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DESENVOLVIMENTO DE BIOFORMULAÇÕES CONTENDO ENDÓSPOROS DE *Bacillus velezensis* S26 PARA CONTROLE BIOLÓGICO DE DOENÇAS E PROMOÇÃO DO CRESCIMENTO VEGETAL

Alessandra Russi

CAXIAS DO SUL
2024

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, como parte dos requisitos para a obtenção de grau de Doutora em Biotecnologia.

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ADP	Ácido dipicolínico
ANOVA	*Analysis of variance*
BAP	*6-benzyl aminopurine*
BC	*Bacterial cellls*
BCA	*Biological control agent*
BE	*Bacterial endospores*
BF	*Bioformulation*
BOD	*Biochemical oxygen demand*
CB	*Culture broth*
CBB	*Composted bagasse biochar*
CFS	*Cell-free supernatant*
CFU	*Colony-forming unit*
CMC	*Carboxymethylcellulose*
COD	*Chemical oxygen demand*
CTC	Capacidade de troca catiônica
DBO	Demanda bioquímica de oxigênio
DI	*Disease incidence*
DQO	Demanda química de oxigênio
ERO	Espécie reativa de oxigênio
DS	*Disease severity*
DSI	*Disease severity index*
END	Endósporos
FAO	*Food and Agriculture Organization*
FBB	*Fresh bagasse biochar*
FI	*Fresh inoculant*
FM	*Fresh mass*
FS	*Foliar spraying*
GTP	Trifosfato de guanosina
LB	Luria-Bertani
MGI	*Mycelial growth rate*
MS	Murashige and Skoog

NAA	*α-naphthalene acetic acid*
NRPS	*Nonribosomal peptide synthetases*
ODS	Objetivo do Desenvolvimento Sustentável
OIV	*International Organisation of Vine and Wine*
PDA	*Potato dextrose agar*
PGPB	*Plant growth-promoting bacteria*
PI	*Percentage of inhibition*
PKS	*Polyketide synthetases*
PM	*Plant mortality*
PNRS	Política Nacional de Resíduos Sólidos
SASP	*Small acid soluble proteins*
SD	*Soil drenching*
SI	*Stored inoculant*
TSS	*Total soluble solids*
TTA	*Total titratable acidity*
UN	*United Nations*
VOC	*Volatile organic compounds*
WP	*Wettable powder*
YPG	*Yeast extract peptone and glucose*

