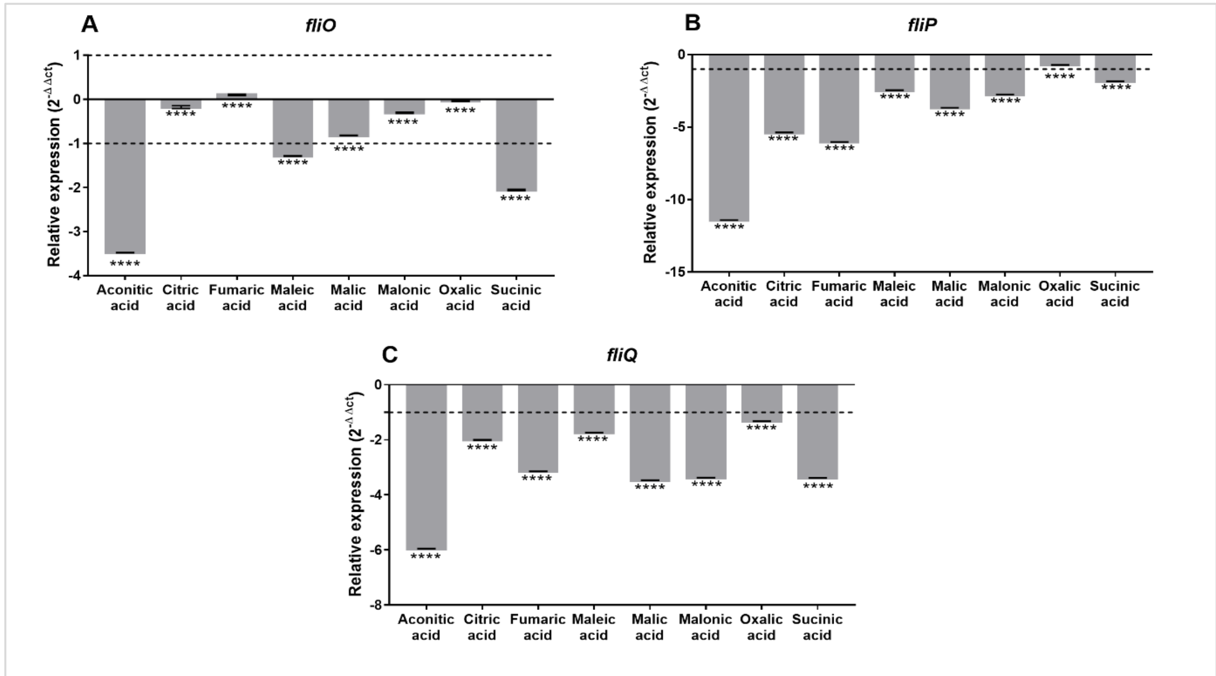

Figure 9: Relative expression of flagellar components of *H. seropedicae* in contact with organic acids of maize exudates as chemoattractant. (**A**) flagellar biogenesis FliO protein. (**B**) Flagellar biosynthetic protein FliP precursor protein. (**C**) flagellar biosynthetic FliQ protein. The dotted line represents the expression value of the control sample. Means marked by asterisk differ from control condition (* p value < 0,01; ** p value < 0.005; *** p value < 0,005; **** p value < 0,0001). Bars represent the standard error (n = 3).

Gene expression related to biofilm formation was analyzed to understand the influence of organic acids of maize exudates on biofilm formation by *H. seropedicae* in glass wool. Aconitic, fumaric, malic, and oxalic acids induced expression of *EpsB* gene 3.96, 0.17, 3.56, and 2.17-fold, respectively. The citric acids, maleic, malonic, and succinic repressed *EpsB* expression at -0.06, -0.42, -4.48, -2.46-fold, respectively. (Figure 10).

