EFEITO DA HIPOMETILAÇÃO NA INTERAÇÃO DE MILHO COM BACTÉRIAS PROMOTORAS DO CRESCIMENTO VEGETAL

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Orientadora: Prof.ª Dr.ª Clícia Grativol Gaspar de Matos

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“A persistência é o caminho do êxito.”

(Charles Chaplin)

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ABREVIATURAS

µg	micrograma
µL	microlitro
BPCVs	Bactérias Promotoras de Crescimento Vegetal
PGPB	Plant growth-promoting bacteria
5-azaC	5-azacitidina
5mC	5-metilcitosina
DAE	Days After Emergence
HAI	Hours After Inoculation
ISSR	Inter Simple Sequence Repeats
DRM2	Domains Rearranged Methyltransferase 2
DML	Demeter-like
MET	Methyltransferase
MBD1	Methyl-CpG Binding Domain Protein 1
MBD7	Methyl-CpG Binding Domain Protein
SAM	S-adenosylmethionine synthetase
SAHA	Suberoylanilide Hydroxamic Acid
DCL	Dicer-like
CLS1	Chlorophyll Synthase 1-2
CSL3-4	Cellulose Synthase-like 3-4
DNG103	DNA Glycosylase 103
DNG101	DNA Glycosylase 101

RESUMO

A interação entre microrganismos e plantas desempenha um papel fundamental no desenvolvimento vegetal e na adaptação ao ambiente. Dentre esses microrganismos, as bactérias promotoras do crescimento vegetal (BPCV) são amplamente estudadas por sua capacidade de aumentar a produtividade agrícola de maneira sustentável. Além dos efeitos diretos na fisiologia vegetal, modificações epigenéticas, especialmente a metilação do DNA, essas bactérias podem influenciar essa interação ao regular a expressão gênica e as respostas adaptativas das plantas. Neste estudo, investigamos como a hipometilação do DNA em raizes de plântulas de milho afeta sua resposta à inoculação com *Herbaspirillum seropedicae,* analisando os impactos no crescimento, metabolismo e microbioma radicular. Nossos resultados demonstraram que o agente hipometilante 5-azaC não interfere no crescimento da bactéria, mas causa alterações fenotípicas significativas, especialmente nas raízes das plântulas. A inoculação bacteriana resultou em crescimento em todos os parâmetros avaliados nas plântulas. A microscopia revelou que a colonização bacteriana ocorre preferencialmente na zona pelúcida das raízes, enquanto a quantificação indicou maior acúmulo de bactérias nas raízes tratadas com 5-azaC. A análise da metilação global revelou que a bactéria modula a metilação da citosina de forma semelhante ao 5-azaC, sugerindo que a inoculação com a bactéria pode influenciar mecanismos epigenéticos da planta. A expressão diferencial de genes relacionados à metilação do DNA, regulação epigenética, crescimento celular e resposta ao estresse reforça a influência da hipometilação na interação planta-microrganismo. Além disso, a análise do bacterioma revelou que o 5-azaC altera significativamente a composição da comunidade microbiana radicular, enquanto a inoculação bacteriana tende a restaurar a microbiota para um estado semelhante ao controle, embora ainda apresente algumas modificações. A análise proteômica identificou 1.818 proteínas nos diferentes tratamentos, destacando modificações significativas nas vias metabólicas, como no metabolismo do carbono e na via do citrato. Essas modificações indicam como a hipometilação do DNA, combinada com a interação com *H. seropedicae*, pode influenciar profundamente os mecanismos celulares e vias metabólicas. Esses achados

ampliam o entendimento sobre os efeitos epigenéticos na interação planta-bactéria e proporcionar novos *insights* para o desenvolvimento de estratégias mais eficientes e sustentáveis.

Palavras chaves: Interação planta-microrganismo; Hipometilação do DNA; Regulação epigenética; 5-azacitidina; Microbioma.

ABSTRACT

The interaction between microorganisms and plants plays a fundamental role in plant development and environmental adaptation. Among these microorganisms, plant growth-promoting bacteria (PGPB) are widely studied for their ability to sustainably enhance agricultural productivity. In addition to their direct effects on plant physiology, these bacteria can influence this interaction through epigenetic modifications, especially DNA methylation, by regulating gene expression and adaptive responses in plants. In this study, we investigated how DNA hypomethylation in maize seedling roots affects their response to inoculation with *Herbaspirillum seropedicae*, analyzing the impacts on growth, metabolism, and root microbiome. Our results demonstrate that the hypomethylating agent 5-azaC does not interfere with bacterial growth but causes significant phenotypic changes, especially in seedling roots. Bacterial inoculation promoted growth in all evaluated seedling parameters. Microscopy revealed that bacterial colonization occurs preferentially in the root hair zone, while quantification indicated a higher accumulation of bacteria in roots treated with 5-azaC. Global methylation analysis revealed that the bacterium modulates cytosine methylation similarly to 5-azaC, suggesting that bacterial inoculation may influence the plant's epigenetic mechanisms. The differential expression of genes related to DNA methylation, epigenetic regulation, cell growth, and stress response reinforces the influence of hypomethylation on plant-microbe interaction. Additionally, bacteriome analysis revealed that 5-azaC significantly alters the composition of the root microbial community, while bacterial inoculation tends to restore the microbiota to a state similar to the control,

although some modifications persist. Proteomic analysis identified 1,818 proteins across different treatments, highlighting significant changes in metabolic pathways, such as carbon metabolism and the citrate cycle. These modifications indicate how DNA hypomethylation, combined with interaction with *H. seropedicae*, can profoundly influence cellular mechanisms and metabolic pathways. These findings expand the understanding of epigenetic effects in plant-bacteria interactions and provide new insights for developing more efficient and sustainable strategies.

Keywords: Plant-microbe interaction; DNA hypomethylation; Epigenetic regulation; 5-azacytidine; Microbiome.

1. INTRODUÇÃO

A interação entre microrganismos e plantas é mais antiga do que a existência da raça humana, uma vez que ambas as espécies existem há muito mais tempo. Desde o início dos tempos, essas duas formas de vida têm se comunicado de maneira complexa e eficiente. No entanto, inicialmente não percebemos a perfeição dessa comunicação e muitas vezes observamos as bactérias como organismos não benéficos, associando-as apenas a patologias e problemas (BERG et al., 2017; DELAUX; SCHORNACK, 2021).

Com o avanço da ciência, compreendemos que nem todas as bactérias causam malefícios; na verdade, muitas delas são benéficas para o crescimento e a saúde das plantas (RODRIGUEZ et al., 2019). Essas bactérias promotoras de crescimento vegetal (BPCV) desempenham papéis fundamentais, como a fixação de nitrogênio, a solubilização de fósforo e produção de hormônios que estimulam o desenvolvimento radicular e foliar (TIMOFEEVA; GALYAMOVA; SEDYKH, 2023).

Apesar dos avanços no conhecimento, ainda entendemos muito pouco sobre como essa comunicação tão antiga, estabelecida e refinada ocorre dentro das plantas. A interação microrganismo-planta é um mecanismo intrincado com sinais químicos e físicos que permite às plantas não apenas crescerem, mas também se adaptam a diversos estresses ambientais como seca, alta salinidade e temperaturas extremas (RODRIGUEZ et al., 2019; SHARIFI; RYU, 2021).

Entender essa interação vai além de um interesse acadêmico, é uma busca pela compreensão dos mecanismos fundamentais da vida. Ao decifrar como as plantas e seus microrganismos associados se comunicam e cooperam, podemos desenvolver novas estratégias agrícolas mais sustentáveis, melhorar a produtividade das culturas e reduzir a necessidade de insumos químicos nocivos ao meio ambiente (SINGH et al., 2020).

Assim, a exploração dessa relação simbiótica e mutualística entre plantas e microrganismos representa uma das fronteiras mais promissoras da biologia vegetal, oferecendo *insights* valiosos para a ciência, agricultura e sustentabilidade global.

1.1. Bactérias Promotoras de Crescimento Vegetal (BPCVs)

As bactérias promotoras de crescimento vegetal (BPCVs) são microrganismos que interagem com as plantas e desempenham um papel crucial no estímulo ao crescimento e promoção da saúde das plantas (CHENG; ZHANG; HE, 2019). Essas bactérias são conhecidas por seus diversos mecanismos de ação que favorecem o desenvolvimento vegetal, sendo cada vez mais valorizadas na agricultura sustentável (CHENG; ZHANG; HE, 2019; SRIVASTAVA et al., 2022).

Algumas BPCVs como Rhodotorula mucilaginosa e Arthrobacter spp. são bactérias eficazes na fixação de nitrogênio, reduzindo a necessidade de adição de fertilizantes químicos e colaborando para o crescimento de plantas como o trigo *(Triticum spp*.) (AASFAR et al., 2024). Este processo de fixação de nitrogênio é essencial para a disponibilizar esse nutriente, fundamental para o crescimento e produtividade das culturas agrícolas. Bactérias como *Azospirillum, Pseudomonas* e *Burkholderia* podem ser usadas para substituir fertilizantes, uma vez que as bactérias fixadoras de nitrogênio formam relações simbióticas com as espécies de plantas leguminosas, estabelecendo-se nas raízes e fornecendo nitrogênio diretamente para a planta hospedeira (CHENG; ZHANG; HE, 2019).

Outra importante função das BPCVs é a solubilização de fósforo como as bactérias *Pantoea sp.* e *Burkholderia cepacia* que são capazes de solubilizar formas insolúveis de fósforo presentes no solo, tornando-o disponível para absorção pelas plantas (LUO et al., 2024). O fósforo é um nutriente vital para vários processos fisiológicos, incluindo a fotossíntese, transferência de energia e a formação de ácidos nucleicos e a sua solubilização é uma estratégia eficaz para melhorar a absorção de nutrientes pelas plantas, reduzindo a dependência de fertilizantes (DADAŞOĞLU; DADAŞOĞLU; ORHAN, 2023; RANDIVE; AGNIHOTRI; BHAGAT, 2024).

As BPCVs podem também produzir fitohormônios, como o ácido indolacético (IAA), que estimula o crescimento radicular e foliar, promovendo o crescimento das raízes e brotos das plantas (FANAI et al., 2024). Esses hormônios são essenciais para o desenvolvimento das plantas, pois estão envolvidos com a regulação de processos tais como a divisão celular, elongação dos caules e formação de raízes laterais (FANAI et al., 2024; RANDIVE; AGNIHOTRI; BHAGAT, 2024).

Ainda, além de promover o crescimento vegetal, as BPCVs têm a capacidade de proteger as plantas contra patógenos. Elas produzem substâncias antimicrobianas que inibem o crescimento de microrganismos prejudiciais e competem com eles por espaço e nutrientes, o que ajuda a reduzir a incidência de doenças e contribui para a saúde geral das plantas (BERG, 2009; RODRIGUEZ et al., 2019; NOMAN et al., 2020).

As BPCVs podem contribuir nas respostas das plantas frente a estresses abióticos, como seca, alta salinidade e temperaturas extremas. As bactérias produzem compostos que aumentam a resistência das plantas tais como osmólitos compatíveis e componentes, antioxidantes que contribuem para a manutenção do equilíbrio osmótico e proteção contra danos causados pelo estresse oxidativo (RODRIGUEZ et al., 2019; TRIVEDI et al., 2020).

O uso de BPCVs na agricultura está ganhando destaque como uma estratégia ecológica e sustentável para aumentar a produtividade das culturas, ao mesmo tempo que reduz a dependência de fertilizantes químicos e pesticidas. A aplicação dessas bactérias no solo ou como inoculantes de sementes tem mostrado resultados promissores no aumento do rendimento das colheitas e na melhoria da saúde do solo (CHAUDHARY et al., 2023).

1.1.1 *Herbaspirillum seropedicae:* Bactéria Promotora de Crescimento Vegetal

Herbaspirillum seropedicae é uma bactéria diazotrófica endofítica, gram-negativa, conhecida por sua capacidade de fixar nitrogênio atmosférico e promover o crescimento das plantas com milho *(Zea mays*), arroz (*Oryza sativa*), sorgo (*Sorghum bicolor*), cana-de-açúcar (*Saccharum officinarum*), banana (*Musa*) e abacaxi *(Ananas comosus)*(TADRA-SFEIR et al., 2011). A fixação de nitrogênio por *H. seropedicae* vem mostrando a menor necessidade de fertilizantes nitrogenados, e assim, promovendo práticas agrícolas mais sustentáveis (CHUBATSU et al., 2012; WALLER et al., 2021).

Como as demais bactérias acima citadas, *H. seropedicae* está envolvida com a produção de hormônios vegetais, como auxinas e citocininas, que aumentam a absorção de nutrientes e melhoraram a eficiência fotossintética, resultando em maior saúde vegetal e produtividade (BRUSAMARELLO-SANTOS et al., 2017) e vem

mostrando a capacidade de minimizar os efeitos de alguns estresses ambientais, aumentando sua capacidade de sobrevivência em condições adversas (AGTUCA et al., 2020; IRINEU et al., 2022).

H. seropedicae é uma bactéria promissora para a agricultura sustentável, devido à sua capacidade de fixar nitrogênio, promover o crescimento das plantas e ajudar na adaptação a estresses ambientais. A pesquisa contínua sobre esta bactéria pode levar a novas estratégias agrícolas que beneficiem tanto os agricultores quanto o meio ambiente (ALVES et al., 2021).

1.1.1.1 Interação de *H. seropedicae* com raízes de Milho

A inoculação de raízes de plantas de milho com *H. seropedicae* tem mostrado melhoras notáveis no crescimento das raízes e da parte aérea dessas plantas. Essas melhorias se traduzem em um aumento da biomassa e produtividade, benefícios observados em diferentes genótipos e sob diversas condições (ÁVILA et al., 2020; ALVES et al., 2021; IRINEU et al., 2022). O uso de *H. seropedicae* aumenta a eficiência do uso de nitrogênio pelas plantas, promovendo um uso mais sustentável dos recursos, especialmente em condições de baixa fertilização nitrogenada (DE OLIVEIRA ARAUJO et al., 2014; MEHNAZ, 2017).

Os mecanismos de ação de *H. seropedicae* incluem a modulação dos níveis hormonais e aumento da assimilação de nitrogênio e carbono, que contribuem para o crescimento inicial das plantas (IRINEU et al., 2022). A interação entre diferentes genótipos de milho e a bactéria é variável, indicando que a diversidade genética do milho pode ser explorada para maximizar essas interações (DE OLIVEIRA ARAUJO et al., 2014; BRUSAMARELLO-SANTOS et al., 2017).

Do ponto de vista agronômico, a combinação da inoculação com *H. seropedicae* e fertilização nitrogenada tem o potencial de reduzir significativamente a necessidade de fertilizantes químicos, promovendo práticas agrícolas mais sustentáveis (DE OLIVEIRA ARAUJO; MERCANTE; VITORINO, 2015; ALVES et al., 2021). A eficácia da inoculação pode variar de acordo com as condições de nitrogênio no solo, sendo mais benéfica em ambientes de baixo nitrogênio (WALLER et al., 2021; KUANG et al., 2022). Essa abordagem integrada não só aumenta a produtividade das culturas, mas também contribui para a saúde do solo e conservação do meio ambiente.

1.2. Produção de milho no Brasil

O milho (*Zea mays*) é um dos principais cereais cultivados no Brasil, desempenhando um papel fundamental na economia e na segurança alimentar do país (DE OLIVEIRA DUARTE; MATTOSO; GARCIA, 2021). O Brasil é o terceiro maior produtor mundial de milho, ficando atrás somente dos Estados Unidos e China respectivamente (BRASIL, 2024). A cultura do milho é essencial para diversos setores, incluindo a alimentação humana, uma vez que o mesmo é uma fonte de energia e nutrientes, com 72% de amido, 10% de proteína e 4% de gordura, um alimento ideal para regiões com deficiências de micronutrientes (RANUM; PEÑA-ROSAS; GARCIA-CASAL, 2014), a produção de ração animal e a indústria de biocombustíveis (POPP et al., 2016; SHURSON, 2017).

Mesmo o Brasil sendo uns dos maiores produtores, há muitas perdas anuais de milho e arroz são significativas, resultando em um prejuízo econômico estimado em US$1,7 bilhão (ABBADE, 2021). Apesar dos avanços, a produção de milho no Brasil enfrenta desafios significativos, incluindo variações climáticas, pragas, doenças e oscilações no mercado internacional (BECERRA-SANCHEZ; TAYLOR, 2021). Em Santa Catarina o vírus do rayado fino do milho, que foi recentemente identificado, indicando a necessidade de monitoramento e controle mais rigorosos (ALBUQUERQUE et al., 2022).

A sustentabilidade da cultura também tem sido uma preocupação crescente, incentivando pesquisas sobre práticas agrícolas mais eficientes, como o manejo integrado de pragas (MIP) e a utilização de estratégias epigenéticas para melhorar a adaptação do milho a condições adversas. O MIP é crucial para a sustentabilidade agrícola, pois reduz o uso de pesticidas químicos, minimizando impactos ambientais e riscos à saúde humana (BARZMAN et al., 2015; ANDERSON et al., 2019; BAKER; GREEN; LOKER, 2020).

Uso de culturas geneticamente modificadas como o milho Bt, são integradas ao MIP para controlar pragas de forma mais eficiente, reduzindo a necessidade de inseticidas convencionais (ANDERSON et al., 2019; GASSMANN; REISIG, 2023; BRYANT et al., 2024) . No entanto, a resistência das pragas a essas culturas é um desafio contínuo (GASSMANN; REISIG, 2023; BRYANT et al., 2024).O MIP pode reduzir significativamente o uso de inseticidas (até 95%) enquanto mantém ou aumenta a produtividade, como demonstrado em experimentos com milho e

melancia 8. No entanto, a resistência das pragas e a necessidade de refúgios não-Bt são desafios que precisam ser geridos (GLASER; MATTEN, 2003; BRYANT et al., 2024).

