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Seleção de espécies de *Bacillus* para o controle biológico do oídio da videira

John Lennon Ferreira dos Santos

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COMITÊ DE ORIENTAÇÃO:

Orientador(a): Prof. D. Sc. Delson Laranjeira (UFRPE)

Coorientador(a): D. Sc. Carlos Alberto Tuão Gava (EMBRAPA)

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Sumário

RESUMO GERAL

O oídio causado por *Erysiphe* (*Uncinula*) *necator (Schw.) Burr*. é uma das principais doenças da videira (*Vitis vinifera* L.) e seu controle é baseado principalmente no uso de fungicidas sintéticos. No entanto, estratégias de controle ecologicamente amigáveis de baixo risco de contaminação do alimento, como os agentes de biocontrole, têm sido empregados. Os objetivos deste trabalho foram: 1. Avaliar e identificar isolados de *Bacillus* antagonistas a *E. necator*; 2. Caracterizar a adaptação a condições de estresse ambiental e nutricional de isolados antagonistas; 3. Caracterizar os mecanismos de ação de isolados de *Bacillus* antagonistas a *E. necator*; 4. Avaliar a aplicação de isolados antagonistas no manejo integrado do oídio da videira. Em um screening inicial utilizando 45 isolados de *Bacillus* spp. da Coleção de Microrganismos da Embrapa Semiárido, seis isolados mostraram uma redução dos sintomas da doença maior que 70,0% em folhas destacadas. Dois experimentos em casa de vegetação mostraram que, quando aplicadas semanalmente, os isolados LCB03, LCB28 e LCB30 apresentaram eficiência de controle superior a 80%. O sequenciamento de 16s rDNA mostrou que LCB03 apresentou 100,0% de homologia com *B. velezensis*, LCB28 99,93% com *B. tequillensis* e LCB30 99,4% com *B. siamensis*. Os isolados apresentaram pequenas diferenças quanto à exigência de fontes de carbono, faixa de pH ideal, concentrações de glicose e diferentes fontes de nitrogênio para seu crescimento em meio de cultura e metabolizaram nitrato e amônio como fontes de nitrogênio. Em um ensaio in vitro, LCB28 e LCB30 inibiram significativamente a germinação de conídios em co-cultivo e pela produção de compostos orgânicos voláteis. O potencial para sintetizar ciclopeptídeo antifúngico foi confirmado pela detecção baseada em PCR de marcadores gênicos para a produção de surfactina, bacilomicina, fengicina e bacilisina no genoma LCB28, e os marcadores para todos esses compostos e iturina no genoma LCB30. A aplicação alternada dos fungicidas triflumizol e difenaconazol com os isolados LCB28 e LCB30 em casa de vegetação reduziram a incidência do oídio em mais de 70% e a severidade em mais de 90% em média. Dois experimentos de campo mostraram que a incidência e a severidade do oídio foram significativamente reduzidas pela aplicação de formulações contendo LCB28 ou LCB30 em uma estratégia de manejo integrado que substituiu a pulverização de fungicidas sintéticos, exceto durante a floração e o crescimento inicial dos frutos, que são fases fenológicas altamente favoráveis. Os resultados dos experimentos indicam que LCB28 e LCB30 têm grande potencial para serem aplicados em programas de manejo integrado do oídio em videira.

Palavras-chaves: *Vitis viniferas* L.; Patógeno biotrófico; Mecanismo de ação; Biocontrole.

GENERAL ABSTRACT

Powdery mildew caused by Erysiphe (Uncinula) necator (Schw.) Burr. is one of the primary diseases of the grapevine (Vitis vinifera L.), and its control is mainly based on the use of synthetic fungicides. However, ecologically friendly control strategies with low risk of food contamination, such as biocontrol agents, have been employed. The objectives of this work were: 1. To evaluate and identify Bacillus isolates antagonistic to E. necator; 2. Characterize the adaptation of the antagonist isolates' to environmental and nutritional stress conditions; 3. Characterize the mechanisms of action of Bacillus isolates antagonistic to E. necator; 4. Evaluate the application of antagonist isolates in the integrated management of grape powdery mildew. In an initial screening using 45 Bacillus spp. from the Embrapa Semi-Arid Microorganism Collection, six isolates showed a reduction in disease symptoms greater than 70.0% on detached leaves. Two experiments in a greenhouse demonstrated that, when applied weekly, the isolates LCB03, LCB28, and LCB30 showed control efficiency greater than 80%. Sequencing of 16s rDNA showed that LCB03 showed 100.0% homology with B. velezensis, LCB28 99.93% with B. tequillensis, and LCB30 99.4% with B. siamensis. The isolates showed minor differences in the requirement of carbon sources, ideal pH range, glucose concentrations, and different nitrogen sources for their growth in culture medium and metabolized nitrate and ammonium as nitrogen sources. In an in vitro assay, LCB28 and LCB30 significantly inhibited conidial germination in co-culture and by producing volatile organic compounds. The potential to synthesize antifungal cyclopeptide was confirmed by PCR-based detection of gene markers for surfactin, bacillomycin, fengycin, and bacillysin production in the LCB28 genome, and the markers for all these compounds plus iturin in the LCB30 genome. Weekly alternating application of triflumizol and difenoconazole with the isolates LCB28 and LCB30 in a greenhouse reduced the incidence of powdery mildew by more than 70% and the severity by more than 90% on average in a greenhouse experiment. Two field experiments showed that powdery mildew incidence and severity were significantly reduced by applying formulations containing LCB28 or LCB30 in an integrated management strategy that replaced the concentration of synthetic fungicides, except during concentration and initial fruit growth, which are highly susceptible phases. The results of the experiments indicate that LCB28 and LCB30 have great potential to be applied in integrated powdery mildew management programs in grapevines.

Key-words: *Vitis viniferas* L.; *B*iotrophic pathogen; Mechanism of action; Biocontrol.

CAPÍTULO I

INTRODUÇÃO GERAL

Prospecção de bactérias antagonistas para o controle biológico de oídio (*Erysiphe necator* Schwin) em videiras no semiárido brasileiro

INTRODUÇÃO GERAL

1. Aspectos gerais da cultura da videira

A videira pertence à classe Magnoliopsida, ordem Vitales, família *Vitaceae*, gênero *Vitis* e apresenta mais de mil espécies, sendo o gênero *Vitis* o único de importância econômica com cerca de 108 espécies adaptadas a diferentes tipos de solo e clima, podendo ser cultivada em áreas tropicais, subtropicais e temperadas (INGLEZ DE SOUZA; MARTINS, 2002).

A videira (*Vitis vinifera* L.) apresenta-se como uma das mais importantes espécies vegetais do mundo, sendo cultivada a milhares de anos com relatos ligados aos vários tipos de atividades agrícolas e religiosas. Esta cultura te como centro de origem a Groelândia em função dos fósseis encontrados de seus ancestrais, entretanto, o seu cultivo foi domesticado nas regiões entre o mar Negro e o Irã, por volta de 7000 – 4000 anos a.C., e foi difundida para Europa Meridional, Oriente Médio e Europa Central (TERRAL et al., 2010).

No Brasil, a videira foi introduzida no período da colonização pelos portugueses em 1532, sendo implantada na Capitania de São Vicente, atual estado de São Paulo (IBRAVIN, 2010), e posteriormente distribuída para as demais regiões do país. Na região nordeste por volta do século XVI já se havia registro de seu cultivo no litoral do estado de Pernambuco e Bahia nas ilhas de Itaparica, BA e Itamaracá, PE (LEÃO; POSSÍDIO, 2000). Posteriormente, seu cultivo foi estendido para o interior desses estados, chegando as regiões de clima semiárido como o Submédio do Vale do São Francisco.

No Submédio do Vale do São Francisco, a produção comercial de videira teve seu início na década de 50, com apoio da Comissão de Desenvolvimento do Vale do São Francisco (CDVA), atual (CODVASF), que fomentou o emprego de tecnologias modernas de produção como a irrigação automatizada, poda racional, desbastes dos cachos, emprego dos fertilizantes, variedades adaptadas e o controle fitossanitário das doenças e pragas da videira, garantido a produtividade elevada dos parreiras instalados na região (SILVA; CORREIA; SOARES, 2009).

Atualmente a viticultura se apresenta como uma das principais atividades agrícolas da agricultura irrigada no Nordeste brasileiro. Nesta região, a produção de uva se concentra principalmente no polo de fruticultura irrigada em Petrolina (PE) e em Juazeiro (BA), situada

na mesorregião do Submédio do Vale do São Francisco. Graças às condições edafoclimáticas como luminosidade, temperatura em conjunto com tecnologias de alta performance aplicada nos vinhedos, a produção da fruta ocorre durante todo o ano, colocando o polo Petrolina (PE) e Juazeiro (BA) como maior produtor nacional de uvas finas, (ALBUQUERQUE et al., 2013; LEÃO; BRANDÃO; GONÇALVES, 2011). A uva se destaca como a terceira fruta mais exportada pelo Brasil, sendo o Submédio do Vale do São Francisco responsável por 99% do total da fruta exportada pelo país (COMEXTAT, 2021).

A viticultura apresenta grande importância econômica no agronegócio brasileiro, sendo que em 2021 a área plantada com essa cultura foi em torno de 75.622 ha, com produção de 1.748.197 t e rendimento de 23.118 kg/ha (FAO, 2021; IBGE, 2022). Neste mesmo ano, o Brasil ocupou a 13º posição na produção mundial da fruta, sendo a China, Itália e Espanha os maiores produtores mundiais (FAO, 2021).

A videira é cultivada em todas as regiões do país, entretanto, duas regiões distintas merecem destaque, a região Sul como a maior produtora de uvas, com 56,37% da produção nacional em 2022 e, a região Nordeste segunda maior produtora, com 30,80% da produção de uva do país. Dentre os estados produtores, o Rio Grande do Sul apresenta-se como principal produtor, com 734.970 t, acompanhado por Pernambuco com 399.132 t e Bahia com 60.804 t de uva (IBGE, 2022).

No Submédio do Vale do São Francisco a cadeia produtiva da videira apresenta relevante papel social para região, com geração de cinco empregos diretos por hectare plantada e, somente no ano de 2018 junto com a manga foram responsáveis pela geração de 100 mil empregos (LEÃO, 2021). Logo, a cadeia produtiva da uva vem sendo constantemente transformada com o emprego de técnicas de melhoramento genético aliado a tecnologias de produção modernas (irrigação, poda, desbastes dos cachos, nutrição, reguladores de crescimento, controle fitossanitário das doenças e pragas) tem possibilitado a introdução de novas cultivares de videira mais adaptadas ao semiárido, com produtividade elevadas e estáveis.

Atualmente no Submédio do Vale do São Francisco é cultivado um número superior a 20 cultivares de uvas de mesa desenvolvidas pela Embrapa e empresas privadas internacionais de melhoramento genético. Entre as cultivares produzidas pela Embrapa, podemos destacar ‘BRS Vitória’ (MAIA et al., 2012), ‘BRS Isis’ (RITSCHEL et al., 2013), ‘BRS Nubia’ (MAIA et al., 2013), ‘BRS Melodia’ (MAIA et al. 2019) e ‘BRS Tainá’ (LEÃO et al. 2020), bem como ‘BRS Magna’ (RITSCHEL et al., 2012). Já as cultivares produzidas por empresas privadas de melhoramento genético se destacam as uvas brancas [Arra 15® (Grapa), Sugar Crisp®, Sweet Globe®, Cotton Candy® (International Fruit Genetics – IFG) e Autumm Crisp® (Sun Word)];

uvas vermelhas [Sweet Celebration®, Candy Snaps® (IFG), Timco® (Sheegene), Scarlotta Seedless® (Sun World)] e uvas negras [Sweet Saphire® (IFG), Sable®, Midnight Beauty® (Sun World)].

No Submédio do Vale do São Francisco, o clima tropical semiárido se caracteriza por apresentar temperatura média anual de 26,5 ºC e insolação de 3.000 horas/ano, onde a videira vegeta continuamente, o que permite o escalonamento da produção para qualquer época do ano (LEÃO; MARQUE; BARROS, 2021). Entretanto, de acordo de com Angelotti et al. (2021), a presença de plantas em distintas fazes fenológica na mesma área favorece a sobrevivência e disseminação do *E. necator* para áreas vizinhas. Logo, a região apresenta um ambiente complexo e de difícil manejo da doença (BUFFARA et al., 2014), que exige o emprego de estratégias inteligentes de controle.

2. Doenças da Videira (Vitis vinifera L.)

Desde o início da agricultura, as doenças se constituem como um dos principais problemas ocasionando perdas na produtividade e na qualidade dos produtos agrícolas. Neste contexto, a videira durante todo seu ciclo está sujeita a uma série de doenças que podem trazer prejuízo ao produtor, sendo que no Brasil a ampla diversidade de locais de produção e clima proporcionam condições favoráveis para o desenvolvimento de uma grande gama de doenças e fitopatógenos que afetam as videiras (ALMANÇA; LERIN; CAVALCANTI, 2015).

Conhecer as doenças que ocorrem nas diferentes regiões produtoras de uva é de extrema importância para que medidas de controle adequadas sejam adotadas. De acordo com Jackson (2008), as doenças podem afetar a fisiologia da planta da videira e influenciar o rendimento e qualidade de seu produto em diferentes graus. No Brasil, as principais doenças da videira são podridão cinzenta (*Botrytis cinerea*), míldio (*Plasmopara vitícola*), oídio (*E. necator*)), doenças de Petri (*Phaeomoniella chlamydospora*), pé-preto da videira (*Cylindrocarpon destructans*), ferrugem (*Phakopsora euvitis*), antracnose (*Elsinoe ampelina*), escoriose (*Phomopsis vitícola*), podridões do cacho (*Melanconium fuligineum e Glomerella cingulata*), cancro-bacteriano (*Xanthomonas citri* pv. *vitícola*), fusariose (*Fusarium oxysporum* f. sp. *herbemontis*), morte-descendente (*Lasiodiplodia* spp., *Neofusicoccum* spp., *Dothiorella* spp. e *Diplodia* spp.) (ALMANÇA; LERIN; CAVALCANTE, 2015; BARBOSA et al., 2016; SÔNEGO; GARRIDO; JUNIOR, 2005).

Em função das condições favoráveis ao desenvolvimento das doenças que ocorrem nas principais regiões produtoras de uva, o míldio e as podridões de cachos têm maior importância na região Sul e Sudeste devido ao alto volume de precipitação, enquanto o oídio e o cancro

bacteriano têm maior incidência no Nordeste, em função do clima seco e temperatura alta (ALMANÇA; LERIN; CAVALCANTI, 2015; SÔNEGO et al., 2005). Ainda segundo Barbosa et al. (2016), dentre as principais doenças da videira, o oídio, encontra-se amplamente distribuído em todas as áreas vitícolas do mundo.

3. Oídio da videira (E. necator Schwin)

O oídio da videira é uma doença causado pelo fungo biotrófico obrigatório *E. necator* (Schwein.) Burrill (Gadoury et al., 2012), pertencem ao reino: Fungi, divisão: Ascomycota, classe: Leotiomycetes, ordem: *Erysifhales*, família: *Erysiphaceae*, gênero: *Erysiphe*. A doença foi relatada pela primeira vez em 1834 por Schweinitz em sua obra "Synopsis fungorum in America boreali media degentium", e tem sua origem na América do Norte como parasita moderado de videiras nativas do gênero *Vitis* L. (CRUZ, 2001). Entretanto, outras hipóteses sobre a origem do patógeno são inferidas em função da existência de videira silvestres em outras áreas do globo como Asia, América Central e noroeste da América do Sul (Colômbia e Venezuela) (GALET; MORTON, 1988).

O patógeno ganhou notoriedade a partir da sua disseminação para áreas viniferas do continente Europeu cujo as variedades pertenciam a espécie *V. vinifera* que é altamente suscetível a infecção causada pelo *E. necator*. O fungo foi registrado primeiramente na Inglaterra em 1845 pelo jardineiro inglês Tucker em videiras contaminadas em Margate, próximo da foz rio Tâmisa (BULIT; LAFON, 1978). A doença foi rapidamente disseminada para outras regiões vitícolas do continente e, em 1950 o oídio da videira já era conhecido em todas as áreas vitivinícolas da França e da Europa por causar grandes prejuízos aos produtores (CRUZ, 2001). No Brasil, o *E. necator* foi relatado pela primeira vez em cultivares de videiras americanas em 1888, e rapidamente disseminado para áreas com as espécies *V. vinifera* que é suscetível a doença.

4. Ciclo de vida E. necator e epidemiologia da doença

O oídio da videira está presente nas diversas regiões produtoras da videira, englobando áreas temperadas, subtropical e tropical. O *E. necator* apresenta duas fazes destintas, a fase teleomórfica (ciclo sexual) e a fase anamórfica (ciclo assexual). O ciclo sexual se caracteriza pela fusão de duas hifas compatíveis que após 24 h dão origem a um ascocarpo esférico, completamente fechado e sem ostíolo, denominado cleistotécios (CRUZ, 2001; GADOURY et al., 1988). Os cleistotécios são formados sobre diferentes partes da planta infectada e fixados ao micélio do oídio por meio de hifas de ancoragem. Eles contem entre quatro a oito ascos na

maturidade e, cada asco de quatro a oito ascósporos hialinos, ovalados a elipsoides (GADOURY et al., 2011; PEARSON, 1988). Em regiões com invernos frios o *E. necator* hiberna na forma de cleistotécio, servindo como fonte de inóculo primário para formação dos chamados brotos bandeiras. De acordo com Cortesi et al. (2004), a doença se multiplica em volta dos brotos bandeiras, pois há uma larga produção de conídios que se dissemina para videiras vizinhas.

A faze anamórfica do oídio ou ciclo assexual se caracteriza pela reprodução vegetativa do *E. necator* e, é composto por um micélio filamentoso hialino, septado que se desenvolve sobre a superfície do órgão parasitado (Cruz, 2001). A relação dos patógenos causadores de oídio com a planta é altamente evoluída e complexa. De acordo com Qiu, Feechan e Dry. (2015), a formação do tubo germinativo durante a germinação conidial se dá quando o conídio entra em contato com tecido vegetal fotossintético suscetível. Posteriormente o tubo primário do conídio se alonga e forma na sua extremidade um apressório multilobado, que se adere à superfície da folha e na sua extremidade inferior da origem a hifa de penetração, órgão responsável pelo rompimento da cutícula e da parede das células epidérmicas (STADNIK; MASSAFERA, 2001). Com base em estudos realizado com *E. necator* a penetração da cutícula e da parede celular epidérmica pela hifa de penetração ocorre por meio da pressão mecânica exercidas pelas hifas e por meio da liberação de enzimas hidrolíticas fúngicas (HEINTZ; BLAICH, 2010; SCHNEE et al., 2013). Uma vez dentro da célula, o ápice da hifa dilata-se ou ramifica-se, formando o haustório globoso, uma estrutura especializada envolta por uma membrana perihaustorial e, é responsável pela absorção de nutrientes do citoplasma da célula e ao mesmo tempo atua na secreção de proteínas efetoras para inibir o sistema de defesa da planta hospedeira (BEDENDO, 2018; QIU; FEECHAN; DRY, 2015). Após estabelecimento do haustório e suprimento continuo do micélio com fotoassimilados, o micélio se espelha na superfície do órgão afetado e produz numerosos haustórios e, entre 5-25 dias da infecção inicial, há a formação dos conidióforos e abundantes cadeias de conídios formados perpendicularmente a célula epidérmica (GADOURY et al., 2012; GAY; BUSHNELL, 1978; JONES et al., 2014).

Nos vinhedos cultivados no Semiárido brasileiro, mais precisamente no Submédio do Vale do São Francisco, o oídio aparece como um dos principais problemas fitossanitários da videira, podendo ocasionar danos na produção que variam de 30% a 80% (CHOUDHURY, 1991). Nessa região a fase anamórfica predomina como principal fonte de inóculo na forma de micélios remanescentes nos ramos e gemas dormentes após a poda. Ainda de acordo com Angelott et al. (2012), a produção escalonada da videira com vinhedos em diferentes estágios favorece a sobrevivência e disseminação constantes do patógeno para áreas vizinhas. Sua

disseminação ocorre por respingos de chuva, mas, principalmente pela ação do vento que desprende os conídios dos conidióforos, transportando-os para áreas distantes (BARBOSA et al., 2016)

O micélio fúngico desenvolve-se sobre todas as partes verdes da planta, causando maiores estragos nos brotos e nos cachos. Os sintomas iniciam-se com manchas cloróticas e evolui para manchas brancas ou acinzentadas, facilmente removíveis, na superfície de folhas sombreadas ou nas bagas jovens. Infecções severas paralisam o crescimento do tecido epidérmico resultando em rachadura das bagas, à medida que os frutos jovens se expandem (GADOURY et al., 2012). Ainda de acordo com os autores, as bagas são suscetíveis num período de 3 a 4 semanas após a antese e posteriormente tornam-se resistente, sendo observadas colônias miceliais difusas, que morrem com o amadurecimento continuo da baga.

Nas inflorescências afetadas podem ocorrer a queda de botões florais, que ficam recobertos pelo crescimento micelial do fungo. Os ramos afetados também podem apresentar manchas na superfície causadas pelo fungo (BARBOSA et al., 2016; NOGUEIRA et al., 2017).

Segundo Amorim et al. (2016), o fungo tem um melhor desenvolvimento em climas secos e frescos com temperaturas entre 20 ºC e 27 ºC, sendo desfavorecido com a ocorrência de chuvas, que promove a retirada da massa micelial do hospedeiro e ocasiona a destruição do fungo. Em regiões semiáridas como o Submédio do Vale do São Francisco, as temperaturas médias situam-se em torne de 27 °C, logo, o *E. necator* tem seu desenvolvimento favorecido, e pode completar seu ciclo em torno de 5 a 7 dias (TAVARES; CRUZ, 2002).

A umidade relativa entre 40 a 60% é ótima para o desenvolvimento, porém, condições de clima seco, quente e com nebulosidade, baixa luminosidade ou luz difusa, favorecem o desenvolvimento da doença (SÔNEGO; GARRIDO; GRIGOLETTI, 2005). Ainda de acorde com os autores em regiões com condições propícias para o desenvolvimento de fungos fitopatogênicos o custo com agroquímicos para o controle destes, pode chegar até a 30% do custo total da produção da uva.

No Submédio do vale do São Francisco e em outras regiões produtoras da videira, o manejo do oídio se dá principalmente pelo uso de fungicidas sintéticos de ação específica pertencentes aos grupos dos inibidores da quinona externa (QoI), inibidores da succinato desidrogenase (SDHI), inibidores da desmetilação de esteróis (DMI) e de fungicidas de ação não específica como o enxofre (GADOURY et al., 2012; GHULE et al., 2018). Entretanto, de acordo com Amorim et al. (2016), os três primeiros grupos citados, são classificados como fungicidas de médio a alto risco para o desenvolvimento de resistência pelos patógenos e, o uso indiscriminado desses produtos causam elevação dos custos de produção, acumulo de resíduo

nos frutos, impacto ambiental e danos à saúde humana, tornando necessário a busca por estratégias sustentáveis e eficazes como o emprego de agente de controle biológicos para o manejo do oídio nas videiras (GHULE et al., 2019; PÉREZ-GARCÍA; ROMERO; DE VICENTE, 2011).