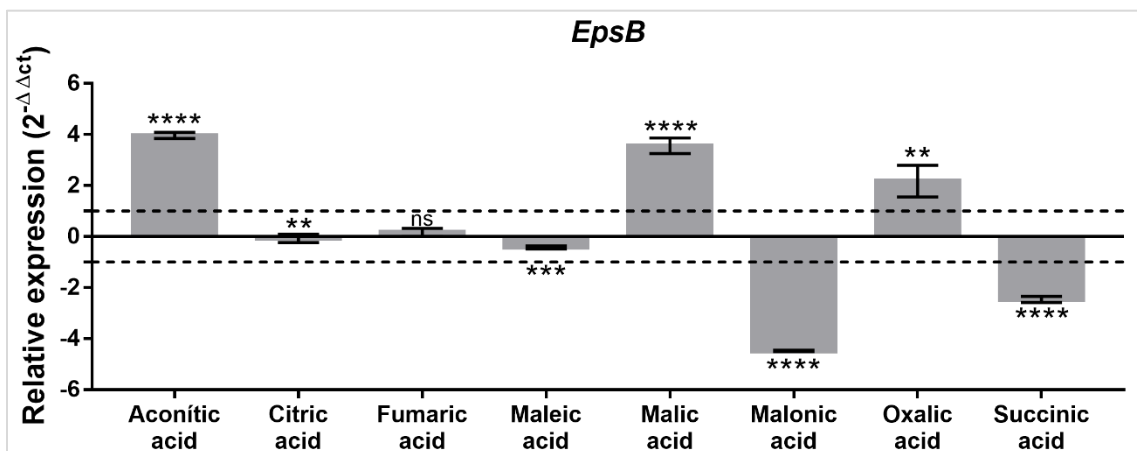

Figure 10: Relative expression of EpsB, involved in the biofilm formation of *H. seropedicae* grown in glass wool using the organic acids of maize exudates as carbon sources for 48h. The dotted line represents the expression value of the control sample. Means marked by asterisk differ from the control condition (** p value < 0.005; **** p value < 0,0001; ns = non-significant). Bars represent the standard error (n = 3).

4. Discussion

As described by Olivares et al. (1997) and James; Olivares (1998), the initial interaction of *H. seropedicae* with maize roots depends on the compounds of the root exudates that mediate the communication between host and bacteria. The previous chapter showed that the inoculation of *H. seropedicae* modulated the enzymes of the citric acid cycle (or tricarboxylic acid cycle) of roots in comparison to plants without inoculation, suggesting that *H. seropedicae* can induce maize roots to produce more organic acids like described by Canellas; Olivares; Canellas, (2019) in response to inoculation.

Here we showed that *H. seropedicae* could use the aconitic, citric, fumaric, malic, and succinic acids as the sole carbon source for growth, as Baldani et al. (1986) described. However, *H. seropedicae* could not use the maleic, malonic, and oxalic acids as carbon sources. These results corroborate other works investigating *H. seropedicae* and its relationship with sugarcane juice, which is rich in organic acids

such as aconitic, citric, fumaric, malic and oxalic acids (BELLONE and BELLONE (2006).

According to the genome annotation of *H. seropedicae* SmR1, this species has all the genes for the citric acid cycle, and pathways replenishing intermediates of the cycle include the glyoxylate cycle (isocitrate lyase and malate synthase), genes encoding the maleate isomerase and malonate decarboxylase, and genes for the glycolytic pathway (PEDROSA et al., 2011). This explains the results where *H. seropedicae* could use the aconitic, citric, fumaric, malic and succinic acids as the sole carbon sources for growth.

Maleic acid is a cis-isomer of the fumaric acid (FELTHOUSE et al., 2001) and, when released by roots, has a significant influence on zinc phytoavailability in calcareous soils (MAQSOOD et al. 2011). Bacteria possessing the enzyme maleate isomerase can isomerize maleic acid to fumaric acid; however, maleate isomerase is unstable even at moderate temperatures (ROA ENGEL et al., 2008). This could explain why *H. seropedicae*, despite having maleate isomerase, could not grow using maleic acid as the sole carbon source.

Malonic acid is a three-carbon dicarboxylic acid synthesized via acetyl-coA carboxylase in seedling root tissue (STUMPF; BURRIS, 1981), with competitive inhibitor activity over succinate dehydrogenase (KIM, 2002). Diverse bacteria can grow on malonic acid as the sole source of carbon and energy, and malonate decarboxylase is the key to the degradation of this compound (DIMROTH; HILBI, 1997). Even though *H. seropedicae* possessed genes to encode malonate decarboxylase (PEDROSA et al., 2011); it was not possible to grow using this compound as a sole carbon source.

In plants, the way of accumulating oxalic acid is the oxidation of glyoxylate, which can be formed in photorespiration or by the action of isocitrate lyase in the TCA

(IGAMBERDIEV; EPRINTSEV, 2016). Some bacteria can degrade oxalic acid using oxalate decarboxylase and oxalate oxidase enzymes, such as the gram-negative *Oxalobacter formigenes* (ANANTHARAM; ALLISON; MALONEY, 1969; BALDINI et al., 2014). However, according to the genome, *H. seropedicae* does not have this enzyme, making it unable to grow using this compound as the sole carbon source.

The organic acids tested could induce a chemotactic response. Maleic, malonic and oxalic acids were not used as a carbon source for growth but could also attract *H. seropedicae*. However, little research has been conducted on the chemotaxis phenotype in *H. seropedicae*.

Tadra-sfeir et al., (2015) demonstrated that *Herbaspirillum seropedicae* SmR1 grown in the presence of the flavonoid naringenin was able to accelerate endophytic colonization in maize seedlings in the first 36h of colonization. Furthermore, transcriptional responses of *H. seropedicae* SmR1 demonstrated that environmental phosphate concentration could modulate the chemotaxis and mobility (GRILLO-PUERTAS et al., 2021).

