UNIVERSIDADE ESTADUAL PAULISTA UNESP

CÂMPUS DE JABOTICABAL

ATIVIDADE ANTAGONISTA DE *Bacillus bombysepticus* JAB01 CONTRA *Sclerotinia sclerotiorum*

Paula Klotz Brandão Rodrigues

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Orientadora: Profa. Dra. Eliana Gertrudes de Macedo Lemos

Coorientador: Prof. Dr. Manoel Victor Franco Lemos

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Genética e Melhoramento de plantas

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Impacto da pesquisa na sociedade, relacionado aos Objetivos do Desenvolvimento Sustentável (ODS)

Impacto Potencial dessa Pesquisa:

A pesquisa desta tese promete um impacto na agricultura e na indústria de controle de fitopatógenos contribuindo com práticas agrícolas sustentáveis no cultivo de soja e de outras plantas hospedeiras do fungo, com potencial para redução do uso de fungicidas no controle da doença mofo branco e consequentemente beneficiar agricultores, consumidores e o meio ambiente, promovendo práticas agrícolas mais responsáveis e eficazes. Ela se concentra na eficácia da bactéria *Bacillus bombysepticus* JAB01 no controle do fungo *Sclerotinia sclerotiorum*. Além disso, a pesquisa destaca a presença de genes com potencial de promover o crescimento vegetal e genes com potencial aplicação biotecnológica, podendo ser utilizados tanto na indústria agrícola quanto em outras áreas.

Potential Impact of this Research:

The research in this thesis promises to have an impact on agriculture and the plant pathogen control industry by contributing to sustainable agricultural practices in the cultivation of soybeans and other plants that host the fungus, with the potential to reduce the use of fungicides to control white mold disease and consequently benefit farmers, consumers and the environment, promoting more responsible and effective agricultural practices.It focuses on the effectiveness of the bacteria *Bacillus bombysepticus* JAB01 in controlling the fungus *Sclerotinia sclerotiorum*. Furthermore, the research highlights the presence of genes with the potential to promote plant growth and genes with potential biotechnological application, which can be used both in the agricultural industry and in other areas.

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Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: ATIVIDADE ANTAGONISTA DE *Bacillus bombysepticus* JAB01 CONTRA *Sclerotinia sclerotiorum*

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DADOS CURRICULARES DO AUTOR

Paula Klotz Brandão Rodrigues- nascida em 08 de junho de 1991, em Barra Mansa, Rio de Janeiro. Iniciou sua graduação no curso de Agronomia em 2011 pela Universidade Federal de Lavras, onde foi monitora da disciplina Estátistica Experimental por 2 anos, participou do Núcleo de estudos em Floricultura e Paisagismo e fez estágio no setor de plantas ornamentais. O curso foi concluído no final do ano 2016. No ano de 2017 iniciou o curso de mestrado em Genética e Melhoramento de Plantas na mesma universidade e obteve o título de mestre no início de 2019. Em março do mesmo ano, iniciou o curso de doutorado em Genética e Melhoramento de Plantas sob a orientação da professora Doutora Eliana Gertrudes de Macedo Lemos.

“Nada do que vivemos tem sentido se não tocarmos o coração das pessoas.”

Cora Coralina

Dedicatória

Dedico esse trabalho ao meu filho Bernardo, que é o grande amor da minha vida. Por você desejo ser uma pessoa melhor todos os dias. Espero que a minha jornada te inspire a enfrentar suas dificuldades, que você acredite no seu potencial, e que adquira muitos conhecimentos ao longo da sua vida. E principalmente seja feliz sendo quem você é, você é incrível meu amor.

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SUMÁRIO

ATIVIDADE ANTAGONISTA DE *Bacillus bombysepticus* JAB01 CONTRA *Sclerotinia sclerotiorum*

RESUMO - A descoberta do potencial antagonista de bactérias possibilitou o uso de biológicos bacterianos como estratégia mais segura no controle de pragas e patógenos. Prospectar microrganismos com atividade de biocontrole e promoção do crescimento vegetal é essencial ao processo de uso de produtos biológicos no controle de fitopatógenos. O objetivo do trabalho foi avaliar o efeito antagônico do isolado bacteriano JAB01 por meio da capacidade de controle *in vitro* de *Sclerotinia sclerotiorum* e avaliar seu potencial como promotor de crescimento vegetal por meio de análises do genoma completo do isolado, buscando genes associados ao biocontrole e promoção de crescimento vegetal. *B. bombysepticus* JAB01 produz substâncias difusíveis e COVs que suprimem o crescimento micelial de *S. sclerotiorum* e inibem a germinação miceliogênica de sua estrutura de resistência. Além disso JAB01 foi capaz de suprimir o desenvolvimento dos sintomas da doença em sementes e folhas destacadas de soja. O isolado foi identificado como *Bacillus bombysepticus* JAB01 por meio de análise filogenômica e ANI e possui diversos genes relacionados a produção de metabólitos secundários com atividade antimicrobiana, sideróforos, enzimas hidrolíticas, solubilização de macronutrientes, fitohormônios, compostos orgânicos voláteis entre outros compostos de interesse biotecnológico.

Palavras chave: Antibiose, Biocontrole de patógenos, Caracterização de moléculas, Mofo Branco.

ABSTRACT- The discovery of the bacterial antagonist potential allowed the use of bacterial biologicals as a safer strategy in the control of pests and pathogens. Characterizing antagonistic activity is an important step towards the process of using bacteria to control phytopathogens. The objective of this work was to evaluate the antagonistic effect of the bacterial isolate JAB01 through the in vitro control capacity of *Sclerotinia sclerotiorum* and to evaluate its potential as a plant growth promoter through analyzes of the complete genome of the isolate, searching for genes associated with biocontrol and promotion of plant growth. B. bombysepticus JAB01 produces diffusible substances and VOCs that suppress mycelial growth of S. sclerotiorum and inhibit mycelial germination of its resistance structure. Furthermore JAB01 was able to suppress the development of disease symptoms in soybean seeds and detached leaves. The isolate was identified as *Bacillus bombysepticus* JAB01 through phylogenomic analysis and ANI and has several genes related to the production of secondary metabolites with antimicrobial activity, siderophores, hydrolytic enzymes, solubilization of macronutrients, phytohormones, volatile organic compounds, among other compounds of biotechnological interest.

Keywords: Antibiosis, Biocontrol of pathogens, Characterization of molecules, White Mold.

CAPÍTULO I – Considerações Gerais

1. INTRODUÇÃO

Doenças causadas por fungos e patógenos oomicetos em plantas são fatores limitantes da produção agrícola. Fungos fitopatogênicos podem causar enormes prejuízos econômicos às culturas, pois podem afetar a quantidade de produção e qualidade do produto, levando a maiores custos para o produtor e podendo levar ao aumento do preço para o consumidor. Além disso, alguns são de difícil controle depois que introduzidos na cultura, devido à capacidade de produzirem estruturas de resistência que sobrevivem por vários anos no solo.

Para reduzir as perdas causadas por microrganismos no rendimento econômico de culturas no agronegócio, o método de controle mais comumente utilizado é o químico. Entretanto, o uso de produtos químicos pode levar ao desenvolvimento de populações resistentes dos patógenos. Além disso, aumentam o custo de produção e podem causar danos ao meio ambiente e a organismos não alvo, inclusive humanos. Sendo então necessária a busca de alternativas com menos efeitos adversos.

Diante dessa demanda, o controle biológico de doenças de plantas assume importância, pois é uma alternativa, ecologicamente sustentável, ao controle químico para o manejo de diversas pragas e patógenos.

Agentes de controle biológico são microrganismos como bactérias, actinomicetos e fungos. Os princípios do controle biológico baseiam-se nas relações antagônicas entre microrganismos. Tal fato se deve à capacidade de inibir ou impedir o desenvolvimento de outros microrganismos que alguns organismos possuem. Esse método de controle de doenças de plantas pode ser obtido por meio de diferentes tipos de interação entre os microrganismos, como o parasitismo, antibiose, competição, entre outros.

Portanto, devido à importância do controle de fitopatógenos, visto o impacto econômico causado a produtores e o impacto ambiental amenizado através do uso de bioprodutos e culturas transgênicas, destaca-se a importância da exploração e caracterização de novas moléculas bioativas.

Agentes de controle biológico, particularmente *Bacillus* foram relatados em diversos trabalhos por proteger as plantas contra fitopatógenos como fungos, bactérias e nematoides. O mofo branco é uma doença causada pelo fungo *Sclerotinia sclerotiorum* que tem grande importância na produção agrícola pois é um fator limitante na produção de muitas culturas de relevância mundial. Até o momento atual, não há informações na literatura sobre o efeito de *Bacillus bombysepticus* sobre o patógeno do solo *Sclerotinia Sclerotiorum*.

Diante desse contexto, o objetivo do trabalho foi avaliar o isolado bacteriano *Bacillus bombysepticus* JAB01 quanto ao seu potencial antagonista contra *Sclerotinia sclerotiorum in vitro* buscando identificar diferentes moléculas bioativas para o desenvolvimento de novos produtos transgênicos e/ou biofungicidas e como promotor de crescimento vegetal.

2. REVISÃO DE LITERATURA

2.1. Uso de biológicos no controle de pragas e patógenos

A agricultura sofre ataques destrutivos de inúmeros patógenos, incluindo fungos, bactérias, vírus e nematóides que levam a perdas e consequentemente à reduções drásticas nos rendimentos e na qualidade do produto final (Pandit et al., 2022). Considerando as perdas causados por esses organismos e do impacto na segurança alimentar, a utilização de métodos de controle eficazes é essencial para garantir a produção agrícola e consequentemente, a disponibilidade de alimentos.

Ao longo dos anos, os produtos químicos contribuíram muito para o controle de pragas e doenças e ainda são a principal forma utilizada para controlar patógenos de plantas (Rahman et al., 2018). No entanto, o uso descuidado de agroquímicos pode levar a resistência e prejudicar a saúde humana, animal e de organismos não alvo. Pois, a exposição a longo prazo a fungicidas sintéticos leva a redução da eficácia devido ao desenvolvimento de mecanismos de resistência por patógenos de plantas, resultando no aumento da aplicação de produtos químicos pelos agricultores, com

consequente acúmulo de resíduos nos vegetais e seus subprodutos que, por sua vez, são responsáveis por efeitos nocivos para a saúde humana e animal (Pal e Gardener, 2006).

Portanto, há uma demanda por novas abordagens, é necessário desenvolver produtos agrícolas mais sustentáveis, de forma segura e ecologicamente correta como uma alternativa ao controle de doenças e pragas. Nesse contexto, uma alternativa compatível com as práticas de agricultura sustentável, seria o uso de produtos biológicos, que possibilitam a produção de alimentos com menos impactos ambientais e à saúde humana e não causam alterações na diversidade da microflora no solo e nas raízes (Fontes e Valadares 2020).

Ao longo dos anos, os produtos biológicos assumiram uma importância significativa tanto no mercado agrícola mundial quanto no mercado brasileiro. Isso se deve, em grande parte, à sua eficácia comprovada e à crescente conscientização sobre os impactos associados ao uso de químicos tradicionais. Um exemplo notável de sucesso no campo dos produtos biológicos é o Bt (*Bacillus thuringiensis*) que produz toxinas inseticidas e tem sido utilizado comercialmente em produtos biológicos há décadas como uma alternativa eficaz ao uso de inseticidas químicos (Sena da Silva et al 2021; Fernandes et al., 2021; Lemes et al., 2017).

O uso de agentes de controle biológico explora os efeitos antagônicos naturais entre microrganismos para controlar os efeitos devastadores causados por patógenos de plantas. Alguns microrganismos do solo possuem a capacidade de inibir ou impedir o desenvolvimento de fitopatógenos e são empregados diretamente como agentes de controle, além de estimular o crescimento de plantas e melhorar o uso de nutrientes no solo (Berg et al., 2017). Antagonistas são organismos que ocorrem naturalmente e possuem o potencial de interferir na infecção, crescimento e sobrevivência de patógenos (Chernin e Chet 2002).

Agentes de controle biológico são microrganismos como bactérias, actinomicetos e fungos. Segundo Chen et al. (2020) microbicidas biológicos podem controlar pragas e doenças em plantas de maneira segura e ecológica, mas sua eficácia precisa ser melhorada e, portanto, é importante encontrar isolados microbianos mais eficazes para desenvolver novos microbicidas biológicos.

De acordo com Köhl et al. (2011), é necessária a triagem de um número elevado de candidatos antagonistas para o desenvolvimento de novos produtos biológicos no controle de fitopatógenos, e tais agentes de controle precisam cumprir distintas exigências além do controle da doença, tais como, serem seguros, devem ser produzidos e disponibilizados a um preço acessível, que seja economicamente viável tanto para os produtores quanto para os consumidores e apresentar amplo mercado consumidor.

Um agente de controle biológico bem sucedido geralmente é caracterizado pela ativação de vários mecanismos, visando sinergicamente o controle do patógeno e/ou seu efeito prejudicial, e não produzem metabólitos tóxicos tanto para humanos/animais quanto para o meio ambiente (Tilocca et al., 2020)

Nas últimas décadas, antagonistas foram direcionados com sucesso para doenças de mudas, raízes, folhas e frutos (Valdivia et al., 2018). Entretanto, apesar das vantagens associadas ao uso de microrganismos como agentes de biocontrole pouco se sabe sobre as consequências às comunidades microbianas do solo, quando um microrganismo é adicionado no solo em elevadas concentrações como agente de biocontrole.

2.2. Uso de bactérias no biocontrole de fungos

As doenças das plantas causadas por patógenos fúngicos são um dos principais fatores limitantes da produção agrícola. De acordo com Bueno et al. (2007), fungos fitopatogênicos podem causar enormes prejuízos econômicos as culturas, devido a sua capacidade de produzirem estruturas de resistência que sobrevivem por vários anos no solo e por isso são de difícil controle depois que introduzidos na cultura.

Ao longo dos anos, os biofungicidas ganharam muito interesse como alternativa aos fungicidas químicos devido à sua natureza ecologicamente correta. Além disso, as plantas transgênicas resistentes a fungos não são aceitas em vários países, e portanto há um interesse crescente no uso de agentes de controle biológico (Validov et al 2007).

Bactérias são utilizadas na promoção direta do crescimento vegetal, e podem atuar indiretamente, como agentes de biocontrole, pelo controle de pragas e patógenos. Consequentemente possibilitam um sistema de produção mais sustentável, diminuindo ou até mesmo substituindo defensivos químicos por produtos biológicos.

O antagonismo entre microrganismos é um fenômeno comum. Fungos e bactérias patogênicos para plantas podem ser afetados por antagonistas fúngicos e bacterianos (Cook e Baker, 1983). Entre os microrganismos utilizados no controle biológico, as bactérias merecem destaque. Muitos estudos demonstraram a atividade antagônica de bactérias que foram capazes de inibir o crescimento micelial e a germinação de esporos de fungos causadores de doenças em plantas (Giorgio et al.,2015; Sabaté et al., 2018, Xu et al., 2020).

De acordo com Perez-Garcia et al. (2011) bactérias formadoras de esporos são potenciais agentes de controle biológico devido a produção de diferentes tipos de compostos inseticidas e antimicrobianos, e a capacidade que algumas possuem de promover o crescimento e induzir sistemas de defesa nas plantas.

A capacidade de controle de doenças de plantas por bactérias antagonistas pode ocorrer por diferentes mecanismos de ação, como antagonismo direto, produção de compostos tóxicos aos patógenos, enzimas hidrolíticas, biossurfactantes, antibióticos, competição por espaço e nutrientes, parasitismo ou, também, pela indução de resistência pela ativação de mecanismos de resistência da planta (Kumar et al., 2012) sendo os gêneros *Pseudomonas* e *Bacillus* os mais estudados devido à capacidade que estas bactérias têm de exercer antagonismo direto pela produção de compostos antimicrobianos (Wu et al., 2014).