As estratégias epigenéticas estão sendo exploradas para melhorar a resiliência do milho a condições adversas, como mudanças climáticas e estresse ambiental. Essas estratégias podem complementar o MIP ao aumentar a capacidade das plantas de resistir a pragas e doenças sem depender exclusivamente de modificações genéticas ou pesticidas (ANDERSON et al., 2019; FAHAD et al., 2021).

1.3. Metilação do DNA em Plantas

A genética e a epigenética são duas áreas de estudo que investigam alterações hereditárias na atividade e função dos genes. Enquanto a genética se concentra nas mudanças na sequência de DNA, a epigenética busca compreender os processos que alteram a leitura do DNA, mas sem modificação da sua sequência. Isso envolve modificações químicas como a metilação do DNA, modificações pós-traducionais das histonas e RNAs não codificantes, modificações epigenéticas hereditárias que podem influenciar o fenótipo da planta. O dinamismo e a plasticidade do epigenoma desempenham um papel importante no desenvolvimento e evolução das plantas em resposta ao ambiente em que estão inseridas (MOORE; LE; FAN, 2013; LUCIBELLI; VALOROSO; ACETO, 2022).

A metilação do DNA consiste na adição de um grupamento metil nas citosinas da região alvo do DNA que está envolvida no controle da expressão gênica e, portanto, é um importante regulador da estrutura e organização funcional da cromatina (MOORE; LE; FAN, 2013; MENG et al., 2015; FEINBERG; LEVCHENKO, 2023).

No tecido vegetal, a metilação do DNA ocorre em regiões simétricas, como CG, não-CG e CHG, além de contextos assimétricos, como CHH, onde H representa qualquer nucleotídeo, exceto guanina (HENDERSON; JACOBSEN, 2007; ZHANG; LANG; ZHU, 2018; SUN et al., 2022). Durante o processo de metilação do DNA, um grupo metil (-CH3) é transferido para a base do DNA, principalmente para a citosina (C) adjacente a uma guanina (G) no carbono 5 C5, resultando na formação da 5-metilcitosina (5mC). A posição 5 da C desempenha um papel crucial na regulação

epigenética e na expressão de genes nucleares, além de contribuir para a estabilidade do genoma (ZHANG; LANG; ZHU, 2018; SUN et al., 2022).

Em plantas, a via de metilação do DNA dependente de RNA, RdDM (*RNA-directed DNA methylation*) se dá por meio de moléculas de RNA não codificantes que adicionam diretamente a metilação do DNA alvo, e pode ocorrer de duas formas: canônica e não canônica. A forma canônica envolve a interação de várias proteínas e enzimas responsáveis por transcrever pequenos RNAs e clivá-los em fitas simples de RNA, resultando na metilação do DNA. Já a forma não canônica é menos comum e está relacionada à metilação de elementos móveis recém-transpostos do DNA (HENDERSON; JACOBSEN, 2007; YANG et al., 2016; LUCIBELLI; VALOROSO; ACETO, 2022).

A metilação do DNA em plantas tem um impacto significativo no desenvolvimento e na resposta a estresses ambientais. Estudos têm mostrado que a metilação diferencial do DNA pode regular a expressão de genes envolvidos na resposta à seca, salinidade e outras condições adversas, permitindo que as plantas se adaptem melhor ao seu ambiente (ZHANG; LANG; ZHU, 2018; SUN et al., 2022)

1.3.1. Metilação do DNA e a Interação Planta-Bactéria

Alterações dos padrões de metilação do DNA podem modular a capacidade das plantas de reconhecer e responder a diferentes tipos de bactérias (CHEN et al., 2022; MARTIN et al., 2024). A colonização de BPCV nas raízes de plantas, como com *H. seropedicae* promove benefícios tais como a disponibilidade de nutrientes essenciais e mecanismos de biocontrole (RONCATO-MACCARI et al., 2003; OLANREWAJU; BABALOLA, 2019; GUPTA; SCHILLACI; ROESSNER, 2022).

Estudos sugerem que a metilação do DNA possui um importante papel na regulação da interação planta-bactéria, influenciando a secreção de compostos, que promovem o crescimento de bactérias benéficas, e modulando a expressão de genes de defesa em resposta a patógenos (DOWEN et al., 2012; VÍLCHEZ et al., 2020). Por exemplo, a desmetilação (eliminação de um grupo metilo, -CH3) ativa do DNA em *Arabidopsis thaliana* controla a secreção de mio-inositol pelas raízes, promovendo o crescimento da planta através da interação com a bactéria benéfica *Bacillus megaterium*. Este processo é essencial para a colonização e atração preferencial de *B. megaterium*, sugerindo um mecanismo epigenético conservado

que regula a mutualidade entre plantas e rizobactérias promotoras de crescimento (VÍLCHEZ et al., 2020; CHEN et al., 2023).

Na interação planta-bactéria patogênica, a metilação do DNA pode modular a resposta da planta, influenciando a expressão de genes e mecanismos de defesa. A metilação diferencial de genes relacionados à imunidade pode determinar a capacidade da planta de reconhecer e responder a diferentes tipos de patógenos, ajustando a intensidade e duração da resposta imune (MARINUS; CASADESUS, 2009; TIRNAZ; BATLEY, 2019).

Em patógenos como *Agrobacterium tumefaciens*, a metilação do DNA, mediada pela metiltransferase CcrM é crucial para a manutenção do genoma e afeta a motilidade, formação de biofilme e viabilidade celular. A metilação influência também a expressão de genes essenciais para a replicação e regulação do ciclo celular, demonstrando uma conexão direta entre a metilação do DNA e a patogenicidade (TIRNAZ; BATLEY, 2019; MARTIN et al., 2024).

A metilação dinâmica do DNA responde a estresses bióticos, como a exposição a patógenos bacterianos, e resulta em regiões metiladas diferencialmente que estão associadas a genes diferencialmente expressos. Em *A. thaliana*, essas mudanças epigenéticas podem regular genes vizinhos em resposta ao estresse, destacando a importância da metilação do DNA na resposta imune das plantas. Alterações na metilação do DNA podem ajudar as plantas a "lembrar" de infecções passadas, permitindo uma resposta mais rápida e eficaz a futuros ataques patogênicos (DOWEN et al., 2012).

Além disso, a interação com bactérias benéficas pode, por sua vez, influenciar o epigenoma da planta. Estudos têm mostrado que a colonização por bactérias promotoras de crescimento pode levar a mudanças epigenéticas, como a metilação do DNA, que beneficiam a planta a longo prazo, melhorando sua capacidade de adaptação a estresses ambientais e aumentando sua produtividade (CHEN et al., 2022).

2. OBJETIVO

2.1. Objetivo geral

Caracterizar os efeitos da modulação epigenética induzida por inibidor químico de metilação do DNA durante a interação entre raízes de plantas de milho e a bactéria *Herbaspirillum seropedicae.*

2.2. Objetivos específicos

- Medir variações no crescimento de *H. seropedicae* e desenvolvimento de plântulas de milho após tratamento com 5-azaC.
- Quantificar a colonização bacteriana durante a interação *H. seropedicae* milho submetida ao tratamento com 5-azaC.
- Caracterizar a colonização e distribuição de *H. seropedicae* em plântulas de milho sob hipometilação do DNA de células radiculares.
- Determinar a expressão diferencial de genes em raízes de milho submetidas ao tratamento com 5-azaC por RT-qPCR.
- Obter biblioteca metagenômica do microbioma radicular de milho após tratamento com 5-azaC e interação com *H. seropedicae*.
- Determinar perfil proteômico das raízes de milho sob hipometilação induzida por 5-azaC e interação com *H. seropedicae.*

3. CAPÍTULOS

3.1. Capítulo-1: Epigenetic modulation of maize growth and microbiome by *Herbaspirillum seropedicae* inoculation and DNA hypomethylation

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ABSTRACT

The interaction between microorganisms and plants plays a fundamental role in plant development and environmental adaptation. Among these microorganisms, plant growth-promoting bacteria (PGPB) are widely studied for their ability to enhance agricultural productivity and sustainably. Beyond their direct effects on plant physiology, epigenetic modifications, particularly DNA methylation that can regulate gene expression and induce plant adaptive responses. This study investigates how DNA hypomethylation affects early interaction of maize (*Zea mays*) and plant-growth promoting bacteria - *Herbaspirillum seropedicae*-, with the focus on differences in plant growth, metabolism, and root microbiome. Our results demonstrate that the hypomethylating agent *5-azacytidine* (5-azaC) does not interfere with bacterial growth, but induces significant phenotypic changes in maize, particularly in root morphology. Bacterial inoculation led to the enhancement of plant growth across all measured biometric parameters. Microscopy analyses revealed preferential bacterial colonization in the mucilage zone of maize roots, while quantification assays indicated higher accumulation of bacteria in roots treated with 5-azaC. Global methylation analysis showed that *H. seropedicae* modulates cytosine methylation in a manner like 5-azaC, suggesting that bacterial inoculation can impact plant epigenetic mechanisms. Analysis of differential expression of genes related to DNA methylation machinery supports the role of hypomethylation in shaping plant-microbe interactions. Moreover, bacteriome profiling demonstrated that 5-azaC significantly

alters the root microbial community, while bacterial inoculation tends to restore microbiota composition to a state resembling the control, although with some persistent modifications. Proteomic analysis identified 1,818 proteins across different treatments, revealing significant alterations in metabolic pathways, particularly carbon metabolism and the citric acid cycle. These changes highlight how DNA hypomethylation, in combination with *H. seropedicae* interaction, can profoundly deploy cellular mechanisms and metabolic processes, offering new insights into early plant-microbe interactions. These findings enhance our understanding of epigenetic regulation in plant-bacterium interactions and may contribute to the development of more effective and sustainable agricultural approaches.

Keywords: Plant-microorganism interaction; DNA hypomethylation; Epigenetic regulation; 5-azacitidine; Microbiome.

3.1.1. INTRODUCTION

The increasing demand for environmentally friendly agricultural practices has driven the search for alternatives that reduce reliance on chemical fertilizers and mitigate their ecological impact [1]. One of the most promising solutions in this context is the use of bioinputs, which encompasses a range of biological agents and natural compounds that can enhance plant growth and productivity while maintaining environmental sustainability. Among bioinputs, plant growth-promoting bacteria (PGPB) have gained noteworthy attention due to their ability to improve crop yield and resilience through various mechanisms [2–4].

Plant growth-promoting bacteria (PGPB) have emerged as key contributors to sustainable agriculture by facilitating nitrogen fixation, phosphate solubilization, and the production of phytohormones that enhance root and shoot development [5,6]. These microorganisms interact with plants at the molecular level, triggering physiological responses that lead to increased stress tolerance and overall growth improvement [7].

At a molecular level, PGPB-mediated plant growth regulation involves complex signaling pathways, gene expression modulation, and metabolic adjustments. The interaction between plants and PGPB can induce changes in gene regulatory

networks, influencing key biological processes such as nutrient uptake, hormone production, and defense responses [7,8].

Maize (*Zea mays*) is a critical staple crop for global food security, providing essential calories and nutrients for both human and livestock consumption. It plays a significant role in the diets of billions, particularly in developing regions[9–11]. As a model cereal with a well-annotated genome, maize also offers unique opportunities to investigate epigenetic regulation in response to microbial interactions[12,13]

In recent years, numerous studies have investigated the effectiveness of PGPB, linking their beneficial effects to direct mechanisms such as biofertilization, biostimulation, biocontrol, and abiotic stress mitigation [14–16]. Despite these advances, a crucial aspect has been little explored: the molecular and epigenetic modifications induced by plant-PGPB interactions [7].

Epigenetic modifications, which regulate gene expression without altering DNA sequence, play a fundamental role in plant development and adaptation to environmental conditions. Among these modifications, DNA methylation is one of the most extensively studied, as it directly influences transcriptional activity and plant responses to external stimuli, including interactions with beneficial microbes [7,17,18].

In plants, DNA methylation occurs in three distinct sequence contexts: cytosine-guanine (CG), cytosine-H-guanine (CHG), and cytosine-H-H (CHH), where H represents adenine (A), cytosine (C), or thymine (T). The molecular mechanisms involved in the maintenance of these methylation patterns are well characterized [7,19,20]. CG methylation is maintained by METHYLTRANSFERASE 1 (MET1), CHG methylation is controlled by CHROMOMETHYLASE 2 (CMT2) or 3, while CHH methylation is regulated by DOMAINS REARRANGED METHYLASE 2 (DRM2) or CMT2. Conversely, active DNA demethylation is catalyzed by DNA glycosylases, including repressor of silencing 1 (ROS1), demeter (DME), and demeter-like enzymes [21].

This epigenetic regulation not only affects transcriptional activity [18,22] but also plays a crucial role in morphological development and phenotypic plasticity [23,24]. Population-wide studies have shown that DNA methylation patterns vary among individuals within a species, leading to extensive phenotypic differences, including biomass accumulation, disease resistance, and environmental adaptation

[25,26]. These variations can be decisive for plant survival and productivity under diverse stress conditions [27–30].

Previous studies with *Arabidopsis thaliana* have demonstrated that PGPB can modulate DNA methylation patterns, influencing root architecture and stress responses, allowing plants to adapt to environmental fluctuations [6,30]. Similar effects have been observed in crops such as *Phytolacca americana*, where epigenetic modifications induced by PGPB enhance nutrient uptake and drought tolerance. Additionally, PGPB-driven epigenetic modifications correlate with increased biomass production and pathogen resistance [6,7].

Given these insights, understanding how DNA methylation modulates plant-PGPB interactions is essential for advancing agricultural biotechnology to developing sustainable crop improvement strategies. This study aims to elucidate the epigenetic mechanisms underlying the response of hypomethylated maize roots to PGPB inoculation, exploring the impact of this interaction on plant growth, metabolism, and adaptation.

3.1.2. MATERIALS AND METHODS

3.1.2.1. Bacterial growth assessment

Herbaspirillum seropedicae strain RAM10 growth in the presence of the DNA methylation inhibitor 5-azacytidine (5-azaC) was assessed using a 96-well microplate assay. Bacterial cultures were initially grown in liquid DYGS medium (2 g of glucose, 2 g of malic acid, 1.5 g of bacteriological peptone, 2 g of yeast extract, 0.5 g of K_2HPO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.5 g of glutamic acid, adjusted to pH 6.0.) at 30°C with agitation at 120 rpm until reaching an optical density at 600 nm (OD_{600}) of 1.0. For the assay, an inoculum corresponding to 10% of the final volume was added to each well, containing DYGS medium and different concentrations of 5-azaC (2.5, 25, and 250 µM). A control without 5-azaC was included to evaluate bacterial growth in the absence of the hypomethylating agent. Bacterial growth was monitored hourly by measuring OD_{600} over a period of 16 hours using a microplate spectrophotometer, with incubation at 30°C under continuous shaking at 120 rpm.

3.1.2.2. Plant growth

The seeds of *Zea mays* (UENF 506-11) were superficially sterilized with 70% ethanol for 5 minutes, followed by immersion in 1.5% sodium hypochlorite for 30 minutes. The seeds were then washed 6 times with ultrapure water and left to soak for 6 hours. For germination, the seeds were sown in Petri dishes (150 × 25 mm) containing 20 mL of agar agar medium (5 g/L agar, pH adjusted to 6), sterilized in an autoclave at 120°C for 15 minutes. The dishes were incubated in B.O.D at 27°C with a 12/12h photoperiod and light intensity of 100 µmol m-2 s -1 for 4 days. After this period, the seedlings were transferred to test tubes (25 × 150 mm) containing three glass spheres (1.6 cm diameter) and 10 mL of Murashige and Skoog (MS) medium at ½ strength, with pH adjusted to 5.8. The medium was sterilized in an autoclave at 120°C for 15 minutes, and the seedlings were maintained in B.O.D at 27°C with a 12/12h photoperiod and light intensity of 100 µmol m-2 s -1 for an additional 3 days, adding up to 7 days of culture.

3.1.2.3. Methylation Inhibitor Treatment

A stock solution of 5-azacitidine (5-azaC) was prepared at 100 mM, dissolved in dimethyl sulfoxide (DMSO). From this stock solution, a 10 mM solution of 5-azaC was prepared in ultrapure water. The 5-azaC solution was added to both the agar agar and MS media at the desired concentrations. The treatment with 5-azaC was applied in three stages: (i) during seed germination, where seeds were sown in Petri dishes containing agar agar medium with 0.25, 2.5 and 25µM 5-azaC; (ii) when transferring the seedlings to MS medium containing 0.25, 2.5 and 25µM 5-azaC; (iii) when the seedlings were inoculated with bacteria, the MS medium was also supplemented with 0.25, 2.5 and 25µM 5-azaC.

3.1.2.4. Bacterial Growth and Inoculation

The *H. seropedicae* strain RAM10, containing the GFP:Tn5 marker in its chromosomal DNA, was cultivated in DYGS liquid medium at 30°C for 24 hours shaking at 120 rpm. After bacterial growth, 5-days-old maize seedlings were

inoculated with the bacterial solution, adjusting the final concentration to 2 × 10^6 bacteria/mL.