Chemotaxis events begin with molecules binding to receptors on the plasma membrane. The *tar*, *tsr*, *trg* and *tap* genes encode these chemoreceptors (PEREIRA et al., 2004). Such genes are known to encode Methyl-accepting Chemotaxis Proteins (MCPs), which are reversibly methylated proteins. *H. seropedicae* has 66 genes encoding *mcp*s in its genome (PEDROSA et al., 2011).

The MCPs are homodimers, which act as starting sites for signal transduction (FALKE et al., 1997; MOWBRAY; SANDGREN, 1998). Therefore, they have a cytoplasmic domain, which is highly conserved between species (MORGAN; BAUMGARTNER; HAZELBAUER, 1993). The *mcps* genes were identified in *Escherichia coli*, acting in sensory transduction (STEWART; DAHLQUIST, 1987). The

abundance of specific chemotaxis receptors varies between species; the *tsr* and *tar* genes are the most found (HAZELBAUER; HARAYAMA, 1983).

The *cheABDRYW* genes are also involved in the chemotaxis signal transduction pathway. Mcp receptors form ternary signaling complexes with cytoplasmic proteins such as CheA, a histidine kinase controlling phosphorylated CheY and CheB concentration. CheY is a small single-domain protein, a response regulator that binds to the FLiM component of the flagellar apparatus after phosphorylation-inducing rotation (WELCH et al., 1993). CheB acts with CheR, a methyltransferase; both are involved in the adaptation process. CheR is an enzyme that transfers methyl groups from S-adenosylmethionine molecules to glutamate residues in the cytoplasmic domains of mcps, performing reversible methylation of these proteins. CheB is responsible for demethylation. The function of *CheW* is essential for forming the ternary complex, CheA-CheW-mcp (LI; HAZELBAUER, 2004; SPRINGER; KOSHLAND, 1977; WU et al., 1996).

Until the work of Kristich; Ordal, (2002) with *Bacillus subtilis*, the function of the CheD protein had not yet been fully elucidated. The work shows CheD homologs of this bacterium found in chemotaxis operons similar to many bacteria and archaea. This suggests that CheD is essential in chemotaxis and sensory transduction for many organisms. The authors showed that CheD catalyzes amide hydrolysis of MCPA chemoreceptor-specific glutaminyl side chains. In addition, there is evidence that CheD deactivates other chemoreceptors, including MCPB and MCPC, which are sites of initiation of signal transduction (Figure 11). *Herbaspirillum seropedicae* has 32 *che*-like genes dispersed in the genome (PEDROSA et al., 2011).

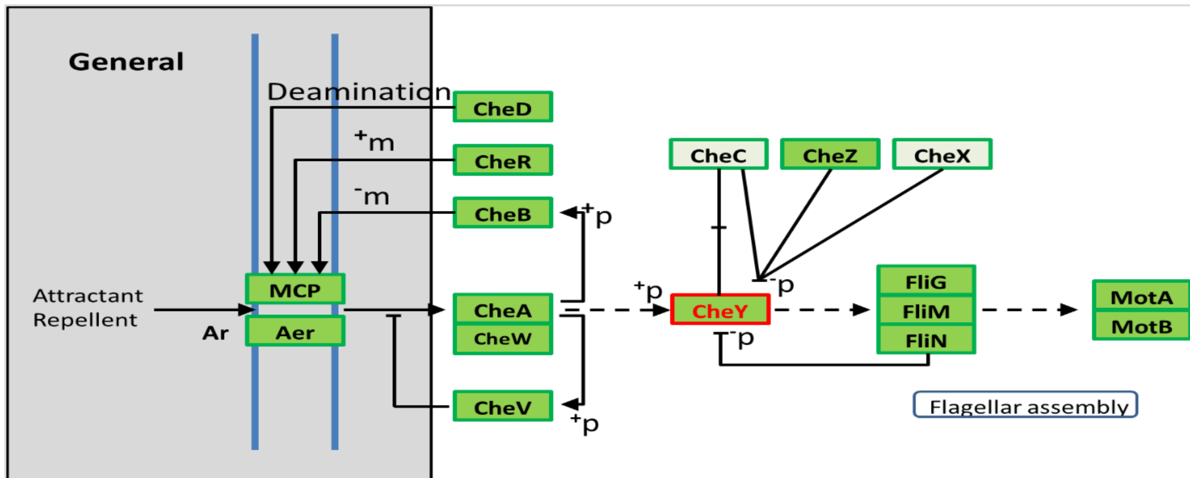

Figure 11: Representation of the consensus of the chemotaxis pathway for bacteria in general. The green squares are the genes present in *H. seropedicae*. Source Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/).