No antagonismo direto, a antibiose possui destaque, sendo essa caracterizada pela produção de um ou mais metabólitos por um organismo, que causam prejuízos sobre o outro (Bettiol, 1991). Segundo Calvo-Garrido et al. (2019), no controle de *Botrytis cinerea* o principal modo de ação dos agentes de biocontrole bacterianos do gênero *Bacillus*, é a antibiose.

De acordo com Souza et al. (2015), bactérias exercem antibiose sobre patógenos por mecanismos tais como, síntese de substâncias antimicrobianas, secreção de enzimas líticas, alteração de pH e/ou síntese de compostos voláteis. Os mecanismos de síntese de metabolitos secundários têm demonstrado eficiência em inibir o crescimento de fungos (Giorgio et al., 2015; Fan et al., 2018; Dunlap et al., 2019).

Em diversos estudos, agentes de controle biológicos foram capazes de controlar fitopatógenos fúngicos pela produção de enzimas que degradam compostos majoritários da parede celular fúngica, como a quitina e glucano (Saravanakumar et al., 2018).

2.3. Mofo branco

O mofo-branco, doença causado pelo fungo necrotrófico *Sclerotinia sclerotiorum* (Lib.) de Bary, que pertence ao Filo Ascomycota, Classe Leotiomycetes, Ordem Helotiales e Família Sclerotiniaceae (Albert et al., 2022), infecta mais de 600 espécies de plantas em todo o mundo, incluindo culturas importantes como soja, feijão, algodão, tomate, girassol, alface, cenoura e canola (Liang e Rollins, 2018).

Os sintomas podem diferir entre as culturas hospedeiras, mas há várias semelhanças, sendo os mais comuns manchas marrom-claras ou branco-acinzentadas. Este é um patógeno do solo que se manifesta em qualquer parte da planta com abundante formação de micélio cotonoso de coloração branca e formação de escleródios, que são estruturas que garantem sua sobrevivência no solo por longos períodos (Smolińska et al., 2018).

De acordo com Brustolin et al. (2015), a sobrevivência e viabilidade de escleródios de *Sclerotinia sclerotiorum* no solo varia muito de acordo com diferentes autores, com valores entre pelo menos dois anos até onze anos e um dos fatores que afeta a longevidade, é a profundidade em que estão enterrados no solo.

No Brasil *S. sclerotiorum* foi relatado pela primeira vez em 1921 em São Paulo, na cultura da batata (Chaves 1964), e desde então tem sido descrito em soja, feijão, algodão, tomate, girassol e crotalária, entre outras espécies (Mathews et al., 2019; Sabaté et al., 2018; Silva et al., 2022; Wong et al., 2022). Na cultura da soja é uma doença desafiadora e significativa pois, pode levar a reduções expressivas de rendimento e qualidade na produção de grãos (Ranjan et al., 2019), uma vez que a infecção pode levar à necrose, anelamento e possivelmente à morte da planta (Kandel et al., 2018).

De acordo com Bolton et al. (2006), a disseminação do patógeno ocorre principalmente, por meio de sementes infectadas com o micélio do fungo ou contaminadas por escleródios. O crescimento micelial, a produção de escleródios (Bloomfield e Alexander, 1967), de enzimas hidrolíticas, que degradam a parede celular, (Sharma et al., 2016) e do ácido oxálico (AO) (Kim et al., 2008, Xu et al., 2018) são fatores de patogenicidade deste fungo.

Os escleródios são uma importante fonte de inóculo para a infecção de plantas pois, podem permanecer viáveis no solo por muitos anos devido a uma camada protetora de melanina que confere resistência a produtos químicos e condições ambientais adversas (Butler et al., 2009). Esses quando em condições favoráveis, podem germinar e formar apotécios (germinação carpogênica), que produzem grande quantidade de ascósporos, fonte primária de infecção, ou micélio (germinação miceliogênica) (Bolton et al., 2006).

As enzimas hidrolíticas, facilitam a colonização da planta. pois atuam na degradação da parede celular vegetal (Albert et al., 2022). O pH do ambiente regula a produção dessas enzimas a nível transcricional (Bolton et al., 2006).

O fungo acidifica o microambiente circundante a um nível desejável para a formação de escleródios e atuação das enzimas degradadoras através da produção e secreção do ácido oxálico (Xu et al., 2015). Hegedus e Rimmer et al. (2005) concluíram que o ácido oxálico é um fator necessário para a patogenicidade de *S. sclerotiorum* pois, em seu estudo mutantes fúngicos que não produziram ácido oxálico perderam sua patogenicidade. Entretanto, Xu et al. (2015) demonstraram que é o

baixo pH, e não o ácido oxálico per se, que estabelece o ambiente ideal para o crescimento, reprodução e patogenicidade do *S. sclerotiorum*.

Estudos com plantas transgênicas que expressam genes que codificam enzimas que podem metabolizar AO, como oxalato oxidases (OXO) e oxalato descarboxilases (OXDC), têm demonstrado maior resistência a *S. sclerotiorum* (Hu et al., 2003; Cunha et al., 2010; Xu et al., 2015) relacionam a proteção aumentada de plantas transformadas geneticamente com OXDC ou OXO a um pH mais alto devido a remoção do oxalato.

Ferraz et al. (2003) relataram que, em várias culturas, a dificuldade no controle do mofo branco se deve a capacidade do patógeno em produzir escleródios, associada à alta variabilidade genética. Além disso, a ampla variedade de hospedeiros limita os métodos de controle, uma vez que o número de culturas não hospedeiras disponíveis para rotação de culturas é limitado (Albert et al., 2022).

O uso de fungicidas sintéticos tem sido um importante método de controle para doenças causadas por *Sclerotinia*, devido à falta de níveis adequados de resistência do hospedeiro (Bardin e Huang, 2001). Além das desvantagens associadas ao controle químico, o uso de produtos químicos e medidas culturais são medidas de controle de baixa eficácia que não conseguem proporcionar o controle completo da doença (Rana et al., 2022).

2.4. Gênero *Bacillus*

O gênero *Bacillus* é um grupo diversificado de bactérias gram-positivas, em forma de bastonetes, produtoras de esporos altamente resistentes a condições desfavoráveis e que permitem fácil formulação e armazenamento dos produtos comerciais (Ingraham e Ingraham, 2011).

Bacillus tem um grande potencial para aumentar a produtividade agrícola e controlar fitopatógenos devido às suas propriedades de promoção de crescimento de

plantas (PGP) e de produção de compostos antimicrobianos de amplo espectro. Diante dessas características, algumas linhagens de *Bacillus* podem ser utilizadas como biofertilizantes ecologicamente corretos e inoculantes biofungicidas na produção agrícola.

Os produtos à base de *Bacillus* representam a classe mais importante de produtos microbianos para uso fitossanitário comercialmente disponível (Fravel 2005). E possuem grande potencial para serem usados como agentes de controle biológico, pois além da sua capacidade de proteger as plantas contra fitopatógenos, como fungos, bactérias e nematóides, sua viabilidade pode ser mantida quando estocados por longos períodos, podendo variar de meses a alguns anos, variando conforme a espécie, formulação do produto e condições de armazenamento. (Petras e Casida, 1985).

A promoção do crescimento em plantas afeta direta e indiretamente o desenvolvimento e a nutrição das plantas e acredita-se que seja o resultado de um conjunto complicado de mecanismos. Estimula o desenvolvimento da planta diretamente, facilitando a absorção de nutrientes, como solubilizando a fixação de fosfato e nitrogênio, produzindo ou regulando hormônios vegetais, como IAA, produzindo compostos voláteis que aumentam a resistência sistêmica induzida pela planta (ISR) e a liberação de enzimas ativas de carboidratos, que têm a capacidade de quebrar substratos que podem ser usados na produção de compostos essenciais e hidrolisar a parede celular de microrganismos fitopatogênicos (Zaid et al., 2022).

A importância do gênero *Bacillus* em programas de controle biológico se deve a alta capacidade de exercer antibiose resultante da produção de compostos antimicrobianos sendo por isso, muito utilizado na prospecção de metabólitos bioativos (Li et al., 2012).

Segundo Nielsen e Sorensen (1997), em torno de 20 a 40% das espécies de *Bacillus* isoladas do solo possuem alguma forma de antagonismo contra fungos patogênicos. De acordo com Ling et al. (2021). *Bacillus* possuem uma ampla gama de efeitos antagônicos em fitopatógenos, pois 5%–8% de todos os seus genes estão envolvidos na síntese de metabólitos secundários como peptídeos, lipopeptídeos, bacteriocinas, e outras substâncias ativas biologicamente

2.5. Compostos antimicrobianos de *Bacillus*

Bacillus spp. produzem uma vasta gama de metabólitos secundários, incluindo compostos orgânicos voláteis e não voláteis. O arsenal de substâncias antimicrobianas conhecidas produzidas por cepas de *Bacillus* inclui peptídeos sintetizados ribossomalmente e peptídeos sintetizados via não ribossomal, que são sintetizados por peptídeos não ribossômicos sintases (NRPs), policetídeos sintases (PKs) e híbridos NRPS/PKS (Wang et al., 2014). Esses incluem toxinas, sideróforos, antibióticos, surfactantes entre outros.

Sideróforos são moléculas orgânicas de baixa massa molecular com um sistema de captação de ferro capaz de quelar moléculas de Fe3+ com alta atividade específica (Carrol e Moore 2018). No meio ambiente, a maioria dos ferros encontrados está na forma de complexos insolúveis de óxido férrico/hidróxido (Ghosh et al., 2020). Sideróforos são secretados no meio ambiente sob condições limitantes de ferro e podem retirá-lo de minerais no solo, água marinha e doce, e de plantas ou outros organismos devido à alta afinidade pelo ferro férrico (Hider e Kong 2010).

Assim, a aquisição bacteriana de ferro por meio de sideróforos pode ser muito eficaz, e sua secreção pode contribuir significativamente para a PGP e para a supressão de doenças, pois a restrição da disponibilidade de ferro pode ser uma estratégia antimicrobiana contra fitopatógenos, já que restringe o acesso a esse elemento essencial (Khatoon et al., 2020)

Vários estudos têm empregado bactérias do gênero *Bacillus* produtoras de sideróforos como potenciais agentes de biocontrole (Ghazy e El-Nahrawy 2021; Kang et al., 2023). Por exemplo, Shen et al. (2022) em seu estudo relataram que *Bacillus siamensis* Gxun-6, uma bactéria produtora de sideróforos, demonstrou atividade antifúngica de amplo espectro contra *Fusarium oxysporum* e outros patógenos de plantas e promove o crescimento de bananeira.

Os antibióticos sintetizados via não-ribossomal incluem principalmente antibióticos lipopeptídicos (LPs), como a surfactina, iturina e fengicina que precisam ser modificados pós-traducionalmente para se tornarem uma forma ativa. Os LPs são compostos naturais de origem bacteriana constituídos por uma cadeia alquila

hidrofóbica ligada a um polipeptídeo hidrofílico para formar uma cadeia cíclica ou estrutura linear. Em função da presença de grupos hidrofílicos e hidrofóbicos na mesma molécula, exibem propriedades surfactantes (Santos et al., 2019).

Esses lipopeptídeos possuem atividades biológicas notáveis como o potencial para o controle biológico de fitopatógenos, e são substitutos potenciais para os surfactantes sintetizados quimicamente, já que podem ser usados como biosurfactantes de baixa toxicidade e alta biodegradabilidade. Existem cepas de *Bacillus* que têm a capacidade de produzir essas três famílias de lipopeptídeos simultaneamente (Farzand et al., 2019).

A surfactina é um lipopeptídeo com aplicação no campo da agricultura devido sua atividade antibacteriana e indução da resistência sistêmica em plantas (Théatre et al., 2021). Apesar de não possuir atividade contra fungos, quando associada à outros lipopeptídeos pode ter um efeito sinergético e aumentar o efeito antifúngico desses compostos (Desmyttere et al., 2019).

Os lipopeptídeos da família iturina inibem fitopatógenos bacterianos e fúngicos. Com destaque em suas propriedades antifúngicas poderosas e baixa toxicidade (Zhang et al., 2022).

A fengicina é um antibiótico que possui ação inibitória contra fungos filamentosos e por isso é usado como biofungicida. De acordo com Sur et al. (2018) o composto pode atuar por dois mecanismos, diretamente pela ligação à membrana celular que causa alterações na bicamada lipídica e consequentemente danifica a estrutura da membrana causando vazamento e lise, e indiretamente pela indução da resistência sistêmica da planta hospedeira. É importante ressaltar que a fengicina possui seletividade com relação à membrana e por isso não causa danos às plantas hospedeiras e bactérias.

As proteínas antimicrobianas sintetizadas via ribossomal incluem metabólitos como bacteriocinas e enzimas de degradação da parede celular, como proteases, celulases, quitinases e outras. As atividades de defesa dessas enzimas foram relatadas contra vários fitopatógenos (Myo et al., 2019; Zhou et al., 2021; Huang et al., 2022).

A quitinase é uma enzima capaz de decompor a quitina, um dos principais componentes das paredes celulares de fungos. A enzima ao degradar a quitina atua interrompendo a integridade das estruturas fúngicas e por consequência inibe o crescimento fúngico (Zavala et al., 2020, Malik et al., 2023).

As proteases são enzimas que hidrolisam proteínas e podem ter um papel no biocontrole ao degradar proteínas essenciais ao crescimento e desenvolvimento de patógenos (Deng et al., 2018). Proteases produzidas por *Bacillus* foram relatadas como inibidoras do crescimento e patogenicidade de fungos fitopatogênicos já que podem perturbar as membranas celulares de patógenos (Guleria et al., 2016).

A atividade de biocontrole por antagonistas do gênero *Bacillus* também pode ser devido à produção de compostos orgânicos voláteis (COV), que podem proteger as plantas diretamente contra fitopatógenos ou indiretamente através da indução de resistência das plantas (Tahir et al. 2017; Zamioudis et al. 2015). Diversos relatos estão disponíveis sobre o efeito de VOCs de *Bacillus* no crescimento de plantas e patógenos. Por exemplo, Myo et al. (2019) relataram que Bacillus velezensis NKG-2 produz enzimas degradadoras da parede celular fúngica e VOCs que mostraram atividade antifúngica e promoveu o de crescimento de plantas de tomate.

Na literatura há relatos de alguns microrganismos do gênero *Bacillus* com atividade antagonista contra *S. sclerotiorum*: (Zhang and Xue 2010; Farzand et al., 2019; Yang et al., 2020; Bajpai et al., 2022; Hu et al., 2022; Cheng et al., 2022). *Bacillus bombysepticus* é uma bactéria formadora de esporos e cristais paraesporais, do grupo *Bacillus cereus*, que causa a doença da septicemia no bicho-da-seda (Bezuidenhout et al., 2023). Até o momento não há relatos de atividade de biocontrole de *Bacillus bombysepticus* sobre *S. sclerotiorum*.

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CAPÍTULO 2 - Antifungal activity of *Bacillus* isolate JAB01 against white mold disease caused by *Sclerotinia sclerotiorum*

Abstract - White mold, caused by the pathogenic fungus *Sclerotinia sclerotiorum*, is a widespread disease that affects more than 600 plant species worldwide, including important crops such as soybeans, cotton, and tomato. The fungus can cause significant reductions in yield and quality of grain production. Biological control is an environmentally safe alternative and is effective against *S. sclerotiorum*, with the *Bacillus* genus being a promising tool for its biological control. In this study, the strain JAB01 was identified as *Bacillus* sp. by morphological examinations and confirmed by 16S rDNA gene sequence analysis. Under *in vitro* assays, *Bacillus* JAB01 effectively suppressed *S. sclerotiorum* growth and sclerotia germination, significantly reducing the disease infection on seeds and leaves. These results suggest that *Bacillus* JAB01 could be a promising biological agent against white mold diseases.