3.1.2.5. Bacterial inoculation and methylation inhibition assay

The seedlings were distributed into the following experimental conditions: 5-azaC treatment (A), where seedlings were treated with 5-azaC during germination and growth, without bacterial inoculation; inoculation with *H. seropedicae* (B), where seedlings were inoculated with *H. seropedicae* without 5-azaC treatment; 5-azaC treatment and inoculation with *H. seropedicae* (AB), where seedlings were treated with 5-azaC during germination and growth and later inoculated with *H. seropedicae*; and the Control (C), where maize seedlings were neither treated with 5-azaC nor inoculated with *H. seropedicae*. To better illustrate the experimental design and methodology, a schematic representation was also created (Figure 1).

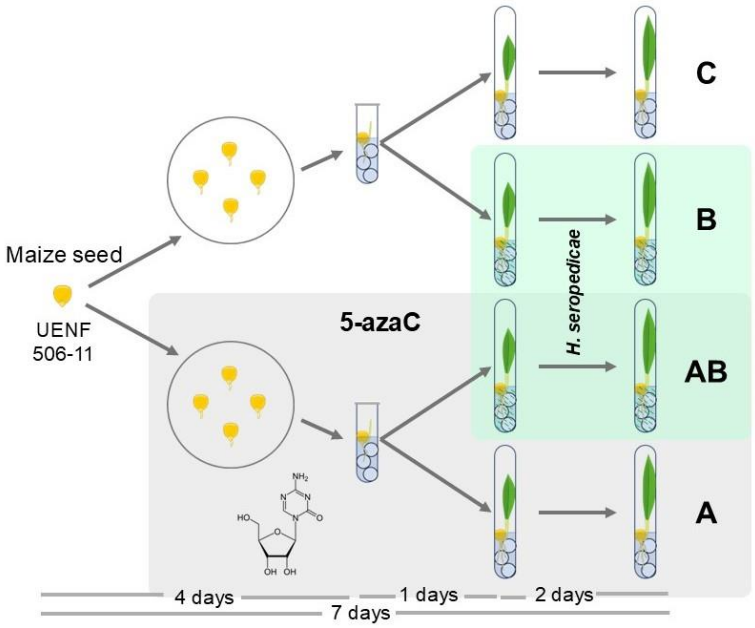


Figure 1. Schematic representation of the experimental design, highlighting the different treatment conditions applied to maize seedlings.

3.1.2.6. Biometric Variable Analysis

The experiment was conducted using a completely randomized design (CRD), with twenty biological replicates for each treatment (C, B, A, AB). The seedlings were

subjected to biometric evaluations, which included measurements of the length (cm) of root and aerial segment fresh and dry weight of the plant.

3.1.2.7. Nucleic Acid Extraction and cDNA Synthesis

Genomic DNA from maize roots was extracted using the CTAB (Cetyltrimethylammonium bromide) method[31,32]. Total RNA was extracted using Trizol® (Invitrogen), following the manufacturer's instructions. Nucleic acid concentration and purity were assessed using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). DNA and RNA integrity was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide. cDNA was synthesized from 5 µg of RNA using the GoScript™ Reverse Kit (Promega), following the manufacturer's protocol.

3.1.2.8. RT-qPCR Analysis

The quantification of *H. seropedicae* followed the protocol described by Da Silva[33], with modifications. For gene expression analysis, RT-qPCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems). Amplifications were carried out using the Applied Biosystems StepOne™ Real-Time PCR System, following the manufacturer's recommendations, in 96-well plates with a final volume of 10 µL. Each reaction contained 0.75 µL of each primer (forward and reverse), 7.5 µL of SYBR Green, 3 µL of cDNA, and 3 µL of ultrapure water. Primers were designed using the OligoAnalyzer tool from Integrated DNA Technologies (IDT) (Table S1). Relative gene expression was quantified using the 2^−ΔΔCT method, as described by[34].

3.1.2.9. Fluorescence and Scanning Electron Microscopy (SEM)

In order to observe bacteria inoculated. For fluorescence microscopy entire roots were placed on glass slides with sterile distilled water and observed under an ECLIPSE Ni (Nikon) fluorescence microscope, equipped with specific filters for GFP

detection (BP 460-490 nm; LP 510-550 nm) and a Prime Vision FL digital photography to image capture system. Observations were performed on longitudinal sections of the pellucid zone of maize roots condictions C, B, A and AB

For maize roots were cut into 1 cm long segments, including the root cap, elongation zone, and root hair zone, and immediately fixed in Karnovsky's solution (4% formaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.4). Samples were then washed with the same buffer (3 times for 10 min), dehydrated in an ethanol series (15%, 30%, 50%, 70%, 90%, and 2x 100% for 10 min each), and dried in a critical point drying device (Baltec CPD 030). The segments were mounted on aluminum stubs, sputter-coated with ionized platinum (Bal-tec SCD 050), and visualized using the scanning electron microscopy (SEM) Zeiss EVO 40 SEM at 15 kV.

3.1.2.10. DNA Methylation Analysis

The DNA extracted from the roots of the different treatments (C, B, A, and AB) was digested into nucleosides using the Nucleoside Digestion Mix from New England Biolabs. The reaction mixture was prepared as follows: 1 µl of DNA, 2 µl of Nucleoside Digestion Mix Reaction Buffer (10X), and 1 µl of Nucleoside Digestion Mix, with the volume adjusted to 20 µl. The mixture was incubated in a thermomixer at 37°C for 24 hours. After this period, the reaction was heated at 70°C for 10 minutes and then centrifuged at 10,000 rpm for 10 minutes at 23°C. From the resulting solution, 18 µl were transferred to a new microtube, and a 200x dilution was prepared for mass spectrometry analysis. The run was conducted as described by Adamczyk[35].

To assess the cytosine methylation pattern in maize DNA, the isoschizomeric enzyme HpaII and MspI were used. Genomic DNA was extracted and diluted in ultrapure water to a final concentration of 25 ng/µL. Approximately 250 ng of DNA from each sample was digested with 5U of HpaII and MspI (Promega) in the presence of 1X reaction buffer and ultrapure water, in a final volume of 50 µL per sample, at 37°C for 2 h. Subsequently, the samples were amplified by PCR using ISSR markers (Table S2). PCR reactions were performed in a final volume of 25 µL containing 5 µL of GoTaq® Flexi buffer (Promega), 0.5 µM primer, 0.15 mM dNTPs, 1U of Taq DNA polymerase (Promega), 3 mM $MgCl_2$, and 15 ng of DNA. The

amplification program consisted of an initial denaturation step at 94°C for 4 min, followed by 45 cycles of denaturation at 94°C for 45 s, annealing at a temperature optimized for each primer (Table S2), extension at 72°C for 2 min, and a final extension step at 72°C for 7 min.

PCR products were separated by electrophoresis in 1X TBE buffer and 2% agarose gels. The gels were stained with SYBR® Safe DNA Gel Stain (Sigma) and visualized using a UV transilluminator. Fragment sizes were estimated using a 100 bp molecular weight marker (Amresco). For quantification of global DNA methylation, PCR amplification patterns were recorded as a binary matrix, where bands of similar size were classified as 1 (band present) or 0 (band absent) (Table S3).

3.1.2.11. Quantification of Fluorescent Inoculum Colony Forming Units (CFUs)

After seven days of growth under the different conditions (C, B, A, AB), the plant roots were collected. For bacterial extraction, one gram of root was macerated in sterile 0.85% saline solution and the resulting suspension was then subjected to serial dilutions.

The quantification of colony-forming units (CFUs) was performed using the Drop Plate technique [36]. The culture medium used was DYGS solid, prepared with the following composition (per 1 L of medium): 2 g of glucose, 2 g of malic acid, 1.5 g of bacteriological peptone, 2 g of yeast extract, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.5 g of glutamic acid, and 15 g of agar, adjusted to pH 6.0.

Serial dilutions were applied to the Petri dishes, which were then incubated at 30°C for 20 hours in a thermostat. The quantification of CFUs was performed using a fluorescence microscope equipped with filters specific for GFP detection (BP 460–490 nm; LP 510–550 nm). Only the fluorescent colonies were counted, ensuring that the quantification was specific to the bacterial inoculum.

3.1.2.12. Metataxonomic Analysis

The DNA extracted from the roots of the different conditions (C, B, A, and AB) was sent to the company “Genone” for sequencing of the 16S rRNA gene using the Illumina MiSeq platform, with three replicates per condition. The quality and quantity

of the extracted DNA were examined using electrophoresis on a 1.8% agarose gel and DNA concentration and purity were determined with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with the primer pairs 338F: 5'-ACTCCTACGGGAGGCAGCA-3' and 806R: 5'- GGACTACHVGGGTWTCTAAT-3' . Both the forward and reverse 16S primers were tailed with sample-specific Illumina index sequences to allow for deep sequencing. The PCR was performed in a total reaction volume of 10 µl: DNA template 5-50 ng, forward primer (10µM) 0.3 µl, reverse primer (10µM) 0.3 µl, KOD FX Neo Buffer 5 µl, dNTP (2 mM each) 2 µl, KOD FX Neo 0.2 µl, and finally ddH2O up to 20µL. After with initial denaturation at 95 °C for 5 min, followed by 20 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 40 s, and a final step at 72 °C for 7 min. The amplified products were purified with Omega DNA purification kit (Omega Inc., Norcross, GA, USA) and quantified using Qsep-400 (BiOptic, Inc., New Taipei City, Taiwan, ROC). The amplicon library was paired end sequenced (2×250) on an Illumina NovaSeq6000.

The qualified sequences with more than 97% similarity thresholds were allocated to one operational taxonomic unit (OTU) using USEARCH (version 10.0). Taxonomy annotation of the OTUs/ASVs was performed based on the Naive Bayes classifier in QIIME2[37] using the SILVA database[38,39] (release 138.1) with a confidence threshold of 70%. Alpha was performed to identify the complexity of species diversity of each sample utilizing QIIME2 software. Beta diversity calculations were analyzed by principal coordinate analysis (PCoA) to assess the diversity in samples for species complexity. One-way analysis of variance was used to compare bacterial abundance and diversity. Linear Discriminant Analysis (LDA) coupled with effect size (LEfSe) was applied to evaluate the differentially abundant taxa.

3.1.2.13. Label-Free Proteomic Analysis

Roots from maize seedlings subjected to treatments C, B, A, and AB after seven days of growth were collected for proteomic analysis. Three biological replicates per treatment (300 mg fresh mass) were ground in liquid nitrogen and resuspended in 1 mL of extraction buffer (10% TCA/acetone, 20 mM DTT). Samples

were vortexed for 30 min at 4°C, incubated at −20°C for 1 h for precipitation, and centrifuged (16,000 g, 30 min, 4°C). The pellet was discarded, and the protein concentration in the supernatant was determined using the Bradford assay (Bio-Rad) with BGG as the standard.

Proteins were solubilized in 7 M urea and 2 M thiourea solution and digested with trypsin using Microcon-30 kDa filter units (Millipore), following the FASP protocol[40] with modifications. Peptides were quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), and 1 µg was injected into a nanoAcquity UPLC system coupled to a SYNAPT G2-Si Q-TOF mass spectrometer (Waters, Manchester, UK), as described by Botini et al. (2021).

Proteomic analysis was performed using ProteinLynx Global SERVER (PLGS) v.3.02 (Waters), and label-free quantification was conducted with ISOQuant v.1.7 (Distler et al., 2014). Differential protein abundance was assessed using a two-tailed Student's t-test, considering proteins as significantly up- or down-accumulated if $p \leq 0.05$ and $\log_2$ fold-change ≥ 0.5 or ≤ -0.5. Functional annotation of differentially expressed proteins was performed in ShinyGO (https://bioinformatics.sdstate.edu/go/) using KEGG pathway analysis to identify key metabolic pathways.

3.1.3. RESULTS

3.1.3.1. Effect of 5-azaC on Bacterial Growth

To determine whether the hypomethylating agent 5-azaC impacts the growth of *H. seropedicae*, bacterial cultures were exposed to different concentrations of the compound. The growth dynamics, illustrated in Figure 2, indicate that 5-azaC does not alter bacterial proliferation within the tested concentration range. Growth was assessed under treatments of 2.5 µM, 25 µM, and 250 µM of 5-azaC, and compared to untreated controls.

These results demonstrate that 5-azaC does not compromise bacterial growth, supporting its suitability for studies focused on epigenetic interactions without unintended effects on bacterial viability.

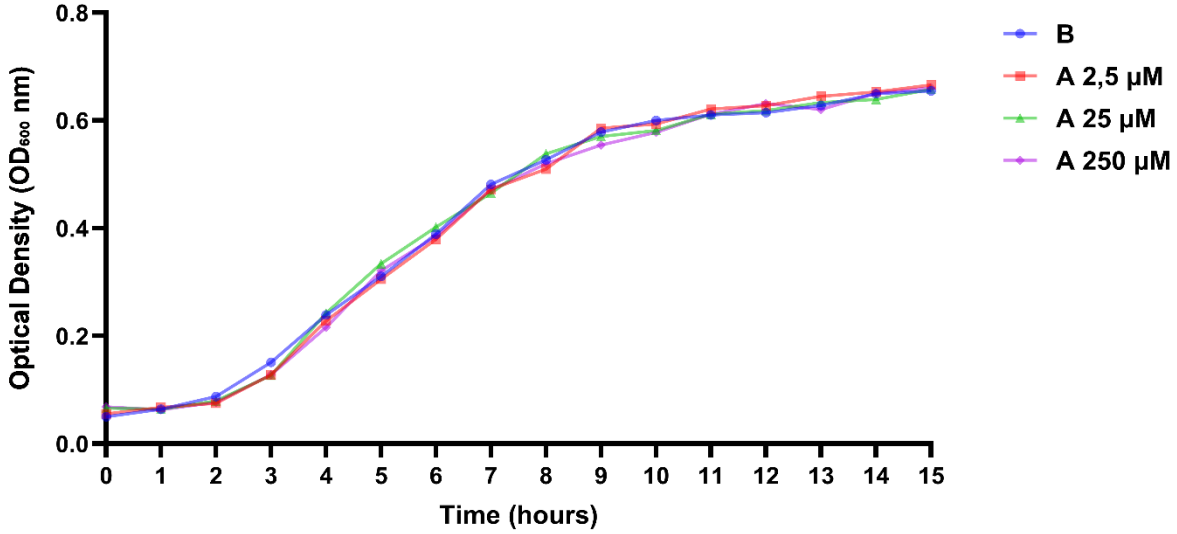


Figure 2. Growth curves of *H. seropedicae* treated with 5-azaC (A). Bacteria were exposed to 2.5 µM, 25 µM, and 250 µM of 5-azaC, and an untreated control group. No significant differences ($p > 0.05$) were detected usinng ANOVA analysis.

3.1.3.2. Effect of 5-azaC on Maize Seedling Growth

Aware that the hypomethylating agent 5-azaC has a significant impact on plant epigenetic mechanisms without compromising the growth and viability of *Herbaspirillum seropedicae*, we tested different concentrations to identify a condition that allows observation of phenotypic alterations associated with hypomethylation and plant-bacteria interaction.

At the concentration of 25 µM, shown in Supplementary Figure S1, root development was severely impaired, while the bacteria did not exhibit significant changes in hypomethylated treatments (A and AB). This result indicates that high doses of 5-azaC primarily affect root architecture, possibly through mechanisms linked to the altered expression of root growth-associated epigenetic genes. Interestingly, the comparison between control (C) and bacteria-only (B) treatments revealed a significant increase in shoot length and fresh and dry masses in treatment B, suggesting a growth-promoting effect by the bacteria in the absence of the hypomethylating agent.

Reducing the concentration to 2.5 µM (Fig. 3) resulted in less harmful but yet with characteristic effects of the hypomethylating treatment. Under this condition, the outcomes plant-bacteria interaction became more evident. A statistically significant increase in shoot length and dry mass was observed in treatments A and AB. These

findings suggest that hypomethylation induced by moderate doses of 5-azaC may create favorable conditions for bacteria to promote initial seedling growth, particularly in the shoot. Finally, at 0.25 µM (Supplementary Figure S2), the effects of the hypomethylating agent were less pronounced compared to higher concentrations. . This concentration appears to be insufficient to induce robust epigenetic changes that are capable of altering significantly seedling development or enhancing plant-bacteria interaction. Based on these results, we selected the 2.5 µM concentration for subsequent analyses, as it showed characteristic hypomethylation effects and demonstrated changes promoted by plant-bacteria interaction, particularly in the seedling shoot.