Our results demonstrated that contact with organic acids induced the genes *tar*, *tsr* and *Hsero_3720* to code for chemotaxis receptors. Only aconitic acid repressed the expression of these genes, and succinic acid repressed the *Hsero_3720* gene. Transcriptional profiling of *H. seropedicae* SmR1 grown with naringenin demonstrated that chemotaxis *tar* and *tsr* genes were expressed (TADRA-SFEIR et al., 2015). Balsanelli et al., (2016) demonstrated that planktonic cells SmR1 strain expressed *Hsero_3720* 4,00-fold and that the same gene was repressed after epiphytic colonization of maize roots. Also, a mutant strain carrying a disruption in *Hsero_3720* exhibited a twofold reduction in attachment capacity compared with the parental strain, proving that the *Hsero_3720* encoded protein is required for efficient sensing and colonization.

Here, the gene expression of *cheA* and *cheD* were induced by the presence of all organic acids tested, except for aconitic acid, indicating that the organic acids activated the expression of *mcps tar*, *tsr* and *Hsero_3720* genes and transmitted the signal to *cheA*; also the *mcps* are deactivated by the deamination from of cheD. *cheB*

was induced only by oxalic acid, indicating that the signal activated by this organic acid induces demethylation of the MCPs.

Studies with *H. seropedicae* SmR1 grown with naringenin also demonstrated genes related to chemotaxis, such as *cheRDBYZ* (TADRA-SFEIR et al., 2015). The expression of *mcp*s was repressed in *H. seropedicae* HRC54 grown in the presence of sugarcane apoplastic fluid (PESSOA et al., 2021).

Our results demonstrated that contact with organic acids induced flagellar formation with a different number of flagella according to the organic acid, resulting in the chemotaxis activity of the cells of *H. seropedicae*. Furthermore, the flagellar gene expression reveals that organic acids, compared with the control sample, repressed the biosynthetic flagellar genes *fliOPQ*.

Herbaspirillum seropedicae genome has at least 46 genes involved in flagella biosynthesis, assembly, and structure (PEDROSA et al., 2011). These genes are found in operons that are divided into three temporally regulated transcriptional classes: early (class1), middle (class 2), and late (class 3) and are regulated in response to environmental changes, being the genes *fliOPQ* belonging to class 2, where it is products are responsible for the assembling of the basal body and secretion apparatus (KOMEDA, 1986; LIU; MATSUMURA, 1994).

The operon *fliLMNOPQR* encodes components of the C-ring and secretion apparatus localized at the proximal portion of the flagella. High levels of *fliA* repress it, which leads to the initiation of *fliA*-dependent transcription from Class 3 promoters (*flgKL*, *fliDST*, *flgMN*, *fliC*, *tar-tap-cheRBYZ*, and *motAB-cheAW*. This coupling of transcription and assembly allows for efficient “just-in-time” expression kinetics (FITZGERALD; BONOCORA; WADE, 2014). The flagellar gene expression obtained here could reflect these *fliA* regulations, where the expression of *fliOPQ* was repressed

by *fliA*, and the expression of late genes of flagellar assemble and chemotaxis, as *tar* and *cheA* were induced by the organic acids (except for aconitic acid).

The flavonoid naringenin repressed genes involved in flagella biosynthesis, assembly, and structure *flhC*, *flhD*, *flgA*, *flgBCDEFGHI*, *flhB*, *fliA*, *fliD*, *fliFGHIJK*, and *fliOPQR* in SmR1 strain (TADRA-SFEIR et al., 2015). The flagellin and flagellar transcription genes, *fliC* and *flhC*, were down-regulated by the presence of the apoplastic fluid of sugarcane (PESSOA et al., 2021).

The biofilm formation is the last step of root epiphytic colonization before entering the root tissues for endophytic colonization (JAMES; OLIVARES, 1998; OLIVARES et al., 1997). Biofilm consists of exopolysaccharides (EPS), proteins, lipids, and other polymeric compounds exuded by bacterial cells, forming a complex matrix aggregate providing a microenvironment for sessile cells (FLEMMING; WINGENDER, 2001).

Colonization of *H. seropedicae* SmR1 within maize roots was not affected by the disruption of the *EpsB* gene, suggesting that attachment, epiphytic and endophytic colonization are not dependent on the *epsB* gene (BALSANELLI et al., 2014). The same results could be observed on the transcriptional profiling of *H. seropedicae* SmR1 attached in wheat roots after 3 days of inoculation, where the *epsB* gene was not expressed (PANKIEVICZ et al., 2016).

Using organic acids from the exudates of maize roots as sole carbon sources demonstrated a different influence on *H. seropedicae* biofilm production *in vitro*. Aconitic acids were the carbon source where *H. seropedicae* produced biofilm and the most expressed *Epsb* gene. These results could be a response of *H. seropedicae* in an endophytic stimulus, like when in a relationship with sugarcane, since the aconitic acid and glucose are the main constituents of sugarcane juice (CONSTANCIO A. ASIS

JR.; KATSUKI ADACHI; SHOICHIRO AKAO, 2004). Furthermore, endophytic diazotroph *Paraburkholderia tropica* strain Ppe8 had genes related to chemotaxis, flagella and exopolysaccharides repressed in response to sugarcane juice (DA SILVA et al., 2018), corroborating this hypothesis.