Keywords: antifungal activity; *Bacillus*; biological control agents; phytopathogenic fungus; VOCs

1 Introduction

White mold, caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, which belongs to the Phylum Ascomycota, Class Leotiomycetes, Order Helotiales, and Family Sclerotiniaceae is a necrotrophic and pathogenic fungus that infects more than 600 plant species worldwide, including important crops such as cotton, tomato, sunflower, beans, and soybeans (Liang and Rollins, 2018).

In Brazil, *S. sclerotiorum* was reported for the first time in 1921 in São Paulo, on the potato crop, and since then it has been described in soybean, beans, cotton, tomato, sunflower, and sun hemp, among other vegetable species (Sousa Melo et al., 2019). On soybean in the country, it is one of the most important diseases and can lead to significant reductions in yield and quality in grain production, as the infection can lead to necrosis, girdling, and possibly death of the plant (McDonald and Boland, 2010). The fungus provokes the development of various rots of roots, stems, and other

plant organs characterized by a formation of soft watery areas due to the ability of *S. sclerotiorum* to synthesize Oxalic Acid (OA) and lytic enzymes (Yaderets et al., 2021). According to (Danielson et al., 2007), yield reductions are caused by reduced seed number and weight resulting from the girdling of stems and disruption of the xylem and phloem.

This soil pathogen with destructive potential manifests itself in any part of the plant with the abundant formation of white cottony mycelium and sclerotia, which are structures that guarantee their survival in the soil for long periods. According to Bolton et al. (2006), the spread of the pathogen occurs mainly through seeds infected with the mycelium of the fungus or contaminated by sclerotia. Mycelial growth, production of sclerotia (Bloomfield and Alexander, 1967), and oxalic acid (Kyoung et al., 2008; Xu et al., 2018) are pathogenic factors of this fungus.

The most commonly used control method is chemical using fungicides. However, the use of chemicals can lead to the development of resistant populations of the fungus (Brent & Hollomon, 2007; Kuang et al., 2011; Firoz et al., 2016). In addition, it can cause damage to the environment acting on non-target organisms, including humans. Therefore, it is necessary to search for alternatives with fewer adverse effects. Faced with this demand, biological control of plant diseases is important, as it is an ecologically sustainable and environmentally safe alternative to chemical control for managing various pests and pathogens (M Mari, F Neri and P Bertolini, 2008).

In recent years, progress has been made in the biological control of *Sclerotinia* stem rot, among different biological approaches, the use of microbial antagonists, like yeasts (Cavalcanti et al., 2020) , fungi (You-you et al., 2017; de Rezende et al., 2020), and bacteria (Fernando et al., 2007; Massawe et al., 2018; Liu et al., 2022) have been reported as efficient against *S. sclerotiorum*. According to several studies *in vivo* and *in vitro* with bacteria with antagonistic activity against *S. sclerotiorum* the species of *Bacillus* genus are in evidence (Wu et al., 2014; Ansary et al., 2018; Massawe et al., 2018). *Bacillus* have the capacity to generate various biologically active compounds with antimicrobial properties like i.e. hidrolytic enzymes, toxins, antibiotics, siderophores, volatile organic compounds (VOCs) and others (Legein et al., 2020; Benchlih et al., 2023).

Given this context, the objective of this work was to evaluate a bacterial isolate of *Bacillus* genus regarding its potential antagonist against *Sclerotinia sclerotiorum in vitro*. The results of the study may provide further information on a promising bacterial biological control agent resulting in an advance in the control of diseases caused by this fungal phytopathogen.

2. Materials and Methods

2.1. Isolates and culture conditions

In a preliminary bioprospecting study with fifty-one bacterial isolates of the internal collection of genomes and metagenomes of the LBMP (Biochemistry of Microorganisms and Plants Laboratory), one isolate (JAB01) was identified as a promising against the fungus *Sclerotinia sclerotiorum*. The bacterial isolates were randomly isolated from the rhizospheric soil samples of various plants collected from different places in São Paulo State University (UNESP), Jaboticabal- São Paulo, Brazil and are stored in -80ºC freezers of the LBMP. For preliminary tests, they have been cultivated in Luria-Bertani (LB) broth at 30ºC and 150 r.p.m for 24 h.

The microorganism used here, *Bacillus sp*. JAB01, was isolated from mimosa soil, and after carrying out its bacterial growth curve, it was cultivated in LB medium at 30ºC and 150 r.p.m for 4 h. The fungus *S. sclerotiorum* was obtained from the Jco Biofertilizer industry and was grown on Potato Dextrose Agar (PDA) for 4 days at 25°C and maintained at 4°C for future use.

2.2. Partial sequencing of the 16S ribosomal region

For the isolate that showed antagonist activity, partial sequencing of the 16S ribosomal RNA was performed in order to confirm the identification of the genus and obtain an approximation of the species. Total DNA extraction was performed using the Insta Gene matrix Bio-Rad Kit according to the manufacturer's instructions. Subsequently, DNA amplification was performed by Polymerase Chain Reaction (PCR) with primers specific for the 16S rRNA gene, fD1 (5'-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G - 3') and rD1 (5'-CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC - 3') (Weisburg et al., 1991), the amplified products were sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing kit on the capillary

sequencer model ABI 3130 - Perkin Elmer. The sequences obtained were submitted to a nucleotide similarity query in the GenBank database accessed through the NCBI website (“National Center for Biotechnology Information”), through the local BLAST tool - “Basic Local Alignment Search Tools”.

2.3. Phase Contrast Microscopy (PCM)

For morphological identification, samples were analyzed with a PCM. The JAB01 isolate was cultured in liquid EMBRAPA (Monnerat et al., 2020) medium for 72 h at 150 rpm and 30°C. After culture, a drop of the bacterium was placed on a slide with a sterile pipette and covered with a coverslip, a drop of immersion oil was added to the coverslip, and visualization of the samples was performed on a Zeiss Z2 Axionvsion microscope at 1000x magnification.

2.4. Bioassay of antagonist activity *in vitro* by diffusible substances

The antagonist activity of the bacterial isolate JAB01 was tested against the phytopathogen by dual-culture plate technique. Discs of *S. sclerotiorum* mycelium (≈ 7 mm Ø) cultured on solid PDA medium were cut using a sterilized punch and placed in the center of PDA Petri dishes. At the end of the plate (≈ 2.5 cm from the center) 10 µl ($1.13x10^8$ CFU/ml) of the bacterial isolate JAB01, previously incubated for 4 h at 30°C in liquid LB medium, was inoculated. The plates were incubated at 25°C in a B.O.D (Bio-Oxygen Demand) for 5 days. Inhibition zones were measured from the edge of the JAB01 colony to the end of fungal mycelial growth. PDA plates inoculated with the fungus disc and 10 µl of sterile LB medium were used as controls. Each treatment was applied to three replicate plates and repeated at least three times.

2.5. Germination and viability of sclerotia and oxalic acid production

For the evaluation of sclerotia germination, an aliquot of 100 µL ($1.13x10^8$ CFU/ml) of bacterial suspension was spread with a Drigalsky loop sterilized in a Petri dish with PDA medium, then two sclerotia were placed at the ends of the plates and incubated at 25°C. Germination was assessed after 7 days. Additionally, sclerotia were submerged for 1 min in a spore suspension ($1.13x10^8$ CFU/ml) of the antagonist, then collected and transferred to a Petri dish containing PDA.

After the germination, the sclerotia were collected and the surface was sterilized with 3 washes with sodium hypochlorite (5%) for 1 min interspersed with sterile water to eliminate the JAB01 inoculum. After disinfection, sclerotia were collected and transferred to Petri dishes containing PDA medium supplemented with 50 mg/L bromophenol blue to assess myceliogenic germination viability and oxalic acid production. Bromophenol blue is an acid-base indicator, which emits blue color at pH ≥ 4.6 and yellow color at pH ≤ 4.6 (Kreft and Kreft, 2009).These experiments had three repetitions and for the control treatments plates with sclerotia non inoculated with JAB01 was used.

2.6. Seed bacterization

Susceptible soybean seeds of BMX Valente, upon surface desinfection, were immersed in bacterial culture ($1.13x10^8$ CFU/ml) JAB01 for 30 min. To assess seed infection, the method established by (Abdullah et al., 2008) with modifications was used. Two mycelial plugs (≈ 7 mm Ø) of *S. sclerotiorum* were placed on both sides of a PDA plate, subsequently, five bacterial seeds were placed forming a row between the mycelial plugs. After incubation for 3 and 7 days at 25 °C, the number of germinated healthy seeds and infected seedlings was recorded. Untreated soybean seeds were used as a control and the experiment was performed in five replicates.

2.7. Pathogenicity on detached leaves

Fully expanded leaves of selected soybean plants were detached, rinsed with sterile distilled water and air dried. The leaves were placed on plastic Petri plates (8 by 8 cm) on damp paper towels to avoid direct contact with water. The leaves that were inoculated with the JAB01 isolate were immersed in a bacterial solution grown under the conditions described above. Plugs of *S. sclerotiorum* fungal hyphae that were prepared as described above were placed on the upper part of the mid rib of the leafs, while their petioles were wrapped with damp cotton to prevent desiccation. For the control treatments, there has been no inoculation of the leaves with JAB01. All treatments were incubated at 25°C and fungal pathogenicity was assessed from 72 h to 144 h post-inoculation (hpi) by the presence of disease symptoms. The experiment was replicated three times.

2.8. Evaluation of the inhibitory activity of VOCs produced by *Bacillus* JAB01 *in vitro* against *S. sclerotiorum*

In addition, the double-plate assay was used to study the antagonistic activity of volatile compounds released by JAB01 against *S. sclerotiorum*. Two Petri dishes were placed opposite each other. The lower Petri dish contained LB agar, which was inoculated with 100 µl of JAB01 ($1.13x10^8$ CFU/ml). The upper Petri dish contained PDA, where a ≈ 7 mm diameter disk of actively growing *S. sclerotiorum* was placed. The upper and lower Petri dishes were sealed with parafilm to prevent the loss of volatiles and incubated at 25°C for four days. Control plates were also prepared in the same way, except that an uninoculated LB plate was used in place of the JAB01 plate.

To further evaluate the effects of *Bacillus* VOCs on *S. sclerotiorum*, ≈ 7 mm diameter fungal plugs were taken from an *S. sclerotiorum* mycelial mat previously exposed to *Bacillus* VOC for 96 h. The plugs were then placed at the center of PDA Petri dishes (7 mm Ø), sealed, and incubated at 25°C. The Petri dishes containing VOC-untreated fungal mycelial plugs served as a control. Growth and development of fungal mycelia were monitored for 96 h by recording mycelial growth (in millimeters) at intervals of 24 h. The *in vitro* mycelial growth inhibition rate (R) was calculated by the equation:

R (%) = D1 - D2/D1 - D0 × 100

where R is the percentage of inhibition of mycelial extension; D1 is the mycelial diameter (mm) of the negative control set; D2 is the mycelial diameter of the treated plate, including the size of the fungal agar plug (mm); and D0 is the original mycelium diameter (7 mm Ø) of the fungal agar plug (Gao et al., 2018). The experiment had three replicates and was replicated at least three times.

2.9. Effects of VOCs on sclerotia germination

To evaluate the effects of JAB01 VOCs on *S. sclerotiorum* sclerotia germination, sclerotia were taken from an *S. sclerotiorum* plate and placed on one side of a split Petri dish. On the other side of the plate, 50 µl of the bacterial solution, grown under

the conditions described above, was spread with a Drigalsky loop and the plates were sealed with parafilm and kept in BOD at 25°C. After 5 days, the mycelial germination of the resistance structure of the fungus and antagonistic activity was considered when there was an inhibition zone. Each treatment had five replicates.

2.10. Effects of VOCs on detached leaves

To evaluate the effects of JAB01 VOCs on soybean leaves inoculated with *S. sclerotiorum*, fully expanded V3 leaves of the BMX Valencia line grown in a greenhouse were cut, washed in sterile distilled water, and a single leaflet was carefully placed on wet paper towels on one side of a 9 cm diameter bipartite Petri dish. Plugs of ≈ 7 mm in diameter were taken from an *S. sclerotiorum* mycelial mat and placed on the upper part of the leaf, while its petioles were wrapped with damp cotton. On the other side of the plate, 60 µl of the bacterial solution, grown under the conditions described above, was spread with a Drigalsky loop and the plates were sealed with parafilm and kept in BOD at 25°C for 9 days. After 9 days, the disease incidence was evaluated, and the antagonist activity was considered according to the presence of disease symptoms. The experiment had five replicates for each treatment.

2.11. Scanning Electron Microscopy (SEM)

The influence of VOCs emitted by *Bacillus* JAB01 on the morphology of *S. sclerotiorum* hyphae was assessed by SEM. Fungal mycelia were extracted from VOC-treated and untreated Petri dishes. Samples were fixed in 2.5% glutaraldehyde (Solarbio, Co. Ltd.) in 0.1M sodium cacodylate buffer for 18 h and postfixed at 4°C for 6 h with the same buffer in 1% osmium tetroxide, followed by dehydration using a number (30 to 100%) ethanol gradient (vol/vol). Samples were dried in a critical point dryer and then metalized in gold and visualized in a Zeiss scanning electron microscope, Evo MA 10, at 10KV.

3. Results

The molecular identification of strain JAB01, by sequencing of the 16S rDNA gene, showed high similarity (99,93%) and coverage (99%) with *Bacillus sp.* (table 1).

TABLE 1 - Alignment of isolate JAB01 with nucleotide sequences from the NCBI database. The first five sequences with the highest degree of similarity are shown.

Scientific Name	Max Score	Total Score	Query Cover	E-value	Per. Ident	Accession
Bacillus sp. 4	2760	2760	99%	0.0	99.93%	AY853168.1
Bacillus thuringiensis strain HER1410	2758	38547	99%	0.0	100.00%	CP050183.1
Bacillus thuringiensis strain FDAARGOS_796	2758	38381	99%	0.0	100.00%	CP053972.1
Bacillus thuringiensis serovar *israelensis* strain BGSC 4Q7rifR	2758	32920	99%	0.0	100.00%	CP051858.1
Bacillus sp. RZ2MS9	2758	38492	99%	0.0	100.00%	CP049978.1

Phase contrast microscopy and SEM revealed bacillary morphology of vegetative cells (Figure 1A-B), endospore production and absence of crystals (Figure 1B).

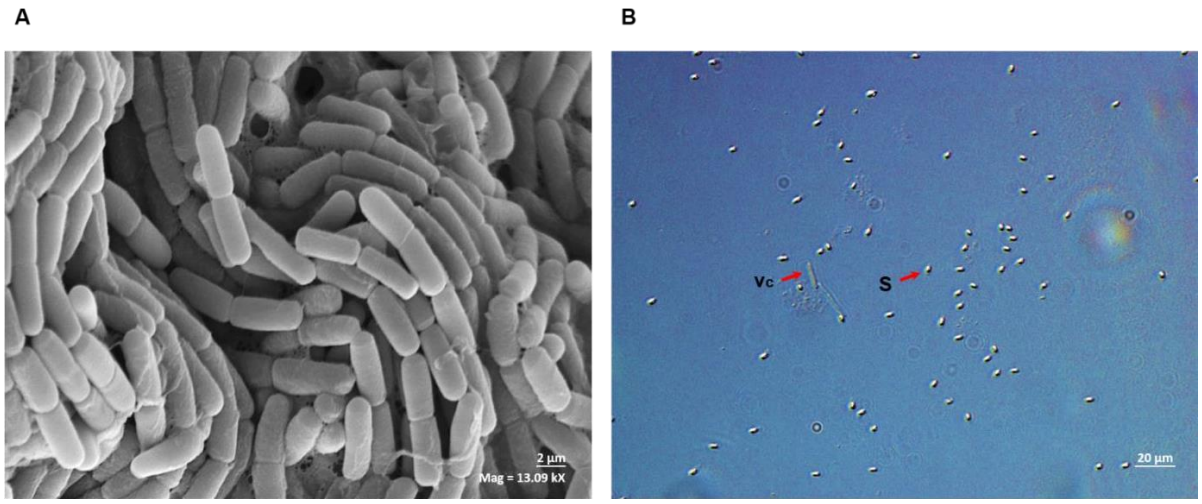

Figure 1. Morphological characteristics of *Bacillus* JAB01. **(A)** JAB01 cells under scanning electron microscopy. **(B)** Spores (S) and vegetative cells (Vc) of JAB01 under phase contrast microscopy at 1000x magnification.