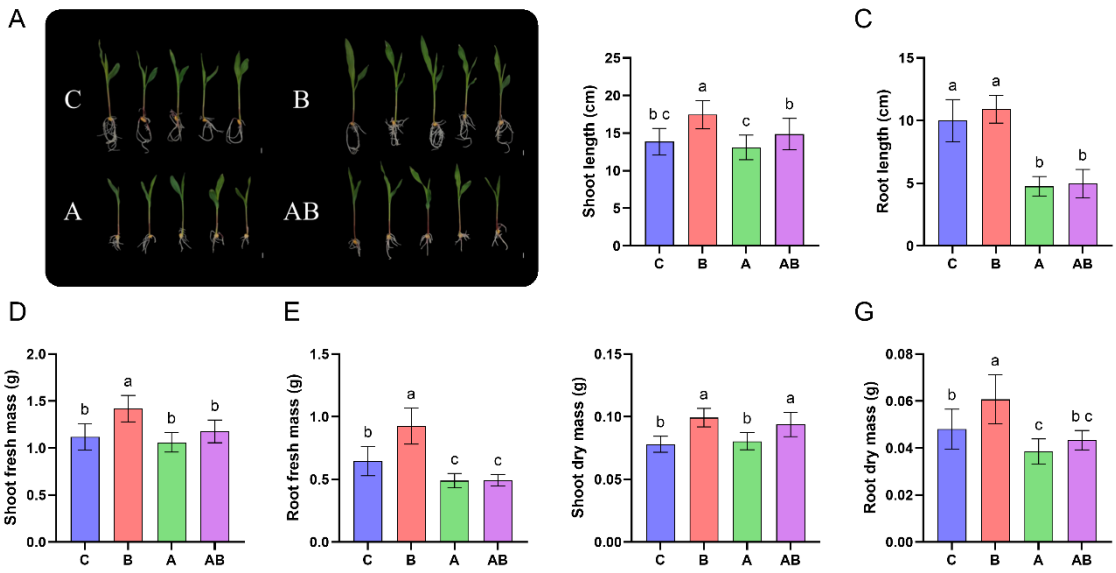


Figure 3. Effect of the methylation inhibitor (5-azaC) on maize seedlings development at 7 DAI. Treatments included 2,5 µM 5-azaC and inoculation with H. seropedicae for 48 HAI. (A) Image showing the effect of the compound on seedling growth (Scale bar = 1 cm). (B, D, F) Measurements of shoot length, fresh mass, and dry mass, respectively. (C, E, G) Measurements of root length, fresh mass, and dry mass, respectively. Letters (a, b, c, d) indicate significant differences between treatments based on Tukey's test. DAI – Days after imbibition; HAI – Hours After Inoculation. In the graphs, the labels C, B, A, and AB correspond to Control, Bacteria, 5-azaC, and 5-azaC + and Bacteria, respectively.

3.1.3.3. Quantification of DNA Methylation Induced by treatments

To understand the effects of the hypomethylating agent 5-azaC on DNA methylation in maize roots, a quantification analysis of global DNA methylation, unmethylated cytosines, hemimethylation at CHG sites, and fully methylated CG sites was performed, as shown in Figure 4. As expected, a significant reduction in DNA

methylation was observed in plants treated with 5-azaC, consistent with its reported role as a DNA methylation inhibitor. Likewise, the bacterial treatment (Herbaspirillum seropedicae) also caused a noticeable reduction in global DNA methylation, suggesting that the plant-bacteria symbiosis significantly impacts DNA methylation patterns.

This reduction in DNA methylation correlates with the observed growth promotion in bacterial treatment (B), indicating that these epigenetic modifications may play a key role in the physiological responses of the plant. Notably, in plants treated with both 5-azaC and bacteria (AB), the levels of cytosine methylation were significantly lower than in plants treated with 5-azaC alone (A). This suggests an additive effect where bacteria may target specific DNA regions for hypomethylation that are distinct from those affected by the chemical inhibitor.

Interestingly, phenotypic differences between treatments A and B (5-azaC and bacteria, respectively) were evident, as observed in Figure 3, despite their similar levels of hypomethylation. This reinforces the hypothesis that bacterial symbiosis induces specific hypomethylation patterns in DNA that are associated with growth promotion.

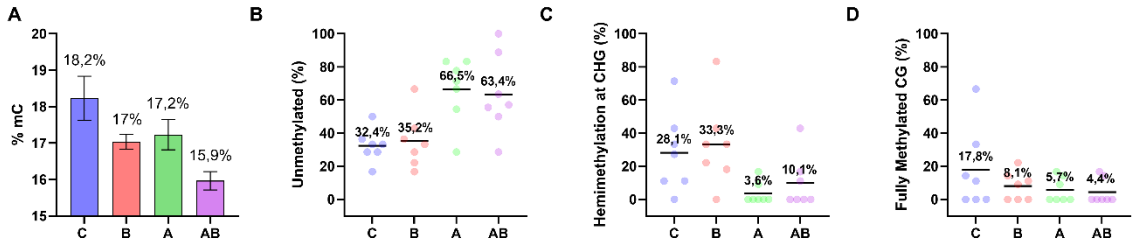


Figure 4. Percentage of cytosine methylation in maize root DNA from seedlings treated with the methylation inhibitor 5-azaC at a concentration of 2.5 µM for 7 DAI and inoculated with *H. seropedicae* for 48 HAI. The graph displays global cytosine methylation (A), unmethylated cytosines (B), hemimethylation at CHG sites (C), and fully methylated CG sites (D). Treatments are represented as follows: C (Control), B (Bacteria), A (5-azaC), and AB (5-azaC + Bacteria). Data are presented as mean percentages with standard error.

When comparing the results obtained through mass spectrometry-based DNA methylation analysis and those from MS-ISSR, differences in the methylation profiles were observed. Specifically, the percentage of unmethylated cytosines (Fig 4B) and hemimethylation at CHG sites (Fig 4C) showed distinct patterns of methylation. However, fully methylated CG sites (Fig 4D) showed a similar pattern with global methylation analysis, particularly in bacterial treatments, suggesting that the bacteria inoculation induced specific hypomethylation at gene loci.

Furthermore, in treatment AB, there is a trend toward intermediate methylation patterns between treatments A and B (Fig 4). This is evidenced by a slight decrease in unmethylated cytosines and a slight increase in hemimethylation at CHG sites compared to treatment A. These results suggest an additive or synergistic interaction between 5-azaC and bacterial treatments in modulating DNA methylation patterns, particularly at CG-rich regions, which may underpin the observed phenotypic effects.

3.1.3.4. Gene Expression Modulation Induced by treatments

To investigate DNA methylation modulation and its relationship with phenotypic characteristics, we performed gene expression analysis in maize seedling roots treated with the DNA methylation inhibitor 5-azaC and inoculated with H. seropedicae. This analysis focused on genes associated with the maintenance and removal of DNA methylation, as well as with epigenetic regulation.

The DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE 2) gene, which encodes a methyltransferase essential for maintaining DNA methylation in previously methylated regions, showed significant expression changes across all treatments (Fig 5A). A marked decrease in expression was observed in the bacterial treatment (B) compared to the control (C).

The DML (DEMETER-LIKE) gene, responsible for active demethylation and epigenetic reprogramming, was differentially expressed in all treatments. Both bacterial treatments (B and AB) showed reduced expression levels, suggesting that plant-bacteria interaction decreases DML expression (Fig 5B). However, no statistically significant changes were observed in the hypomethylation treatment (A).

The MET (METHYLTRANSFERASE) gene, associated with CpG island methylation maintenance, hormonal regulation, stress response, and development, exhibited significantly reduced expression, particularly in the bacterial (B) and combined bacterial-hypomethylation (AB) treatments (Fig 5C).

The MBD1 and MBD7 (METHYL-CPG BINDING DOMAIN PROTEINS) genes, which encode proteins that bind methylated DNA and are involved in transcriptional repression and interactions with other epigenetic factors, exhibited distinct expression patterns (Fig 5D.E). MBD1 showed reduced expression in B, A, and AB treatments, while MBD7 showed positive regulation in the AB treatment, contrasting with the negative regulation observed in B and A treatments.

Genes involved in RNA processing and epigenetic regulation were also assessed. The SAMS (S-ADENOSYLMETHIONINE SYNTHETASE) gene, which participates in the biosynthesis of S-adenosylmethionine (SAM), a critical methyl donor for DNA, RNA, and protein methylation, exhibited reduced expression in treatments B, A, and AB compared to the control (Fig 5F). The SAHH (S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE) gene, an inhibitor of histone deacetylases, did not show any significant expression changes (Fig 5G). The DCL (DICER-LIKE) gene, essential for siRNA and miRNA biogenesis, was downregulated in all treatments (Fig 5H). The bacterial treatment (B) showed the lowest expression levels, while A and AB were also negatively regulated but less so compared to B.

Furthermore, genes related to RNA directed DNA Methylation (RdDM) pathway, cell growth, development, and stress response, such as CLASSY (CLS) 1-2 and CLASSY 3-4, were analyzed. The CLS 1-2 gene exhibited differential expression in all treatments (Fig 5I). Treatment B showed reduced expression, while A and AB were positively regulated, with AB showing the highest expression. The CSL 3-4 gene exhibited increased expression in all treatments compared to the control, with treatment A being the most significantly expressed (Fig 5J).

Finally, genes DNG101 and DNG 103 homologous of AtROS1 tuning the level of demethylase activity in response to methylation alterations, thus ensuring epigenomic stability, showed significant reductions in expression; DNG 101 exhibited decreased expression across treatments B, A, and AB, while DNG 103 also showed reduced expression but with less variation between treatments (Fig 5K.L). The AB treatment had the highest expression among the treatments for DMG 103.

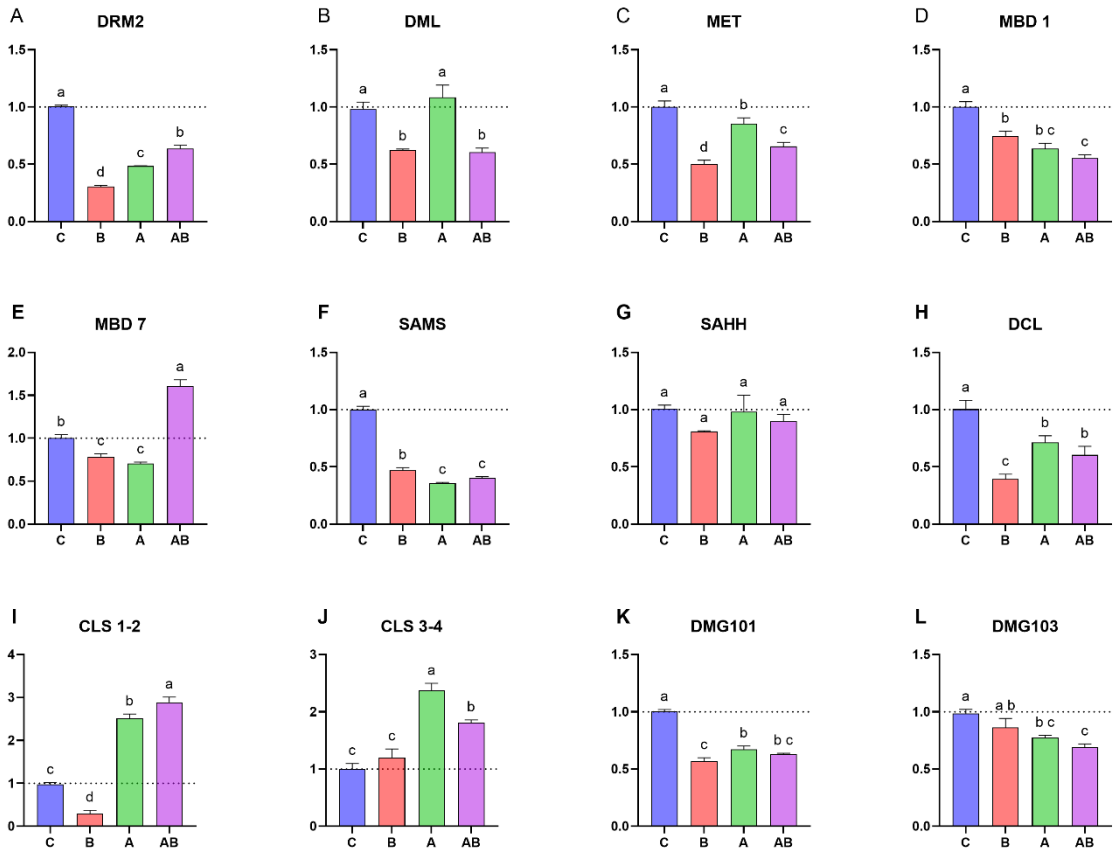


Figure 5. Expression of genes related to DNA methylation and epigenetic modulation (A–E); genes involved in RNA processing and epigenetic regulation (F–H); and genes associated with cell growth, development, and stress response (I–L) in maize seedlings treated with the methylation inhibitor 5-azaC at a concentration of 2.5 µM for 7 DAI and inoculated with *H. seropedicae* for 48 HAI. Letters (a, b, c, d) indicate significant differences between treatments based on Tukey's test. Treatments are represented as follows: C (Control), B (Bacteria), A (5-azaC), and AB (5-azaC + Bacteria).

3.1.3.5. Fluorescence Analysis in Maize Roots Induced by 5-azaC Treatment and *H. seropedicae*

To investigate the interaction between maize seedlings and *H. seropedicae,* fluorescence microscopy was performed in order to detect the bacterial strain used (RAM10) that contains a chromosomal GFP fluorescent marker, allowing the visualization of bacterial colonization on the roots to confirm the successful interaction.

As shown in Figure 6, a characteristic colonization was observed near the root hairs in treatments B (Bacteria) and AB (5-azaC and Bacteria), while no fluorescence was detected in the control (C) or in the treatment with 5-azaC alone (D), as

expected. These results confirm that H. seropedicae RAM10 was able to colonize maize roots although the presence of the bacterial inoculum.

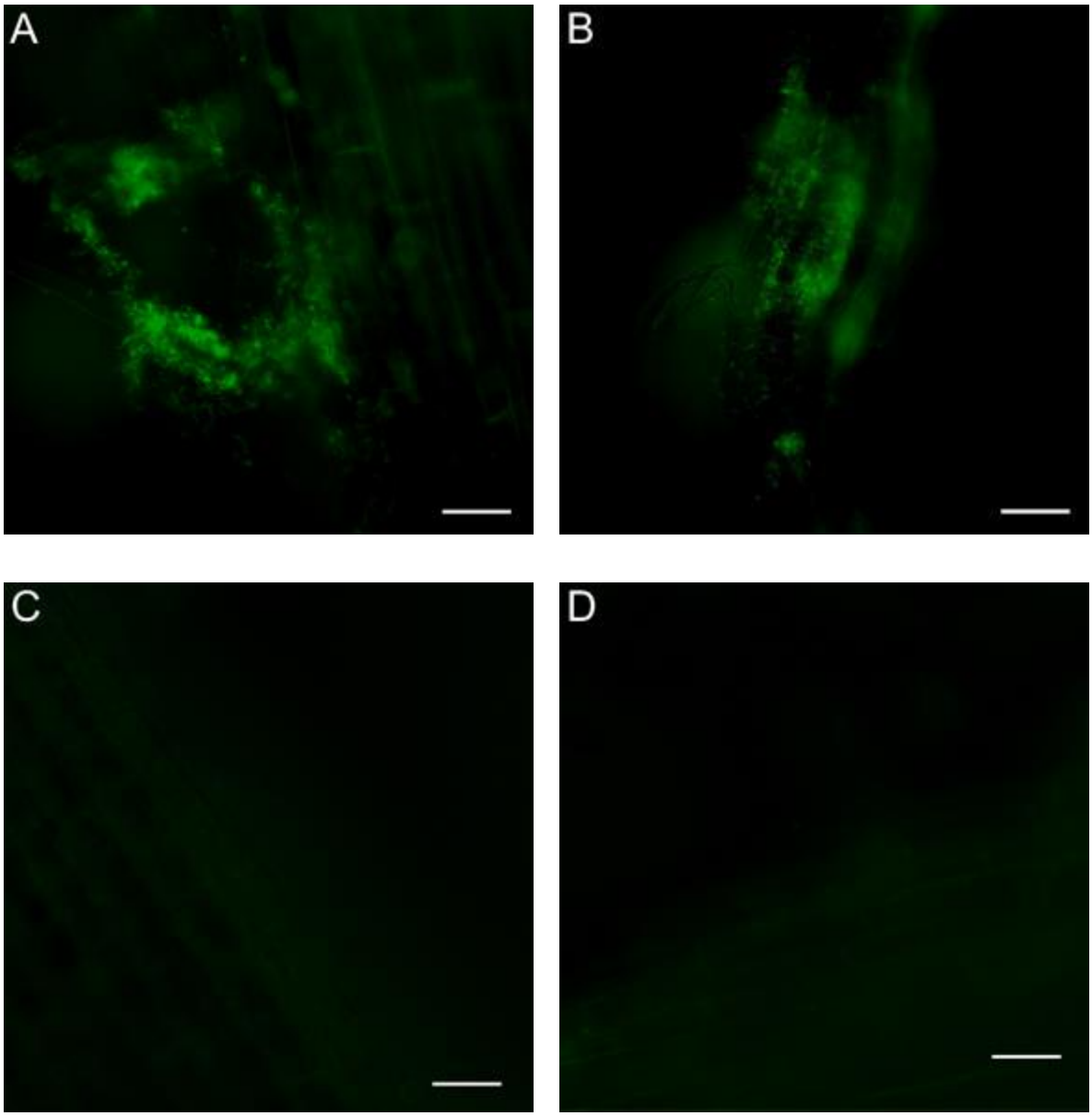


Figure 6. Fluorescence microscopy analysis of maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAI and inoculated with H. seropedicae expressing the fluorescent marker GFP (RAM10) for 48 HAI. (A) Bacteria-only treatment, (B) 5-azaC + Bacteria, (C) Control, and (D) 5-azaC-only treatment. Longitudinal sections of the pellucid zone. Scale bar: 40 µm.

Given this colonization pattern, a follow-up methodology was implemented to quantify the bacterial inoculum using CFU counts. Since only the GFP-tagged *H. seropedicae* strain fluoresces, this method allowed specific quantification of the inoculated bacteria.

Figure 6 demonstrates the quantification of bacterial CFUs and their respective dilutions (Fig. 7A.B) and the total bacterial quantification via RT-PCR (Fig.

7C). Treatments B and AB showed significant CFU presence, confirming the absence of contamination in the experiment. Additionally, the comparison between treatments B and AB revealed a statistically significant increase in bacterial colonization in the AB treatment (5-azaC + and Bacteria), suggesting that the hypomethylation induced by 5-azaC eased bacterial interaction and colonization.