This study attempts to understand the *H. seropedicae* strain HRC54 response to organic acids exuded by maize roots by phenotypic observation and expression trends of genes. The data presented here are pioneers in observing the effects of chemoattraction by maize root exudates on this species and can contribute to new research about other compounds exuded and how it modulates the rhizospheric competence of *H. seropedicae*. The scheme in Figure 12 represents the integration of the transcriptional data obtained by this study related to chemotaxis, flagella biosynthesis, and biofilm in *H. seropedicae* HRC54 in response to organic acids.

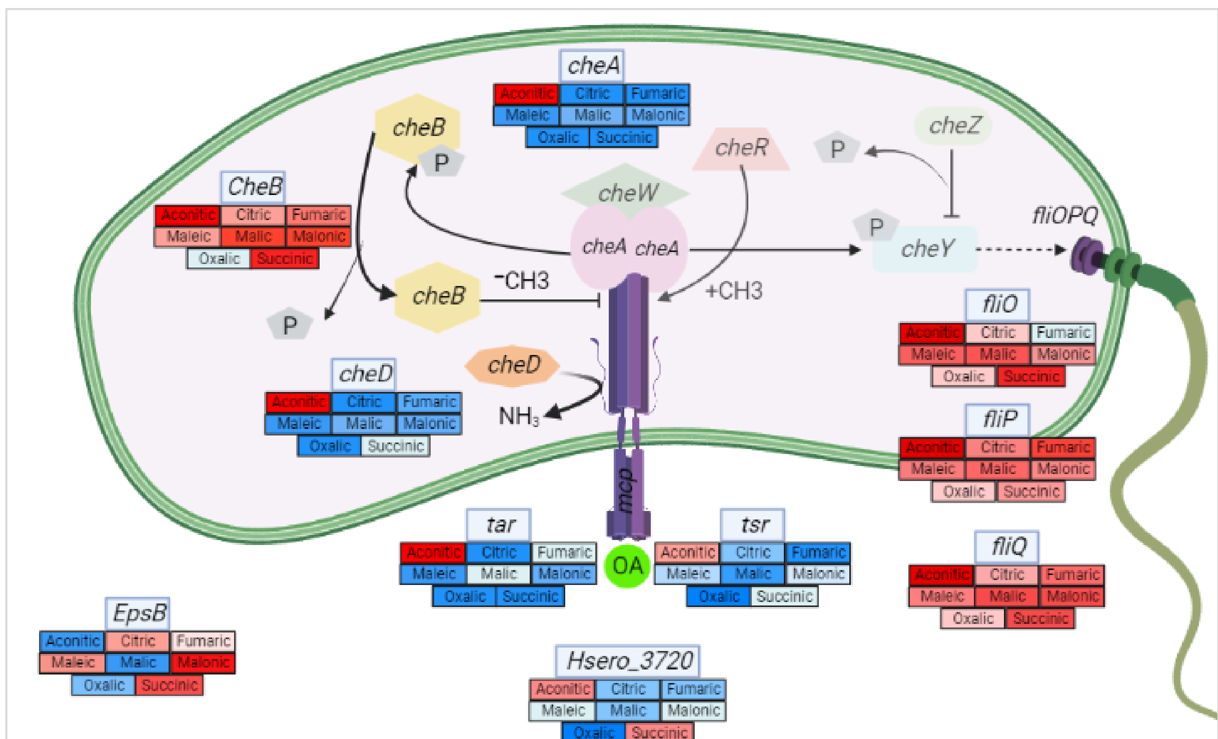

Figure 12: Integration of the transcriptional profile related to chemotaxis, flagellar biosynthesis, and biofilm formation by *H. seropedicae* in response to organic acids. Genes followed by organic acids groups in blue and red represented expression up and downregulated, respectively. OA: Organic acid.

5. Conclusion

The data obtained in this work reveals that organic acids exuded by maize roots can induce the chemotactic movement in *H. seropedicae* by activating methyl-accepting proteins and the transduction of the signals received. In addition, some organic acids also can modulate biofilm formation.

This work results contribute to understanding the rhizosphere communication between maize roots and *H. seropedicae* before the endophytic colonization.

REFERENCES

ANANTHARAM, V.; ALLISON, M. J.; MALONEY, P. C. Oxalate: formate exchange the basis for energy coupling in oxalobacter*. **THE JOURNAL OF BIOLOGICAL CHEMISTRY**, v. 264, n. 13, p. 7244–7250, 1969.

BACILIO-JIMÉNEZ, M. et al. Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. **Plant and Soil**, v. 249, n. 2, p. 271–277, 2003.

BADRI, D. V.; VIVANCO, J. M. Regulation and function of root exudates. **Plant, Cell and Environment**, v. 32, n. 6, p. 666–681, 2009.