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RESUMO

Os bioinsumos têm ganhado destaque em resposta aos efeitos prejudiciais ocasionados pelo uso excessivo de fertilizantes químicos e pesticidas ao meio ambiente e à saúde humana. Nesse contexto, o gênero *Bacillus* é constituído por bactérias Gram-positivas, formadoras de endósporos e com ação antagonística contra vários fitopatógenos. Essas bactérias também atuam estimulando o desenvolvimento vegetal e promovendo a indução de mecanismos de resistência em plantas. Consequentemente, o emprego desses micro-organismos, na forma de endósporos, pode contribuir para prolongar a estabilidade e a vida de prateleira de bioformulações. O presente estudo teve como objetivo determinar o potencial inibitório de suspensões de endósporos de *Bacillus velezensis* S26 no controle da podridão cinzenta causada por *Botrytis* spp., da antracnose ocasionada por *Colletotrichum* spp. e do pé-preto, cujo agente causal inclui o fungo *Dactylonectria macrodidyma*. Somado a isso, buscou-se verificar a ação de *B. velezensis* S26 na promoção do crescimento em morangueiros e videiras, bem como seu emprego na elaboração de formulações líquidas e secas usando subprodutos agroindustriais. Inicialmente, os ensaios foram conduzidos visando promover a esporulação bacteriana, em frascos de cultivo mantidos sob agitação. Posteriormente, avaliou-se o potencial antagonístico das suspensões de endósporos *B. velezensis* S26 contra isolados de *Botrytis* spp. e *Colletotrichum* spp., tanto *in vitro* quanto *in vivo*. Os ensaios *in vivo* foram realizados em frutos e plantas de videira e morangueiro sob condições ambientais controladas. Em seguida, foram elaboradas quatro bioformulações constituídas por lixiviado da compostagem de bagaço de uva e soro de leite, isoladamente ou em combinação. A eficácia dessas formulações foi determinada tanto em condições controladas quanto em uma estufa comercial de morangos. De forma similar, foi desenvolvida uma formulação seca a partir de biochares provenientes da pirólise do bagaço de uva fresco e do bagaço de uva compostado. O potencial de promoção do crescimento dessas formulações foi testado no porta-enxerto de videira SO4 (*Vitis berlandieri* × *V. riparia*), cultivado em substrato convencional e substrato contendo altos níveis de cobre. Também, examinou-se o efeito desses biochares associados com endósporos *B. velezensis* S26 na supressão do pé-preto da videira. Os resultados demonstraram *B. velezensis* S26 apresenta capacidade de controlar, de forma eficaz, a antracnose e a podridão cinzenta em morangueiros e videiras. Essa atividade inibitória foi mantida após seis meses de armazenamento. Além disso, os ensaios em frascos de cultivo e biorreatores possibilitaram induzir a esporulação de *B. velezensis* S26, utilizando lixiviado de compostagem e soro como meio de cultura. O emprego dessas formulações líquidas contribuiu para a promoção do crescimento de morangueiros micropropagados, bem como para o biocontrole de podridões de frutos em uma estufa comercial de morangos, com um desempenho similar ao bioproduto Duravel WP®. Finalmente, os tratamentos utilizando biochar enriquecido com endósporos de *B. velezensis* S26 contribuíram para minimizar o estresse ocasionado pelo excesso de cobre no solo, aumentar a biomassa de plantas de SO4 micropropagadas e controlar o pé-preto da videira. Portanto, *B. velezensis* S26 é um agente promissor no controle biológico da antracnose, podridão cinzenta e pé-preto em morangueiros e videiras, não apenas reduzindo a incidência e severidade dessas doenças, mas também, estimulando o crescimento das plantas. Dessa forma, a incorporação de endósporos de *B. velezensis* S26 em bioformulações líquidas e biochares possibilita a preservação tanto da viabilidade celular durante o armazenamento quanto da capacidade de supressão de doenças fúngicas e de estímulo ao desenvolvimento vegetal, contribuindo para uma agricultura sustentável e agregando valor a subprodutos agroindustriais.

Palavras-chave: Antracnose, bioformulação, esporulação, podridão cinzenta, pé-preto, promoção do crescimento.

ABSTRACT

The bioinputs has gained prominence in response to the harmful effects caused by synthetic fertilizers and pesticides on the environment and human health. The genus *Bacillus* consists of Gram-positive, endospore-forming bacteria with antagonistic potential against various phytopathogens. These bacteria also stimulate plant development and induce resistance mechanisms in plants. Consequently, the use of endospores can contribute to extending the shelf life and stability of bioformulations. This study aimed to determine the inhibitory potential of *Bacillus velezensis* S26 endospores suspensions in controlling gray mold caused by *Botrytis* spp., anthracnose caused by *Colletotrichum* spp., and black foot rot, with the causal agent being the fungus *Dactylonectria macrodidyma*. Additionally, the study sought to investigate the action of *B. velezensis* S26 endospores suspensions in promoting the growth of strawberries and grapevines and to develop liquid and dry formulations using agro-industrial by-products. Initially, trials were conducted to induce and optimize bacterial sporulation in shaking-flasks. Subsequently, the antagonistic potential of *B. velezensis* S26 endospore suspensions against isolates of *Botrytis* spp. and *Colletotrichum* spp. was evaluated both *in vitro* and *in vivo*. *In vivo* trials were conducted on fruits and plants of grapevine and strawberry under controlled environmental conditions. Four bioformulations were then developed, consisting of leachate from grape marc composting and whey, either individually or in combination. The effectiveness of these formulations was determined under controlled conditions and in a commercial strawberry greenhouse. Similarly, a dry formulation was developed from two biochars derived from the pyrolysis of fresh grape pomace and previously composted grape pomace. The growth-promoting potential of these formulations was tested on the grapevine rootstock SO4 (*Vitis berlandieri* × *V. riparia*), cultivated in conventional substrate and copper-treated substrate. The effect of biochars associated with *B. velezensis* S26 endospores on black foot rot suppression was also examined. The results demonstrated that *B. velezensis* S26 endospores can effectively control anthracnose and gray mold in strawberries and grapevines. This inhibitory capacity was maintained even after six months of storage. Furthermore, cultivation in shaking-flasks and bioreactors led to *B. velezensis* S26 sporulation using compost leachate and whey as culture media. The use of these culture suspensions as liquid formulations provided performance similar to the commercial bioproduct Duravel WP® in a commercial strawberry greenhouse and promoted the growth of micropropagated strawberry plants. Finally, treatments using biochar enriched with *B. velezensis* S26 endospores contributed to minimize copper-induced stress, increase the biomass of micropropagated SO4 plants, and control grapevine black foot rot. Therefore, B. *velezensis* S26 emerges as a promising agent in the biocontrol of anthracnose, gray mold, and black foot rot, not only reducing the incidence and severity of these diseases but also stimulating the growth of these plants. As a result, the incorporation of *B.* velezensis S26 endospores into liquid bioformulations and biochars allows for the preservation of both cellular viability during storage and the ability to suppress fungal diseases and stimulate plant development, contributing to sustainable agriculture and adding value to agro-industrial waste and by-products.