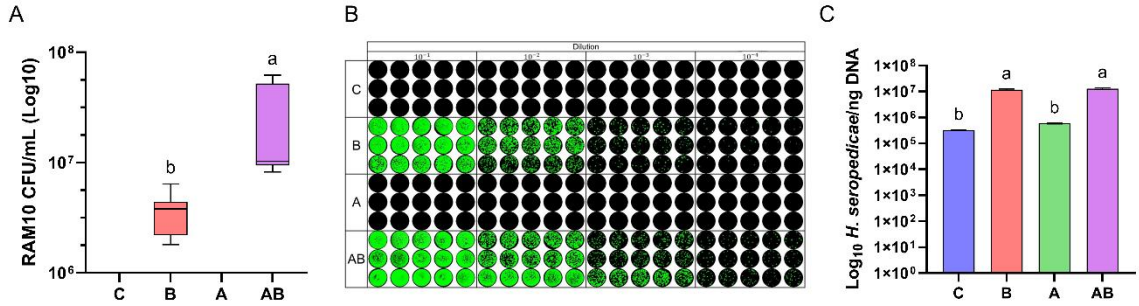


Figure 7. Quantification of bacterial inoculum in maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAI and inoculated with *H. seropedicae* for 48 HAI. (A) Quantification of colony-forming units (CFU/mL). (B) Visualization of CFUs and their serial dilutions. (C) Total bacterial quantification of H. seropedicae via RT-PCR. Treatments are represented as follows: C (Control), B (Bacteria), A (5-azaC), and AB (5-azaC + Bacteria). Different letters (a, b, c, and d) indicate significant differences among treatments based on Tukey's test.

RT-PCR quantification confirmed the higher abundance of *H. seropedicae* in treatments inoculated with bacteria (B and AB). However, RT-PCR also quantified endogenous *Herbaspirillum* strains already present in the maize microbiome. Control (Fig. 2) demonstrated that 5-azaC does not inhibit the growth of *Herbaspirillum* in the maize microbiome, even at varying concentrations, supporting the conclusion that the bacterial increase observed in treatment AB is due to the enhanced interaction between plant-inoculated bacteria.

These findings suggest that hypomethylation induced by 5-azaC facilitates the colonization of exogenous bacteria like *H. seropedica*e in maize roots. In contrast, treatment B (Bacteria) showed less bacterial colonization, likely due to a stronger interaction between maize and its endogenous microbiota in the absence of the methylation inhibitor. The results highlight the potential of epigenetic modulation to enhance plant-microbe interactions, particularly with beneficial exogenous bacteria.

3.1.3.6. Bacterial Distribution in Maize Roots After Treatments

Following fluorescence microscopy, which confirmed the interaction between maize seedlings and H. seropedicae, scanning electron microscopy (SEM) was performed to visualize bacterial distribution among the treatments and examine the root microbiota. This analysis aimed to provide a detailed view of the spatial distribution of microorganisms in the root tissues under different treatments.

As shown in Figure 8, no bacterial presence was detected in the control seedlings (C). SEM analysis demonstrated that the superficial sterilization of the seeds effectively removed external microbiota, leaving only the internal microbiome of the roots. This confirmed the absence or minimal presence of surface-associated bacteria in control roots.

In the bacterial treatment (B), bacteria were distributed throughout the roots, including the root cap, elongation zone, and pellucid zone (Fig. 8). This uniform colonization aligns with observations from fluorescence microscopy.

Interestingly, in the treatment with 5-azaC alone (A), SEM revealed a substantial fungal presence, particularly in the elongation zone (Fig. 8). This fungal colonization was also observed under bright-field microscopy (Figure S3), suggesting that the fungi were part of the internal microbiome of the root and not due to external contamination. These findings indicate that hypomethylation induced by 5-azaC may disrupt fungal regulation within the maize root microbiome, allowing for increased fungal growth.

In the combined treatment (AB), bacteria from the inoculum were less prevalent in the root cap but they were prominently localized in the elongation and pellucid zones (Fig. 8). Notably, no fungal presence was observed in the AB treatment, indicating that the bacterial inoculum may have inhibited or altered the root's fungal microbiota in the root. This suggests a complex interaction between the internal microbiome, the introduced bacteria, and the hypomethylation effects induced by 5-azaC.

These results highlight the influence of epigenetic modulation on composition of the root microbiota and the potential for H. seropedicae to modify or regulate internal fungal populations when introduced in combination with 5-azaC.

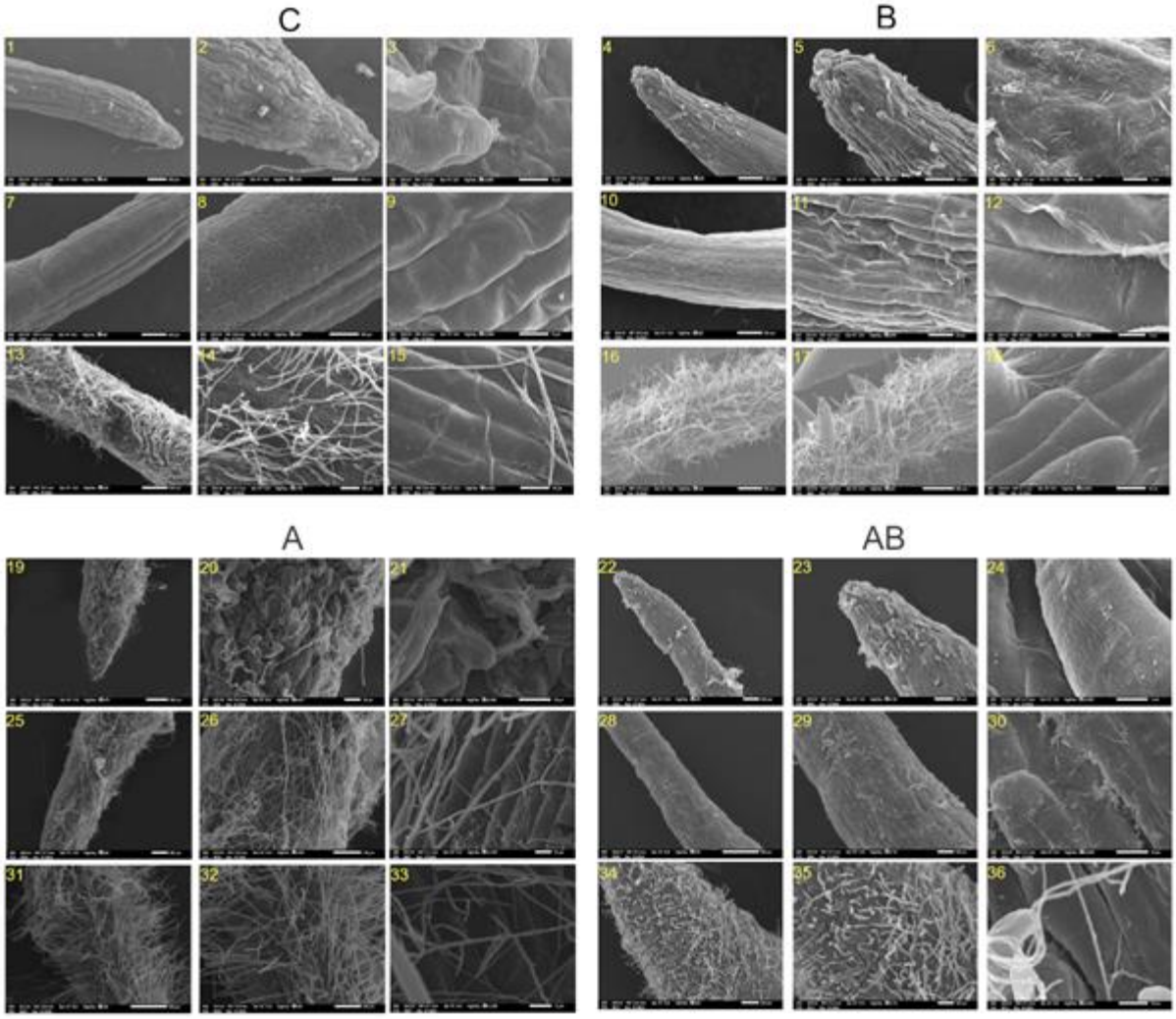


Figure 8. Scanning electron microscopy of maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAI and inoculated with H. seropedicae for 48 HAI. The analysis highlights three root zones: root cap (images 1–6 and 19–24), elongation zone (images 7–12 and 25–30), and root hair zone (images 13–18 and 31–36). Treatments are as follows: Control (C) includes images 1–3, 7–9, and 13–15; Bacteria (B) includes images 4–6, 10–12, and 16–18; 5-azaC (A) includes images 19–21, 25–27, and 31–33; and 5-azaC + Bacteria (AB) includes images 22–24, 28–30, and 34–36.

3.1.3.7. Metataxonomic Analysis of Maize Root Microbiome under the Influence of 5-azaC and *H. seropedicae*

A metataxonomic analysis was performed to understand how the native seed microbiota was altered by 5-azaC and *H. seropedicae*. The Venn diagram (Fig. 9A) shows unique and shared OTUs/ASVs across treatments. While groups C, B, and AB had similar values, the 5-azaC treatment (A) exhibited more than twice the number of unique microbial taxa, suggesting that DNA hypomethylation significantly reshapes the composition of the root microbiome.

Alpha diversity analysis (Fig. 9B) confirmed significant differences, with the A group showing a marked increase in diversity, whereas C, B, and AB displayed no significant variations. After 48 hours, bacterial interaction in AB did not significantly alter diversity, indicating a stabilizing effect.

Beta diversity analysis using PCA (Fig. 9C) revealed distinct microbial profiles among treatments. The control group (C) clustered separately, while B and AB overlapped, suggesting a shared microbial composition. The A treatment formed an isolated cluster, reinforcing the impact of DNA hypomethylation on the structure of the microbiome.

Hierarchical clustering via heatmap analysis (Fig. 9D) highlighted specific enriched bacterial taxa in response to 5-azaC. The AB treatment closely resembled control, with slight alterations, indicating that H. seropedicae strongly modulated the microbiome shifts induced by 5-azaC.

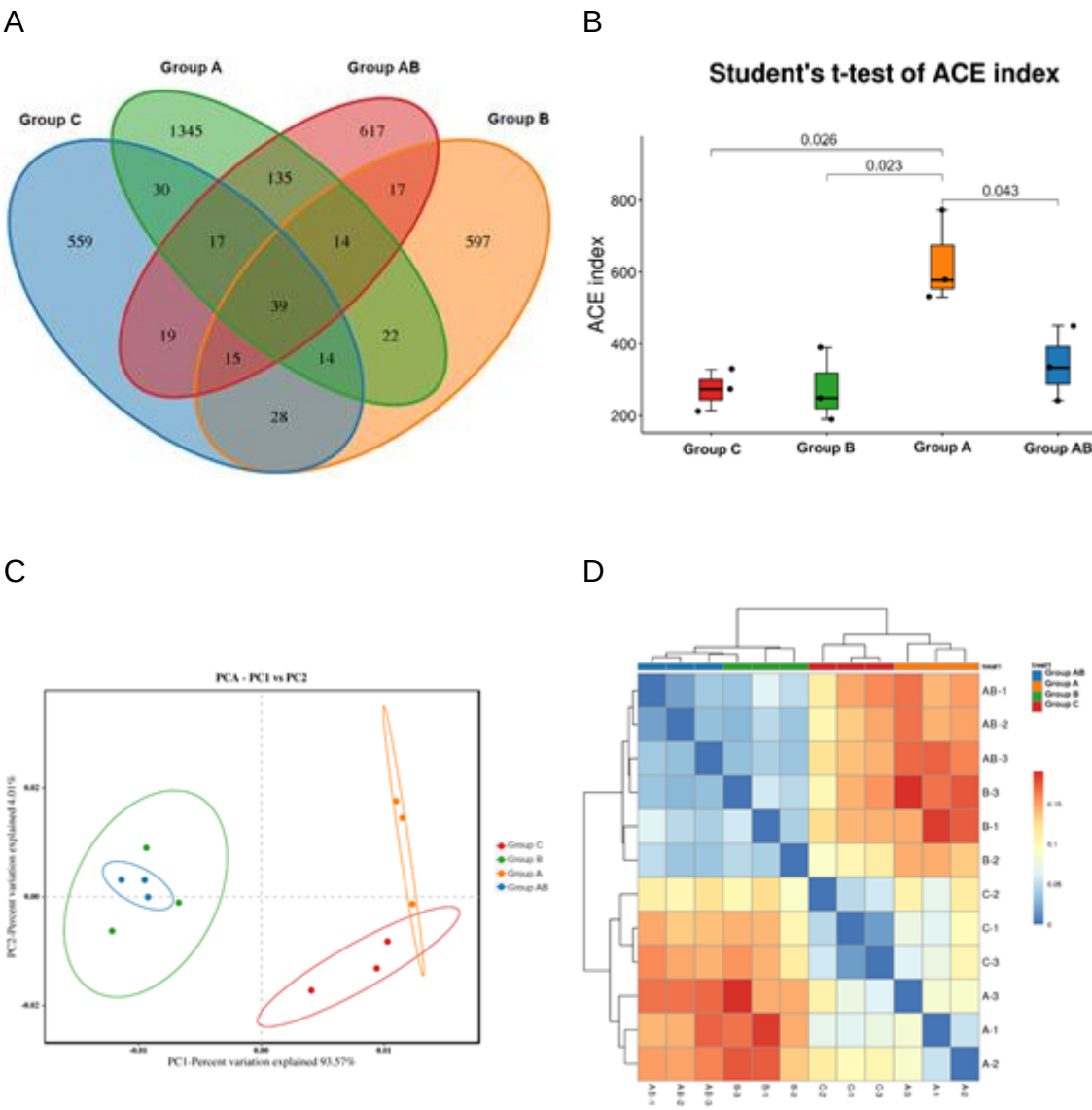


Figure 9. Variance analysis of the microbial community in maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAI and inoculated with H. seropedicae for 48 HAI. (A) Venn diagram showing OTU/ASV analysis results. (B) Boxplot representing alpha diversity among treatments. (C) PCA plot illustrating beta diversity analysis (D) Sample Clustering HeatMap Analysis. Treatments are represented as follows: C (Control), B (Bacteria), A (5-azaC), and AB (5-azaC + Bacteria).

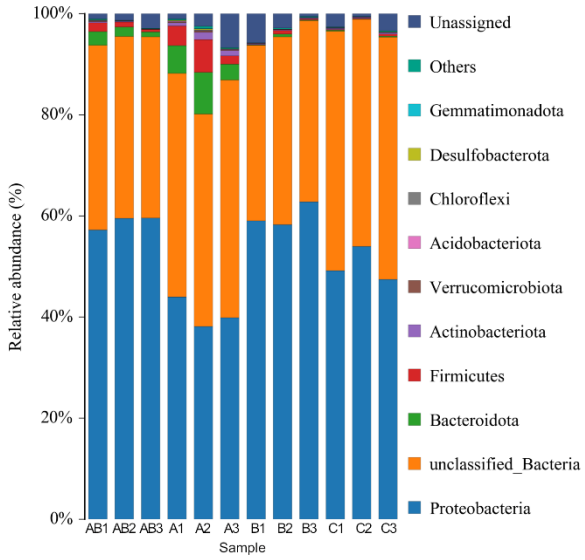
The taxonomic distribution of bacteria in maize seedling roots (Fig. 10) illustrates how treatments influenced microbiome composition. At the phylum level (Fig. 10A), Proteobacteria dominated across all treatments, but relative abundances varied significantly. Treatments B and AB showed an increase *in Proteobacteria*, expected due to *H. seropedicae* inoculation. In contrast, the 5-azaC treatment (A) led to the decrease in Proteobacteria and an increase in *Bacteroidota*, suggesting a microbial response to DNA hypomethylation.

At the class level (Fig. 10B), roots treated with 5-azaC exhibited a higher proportion of Actinobacteria and Clostridia compared to other treatments. The presence of *H. seropedicae* in B and AB was confirmed by an enrichment of Gammaproteobacteria.

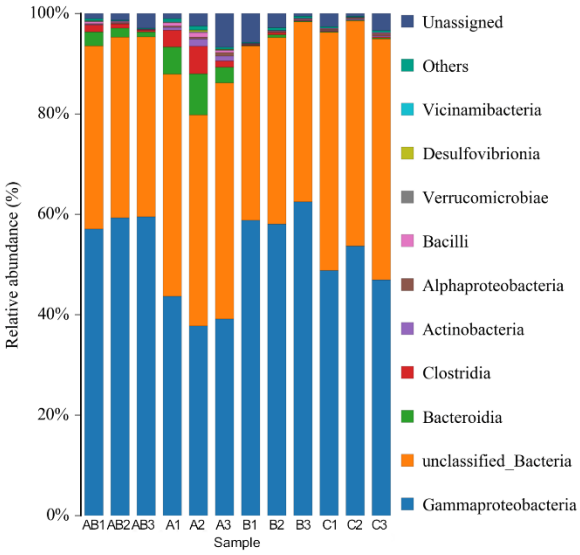
At the family level (Fig. 10C), treatment A showed a drastic reduction in *Oxalobacteraceae*, nearly disappearing, while *Rikenellaceae, Prevotellaceae*, and *Lachnospiraceae* increased. These taxa were barely detected in C and B, and are present at lower levels in AB compared to A.

At the genus level (Fig. 10D), *Herbaspirillum* was notably enriched in AB and B, as expected due to *H. seropedicae* inoculation. Interestingly, *Herbaspirillum* presence was almost absent in A, despite previous qPCR analysis detecting it in this treatment. Additionally, *Muribaculum and Alistipes*, present in A but absent in C and B, appeared suppressed in AB.