5. Interação E. necator – Vitis vinifera L.

O *E. necator*, por ser um patógeno biotrófico obrigatório, precisa estabelecer uma relação infeciosa bem sucedida com a planta hospedeira para completar seu ciclo de vida (STADNIK; MAZZAFERA, 2001). Entretanto, a planta desencadeia uma série de reações para impedir que o processo infecioso aconteça. Segundo Qiu, Feechan e Dry (2015), as plantas restringem a invasão e o crescimento de patógenos biotróficos por meio da estratégia de resistência a penetração e resistência mediada pela morte celular programada (MCP).

Primeira interação que o *E. necator* enfrenta para estabelecimento da infecção em plantas de videira é a presença de uma camada cuticular composta de cutina (polímero complexo de ácidos graxo epóxi e hidroxi), que necessita ser rompida para infectar as células epidérmicas (SCHNEE et al., 2013). Segundo os autores a parede celular dos conídios de *E. necator* são constituídas por proteínas hidrolíticas (esterases e cutinases) que atuam na hidrolise da cutina da célula vegetal formando monômeros de cutina e, contribui para fixação do conídio, bem como, na formação do tubo germinativo e diferenciação do apressório. Entretanto, cada célula da planta tem sensores que percebem a presença do patógeno e sinalizam para disparar o sistema de defesa (ZIPFEL et al., 2014).

As plantas no geral, incluindo a videira se defendem das investidas dos patógenos por dois mecanismos de defesa, a imunidade desencadeada por padrão molecular associado ao patógeno (PAMP) (PTI) e por imunidade desencadeada por efetores (ETI) (DRY et al., 2009). O PTI é acionado quando receptores de reconhecimento de padrão (PRR) presentes nas membranas plasmáticas da célula vegetal percebe a presença de moléculas constituintes do patógeno na superfície do tecido vegetal (BOLLER; FELIX, 2009).

A parede celular de *E. necator* é constituído de quitina (PAMP) e quando em contato com a superfície da planta, são detectadas por (LysM-RKs) receptor de quinases LysM (motivo de lisina) desencadeando respostas de defesa da videira, tais como fosforilação das proteínas quinases ativada por mitógeno (MAPKs) e expressão de genes de defesa (BRULE et al., 2018). Em estudo realizado com aplicação de quitina em videiras, os autores identificaram dois genes VvLYK1-1 e VvLYK1-2, ortólogos a AtCERK1 e OsCERK1, os quais são genes codificadores de proteínas responsáveis pela percepção e sinalização de defesa contra quitina em *Arabidopsis*

thaliana e *Oriza sativa* (SHINYA et al., 2014). Os genes VvLYK1-1 e VvLYK1-2 foram definidos como receptores de quinases (RLKs) responsáveis pela percepção e ativação da defesa por quitina, ativação de MAPK conferindo resistência a *E. necator* (BRULE et al., 2018).

Como regra, PTI se constitui na primeira barreira de proteção a maioria dos patógenos de plantas, entretanto, patógenos evoluíram desenvolvendo proteínas efetoras contra hospedeiros específicos, logo, tornam-se virulentos ao hospedeiro após suprimirem a via de PTI, porém, as plantas também evoluíram e desenvolveram (proteínas de resistência R) receptores que reconhecem esses efetores, o que culmina na ativação da via de ETI (QIU; FEECHAN; DRY, 2015). *E. necator* secreta efetores dentro de células vegetais por meio da estrutura do haustório. De acordo com Moroldo et al. (2008) plantas de videira possui genoma que codifica uma grande família de genes de sítios de ligação de nucleotídeo com domínios de repetição rica em leucina (NBS-LRR). Esses genes são altamente eficazes durante o processo de reconhecimento de moléculas efetoras microbianas secretadas por patógenos biotróficos na infecção.

As proteínas NBS-LRR são as proteínas de resistência mais caracterizadas atuando como receptores imunes intracelulares que reconhecem de forma direta ou indireta efetores (proteínas avirulentas - avr) dos patógenos específicos e desencadeiam respostas de defesa das plantas (GOYAL *et al*., 2020). Em videira as reações de ETI ao oídio se caracteriza pela indução de morte celular programada (reação de hipersensibilidade) de células epidérmicas, interrompendo o desenvolvimento do fungo após a penetração bem sucedida (GADOURY et al., 2012; QIU; FEECHAN; DRY, 2015).

Vários loci (RUN1; RUN2; REN1; REN2; REN3; REN4; REN5; REN6; REN7) foram identificados em videiras nativas da América do Norte, China e Ásia Central contendo genes R com fortes resistência a *E. necator*. Conforme Qiu, Feechan e Dry (2015) o locus RUN1/RPV1 contém sete genes R putativo do receptor do tipo toll/interleucina-1 (TIR)-NB-LRR. De acordo com os autores, apenas o gene MrRGA10 no grupo (TIR-NB-LRR) conferiu forte resistência a *E. necator* em uma gama de cultivares de videiras suscetíveis como Shiraz, Tempranillo e Portan. Já Goyal et al. (2020), estudando o genoma completo de diferentes variedades de videira mostraram que os genes NBS-LRR estão presentes em vários loci REN e RUN, e encontraram 20 e 14 genes NBS-LRR expressando respostas de resistência a infecção de oídio em cultivares suscetíveis Carignan e Thompson seedless, respectivamente.

Também em *V. viniferas* melhorada com genes R de *V.* rotundifolia, um locus dominante definido RUN1 apresentou resistência completa em *V. viniferas* (PAUQUETE et al., 2001; DRY et al., 2010). Outras duas cultivares de *V. viniferas*, Kishmish vatkana e Dzhandzhal

kara, provenientes do Uzbequistão na Ásia Central contendo o locus dominante REN1 no cromossomo 13 conferiram resistência ao *E. necator* por meio do processo de MCP (HOFFMANNE et al., 2008; COLEMANE et al., 2009).

Na videira inúmeros genes que atuam codificando proteínas de defesa, como proteínas relacionadas a patogênese (PR) quinase e B-1,3 -glucanase, proteína tipo germinativa (GLPs) e estilbeno sintases, são ativadas em respostas das infecções causadas por oídios em videiras suscetíveis (FICKE et al., 2004; FUNG et al., 2008). Estudos mostraram que bagas de uva ontogenicamente resistente expressaram o gene da família GLP, VvGLP3, após a inoculação com *E. necator* e confirmaram que esse gene só é induzido em videira por infecção causada por oídio, indicando um papel na defesa da planta (FICKE et al., 2004; GODFREYE et al., 2007).

De acordo com Gadoury et al. (2012), múltiplas vias de defesa são acionadas em resposta a infecção por oídio, entretanto, nenhuma dessas proteínas ou vias de defesas são capazes de restringir completamente o crescimento e desenvolvimento do *E. necator* em videiras suscetíveis.

6. Controle Biológico do oídio na cultura da videira.

De acordo com Cook e Baker (1983), o controle biológico de doenças de plantas consiste na redução da concentração do inóculo ou das atividades determinantes da doença provocada por um patógeno, realizada por um ou mais organismos que não seja o homem.

O controle biológico basear-se na relação antagônica entre microrganismo e fitopatógeno (LIU; LUO; LONG, 2013). Nesse tipo de controle, os microrganismos possuem diferentes mecanismos, os quais podem atuar garantindo maior estabilidade de controle e amplo espectro de ação. Os principais mecanismos de ação são antibiose, indução de resistência, competição, parasitismo, predação e promoção de crescimento (BETTIOL; GHINI, 2005; MEDEIROS; SILVA; PASCHOLATI, 2018).

O controle biológico de doença é notavelmente uma alternativa importante para a redução da aplicação de produtos químicos e de resíduos presentes em produtos alimentares. De acordo com (MEDEIROS; SILVA; PASCHOLATI, 2018), o uso de produtos químicos ainda é muito mais difundido em comparação ao uso dos produtos biológicos, entretanto, nos últimos anos, a necessidade de diversificação de ferramentas para o manejo de doenças e a disponibilidade de produtos eficazes e de qualidade, fizeram o controle biológico ganhar a atenção dos produtores passando a fazer parte do manejo de doenças das principais culturas nacionais.

De acordo Van Lenteren et al. (2020) nesses últimos dez anos o uso de agentes antagonistas tem ganhado alta expressividade e, as áreas tratadas com agentes de controle biológicos já ultrapassa mais de 100 milhões de hectares em todo mundo, se somada as áreas tratadas com controle biológicos, natural, conservacionista, clássico e aumentativo.

Somente no Brasil, mais de 33 milhões de hectares são manejadas com agentes de controle biológico, o país é o quarto com melhor performance de produção de produtos biológicos e, atualmente já existe cerca de 616 produtos com princípio biológico registrados pelo MAPA (CROP LIFE BRASIL, 2023; AGROFITO, 2023). De acordo com Bettiol (2022), existem cerca de 130 empresas envolvidas na produção dos bioinsumos no Brasil com movimentação de cerca de R$ 1,8 bilhões na safra de 2021.

Alguns produtos já tem registros no MAPA e são empregados para controle biológico do oídio da videira, entre os quais podemos citar o Sonata (*Bacillus pumilus* linhagem QST 2808), Bio-imune (*Bacillus subtilis* BV02), Duravel (*Bacillus amyloliquefaciens* MBI600), Eco-shot (*Bacillus amyloliquefaciens* cepa D-747), ambos tendo como ingrediente ativo as bactérias do gênero *Bacillus* (AGROLINKFITO, 2023). Somando a esses avanços, as pesquisas continuam testando diferentes microrganismos com ação antagônica ao *E. necator*, por exemplo: *Saccharomyces cerevisiae*, *Trichoderma* spp., *Ampelomyces quisqualis*, *Pseudomonas fluorescens* (KUSHARE; KOLASE; MAHADEVASWAMY, 2020; SAWANT et al., 2017; SINGH et al., 2017).

Conhecer os principais mecanismos de ação usados por cada agente de controle, bem como, entender a forma e o momento em que esses mecanismos iram agir sobre cada etapa do processo infeccioso do *E. necator* irá auxiliar no momento da escolher e aplicação do agente de controle biológico (ACB) no vinhedo.

Devido apresentar excelentes resultados como agentes de biocontrole, atualmente o gênero *Bacillus* é um dos mais estudados para o controle de fitopatógenos de parte aérea e do solo, como também, é o mais importante ACB atualmente comercializado (SHAFI et al., 2017). Bactérias desse gênero possuem diferentes modos de ação como competição por espaço e nutrientes, antibiose, formação de biofilme, indução de resistência à hospedeira e promoção de crescimento da planta (SANTOYO; OROZCO-MOSQUEDA; GOVINDAPPA, 2012; VILLARREAL-DELGADO et al., 2018). Foi recentemente estabelecido que 5 a 8% de seu genoma é dedicado a biossíntese de metabólitos secundários e enzimas líticas (WANG et al., 2022; WANG et al., 2021). Espécies de *Bacillus* são a fonte de diversos metabólitos antibióticos, como subtilina, bacitracina, polimixina e lipopeptídeos cíclicos das famílias iturina, fengicina e surfactina (CORTÉS-CAMARGO et al., 2021; MILJAKOVIĆ et al., 2020).

Esses compostos podem ser a fonte responsável pela inativação dos esporos de *E. necator*, que segundo Sellitto et al. (2021) é um importante alvo a ser inativado para melhor proteção das videiras contra infecções secundárias. Logo, o ideal ACB deverá ser efetivo contra os conídios, que se constituem o órgão responsável pelo início da infecção, no momento do contato com o tecido vegetal suscetível. Conídios, tubo germinativo, apressório e hifa de *E. necator* liberam enzimas hidroliticas (cutinase e esterase) na tentativa de desintegrar a cutícula e penetrar as células epidérmicas da planta (SCHNEE et al., 2013), assim, ACB capaz de ativar vias de defesas das plantas para reconhecimento e inibição da infecção é desejado.

Inúmeros trabalhos *in vitro* e *in vivo* têm demostrados o efeito de compostos de *Bacillus* sobre a germinação de conídios de diferentes patossistemas. Por exemplo, Jiao et al. (2021) mostraram que os lipopeptídeos cíclicos (LPCs) bacilomicina D e fengicina da cepa *B. amyloliquefaciens* YN201732 inibiram a germinação de conídios de *Erysiphe cichoracearum*, contribuindo fortemente para prevenção e controle do oídio do tabaco. Hafes et al. (2020), observaram a inibição da germinação de conídios e micélio enrugados e degradados após aplicação de *B. subtilis* sobre oídio do pepino (*Podosphaera xanthii*). Xie et al. (2021) mostraram recentemente que metabólitos de *B. subtilis* TP-08 inibiu a germinação de conídios e o desenvolvimento de apressórios de *Blumeria graminis* f. sp. *tritice* e induziu resistência a doenças no trigo.

De acordo com Reddy e Hynes, (1994), resultados de pesquisas microbianas obtidos sob condição controladas, quase sempre, não condiz com os resultados alcançados em experimento no campo, pois, diversos são os fatores ambientais externos que atuam afetando a capacidade antagônica dos agentes de controle biológicos (ZHANG et al., 2017). Desta forma, isolados de *Bacillus* também têm sido testados quanto a eficiência de controle do oídio da videira em condições de casa de vegetação e campo por diferentes estudos.

Maachia et al. (2010, 2015), avaliando a eficiência de isolados de *Bacillus* sp. ao oídio (*E. necator*) em casa de vegetação, verificaram que os isolados B27 e B29, promoveram redução significativa da severidade da doença, e induziram acumulo de compostos fenólicos em folhas de videira. Kushare, Kolase e Mahadevaswamy (2020), observaram eficiência de controle positiva de *B. subtilis* e mais três antagonistas diferentes sobe a severidade do *E. necator* na cv. Thompson seedless em condição de campo sob duas estações de cultivo. Kanitkar et al. (2020), em experimento de campo, avaliaram a ação de Milastin K (*B. subtilis* KTSB-1015) quando aplicado só ou em consorcio com diferentes fungicidas triazólicos contra oídio da videira (var. Tas-AGanesh) e observaram eficiência de controle sobre as folhas (99,98 %) e cachos (92,31 %). Da mesma forma, Malviya et al. (2022), relataram uma redução da

severidade de oídio em folhas (24,62 %) e cachos (26,77 %) da videira cv. Fantasy Seedless após três aplicações de Bio-Care (*B. subtilis*) mais uma aplicação de enxofre.

Além das bactérias do gênero *Bacillus*, fungos filamentosos e leveduriformes antagonistas tem sido mostrado para inibir as infecções de *E. necator* em videiras. Por exemplo, Sawant et al. (2017) em estudo de seleção de isolados de *Trichoderma* spp. contra *E. necator* em ensaio de campo, demostraram que a cepa *Trichoderma* NAIMCC-F-01938 inibiu (43%) a severidade do oídio em videira e quando integrado com o enxofre maximizou em 30% o controle da doença. Ainda observaram em teste de co-cultura, que as hifas de *Trichoderma* cresceram e se enrolaram em torno dos conídios de *E. necator*, causando distorção da estrutura conidial e superando as colônias de oídio.

Ghule et al. (2019), identificando e avaliando o parasitismo e biocontrole de três fungos micoparasitas (*Lecanicillium antillanum, Acremonium sclerotigenum* e *Sarocladium terrícola*) contra oídio em videira observaram um crescimento micelial dos micoparasitas sobre colônias de oídio e penetração dos conídios causando colapso total. Em condições de viveiro e campo ambos os isolados mostraram redução consistente do oídio e atingiram 41,76 % a 65,61 % de redução da doença.

Sawant et al. (2020), investigando a capacidade de indução de resistência sistêmica (SRI) contra *E. necator* em plantas de videira por quatro isolados de *Trichoderma sperelloides*, registraram aumento dos teores de fenóis totais; atividades de quitinase, β-1,3-glucanase, polifenol oxidase e peroxidase em folhas de videiras tratadas e reduziram a severidade do oídio em cerca de 30% em comparação com o controle fungicida.

Alguns poucos trabalhos com fungos antagonistas do gênero *Ampelomyces* e fungos leveduriformes têm mostrado a eficiência no controle biológico do oídio da videira. Por exemplo, Legler et al. (2015), evidenciaram a eficiência de controle de *Ampelomyces* cepa RS1-a, quanto a inibição da formação de conídios, parasitismo de ascocarpos, bem como redução da severidade de *E. necator* em videiras cv. Barbera *in vitro* e no campo.

Singh et al. (2017), estudando o potencial de isolados de *Ampelomyce quisqualis*, *Trichoderma hazianum* e *Saccharomyces cerevisiae* para prevenir, controlar ou curar a doença do oídio em videiras em campo aberto, observaram que os três micoparasitas reduziu significativamente a severidade da doença e atrasaram o desenvolvimento da infecção por *E. necator*. Também os autores constataram através de microscopia que hifas, conídios e conidióforos de *E. necator* sobre folhas tratadas encontravam-se morfologicamente anormal. Falk et al. (1995), avaliaram *A. quisqualis* quanto à patogenicidade e virulência sobre *Uncinula*

necator em videras cv. Riesling e hibrido interespecífico *V. Aurore* e observaram redução na severidade e atraso no desenvolvimento da doença.

Os objetivos deste trabalho foram: 1. Avaliar e identificar isolados de *Bacillus* antagonistas a *E. necator*; 2. Caracterizar a adaptação a condições de estresse ambiental e nutricional de isolados antagonistas; 3. Caracterizar os mecanismos de ação de isolados de *Bacillus* antagonistas a *E. necator*; 4. Avaliar a aplicação de isolados antagonistas no manejo integrado do oídio da videira.

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CAPÍTULO II

Selecting *Bacillus* strains antagonist to *Erysiphe necator* (Schw.) Burr. the causal agent of grape powdery mildew

Submissão: Biocontrol Science and Technology

Selecting *Bacillus* isolates antagonist to *Erysiphe necator* (Schw.) Burr. the causal agent of grape powdery mildew

John Lennon Ferreira dos Santos[a], Carlos Alberto Tuão Gava[b*], Delson Laranjeira[a]

[a] Universidade Federal Rural de Pernambuco - UFRPE, Programa de Pós-Graduação em Fitopatologia - Recife, PE, Brazil

[b] Embrapa Semiárido, Petrolina, PE, Brazil; Corresponding author

* Corresponding author address:

Embrapa Semiárido

Laboratório de Controle Biológico

Caixa Postal 23 – Petrolina, PE – Brazil

CEP 56.302-970

e-mail: carlos.gava@embrapa.br

Selecting Bacillus isolates antagonist to Erysiphe necator (Schw.) Burr. the causal agent of grape powdery mildew

Abstract

Grape powdery mildew (GPM) control is based on the preventive use of sulfur and curative application of synthetic fungicides, increasing the risk for producers' health and environmental and fruit contamination. This work aimed to select *Bacillus* isolates isolated from the Brazillian tropical semi-arid region antagonists to GPM. An initial screening was performed on detached leaves of grapevine cv. Sugraone with 45 native isolates of *Bacillus* spp. The isolates were sprayed on ten leaves that were subsequently inoculated with 20 microliters of suspension with conidia of *E. necator* (10^6 conidia mL^{-1}). After inoculation, the leaves were kept in BOD (25 ºC; 70% RH) and evaluated for seven days. Two greenhouse experiments were performed with weekly sprayings of suspensions of six isolates of *Bacillus* spp in cv. Sugraone. In detached grape leaves, six isolates of *Bacillus* spp. showed disease symptom reduction higher than 70.0%. Two greenhouse experiments showed that the bacterial isolates LCB03, LCB28, and LCB30 showed control efficiency >80%, similar to a commercial formulation with *Bacillus amyloliquefaciens* QST713. 16s rDNA sequencing showed that strain LCB03 was close to *B. velezensis* (100.0%), while LCB28 has high homology to *B. tequillensis* (99.93%), and LCB30 had 99.71% homology with *B. siamensis*. As an average for both greenhouse experiments, weekly application of *B. velezensis* LCB03, *B. tequilensis* LCB28, and *B. siamensis* LCB30 reduced the average incidence by around 50% and GPM severity by close to 90%. The three *Bacillus* isolates are potential GPM biocontrol agents and, when continuously applied, result in high control efficiency.

Key-words: Detached leaf test; Biotrophic pathogen; Greenhouse efficiency; Biological control; *Vitis vinifera* L.

1 Introduction

Table grape (*Vitis vinifera* L) is one of the main crops produced in the São Francisco River valley in the Northeastern region of Brazil. The tropical semi-arid climate in the region allows continual plant growth, and management technology based on pruning, irrigation, and plant growth regulators allows fruit production throughout the year (Camargo et al., 2008). In the tropics, powdery and downy mildew are primary grape diseases, and the co-existence of grape plants in different development stages all over the year complicates disease management (Buffara et al., 2014). GPM is caused by the obligate pathogen heterothallic fungus *Erysiphe*

necator (Schw.) Burr. (Braun and Takamatsu, 2013). In temperate regions, powdery mildew epidemy is triggered by rains at the beginning of the growing season that cause ascospore dispersal and germination (Jarvis et al., 2002). However, sexual stages do not occur in tropical regions where primary inocula originate from infection of prophylls within the bud, branches, and conidia transported from neighboring fields (Bettiga et al., 2013; Buffara et al., 2014). In the tropics, GPM epidemic outbreaks are favored by warm temperatures at night, higher relative humidity, and cloudy weather without rain (Bettiga et al., 2013; Jarvis et al., 2002).

The complex epidemiological environment imposed by the regional grape production system led to the extensive use of synthetic fungicides belonging to the chemical groups of triazoles, strobilurins, and sulfur (Gadoury et al., 2012; Sawant et al., 2017) to control GPM. Its' constant use increases the risks of fruit and environment contamination and pathogen resistance. Therefore, grape producers have been searching for healthier and environmentally friendly approaches. Applying antagonist microorganisms can be a potential alternative in the integrated management of GPM. *Bacillus* spp. isolates have been studied as biocontrol agents for shoot and root plant pathogens, becoming the most important biological control agents (BCA) currently commercialized (Shafi et al., 2017). Many studies have shown that *Bacillus*-based biofungicides can control grapevine diseases (Pertot et al., 2017), including *E. necator* in different grape production regions (Sawant et al., 2017, 2016). However, climate and cultural practices can undermine its control potential, and continual efforts have been made to obtain more efficient isolates (Fira et al., 2018).

Bacillus spp. are versatile bacteria able to colonize different plant and soil niches (Zeigler and Perkins, 2021). The genus comprises a group of rhizosphere and phyllosphere-competent isolates able to establish epiphytic and endophytic colonization of plant tissues and potentially become efficient biocontrol agents against GPM (Silva et al., 2021). This work aimed to select *Bacillus* isolates isolated from different sources to be applied against the obligatory biotrophic pathogen *E. necator*, the causative agent of grape powdery mildew.