The results demonstrated that in *in vitro* plate assays *Bacillus JAB01* exhibited antagonistic activity by the production of diffusible secondary metabolites since an inhibition zone was created among the microrganisms, as well as volatile organic compounds (VOCs). The results of the dual culture assays showed that JAB01 was able to inhibit the mycelial growth of *S. sclerotiorum*, a 67% reduction compared to the control (Figure 2D). When the bacterial isolate was placed over the mycelial plugs of the fungus there was a complete inhibition of the disease. JAB01 also produced VOCs with strong inhibition of *S. sclerotiorum* growth by suppressing fungal mycelium growth by 78.43% compared to the control (Figure 4A - C).

The inhibitory activity observed against *S. sclerotiorum* mycelial growth has prompted further investigation into whether the products produced by *Bacillus* can also affect sclerotia germination and viability.

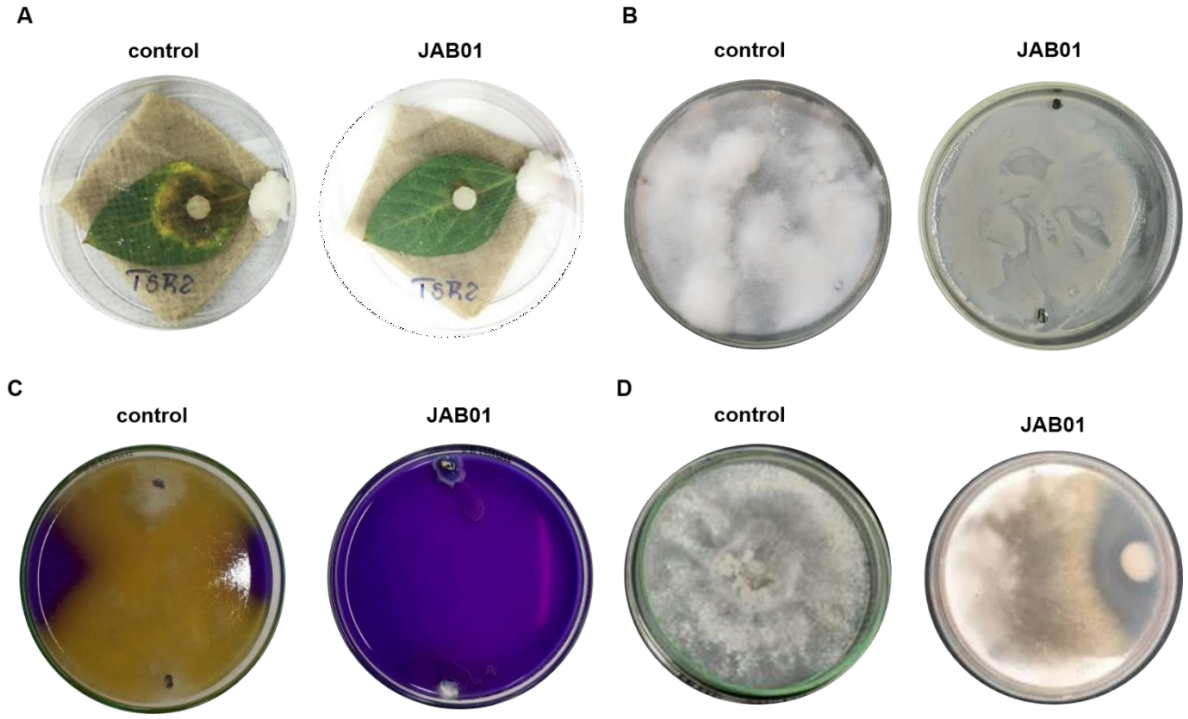

FIGURE 2. Antagonistic effects of diffusible substances produced by bacterial isolate JAB01 on mycelial growth of *Sclerotinia sclerotiorum*. **(A)** Effect of JAB01 for protection of soybean leaves against *Sclerotinia sclerotiorum*. **(B)** Myceliogenic germination of sclerotia on PDA plates. **(C)** Myceliogenic germination of sclerotia and oxalic acid production on PDA suplemmented with bromopheenol blue. **(D)** Mycelial growth of *S. sclerotiorum* on PDA plates.

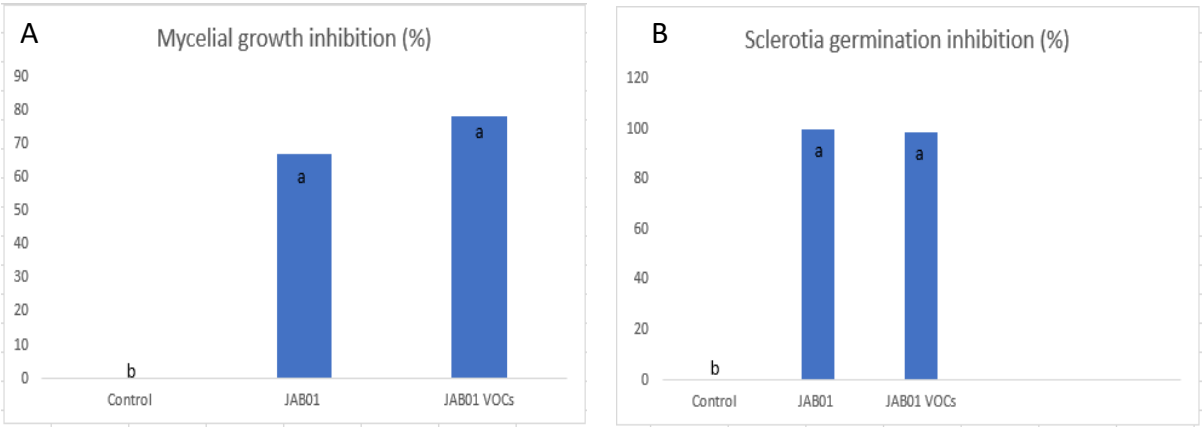

FIGURE 3 Fungistatic effects of difusible substances and VOCs produced by *Bacillus* JAB01 on mycelial growth and sclerotia germination **(A)** Rate (percentage) of inhibition

of *Sclerotinia sclerotiorum* mycelial growth. **(B)** Rate (percentage) of inhibition of sclerotia germination. Different letters represent a significant difference between treatments.

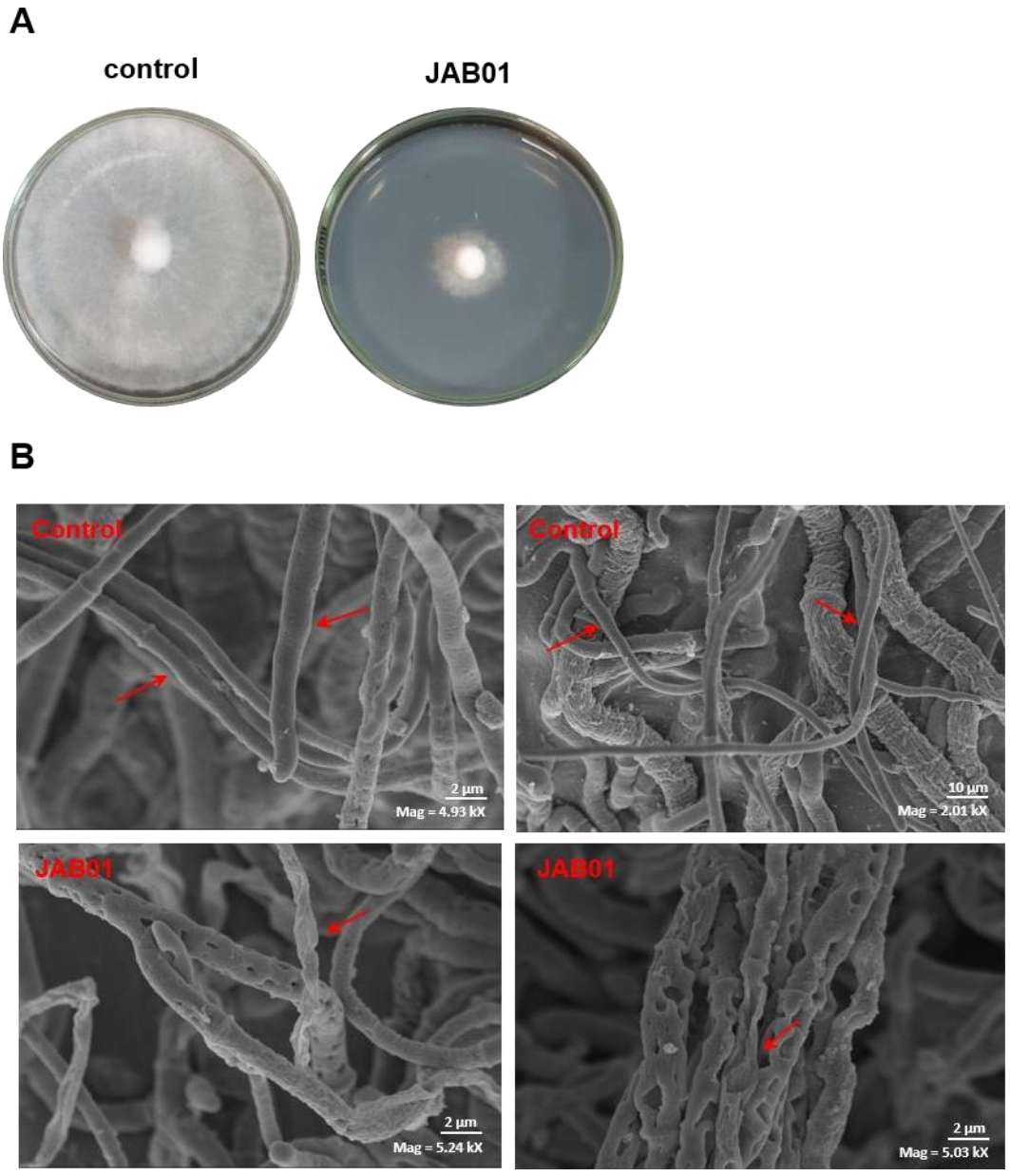

Figure 4. (A) Effects of VOCs of JAB01 on mycelial growth of *Sclerotinia sclerotiorum* on PDA. (B) Mycelium of *Sclerotinia sclerotiorum* on Scanning Electron Microscopy.

Sclerotia exposed to VOCs produced by JAB01 in the split plates showed halos of mycelial inhibition, indicating that the isolate was also capable of inhibiting the mycelial growth of the fungal resistance structure by producing volatile compounds.

The diffusible secondary metabolites produced by *Bacillus* also exhibited fungistatic effects against sclerotia of *S. sclerotiorum*, as evidenced by the complete inhibition of sclerotia germination when the resistance structures were inoculated with *Bacillus* on Petri plates, unlike the control plates where the sclerotia germinated normally (Figure 2B). Additionally, the absence of yellow coloration in plates PDA supplemented with bromophenol blue containing treated sclerotia indicated that there was no production of oxalic acid (Figure 2C).

The results of seed bacterization show that seed inoculation with *Bacillus* JAB01 provided complete protection against seed rot caused by *S. sclerotiorum* infection. When the seeds were treated with JAB01, at least 80% of the resulting seedlings remained healthy. In contrast, the control treatment without bacterial inoculation resulted in seedlings being infected by the fungus within 10 days of incubation (Figure 5).

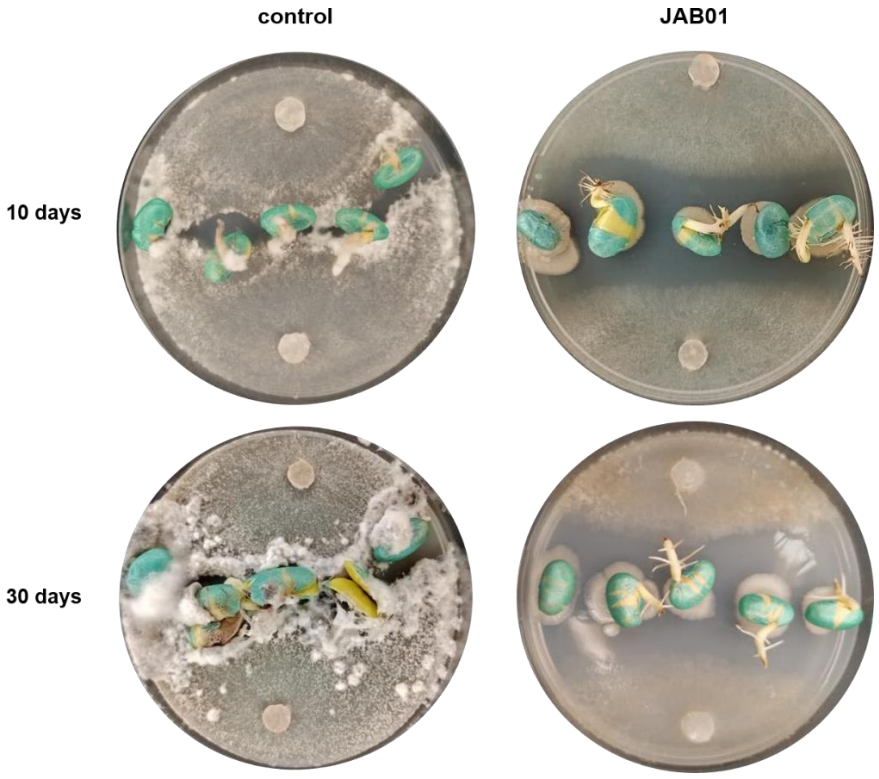

Figure 5. Protective effect of JAB01 on soybean seeds against *Sclerotinia sclerotiorum*. Soybean seeds inoculated by immersion with JAB01 were germinated in Petri dishes with solid PDA medium in the presence of *S. sclerotiorum* at the ends of the plate. Evaluation conducted at 10 and 30 days.

In detached leaf assays, it was observed that the untreated fungal hyphae plug caused brownish lesions on the leaves of the host plants, whereas the treated fungal disc exhibited reduced lesion development. The metabolites produced by *Bacillus* JAB01 significantly decreased the size and incidence of the lesions, as evidenced by absence or limited lesion formation on all tested host plant leaves 72 hours after infection, compared to the untreated control (Figure 6). Similarly, the pathogenicity assay involving the volatile organic compounds (VOCs) produced by JAB01 on detached leaves of soybean plants yielded similar results. Exposure to JAB01 VOCs also impeded the progression of the disease by reducing lesion size compared to the untreated control.