A



B



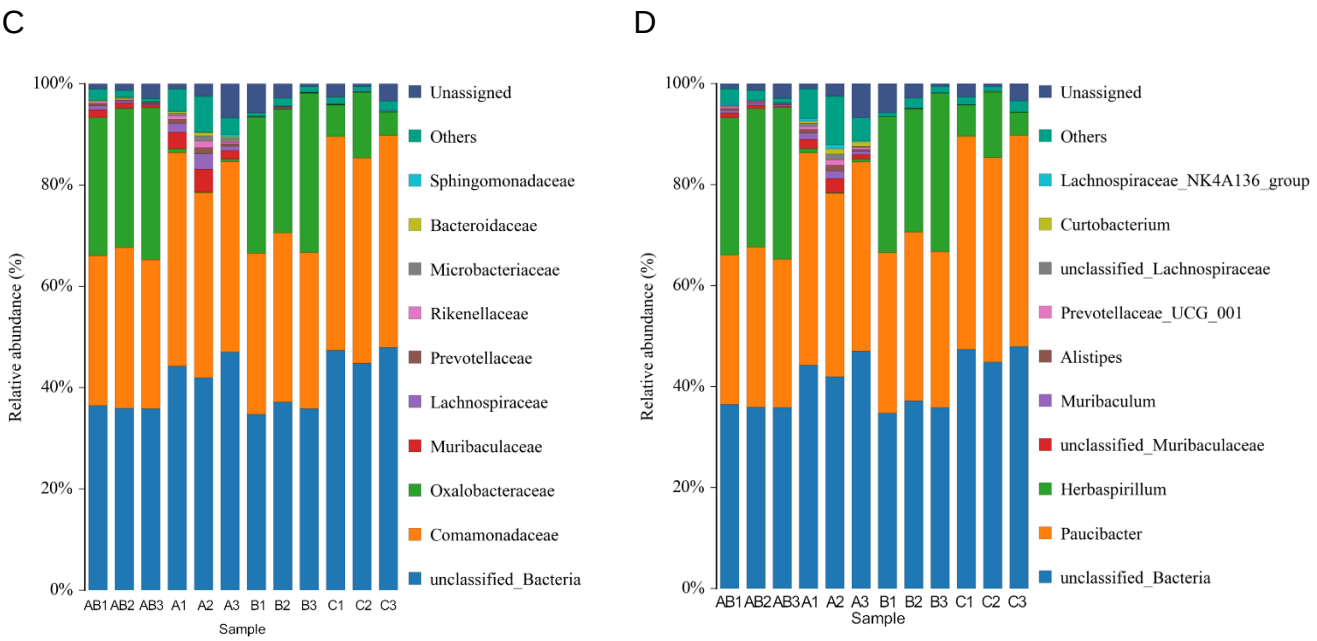


Figure 10. Taxonomic classification of bacterial communities in maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAI and inoculated with H. seropedicae for 48 HAI. Taxonomic levels are represented as follows: (A) Phylum, (B) Class, (C) Family, (D) Genus. Treatments are represented as follows: C (Control), B (Bacteria), A (5-azaC), and AB (5-azaC + Bacteria).

The functional characterization of the root microbiome (Fig. 11) provided further insights into how treatments influenced root-associated bacteria. Aerobic (Fig. 11A), anaerobic (Fig. 11B), and facultative anaerobic bacteria (Fig. 10C) were enriched in treatment A, suggesting potential changes in root exudates or microbial interactions in response to DNA hypomethylation.

Biofilm-forming bacteria (Fig. 11D) were particularly enriched in B and AB, indicating that H. seropedicae may promote microbial aggregation and root colonization and in contrast, treatment A showed a reduced enrichment. Mobile genetic elements (Fig. 11E) and stress-tolerant bacteria (Fig. 11F) were more abundant in B and AB, likely due to H. seropedicae treatments.

The distribution of Gram-negative (Fig. 11G) and Gram-positive (Fig. 10H) bacteria varied significantly, with Gram-negative bacteria dominating in C, B, and AB, consistent with the presence of H. seropedicae.

Interestingly, the analysis showed an increase in bacteria with pathogenic potential in B and AB (Fig. 11I), whereas treatment A lacked these bacteria in the roots. This suggests that 5-azaC modulates plant-microbe interactions, potentially suppressing the colonization of harmful microorganisms.

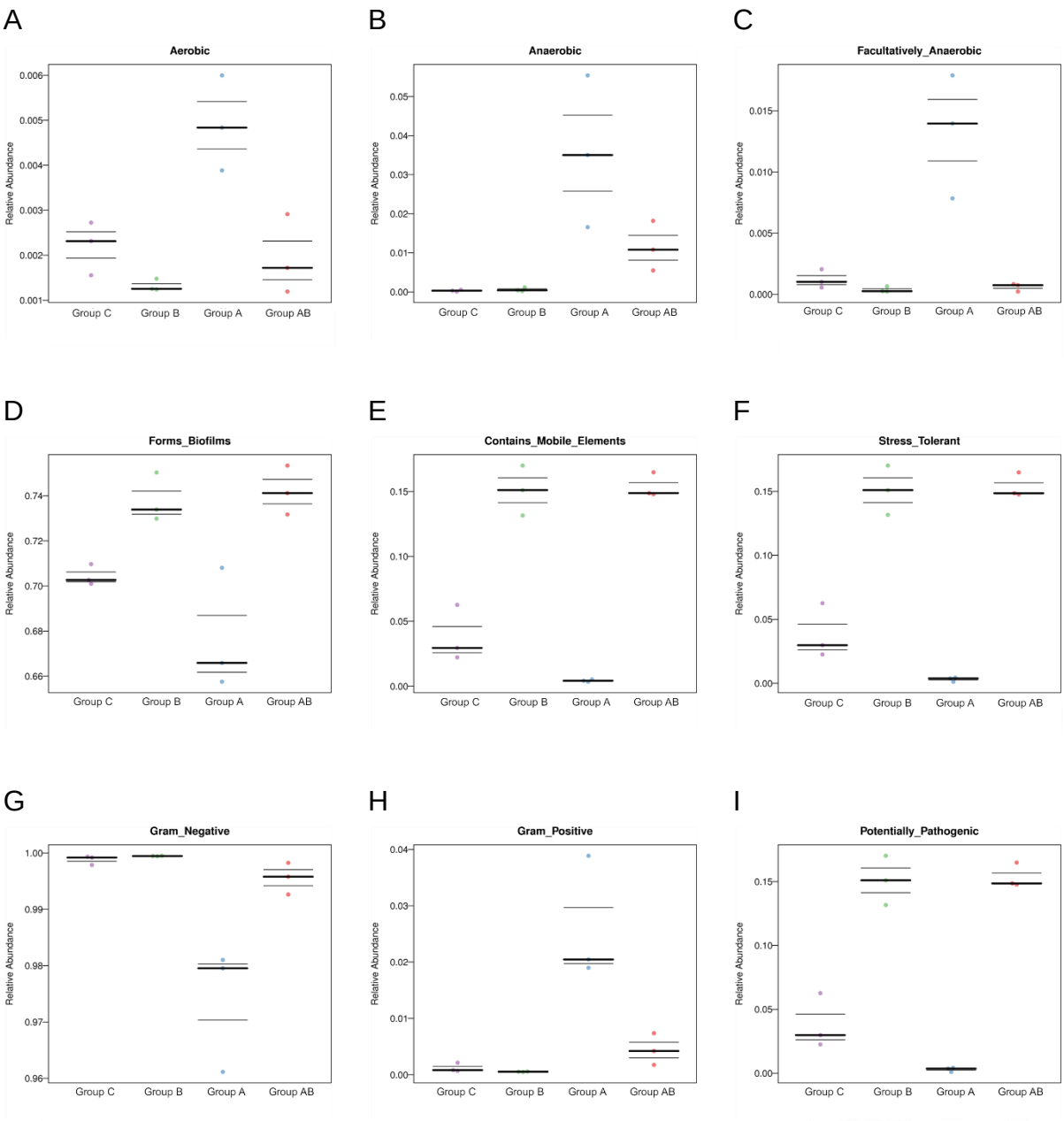


Figure 11. Characteristics of microbial groups in maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAI and inoculated with *H. seropedicae* for 48 HAI. (A) Aerobic bacteria, (B) Anaerobic bacteria, (C) Facultative anaerobes, (D) Biofilm-forming bacteria, (E) Presence of mobile genetic elements, (F) Stress tolerance, (G) Gram-negative bacteria, (H) Gram-positive bacteria, and (I) Pathogenic potential. Treatments are represented as follows: C (Control), B (Bacteria), A (5-azaC), and AB (5-azaC + Bacteria).

3.1.3.8. Proteomic Modulations Induced by Treatments

To understand how alterations in DNA methylation, gene expression, and root microbiome composition impact the proteome of the root, a label-free proteomic analysis was performed to compare the different treatments. In total, 1,818 proteins

were identified across the treatments. Comparisons were made between the treatments B/C, A/C, AB/A, and AB/B, allowing the identification of differentially accumulated proteins (DAPs). In Figure 12, up- (blue arrows) and down-accumulated proteins (red arrows) are highlighted for each comparison, with the proteins exclusive to a single treatment being excluded from the comparative analyses.

Our analysis revealed differences in the concentration of the proteins the A/C and AB/B comparison. In the A/C comparison, 1,789 proteins were identified, with 54 up-accumulated, 66 down-accumulated, and 29 exclusive proteins (17 from A and 12 from C). In the AB/B comparison, 1,783 proteins were identified, with 96 up accumulated, 59 down accumulated, and 32 exclusive proteins (13 from AB and 19 from B).

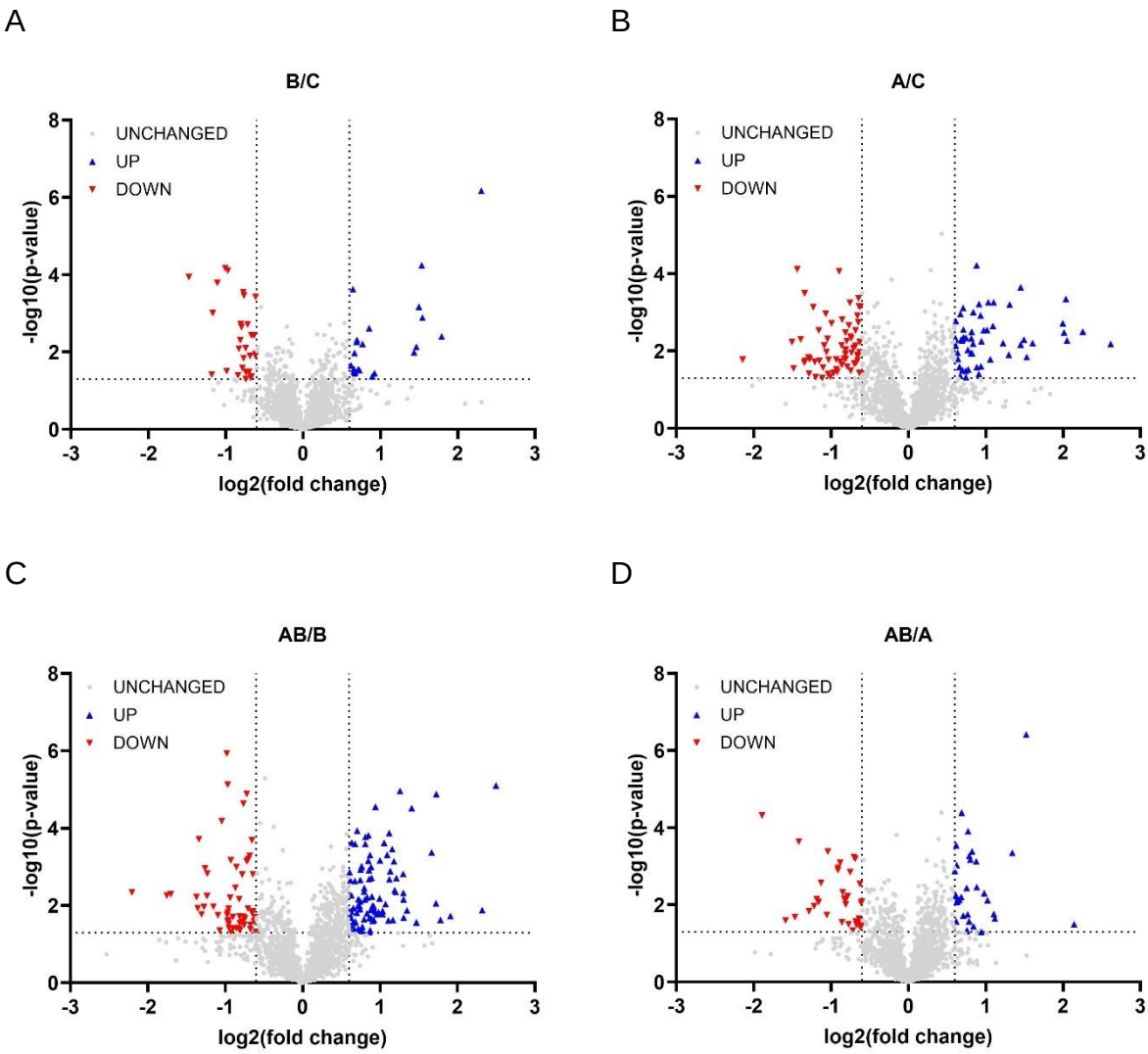


Figure 12. Differentially accumulated proteins in maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAI and inoculated with *H. seropedicae* for 48 HAI. Volcano plot of differentially accumulated proteins. Blue dots: up-accumulated proteins (log2 FC ≥ 0.5, P ≤ 0.05). Red dots: down-accumulated proteins (log2 FC ≤ -0.5, P ≤ 0.05). (A) Comparison B/C. (B) Comparison A/C. (C) Comparison AB/A. (D) Comparison AB/B.

To determine the functional annotation of the differentially accumulated proteins, a KEGG functional enrichment analysis was conducted using ShinyGO. Tthe differentially accumulated proteins with their respective functional annotations for both up-regulated and down-regulated proteins in the B/C, A/C, AB/A, and AB/B comparisonsare shown in Figure 13

It was observed that metabolic pathways such as the citric acid cycle (TCA) displayed differential regulation across comparisons. In the B/C treatment, this pathway was both up-regulated and down-regulated, while in the AB/B treatment, a down-regulation (negative regulation) was observed. The phenylpropanoid biosynthesis pathway was regulated in almost all comparisons, with proteins being both up- and down-accumulated, except in the AB/B comparison, where no positive regulation was observed.

Carbon metabolism was also differentially expressed in various comparisons, except the A/C and AB/A comparisons. Carbon fixation in photosynthetic organisms was down-regulated only in the AB/A comparison. Furthermore, the 2-Oxocarboxylic acids metabolism pathway was up-regulated in the B/C comparison and down-regulated in the AB/B comparison, suggesting that the interaction between the plant and the bacterium without hypomethylation may regulate this pathway.

Another key finding was the positive regulation of proteins related to nitrogen fixation, observed in the AB/A and AB/B comparisons. This result indicates that hypomethylated plants inoculated with the bacterium exhibited higher expression of these proteins. Lastly, in the B/C comparison, a negative regulation of a plant-pathogen interaction-related protein was observed, suggesting an impact of the bacterial interaction in plant response.

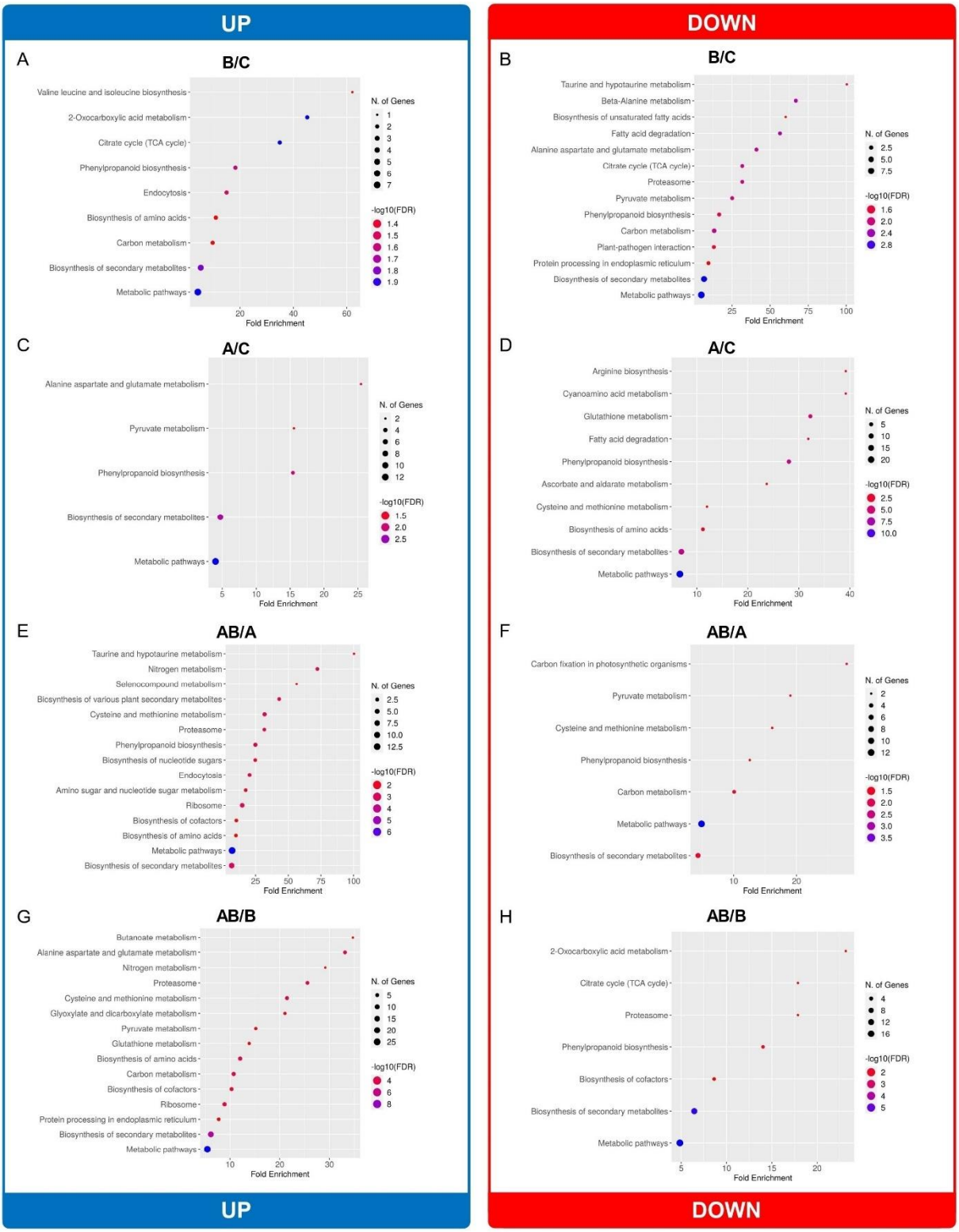


Figure 13. Differentially accumulated proteins in maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAI and inoculated with H. seropedicae for 48 HAI. Comparisons: B/C (A,B), A/C (C,D), AB/A (E,F), AB/B (G,H). KEGG functional enrichment of differentially accumulated proteins, with bubble maps showing up-regulated (A,C,E,G) and down-regulated (B,D,F,H) pathways.