2 Materials and Methods

2.1 Inoculum of E. necator

Powdery mildew inoculum was obtained from naturally infected leaves of *V. vinifera* cv. Sugraone and cv. Thompson Seedless from a vineyard in the Experimental Farm of Embrapa (Petrolina, Brazil). A conidial suspension of *E. necator* was extracted from heavily sporulated lesions with a brush, standardized at 10^7 conidia mL^{-1}, and inoculated onto plantlets of grape cv. Sugraone. The plants were kept at 26 (±1) °C and 70% relative humidity (RH) in a growth

chamber (BOD) with 12 hours of photoperiod. After the initial symptoms were observed, infected plants were transferred to a greenhouse.

2.2 Bacillus isolates and production of a technical-grade formulation

Forty-five *Bacillus* isolates maintained in the Collection of Microorganisms of Agriculture Interest of the Embrapa Semiárido (CMISA) were evaluated for their ability to reduce the incidence and severity of lesions caused by *E. necator*. The bacterial isolates were maintained at -80 °C, subcultured after thawing in nutrient yeast dextrose agar (NA), and kept at 27 °C for 48 h. The bacterial isolates were grown in Luria broth (LB) media (Himedia) for 24 h in an orbital shaker (120 rpm) and standardized to an optical density (OD) of 0.5 at 595 nm. Technical grade formulations (TGF) of the *Bacillus* isolates were prepared by adding cell suspensions to a preparation containing previously autoclaved natural polymer at 1.2% (patent pending) and were used in greenhouse experiments.

2.3 Prescreening Bacillus isolates against GPM

A fast-throughput experiment using detached grape leaves was conducted to screen antagonist isolates. Fully developed grape leaves were collected from grape plants cv. 'Sugraone' grown in Embrapa experimental farm (Petrolina, Brazil). Immediately, their petioles were inserted into microtubes containing sterilized distilled water and transported to the laboratory. The leaves were superficially sterilized using cotton soaked in sodium hypochlorite solution (1.0% v/v) and placed in an aseptic chamber with UV-C light for 5 minutes on each side.

Three treatments were applied in addition to the *Bacillus* isolates: control (autoclaved distilled water); micronized sulfur (Kumulus DF, BASF) 1.0 g L^{-1}; *B. amyloliquefaciens* QST713 (Serenade, Bayer CropScience) 10^7 endospores L^{-1}. Bacterial suspensions were sprayed on ten leaves per treatment using a bench atomizer. After evaporating the excess liquid, they were inoculated with 20 μL of a conidia suspension of *E. necator* with 10^7 conidia mL^{-1} at three points on the abaxial leaf surface (two at the base one at the apex of the leaf). After the treatments were applied, the leaves were kept in a BOD (25 °C; 70% RH) with 12 hours of photoperiod, and the diameter of the lesions caused by GPM was measured seven days after inoculation using a caliper. Given the large number of isolates tested, the experiment was divided into three portions containing all reference treatments. The experiment was carried out in a completely randomized design, with 10 repetitions (each plant considered a repetition). All isolates were tested at least twice using an independent leaf group.

2.4 Control efficiency in greenhouse experiments

Healthy grape seedlings cv. 'Sugraone' were planted in pots containing 5 kg Yellow Argisol soil collected from the experimental farm of Embrapa Semiárido (09°09'S; 40°22'W) and mixed with 10% (w/w) manure. Two experiments were conducted in greenhouse conditions (26.5 ±2.0ºC, RH 60 ±10%) with forced ventilation. Soil water content was monitored by weighing pots every two days, and irrigation was conducted using a drip irrigation system (flow rate 4.1 L h^{-1}). Ten plants were sprayed with different treatments when their branches reached five leaves with an average transect larger than 5.0 cm. The treatments were: 1. a control treatment sprayed only with distilled water; 2. micronized sulfur at 1.0 g L^{-1}; 3. *B. amyloliquefaciens* QST713 (Serenade, Bayer CropScience) 10^7 endospores mL^{-1}; 4-9. Suspensions containing 10^7 cells mL^{-1} of *Bacillus* sp. LCB03, LCB05, LCB28, LCB30, LCB42, and LCB45.

Treatment spraying was performed using an electric handheld sprayer with a standard hollow cone nozzle (flow rate 120 mL min^{-1}). After spraying, the plants were divided into groups containing all treatments and evenly distributed in the greenhouse. The inoculation of the pathogen occurred naturally, distributing two plants with high incidence and severity of powdery mildew to each group (Punja et al., 2019). The position of the inoculum-producing plants was changed daily throughout the experiment to ensure the homogeneous distribution of GPM inoculum. The treatments were applied weekly for four weeks. GPM's incidence (number of symptomatic leaves) and severity (injured area) were evaluated weekly by measuring symptomatic areas with a digital caliper. The experiment was repeated twice using a different set of plants and carried out in a completely randomized design, with 10 repetitions (each plant considered a repetition).

2.5 Identification of bacterial isolates

Three selected bacterial isolates were identified by 16S-rDNA gene sequencing. The bacteria were cultivated in LB medium and kept for 42 hours in an orbital shaker at 120 rpm. After the growth period, the DNA of the isolates ware extracted using the genomic DNA extraction kit Wizard Genomic DNA Purification kit (Promega), following the manufacturer's instructions. PCR amplification was performed using a universal primer: forward PA-5′-AGAGTTTGATCCTGGCTCAG-3′ and PH-5′-AAGGAGGTGATCCAGCCGCA-3′ (Edwards et al., 1989). PCR products were purified and sequenced using the Sanger sequencing method (Macrogen, Korea). The isolate's 16S rDNA gene sequence was analyzed using BLAST and EzTaxon-edatabase–EzBioCloud tools (Yoon et al., 2017). Sequences with the highest identity were selected and aligned using Clustal-W (Thompson et al., 2003). A maximum-

likelihood method-based phylogenetic tree was constructed using MEGA11 (Kumar et al., 2016). Its neighbor-joined data were bootstrapped to evaluate the tree's topology using 500 randomized data sets (Felsenstein, 1985). The 16S-rDNA sequences of the identified isolates were submitted to GenBank.

2.6 Data treatment and statistical analysis

1. Prescreening experiments

The injured area of leaves was applied to calculate the relative control efficiency compared to the control treatment. Relative control efficiency (E%) was estimated based on the reduction of the injured leaf area using the equation $E\% = (A_c - A_{ti})/A_c \times 100$, in which A= leaf injured area; C= control treatment; Ti= treatments. Relative efficiency data were used to classify the isolates in the prescreening experiments using an arbitrary scale in which they were classified into four groups: 1. E%< 25%; 2. E% 25-50; 3. E% 50-69; and 4. E% ≥70. The experiment was repeated twice.

2. Greenhouse experiments

Weekly leaf incidence and severity were used to calculate the area under the disease progress curve (AUDPC), according to Madden (2007). The apparent infection growth rate (r) was estimated using the procedure defined by (Kushalappa and Ludwig, 1982). The control efficiency (E%) was estimated based on the percentage reduction of the injured leaf area at the end of the experiment.

3. c. Analysis of variance and mean comparison

An initial ANOVA that included the experiments as a factor was performed in those experiments with repetition in the greenhouse experiment. This procedure was performed to identify if there were significant interactions between treatments and experiment repetition. There was a significant interaction between the two experiments, and the data were analyzed separately. Percentage data were arcsine transformed while AUDPC was square root transformed to check for normality and homogeneity of the data to apply ANOVA, but the results were presented as the original unities. ANOVA was followed by Tukey's multiple comparison test ($p < 0.05$) using Statistica for Windows v. 12 (StatSoft, 2013).

3 Results

3.1 Prescreening of Bacillus isolates against GPM

In spite of being kept in growth chambers with controlled RH, some detached leaves quickly dehydrated and had to be removed from the experiment. However, their petioles were immediately inserted into microtubes containing ADE after cutting to reduce dehydration. However, disease evaluations were carried out only for seven days after inoculation to avoid the effect of leaf dehydration, resulting in small lesioned areas.

All detached grape leaves showed GPM symptoms after inoculation, with the control treatment having an average injured area of 28.69%. Among the tested isolates, 66.8% did not exhibit more than 25% reduction in the injured area compared to the control treatment. Only the six *Bacillus* isolates (13.3%) highlighted in Figure 1 and Table 1 demonstrated a relative control efficiency of 70% or higher on detached grape leaves. These isolates were selected for further testing as potential BCA against the GPM. It is worth noting that *Bacillus* isolates LCB03 and LCB45 had a severity level similar to that of the reference treatments, resulting in a relative efficiency of 79.7% and 79.4%, respectively.

Figure 1 – Distribution of isolates into the different classes of relative control efficiency by co-inoculating *E. necator* conidial suspension and *Bacillus* isolates. The table on the left shows the GPM severity (means ±SD) in detached leaves and the average reduction observed for the isolates with control efficiency higher than 70%.

3.2 Control efficiency in greenhouse experiments

In the first experiment, we evaluate the effect of weekly spraying of a TGF containing six experimental isolates over the occurrence of GPM in the grape cultivar Sugraone. Applying the antagonists significantly reduced GPM incidence ($F_{8;\ 27}$= 16.8385; p< 0.001) and severity ($F_{8;\ 27}$= 13.0813; p< 0.001). The control treatment showed 68.8% disease incidence on the fifth day, reaching 100% on the 12th after introducing the inoculum source. Thus, control data from the experiment were excluded from ANOVA and post hoc tests. The treatments also significantly affected the AUDPC ($F_{8;\ 27}$= 3,661; p=0,023). The sulfur treatment delayed symptoms development until the eighth day, resulting in a lower disease growth ratio, severity, and AUDPC. All *Bacillus* isolates except for LCB42 significantly reduced GPM incidence by the Tukeys' test (p< 0.05). According to Tukey's test, treatments with LCB03, LCB28, LCB30, and LCB45 showed control efficiency similar to QST713 and sulfur (Table 2).

Table 2 – Effect of weekly application of *Bacillus* isolates on the disease incidence, severity, area under the disease progress curve (AUDPC), apparent disease growth rate (r), and control efficiency of powdery mildew in plants of grape cv "Sugraone" in greenhouse conditions.

The application of the TGF containing the BCAs during the second greenhouse experiment had a significant impact on GPM incidence ($F_{8; 27}$= 22.2608; p< 0.001) and severity ($F_{8; 27}$= 28.8802; p< 0.001). The control treatment showed a disease incidence of 98.9% and a severity of 47.40% (Table 3). The statistical analysis showed that all treatments effectively reduced the GPM apparent growth rate (r) in grape plants compared to the control treatment, significantly decreasing disease severity and AUDPC. While treatments with LCB03 and LCB28 showed similar disease incidence to the sulfur and QST713 treatments, LCB30 and LCB45 treatments had a lower AUDPC, which was similar to the sulfur treatment based on Tukey's test results ($p<0.05$).

Table 3 – Suppression of GPM by the weekly application of *Bacillus* isolates on cv "Sugraone" plants in greenhouse conditions. The table shows the disease incidence, severity, area under the disease progress curve (AUDPC), apparent disease growth rate (r), and control efficiency of GPM.

In the second experiment, the disease severity curves revealed that sulfur and LCB28 treatments prolonged the onset of initial symptoms until day 13 (Figure 2). The incidence curves also indicated that LCB05 was ineffective in preventing *E. necator* infection after the initial exposure to the inoculum source, as disease symptoms were observed on the 5th day and rapidly increased. In contrast, LCB03 and LCB30 consistently delayed symptom development and demonstrated a reduced infection rate. However, the most noteworthy findings were observed in the disease severity evolution curves, which demonstrated a significant reduction in leaf area damage caused by GPM throughout the entire experiment due to applying the BCAs.

Figure 2 – Severity (McKinley index) of grape PM in two greenhouse experiments treated with weekly spraying of technical grade preparations of *Bacillus* strains.

3.3 Identification of the bacteria isolates

The strain LCB03 was isolated from grape rhizosphere sample collected from a grapevine cultivated in a sandy Ultisol, and LCB28 and LCB30 were isolated respectively from the rhizosphere and root tissue of *Passiflora edulis* Sims (Passifloraceae: Malpighiales) cultivated in the same soil in the experimental farm of Embrapa (Petrolina, -9.09641, -40.29883). They are gram-positive and spore-forming bacteria with rod-shaped cells. BLAST

and EzTaxon-edatabase–EzBioCloud (https://www.ezbiocloud.net/identify) analysis were used to compare the 1500-bp 16S rDNA gene sequence. The results showed that all three antagonistic are closely related to *Bacillus* (Firmicutes; Bacilli; Bacillales; Bacillaceae). Contig blasting showed that LCB03 was 100.0% related to the type strain of *B. velezensis* CR-502 (AY603658) Ruiz-García et al. (2005), while LCB28 showed 99.93% nucleotides identity shared with *B. tequilensis* KCTC13622 (AYTO01000043) Gatson et al. (2006), and LCB30 shared 99.71% homology with *B. siamensis* KCTC13613 Sumpavapol et al (2010).

Phylogenetic analysis of the 16S rDNA sequences strain using maximum-likelihood relatedness suggested that LCB03 clustered in one group with *B. velezensis* (Figure 2), while LCB30 grouped within a subcluster together with *B. siamensis* KCGC13613 and *B. amyloliquefasciens* DSM7. LCB28 clustered with three species: *B. tequilensis* KCTC13622, *B. stercoris* JCM30051 (Adelskov and Patel) Dunlap et al., and *B. spizizenii* NRRLB23049 (Nakamura et al.) Dunlap et al. The 16S-rDNA sequences of the isolates were deposited in GenBank with accession numbers *B. velezensis* LCB03 OP453366, *B. tequilensis* LCB28 OP453367, and *B. siamensis* LCB30 OP454462.

Figure 2 - Phylogenetic tree of the *Bacillus* isolates based on the sequence of 16S rDNA gene sequence analysis constructed using the neighbor-joining method. The tree was rooted using the 16S rDNA sequence of *Rosselomorea marisflavi* (Bacillaceae; Rossellomorea), and the level of bootstrap support (1000 repetitions) is indicated at all nodes.

4 Discussion

This study aimed to identify *Bacillus* isolates antagonistic to the causal agent of grape powdery mildew *E. necator*. The isolates were initially isolated from various plants and soils in the Brazilian semi-arid region and have previously shown in vitro and in vivo antagonism against pathogens such as *Fusarium* spp. and *Sclerotium rolfsii* (Sá et al., 2019a, 2019b), *Lasiodiplodia theobromae* (non-published data), and *Meloydogine enterolobii* (Carvalho-Júnior et al., 2021). Since the experiments required living tissue, testing potential biocontrol agents against the obligatory biotrophic *E. necator* proved challenging. We used detached leaf assays to overcome this obstacle, but the prescreening assays were limited to the period before the grape leaves showed senescence signals. Despite these limitations, six isolates were found to be effective against E. necator, reducing over 70% of the lesioned area caused by conidia inoculation. These isolates' control efficiency was similar to that of the commercial strain *B. amyloliquefaciens* QST713.

During greenhouse experiments, three *Bacillus* isolates that were selected in the prescreening process demonstrated a significant reduction in the development of GPM in potted grape plants when applied before exposure to *E. necator* inocula. Although there was a variation between the two experiments, the average GPM incidence observed for LCB03, LCB28, and LCB30 isolates was half that of the control treatment, and they reduced disease severity by nearly 90%. These isolates proved as effective as the weekly spraying of micronized sulfur and the commercial strain QST713. Previous studies have also indicated that *Bacillus* isolates, such as *B. subtilis* GLB191 and GLB197, could provide effective control against grape downy mildew (Zhang et al., 2017) and GPM (Maachia et al., 2015) when applied preventively. However, most treatments showed reduced control efficiency over time, except for sulfur, LCB03, and LCB28. A similar reduction in disease control efficiency during the experiment was observed from the disease incidence data in a study applying mycoparasites for the biological control of GPM in India (Ghule et al., 2019). Similarly, analyzing the incidence curve of cucumber PM caused by *Podosphaera xanthii* (Sarhan et al., 2020) also showed that while a synthetic fungicide showed a linear decay of control efficiency, the application of BCA treatments, including *Bacillus subtilis*, showed an inverted parabola pattern.

Bacillus is a phenotypic and phylogenetically diverse genus characterized by the ability to make endospores in unfavorable growth conditions and showing differences in nutritional requirements and growth conditions (Zeigler and Perkins, 2021). The 16S rDNA sequence analysis showed that LCB03 was similar to *Bacillus velezensis*, a species already used in commercial biofungicides formulations (Wang et al., 2022). The strain LCB30 was closely related to *B. siamensis* (Sumpavapol et al., 2010), known to produce lipopeptides with a broad antifungal spectrum (Xu et al., 2018; You et al., 2021). Meanwhile, strain LCB28 was closely related to *B. tequillensis.* All these species belong to the Subtilis Clade, gathering *Bacillus* species recognized to stimulate plant growth, biocontrol of plant disease, increase soil nutrient availability, induce plant tolerance to abiotic stress, and induce plant resistance to plant pathogens (Wang et al., 2022).

Achieving efficient control using bacteria-based biofungicide as a sole strategy in open field experiments is complex because field reinfection can occur by inoculum entry from neighboring plots, as well as, antagonists would face adverse climatic conditions of high temperatures, solar radiation high and low relative humidity directly influencing the development and action of ACB at the site of infection (Ghule et al., 2019). This study did not evaluate the mechanisms of action of the *Bacillus* isolates, but previous works have already shown that *Bacillus* species produce hydrosoluble and volatile antifungal compounds and lytic

enzymes, and can elicit plant defense responses (Wang et al., 2022). Nevertheless, new studies are necessary to define formulations and application strategies for inserting the isolates in an integrated GPM management program.

The results obtained in this work showed that the isolates could be potential biocontrol agents of GPM and that the continual application of the antagonist likely would result in high control efficiency. In our experiments, *B. velezensis* LCB03, *B. tequilensis* LCB28, and *B. siamensis* LCB30 reduced the average incidence to around 50% and GPM severity to 90%.

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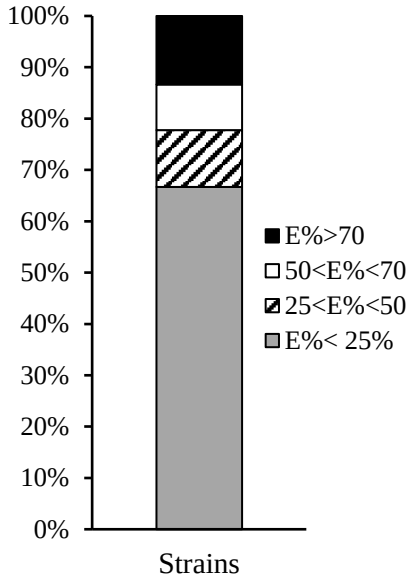

Treatments	Disease (cm^2)	Efficiency (E%)
Control	28.69 (11.22)	0.00
Sulfur	1.14 (0.22)	99.52
QST713	4.31 (1.56)	84.98
LCB03	5.83 (2.85)	79.66
LCB45	5.91 (2.51)	79.40
LCB30	7.56 (2.60)	73.66
LCB05	7.78 (2.79)	72.87
LCB28	8.63 (1.37)	70.00
LCB42	8.80 (2.51)	70.00

Figure 1 – Distribution of isolates into the different classes of reduction in symptomatic leaf area produced by co-inoculation of *E. necator* conidial suspension and *Bacillus* isolates. The table on the right shows the PM severity (means ±SD) in detached leaves and the average reduction observed for the isolates with control efficiency higher than 70%.

Table 2 – Effect of weekly application of *Bacillus* isolates on the disease incidence, severity, area under the disease progress curve (AUDPC), apparent disease growth rate (r), and control efficiency of powdery mildew in plants of grape cv "Sugraone" in greenhouse conditions.

Treatments	Incidence (%)	Severity (%)	AUDPC	r	Efficiency (%)
Control	100.00	32.50 (3.30) a	132.50 (4.45) a	2.55 (0.27) a	
QST713	80.00 (12.58) a	8.00 (4.64) b	29.50 (15.56) c	0.57 (0.33) b	80.49 (11.31) a
Sulfur	30.00 (3.52) b	3.37 (1.16) c	22.99 (3.36) c	0.26(0.07) b	93.80 (7.38) a
LCB03	70.00 (12.91) a	3.67 (1.89) c	20.17 (7.21) c	0.40 (0.23) b	88.75 (4.51) a
LCB05	75.00 (18.93) a	10.33 (3.32) b	26.88 (9.05) c	0.80 (0.27) b	66.77 (6.07) b
LCB28	70.00 (8.16) a	4.67 (1.55) c	36.67 (20.80) bc	0.46 (0.17) b	85.72 (12.54) a
LCB30	75.00 (9.57) a	6.00 (3.89) bc	48.50 (10.74) b	0.65 (0.24) b	81.63 (8.85) a
LCB42	85.00 (4.71) a	19.33 (2.09) ab	99.17 (20.64) ab	1.77 (0.20) ab	38.69 (4.59) c
LCB45	60.00 (9.57) a	4.67 (0.85) c	22.83 (3.97) c	0.37 (0.07) b	85.43 (11.96) a

*Treatments with the same letters in the columns did not differ by Tukey test ($p < 0.05$).

Table 3 – Suppression of GPM by the weekly application of *Bacillus* isolates on cv "Sugraone" plants in greenhouse conditions. The table shows the disease incidence, severity, area under the disease progress curve (AUDPC), apparent disease growth rate (r), and control efficiency of GPM.

Treatments	Incidence (%)	Severity (%)	AUDPC	r	Efficiency (%)
Control	98.88 (1.73) a	47.40 (12.21) a	191.30 (28.53) a	2.18 (0.58) a	-
QST713	16.52 (5.72) c	2.50 (1.18) b	22.88 (11.73) bc	0.14 (0.05) b	94.76 (1.80) a
Sulfur	6.44 (3.72) c	2.99 (1.01) b	18.03 (3.91) c	0.11 (0.05) b	92.74 (2.88) a
LCB03	30.00 (12.91) bc	3.78 (1.16) b	44.13 (16.70) b	0.18 (0.05) b	81.85 (6.76) a
LCB05	40.00 (8.16) b	2.32 (0.92) b	26.93 (14.64) b	0.16 (0.04) b	89.92 (6.46) a
LCB28	27.00 (9.57) bc	3.54 (0.57) b	27.76 (8.75) b	0.10 (0.03) b	87.90 (5.27) a
LCB30	45.00 (9.57) b	3.04 (0.16) b	14.82 (1.38) c	0.18 (0.01) b	89.91 (6.46) a
LCB42	93.33 (4.71) ab	3.72 (1.21) b	31.35 (10.05) b	0.17 (0.06) b	88.71 (3.63) a
LCB45	55.00 (18.93) b	3.66 (1.51) b	20.95 (6.36) bc	0.15 (0.08) b	79.62 (2.88) a

* Treatments with the same letters in the columns did not differ by Tukey test ($p < 0.05$).