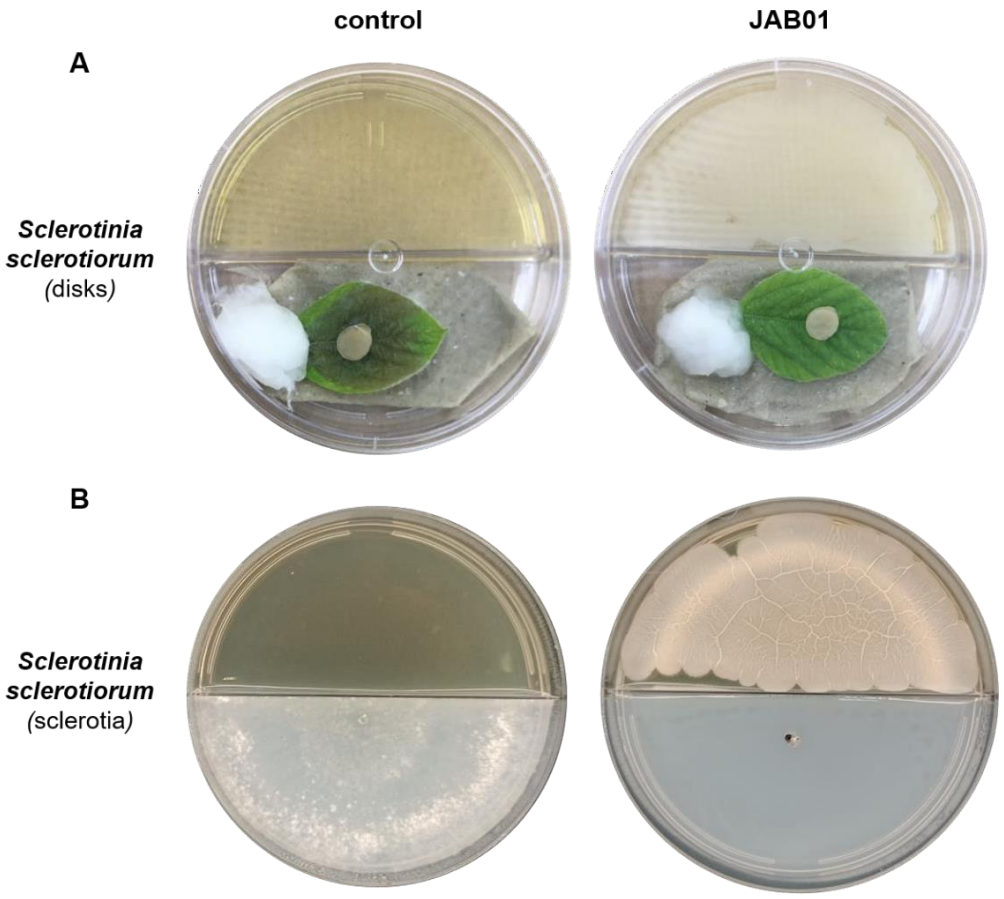

Figure 6. *In vitro* effects of VOCs on the growth of *Sclerotinia sclerotiorum*. (A) Soybean leaves infested with PDA discs of *Sclerotinia sclerotiorum*, one end of the Petri dish, and bacterial isolate JAB01 on solid LB medium, the opposite end of the dish. (B) Sclerotia was grown on PDA medium and JAB01 was inoculated on a solid LB medium at the opposite end of the Petri dish. For the controls, JAB01 was not inoculated.

To investigate the effects of the VOCs emitted by *Bacillus* JAB01 on *S. sclerotiorum* hyphae, scanning electron microscopy (SEM) analyses were conducted. The scanning electron images captured from the VOC-untreated control exhibited healthy, dense, and cylindrical hyphae (Figure 4B). However, the fungal hyphae treated with the VOCs of *Bacillus* displayed signs of dryness and an increase in the number and size of pores in certain parts. These observations revealed that the VOCs

emitted by B. JAB01 have a discernible impact on the morphology and structure of S. sclerotiorum hyphae, leading to notable changes compared to the untreated control.

4. Discussion

In this study, the 16S rDNA partial gene sequencing of the isolate that showed antagonistic performance against *S. sclerotiorum* confirmed that the isolate belongs to the *Bacillus* genus. Since sequencing 16S rDNA gene sequencing isn't reliable at the species level and *Bacillus* is a bacterial genus with great genomic diversity, its taxonomic affiliation is complex. Thus, here JAB01 was affiliated, at the genus level, to Bacillus, supported by its microscopic traits, i.e., bacillary morphology and endospore production. However, more analyzes are needed, at the genome level, to certify the specie of strain JAB01.

Although there are reports in the literature of the presence of parasporal crystals in *Bacillus bombysepticus*, according to the results of contrast microscopy it was not possible to observe the presence of crystals in *Bacillus bombysepticus* JAB01 cells.

An approach based on biological control is essential for food production. The genus *Bacillus* has many microorganisms being used as biocontrol agents because they are known for their production of a wide range of biologically active molecules (Tserkovniak and Kurdish, 2009). According to Ashwini & Srividya (2013), *Bacillus* species offer several advantages over other organisms due to their ability to form endospores and hence can tolerate extreme pH, temperature, and osmotic conditions.

In this study, the focus of our attention was on the antifungal activity of the isolate *Bacillus* JAB01 from mimosa soil against white mold disease. This strain produced diffusible substances and VOCs, which suppressed the mycelial growth of *S. sclerotiorum*, inhibited sclerotia germination and slowed disease progression on detached leaves.

Our results are like the findings of (Massawe et al., 2018), which related that *Bacillus velezensis* VM11 could produce diffusible compounds and VOCs that inhibited mycelial growth of *S. sclerotiorum*.

The zone of inhibition formed in the dual culture bioassay plates (Figure 2D) indicated the presence of biologically active metabolites that diffused in an agar medium. Our findings are in agree with other studies that revealed the antagonistic activity exerted by *Bacillus* spp (Abdullah et al., 2008). In the present study, the growth suppression during dual culture assay was less than the volatile inhibition; these results are like the findings of (Chaurasia et al., 2005).

The results about germination and production of oxalic acid indicate that JAB01 can suppress the myceliogenic germination of the resistance structure and the oxalic acid secretion. At the moment of infecting plants, *S. sclerotiorum* secrets oxalic acid to acidify the surrounding ambient creating the ideal conditions for cell wall degrading enzyme activity. According to (Kamal et al., 2015) , the secretion of oxalic acid and a battery of acidic lytic enzymes kill cells ahead of the advancing mycelium and causes the death of cells at the injection sites. So oxalic acid is important to sclerotia production and pathogenesis. (Rollins and Dickman, 2001) showed that under neutral or alkaline pH sclerotial formation is inhibited.

The sclerotia are the resistance structure of the fungus, which can withstand extremely adverse conditions and persist in the soil for many years until favorable conditions appear, making it difficult to control the disease (Giorgio et al., 2015). Sclerotia can germinate either myceliogenically or cryogenically with favorable environmental conditions. Myceliogenic germination produces infective hyphae (Willetts and Wong, 1971). The observed inhibition of sclerotia germination may turn to be a very useful and valuable factor in the development of biological methods of plant protection.

Our results are in agree with the findings of (Tyagi et al., 2020) and (Haddad et al., 2017). These results might lead to a promising control approach given the sclerotia's importance. Besides that, the absence of oxalic acid secretion also deserves attention since it can affect the virulence and pathogenicity of the fungus. In the literature, there are related resistant transgenic plants based on oxalic acid degradation (Yang et al., 2019). So, it's necessary to do more tests to understand the nature of this suppression.

Our observations regarding seed bacterization (Figure 4) are according with other reports where seed treatment with *Bacillus* isolates had a positive effect on

suppressing seedling diseases caused by several phytopathogens (Abbas et al., 2019; Ashwini & Srividya, 2013; Moustafa et al., 2007).

The results of the detached leaf tests are in agreement with other studies in the literature, in which *Bacillus* isolates were able to reduce the progression of the disease on leaves of different hosts (Wu et al., 2014; Rahman et al., 2016).

The wide arsenal of antimicrobial substances produced by *Bacillus* species known to inhibit phytopathogens includes secondary metabolites like lipopeptides, volatile compounds (Gao et al., 2018), and hydrolytic enzymes (Oztopuz et al., 2019).

The fungal cell wall is rich in cellulose and chitin. The activity of cell wall-degrading enzymes is considered an important mechanism of microbial antifungal action (Sadeghy and Hatamy, 2014). Thus, hydrolytic enzymes produced by biocontrol agents, such as chitinases, glucanases, and proteases, are important mechanisms involved in the biocontrol of fungal plant pathogens. So, the mechanism involved in the *in vitro* disease suppression might involve the production of these degradation enzymes.

The data obtained in the present study corroborate with previous studies in which species of the genus *Bacillus* were able to control fungal pathogens *in vitro* by the volatile organic compounds (Yuan et al., 2012; Gao et al., 2017; Tahir et al., 2017; Myo et al., 2019). For example, (Wu et al., 2014) related the antagonistic effect of VOCs produced by *Bacillus amyloliquefaciens* strain NJZJSB3 that adversely affected the growth of *S. sclerotiorum* due to its antifungal activity. (Gao et al., 2017) related that the VOCs generated by *B. velezensis* provided the inhibition of the mycelial growth of several phytopathogens. According to (Li et al., 2015), COVs can act as biofumigant and have some advantages compared to some biological and chemical fungicides, as the absence of residue and pollution problems, given that there is no need for spraying.

We hypothesized that the VOC of *Bacillus* might have fungicidal effects toward *S. sclerotiorum* hyphae but, when regrown on a fresh PDA medium, hyphae were observed to regain their growth momentum similar to the control. This suggests that while the VOCs emitted by *Bacillus* JAB01 have an inhibitory effect on the hyphae development, they do not have a long-lasting or permanent effect on the growth of *S.*

sclerotiorum hyphae since the hyphae were able to recover and grow once transferred to a favorable growth medium

Besides that, VOCs produced by the *Bacillus* JAB01 had effects on sclerotia germination, since healthy sclerotia lost their viability *in vitro* when cocultured with the antagonist. These findings are similar to some work in literature (Fernando et al., 2005; Massawe et al., 2018).

Given the result in SEM microscopic observations (Figure. 3B), we believe that the observed damages to the hyphae may result in weaker infection and colonization in host plants. So, the antagonistic activity observed on the detached leaves may be caused by mycelium weakened by exposure to VOCs, explaining the smaller lesions on soybean leaves. These results lead us to believe that JAB01 has great potential to control the disease in host plants. Therefore, we highlight the need for field studies to evaluate the potential of our isolate *in vivo*.

In light of these promising findings, it is important to emphasize the necessity of conducting field studies to evaluate the potential of *Bacillus* JAB01 *in vivo*. Field studies would provide valuable information about the effectiveness of *Bacillus* JAB01 as a disease control agent under field conditions. Such studies would further validate the potential of this isolate and guide its practical application in agricultural settings.

5. Conclusion

In conclusion, the isolate JAB01 effectively suppressed *S. sclerotiorum* growth and sclerotia germination *in vitro* assays. Besides that, the *Bacillus* significantly reduced the disease infection on seeds, so it has the potential to be used as a seed treatment biocontrol agent. In future work, the isolate JAB01 should be tested in field trials under different environmental conditions.

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CAPÍTULO 3 Genomic features of *Bacillus bombysepticus* JAB01, a potential biocontrol agent of white mold disease

Abstract - We have previously confirmed that *Bacillus bombysepticus* JAB01 has an fungistatic effect on *S. sclerotiorum* in *in vitro* experiments. *B. bombysepticus* strain JAB01 was isolated from the rhizosphere of mimous plants and identified through whole-genome sequencing. In this work, a study was carried out on the genetic basis involved in the control of the phytopathogen *S.sclerotiorum* and a comparative analysis of the Bb JAB01 whole genome with other *Bacillus* species that had been already reported with biocontrol effects. Clusters responsible for the synthesis of petrobactin, fengycin, bacillibactin and cericidin were detected in JAB01 genome. Genome mining showed that this strain has genes related to biocontrol and growth promotion.like phosphate solubilization, siderophores, indole acetic acid (IAA), and forming biofilms that promote plant growth and facilitate biocontrol. These results suggest that this bacterial strain provides good protection against white mold via direct and indirect modes of action and could thus be a valuable biocontrol agent.

Keywords: biological control, carbohydrate active enzymes, comparative genomics, genome sequencing

1. INTRODUCTION

White mold disease caused by the fungus *Sclerotinia* sclerotiorum, is one of the most prevalent diseases in soybean and can lead to several losses in the production of the crop. The soil-borne pathogen *S. sclerotiorum* is an important necrotrophic fungus infecting a wide host range of agriculture crop like soybean, bean, letucce, sunflower, potato and others (Smolińska and Kowalska 2018).

The biocontrol potential of bacterial isolates is given by the production of an array of antimicrobial compounds like antibiotics, COVs, iron chelating siderophores and cell wall degrading enzymes (Carmona-Hernandez et al., 2019) and some still

have Plant growth promoting traits (PGPT) like the production of índole acetic acid, siderophores, phosphate solubilization and others.

Growth promotion in plants impact both plant development and nutrition directly and indirectly and is believed to be the result of a complicated set of mechanisms. It stimulates plant development directly by facilitating nutrient absorption such as solubilizing of phosphate and nitrogen fixation, producing or regulating plant hormones such as IAA, producing volatile compound that enhances plant induced systemic resistance (ISR) and the release of carbohydrate-active enzymes, which have the capacity to breakdown substrates that can be used in the production of essential compounds and hydrolyze the cell wall of plant pathogenic microorganisms (Zaid et al., 2022).

In a previously study, a collection of bacteria were screened for their antagonistic activity against *S. sclerotiorum* aiming to avoid the use of chemical fungicides to control the white mold disease. One of them, identified as *Bacillus bombysepticus* JAB01 was the most promising and was tested further using *in vitro* assays, as well as other tests. In this study, JAB01 was effective in suppressing the development of the phytopathogen and its resistance structure *in vitro* and, therefore, was considered as a potential biological control agent for white mold. The results suggest that the isolate has more than one biocontrol mechanism, diffusible substances and volatile organic compounds, and a wide range of metabolites that may be linked to antagonistic activity.

Therefore, a study on the genes contained in the *Bacillus bombysepticus* JAB01 genome may bring information about the genetic basis involved in the fungal control of *S.sclerotiorum*. Considering the importance of controlling diseases caused by *S.sclerotiorum*, the complete sequencing of the JAB01 genome was carried out and its molecular arsenal was studied for plant growth promotion, biocontrol activity, secondary metabolites and cell wall degradation enzymes.

2. MATERIALS AND METHODS

2.1. Whole genome sequencing of *B. bombysepticus* JAB01

The *Bacillus bombysepticus* JAB01 genome was sequenced using Ilumina Novaseq6000 and Oxford Nanopore (MinION) technologies. For genome assembly, the Novaseq6000 pair-end fastq files were trimmed to adapters and potential contaminating human sequences were mapped using Bowtie2 using the human reference genome. Additionally, the fastq files from the MinION run (SQK-LSK110 kit, Oxford Nanopore) were trimmed for low quality reads with NanoFilt and adapters trimmed using Porechop. All trimmed reads from Illumina and Nanopore were then used for hybrid assembly using the MaSuRCa assembler according to developer's instructions. The filtered reads were combined to create a single contig with no gaps and the Fasta file contains a final assembly. These files were used for gene prediction and mannual annotation, annotation with NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Seeman, 2014). Construction and visualization of the genome map was performed using DNAPlotter (Carver et al., 2009) and CGView.

2.2. Genome annotation and identification of genes of interest in *B. bombysepticus* JAB01

The functional annotation of the encoded proteins by JAB01 and the predicted genes were inferred done on eggNOG-mapper (v. 2.1.6) (Cantalapiedra et al., 2019) that is based on the EggNOG orthology system (Huerta-Cepas et al., 2016). That contains functional information from many sources including a Cluster of Orthologous Groups of proteins (COGs), KEGG pathways, and others.

The antiSMASH (v. 7.0) server was used for automatic annotation of secondary metabolite biosynthetic gene clusters (BCGs).

The dbCAN3 (Zheng et al., 2023) automated carbohydrate-active enzyme annotation web server, was used to determine the Carbohydrate-Active enZYmes (CAZYmes) We consider CAZYme when a protein is predicted at least two true annotations for CAZyme.

2.3. Phylogenomic analysis and Average Nucleotide Identity (ANI)

To reconstruct and position Bb JAB01 within the *Bacillus* genus (**Supplementary table S1**), we conducted a phylogenetic analysis using GToTree v1.1.10 (Lee 2019). The phylogenetic analysis was performed using 1050 *Bacillus* genomes available in GenBank. We utilized the hidden Markov model (HMM) single-copy gene set and employed the maximum likelihood (ML) method for tree reconstruction, generated from 1000 bootstrap replicates. The phylogenomic tree was edited and annotated with iTol (Interactive Tree Of Life) (v. 6.7.5) (Letunic and Bork 2021). In addition, the Orthologous Average Nucleotide Identity Tool software (OAU) (Yoon et al., 2017) was used to determine the ANI values among biocontrol *Bacillus* genomes.