BALDANI, J. I. et al. Characterization of *Herbaspirillum seropedicae* gen. nov. sp. nov. a Root- Associated Nitrogen-Fixing Bacterium. **INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY**, v. 36, n. 1, p. 86–93, 1986.

BALDINI, J. I. et al. The Prokaryotes - Alphaproteobacteria and Betaproteobacteria, **The Family Oxalobacteraceae. In: ROSEMBERG, E., et al. The Prokaryotes - Alphaproteobacteria and Betaproteobacteria 2014**. 4th edition ed. Springer Reference, 2014. p. 919–974.

BALSANELLI, E. et al. Exopolysaccharide biosynthesis enables mature biofilm formation on abiotic surfaces by *Herbaspirillum seropedicae*. **PLoS ONE**, v. 9, n. 10, 2014.

BALSANELLI, E. et al. Molecular adaptations of *Herbaspirillum seropedicae* during colonization of the maize rhizosphere. **Environmental microbiology**, v. 18, n. 8, p. 2343–2356, 2016.

BARBOSA, M. S. et al. Root exudate-supplemented inoculant of *Azospirillum brasilense* Ab-V5 is more effective in enhancing rhizosphere colonization and growth of maize. **Environmental Sustainability**, v. 3, n. 2, p. 187–197, jun. 2020.

BASTIÁN, F. et al. Production of indole-3-acetic acid and gibberellins A 1 and A 3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically-defined culture media. **Plant Growth Regulation**, v. 24, p. 7–11, 1998.

BAUDOIN, E.; BENIZRI, E.; GUCKERT, A. Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. **Soil Biology and Biochemistry**, v. 35, n. 9, p. 1183–1192, 2003.

BOTTINI, R.; CASSÁN, F.; PICCOLI, P. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. **Applied Microbiology and Biotechnology**, v. 65, n. 5, p. 497–503, 2004.

CANELLAS, N. O. A.; OLIVARES, F. L.; CANELLAS, L. P. Metabolite fingerprints of maize and sugarcane seedlings: searching for markers after inoculation with plant growth - promoting bacteria in humic acids. **Chemical and Biological Technologies in Agriculture**, v. 6, n. 14, p. 1–10, 2019.

CONSTANCIO A. ASIS JR.; KATSUKI ADACHI; SHOICHIRO AKAO. N2 fixation in sugarcane and population of n2-fixing endophytes in stem apoplast solution. **Philippine Journal of Crop Science**, v. 29, n. 2, p. 45–58, 2004.

CRUZ, L. M. et al. 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (. **APPLIED AND ENVIRONMENTAL MICROBIOLOGY**, v. 67, n. 5, p. 2375–2379, 2001.

DA SILVA, P. R. A. et al. Sugarcane apoplast fluid modulates the global transcriptional profile of the diazotrophic bacteria *Paraburkholderia tropica* strain Ppe8. **PLoS ONE**, v. 13, n. 12, p. 1–19, 2018.

DÖBEREINER, J.; BALDANI, V. L. D.; BALDINI, J. I. **Como Isolar e identificar bactérias diazotróficas de plantas não-leguminosas**. EMBRAPA-SPI, 1995. v. 136

ESTRADA, G. A. et al. Selection of phosphate-solubilizing diazotrophic *Herbaspirillum* and *Burkholderia* strains and their effect on rice crop yield and nutrient uptake. **Plant and Soil**, v. 369, n. 1–2, p. 115–129, 2013.

FALKE, J. J. et al. The two-component signaling pathway of bacterial chemotaxis: a molecular view of signal transduction by receptors, kinases, and adaptation enzymes. **Annual Review of Cell and Developmental Biology**, v. 13, n. 1, p. 457–512, 1997.

FELTHOUSE, T. R. et al. **MALEIC ANHYDRIDE, MALEIC ACID, AND FUMARIC ACID**. In Kirk-Othmer Encyclopedia of Chemical Technology, 2001, v. 15, p. 1–49.

FITZGERALD, D. M.; BONOCORA, R. P.; WADE, J. T. Comprehensive Mapping of the *Escherichia coli* Flagellar Regulatory Network. **PLoS Genetics**, v. 10, n. 10, p. 1–18, 2014.

GRILLO-PUERTAS, M. et al. Transcriptional Responses of *Herbaspirillum seropedicae* to Environmental Phosphate Concentration. **Frontiers in Microbiology**, v. 12, n. 666227, p. 1-15, 2021.

HAZELBAUER, G. L.; HARAYAMA, S. Sensory transduction in bacterial chemotaxis. **International Review of Cytology**, v. 81, p. 33–70, 1983.

IGAMBERDIEV, A. U.; EPRINTSEV, A. T. Organic acids: the pools of fixed carbon involved in redox regulation and energy balance in higher plants. **Frontiers in Plant Science**, v. 7, n.1042, p. 1-15, 2016.