3.1.3.9. Metabolic pathways altered by 5-azaC treatment and bacterial inoculation

Proteomic analysis of proteins differentially accumulated revealed significant variations in the metabolic pathways among the conditions C, B, A, and AB (Table 1).

In the Citrate cycle (TCA cycle), the comparison B/C showed that DIHYDROLIPOYLLYSINE-RESIDUE SUCCINYLTRANSFERASE (Zm00001eb085130_P001) and ACONITATE HYDRATASE (Zm00001eb105870_P004) were upregulated, whereas MALATE DEHYDROGENASE (Zm00001eb045790_P003) and DIHYDROLIPOYLLYSINE-RESIDUE SUCCINYLTRANSFERASE (Zm00001eb421290_P001) were downregulated. Interestingly, DIHYDROLIPOYLLYSINE-RESIDUE SUCCINYLTRANSFERASE exhibited both up- and downregulation depending on the comparison. Additionally, in the AB/B comparison, ACONITATE HYDRATASE (Zm00001eb105870_P004) was exclusively downregulated.

In the carbon metabolism pathway, the enzymes D-3-PHOSPHOGLYCERATE DEHYDROGENASE (Zm00001eb040890_P001), MALATE DEHYDROGENASE (Zm00001eb045790_P003), PYRUVATE KINASE (Zm00001eb060760_P002), GLUCOSE-6-PHOSPHATE 1-DEHYDROGENASE (Zm00001eb079220_P001), and MALIC ENZYME (Zm00001eb285890_P009) were upregulated in the AB/B comparison. Conversely, 3-HYDROXYISOBUTYRYL-COA HYDROLASE (Zm00001eb409740_P002) was downregulated in the AB/A comparison. Moreover, MALATE DEHYDROGENASE (Zm00001eb134330_P001) and MALIC ENZYME (Zm00001eb283570_P002) were also downregulated in the AB/A comparison regarding carbon fixation in photosynthetic organisms.

The DIHYDROXY-ACID DEHYDRATASE CHLOROPLASTIC (Zm00001eb175970_P003) enzyme, belonging to the 2-oxocarboxylic acid metabolism, was upregulated in the B/C comparison and downregulated in the AB/B comparison. In the nitrogen metabolism pathway, the enzymes CYANATE HYDRATASE (Zm00001eb046120_P002) and CARBONIC ANHYDRASE (Zm00001eb158800_P001) were upregulated in the AB/A comparison. Additionally, GLUTAMINE SYNTHETASE (Zm00001eb054990_P003; Zm00001eb399860_P004) was upregulated in the AB/B comparison. In the plant-pathogen interaction pathway, the enzymes HISTIDINE KINASE/HSP90-LIKE ATPASE DOMAIN-CONTAINING

PROTEIN (Zm00001eb315880_P002) and HEAT SHOCK PROTEIN 90-2 (Zm00001eb316410_P002) were downregulated in the B/C comparison.

In the phenylpropanoid biosynthesis pathway, we identified 11 different PEROXIDASE-RELATED proteins, with PEROXIDASE 42 being the only one with a distinct annotation. These peroxidases were upregulated in two instances in B/C (Zm00001eb109960_P001; Zm00001eb276250_P002), three in A/C (Zm00001eb109960_P001; Zm00001eb276250_P002; Zm00001eb330550_P002), three in AB/A (Zm00001eb111430_P002; Zm00001eb195200_P001; Zm00001eb281180_P002), and three in AB/B Zm00001eb017950_P001; Zm00001eb083140_P001; Zm00001eb291850_P001), while being downregulated once in B/C (Zm00001eb354680_P001), five times in A/C Zm00001eb017950_P001; Zm00001eb083140_P001; Zm00001eb281180_P002; Zm00001eb291850_P001; Zm00001eb354680_P001), and twice in AB/A (Zm00001eb251340_P001; Zm00001eb282430_P002). Furthermore, PHENYLALANINE AMMONIA-LYASE (Zm00001eb185260_P001; Zm00001eb247650_P001) was downregulated in the B/C and A/C comparisons.

Tabele 1. Differentially regulated enzymes in selected pathways in maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 μM) for 7 DAI and inoculated with H. seropedicae for 48 HAI. Comparisons: B/C, A/C, AB/A, AB/B. "Up" indicates upregulation and "down" indicates downregulation

ID	Proteins	B/C	A/C	AB/A	AB/B	
Zm00001eb175970_P003	Dihydroxy-acid dehydratase chloroplastic	UP↑			DOWN↓	2-Oxocarboxylic acid metabolism
Zm00001eb134330_P001	Malate dehydrogenase			DOWN↓		Carbon fixation in photosynthetic organisms
Zm00001eb283570_P002	Malic enzyme			DOWN↓		Carbon fixation in photosynthetic organisms
Zm00001eb040890_P001	D-3-phosphoglycerate dehydrogenase				UP↑	Carbon metabolism
Zm00001eb045790_P003	Malate dehydrogenase				UP↑	Carbon metabolism
Zm00001eb060760_P002	Pyruvate kinase				UP↑	Carbon metabolism
Zm00001eb079220_P001	Glucose-6-phosphate 1-dehydrogenase				UP↑	Carbon metabolism
Zm00001eb285890_P009	Malic enzyme				UP↑	Carbon metabolism
Zm00001eb409740_P002	3-hydroxyisobutyryl-CoA hydrolase			DOW↓		Carbon metabolism
Zm00001eb045790_P003	Malate dehydrogenase	DOWN↓				Citrate cycle
Zm00001eb085130_P001	dihydrolipoyllysine-residue succinyltransferase	UP↑			DOWN↓	Citrate cycle (TCA cycle
Zm00001eb421290_P001	Dihydrolipoyllysine-residue succinyltransferase	DOWN↓			UP↑	Citrate cycle (TCA cycle
Zm00001eb105870_P004	Aconitate hydratase	UP↑			DOWN↓	Citrate cycle (TCA cycle)
Zm00001eb046120_P002	Cyanate hydratase			UP↑		Nitrogen metabolism
Zm00001eb054990_P003	Glutamine synthetase				UP↑	Nitrogen metabolism
Zm00001eb158800_P001	Carbonic anhydrase			UP↑		Nitrogen metabolism
Zm00001eb399860_P004	glutamine synthetase				UP↑	Nitrogen metabolism
Zm00001eb017950_P001	Peroxidase		DOWN↓		UP↑	Phenylpropanoid biosynthesis
Zm00001eb083140_P001	Peroxidase		DOWN↓		UP↑	Phenylpropanoid biosynthesis
Zm00001eb109960_P001	Peroxidase	UP↑	UP↑			Phenylpropanoid biosynthesis
Zm00001eb111430_P002	Peroxidase			UP↑		Phenylpropanoid biosynthesis
Zm00001eb185260_P001	Phenylalanine ammonia-lyase	DOWN↓				Phenylpropanoid biosynthesis
Zm00001eb195200_P001	Peroxidase			UP↑		Phenylpropanoid biosynthesis
Zm00001eb247650_P001	Phenylalanine ammonia-lyase	DOWN↓	DOWN↓			Phenylpropanoid biosynthesis
Zm00001eb251340_P001	Peroxidase			DOWN↓		Phenylpropanoid biosynthesis
Zm00001eb276250_P002	Peroxidase	UP↑	UP↑			Phenylpropanoid biosynthesis
Zm00001eb281180_P002	Peroxidase		DOWN↓	UP↑		Phenylpropanoid biosynthesis
Zm00001eb282430_P002	Peroxidase			DOWN↓		Phenylpropanoid biosynthesis
Zm00001eb291850_P001	Peroxidase		DOWN↓		UP↑	Phenylpropanoid biosynthesis
Zm00001eb330550_P002	Peroxidase 42		UP↑			Phenylpropanoid biosynthesis
Zm00001eb354680_P001	Peroxidase	DOWN↓	DOWN↓			Phenylpropanoid biosynthesis
Zm00001eb315880_P002	Histidine kinase/HSP90-like ATPase domain-containing protein	DOWN↓				Plant-pathogen interaction
Zm00001eb316410_P002	Heat shock protein 90-2	DOWN↓				Plant-pathogen interaction

3.1.4. DISCUSSION

The compound 5-azaC is a widely used methylation inhibitor for studying the processes involved in plant development under hypomethylation conditions [41,42]. This compound is a cytosine analog and can be randomly incorporated into the newly synthesized DNA strand, replacing natural cytosine. Consequently, it interferes with the activity of DNA methyltransferases, preventing normal DNA methylation and leading to genomic hypomethylation in different regions [43–45]. These effects make 5-azaC a valuable tool for investigating the role of DNA methylation in regulating plant growth and development.

To study the interaction between hypomethylated plants and plant growth-promoting bacteria, a bacterial growth curve was performed to assess whether the

compound interfered with the bacterial development. We observed that the interaction between 5-azaC and *H. seropedicae* did not inhibit bacterial growth at the tested concentrations, as shown in Figure 1. However, *Escherichia coli RecA* and *lexA* mutants were highly sensitive to 5-azaC [46,47], and *Bacillus subtilis* also exhibited growth inhibition [46]. Additionally, biofilm formation in Streptococcus pneumoniae was inhibited by 5-azaC [48], demonstrating that the hypomethylating effect can impact certain bacterial groups, which was not the case for H. seropedicae.

The effect of 5-azaC on maize seedling development resulted in reduced root growth, fresh mass, and dry mass, like the findings in soybean [48,49]. However, in soybean, these effects were observed at higher concentrations, whereas in our study, effects at 100 µM were comparable to those at 2.5 µM, indicating that the inhibitor has a stronger morphological effect on monocotyledons than on dicotyledons. In Populus nigra, 5-azaC also reduced plant development [42]. In bamboo, hypomethylation promoted greater lateral root development, contrary to our results, where root growth was reduced [50]. In Arabidopsis seedlings, hypermethylation treatment affected fresh and dry mass and increased flowering [51].

The early bio-stimulation effect of *H. seropedicae* inoculation was observed in other studies involving maize and this bacterium, where increased fresh and dry mass in both root and shoot was reported [51,52]. In rice, however, no biomass increase due to H. seropedicae was noted, suggesting that *H. seropedicae* promotes biomass accumulation differently depending on the plant species.

Global methylation quantification demonstrated that 5-azaC treatment had a hypomethylating effect (Fig. 4). Bacterial treatment modulated methylation similarly to 5-azaC, as observed in the group treated solely with the bacterium. When assessing the combined effect of the methylation inhibitor and bacteria, different genomic regions were affected, indicating an interaction between these treatments. When analyzing the demethylation pattern, we found that bacteria did not induce CHG hemimethylation, with 5-azaC being the primary modulator of this pathway. Interestingly, bacterial treatment tended to increase CHG methylation levels, as observed in our data. In total CG methylation, both bacterial treatment and 5-azaC treatments, including the combined treatment, reduced methylation levels.

Studies in bamboo (*Bambusa mimoso*) found that 5-azaC reduced CG and CHG methylation levels [50]. In Arabidopsis thaliana, both zebularine and 5-azaC reduced methylation levels [50][53]. The interaction between plants and plant growth-promoting bacteria was shown to modulate methylation [7]. In plant-pathogen interactions, hypomethylation was observed as a plant response [54–56].

Expression analyses of genes involved in epigenetic regulation showed that H. seropedicae and 5-azaC significantly influence the expression of epigenetic genes in maize roots. The reduction in DRM2 and MET expression in bacterial treatments suggests an impact on DNA methylation maintenance [57–60], possibly reducing global methylation. Simultaneously, lower DML expressions in B and AB treatments suggest that bacteria may affect active demethylation mechanisms, reinforcing the hypothesis of epigenetic reprogramming [61,62].

MBD1 and MBD7, linked to recognition of methylated cytosines, showed distinct patterns, with MBD7 being induced in the AB treatment, suggesting an epigenetic adjustment to hypomethylation and bacterial interaction [63–65]. The reduced SAMS expression across all treatments indicates a possible decrease in methyl group availability through SAM, potentially affecting both DNA methylation and broader regulatory processes [66,67]].

Lower DCL expression suggests reduced siRNA and miRNA biogenesis, which may alter post-transcriptional regulation of essential genes for growth and stress responses [68–70]. Additionally, the differential regulation of CLS 1-2 and CLS 3-4 points to impacts on RdDM lays a crucial role in transposon silencing, genome stability, and regulation of gene expression, particularly during developmental transitions and stress responses, with a potential compensatory adjustment in the AB treatment [71–74].

Finally, the reduced expression of DNG 101 and DNG 103, homologous of AtROS1, may be associated with the repression of demethylation in the genome [75–77].

The colonization of maize roots by *H. seropedicae* was demonstrated through the quantification of the inoculum using a fluorescence-based colony-forming unit (CFU) methodology, developed in this study. Validation of this approach was performed using specific primers for *H. seropedicae* quantification. These primers were originally designed by Da Silva et al. [33] to evaluate the inoculation of this bacterium in sugarcane. In the aforementioned study, an initial increase in the

bacterial population was observed, followed by stabilization over time, approaching control levels.

Fluorescence microscopy confirmed bacterial colonization in the roots, as fluorescent bacilli were detected exclusively in the inoculated treatments. *H. seropedicae* was capable of colonizing both the surface and internal tissues of maize roots as early as 30 min and 24 h post-inoculation [78]. This diazotrophic endophytic bacterium colonizes the internal tissues of the host plant by entering through root fissures. After infection, it spreads and colonizes other tissues [79]. Scanning electron microscopy revealed a more intense colonization in the root cap region of H. seropedicae-treated roots. However, in hypomethylated and inoculated roots, H. seropedicae presence was barely noticeable, possibly due to structural modifications induced by 5-azaC [49,79].

In the control samples, we verified that the seed decontamination methodology effectively removed most native rhizosphere bacteria, as neither bacteria nor fungi were detected in the control roots. However, in roots treated with the hypomethylating agent 5-azaC, a significant presence of fungi was observed, suggesting that this compound alters the root microbiome. Interestingly, in hypomethylated roots inoculated with *H. seropedicae*, fungi were not detected, indicating that this bacterium may have an inhibitory effect on fungal proliferation.

Metataxonomic analysis of maize roots treated with 5-azaC and inoculated with H. seropedicae revealed significant modifications in the microbiota. The 5-azaC treatment (A) caused major shifts in microbial composition, increasing diversity and altering taxonomic groups, particularly with a decrease in *Proteobacteria* and an increase in *Bacteroidota* [80,81]. These findings suggest that DNA hypomethylation significantly affects the bacterial community. In contrast, the AB treatment did not show such drastic changes, indicating that the presence of *Herbaspirillum* may mitigate the effects of hypomethylation.

Functional analysis indicated that hypomethylation favored aerobic and anaerobic bacteria, whereas *Herbaspirillum* enhanced biofilm-forming bacteria and those with increased stress resistance, suggesting that *H. seropedicae* inoculation may promote bacterial aggregation and root colonization. Moreover, 5-azaC treatment was associated with a reduction in potentially pathogenic bacteria, suggesting a protective effect [82–84]. These findings highlight the complex interplay

between epigenetic modulations and microbiota, with implications for plant microbiome manipulation and the promotion of beneficial interactions.

Proteomic analysis of maize roots treated with 5-azaC and inoculated with *H. seropedicae* revealed significant alterations in the proteome composition, indicating modulations induced by both DNA hypomethylation and bacterial presence. The results showed that inoculation with *H. seropedicae* in the B/C treatment increased the number of proteins involved in metabolic processes [85,86], while treatment with 5-azaC in A/C led to an increase in proteins associated with stress response and phenylpropanoid biosynthesis [87,88]. The presence of *Herbaspirillum* in the AB treatment exhibited an intermediate proteomic modulation, with some effects of 5-azaC attenuated. This also suggests a synergistic interaction between the plant and bacteria, favoring the expression of nitrogen fixation-related proteins, as observed in the AB/A and AB/B comparisons.