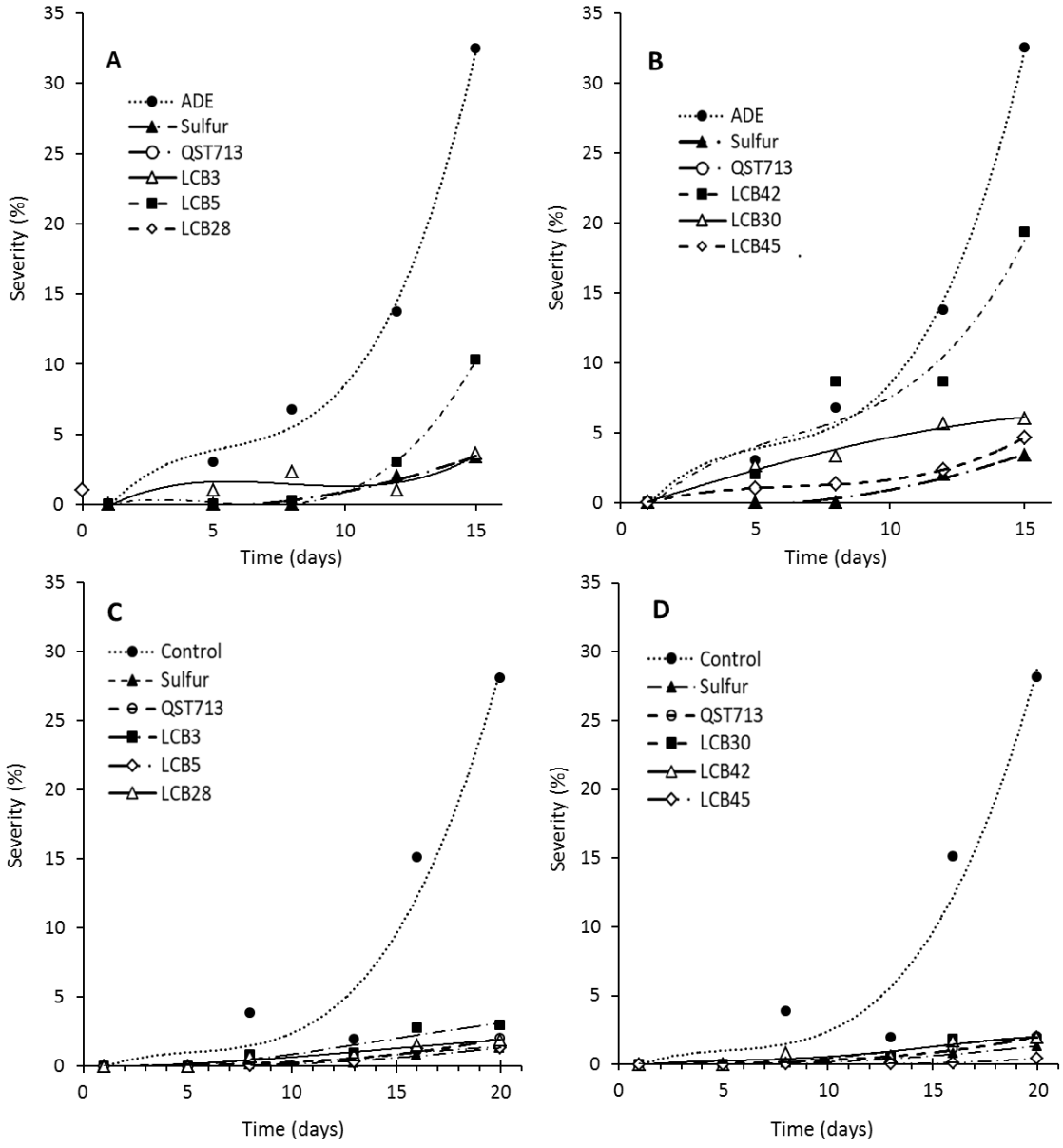

Figure 2 – Severity (McKinley index) of grape PM in two greenhouse experiments treated with weekly spraying of technical grade preparations of *Bacillus* strains.

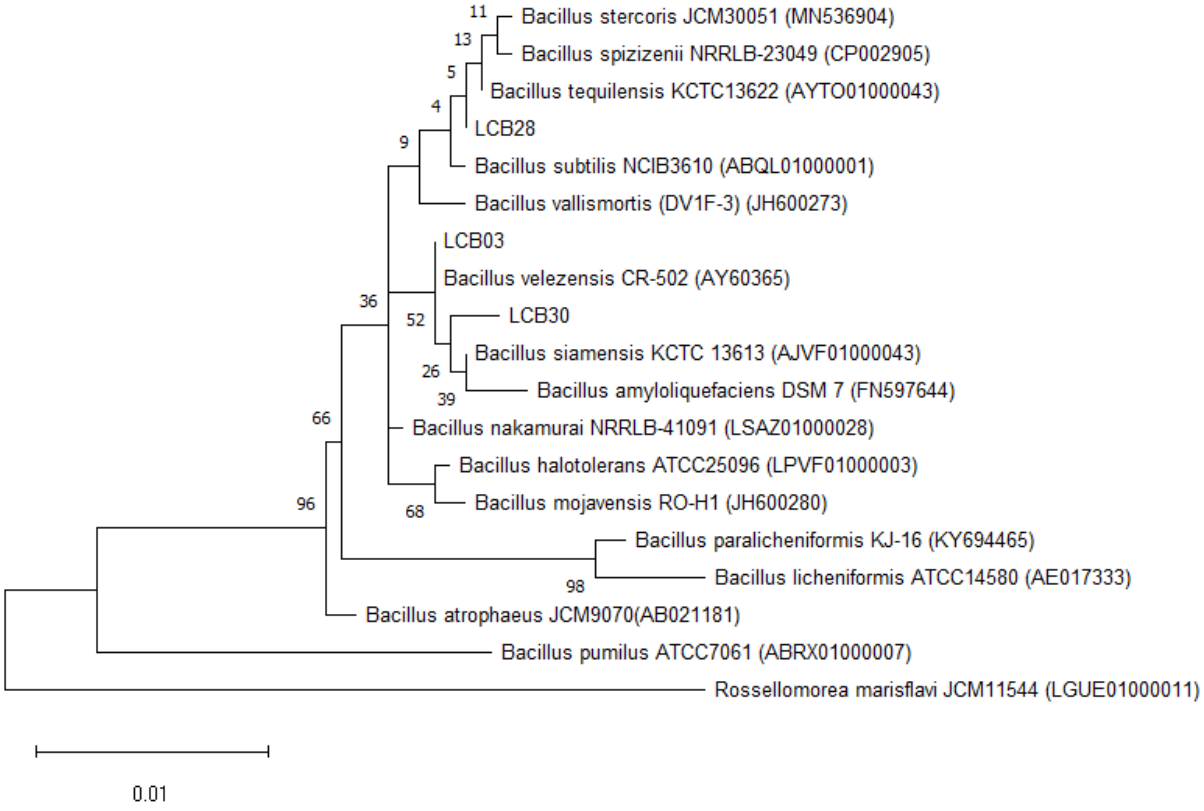

Figure 3 - Phylogenetic tree of the *Bacillus* isolates based on the sequence of 16S rDNA gene sequence analysis constructed using the neighbor-joining method. The tree was rooted using the 16S rDNA sequence of *Rosselomorea marisflavi* (Bacillaceae; Rossellomorea), and the level of bootstrap support (1000 repetitions) is indicated at all nodes.

CAPÍTULO III

Partial characterization and mechanism of action of *Bacillus tequilensis* LCB28 and *B. siamensis* LCB30 against grape powdery mildew

Submissão: Biological Control

Partial characterization and mechanism of action of Bacillus tequilensis LCB28 and B. siamensis LCB30 against grape powdery mildew

John Lennon Ferreira dos Santos[a], Gabriela Alves Gonçalves[b], Kezia Costa Escobar[c], Taise Oliveira Passos[d], Carlos Alberto Tuão Gava[e]*, Delson Laranjeira[a]

[a]Universidade Federal Rural de Pernambuco - UFRPE, Programa de Pós-Graduação em Fitopatologia - Recife, PE, Brasil

[b]Universidade Federal do Vale do São Francisco – UNIVASF– Petrolina, PE, Brasil

[c]Universidade Estadual de Pernambuco – UPE – Campus Petrolina, PE, Brasil

[d]Embrapa Semiárido, Embrapa/CNPQ Fellowship Program; Petrolina, PE, Brasil

[e]Embrapa Semiárido, Petrolina, PE, Brasil; Autor correspondente

* Corresponding author address:

Embrapa Semiárido

Laboratório de Controle Biológico

Caixa Postal 23 – Petrolina, PE – Brazil

CEP 56.302-970

e-mail: carlos.gava@embrapa.br

Partial characterization and mechanism of action of *Bacillus tequilensis* LCB28 and *B. siamensis* LCB30 against grape powdery mildew

Abstract

Grape powdery mildew control (GPM) is based on the continuous application of synthetic fungicides. Biocontrol based on the application of antagonistic microorganisms is an environmentally friendly and food-safe alternative to be included in its integrated management. This work aimed to evaluate the physiological characteristics and mechanism of action of *Bacillus tequilensis* LCB28 and *B. siamensis* LCB30 to control GPM. Both isolates produced water-soluble and volatile antifungal compounds that inhibit conidia germination *in vitro*. The potential to synthesize antifungal cyclopeptide was confirmed by the PCR-based detection of gene markers for producing surfactin, bacyllomicin, fengycin, and bacilysin in the LCB28 genome, and the markers for all these compounds and iturin in the LCB30 genome. Although LCB28 showed a slightly lower exigence of carbon to growth in the culture medium, LCB30 grew in low nitrogen concentration and produced large surfactant activity. Based on the results of two potted plant experiments, *B. tequilensis LCB28 and B. siamensis* LCB30 have proven potential to become effective biocontrol agents (BCA) against *E. necator* in grapevine cv Red Globe. *E. necator* sporulation and mycelial growth were partially inhibited in leaves treated with both bacterial isolates during the experimental period. Furthermore, our analysis revealed a high percentage of non-viable conidia (GII% > 80) in the lesion surfaces treated with both isolates, which was statistically similar to the fungicide triflumizole. This result indicates that secondary infections will be compromised even if the pathogen completes its infectious process. The diversity of potential antimicrobial synthesis, biofilm production, ability to metabolize diverse carbon sources, and a low nutrient requirement to grow supports their adaptation to thrive within the grape phyllosphere environment and their potential as biocontrol agents against the GPM caused by *E. necator*.

Key-words: *Vitis vinifera* L.; *Erysiphe necator*; Antibiosis, Co-cultivation, Biocontrol

1 Introduction

Most grape varieties belong to the species *Vitis vinifera* L. (Gadoury et al., 2012), and they are susceptible to many cryptogamous diseases that cause severe economic losses (Liu et al., 2019; Sawant et al., 2017). Grape powdery mildew (GPM) is a severe problem in all grape-growing regions (Mieslerová et al., 2022). GPM pathosystem is a highly complex and evolved interaction between grape plants and the obligate biotrophic ascomycete *Erysiphe necator* (Schw.) Burr. (Erysiphales, Erysiphaceae) (Braun and Takamatsu, 2013).

Rains at the beginning of the grape growing season trigger ascopores release from cleistothecia starting powdery mildew epidemics in temperate regions. However, sexual stages do not occur in tropical areas (Jarvis et al., 2002). In the tropics, primary inoculum originates from infection of prophylls within the bud and branches and wind-dispersed conidia from neighboring fields. Epidemic outbreaks are favored by seasonal warm and humid nights and cloudy weather, and all green tissues can be infected (Bettiga et al., 2013; Jarvis et al., 2002). *E. necator* conidia germinate on the surface of the susceptible host to form a germ tube ending in a lobed appressorium with a penetration peg, which drives the penetration of epidermal cell walls, followed by the production of a haustorium (Gadoury et al., 2012). GPM colonies proliferate, creating a superficial mycelium-bearing hyphal appressoria with penetration pegs that taps into further host cells, culminating with the production of conidial chains of a network of necrotic plant cells (Mieslerová et al., 2022).

GPM management is primarily based on spraying synthetic fungicides (Gadoury et al., 2012, Sawant et al., 2017). However, growing concerns over environmental pollution, pathogens resistance, and the contamination of fruits with pesticide residues have driven the search for environment-friendly and food-safe alternatives. Plant disease management by applying antagonistic microorganisms has shown to be a reliable alternative. Among these microorganisms, *Bacillus* species have been successfully applied to control plant pathogens in various crops, including powdery mildew (Shafi et al., 2017). Besides the low cost for industrial production and success in field applications, endospore production increases the storability of simple formulations, making *Bacillus*-derived biofungicides the most important bioproduct currently marketed (Lahlali et al., 2022).

Bacillus species are the source of diverse antibiotic metabolites such as subtilin, bacitracin, polymyxin, and cyclic lipopeptides of the iturin, fengycin, and surfactin families (Cortés-Camargo et al., 2021; Miljaković et al., 2020). It was recently established that 5 to 8% of their genome is dedicated to the biosynthesis of secondary metabolites and lytic enzymes (Jiao et al., 2021; Wang et al., 2022), which were implied in controlling pathogens in different pathosystems (Fan et al., 2017; Li et al., 2019; Zhou et al., 2020). For example, Xie et al. (2021) showed that metabolites from *B. subtilis* TP-08 inhibited conidia germination and appressorium development of *Blumeria graminis* f. sp. *tritice* and induced wheat resistance to powdery mildew. Antibiosis was also involved in the preventive and curative effect of *B. amyloliquefaciens* YN201732 against tobacco powdery mildew caused by *E. cichoracearum* (Jiao et al., 2021).

According to Caulier et al. (2019), lipopeptides can remain active under high temperatures, ultraviolet radiation, and different pH, being able to resist the action of hydrolysis by peptidases and proteases. Besides antibiosis, the production of lytic enzymes, competition, and resistance induction have been associated with mechanisms of action of *Bacillus* species against plant diseases (Dong et al., 2023; Miljaković et al., 2020). Among the antibiotics produced by *Bacillus* spp., cyclic lipopeptides have been reported as prominent plant protectors (Caulier et al., 2019; Li et al., 2019) on different pathosystems (Fan et al., 2017, Li et al., 2019, Jiao et al., 2021). This work aimed to partially characterize the potential ecological fitness and the mechanism of action of two *Bacillus* isolates previously selected as efficient biocontrol agents of GPM caused by *E. necator* (Santos, 2023).

2 Material and methods

2.1 Production of E. necator inoculum

Powdery mildew inoculum was obtained from naturally infected leaves of *Vitis vinifera* cv. Sugraone and cv. Red Globe, as described in Santos (2023). Briefly, a conidial suspension of *E. necator* was extracted from heavily sporulated lesions, standardized at 10^7 conidia mL^{-1}, and inoculated onto plantlets of grape cv. 'Sugraone'. The plants were kept at 26 (±1) °C and 70% relative humidity (RH) in a BOD with 12 hours of photoperiod. After the initial symptoms were observed, infected plants were transferred to a greenhouse (26 ±4 °C; 70% average RH).

2.2 Bacillus isolates storage and formulation

Cell suspensions of *Bacillus tequilensis* LCB28 (GenBank accession number OP45337) and *B. siamensis* LCB30 (OP454462) that previously showed efficiency in controlling GPM, Santos (2023), were maintained at -80 °C in a 15% (w/v) glycerol solution. After thawing, they were subcultured in nutrient agar (NA, Himedia India) and kept at 27 °C for 48 h. Routinely, the bacterial isolates were grown in Luria-Bertany (LB) medium (Himedia) for 24 h in an orbital shaker (120 rpm) and standardized to an optical density (OD) of 0.5 at 595 nm.

For greenhouse experiments, technical grade formulations (TGF) were prepared by adding cell suspensions to a preparation containing previously autoclaved natural polymers solution at 1.2% (patent pending).

2.3 In vitro and in vivo antagonism

2.3.1. Inhibition of germination of conidia of *E. necator*

The bacterial isolates were grown in 100 mL LB broth in a 250 mL Erlenmeyer flask for 48h. Half of the bacterial suspensions were stored at 6 ºC, while the second half was centrifuged at 12.000g for 20 min and filtered in 0.22 μm cellulose nitrate filter, obtaining a filtered bacterial extract (FBE). A 20 mL FBE aliquot was autoclaved at 121 ºC for 15 mins (AFBE).

The bioassay evaluating the inhibition of conidia germination was performed on 96 wells plates using a modified version of the method described by Burruano et al. (2008). Each well received a suspension containing 100 μl of the conidial suspension with 10^6 conidia mL^{-1} and 100 μl of bacterial suspension (DO_{595}= 0.5), FBE, AFBE, or sterilized distilled water, as the control treatment. Conidia were examined under light microscopy for germ tube emission, counting 100 spores per well and three wells per treatment. After inoculation, the plates were maintained in BOD (26 °C; 24 h in the dark) to stimulate germ tube emission. Conidia were considered germinated when the presence of a germ tube was observed. Chloramphenicol (10 μg $1mL^{-1}$) was added to the filtered extract to prevent the growth of opportunistic bacteria during the experiments.

The experiment was repeated three times, and the conidial germination inhibition index (GII) was calculated according to the formula proposed by Manici et al. (1997):

$$GII = \frac{T_i - T_c}{T_c} x100$$

Where Tc = conidia germinated in the Control and Ti= conidia germinated in the treatments.

2.3.2. Production of volatile organic compounds

Production of antifungal volatile organic compounds (VOCs) was evaluated using the sealed plate method (Arrarte et al., 2017). The treatments were: a control treatment (ADE), LCB28, and LCB30. The *Bacillus* isolates were cultivated in nutrient agar medium (NA) (Himedia; Mumbai) in a Petri dish and incubated in BOD (28 °C; 24h) to ensure high bacterial biomass and the production of volatile compounds. Plates in the control treatment received only autoclaved distilled water (ADW).

Leaves with fresh lesions and high conidial sporulation were carefully printed on a plate containing agar-agar medium (agar 20 g; 1.0 L H_2O) and disposed of in the top plate, leaving only the conidia adhered to the medium. The plates were fixed and impermeabilized with parafilm M tape, and incubated for 16 h in the dark in BOD at 26 ºC and 70% RH. Conidia germination was observed in an optical microscope (400x), and the germination inhibition index (GII) was calculated as described in item 2.3.1. The experiment was conducted using three replications and was repeated three times.

2.4 Characterization of the Bacillus isolates

The assays were carried out by inoculating 10 μL of an overnight culture of the isolates grown in LB medium (OD_{595} 0.5) to 96-well microplates with LB medium modified to test different environmental and nutritional stressors. A blank treatment consisted of a non-inoculated LB medium amended with the additives to correct their effect over the OD. Overnight cultures' absorbance changes were determined with a Multiskan GO reader (Thermo Scientific) at 595 nm with 15 s agitation before measurement. The experiments were replicated five times for each microplate and repeated twice using fresh-grown bacterial cultures.

2.4.1. Bacterial growth using different C sources

The ability to metabolize different carbon sources was evaluated using the commercial API® 20 NE kit (BioMérieux, France). The bacterial suspensions were calibrated (1×10^9 UFC mL^{-1}) and placed in wells containing carbon sources (D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid).

The interpretation of the observed results followed the recommendations provided by the manufacturer.

2.4.2. Effect of pH and nutrient requirement of *Bacillus* isolates

The pH of the LB medium was adjusted from 7.0 (control) to 4.0 using hydrochloric acid 6.0 N, and from 7.0 to 9.0 with KOH 1.0 N. The ability to grow using non-organic sources of nitrogen was tested using the same concentrations of potassium nitrate (K_2NO_3) and ammonium sulfate ($(NH_4)_2SO_4$). Carbon and nitrogen requirements were evaluated using growing yeast extract and glucose concentrations at 0; 100; 500; 1000; 2500; 5000; and 10000 mg L^{-1}. The N source in the medium was altered to KNO_3 1.0% (w/v) while testing the glucose requirement. Minimal nutritional requirements were established as the minimal C and N concentrations required to achieve a 25% increase in OD_{595} overnight. The optimal pH growth range was defined as the pH range achieving more than 80% of the maximal OD_{595}.

2.4.3. Biofilm production

Biofilm production was evaluated using a methodology adapted from (Fall et al., 2004), replacing the original culture medium with liquid LB medium (10 g tryptone, 5 g yeast extract, and 5 g KCl per liter). Two-hundred microliters of LB medium previously inoculated with one of the *Bacillus* isolates was transferred to a 96-wells polystyrene microplate and incubated at 27(±2) °C overnight. The bacterial suspension was extracted, and the microplates were gently washed using sterile autoclaved water. Bacterial cells adhered to the tube walls were stained by adding 200 μL of a crystal violet 0.1% (w/v) solution for 20 minutes, followed by washing with distilled water. The remaining crystal violet absorbed by the biofilm was solubilized using 200 μL acetic acid 0.1 N. The absorbance was determined using a Multiskan GO reader (Thermo-Fischer Scientific) at 550 nm with 15 s of agitation before measurement.

2.4.4. Production of surfactant

Biosurfactant activity was tested using the emulsification index (Iqbal et al., 1995) and droplet collapse (Youssef et al., 2004). Three mL of the supernatants were transferred to assay tubes added with olive oil and vortexed for 2 minutes to evaluate the emulsification index (E_{24}). The emulsion was equilibrated overnight at room temperature, and the emulsification layer was measured. Aliquots from a culture of each strain were analyzed in triplicate in two separate experiments.

In the droplet collapse assay, the supernatant bacterial culture grown in LB liquid medium was mixed with tartrazine black 0.02% (w/v), and 20 μL droplets were spotted on a parafilm surface. Distilled water and BM medium were used as a negative control, while Triton X-100 0.1% as a positive control. Biosurfactant presence was confirmed based on changes in the area (A) of the droplets.

2.5 Antimicrobial peptides biosynthesis genes

Six sequences were chosen from the coding regions of *bmyB* (bacillomycin L synthetase B), *fenD* (fengycin synthetase), *ituC* (iturin A synthetase C), *srfAA* (surfactin synthetase subunit 1), *bacA* (bacilysin biosynthesis protein), and *spaS* (lantibiotic subtilin) (Mora et al., 2011). PCR was carried out in a total volume of 10 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (Invitrogen Technologies), 0.2 μM of each primer, 1.0 U of Taq DNA polymerase (Biotools), and 1.0 μl of genomic DNA. The cycling conditions for the amplification of all targets were as follows: 95 ºC for 4 min, 40 cycles of 94 ºC for 1 min, annealing temperature for 1 min, and 70 ºC for 1 min. A final extension step at 70 ºC for 5 min was followed by a 4 ºC soak. The annealing temperature was set to 58 ºC for *fenD*, *ituC*, *srfAA*, *bacA*, and spaS, and 55 ºC for *bmyB*. The amplification products were analyzed in a 1.8 % agarose gel in 1× Tris-acetate EDTA (TAE), run for 45 min at 90 V, and viewed after staining with Sybrgreen (Bio-Rad Laboratories, USA). Size comparisons were made with a 1-kb plus ladder (Invitrogen, USA).