JAMES, E. K.; OLIVARES, F. L. Infection and Colonization of Sugar Cane and Other Graminaceous Plants by Endophytic Diazotrophs. **Critical Reviews in Plant Sciences**, v. 17, n. 1, p. 77-119, 1998.

JIN, Y. et al. Role of maize root exudates in promotion of colonization of *Bacillus velezensis* Strain S3-1 in rhizosphere soil and root tissue. **Current Microbiology**, v. 76, n. 7, p. 855–862, 2019.

KIM, Y. S. Malonate metabolism: biochemistry, molecular biology, physiology, and industrial application. **Journal of Biochemistry and Molecular Biology**, v. 35, n. 5, p. 443–451, 2002.

KOMEDA, Y. Transcriptional control of flagellar genes in *Escherichia coli* K-12. **JOURNAL OF BACTERIOLOGY**, v. 168, n. 3, p. 1315–1318, 1986.

KRISTICH, C. J.; ORDAL, G. W. Bacillus subtilis *CheD* is a chemoreceptor modification enzyme required for chemotaxis. **Journal of Biological Chemistry**, v. 277, n. 28, p. 25356–25362, 2002.

LAMBRECHT, M. et al. Indole-3-acetic acid: a reciprocal signaling molecule in bacteria-plant interactions. **Trends in microbiology**, v. 8, n. 7, p. 298–300, 2000.

LI, M.; HAZELBAUER, G. L. Cellular stoichiometry of the components of the chemotaxis signaling complex. **Journal of Bacteriology**, v. 186, n. 12, p. 3687–3694, jun. 2004.

LIMA, L. DA S. et al. Root exudate profiling of maize seedlings inoculated with *Herbaspirillum seropedicae* and humic acids. **Chemical and Biological Technologies in Agriculture**, v. 1, n. 1, p. 1–18, 2014.

LIU, X.; MATSUMURA, P. The *FlhD*/*FlhC* Complex, a Transcriptional Activator of the *Escherichia coli* Flagellar Class II Operons. **JOURNAL OF BACTERIOLOGY**, v. 176, n. 23, p. 7345–7351, 1994.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-$^{\Delta\Delta}$CT method. **Methods**, v. 25, p. 402–408, 2001.

MORGAN, D. G.; BAUMGARTNER, J. W.; HAZELBAUER, G. L. Proteins antigenically related to methyl-accepting chemotaxis proteins of *Escherichia coli* detected in a wide range of bacterial species. **Journal of Bacteriology**, v. 175, n. 1, p. 133–140, 1993.

MOWBRAY, S. L.; SANDGREN, M. O. J. Chemotaxis receptors: A progress report on structure and function. **Journal of Structural Biology**, v. 124, n. 2–3, p. 257–275, 1998.

NARDI, S. et al. Soil organic matter mobilization by root exudates. **Chemosphere**, v. 41, n. 5, p. 653–658, 2000.

OLIVARES, F. L. et al. Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirillum*. **New Phytologist**, v. 135, n. 4, p. 723–737, 1997.

OLIVARES, F. L. **Taxonomia, ecologia e mecanismos envolvidos na infecção e colonização de plantas de cana-de-açúcar (*Saccharum sp.* híbrido) por bactérias endofíticas do gênero *Herbaspirillum*.** Tese de Doutorado—Seropedica: Universidade Federal Rural do Rio de Janeiro-UFRRJ, 1997.

OOSTHUIZEN, M. C. et al. Novel method for the proteomic investigation of a dairy-associated *Bacillus cereus* biofilm. **FEMS Microbiology Letters**, v. 194, n. 1, p. 47–51, 2001.

PANKIEVICZ, V. C. S. et al. RNA-seq transcriptional profiling of *Herbaspirillum seropedicae* colonizing wheat (*Triticum aestivum*) roots. **Plant Molecular Biology**, v. 90, n. 6, p. 589–603, 2016.

PEDROSA, F. O. et al. Genome of *Herbaspirillum seropedicae* strain SmR1, a specialized diazotrophic endophyte of tropical grasses. **PLoS Genetics**, v. 7, n. 5, 2011.

PEREIRA, M. et al. Chemotaxis and flagellar genes of *Chromobacterium violaceum*. **Genetics and Molecular Research**, v. 3, n. 1, p. 92–101, 2004.

PEREIRA, T. P. et al. Real-time PCR quantification of the plant growth-promoting bacteria *Herbaspirillum seropedicae* strain SmR1 in maize roots. **Molecular Biotechnology**, v. 56, n. 7, p. 660–670, 2014.

PESSOA, D. D. V. et al. Validation of reference genes for RT-qPCR analysis in *Herbaspirillum seropedicae*. **Journal of Microbiological Methods**, v. 127, p. 193–196, 2016.