Functional analysis using KEGG revealed significant alterations in metabolic pathways among different treatments (C, B, A, and AB), suggesting that bacterial inoculation and epigenetic modifications impact distinctly, the plant metabolism.

In the citrate cycle (TCA), a complex modulation of the involved enzymes was observed. The upregulation of DIHYDROLIPOYLLYSINE-RESIDUE SUCCINYLTRANSFERASE and ACONITATE HYDRATASE in the B/C comparison suggests an increase in metabolic activity in plants treated with *H. seropedicae.* Conversely, the downregulation of MALATE DEHYDROGENASE and another isoform of DIHYDROLIPOYLLYSINE-RESIDUE SUCCINYLTRANSFERASE may indicate a rerouting of energy flow, potentially influenced by the presence of the bacterium. Interestingly, the differential regulation of DIHYDROLIPOYLLYSINE-RESIDUE SUCCINYLTRANSFERASE among different comparisons suggests an adaptive metabolic response to the treatment. It is noted that citric acid synthase expression in maize during germination and in response to light is regulated by promoter methylation of corresponding genes [89].

In carbon metabolism, the upregulation of enzymes such as D-3-PHOSPHOGLYCERATE DEHYDROGENASE, MALATE DEHYDROGENASE, and PYRUVATE KINASE in the AB/B comparison indicates an activation of glycolytic pathways and secondary metabolism, possibly as a response to the combined treatment of 5-azaC and bacterial inoculation [90,91]. The downregulation of 3-

HYDROXYISOBUTYRYL-COA HYDROLASE in the AB/A comparison may indicate an adjustment in amino acid degradation and intermediate carbon metabolism [92].

In nitrogen metabolism, the upregulation of CYANATE HYDRATASE and CARBONIC ANHYDRASE in the AB/A comparison suggests an increase in the availability of assimilable nitrogen forms, which may be related to the combined effect of 5-azaC and bacterial inoculation [93,94]. The increased expression of GLUTAMINE SYNTHETASE in the AB/B comparison supports the hypothesis of enhanced nitrogen fixation and utilization in these treatments [95].

Regarding plant-pathogen interactions, the downregulation of HISTIDINE KINASE/HSP90-LIKE ATPASE DOMAIN-CONTAINING PROTEIN and HEAT SHOCK PROTEIN 90-2 in the B/C comparison may indicate an alteration in stress response and plant recognition of bacterial treatment [96–99].

Phenylpropanoid biosynthesis was also significantly altered, with various differentially expressed peroxidases. The upregulation of some peroxidases in B/C and AB/B may be associated with an increase in antioxidant defense and cell wall reinforcement, while their downregulation in other comparisons may indicate a physiological adjustment to experimental conditions[100–102]. The downregulation of PHENYLALANINE AMMONIA-LYASE in the B/C and A/C comparisons reinforces the hypothesis that bacterial inoculation and epigenetics influence the synthesis of phenolic compounds and plant responses to the environment[103–105] .

These results highlight that the interaction between epigenetics and PGPB bacteria directly impacts essential metabolic processes. The increased activity of enzymes related to carbon and nitrogen metabolism suggests a potential improvement in plant metabolic efficiency, particularly in combined treatments. On the other hand, the altered expression of defense-related proteins suggests that epigenetic regulation may play a role in plant adaptation to microbial environments.

3.1.5. CONCLUSIONS

We concluded that 5-azaC, at the tested concentrations, does not interfere with the development of *H. seropedicae*. We also demonstrated that it is possible to inoculate a plant growth-promoting bacterium while the plants are under the effect of the hypomethylating agent.

The interaction between plants and bacterium induces global DNA hypomethylation in specific regions, promoting better plant development. However, the hypomethylation caused by 5-azaC does not result in the same observed improvement and modulates different methylation patterns compared to the modifications induced by the bacterium.

Variations in gene expression and differences in expression profiles indicate that the methylation modulation induced by the bacterium and 5-azaC has distinct effects among the treatments.

The bacterial inoculum tends to colonize the roots of hypomethylated seedlings more efficiently. Additionally, treatment with 5-azaC alters the root microbiota, making it more susceptible to fungal colonization. However, the inoculation of *H. seropedicae* in hypomethylated seedlings leads to a significant reduction in fungal growth in the roots.

5-azaC drastically modifies the root microbiota of hypomethylated seedlings, while inoculation with *H. seropedicae* tends to restore the microbiota to a state like the control, although some persistent modifications remain.

Proteomic analysis revealed that hypomethylation induced by 5-azaC alters protein expression in plant roots. Distinct proteomic profiles were observed among the treatments, suggesting that epigenetic regulation influences essential biological pathways. The interaction between plant-bacterium and hypomethylated plant-bacterium exhibits unique proteomic signatures, highlighting the complexity of the molecular mechanisms involved in response to treatment.

These findings contribute to a better understanding of the role of DNA methylation in regulating pathways associated with pathogen defense responses, adaptation to abiotic stress, and seedling development in maize. Furthermore, the proteomic data provides new insights into how hypomethylation affects plant-microorganism interactions.

Our results demonstrate that, regardless of 5-azaC treatment*, H. seropedicae* is capable of colonizing maize roots. Furthermore, inoculum quantification suggests a greater penetration and establishment capacity of the bacterium in hypomethylated plants, highlighting a potential epigenetic impact on plant-microorganism interactions.

3.1.6. Supplementary Material

Table S1: Primers used for qRT-PCR analysis

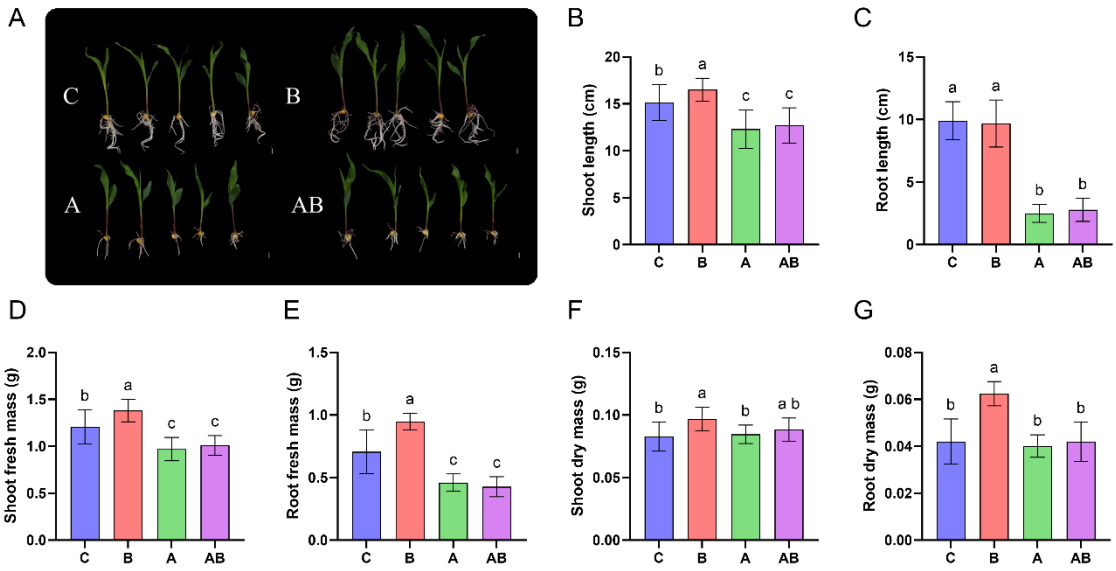
Primer	Foward	Reverse
ZmTubAlfa3	GCGCACCATCCAGTTCGT	CTGGTAGTTGATTCCGCACTTG
ZmDRM2_F	CAAGCACAGGGAAGTAGAGG	GATCTGTCCACTCGTCTTGAC
ZmSAMb_F	TGTTTGGGTATGCGACTGAC	TCCATTCTTGCGAACCTCC
ZmSAHH	TTCCGTCACCAAGAGCAAG	GACATCACCGTATCCGCAG
ZmMBD1	AGGAAATTAAGAACAAGAGGCAAC	CCTTGACTTTCTCGCTAATGC
ZmMBD7	GTGATTATGGGCGGTGACTAC	GGCTTTTGTACGCTGGATTTG
ZmDML	CCTACCCCATACTTATTGGAA	TTGCTAAAATCGCCTCCCA
ZmMET	GCCAACACATTCCGAAACG	CCCGTACAGTCCTTTCCAC
ZmDCL	CCTTGATAGTGGGTGTGCTAC	TCTAATCCTTCGGCTTGCTG
ZmCLS 1-2	CCATCTTCCGCTGATAGTCAAG	TGCTCTCATGAACGACTTCTG
ZmCLS 3-4	CGTGGGAAGCATGAATTTGTT	TTTCACGCCTTTGTCATTTGG
ZmDNG 103	CCATGCTGTGACCCTCAAATG	CTCTGCAGTACAATTCTGGCAC
ZmDNG 101	CCAGATGATCCCTGCCATATCTTC	GGCATCGATCGARRGTGCAGTTTC
Hs54C	ATTCACGCTCCCTCGACGAC	CGGGCTTGGCGTTGGTGACG

Table S2: ISSR primers used for cytosine methylation pattern analysis

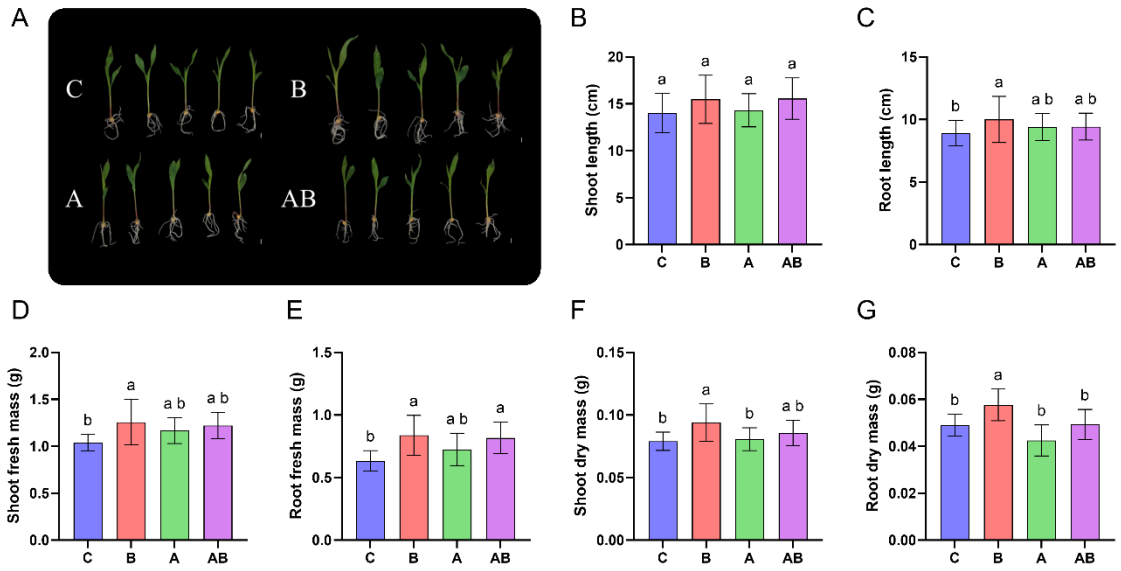
Primer	Loci	Sequencia (5'-3')	Ta (°C)
ISSR-06	UBC- 809	AGAGAGAGAGAGAGAGG	48
ISSR-10	UBC- 823	TCTCTCTCTCTCTCTCC	48
ISSR-14	UBC- 829	TGTGTGTGTGTGTGTGC	53
ISSR-15	UBC- 830	TGTGTGTGTGTGTGTGG	52
ISSR-21	UBC- 841	GAGAGAGAGAGAGAGAYC	48
ISSR-25	UBC- 847	CACACACACACACACARC	53
ISSR-31	UBC- 859	TGTGTGTGTGTGTGTGRC	54

Table S3 Strategy used to interpret different banding patterns and quantify genome methylation percentage

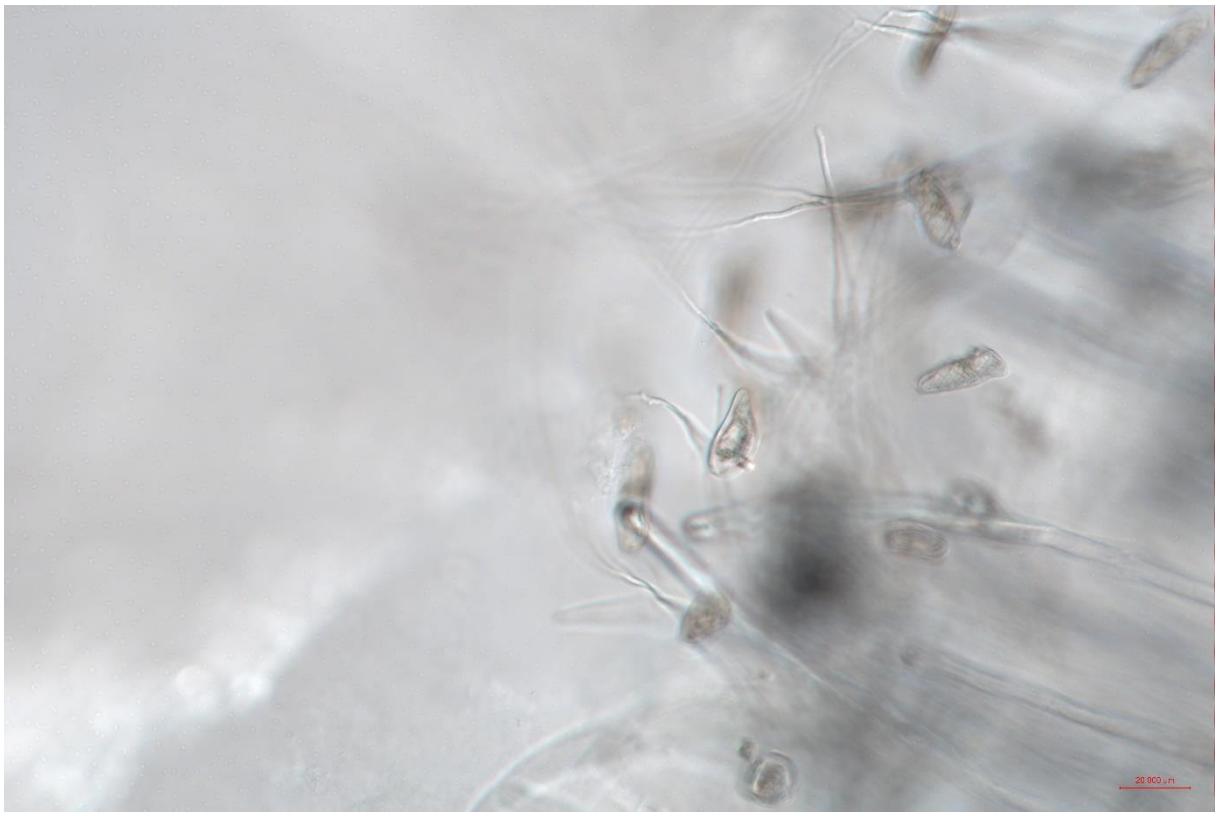
Banding Pattern DNA/ Hpa II/ MspI	Restriction Sites	Interpretation
1/1/1	$^{5'}$CCGG3'	Unmethylated cytosines
1/0/1	$^{5'}$CmCGG$^{3'}$	Fully methylated CG sites
1/1/0	$^{5'm}$CCGG$^{3'}$	Hemimethylation at CHG sites
1/0/0	$^{5'm}$C^{m}CGG^{3} $^{'5'}$CCNG$^{3'}$	Methylation on both cytosines or unknown mutation



Supplementary Figure S 1 Effect of the methylation inhibitor (5-azaC) on maize seedlings development at 7 DAE. Treatments included 25 µM 5-azaC and inoculation with H. seropedicae for 24 HAI. (A) Image showing the effect of the compound on seedling growth (Scale bar = 1 cm). (B, D, F) Measurements of shoot length, fresh mass, and dry mass, respectively. (C, E, G) Measurements of root length, fresh mass, and dry mass, respectively. Letters (a, b, c, d) indicate significant differences between treatments based on Tukey's test. DAE – Days After Emergence; HAI – Hours After Inoculation. In the graphs, the labels C, B, A, and AB correspond to Control, Bacteria, 5-azaC, and 5-azaC + Bacteria, respectively



Supplementary Figure S2 Effect of the methylation inhibitor (5-azaC) on maize seedlings development at 7 DAE. Treatments included 0,25 µM 5-azaC and inoculation with H. seropedicae for 24 HAI. (A) Image showing the effect of the compound on seedling growth (Scale bar = 1 cm). (B, D, F) Measurements of shoot length, fresh mass, and dry mass, respectively. (C, E, G) Measurements of root length, fresh mass, and dry mass, respectively. Letters (a, b, c, d) indicate significant differences between treatments based on Tukey's test. DAE – Days After Emergence; HAI – Hours After Inoculation. In the graphs, the labels C, B, A, and AB correspond to Control, Bacteria, 5-azaC, and 5-azaC + Bacteria, respectively.



Supplementary Figure S3 Supplementary Figure S4. Microscopy analysis of maize seedling roots treated with the DNA methylation inhibitor 5-azaC (2.5 µM) for 7 DAE. Fungi present in the longitudinal sections of the pellucid zone.

3.1.7. REFERENCES

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