2.4. Comparative genomics analysis

In this study a total of twenty nine biocontrol *Bacillus* strains were used including the sequence of *Bacillus bombysepticus* JAB01 strain. RefSeq GenBank format (.gbff) files of the other twenty eight *Bacillus* strains (*Bacillus thuringiensis* sv. kurstaki HD73, *Bacillus thuringiensis* BMB171, *Bacillus thuringiensis* HS18-1, *Bacillus thuringiensis* HD12, *Bacillus thuringiensis* Bt185, *Bacillus thuringiensis* Bt407, *Bacillus thuringiensis* sv. kurstaki HD-1, *Bacillus velezensis* UCMB5113, *Bacillus velezensis* UCMB5033, *Bacillus velezensis* GB03, *Bacillus velezensis* G341, *Bacillus velezensis* B25, *Bacillus amyloliquefaciens* CC178, *Bacillus amyloliquefaciens* UMAF6639, *Bacillus amyloliquefaciens* UMAF6614, *Bacillus amyloliquefaciens* plantarum NJN6, *Bacillus amyloliquefaciens* plantarum At1, *Bacillus amyloliquefaciens* UCMB-5007, *Bacillus subtilis* XF -1, *Bacillus subtilis subtilis* BSD-2, *Bacillus subtilis* HJ5, *Bacillus subtilis* SG6, *Bacillus subtilis* SZMC 6179J, *Bacillus* sp. RZ2MS9, *Bacillus* sp. Pc3, *Bacillus* sp. JS, *Bacillus* sp. BH072 and *Bacillus bombysepticus* Wang with ‘complete genome’ assembly level of the latest RefSeq annotations, annotated with PGAP, were obtained from the public database of NCBI.

2.5. Plant Growth Promotion traits and functional enrichment

The KEGG orthology (KOs) annotated for the biocontrol *Bacillus* genomes were mapped against the plant growth promotion traits (PGPTs) ontology with the PGPT-Pred tool, available on the web resource for plant-associated bacteria, PLaBAse (http://plabase.informatik.uni-tuebingen.de/pb/plabase.php) (Patz et al., 2021). The class of PGPTs were aggregated in the class level 5 to determine functional enrichment of classes. The enrichment was measured by the Fisher's test, considering the ratio of PGPT in the class for each genome in relation to others. Classes with p-value ≤ 0.1 are considered significant.

3. RESULTS

After obtaining the sequencing and assembly results, the genome was deposited in GenBank (accession number). The main features of the *B. bombysepticus* JAB01 genome are summarized in (Figure 1).

The 6.094.706 bp long JAB01 genome is composed of a circular chromosome of 5.374.157 pb and four circular plasmids (121263 pb, 60442pb, 220629pb, and 315703pb) with an average GC content of 35% (Fig. 1 and Table 1). The NCBI Genome Annotation report reveals that the chromosome contains a total of 6215 genes, 6061 of which encode CDS, 154 RNA genes. According to the annotations results, 5833 CDSs were attributed to putative biological functions, while 228 CDSs were characterized as hypothetical proteins with unknown function.

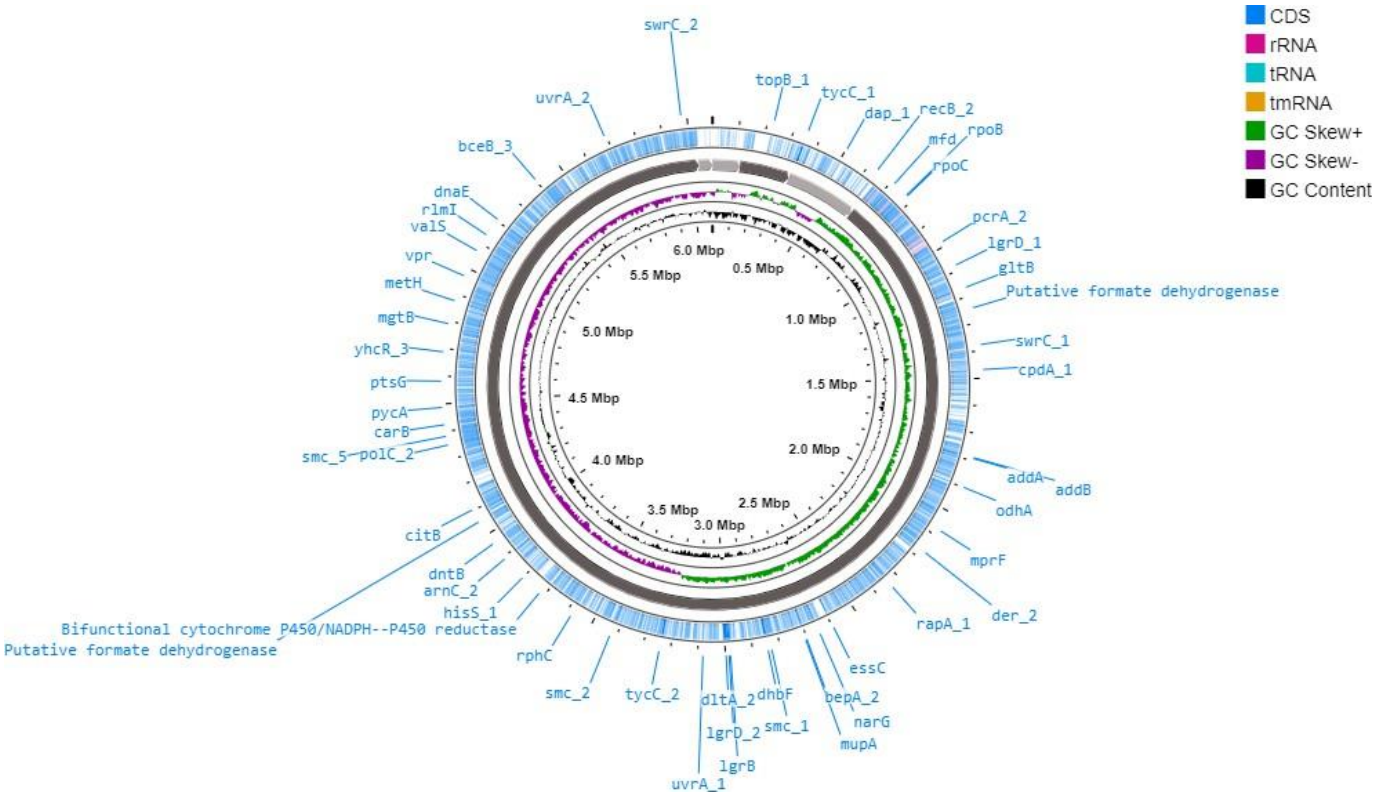

Figure. 1. The whole genome of *Bacillus bombysepticus JAB01*

Genome characteristics	Chromosome	Plasmid 1	Plasmid 2	Plasmid 3	Plasmid 4
NCBI Acession	OP097865				
Size (pb)	5374157	121263	60442	220629	315703
Pseudo genes	228				
Genes	6215				
CDS	6061				
ncRNA	5				
rRNA genes	14				
tRNA	107				

Table 1. General genome features of *Bacillus bombysepticus JAB01*

According to the results of the phylogenomic tree (Figure 2.) JAB01 is positioned in the same clade as *Bacillus bombysepticus* Wang indicating high similarity between the two strains, this result was confirmed by the ANI test, in which the average

nucleotide identity between the two strains was 99.04% and therefore we confirm that JAB01 is a *Bacillus bombysepticus*. It was also possible to infer that JAB01 has similarity with strains of *Bacillus thuringiensis* and *Bacillus cereus*.

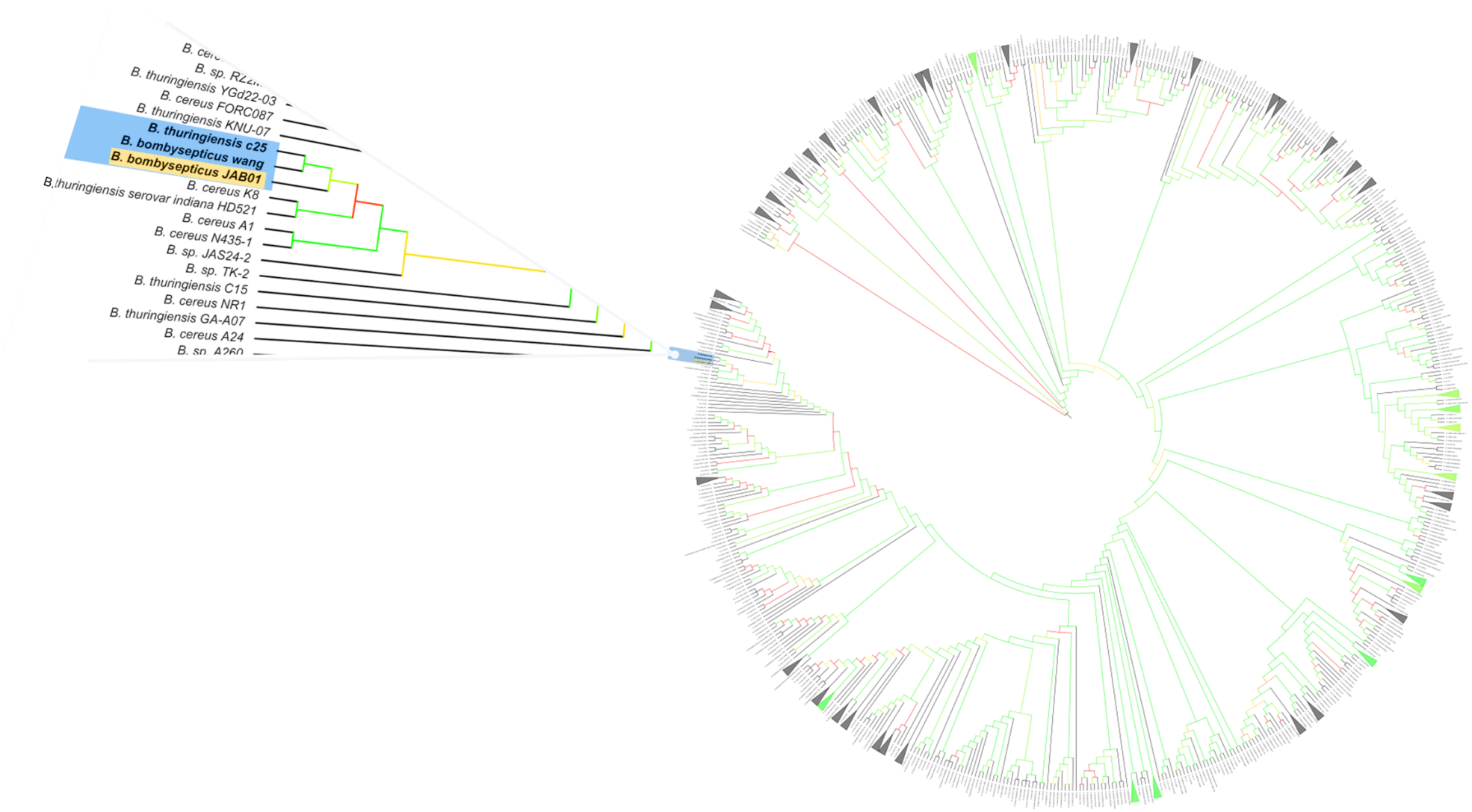

Figure 2. Phylogenomic tree

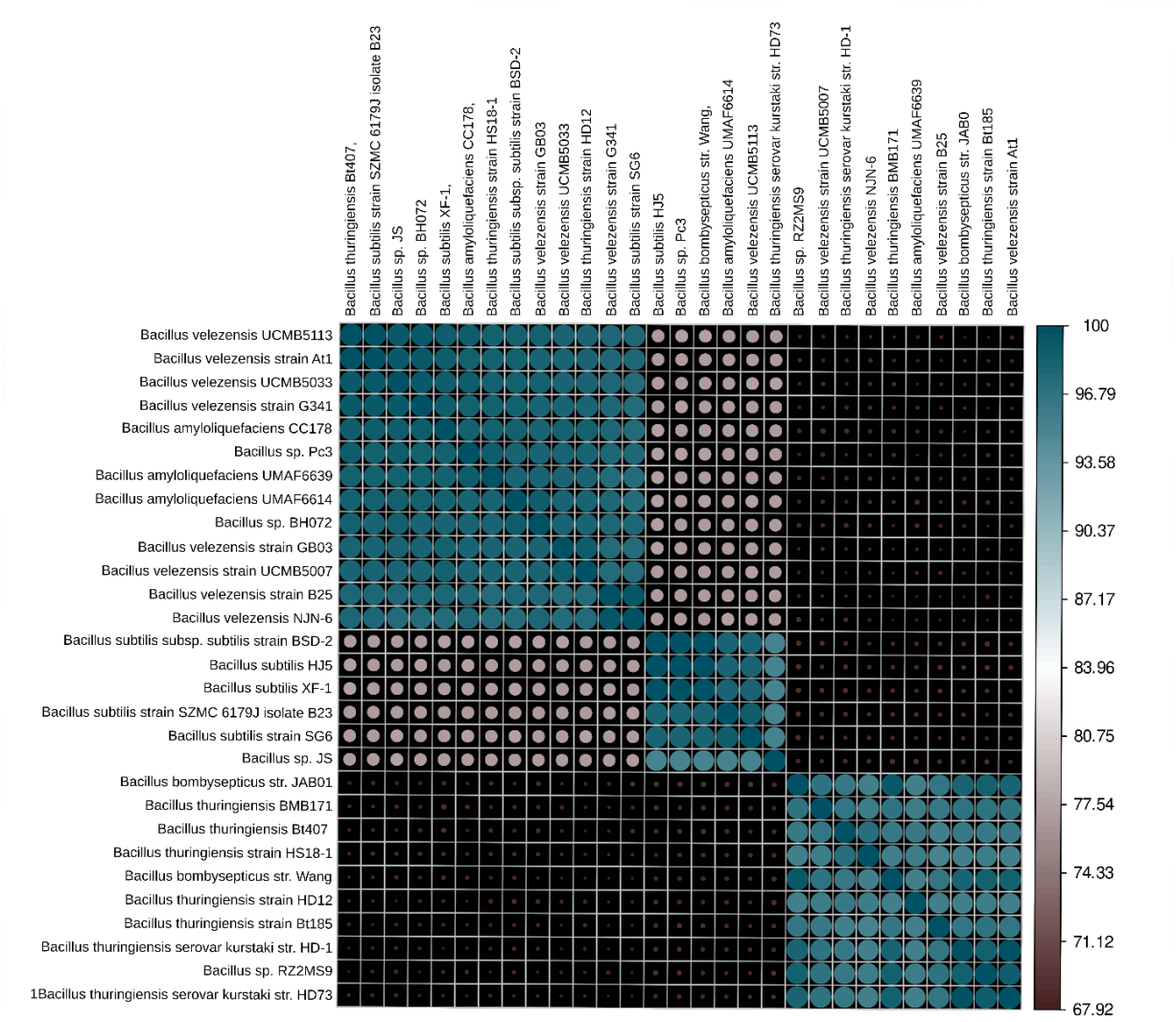

Figure 3. Heatmap ANI

The genome has 4859 genes annotated by COG database, among all COG categories the ones with a larger proportion of genes are: function unknown with 1407 genes, amino acid transport and metabolism 485 genes, transcription with 471 genes, Inorganic ion transport and metabolism with 319 genes and carbohydrate transport with 295 genes. Besides that, JAB01 has 104 genes related to secondary metabolites biosynthesis, transport and catabolism metabolism and 152 to defense mechanism (Figure 4). And the categorie that had the lowest number of genes is the one related to chromatin dynamics and structure.