Keywords: Anthracnose, bioformulation, black foot, sporulation, gray mold, growth promotion.

1 INTRODUÇÃO

O Brasil se destaca na fruticultura tropical e subtropical, ocupando as 11ª e 17ª posições no ranking dos maiores produtores mundiais de uvas e morangos, respectivamente (AGRIANUAL, 2020; FAOSTAT, 2021). A produção vitícola nacional em 2023 foi superior a 1,4 milhões de toneladas, abrangendo uma área de aproximadamente 75 mil hectares. Entre os principais estados produtores de uva, destacam-se o Rio Grande do Sul, Pernambuco e São Paulo (IBGE, 2023). Por outro lado, a produção média anual de morangos é de aproximadamente 165 mil quilos em uma área de 4,5 mil hectares cultivados no país. Essa produção vem crescendo nos últimos anos, principalmente nos estados de Minas Gerais, Paraná e Rio Grande do Sul (Antunes et al., 2021).

No entanto, a produtividade dessas culturas tem sido afetada tanto por fatores bióticos quanto abióticos. Dentre os fatores bióticos, destacam-se diferentes espécies dos fungos *Colletotrichum* e *Botrytis* que podem ocasionar doenças como a antracnose e a podridão cinzenta, respectivamente. Esses patógenos podem afetar as plantas no campo, permanecendo latentes até a ocorrência de condições favoráveis ao seu desenvolvimento, ou ainda, infectar os frutos durante a fase de pós-colheita. Ambas as doenças podem afetar folhas, ramos e frutos, resultando em perdas na quantidade e qualidade da produção. Mundialmente, essas perdas são bastantes significativas, atingindo valores de 10 a 100 bilhões de dólares ao ano (Amarouchi et al., 2021).

Diferentemente, o pé-preto é uma doença ocasionada por vários gêneros e espécies de fungos, incluindo *Dactylonectria macrodidyma*, que ocasiona sérios danos à viticultura. Esse patógeno ataca as raízes da videira através de lesões ocasionadas por práticas de cultivo e agentes bióticos, como nematoides. Plantas em condições de estresse ambiental, decorrentes de danos por frio, deficiência nutricional e plantio em solos contaminados com cobre, são mais suscetíveis ao ataque desse fitopatógeno. De acordo com Aleinikova et al. (2022), as perdas globais ocasionadas pelo pé-preto, associadas a outras doenças do tronco da videira, atingem mais 1,5 bilhão de dólares ao ano.