2.6 Control efficiency in greenhouse experiments

Healthy grape seedlings cv. ' Red Globe ' were planted in pots containing 5 kg Yellow Argisol soil, which was sieved and mixed with 10% (w/w) manure. Ten plants were sprayed weekly with different treatments when they reached five leaves with an average transect larger than 5.0 cm. The treatments were: 1. control treatment sprayed only with distilled water; 2. Triflumizole at 0.80 g L^{-1}; 3. TGF containing 10^7 cells mL^{-1} of LCB28; and 5. LCB30.

Treatment spraying was performed using an electric handheld sprayer with a standard hollow cone nozzle (flow rate 120 mL min^{-1}). After spraying, the plants were divided into groups containing all treatments and evenly distributed in the greenhouse. The inoculation of the pathogen occurred naturally, distributing two plants with high incidence and severity of powdery mildew to each group (Punja et al., 2019). The position of the inoculum-producing plants was changed daily throughout the experiment to ensure

the homogeneous distribution of GPM inoculum. The experiment was repeated twice, and the data were pooled to ANOVA.

2.6.1. Evaluating the incidence and severity of GPM

GPM incidence and severity were evaluated weekly in all plants' leaves. Disease severity was assessed as the percentage of injured leaf area using a diagrammatic scale described by Buffara et al. (2014). The incubation period (IP) was defined as the period until detecting the first symptomatic leaf in the treatments. Severity data were used to calculate the area under the disease progress curve (AUDPC), according to Madden et al. (2007). The apparent infection growth rate (r) was estimated using the procedure defined by (Kushalappa & Ludwig, 1982). The control efficiency (E%) was estimated based on the percentage reduction of the injured leaf area at the end of the experiments.

2.6.2. Characterization of GPM damage and inoculum production

At the end of the second experiment, leaves with fresh wounds were collected from plants of all treatments to evaluate their effect on GPM development using the procedure described by Li et al. (2019). Leaf fragments with GPM lesions were cut with a scalpel and boiled in 96% ethanol for 10 min. Discolored fragments were dipped for two minutes in a dye solution containing 10 mL lactic acid (85% W/w), 10 mL glycerol (99%), 40 mg trypan blue, 10 mL ethanol, and 20 mL distilled water. They were rinsed with distilled water, and excessive dye was removed with 96% ethanol. Dyed fragments were observed under an optical microscope (ZM 200, Leica Optical Systems).

2.6.3. Conidia viability after treatment with ACB and fungicides

Leaves with sporulated lesions were collected from all treatments and immediately processed in the laboratory to determine the viability of *E. necator*. Conidia viability was evaluated by printing the lesions in agar-agar as described in item 2.3. Conidia germination was counted in an optical microscope (400×), and data were used to estimate the conidial germination inhibition index (GII).

2.7 Data processing and statistical analysis

2.7.1. Characterization of bacteria isolates

Nutrient and pH requirements and the effect of salt and osmolites on bacterial growth were estimated by the equation $G\% = \frac{(ODT_{24} - ODT_{\emptyset})}{(ODC_{24} - ODC_{\emptyset})} \times 100$, where *ODC* and *ODT* are the optical density for the control and the amended medium, respectively, at zero (∅)

and 24 hours. The emulsification index in the surfactant production assays was calculated by $E_{24} = \frac{E}{T} \times 100$, where E is the emulsion's height, and T is the mixture's total height. Similarly, a drop collapse index was calculated by $C = \left(1 - \left(\frac{D_t}{D_m}\right)\right) \times 100$, where D is the droplet diameter with strain filtrate (t) and the culture medium (m).

2.7.2. GPM control experiments

Data were evaluated for the analysis of homoscedasticity and homogeneity of variance, respectively. Percentage data were arcsin transformed using the equation, and AUDPC data were $\log_{10}$ transformed. However, tables and figures show the original mean and standard deviation. Data were submitted to ANOVA, and the treatment means were compared using the Tukeys' test ($p>0.05$).

3 Results

3.1 Effect of Bacillus isolates over conidial germination

3.1.1. Co-cultivation

The effect of co-cultivation and filtered extracts of *Bacillus* isolates LCB28 and LC30 were evaluated for their ability to inhibit *E. necator* conidial germination in *in vitro* bioassays. In the first bioassay, the conidia were extracted from freshly collected symptomatic leaves and co-cultivated in liquid culture medium with LCB28 and LC30. Co-cultivation resulted in 97.8% germination inhibition when conidia were paired with LCB28 and 78.2% when co-cultivated with LCB30 (Fig. 1). Most conidia exposed to both actively growing bacteria showed altered morphology. Alterations were observed by apparent cytoplasm aggregation and organelles morphology alteration (Fig. 2a).

Fig. 1. Effect of the co-cultivation and the inoculation into filtered crude extract (FCE), autoclaved FCE (AFCE), and volatile organic compounds (VOCs) of *Bacillus sp.* strain LCB28 and LCB30 on the conidial germination (GII) of *E. necator*.

3.1.2. Effect of filtered extracts

E. necator conidia germinated profusely in the control treatment, but LCB28 and LCB30 produced antifungal compounds that reduced conidia germination. Incubation in FBE resulted in lower GII and cell wall and membrane alteration, and protoplasm showed a coagulated appearance. Similarly, there was lower conidial germination when incubated

in autoclaved FBE, indicating that the antifungal compounds are thermotolerant. However, while AFBE resulted in similar conidial germination, FBE of the LCB28 strain showed the highest GII (Fig. 1).

Fig. 2. Optical microphotography showing morphological alterations caused by co-inoculation and cultivation of *E. necator* conidia with active growing *Bacillus* sp. isolates and their extract. a. conidia co-cultivated with LCB28 showing degraded cell wall and disintegrated protoplasm; b. EB LCB30 (conidia with disintegrated protoplasm); c. Control.

3.1.3. Production of VOCs

Conidial germination was abundant in the control treatment in the sealed plate test evaluating VOC production. In this assay, the bacteria were pre-incubated for 24 h to allow maximal production of VOCs given the short duration of the experiment. In this condition, there was a significant reduction in the germ tube emission of conidia exposed to the VOCs produced by both isolates. LCB30 showed a higher GII of 54.63%, while LCB28 showed a lower GII of 34.0% (Fig. 1).

3.2 PCR detection of antimicrobial peptides genes

A PCR test based on oligonucleotide primers designed to detect genetic markers was applied to identify the potential of the isolates to produce six antimicrobial peptides known to be produced by *Bacillus* species. Among the six antibiotics screened, the subtilisin (*spaS*) marker was not detected in either strain. The PCR runs showed that four markers were amplified for *B. tequilensis* LCB28: bacyllomicin (*bmyB*), fengycin (*fenD*), bacilysin (*bacA*), and surfactin (*srfAA*) (Fig. 3A), while *B. siamensis* LCB30 showed the same markers plus *ituC*, a gene associated with iturin production.

Fig. 3 – Electrophoresis of PCR products showing the distribution pattern of antimicrobial peptides markers of *B. tequilensis* LCB28 (A) and *B. siamensis* LCB30 (B). The codes refer to markers for the gens: *bmyB* (bacillomycin L synthetase B), *fenD* (fengycin synthetase), *ituC* (iturin A synthetase C), *srfAA* (surfactin synthetase subunit 1), *bacA* (bacilysin biosynthesis protein), and *spaS* (lantibiotic subtilin).

3.3 Partial characterization of the Bacillus isolates

3.3.1. Nutrient and pH requirement

The two isolates were able to metabolize most carbon and nitrogen sources tested. However, LCB30 did not metabolize arabinose, and both isolates did not grow using capric acid, adipic acid, and phenyl-acetic acid (Table 1).

Table 1 – Ability of *Bacillus* spp. isolates to metabolize different carbon sources.

Both isolates grew at a relatively low concentration of glucose and yeast extract and grew in culture media containing glucose and using $(NH_4)_2SO_4$ and K_2NO_3 as the sole nitrogen source, doubling its OD at 24 h in relatively low concentrations of these compounds (Table 2). However, LCB28 showed the lowest requirements of C and N to double the OD_{595} in 24 hours. The isolates could grow at pH 5.0 and showed higher tolerance to slightly alkaline conditions, obtaining 80% of the maximal growth at an extensive range (5.0 - 8.0).

3.3.2. Biofilm production

Both isolates produced biofilm on the plastic surfaces of the 96-well plates (Table 2). However, while LCFB28 and LCB30 were able to grow in slightly alkaline conditions, there was no detection of planktonic cells at pH 8.0. On the other side, LCB30 showed the highest production of surfactants both by the drop-collapse and the emulsification indexes (E_{24}) techniques (Table 2).

Table 2 – Partial characterization of *Bacillus* spp. isolates antagonist to *E. necator* on the minimal requirement of carbon and nitrogen, use of a mineral form of nitrogen, optimal growth pH range, biofilm, and surfactant production.

3.4 GPM control in greenhouse experiments

Two experiments using potted grape plants (cv. Red Globe) were conducted to evaluate GPM control efficiency. All treatments did significantly interfere with incidence ($F_{3;\ 44}$= 53.054; $p< 0.001$), apparent disease progress ratio ($F_{3;\ 44}$ = 65.493; $p< 0.001$), incubation period ($F_{3;\ 44}$ = 16.059; $p< 0.001$), and severity ($F_{3;\ 44}$ = 81.85; $p< 0.001$). In general, the treatments significantly affected all variables analyzed compared to the control (Table 3). However, they more effectively reduced the injured area registered during the experiments. The weekly application of triflumizole showed a higher incubation period according to the Tukeys' test ($p< 0.05$). The disease progress rate was

significantly lower than control to all treatments (Tukeys' test, $p<0.05$), which, together with the extension in IP, resulted in significantly lower disease severity and AUDPC according to Tukey's test ($p< 0.05$). While triflumizole reduced 78.2% of incidence, LCB28 and LCB30 showed 50.6% and 66.1%, respectively. On the other hand, the treatments showed more than a 90% reduction in the average injured areas of leaves.

Table 3 – Incidence (%), severity (McKinleys' disease index), the incubation period (IP), apparent disease progress ratio (r), and area under disease progress curve of GPM in potted grape plants in greenhouse conditions with natural infection. All data are the average of two experiments.

2.4.1. Conidia viability in grape leaf lesions

Mycelial growth and conidia production in the GPM lesions at the end of the greenhouse experiment were examined in microscopy. The control treatment showed a large number of cells with necrosis symptoms characterized by the absorption of trypan blue by necrotic cells. Many conidia were recovered from lesions in the control treatment (Fig. 3), and the conidial germination rate in the control treatment was 76.3% (±3.05), on average. In contrast, no conidia were recovered from the lesions collected from plants treated with triflumizole.

The application of both bacteria isolates showed similar results, with a strong reduction in the germination of *E. necator* conidia recovered from the lesions. The germination inhibition index obtained for *B. tequilensis* LCB28 was 89.79% (±4.85) and for *B. siamensis* LCB30 91.58 (±5.82)

Fig. 4. Photomicrography (400× magnification) of leaf tissues from GPM lesions on grape leaves colonized by *E. necator*: A. control treatment with a profuse mesh of fresh mycelia and conidiation; b. Lesions from plants treated with *B. tequilensis* LCB28 with fewer conidiophores and conidia; c. The surface of lesions from plants treated with *B. siamensis* LCB30 with a rarefied mesh of mycelia and conidiophore and a lower number of lesioned cells; d. Smaller lesions from plants treated with triflumizole, with no conidiophore and conidia production.

4 Discussion

Bacillus is a phenotypic and phylogenetically diverse genus characterized by producing endospores in unfavorable environments and showing wide nutritional and

growth requirements plasticity (Zeigler and Perkins, 2021). In a previous study, using 16S rDNA sequence analysis, we identified the *B. tequilensis* LCB28 and *B. siamensis* LCB30 isolates as potential biocontrol agents against GPM (Santos et al., 2023). *B. siamenis* is a species phylogenetically close to *B. amyloliquefasciens* (Sumpavapol et al., 2010), and both are known to produce volatile and cyclic lipopeptides with a broad antifungal spectrum (Xu et al., 2018). *B. tequilensis* is phylogenetically close to *B. subtilis*, and an emergent interest in the species has been driven by its ability to control diverse fungal pathogens (Kwon et al., 2022; Zhou et al., 2021).

In this work, both *B. tequilensis* LCB28 and *B. siamensis* LCB30 significantly reduced the germination of *E. necator* conidia in the experiments using co-inoculation, filtrated, and autoclaved extracts. They also inhibited conidia germination by producing volatile organic compost. Considering germination tube emission and haustoria production a crucial phase in initiating the infection process in the abaxial surface of the phylloplane, bacterial growth and disruption of germ tube emission may become essential characteristics in a biocontrol agent of GPM. *Bacillus* species can synthesize and secrete antifungal metabolites (Cortés-Camargo et al., 2021; Miljaković et al., 2020). In fact, their ability for indole-acetic acid (IAA) synthesis, the use of diverse carbon sources, and a multicellular lifestyle involving motility, biofilm formation, quorum sensing, competence, and sporulation suggest that these species are adept at colonizing plant surfaces (Cuellar-Gaviria et al., 2023; Gorai et al., 2021). Jiao et al. (2021), e. g., showed that inhibiting the conidia germination of *Erysiphe cichoracearum*, the cyclic peptides bacillomycin D and fengycin are an essential mechanism applied by *B. amyloliquefaciens* YN201732 to control tobacco PM. In this work, even after heat treatment (121 °C, 15 min), the filtrate extracts of both isolates maintained antifungal activity, indicating the presence of thermotolerant cyclic peptides.

In addition to antibiosis, these *Bacillus* spp. can produce lytic enzymes that degrade the cell wall of pathogenic fungi, such as chitinases and β-1,3-glucanase (Jeong et al., 2017). This study did not evaluate the production of lytic enzymes, however, microscopic observations showed that conidia had degraded cell membranes and disintegrated protoplasmic content. A similar effect on conidia, conidiophores, and hyphae of *Podosphaera xanthii* was observed through microscopy by Hafez et al. (2020) after applying *Bacillus subtilis* for biological control of powdery mildew in cucumber. In another experiment, LCB28 and LCB30 produced volatile organic compounds that inhibited the conidial germination of *E. necator*. Volatile antifungal compounds could be

a mechanism to inactivate *E. necator* conidia, which is important for protecting grapevines against secondary infections (Sellitto et al., 2021).

The potential production of antimicrobial peptides by LCB28 and LCB30 was confirmed by markers linked to six of the most known antimicrobials synthesized by *Bacillus* species. *B. tequilensis* LCB28 fit in one of the common genotypes described by Mora et al. (2011), testing positive or surfactin, bacyllomicin, fengycin, and bacilysin markers, while *B. siamensis* LCB30 showed a fifth marker for iturin (*ituC*). According to the author, the dominance of these particular genes in *Bacillus* isolates associated with plants reinforces the competitive role of cyclopeptides in their biocontrol fitness. Bacillus species' ability to produce more than one peptide has commonly been reported (Caulier et al., 2019; Jiao et al., 2021; Li et al., 2019). In the studies conducted by Mora et al. (2011) in the Mediterranean region of Spain, the authors reported that most isolates tested had between two and four antimicrobial peptide genes, whereas isolates with five or more genes were seldom detected. However, Vinodkumar et al. (2017) and Dong et al. (2023) reported *Bacillus* sp. isolates with positive for 12 markers for antifungal secondary metabolites.

Biosurfactants are amphiphilic molecules that reduce water's surface tension, which allows its detection by simple methods, and are effective antibiotics. The most known biosurfactants produced by *Bacillus* sp. were classified into two main groups, lipopeptides, and glycolipopetides (Goswami & Deka, 2021). They act on phospholipids and sterols on the cell membrane, disturbing its' permeability and causing cytotoxicity (Kaspar et al., 2019). Both methods applied in this study showed that *Bacillus* isolates LCB28 and LCB30 produced biosurfactants, and it could be a second mechanism of action interacting with the antibiotics to inhibit mycelial growth or cause conidial germination of *E. necator* recovered from the surface of injured leaves observed in the microscopic level (Goswami & Deka, 2021). Lipopeptides shown to be potentially produced by LCB28 and LCB30 by the genetic markers have an amphiphilic nature and act damaging fungal wall integrity (Wang et al., 2022). These peptides can be a key mechanism of action in the antagonism as defined for the antagonistic interaction of *B. subtillis* and *Podosphaera fusca*, the causal agent of cucurbit powdery mildew (Romero et al., 2007).

According to Santos et al. (2021), an essential requirement to develop a successful biocontrol system is that the ACB has a high degree of rhizosphere and phyllosphere competence. Colonizing leaf and root tissues is undoubtedly an object of intense research.

In contrast to the rhizosphere, the phyllosphere is an oligotrophic environment where nutrients are scarce and show a heterogeneous nature (Vorholt, 2012). Therefore, together with adaptations to resist abiotic stress, metabolic adaptation to explore environments with low and diverse carbon (C) and nitrogen (N) availability, including inorganic forms of N, could increase the success of a potential biocontrol agent to multiply and occupy the niches available on leaves surface (Beattie, 2011; Jacobs et al., 2005). *B. siamensis* LCB30 showed a lower C requirement and versatility in using inorganic sources of N. LCB30 also grew preferentially on slightly acidic to slightly alkaline pH conditions, while LC28 showed a growth decline at a pH higher than 7.0. Both species were able to produce biofilm, but LCB28 produced biofilm in a higher range of pH, which could be favorable to colonizing an environment exposed to abiotic stressors (Chaudhry et al., 2021). Altogether, these characteristics suggest LCB30 is well adapted to occupy niches on leaf surfaces.

Based on the results, *B. tequilensis* LCB28 and *B. siamensis* LCB30 proved an effective biocontrol agent against *E. necator* in grapevine cv Red Globe grown in a greenhouse. This success is likely linked to their ability to produce diverse water-soluble and volatile antifungal compounds, as shown by the PCR-based detection of cyclopeptide gene markers. *E. necator* sporulation and mycelial growth were partially inhibited in leaves treated with both bacterial isolates during the experimental period. Furthermore, our analysis revealed a high percentage of non-viable conidia (GII% > 80) when we examined the conidia viability in the lesion surfaces, indicating that secondary infections will be compromised even if the pathogen completes its infectious process. The diversity of potential antimicrobial synthesis, biofilm production, ability to metabolize diverse carbon sources, and a low nutrient requirement to grow supports their adaptation to thrive within the grape phyllosphere environment and their potential as biocontrol agents against the GPM caused by *E. necator*.

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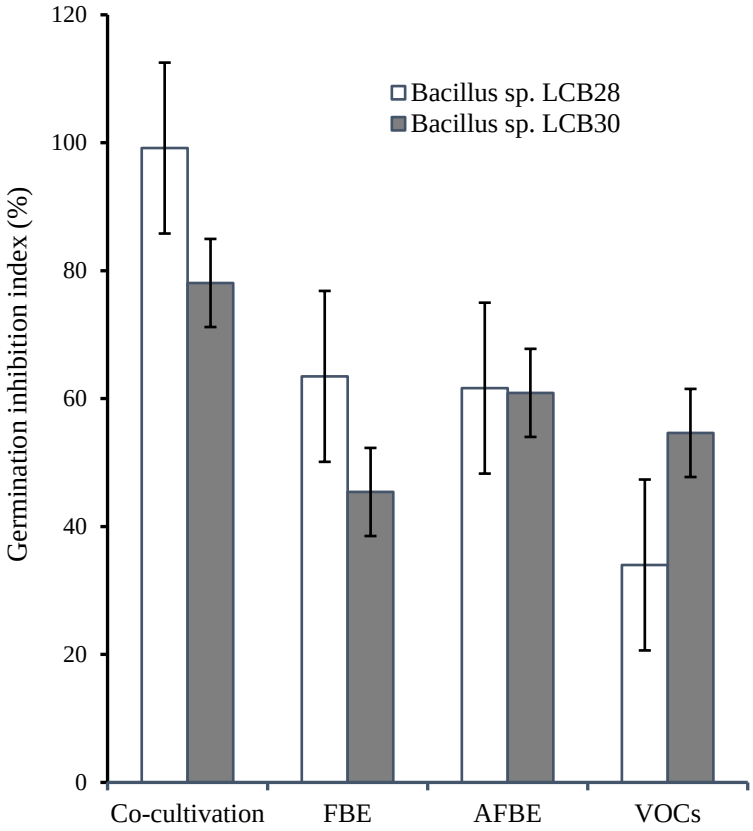

Fig. 1. Effect of the co-cultivation and the inoculation into filtered crude extract (FBE), autoclaved FBE (AFBE), and volatile organic compounds (VOCs) of *Bacillus* isolates LCB28 and LCB30 on the conidial germination (GII) of *E. necator*.

Table 1 – Ability of *Bacillus* spp. isolates to metabolize different carbon sources.

Bactérias	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
LCB28	+	+	+	+	+	+	+	-	-	+	+	-
LCB30	+	-	+	+	+	+	+	-	-	+	+	-

GLU: D-glucose; ARA: L-arabinose; MNE: D-manose; MAN: D-manitol; NAG: N-acetil-glucosamine; MAL: D-maltose; GNT: gluconate; CAP: ácido cáprico; ADI: adipic aid; MLT: ácido málico; CIT: trissodium citrate; PAC: phenyl-acetic acid.

Table 2 – Partial characterization of *Bacillus* spp. isolates antagonist to *E. necator* on the minimal requirement of carbon and nitrogen, use of a mineral form of nitrogen, optimal growth pH range, biofilm, and surfactant production.

Bacillus spp.	Glucose	N-Org	NH_4	NO_3	pH[b]	Biosurfactant		Biofilm	
	(mg L^{-1})[a]		OD_{595}			E[c]	A_{24}[d]	pH[e]	OD_{595}[f]
LCB28	420.0 (22.0)	215.0 (29.0)	0.501	0.433	5 – 8	16.57(4.8)	2.61 (1.4)	5 - 7	0.147 (0.01)
LCB30	550.0 (27.0)	335.0 (15,2)	0.385	0.207	5 – 8	19.00(0.9)	4.76 (1.7)	5 - 7	0.138 (0.03)

[a]Mininimal concentration (mg L^{-1}) required to double the OD_{595} in 24 h; [b]pH range for optimal growth; [c]E24: emulsification index; [d]A_{24}: Scattering area (mm^2); [e]pH range producing biofilm; [f]OD_{595} obtained obtained at pH 6.0.

Table 3 – Incidence (%), severity (McKinleys' disease index), the incubation period (IP), apparent disease progress ratio (r), and area under disease progress curve of grape powdery mildew in potted grape plants in greenhouse conditions with natural infection. All data are the average of two experiments.