PESSOA, D. D. V. et al. *Herbaspirillum seropedicae* strain HRC54 expression profile in response to sugarcane apoplastic fluid. **3 Biotech**, v. 11, n. 6, 2021.

ROA ENGEL, C. A. et al. Fumaric acid production by fermentation. **Applied Microbiology and Biotechnology**, v. 78, p. 379-389, 2008.

RODRIGUES-NETO, J.; MALAVOLTA JR., V.; VICTOR, O. Meio simples para o isolamento e cultivo de *Xanthomonas campestris* pv. *citri* tipo B. **Summa Phytopathol**, v. 12, p. 16, 1986.

ROLFE, S. A.; GRIFFITHS, J.; TON, J. Crying out for help with root exudates: adaptive mechanisms by which stressed plants assemble health-promoting soil microbiomes. **Current Opinion in Microbiology**, v. 49, p. 73-82, 2019.

ROVIRA, A. D. Plant root exudates. **The Botanical Review**, v. 35, n. 1, p. 35–57, 1969.

SASSE, J.; MARTINOIA, E.; NORTHEN, T. Feed your friends: do plant exudates shape the root microbiome? **Trends in Plant Science**, v. 23, n. 1, p. 25–41, 2018.

SINGH, P.; KUMAR, V.; AGRAWAL, S. Evaluation of phytase producing bacteria for their plant growth promoting activities. **International Journal of Microbiology**, v. 2014, p. 7, 2014.

SPRINGER, W. R.; KOSHLAND, D. E. Identification of a protein methyltransferase as the *cheR* gene product in the bacterial sensing system. **Proceedings of the National Academy of Sciences**, v. 74, n. 2, p. 533–537, 1977.

STEWART, R. C.; DAHLQUIST, F. W. Molecular components of bacterial chemotaxis. **Chemical Reviews**, v. 87, n. 5, p. 997–1025, 1987.

STUMPF, D. K.; BURRIS, R. H. Biosynthesis of malonate in roots of soybean seedlings. **Plant Physiol**. v. 68, p. 992-995, 1981.

TADRA-SFEIR, M. Z. et al. Genome wide transcriptional profiling of *Herbaspirillum seropedicae* SmR1 grown in the presence of naringenin. **Frontiers in Microbiology**, v. 6, n. 491, p. 1-8, 2015.

WEBER, O. B.; FREIRE, F. DAS C. O. Contribuição de bactérias diazotróficas na cultura da bananeira: perspectivas de utilização na produção integrada. **Boletim de Pesquisa e Desenvolvimento**, v. 16, p. 29p, 2003.

WELCH, M. et al. Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. **Proceedings of the National Academy of Sciences**, v. 90, p. 8787–8791, 1993.

WONG-NG, J.; CELANI, A.; VERGASSOLA, M. Exploring the function of bacterial chemotaxis. **Current Opinion in Microbiology**, v. 45, p. 16–21, 2018.

WU, J. et al. The Receptor Binding Site for the Methyltransferase of Bacterial Chemotaxis Is Distinct from the Sites of Methylation. **Biochemistry**, v. 35, n. 15, p. 4984–4993, 1996.

XIE, S. et al. Maize Root Exudates Recruit *Bacillus amyloliquefaciens* OR2-30 to Inhibit *Fusarium graminearum* Infection. **Phytopathology**, v. 112, n. 9, p. 1886–1893, 2022.

YE, J. et al. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. **BMC Bioinformatics**, v. 13, n. 134, p. 1-11, 2012, Disponível em: <http://www.biomedcentral.com/1471-2105/13/134>.

CONCLUSÕES GERAIS

Conclusões gerais

A quimiotaxia é o fenômeno onde microorganismos atraídos por sinais químicos são guiados a seguir em direção à fonte desses estímulos. Plantas podem exsudar compostos com função de repelir microorganismos indesejados e ou atrair microorganismos benéficos. Tal mecanismo é a base inicial para a interação microorganismo-planta no solo.

Os resultados desse trabalho expõem que a inoculação de *H. seropedicae* em plantas de milho tendem a induzir mudanças metabólicas nas raízes, levando a mudanças físico-químicas na superfície da raiz e na rizosfera, que favorecem a colonização por *H. seropedicae*, quanto a outros grupos de bactérias naturais da semente com características de promotores do crescimento vegetal.

Pela primeira vez, um estudo mostrou diretamente os efeitos quimiotáticos dos compostos exsudados por raízes de milho sobre *H. seropedicae*, e as alterações moleculares e fenotípicas da bactéria, como resposta aos estímulos químicos gerados pelo contato com os compostos liberados pelas plantas.

Conclui-se que os ácidos orgânicos exsudados por raízes de milho podem mediar a comunicação e a interação entre a planta e *H. seropedicae*. Mais precisamente, é possível utilizar esses compostos como aditivos para a formulação de novos bioinoculantes a fim de melhorar a competência rizosférica de *H. seropedicae* e garantir o sucesso da inoculação.