Dentre os fatores abióticos prejudiciais à fruticultura, destaca-se a contaminação do solo de vinhedos por cobre, em decorrência do uso excessivo de fungicidas cúpricos. Essa condição impacta negativamente a viticultura, levando a um crescimento deficiente, baixo vigor e produtividade das plantas. Somado a isso, as videiras se tornam mais suscetíveis a patógenos veiculados pelo solo, como o pé-preto, a fusariose, entre outras doenças. Dessa forma, o manejo convencional de doenças fúngicas realizado por meio da aplicação de fungicidas químicos, não

apenas ocasiona contaminação do solo e da água, mas também, resulta em desequilíbrio ecológico, seleção de patógenos resistentes, redução das populações de micro-organismos benéficos e acúmulo de resíduos químicos em frutos. Em virtude da contaminação de alimentos com fungicidas sintéticos, alguns países europeus proibiram a aplicação de pesticidas na fase de pós-colheita.

Nesse contexto, torna-se essencial a adoção de métodos alternativos de prevenção e manejo de doenças fúngicas, como o controle biológico. O biocontrole pode ser implementado por meio de estratégias que favoreçam os micro-organismos benéficos existentes no ambiente ou pela introdução de micro-organismos antagonistas a fitopatógenos. Assim, bactérias pertencentes ao gênero *Bacillus*, se caracterizam por inibir o desenvolvimento de muitos fungos patogênicos, induzir mecanismos de defesa vegetal e promover o crescimento de plantas. Essas bactérias, também, apresentam capacidade de produzir esporos de resistência, viabilizando o desenvolvimento de bioformulações com vida de prateleira estendida.

Idealmente, os bioinsumos devem apresentar baixo custo de produção, manter a viabilidade dos micro-organismos durante o armazenamento e, sempre que possível, utilizar resíduos ou subprodutos de processos agrícolas, industriais e comerciais como matéria-prima. Essa abordagem não apenas agrega valor a esses materiais, mas também contribui para a minimização dos impactos ambientais decorrentes de práticas inadequadas de tratamento e disposição final. Dessa forma, é possível promover a economia circular, reduzindo tanto os custos financeiros quanto os impactos ambientais.

A implementação de práticas agrícolas sustentáveis, associada ao resuso de resíduos e subprodutos agroindustriais, desempenha um importante papel na concretização dos Objetivos de Desenvolvimento Sustentável (ODS) propostos pela Organização das Nações Unidas (ONU). Essa abordagem abrange diversos objetivos, incluindo o ODS 2 (Fome Zero e Agricultura Sustentável), o ODS 11 (Cidades e Comunidades Sustentáveis), o ODS 13 (Ação contra a Mudança Global do Clima) e o ODS 15 (Vida Terrestre), contribuindo para enfrentar desafios globais e promover um futuro mais sustentável. No âmbito do ODS 2, que visa garantir segurança alimentar e promover a agricultura sustentável, este estudo contribui disponibilizando alimentos mais seguros e livres de contaminantes químicos. O alinhamento com o ODS 11 ocorre mediante o emprego do controle biológico que busca atender às demandas da sociedade, respeitando a biodiversidade e contribuindo para comunidades mais resilientes e sustentáveis. O cumprimento do ODS 13 é obtido por meio do reuso de subprodutos sólidos e líquidos, promovendo o sequestro de carbono e a redução das emissões de gases do

efeito estufa. Por fim, o ODS 15 é alcançado com a preservação de ambientes terrestres e a produção agroecológica de alimentos.

Dessa forma, visando à adoção de práticas agrícolas menos impactantes e à preservação de ecossistemas naturais, o presente trabalho teve como objetivo desenvolver bioformulações contendo endósporos de *Bacillus velezensis* linhagem S26 destinadas ao controle biológico dos fungos *Botrytis* spp., *Colletotrichum* spp. e *D. macrodidyma,* bem como à promoção do crescimento de morangueiros e videiras, empregando subprodutos de agroindústrias locais.