Treatment	Incidence (%)	Severity (Mackinleys' DI)	IP (days)	r (%/day)	AUDPC
LCB28	47.88 (20.35) b	1.58 (0.57) b	12.25 (6.5) a	0.04 (0.02) b	39.16 (12.67) b
LCB30	32.90 (19.63) b	1.27 (0.66) b	16.75 (9.27) a	0.03 (0.02) b	29.35 (10.43) b
Triflumizole	21.10 (13.57) b	0.87 (0.29) b	25.60 (4.59) b	0.01 (0.01) b	21.60 (4.59) b
Control	96.91 (4.64) a	29.62 (6.84) a	7.33 (1.67) a	1.11 (0.46) a	562.60 (76.61) a

Percentage data were *arcsin* transformed, AUDPC and IP were $\sqrt{x}$ transformed for ANOVA, but shown as untransformed mean values. Numbers with the same letters in the columns did not differ by the Tukey test ($p < 0.05$).

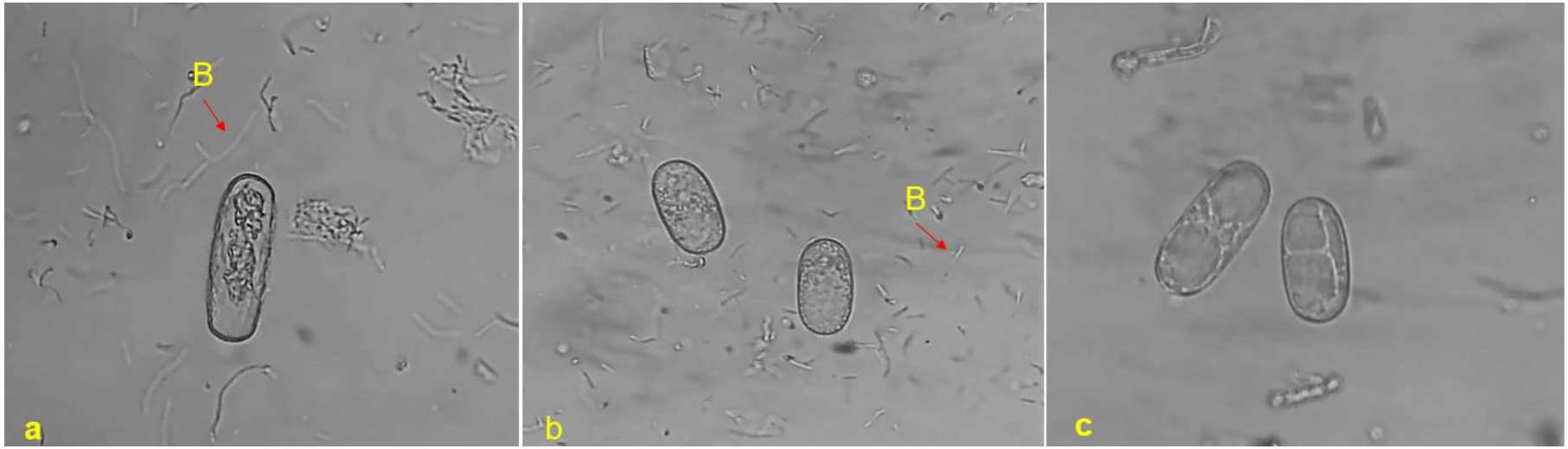

Fig. 2. Optical microphotography showing morphological alteration on the conidia of *E. necator* co-cultivated with *B. tequilensis* LCB28 and *B. siamensis* LCB30. a. Conidium co-inoculated with LCB28 showing alteration in the cell wall and protoplasm; note the lack of vacuoles and organelles disintegration; b. Conidia exposed to LCB30 also show protoplasmatic and organelles alteration and the lack of vacuoles; c. Conidia exhibiting normal morphological appearance from the control treatment. B. bacteria cells.

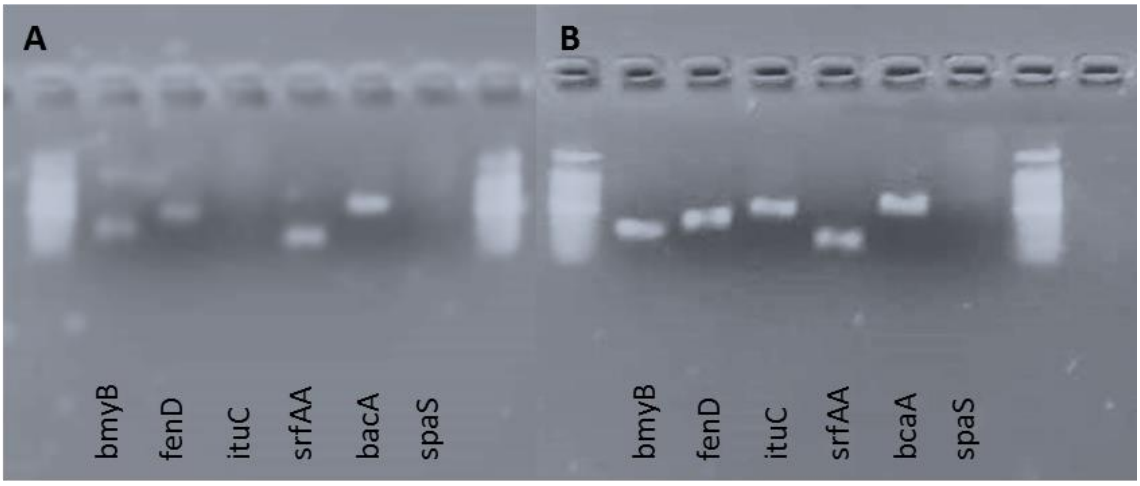

Fig. 3 – Electrophoresis of PCR products showing the distribution pattern of antimicrobial peptides markers of *B. tequilensis* LCB28 (A) and *B. siamensis* LCB30 (B). The codes refer to markers for the gens: *bmyB* (bacillomycin L synthetase B), *fenD* (fengycin synthetase), *ituC* (iturin A synthetase C), *srfAA* (surfactin synthetase subunit 1), *bacA* (bacilysin biosynthesis protein), and *spaS* (lantibiotic subtilin).

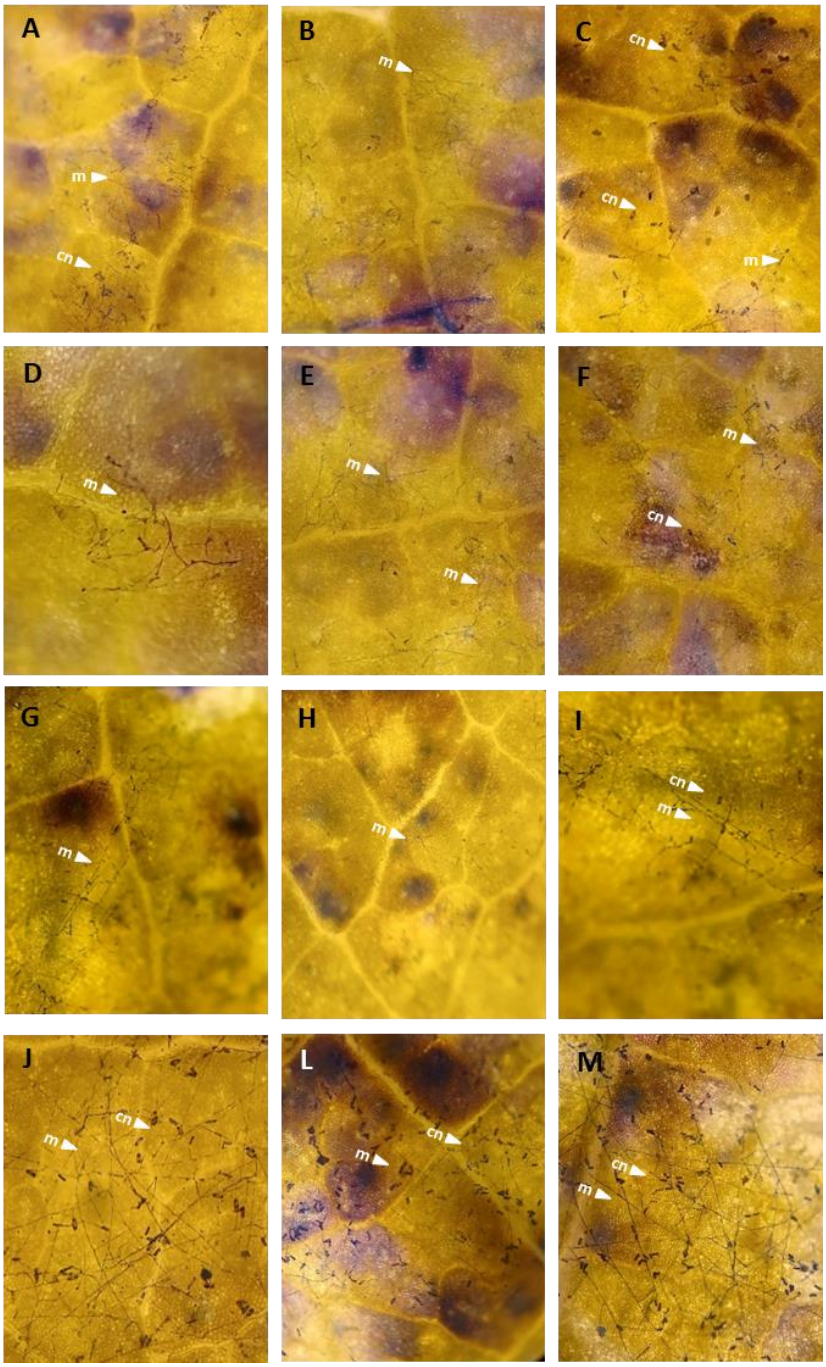

Fig. 4. Photomicrography of leaf tissues from GPM lesions on grape leaves colonized by *E. necator*. A-C. Lesions from plants treated with *B. tequilensis* LCB28 with few conidia and lesioned cells (dyed with trypan blue); D – F. Surface of lesions from leaves treated with *B. siamensis* LCB30 with a rarefied mesh of mycelia and conidia and a lower number of lesioned cells; G – I. Smal lesions in leaves treated with triflumizole, with no conidia cluster; J – M. Control treatment with a profuse mesh of fresh mycelia and conida clusters. Arrow heads indicates the presence of mycelia (m) ou conidia (cn)of *E. necator*.

CAPÍTULO IV

Applying *Bacillus tequilensis* LCB28 and *B. siamensis* LCB30 in the integrated management of grape

Submissão: Crop Protection

Applying Bacillus tequilensis LCB28 and B. siamensis LCB30 in the integrated management of grape powdery mildew

John Lennon Ferreira dos Santos[a], Gabriela Alves Gonçalves[b], Kezia Costa Escobar[c], Taise Oliveira Passos[d], Carlos Alberto Tuão Gava[e]*, Delson Laranjeira[a]

[a]Universidade Federal Rural de Pernambuco - UFRPE, Programa de Pós-Graduação em Fitopatologia - Recife, PE, Brasil

[b]Universidade Federal do Vale do São Francisco – UNIVASF– Petrolina, PE, Brasil

[c]Universidade Estadual de Pernambuco – UPE – Campus Petrolina, PE, Brasil

[d]Embrapa Semiárido, Embrapa/CNPQ Fellowship Program; Petrolina, PE, Brasil

[e]Embrapa Semiárido, Petrolina, PE, Brasil; Autor correspondente

* Corresponding author address:

Embrapa Semiárido

Laboratório de Controle Biológico

Caixa Postal 23 – Petrolina, PE – Brazil

CEP 56.302-970

e-mail: carlos.gava@embrapa.br

Applying *Bacillus tequilensis* LCB28 and *B. siamensis* LCB30 in the integrated management of grape powdery mildew

Abstract

Grape powdery mildew (GPM) caused by *Erysiphe (Uncinula) necator* (Schw.) Burr. is a severe problem in all grape-growing regions of the world, and its control is based on synthetic fungicides. Consumers' concerns about food contamination require efforts to achieve a zero-residue production system involving complementary strategies, such as biological control. This work aimed to evaluate the application of antagonist *Bacillus* isolates in an integrated management approach. Applying *B. tequilensis* LCB28 and *B. siamensis* LCB30 significantly reduced GPM incidence and severity in two greenhouse experiments. The fungicide difenoconazole was highly incompatible with both isolates, significantly decreasing their growth in culture medium at the minimal dose. Meanwhile, the concentration of triflumizole to affect the isolates growth was higher than that recommended by the manufacturer and it was considered compatible. Alternate application of these fungicides and LCB28 and LCB30 in greenhouse reduced GPM incidence by more than 70% and severity by more than 90% in average. The treatments significantly reduced conidial production and viability on the lesions' surface. Two field experiments showed that GPM incidence and severity were significantly reduced by applying formulations containing *B. tequilensis* LCB28 or *B. siamensis* LCB30 in an integrated management strategy that substituted the spraying of synthetic fungicides except during flowering and initial fruit growth that are highly favourable phenological phases.

Key-words: *Vitis vinifera* L.; *Erysiphe necator*; Field Efficiency; Application strategy; Biological control

1. Introduction

Most table grape varieties (*Vitis vinifera* L.) are susceptible to many cryptogamic diseases causing severe economic losses (Li et al., 2019; Sawant et al., 2017). Grape powdery mildew (GPM), i. e., is a severe problem in all grape-growing regions of the world ((Mieslerová et al., 2022). GPM is caused by the obligate biotrophic ascomycete *Erysiphe necator* (Schw.) Burr.) (Erysiphales, Erysiphaceae). Pathogenesis interaction between *E. necator* and grapevine is highly evolved and complex (Braun and Takamatsu, 2013).

Rains at the beginning of the grape growing season trigger ascospores released from cleistothecia starting powdery mildew epidemics in temperate regions. However, sexual stages do not occur in tropical areas (Jarvis et al., 2002). In the tropics, primary inocula originate from infection of prophylls within the bud, branches, and wind-dispersed conidia from neighboring Fields. Epidemic outbreaks are favored by seasonal warm and humid nights and cloudy Weather, and all green tissues can be infected (Bettiga et al., 2013; Jarvis et al., 2002). In the tropics, epidemics are severe under mild temperatures, high air humidity, and cloudy weather (Bettiga et al., 2013; Ghule et al., 2019). Conidium germinates on the surface of a susceptible host to form a germ tube ending in a lobed appressorium with a penetration peg which drives the penetration of epidermal cell walls, followed by the production of a haustorium (Gadoury et al., 2012). GPM colonies proliferate and originate a superficial mycelium hyphal mesh bearing appressoria with penetration pegs that taps into further host cells and produce conidial chains, culminating with the production of a network of necrotic cells (Mieslerová et al., 2022).

In general, control of grape powdery mildew is mainly done with synthetic fungicides belonging to the chemical groups of triazoles, strobilurins, and sulfur (Gadoury et al., 2012, Sawant et al., 2017). However, their application during fruit ripening increases the risk of contamination above the legal maximum residue limit (MRL). Therefore, an extensive effort has been made to achieve a zero-residue production system. This system includes increasing complementary strategies to chemical control, such as biological control (Brader et al., 2017; Furuya et al., 2011). Consequently, the market for microbial biocontrol agents (BCA) based pesticides has grown substantially (Koul, 2023).

Biofungicides applications are slowly becoming part of integrated disease management programs (IDM) in the fruit and vegetables segment (F&V) combined with synthetic and alternative fungicides. Insecurities have restricted its general use given the high production costs in the F&V segment and the risk that the sole application of biological control agent (BCA) does not reach the expected control efficacy in the field (Abbey et al., 2019). Therefore, combining BCA with plant-derived and synthetic fungicides is recommended to maximize disease control, allowing cut fungicide application (Berrie et al., 2022; Berrie and Xu, 2021). For example, Furuya et al. (2011) reported that *B. subtilis* KS1 was tolerant to a group of fungicides used against *P. vitícola* in grapes. Similarly, Xu et al. (2022) reported a synergistic effect of *B. amyloliquefasciens* SDTB009 and the fungicide difenoconazole, suggesting their use both in rotation and as a mixture to control Fusarium wilt in tomato (*Solanum lycopersicum* L).

Bacteria of the genus *Bacillus* have been studied as biocontrol agents for aerial and soil-born plant pathogens, becoming the most important BCA currently marketed (Shafi et al., 2017). These BCA produce several antibiotic compounds able to control a broad spectrum of plant pathogens (Caulier et al., 2019; Li et al., 2019). In previous studies, PCR-based detection of gene markers showed that *B. tequilensis* LCB28 and *B. siamensis* LCB30 could produce antimicrobial cyclopeptides, and they produced hydrosoluble and volatile antifungal compounds that inhibited conidial germination *in vitro* (Santos, 2023). The authors also showed a significant reduction in GPM incidence and severity in greenhouse experiments and that applying these potential BCAs reduced the germination of conidia recovered from grape leaves. Therefore, this work aimed to evaluate the control efficiency of GPM by combining the application of antagonist *Bacillus* isolates and chemical fungicides in an integrated management approach.

2. Material and methods

2.1. Production of the inoculum of E. necator

Powdery mildew inoculum was obtained from naturally infected leaves of *Vitis vinifera* cv. 'Sugraone' and cv. Thompson Seedless from a vineyard in the Experimental Farm of Embrapa (Petrolina, Brazil). A conidial suspension of *E. necator* was extracted from heavily sporulated lesions, standardized at 10^7 conidia mL^{-1}, and inoculated onto plantlets of grape cv. 'Sugraone'. The plants were kept at 26 (±1) °C and 70% relative humidity (R.H.) in a growth chamber with 12 hours of photoperiod. After the initial symptoms were observed, infected plants were transferred to a greenhouse.

2.2. Bacillus isolates cultivation and formulation

Bacillus tequillensis LCB28 and *B. siamensis LCB30* were maintained at -80 °C. They were inoculated in nutrient yeast dextrose agar (NA, Himedia India) after thawing, and kept at 27 °C for 48 h. The bacterial isolates were grown in Luria broth (L.B.) media (Himedia) for 24 h in an orbital shaker (120 rpm) and standardized to an optical density (O.D.) of 0.5 at 595 nm. For greenhouse experiments, technical grade formulations (TGF) were prepared by adding bacterial suspensions to a preparation containing previously autoclaved natural polymers solution at 1.2% (patent pending).

2.3. Control efficiency in greenhouse experiments

Two experiments were executed using two independent groups of potted grape plants cv. 'Sugraone' in greenhouse conditions (26.5 ±4.0ºC, R.H. 60 ±22.5%) with forced ventilation. Ten plants were sprayed with different treatments when their branches reached five leaves with an average transect larger than 5.0 cm. The treatments were: 1. Control treatment sprayed only with distilled water; 2. Micronized sulfur at 1.0 g L^{-1}; 3. *B. amyloliquefaciens* QST713 (Serenade, Bayer CropScience) 10^7 endospores mL^{-1}; 4. *B. tequilensis* TGF LCB2810^7 cells mL^{-1}; 5. *B. siamensis* LCB30 TGF 10^7 cells mL^{-1}. The treatments were applied weekly for four weeks using an electric handheld sprayer with a standard hollow cone nozzle (flow rate 120 mL min^{-1}). The inoculation of the pathogen occurred naturally, distributing two plants with high incidence and severity of powdery mildew groups containing six plants (Punja et al., 2019). The position of the inoculum-plants were changed daily throughout the experiment to ensure a homogeneous distribution of GPM inoculum. The experiment was carried out in a completely randomized design.

2.3.1. Evaluating the incidence and severity of GPM

GPM incidence and severity were evaluated weekly in all plants' leaves. Disease severity was assessed as the percentage of injured leaf area using a diagrammatic scale described by Buffara et al. (2014). The incubation period (I.P.) was defined as the interval to detect the first symptomatic leaf in the treatment. The area under the disease progress curve (AUDPC) was calculated using disease severity data according to Madden et al. (2007). The apparent infection growth rate (r) was estimated using the procedure defined by (Kushalappa and Ludwig, 1982) and the control efficiency (E) was estimated based

on the percentage reduction of the injured leaf area at the end of the experiments using the equation:

$$E = \frac{(A_C - A_{Ti})}{A_C} \times 100$$

Where A= leaf injured area; C= control treatment; Ti= treatments.

2.4. In vitro compatibility of Bacillus and fungicides

The compatibility of *B. tequilensis* LCB28 and *B. siamensis* LCB30 with triflumizol and difenoconazole was evaluated using the poisoned food technique. In the first experiment, the fungicides were prepared in five concentrations: 0.1; 0.5; 1.0; 1.5; and 2.0 times the concentration recommended by the manufacturer. After growth in liquid L.B., the isolates inoculated in melted NA (45-50 ºC), which was transferred to Petri dishes. Filter paper discs (5 mm) were soaked in the fungicide solution and five disks, each represented by a concentration were placed on plates inoculated with the two isolates. The plates were incubated in a growth chamber (28°C) for 24h in the dark. After 24 h, the compatibility between bacterial isolates and fungicides at different doses was determined by observing the formation of an inhibition halo or bacterial growth in the culture medium.

In the second experiment, the bacteria were grown in L.B. media supplemented with the same fungicides concentrations and incubated for 24 h at room temperature (24 ±2 ºC). Bacterial growth was evaluated by measuring their optical density (590 nm) in a Multiskan GO reader (Thermo Scientific) at 595 nm with 15 s agitation before measurement. The experiments were carried out in a completely randomized design with three replications per dose and repeated twice.

2.5. Combining Bacillus isolates and fungicides to control GPM in greenhouse

Grape plants cv. 'Redglobe' were planted into pots containing 5 kg of soil mixed with cow manure (2.5% w/w) and kept in a greenhouse (26.8 ±5.2 °C and 60 - 85% R.H.). Daily irrigation was applied using an automated dripping irrigation system. The experiment started when the plants reached five fully expanded leaves, weekly alternating the application of the synthetic fungicides and the *Bacillus* isolates during five weekly. The treatments were: control sprayed with ADW; LCB28; LCB30; triflumizol 0.80g per L^{-1}; difenoconazole 0.12mL L^{-1}; alternating LCB28 and triflumizol (LCB28 + triflumizol); LCB28 + difenoconazol; LCB30 + triflumizole; LCB30 + difenoconazol.

Treatment spraying was performed using an electric handheld garden sprayer equipped with a standard hollow cone nozzle (flow rate 120 mL min^{-1}). The plants were divided into groups containing all treatments and evenly distributed in the greenhouse. The inoculation of the pathogen occurred naturally, distributing two plants with high incidence and severity of powdery mildew to each group (Punja et al., 2019). The position of the inoculum-producing plants was changed daily throughout the experiment to ensure the homogeneous distribution of GPM inoculum. The experiment was carried out twice in a completely randomized design, with six replicates (one plant per replicate) and all the leaves of each plant were evaluated.

2.5.1. Conidia viability

Leaves with sporulated lesions from plants treated with ACB, fungicides, and their combination were collected and immediately processed for conidia extraction with the aid of a brush and the suspension was standardized at 10^7 conidia mL^{-1} to determine the viability of *E. necator*. Conidia viability was evaluated by inoculating conidia from sporulated leaf fragments in agar-agar as described in item 2.3 and calculating germination inhibition index (GII). Conidia germination was counted in a microscope.