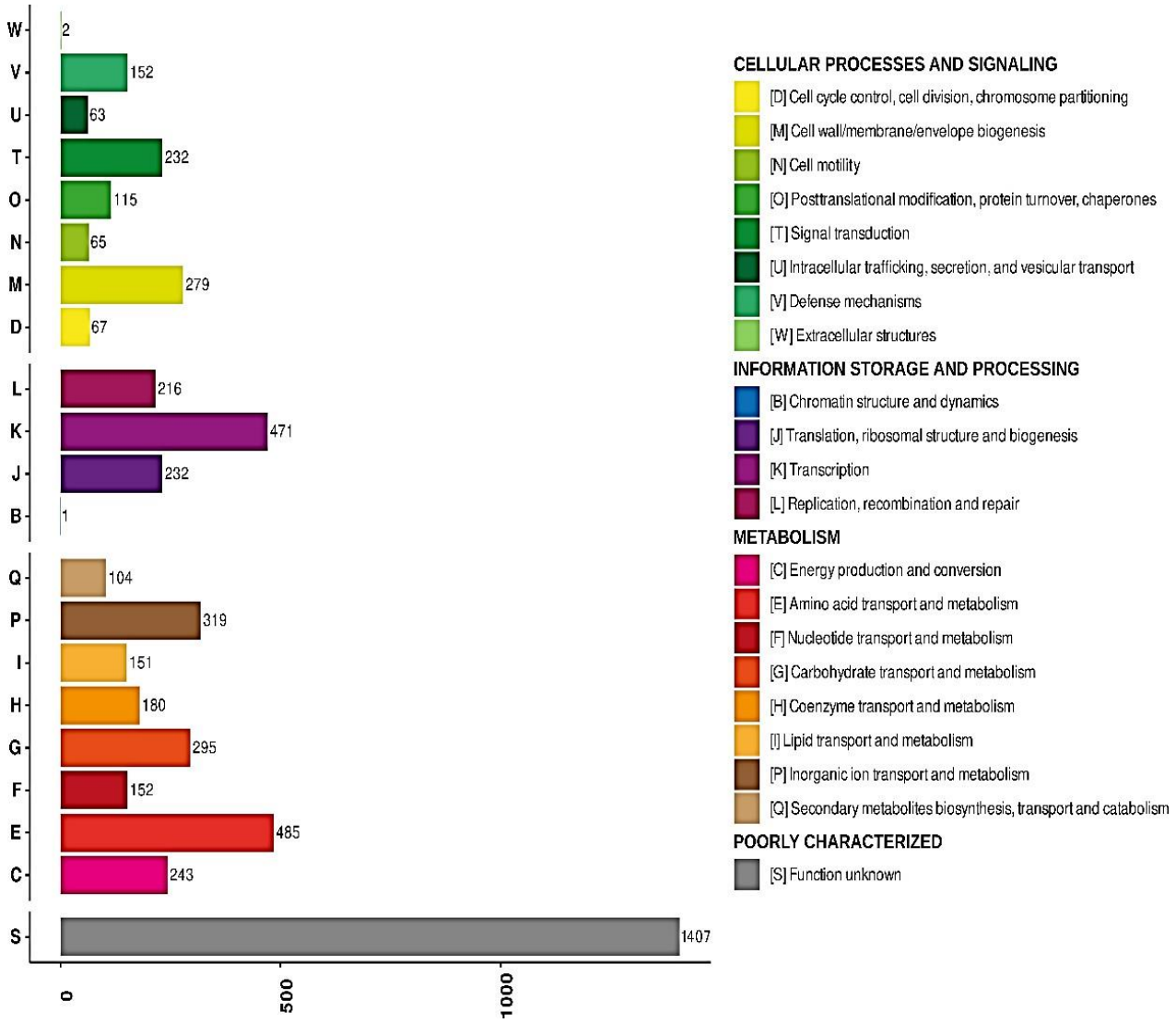

Figure 4. Functional annotation of *B. bombysepticus* JAB01 genes according to the COG database.

The annotation of the KEGG database identified 3436 genes with Metabolism functions, 467 linked to Environmental Information Processing, 302 to Cellular proccess, 273 to Genetic Information Processing, 237 to Human disease and 101 to Organismal Systems 101. metabolic maps were those related to amino acid and carbohydrate metabolism, 416 and 401 respectively (Figure 5).

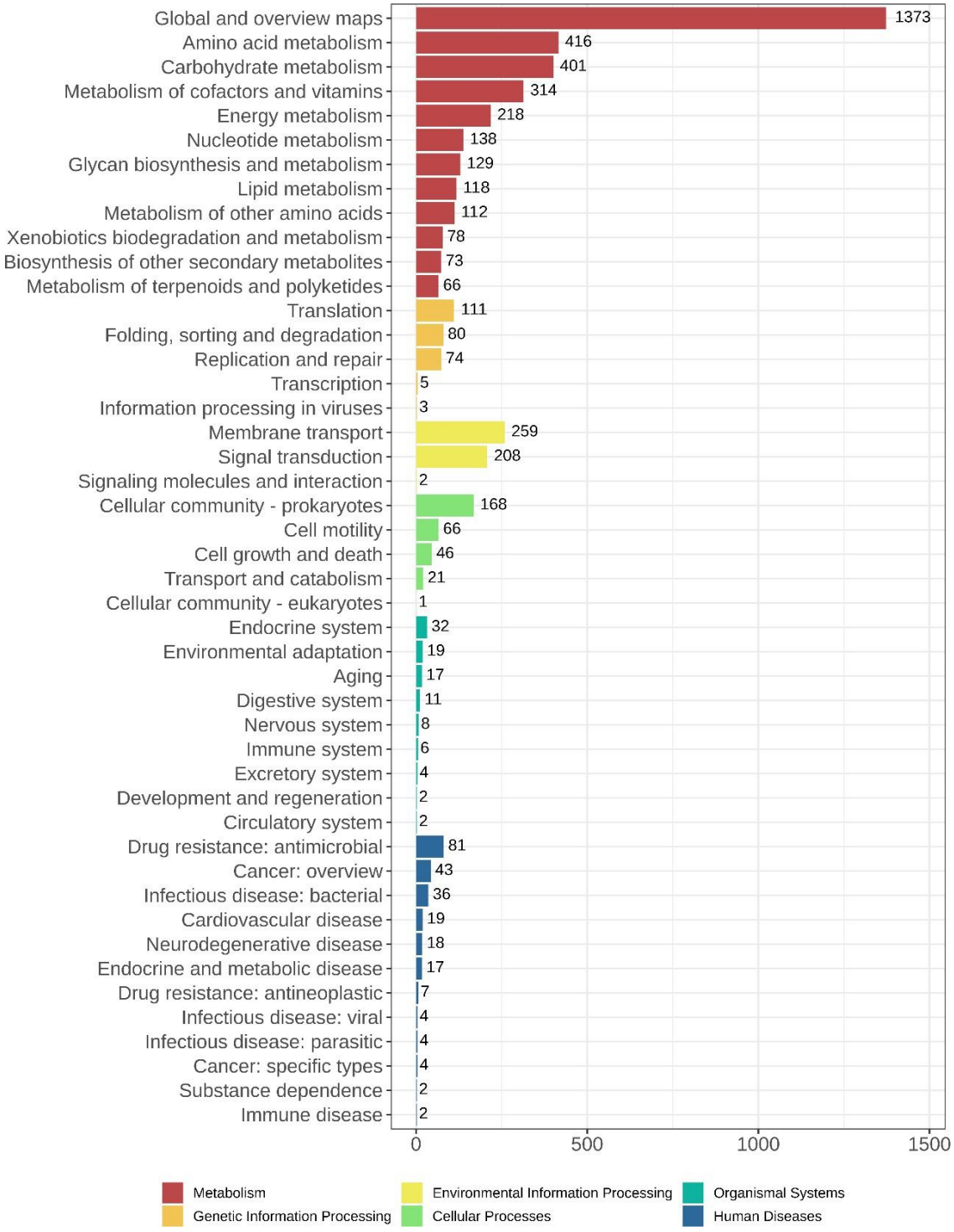

Figure 5. Genome annotation of *Bacillus bombysepticus* JAB01 genes by KEEG

The search for predicted proteins in the JAB01 genome for CAZymes showed that JAB01 has 42 glycosyltransferases enzymes (GTs), 31 glycoside hydrolases enzymes (GHs), 18 carbohydrate esterase (CEs), 6 auxiliary activities (AAs), one polysaccharide lyases enzymes (PLs) and 10 carbohydrate-binding modules proteins (CBMs), which play an important role in enhancing enzyme-substrate binding. Most CAZyme proteins predicated in the JAB01 genome (25%) have amino-terminal with a signal peptide that allows the enzyme to be exported across the membrane, indicating that they are secreting enzymes.

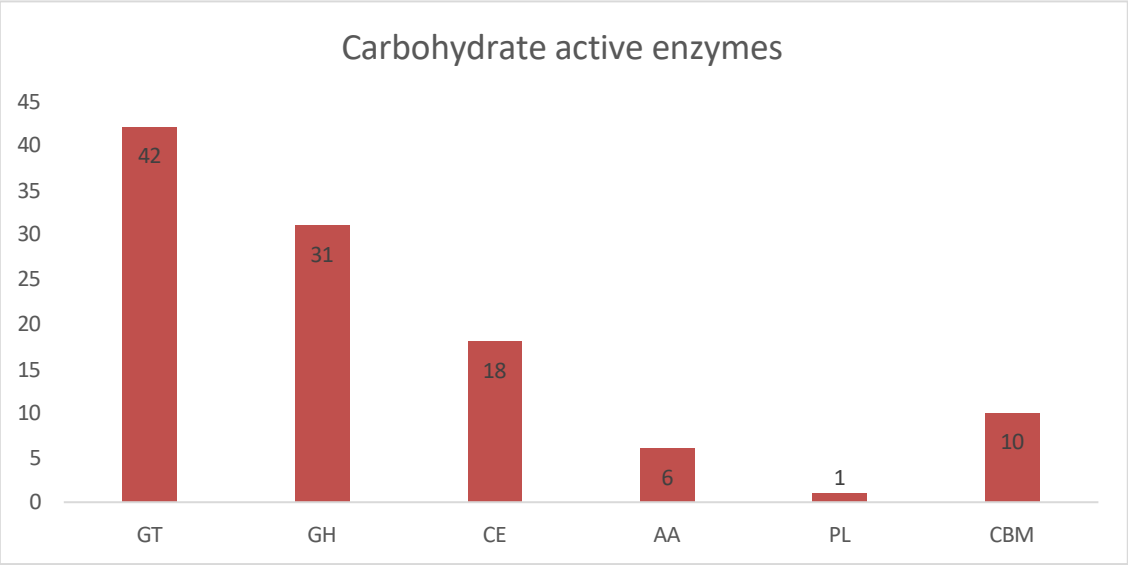

Figure 6. Enzyme classes of carbohydrates active enzymes predicted in JAB01

By mining the Bb genome JAB01, using antiSMASH software version 7.0, 11 groups of secondary metabolites were identified, 3 encoding NRPS, 1 NRPS like, 1 LAP, 3 RiPP like, 1 siderophore, 1 betalactone, 1 terpene, and 1 lanthipeptide (Table 2). These biosynthetic clusters revealed 100% similarity to Petrobactin type siderophore, 40% to betalactone type fengycin, 85% to NRPS type Bacillibactin, 17% to terpene type Molybdenum cofactor, and 94% to lanthipeptide Cerecidin in antiSMASH database and comprised core biosynthetic, additional biosynthetic, transport-related, regulatory, and other genes.

Region	Type	From	To	Most similar known cluster		Similarity (%)
1.1	NRPS like	401,414	443,632			
1.2	Lap Ripp like	1,223,034	1,246,540			
1.3	siderophore	1,836,419	1,850,126	Petrobactin	Other	100
1.4	NRPS	2,165,177	2,210,793	Bacillibactin	NRP	85
1.5	NRPS	2,301,725	2,366,593			
1.6	betalactone	2,383,737	2,408,975	Fengycin	NRP	40
1.7	RiPP like	2,460,640	2,470,110			
1.8	RiPP like	2,532,634	2,542,882			
1.9	NRPS	2,557,607	2,604,623			
1.1	terpene	3,372,733	3,394,586	Molybdenum cofactor	Other	17
1.11	lanthipeptide class ii	5,002,096	5,025,254	Cerecidin	RiPP/Lanthipeptide	94

Table 2. Predicted biosynthetic clusters of genes for secondary metabolites in *B. bombysepticus* JAB01 antiSMASH version 7.0

According to the PGPT annotation, JAB01 has genes related to direct plant growth promotion effects such as biofertilization, bioremediation and phytohormone plant signal production (Figure 5). Among the genes related to biofertilization, the most abundant are the genes related to phosphate solubilization, potassium solubilization and iron acquisition, with 219, 183 and 145 genes respectively. Regarding the genes related to Bioremediation, heavy metal detoxification has the highest number of genes (281) and regarding the production of phytohormones, plant vitamin production is the biggest one with 245 genes.

In relation to the class of indirect effects, JAB01 has genes related to colonizing plant system, competitive exclusion, stress control biocontrol and plant immune response stimulation. Specifically, Bb JAB01 has 1137 genes associated with stress control – biocontrol. Within this set, there are genes related to neutralizing biotic stress, neutralizing abiotic stress and universal stress response. The genes involved in neutralizing biotic stress have effects related to bactericidal compounds antibiotics, fungicidal compounds antibiotics, biotic stress resistance volatiles, biotic stress resistance phenazine derivatives and insecticidal compounds, being the first one the most numerous. Within the gene pool associated with neutralizing abiotic stress, the most numerous category is associated with neutralizing salinity stress. The second

most numerous category is associated with neutralizing nitrosative oxidative stress and ROS (Reactive Oxygen Species) scavenging. In addition to salinity stress and nitrosative oxidative stress, JAB01 also possesses genes related to neutralizing osmotic stress, herbicide stress, high temperature tolerance, low temperature tolerance, and neutralizing acidic stress.