2 OBJETIVOS

2.1 Objetivo geral

Desenvolver uma formulação líquida contendo endósporos de *Bacillus velezensis* S26, com o objetivo de promover o controle biológico de *Botrytis* spp. e *Colletotrichum* spp. em morangueiros e videiras. De forma similar, elaborar uma formulação seca com endósporos bacterianos para promover o biocontrole de *Dactylonectria macrodidyma*, estimular o crescimento vegetal e atenuar os efeitos nocivos do cobre em videiras.

2.2 Objetivos específicos

a) Avaliar o efeito do pH, temperatura, meio de cultura, tempo de incubação, concentração do inóculo e adição de nutrientes na esporulação de *B. velezensis* S26 em frascos mantidos sob agitação;

b) Otimizar a esporulação de *B. velezensis* S26 em meios de cultura elaborados com lixiviado de compostagem e soro, empregando as condições ótimas de cultivo previamente determinadas;

c) Replicar em biorreator as melhores condições para esporulação de *B. velezensis* S26 utilizando o meio LB e os meios elaborados com lixiviado de compostagem e soro;

d) Avaliar o antagonismo de compostos difusíveis e voláteis sintetizados por células vegetativas e endósporos de *B. velezensis* S26 no crescimento micelial de *Botrytis* spp. e *Colletotrichum* spp.;

e) Analisar o efeito da aplicação de suspensão de endósporos, suspensão de células vegetativas, bem como do filtrado do cultivo de *B. velezensis* S26 na inibição da germinação de conídios de *Botrytis* spp. e *Colletotrichum* spp.;

f) Verificar a viabilidade dos endósporos de *B. velezensis* S26 em diferentes formulações durante o armazenamento à temperatura ambiente e sob refrigeração;

g) Determinar a ação antagonística de uma suspensão de endósporos de *B. velezensis* S26 no controle da antracnose e da podridão cinzenta em morangos;

h) Analisar o potencial inibitório de uma suspensão endósporos de *B. velezensis* S26 no controle da antracnose e da podridão cinzenta em bagas, discos foliares e plantas micropropagadas de videira (*Vitis vinifera* e *V. labrusca*);

i) Desenvolver uma formulação líquida contendo endósporos de *B. velezensis* S26 utilizando suprodutos de agroindústrias;

j) Avaliar o potencial da formulação líquida na promoção do crescimento e no biocontrole da antracnose e da podridão cinzenta em uma estufa comercial de morangos;

k) Elaborar uma formulação seca contendo endósporos de *B. velezensis* S26 incorporados em biochar de bagaço de uva;

l) Determinar a eficácia da formulação seca no controle de *Dactylonectria macrodidyma* e na promoção do crescimento do porta-enxerto de videira SO4 (*Vitis berlandieri* × *V. riparia*), cultivado tanto em substrato não contaminado quanto com altos níveis de cobre.

3 REVISÃO DA LITERATURA

Ao longo deste capítulo serão abordados aspectos genéticos, botânicos e fisiológicos, bem como os principais cultivares empregados comercialmente nas culturas do morangueiro e da videira. Além disso, serão discutidas doenças, como a antracnose e a podridão cinzenta, que afetam ambas as culturas, principalmente, na etapa de pós-colheita e o pé-preto que ataca o sistema radicular de videiras submetidas a estresses ambientais, como cultivo em solos contaminados por fungicidas cúpricos. Finalmente, serão descritas estratégias para o controle biológico dessas doenças e promoção do crescimento vegetal, destacando a utilização de endósporos bacterianos e sua aplicabilidade na elaboração de bioformulações.

3.1 Morangueiro

O morangueiro (*Fragaria* × *ananassa* Duchesne) é uma planta originária da hibridização natural entre as espécies de *Fragaria chiloensis* Mill. e *F. virginiana* Duch. Geneticamente, as plantas podem ser diploides, tetraploides, hexaploides, octoploides e, até mesmo, decaploides (Vignolo et al., 2016). Botanicamente, o morangueiro é uma angiosperma dicotiledônea, pertencente à família Rosaceae, subfamília Rosoideae e gênero *Fragaria*, o qual é constituído por 24 espécies distintas (Vignolo et al., 2016). As plantas são herbáceas com sistema radicular longo e fasciculado, o caule é um rizoma com entrenós curtos, dos quais partem folhas compostas e formadas por três folíolos (Gonçalves et al., 2016). O fruto do morangueiro é um aquênio, conhecido erroneamente como semente do morango, que apresenta dimensões reduzidas, coloração variando de amarelo a avermelhada e, quando associado ao receptáculo floral desenvolvido, forma o pseudofruto ou infrutescência denominado morango (Fillinger e Yigal, 2016; Vignolo et al., 2016).