2.5.2. Effect of treatments on lesions colonization

In the 32nd day after starting the experiment, symptomatic leaves were collected from plants of all treatments to evaluate their effect on GPM development using the procedure described by Li et al. (2020). Leaf fragments containing GPM lesions were cut with a scalpel and boiled in 96% ethanol for 10 min. Discolored fragments were dipped for two minutes in a dye solution containing 10 mL lactic acid (85% W/w), 10 mL glycerol (99%), 40 mg trypan blue, 10 mL ethanol, and 20 mL distilled water. They were rinsed with distilled water, and excessive dye was removed with 96% ethanol. Dyed fragments were observed under an optical microscope (ZM 200, Leica Optical Systems), and the lesions were evaluated by observing the development of the mycelium, the production of conidiophores and conidia.

2.6. Applying Bacillus isolates against GPM in the field

Two experiments were conducted in the Experimental Farm of Embrapa Semiárido (Petrolina, Brazil: -9.13740, -40.30212). The first experiment was carried out in five-year-old vines cv. 'Sugraone', cultivated in trellis with 3.0 × 1.0 m spacing,

between April and July (2021). The second experiment was conducted in a four-year-old vineyard cultivated with the cv. 'Crimson' between August and November (2021). Climate variables were monitored using an automatic weather station installed at 120 and 180 m from the experiments.

2.6.1. Preparing tank mix and application

One percent *of Bacillus* experimental and commercial formulations containing 10^9 cells mL^{-1} were dispersed in distilled water added with 0.05% (v/v) silicon surfactant (polysiloxane polyether copolymer) (Silwet® L-77 272 AG, Rhizobacter SA, Argentina) adjusted to pH 6.0. Spraying was performed using a backpack sprayer (Jacto PJH20L, Jacto SA) equipped with a standard solid cone nozzle. The spray volume applied to each plot was equivalent to 400.0 L ha^{-1}. Spraying devices were previously washed with neutral detergent, 70% ethanol solution (v/v), and distilled water.

2.6.2. Vineyard management and treatments

The vineyards were monitored weekly for the occurrence of insect pests and sprayed twice with abamectin (avermectin) and one time with spinetoram to control thrips (*Frankliniella schultzei*) and two-spotted spider mite (*Tetranichus urticae*), respectively. The experiments were conducted in randomized block design, with four replications. The experimental plots contained 15 plants per plot with 3.0 × 1.0 m spacing (67,5 m^2). The BCA TGFs were introduced in the integrated management of GPM in substitution to synthetic fungicides during phenological phases with lower infection risk in the region (Table 1). The treatments were: 1 - control treatment without fungicides; 2. IDM with *B. tequilensis* LCB28; 3 - IDM with *B.* siamensis LCB30; 4 - 1.0% commercial formulation of *B. amyloliquefaciens* QST713; 5 - conventional fungicide treatment. The treatments were sprayed weekly, starting from bud sprouting (Table 1). Given the high inoculum pressure and infection risk, synthetic fungicides were sprayed during the flowering and initial phase of fruit development (until olive size berries) (Table 1). The biological treatments were returned during grape berries' growth and maturation phases. In the first experiment, extraordinary pulverization was performed before flowering. Preventive sprays of micronized sulfur were applied weekly in the conventional treatment until the first symptomatic leaf was detected. Afterward, weekly sprays of the fungicides were applied alternating the active ingredients cyproconazole (Alto100, Syngenta S.A.) 20.0

mL 100 L^{-1}, boscalid + kresoxim-methyl 500 mL 100 L^{-1} (Collis, Basf S.A.), and triflumizole 50 mL 100 L^{-1} (Trifmine, Ihara S. A.).

Table 1. Timing the application of the biofungicides treatments during the different phenological phases in the integrated management of grape powdery mildew in the field experiments.

2.6.3. Incidence and severity of GPM

Evaluation of GPM incidence and severity was conducted in three central plants of each plot according to the following criteria: one basal, median, and apical branch on each side of the plant (6 branches per plant); 3 basal leaves, four central leaves, and three apical leaves were evaluated per branch, recording the number of symptomatic leaves in each location. Disease severity was assessed as the percentage of injured leaf area using a diagrammatic scale adapted from Horsfall and Barratt, described in (Madden et al., 2007).

2.7. Data processing and statistical analysis

2.7.1. Fungicide tolerance in vitro

The effect of fungicide on bacterial growth was estimated as the ratio of the treatments OD_{600} over the control, named proportional growth (P.G.). The data obtained were submitted to a non-linear analysis to estimate the concentration to reduce P.G. to 50% and 90%, using the model:

$$PG = \frac{e^{(\beta_0+\beta_1 IA)}}{1 + e^{(\beta_0+\beta_1 IA)}}$$

Where P.G. is the proportional bacterial growth, I.A. is the concentration active ingredient of the fungicide, β0 and β1 are the intercept and the slope estimated for the model. Fungicide concentration was defined as a proportion of the manufacturer's recommended dose. The fungicide doses needed to reduce bacterial growth by 50% within 24 hours were determined by solving the non-linear equations obtained.

2.7.2. GPM control experiments

Severity data (MacKinley disease index) from all experiments were applied to calculate the area under the disease progress curve (AUDPC), according to Madden

(2007). The control efficiency (E) was calculated based on the percentage reduction in disease severity at the end of the experiment using the equation:

$$E = {(C_{ij} - T_{ij})}/{C_{ij}}\, x100$$

Where, C_{ij} and T_{ij} are the control and treatments with *Bacillus* isolates, respectively.

The apparent growth infection rate (*r*) was estimated using the procedure defined by Kushalappa and Ludwig (1982). Data were evaluated for the analysis of homoscedasticity (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test), respectively. A initial ANOVA was conducted using the treatments and experiments as factors, and when interaction was significant, they were analyzed separately. Data from the greenhouse experiments with the alternated application of BCA and fungicides did not show a normal distribution and were submitted to non-parametric analysis using the Kruskal-Wallis test ($p< 0.05$). In the first greenhouse and the field experiments, incidence and severity data obtained were arcsin transformed using the equation, and AUDPC data were $\log_{10}$ transformed. However, tables and figures show the original mean and standard deviation.

3. Results

3.1. Control efficiency in greenhouse experiments

Weekly spraying of BCA TGFs significantly affected GPM incidence ($F_{8;\ 27}$= 16.8385; $p< 0.001$) and severity ($F_{8;\ 27}$= 13.0813; $p< 0.001$). The control treatment showed 100% disease incidence at the end of the first experiment; thus, data were excluded from ANOVA and post hoc tests. Treatments also significantly reduced AUDPC ($F_{8;\ 27}$= 3,661; p=0,023). The sulfur treatment delayed symptoms development until the eighth day, resulting in a lower disease growth ratio, severity, and AUDPC. According to Tukey's test, treatments with LCB28 and LCB30 showed control efficiency statistically similar to QST713 and sulfur (Table 2).

All biological treatments significantly reduced damage caused by GPM in the second greenhouse experiment (Table 2). Sulfur and LCB28 treatments extended the period for developing the first symptoms until day 13. The isolate LCB30 continually showed a reduced infection rate. However, the most significant results were observed in the disease severity evolution with reduction of leaf area damage caused by GPM.

Table 2. Incidence, severity, area under the disease progress curve (AUDPC), apparent disease growth rate (r), and control efficiency of powdery mildew in leaves of grape cv "Sugraone" after the application of *Bacillus* isolates under greenhouse conditions.

3.2. In vitro compatibility of LCB28 and LCB30 to fungicides

There was no inhibition halo when LCB28 and LCB30 were exposed to triflumizole in the disk assay. However, difenoconazole inhibited the growth of both isolates at the lower dose tested. Data collected in the experiment they were grown in liquid medium added with increasing fungicides concentrations (poison food technique) adjusted to a non-linear model with a determination quotient (r^2) higher than 0.70 (Fig. 1). The experiment measured bacterial growth as a percentage of the growth achieved without fungicide. Both isolates were highly tolerant do triflumizole, requiring a concentration of 1.18 g L^{-1} to inhibit 50% of their growth (DL50) for LCB28, and 1.36 g L^{-1} for LCB30. These doses are 50 and 69% higher than recommended by the manufacturer (0.80 g L^{-1}). Meanwhile, they were susceptible to difenoconazole showing a DL50 of 0.03 and 0.15 g L^{-1} to inhibit 50% $OD_{600,}$ respectively. These doses were 15.0 and 63.0% of the amount recommended by the manufacturer (0.24 mg L^{-1}).

Figure 1. Effect of triflumizole and difenoconazole on the growth of *B. tequilensis* LCB28 and *B. siamensis* LCB30. Bacterial growth data are presented as the proportion of the DO_{600} observed 24h after inoculation in L.B. media added with fungicide doses. The fungicide doses are presented as the proportion of the dose recommended by the manufacturer.

3.3. Alternating synthetic fungicides and BCA in greenhouse

In the first experiment, all treatments significantly reduced disease incidence compared to the control treatment according to the Kruskal-Wallis test [Degrees of freedom (D.F.)= 8; K-W statistic= 20.051; p=0.01] (Table 3). The treatments significantly increased the incubation period (DF= 8; K-W= 23.054; p=0.003) and apparent disease growth rate (*r*) (DF= 8; K-W= 23.218; p=0.003). All plants in the control treatment showed GPM symptoms on the 7th day, while applying sole LCB28 or LCB30 extended the infection period to 15.5 and 21.33, respectively. Weekly alternating the application of triflumizole with LCB28 or LCB30 significantly increased IP to 25.17 and 26.40 days. While the application of LCB28 and difenoconazole also significantly increased IP, alternate application of LCB30 with DFCZ showed IP statistically similar to the control.

All treatments significantly reduced the apparent infection rate. Even under highly favorable conditions for powdery mildew infection, the incidence rate ranged from 40% to 18% in plants treated with ACB and fungicide combinations. Disease growth rate in the control treatment was 1.0 cm day^{-1}, therefore resulting in significantly higher disease severity (DF= 8; K-W= 15.733; p=0.043) and area under the disease progress curve (AUDPC) (DF= 8; K-W= 23.526; p=0.003) compared to treatments with ACB plus fungicides. In the control treatment, disease incidence rapidly increased between the 5th and 7th day, reaching 70% on the 7^{th} day and 93% at the 15^{th} day after introducing plants used as the inoculum source.

Table 3. Effect of the alternating application of formulation containing *Bacillus* spp. with compatible (triflumizole) and non-compatible (difenoconazole) fungicides on grape powdery mildew in potted Redglobe plants in a greenhouse experiment.

All treatments significantly reduced GPM incidence (DF= 8; K-W= 29.6153; p<0.001) and severity (DF= 8; K-W= 28.157; p<0.001) in the second experiment. Both combinations of fungicides with LCB28 and LCB30 isolates showed a statistically equal incidence of fungicides (Table 3). GPM symptoms development was delayed by the treatments alternating the fungicides and bacteria (DF= 8; K-W= 26.113; p=0.001), which were statistically similar to the fungicides alone. The apparent disease growth rate (r) also was significantly reduced by the weekly spraying of the BCAs and fungicides and their combination (DF= 8; K-W= 24.900; p= 0.002) (Table 2). As a result, all treatments showed a significantly lower AUDPC (DF= 8; K-W= 8.699; p<0.001). Analyzing the disease incidence showed that LCB30 increased I.P. by 73,8% (DF= 8; K-W= 26.113; p= 0.001), while its combination with difenoconazole or triflumizole almost doubled the incubation period. Figure 2 shows the visual differences observed among the treatments. They corroborate the results in Table 2, showing that the control treatment exhibited large discolored lesions with profuse mycelial growth and sporulation characteristics of GPM. Meanwhile, although all treatments showed GPM incidence, the lesions were significantly smaller.

Figure 2. Effect of the alternate application of synthetic fungicide and *Bacillus* isolates on GPM symptoms in leaves of grape cv. Redglobe. a. Control b. LCB28 only; c. LCB30 only; d. Triflumizole (TFMZ); e. Difenoconazole (DFCZ); f. LCB28 + DFCZ; g. LCB28 + TFMZ, h. LCB30 + DFCZ, i. LCB30 + TFMZ.

3.3.1. GPM lesions colonization and conidia viability

All treatments and alternate combinations reduced *E. necator* mycelial growth and conidial production on the surface of GPM lesions in grape leaves at the end of the second greenhouse experiment (Figure 3). Control treatment showed a dense mesh of *E. necator* hyphae and many conidiophores and conidia on the lesion's surface examined in microscopy (Fig. 3a). Both fungicides reduced mycelial and conidia detection in the lesion surfaces (Fig. 3d and e). Treatments with LCB28 reduced mycelia and conidia density (Fig. 3b), and its combination with TFMZ showed a pronounced effect (Fig. 3g). However, LCB30 produced the most pronounced reduction of mycelia density and conidia numbers (Fig 3c and 3i) in the lesion surface, either applied alone or alternating with the fungicides.

Figure 3. Photomicroscopy (400×) of GPM lesions showing the colonization of *E. necator* in Redglobe leaves treated with *Bacillus* isolates and fungicides (HF= hyphae; CNF= conidiophore, CN= conidium). a. Control b. LCB28 only; c. LCB30 only; d. Triflumizole (TFMZ); e. Difenoconazole (DFCZ); f. LCB28 + DFCZ; g. LCB28 + TFMZ, h. LCB30 + DFCZ, i. LCB30 + TFMZ.

In the study, it was found that all treatments had a significant impact on reducing the germination of conidia from leaf lesions. The germination inhibition index ranged from 64.5% to around 90%, with the highest inhibition seen in the combination of biocontrol agents and LCB30 and DFCZ. Notably, no conidia were obtained in the treatment where triflumizole was exclusively applied or rotated with LCB30. This was observed even when printing the GPM colonies of leaf lesions.

3.4. Control efficiency of Bacillus isolates in the field experiment

Climate variables were more favorable for GPM epidemics during the first experiment between April and July (2021). The average daily temperature in the period was 25.9 (±1.83) ºC, average relative air humidity was 57.3% (±10.3). Maximal temperature (32.15 ±2.3 ºC) reached 35.0 ºC only 12 days in the period, while minimal temperature (20.9 ±1.84 ºC) reached below 20.0 ºC for 36 days, and nebulosity was higher than 50% for 32 days. In these conditions, there was a rapid increase in disease incidence in almost all treatments in experiment 1, except for plants exclusively treated with conventional fungicides (Table 4). The treatments that applied biological control spraying in the GPM management received an extra fungicide spray in the budding-flowering

period in this experiment, given the highly favorable climate conditions and the fast incidence increase in the control treatment. ANOVA showed that there was a significant effect of the treatments on disease incidence ($F_{4;53}$= 15.2089; p< 0.001), severity ($F_{4;53}$= 9.0409; p< 0.001), AUDPC ($F_{4;53}$= 10.3187; p< 0.001), and disease progress rate ($F_{4;53}$= 6.6121; p< 0.001). According to Table 4, the treatments with IDM based on the use of *Bacillus* isolates reduced disease incidence significantly compared to the control treatment, as confirmed by the Tukeys test. Synthetic fungicides were the most effective in reducing GPM severity, but treatments utilizing IDM based on the commercial and experimental formulation were still significantly similar and lower compared to the control (p< 0.05). Additionally, the AUDPC was significantly lower in the biocontrol treatments than in the control treatment.

Table 4. Effect of partial substitution of synthetic fungicides by *Bacillus* isolates in the integrated disease management (IDM) of GPM on disease incidence, severity, the area under the disease progress curve (AUDPC), and apparent disease growth rate (*r*) of powdery mildew by sprayed table grape in two experiments under field conditions.

The second experiment was executed between August and November (2021) when climate conditions were slightly less favorable for GPM epidemics. The average daily temperature was 26.4 (±1.82) ºC during the experiment, and the average relative air humidity was 57.19% (±10.1). The average maximal temperature (32.15 ±2.5 ºC) reached 35.0 ºC 21 days, while minimal temperature (21.4 ±2.4 ºC) reached below 20,0 ºC for 20 days, and nebulosity was lower than 50% for 20 days. Overall, there was a lower GPM incidence and severity in this experiment. Anova showed there was a significant effect of the treatments over the incidence ($F_{4;53}$= 79.163; p< 0.001), severity ($F_{4;53}$= 28.835; p< 0.001), AUDPC ($F_{4;53}$= 14.879; p< 0.001), and r ($F_{4;53}$= 13.191; p< 0.001). All treatments significantly reduced disease incidence, severity, and all the variables evaluated (Tukey test; p<0.05). The reduction in disease severity promoted by applying the experimental isolates in the integrated management proposed in this experiment was 69.3% for LCB28 and 73.2% for LCB30.

4. Discussion

Biofungicides formulations based on microbial agents have been widely introduced as a tool in the integrated management of plant disease, and *Bacillus* species are active ingredients in products to control aerial and soil-born plant pathogens (Shafi et

al., 2017). Their success is associated with the production of endospores, allowing large shelf life of commercial formulations, and their ability to produce several antibiotic compounds to control a broad spectrum of plant pathogens (Caulier et al., 2019; Li et al., 2019). In this work, we conducted two greenhouse experiments to evaluate the control efficiency of two *Bacillus* isolates previously selected for their *in vitro* and *in vivo* antagonism against *E. necator* (Santos, 2023). Weekly application of the bacterial TGF significantly reduced GPM incidence and mainly disease severity. There was a remarkable difference in GPM incidence among the experiments, given the different seasons in which they were conducted. The first experiment was conducted during the mild winter season in the region, characterized by cloudy days and mild temperatures at night, while the second was performed during summer, characterized by lower air humidity and high temperature during the day (Alvares et al., 2013). The driest climate could have interfered with conidial production and dispersion from the infested plants used as inoculum sources (Mieslerová et al., 2022). However, the significant reduction of disease severity proved their antagonism against *E. necator*, and their results were statistically similar to a commercial *Bacillus* strain.

Although the search for environmental and food secure alternative to disease management has significantly increased, table grape has a high production cost which *per se* make producers risk averse. The integrated management of grapevine diseases could benefit from a strategy that combines efficient antagonists and synthetic fungicides. This study aimed to evaluate the compatibility of LCB28 and LCB30 with two active ingredients commonly used against GPM. The results of antibiosis test discs and poison food experiments showed that triflumizole had a minimal impact on bacterial growth. To inhibit 50% of bacterial growth, a higher dose than the manufacturer's recommendation was required. On the other side, difenoconazole was highly toxic to both isolates, inhibiting their growth at the lower dose tested.

Testing the hypothesis that the alternate application of synthetic and BCA-based fungicides could effectively control GPM, we conducted two greenhouse experiments using exclusive applications of the synthetic fungicides difenoconazole (Score) and triflumizole (Trifimine), only the antagonist isolates, and alternating the weekly applications of the fungicides and BCA. All treatments significantly affected the epidemiological variables for powdery mildew registered in both experiments. On average, LCB28 and LCB30 reduced disease incidence by 51% and 66% when applied alone, respectively, while synthetic fungicides reduced incidence by 68.4% for

difenoconazole and 78.2% for triflumizole. More importantly, the alternate applications of the fungicides and BCA significantly increased the incubation period and reduced the apparent disease growth ratio. Consequently, on average, alternate treatments reduced more than 70% of GPM incidence and more than 95% severity.

Microscopic observation of *E. necator* on the leaves' surface showed that both fungicides reduced mycelium growth and conidia production. While there was a profuse mesh of hyphae with many conidia in the control treatment, the biocontrol treatments and their combination with fungicide showed a rarefied mycelia growth and a low number of conidia. Besides, germination of conidia recovered from sporulated lesions was significantly inhibited by the treatments with the biocontrol agents and their combination with the fungicides. Therefore, even completing the infectious process, secondary infections could be compromised by low conidia viability. *Bacillus* sp. can inhibit disease development by preventing spore germination, germ tube elongation, and penetration (Cuellar-Gaviria et al., 2023; Wang et al., 2022; You et al., 2021). In a previous study, Santos (2023) showed that LCB28 and LCB30 inhibited conidial germination *in vitro* by producing thermotolerant antifungal compounds. In fact, PCR-based analysis showed that LCB28 presented four markers for genes codifying for bacyllomicin, fengycin, bacilysin, and surfactin, and LCB30 showed the same markers plus the marker for iturin production.

Other studies with *Bacillus* formulations have shown their efficiency in controlling powdery mildew in different crops. Results similar to ours were obtained with the application of *B. subtilis* and *B. pumilus* to prevent powdery mildew caused by *Podosphaera xanthii* on cucumbers and squash in a greenhouse (Hafez et al., 2020). The alternate application of Milastin K, a biofungicide based on *B. subtilis* KTSB-1015, and triazole fungicides significantly reduced GPM in India (Kanitkar et al., 2020). In this study, the alternate application of the isolates with either a compatible (triflumizole) or an incompatible fungicide (difenoconazole) resulted in high control efficiency in a greenhouse. Although there was no synergistic effect, their alternate applications allowed reducing 50% of the use of both fungicides with similar control efficiency.

The seasonal climate remarkably affected the incidence of GPM and control efficiency. Weather conditions during the first field experiments were more favorable to powdery mildew outbreaks. As a result, the disease development ratio grew fast after the midterm of the production cycle. Nevertheless, the integrated management of GPM based on the partial substitution of synthetic fungicides by *Bacillus isolates*, as described in this work, showed promising results in both experiments. Control efficiency of GPM IDM

with the commercial strain *B. subtilis* QST713 and the experimental isolates resulted in incidence and severity statistically similar to the exclusive application of synthetic fungicides in the two experiments. At the end of the experiment, the partial substitution of synthetic fungicides by formulations containing LCB28 and LCB30, as proposed in this work, significantly reduced GPM incidence and severity.

When applied weekly in the field experiments, an IDM strategy based on the partial substitution of synthetic fungicide by biological treatments strongly reduced GPM incidence and severity. However, average control efficiency was lower than 80%, showing that new developments are necessary to allow the general use of biofungicides in GPM management. Achieving efficient control, BCA applied as a sole strategy in open field experiments is complex because field reinfection can occur by inoculum entry from neighboring plots, and the microbial antagonists would face adverse climate conditions(Ghule et al., 2019). Compared to the rhizosphere, the phyllosphere is an oligotrophic environment where nutrients are scarce and show a heterogeneous nature requiring the BCA to adapt to the habitat (Vorholt, 2012). Besides biotic and abiotic stressors, refining the application strategy could improve control efficiency. An association with disease prevision models based on phenological and climate variables could improve the decision process about synthetic or biological alternatives.

Based on our results in this work, using an alternated application of ACB and both a compatible or an incompatible fungicide proved to be an effective measure against *E. necator* in grapevine in the greenhouse. This effect is likely linked to their ability to produce thermotolerant cyclopeptides and volatile antifungal compounds. The experimental period in the greenhouse assay saw a partial inhibition of sporulation and mycelial growth in leaves with sporulated lesions that were treated with bacterial isolates, explaining the alteration of the epidemics variables measured. Promising results were obtained in field experiments when formulations containing *B. tequilensis* LCB28 or *B. siamensis* LCB30 were applied in an integrated management strategy that partially substituted the spraying of synthetic fungicides.

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Table 1. Application of the biofungicides treatments during the different grape phenological phases in the integrated management of grape powdery mildew in the field experiments.