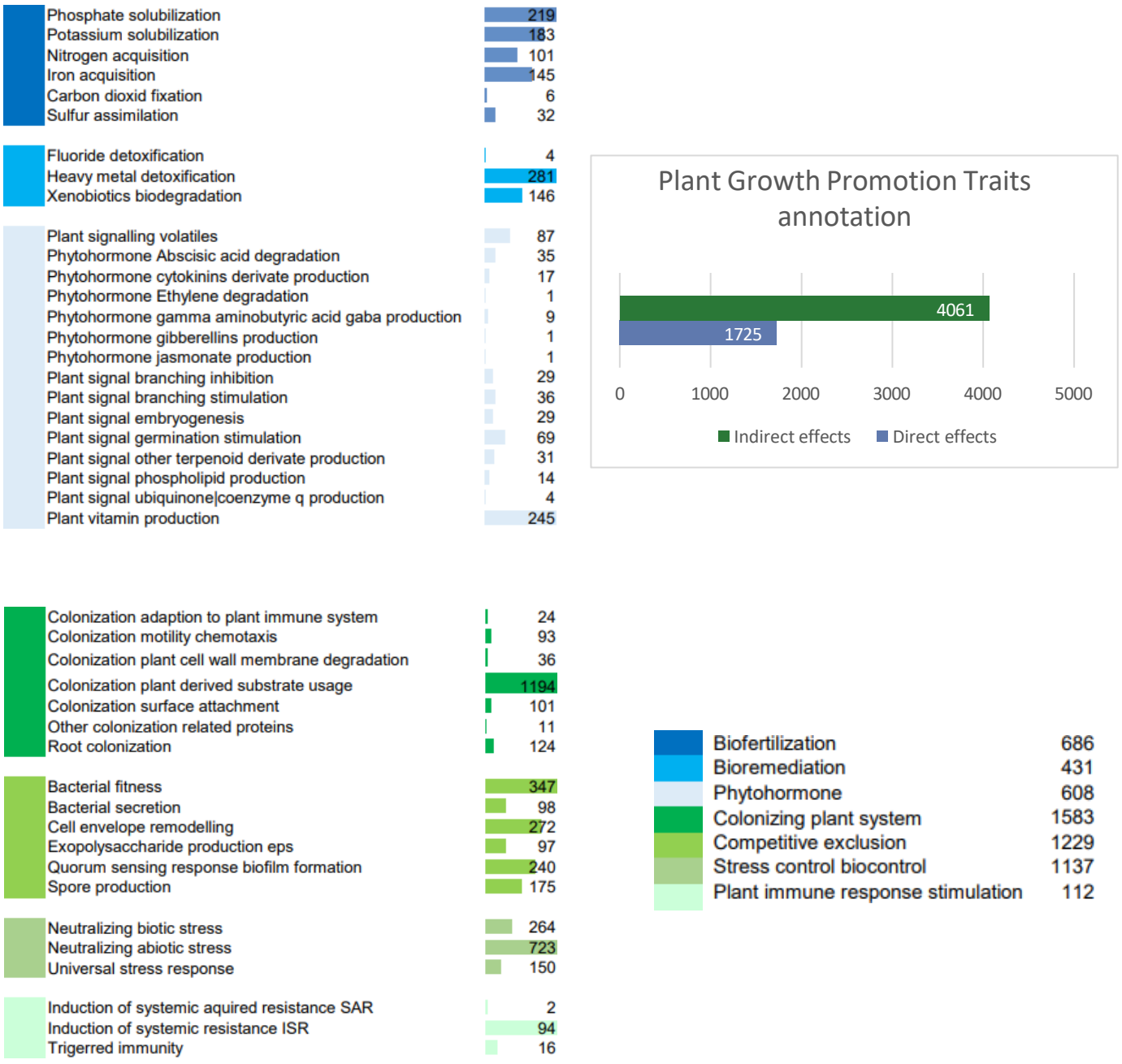

Figure 7. Plant growth promotion traits annotation of JAB01 genes

According to the enrichment analysis, the genes enriched in the JAB01 genome are those related to the following functions: K solubilization organic acid metabolism and P solubilization organic acid metabolism in the category of biofertilization, heavy metal zink resistance in the bioremediation category and plant signal volatile related metabolism in the phytohormone production category, all of these are related to direct effects. As for indirect effects, enrichment was observed in the categories of colonizing plant system and Stress control and biocontrol, with genes related to plant derived carbohydrate transport, plant derived nucleoside metabolism, plant derived organic acid utilization, biotic stress resistance volatiles, neutralizing osmotic stress, high temperature tolerance, and stress signaling proteins. No enrichment was found on the categories of plant immune response stimulation and competitive exclusion

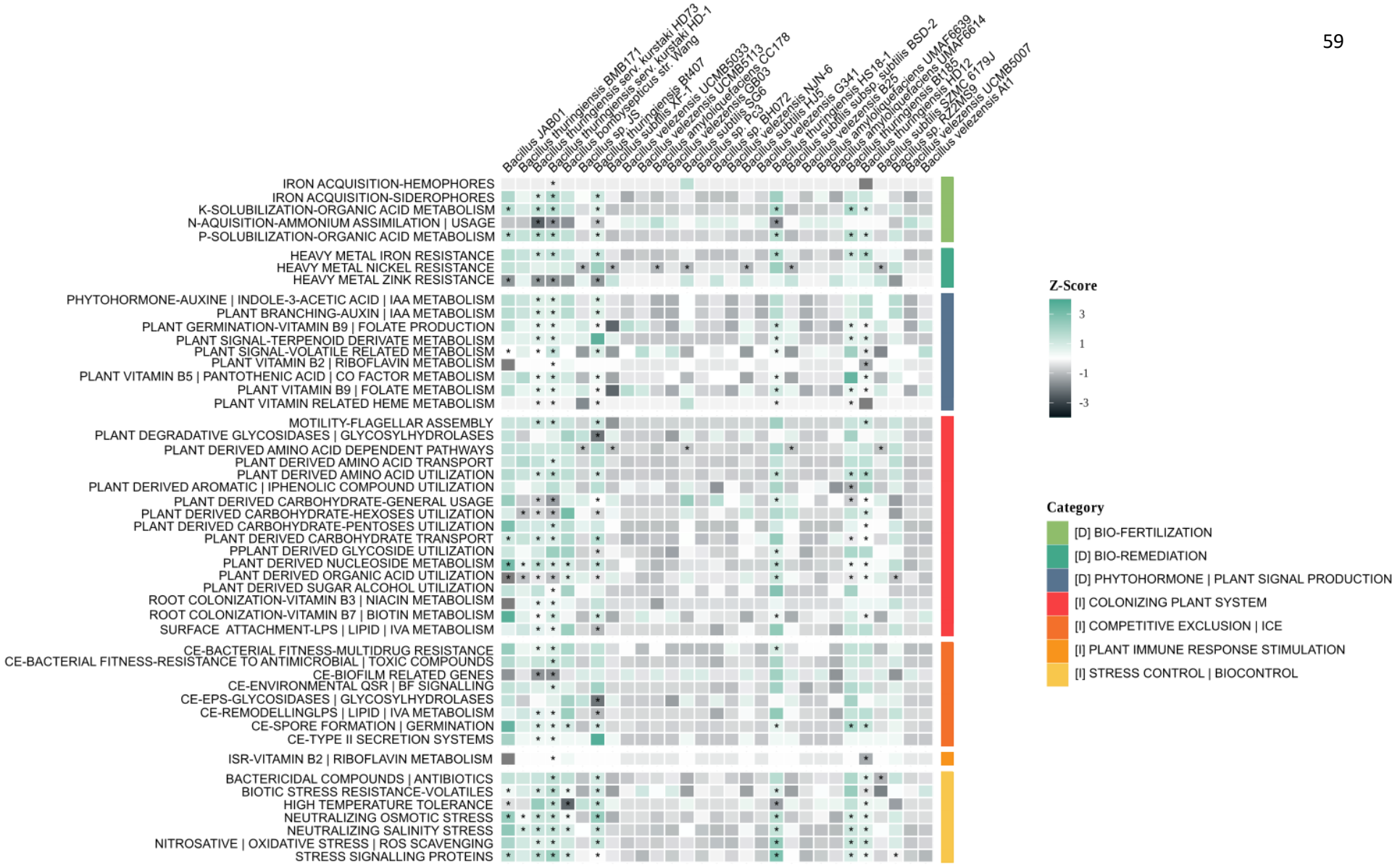

Figure 6. Enrichment analysis of PGPT annotation of twenty nine *Bacillus* strains

4. Discussion:

The bacterial isolate JAB01 isolated from mimous plant soil showed growth inhibition of *Sclerotinia sclerotiorum* and was identified as *Bacillus spp.* JAB01 based on 16s DNA (chapter 2 article). In the present study, insilico analyzes were carried out, in reference databases, aiming at the taxonomic identification of JAB01 at the species level. In addition, it also involved the search for genes related to biocontrol and plant growth promotion.

The isolate was identified as *Bacillus bombysepticus* JAB01 based on whole genome sequencing and phylogenomic analysis. To date, there are no reports of *B. bombysepticus* antagonizing *S. sclerotiorum*.

The complete genome of JAB01 revealed 6092194 bp size with plasmids, larger size when compared to a genome of the same species, *Bacillus bombysepticus* Wang, that has 5873592 bp. Larger genomes usually have greater genetic diversity since they have a greater capacity to store a variety of genes, including those involved in different metabolic pathways and biological functions (Lynch et al., 2001). And this can result in a greater ability to adapt to different environments and conditions.

Higher numbers of genes from the COG and KEGG annotations were associated with amino acid and carbohydrate metabolism. This fact leads us to believe in the adaptive capacity of JAB01 since it suggests that JAB01 has the genetic potential to efficiently utilize and adapt to different sources of amino acids and carbohydrates in diverse environments. In addition, JAB01 genome has genes responsible for the synthesis of carbohydrate active enzymes.

These results corroborate that JAB01 has the enzymatic machinery to efficiently utilize and process various carbohydrates. Among them there are genes encoding for possible antifungal and antibacterial CAZymes, such as chitinase (GH18), glucosidase (GH4), endoglucanase (GH5), lysozyme (GH23,73), which also have the potential to inhibit plant pathogen. The defense related activities of these enzymes have been proven against various phytopathogens due to it´s ability to degrade pathogen cell walls or interfere with pathogen growth and colonization (Myo et al., 2019; Zhou et al., 2021; Huang et al., 2022)

Several gene clusters related to secondary metabolism were revealed in the genome of *Bacillus bombysepticus JAB01*. In total, we estimate that *B. bombysepticus* JAB01 dedicates 3,3 kb to the synthesis of secondary metabolites. These findings indicate that strain JAB01 has genes related to the production of bioactive compounds and it may produce bacteriostatic and fungistatic compounds which can be advantageous in competitive environments to counteract other microrganisms since they are an important molecular source for antagonism and plant-growth promotion.

In the literature, several works related the biocontrol activity of *Bacillus* bacteria against phytopathogenic fungus to the production of secondary metabolites such as Fengycin (Liu et al. 2011), Surfactin (Gao et al., 2017) and Iturin (Zhang et al., 2022). Our genome did not contain the Surfactin and Iturin gene clusters, unlike closely related strains. Although it has some genes from the cluster responsible for Fengycin synthesis, biosynthetic genes for the fenA-E cluster were absent. Such incomplete fengycin cluster is also found in *Bacillus bombysepticus Wang, Bacillus thuringiensis HD1, Bacillus thuringiensis HD73, Bacillus thuringiensis BMB171 e Bacillus thuringiensis 407*.

The products of the Petrobactin and Bacillibactin gene cluster, may act as siderophores, that are low molecular weight molecules capable of make estable bonds with iron (Carrol e Moore 2018) and therefore enhance the ability of Bb JAB01 to obtain iron from the rhizosphere or other enviroments. This action can result into competition advantages since it can deprive bacterial or fungal competitors from essential iron. Moreover, when siderophore-producing bacteria are associated with plants, they can promote plant growth by supplying iron.

Based on a comparative genomic study with related species, the cluster (11) encoding Lanthipeptide Cerecidin was uniquely identified in the Bb JAB01 genome. The lanthibiotic Cerecidin from *Bacillus cereus* strain As11846 presented bactericidal activity against a broad spectrum of Gram-positive bacteria and showed eficcacy against multidrug resistant pathogens like Staphylococcus aureus and others (Wang et al., 2004). So this compound might be useful against bacterial phytopathogens and to human health.

We found three clusters (3, 8, 9) encoding terpene and T3PKs with no previously reported description in the antiSMASH database (Fig. 7), so this could lead to the production of new bioactive compounds.

The results of PGPT annotation suggests that JAB01 has genetic mechanisms to establish beneficial interactions with plants, protecting them from stressors, and enhancing their defense mechanisms, to mitigate the toxic effects of heavy metals in the environment and to combat both biotic and abiotic stresses such as changes in osmotic pressure, exposure to herbicides and extreme temperatures.

P and K are among the most important nutrients for plant growth and development, as they affect key metabolic processes and limitate crop yields (Bagyalakshmi et al., 2017; Gupta et al., 2018). These macronutrients are present in the soil in inorganic and organic form and only a part is avaible to plants (Peix et al., 2001). The nutritional requirements of plants have been achieved by chemical fertilizers, however these increase the production cost and affect the environment (Kour et al., 2021).

The enrichment of genes related to the metabolism of organic acids suggests that *Bacillus* JAB01 can solubilize phosphorus and potassium, this is a process that helps convert insoluble forms of nutrients into soluble forms, thus facilitating their availability and absorption by plants and therefore could reduce the demand for phosphate and phosphorous fertilizers and consequently promote the preservation of natural resources. *Bacillus* spp. are directly related to nutrient uptake and the subsequent growth promotion in different plants (Shahzad et al., 2017; Park et al., 2017; Bahadir et al., 2018).

Acording to Nosalova et al. (2023) the exposure to environmental pollutants like heavy metals affects the native soil and water microbiota, since they have toxic effects, influencing key processes and changing the community composition. Zinc is an essential metal to human, plants and microrganisms metabolism, however in high concentration is toxic. The enrichment of zinc resistance genes suggests that *Bacillus* JAB01 has mechanisms to resist and tolerate high zinc concentrations, like genes that encode for specific proteins involved in zinc detoxification. These could give competitive advantages to our bacteria like, access to zinc resources that would be

inaccessible to other organisms due to toxicity. Additionaly *Bacillus* JAB01 could be utilized in bioremediation processes to clean up zinc-contaminated environments. However, the presence of metal resistance genes must be evaluated in relation to the potential risks associated with the spread of antibiotic resistance, since in some cases there is a correlation between these characteristics (Komijani et al., 2021).

According to Xiong et al. (2022), climate change has the potential to threaten food security through its adverse effects on soil properties, which can alter nutrients and their bioavailability in soils. In addition to the fact that higher temperatures influence production stability and are a major environmental factor that has a large impact on plants and their interactions with pathogens (Hua and Dong 2022).

With global climate change, the challenge that most plant breeding programs face in the 21st century is to increase crop production by obtaining climate-resilient crops like cultivars adapted to extreme temperature conditions (Kakoulidou et al., 2021). Given that context, the enrichment of genes related to high temperature tolerance and stress signaling proteins may contribute to increased tolerance to adverse conditions and give the ability to *Bacillus bombysepticus* JAB01 to withstand and thrive under elevated temperature conditions. Thus, *Bacillus bombysepticus* JAB01 may be a source of interest genes to breeders.

The diversity of genes, related to carbohydrate and amino acid metabolism, secondary metabolite biosynthesis and growth plant traits, in the JAB01 genome reinforce the hypothesis that our bacteria has the potential to produce numerous metabolites that can improve the genetic adaptation to the various environment, in addition to increasing nutrient availability to plants and therefore promote plant growth and protection against phytopathogens.

However, further experimental studies would be necessary to confirm the production and specific bioactivities of these metabolites, as well as to explore their potential applications in biocontrol and plant growth promotion.

In addition, it has genes related to the production of enzymes and compounds of interest that go beyond biocontrol and that can be isolated and used in other areas. Therefore, it has great biotechnological potential.

5. Conclusion

This study highlighted that *Bacillus bombysepticus* JAB01 has the potential of producing vegetable hormones like IAA, iron-chelating compounds like siderophores, solubilization compounds, antimicrobial compounds and lytics enzymes like chitinase and celulase, since it has genes responsible for the production of them. Therefore, we present it as a possible viable environmental solution to control *S. sclerotiorum* and other phytopathogens.

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CAPÍTULO IV – Considerações finais

O desenvolvimento de práticas de controle de fitopatógenos, sustentáveis e eficazes pode reduzir a dependência de produtos químicos, promovendo cultivos mais seguros além de reduzir os custos de produção. O estímulo do crescimento das plantas pode levar a culturas mais saudáveis e produtivas, o que é essencial para a agricultura sustentável e a segurança alimentar.

Bacillus bombysepticus JAB01 é uma bactéria que foi prospectada no solo de plantas de mimosa e demonstrou ser eficiente tanto na inibição do crescimento micelial quanto na germinação miceliogênica de escleródios de *Sclerotinia sclerotiorum* e na proteção de sementes e folhas destacadas de soja em condições *in vitro.* As perspectivas futuras são ensaios em condições de campo para avaliar se *B. bombysepticus* JAB01 consegue proteger plantas hospedeiras, da doença causada pelo fitopatógeno.

Utilizando recursos genômicos, o trabalho reforça o potencial de *Bacillus bombysepticus* JAB01 como agente de controle biológico do mofo branco e fonte de genes relacionados a promoção do crescimento vegetal. Dentre eles, destacam-se genes associados à biofertilização e tolerância a condições adversas como altas temperaturas. A identificação de genes relacionados à produção de metabólitos secundários com atividade antimicrobiana, sideróforos, enzimas hidrolíticas, solubilização de macronutrientes, fitohormônios e compostos orgânicos voláteis abre oportunidades para o desenvolvimento de produtos biotecnológicos avançados na agricultura. Isso pode levar ao desenvolvimento de novos produtos comerciais.

Portanto, esta pesquisa poderá servir como base para futuras investigações da interação de *Bacillus bombysepticus* e *Sclerotinia sclerotiorum*, bem como na área de controle biológico e promoção do crescimento vegetal.