Phenology	Duration (days)		Spraying			
			Experiment 1		Experiment 2	
	Exp. 1[1]	Exp. 2[2]	Biof.[3]	Synth.	Biof.	Synth.
Pruning – budding [4]	12	12	02	-	02	-
Budding – flowerin [5]	21	21	03	01*	03	
Flowering – olive berries	21	21	-	05	-	05
Olive berries – softening	18	15	03	-	03	-
Maturation – harvest	28	28	04	-	04	-
Total	100	92	12	06	13	05

[1] Experiment 1 using cv. "Sugraone" conducted from June to September; [2] Experiment 2 using cv. "Crimson" conducted from August to November; [3] Biofungicides – commercial and experimental *Bacillus* formulations; [4] Fifty percent branches with completely open primordial leaves;[2] Fifty percent flowers blossomed. * Given highly favorable climate conditions, one extra synthetic fungicide spraying was added.

Table 2. Incidence, severity, area under the disease progress curve (AUDPC), apparent disease growth rate (r), and control efficiency of powdery mildew in potted grape plants cv 'Sugraone' after the application of *Bacillus* isolates under greenhouse conditions.

Treatments	Incidence (%)	Severity (DI)[1]	AUDPC[2]	*r*[3]	Efficiency (%)
	Experiment 1				
Control	100.00	31.20 (7.45) a	126.50 (12.43) a	1.95 (0.12) a	
LCB28	70.80 (8.02) a*	4.80 (1.31) b	38.76 (7.46) bc	0.51 (0.10) b	84.61 (12.54) a
LCB30	73.60 (5.1) a	6.30 (1.42) b	51.47 (4.43) b	0.61 (0.04) b	79,81 (8.85) a
QST713	79.80 (6.41) a	7.26 (1.26) b	31.20 (5.36) c	0.47 (0.13) b	76.73 (11.31) a
Sulfur	28.20 (7.84) b	3.90 (2.08) b	24.49 (5.60) c	0.23(0.07) b	87.50 (7.38) a
	Experiment 2				
Control	96.00 (4.42) a	46.10 (7.63) a	176.40 (18.30) a	1.86 (0.58) a	-
LCB28	23.30 (2.87) bc	4.44 (0.97) b	32.75 (3.35) b	0.11 (0.03) b	89.29 (2.44) a
LCB30	31.10 (8.19) b	5.20 (0.92) b	17.28 (1.38) c	0.21 (0.01) b	87.37 (4.79) a
QST713	15.60 (5.06) bc	3.60 (1.35) b	25.17 (4.33) bc	0.12 (0.05) b	91.24 (3.09) a
Sulfur	6.30 (2.87) c	5.10 (0.98) b	23.75 (2.09) c	0.09 (0.05) b	87.59 (5.12) a

*Treatments with the same letters in the columns did not differ by Tukey test ($p < 0.05$). The control treatment showed no variance and was exclude from ANOVA. [1]DI refers to the MacKinley disease index; [2]AUDPC is the area under disease progress curve; [3]*r* is the apparent disease growth rate (Kushalappa and Ludwig, 1982).

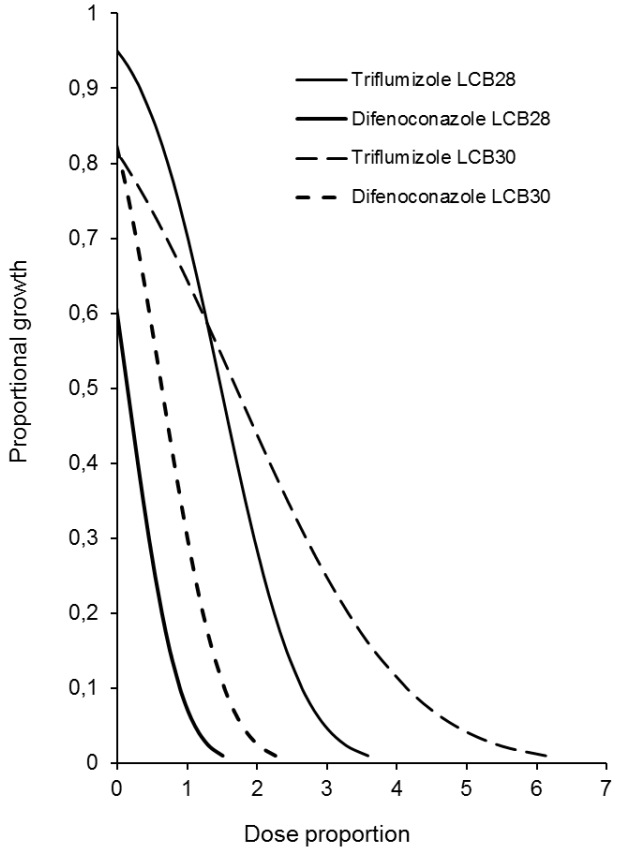

Figure 1. Effect of triflumizole and difenoconazole on the growth of *B. tequilensis* LCB28 and *B. siamensis* LCB30. Bacterial growth data are presented as the proportion of the DO_{595} observed 24h after inoculating the bacterial isolates in L.B. media added with fungicide doses. The fungicide doses are shown as the proportion of the dose recommended by the manufacturer.

Table 3. Effect of the alternate application of TGF containing *Bacillus* spp. with compatible (triflumizole) and non-compatible (difenoconazole) fungicide on the evolution of grape powdery mildew in potted plant with cv. Redglobe in a greenhouse experiment.

Treatments	Incidence (%)	Severity (DI)[1]	r^2	IP[3]	AUDPC[4]
	Experiment 1				
Controle	97.33 (4.13) a*	33.20 (7.58) a	1.22 (0.42) a	7.00 (0.0)	625.67 (75.33) a
LCB28	53.33 (9.87) b	1.77 (0.52) b	0.04 (0.02) b	9.00 (3.10) a	46.15 (10.76) b
LCB30	28.17 (10.50) b	0.96 (0.19) b	0.02 (0.01) b	12.17 (4.83) a	28.20 (5.45) b
Difeconazol	21.17 (10.28) b	0.86 (0.23) b	0.02 (0.01) b	18.83 (5.27) a	23.35 (4.66) b
Triflumizol	16.50 (13.41) b	0.84 (0.32) b	0.01 (0.01) b	28.67 (9.75) b	21.29 (5.84) b
LCB28 + DFCZ	31.83 (7.89) b	1.14 (0.89) b	0.03 (0.03) b	20.00 (13.37) b	32.04 (18.40) b
LCB28 + TFMZ	41.50 (12.57) b	1.41 (0.82) b	0.04 (0.02) b	16.00 (10.56) a	40.67 (17.06) b
LCB30 + DFCZ	28.00 (15.14) b	1.19 (0.52) b	0.02 (0.01) b	15.00 (11.17) a	33.99 (15.45) b
LCB30 + TFMZ	29.67 (7.79) b	0.99 (0.45) b	0.02 (0.02) b	22.17 (11.81) b	27.94 (10.62) b
	Experiment 2				
Controle	96.50 (5.43) a	26.04 (13.05) a	1.00 (0.52) a	7.00 (0.00)	499.37 (50.82) a
LCB28	42.50 (7.25) b	1.40 (0.60) b	0.03 (0.02) b	15.50 (7.61) a	32.16 (10.93) b
LCB30	37.67 (6.25) b	1.45 (0.88) b	0.04 (0.04) b	21.33 (10.73) b	30.49 (14.37) b
Difeconazol	40.00 (3.98) b	4.90 (5.48) b	0.17 (0.21) b	14.17 (10.50) a	84.31 (18.93) b
Triflumizol	25.67 (6.31) b	0.91 (0.27) b	0.02 (0.01) b	22.67 (3.61) b	21.90 (3.44) b
LCB28 + DFCZ	20.83 (7.10) b	0.83 (0.37) b	0.02 (0.02) b	23.33 (8.94) b	21.66 (5.54) b
LCB28 + TFMZ	18.20 (11.59) b	0.88 (0.47) b	0.02 (0.02) b	26.40 (9.48) b	21.87 (7.46) b
LCB30 + DFCZ	29.40 (8.62) b	1.22 (0.57) b	0.03 (0.03) b	18.60 (9.37) b	28.24 (13.22) b
LCB30 + TFMZ	26.50 (8.83) b	0.91 (0.17) b	0.02 (0.01) b	25.17 (7.31) a	21.45 (3.30) b

*Treatments with the same letters in the columns did not differ by Kruskal-Wallis test ($p < 0.05$). [1] DI refers to the MacKinley disease index; [2] *r* is the apparent disease growth rate (Kushalappa and Ludwig, 1982); [3] IP is the incubation period; [4] AUDPC is the area under disease progress curve.

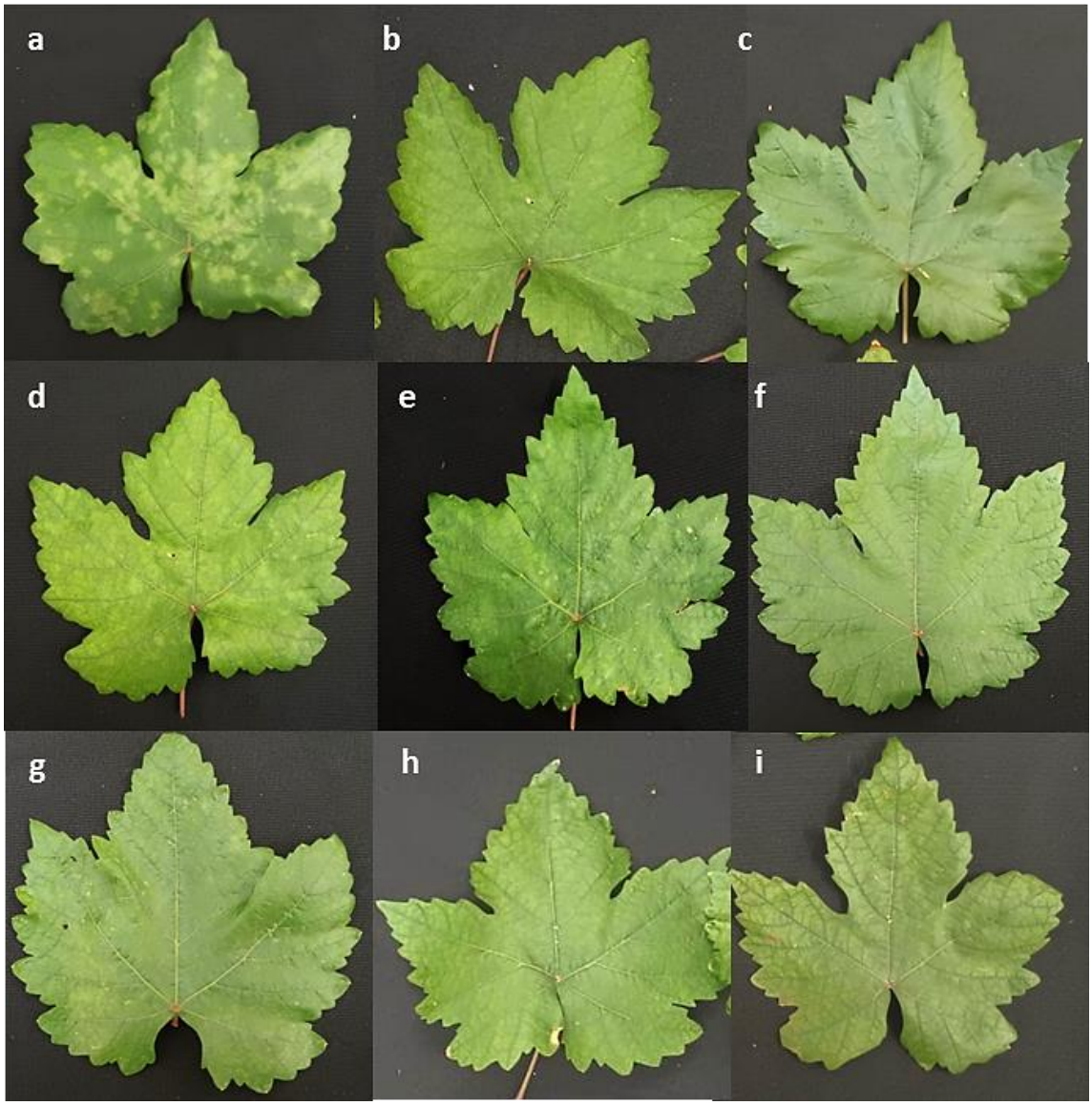

Figure 2. Effect of the alternate application of syntetic fungicide and *Bacillus* isolates on GPM symptoms in leasves of grape cv. Redglobe. a. Control b. LCB28 only; c. LCB30 only; d. Triflumizole (TFMZ); e. Difenoconazole (DFCZ); f. LCB28 + DFCZ; g. LCB28 + TFMZ, h. LCB30 + DFCZ, i. LCB30 + TFMZ.

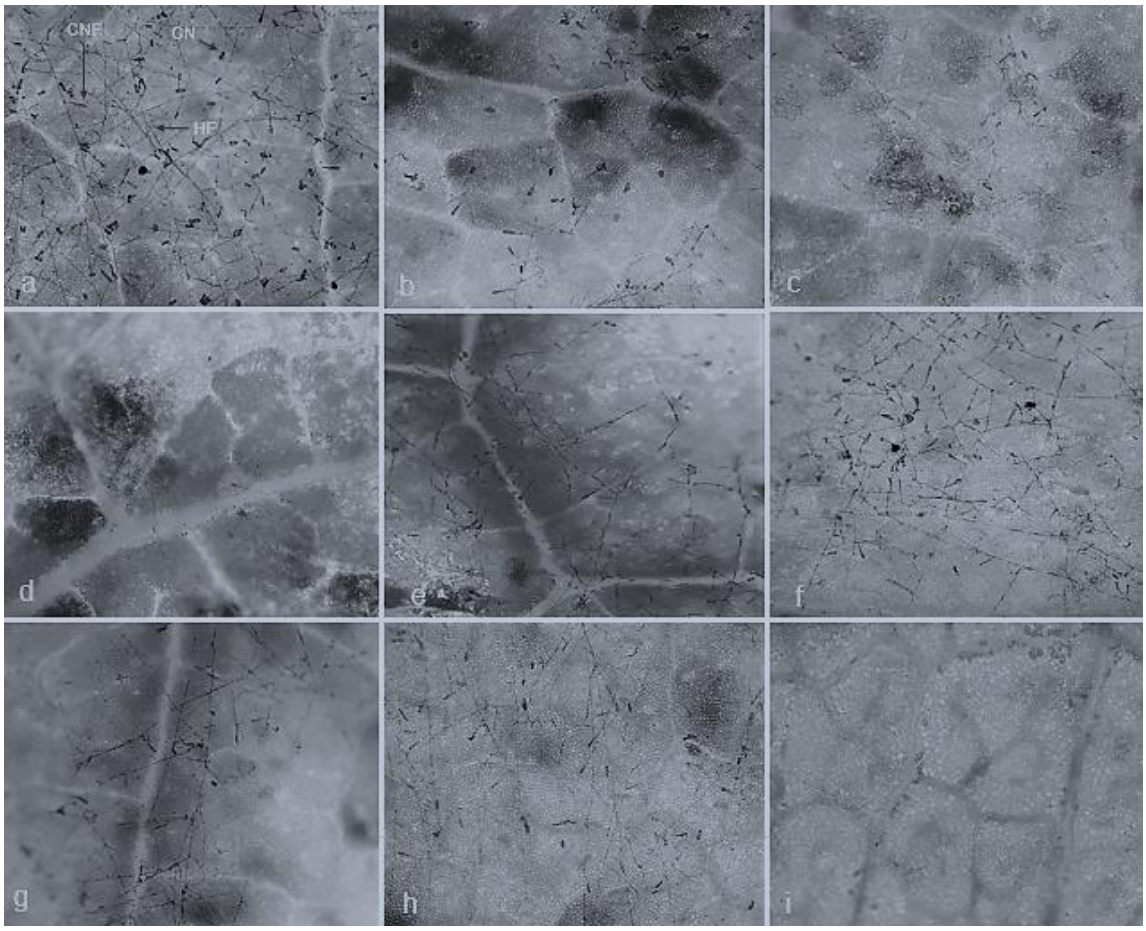

Figure 3. Photomicroscopy (400× magnification) of GPM lesions showing surface colonization by *E. necator* in Redglobe leaves treated with *Bacillus* isolates and fungicides (HF= hyphae; CNF= conidiophore, CN= conidium). a. Control b. LCB28 only; c. LCB30 only; d. Triflumizole (TFMZ); e. Difenoconazole (DFCZ); f. LCB28 + DFCZ; g. LCB28 + TFMZ, h. LCB30 + DFCZ, i. LCB30 + TFMZ. The contrast in the photomicroscopy was enhanced to improve the visualization of mycelia and conidia.

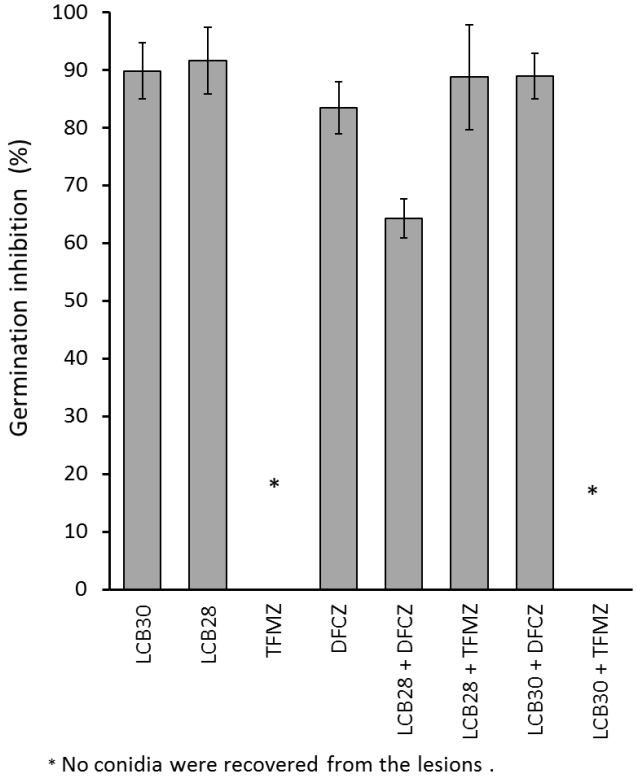

Figure 4. Effect of the alternate application of the fungicides difenoconazole (DFCZ) and triflumizole (TFMZ) and *B. tequilensis* LCB28 and *B. siamensis* LCB30 on the germination of *E. necator* conidia from leaves of grape cv. Redglobe in a greenhouse experiment. Germination inhibition index was estimated using the equation $(C - T_i)/C$, where T and C are the conidia germination at the control and treatments.

Table 4. Effect of partial substitution of synthetic fungicides by *Bacillus* isolates in the integrated disease management (IDM) of GPM on disease incidence, severity, the area under the disease progress curve (AUDPC), and apparent disease growth rate (*r*) of powdery mildew by sprayed table grape in two experiments under field conditions.

	Incidence (%)	Severity (DI)*	r**	AUDPC
	Experiment 1 (Sugraone)			
Control	31.80 (4.96) c	18.15 (1.52) c	0.24 (0.08) b	217.45 (28.75) c
IDM LCB28	8.75 (3.55) b	5.57 (1.49) b	0.21 (0.05) b	74.99 (16.10) b
IDM LCB30	6.75 (3.35) b	4.86 (1.91) b	0.17 (0.05) ab	94.86 (37.09) b
IDM QST713	5,33(2.48) b	3.28 (0.82) b	0.18 (0.04) ab	63.91 (14.80) b
Conventional	3.83 (0.92) a	1.16 (0.05) a	0.01 (0.003) a	33.28 (4.62) a
	Experiment 2 (Crimson)			
Control	20.92 (3.26) b	6.73 (1.24) b	0.19 (0.05) b	173.74 (17.75) b
IDM LCB28	4.58 (1.45) a	0.72 (0.31) a	0.02 (0.00) a	76.4 (2.00) a
IDM LCB30	6.00 (1.81) a	1.02 (0.43) a	0.02 (0.01) a	48.47 (3.29) a
IDM QST713	7.08 (2.18) a	0.70 (0.27) a	0.02 (0.00) a	36.18 (1.78) a
Conventional	3.33 (1.99) a	1.93 (0.81) a	0.03 (0.01) a	13.59 (5.01) a

*Treatments with the same letters in the columns did not differ by Tukeys' test ($p < 0.05$).
*DI refers to the MacKinley disease index; **r is the apparent disease growth rate (Kushalappa and Ludwig, 1982)

CAPÍTULO V

CONCLUSÕES GERAIS

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1. Isolados de *Bacillus* nativas do semiárido pertencente à coleção de microrganismos de interesse agronômico da Embrapa apresentaram antagonismo *in vivo* contra *Erysiphe necator*, causador do oídio da videira em experimento em folhas destacadas.
2. Os isolados de *Bacillus spp.* LCB03, LCB28 e LCB30 são eficientes ACB do oídio da videira em casa de vegetação.
3. *B. tequilensis* LCB28 e *B. siamensis* LCB30 apresentam eficiência de controle semelhantes a cepa comercial *B. amyloliquefacies* QST713 e aos fungicidas sintéticos usados no controle do oídio da videira em casa de vegetação.
4. Todas as três isolados de *Bacillus*, são capazes de crescerem em condições de estresse abiótico com baixas concentrações de carbono, nitrogênio e níveis variado de pH.
5. *Bacillus tequilensis* LCB28 e *B. siamensis* LCB30 produzem compostos antifúngicos termotolerantes e compostos orgânicos voláteis que inibem a germinação de conídios de *E. necator*.
6. *Bacillus tequilensis* LCB28 e *B. siamensis* LCB30 são produtores de ciclopepitídeos antibióticos como bacilomicina, fengicina, sufactina, bacilisina e iturina.
7. *B. tequilensis* LCB28 e *B. siamensis* LCB30 são compatíveis com o fungicida triflumizol e incompatível com o fungicida difenoconazol.
8. A aplicação alternada dos ACB com os fungicidas triflumizol e difenoconazol reduz em 50% o uso dos fungicidas em experimento de casa de vegetação.
9. A aplicação de *B. tequilensis* LCB28 e *B. siamensis* LCB30 em uma estratégia de manejo integrado na qual a aplicação de fungicidas foi restrita aos períodos de plena floração e de crescimento inicial dos frutos reduziu significativamente a severidade e incidência de oídio da videira em dois experimentos de campo.
10. A estratégia de manejo integrado proposta permitiu reduzir o número de aplicações de fungicidas sintéticos em 70% e os isolados experimentais apresentaram eficiência de controle similar a um produto comercial contendo uma estirpe de *Bacillus* como ingrediente ativo.
11. Os resultados obtidos nos diversos experimentos mostram que *B. tequilensis* LCB28 e *B. siamensis* LCB30 são potenciais ACB do *E. necator* que podem ser integrados ao manejo integrado da videira após ajustes na sua formulação e estratégia de aplicação.