O morango se caracteriza por seu elevado valor nutricional, contendo altas concentrações de sais minerais, compostos fenólicos, carotenoides, tocoferóis e ácido ascórbico (Vizzotto, 2016). Esses compostos conferem ao morango propriedades antioxidantes, contribuindo para prevenção de doenças crônicas, degenerativas e cardiovasculares (Kowalska et al., 2018; Tumbarski et al., 2019). O pseudofruto pode ser consumido *in natura*, desidratado, congelado ou empregado na produção de sucos, geleias, doces, iogurtes, sorvetes, chás, entre outros (Madail, 2016).

As variedades de morangueiro podem ser classificadas em variedades de dia curto, neutro e longo, dependendo da demanda por radiação solar (Gonçalves et al., 2016). Nesse sentido, o fotoperíodo é um fator determinante na indução da floração do morangueiro. Assim, cultivares de dia curto, como Oso Grande, Camarosa, Ventana, Camino Real são sensíveis ao fotoperíodo e necessitam de 8 a 12 horas diárias de insolação para florescer e frutificar. Por outro lado, cultivares de dia longo, como Summer Plant, necessitam de fotoperíodo superior a 12 horas para emissão de flores. Finalmente, em cultivares de dia neutro, como San Andreas, Diamante, Monterey, Portola, Aromas e Albion, a indução floral é independente do fotoperíodo (Gonçalves et al., 2016).

Os principais cultivares de morangueiro utilizados no país incluem Oso Grande, típico da região Sudeste, e cultivares como Camarosa, Aromas, Albion e San Andreas empregados nos estados do Sul (Oliveira e Antunes, 2016). Dentre os cultivares de morangos, destaca-se San Andreas, desenvolvido pela Universidade de Califórnia em 2008 (Antunes et al., 2011) e caracterizado pelo alto vigor e moderada resistência ao oídio, antracnose, podridão da coroa, murcha de *Verticillium*, podridão de *Phytophthora* e mancha-comum (Oliveira e Antunes, 2016). Similarmente, Albion é um importante cultivar de morangos de dia neutro, resultante do cruzamento entre ‘Diamante’ e uma seleção originária da Califórnia. A produção desse morangueiro é uniforme ao longo do ano e as plantas apresentam boa tolerância a podridões radiculares e à murcha (Oliveira e Antunes, 2016).

3.2 Videira

A videira é uma planta perene, caducifólia, trepadeira e sarmentosa pertencente à família Vitaceae (Kishino et al., 2007). A família Vitaceae apresenta 11 gêneros e aproximadamente 450 espécies. O gênero *Vitis* é o mais importante, sendo constituído por aproximadamente oitenta espécies distribuídas nos diferentes continentes (Galet, 2000). Esse gênero é dividido em dois subgêneros: *Muscadinia* Planch ($2n = 40$) e *Euvitis* Planch ($2n = 38$), cujas espécies estão agrupadas de acordo com morfologia, anatomia e origem geográfica (Denega et al., 2010).

As videiras pertencentes ao subgênero *Euvitis* apresentam características como caule com casca solta, ausência de lenticelas, gavinhas ramificadas, cachos grandes, casca fina e frutos açucarados. Esse subgênero é constituído por aproximadamente trinta espécies, incluindo *Vitis vinifera* Linneu, *V. labrusca* Linneu, *V. rupestris* Scheele, *V. riparia* Michaux, *V. berlandieri* Plachon, *V. caribae* De Candolle, *V. cinerea* Engelmann, *V. aestivalis* Michaux, *V. amurensis* Ruprecht e *V. candicans* Engelmann (Schuck et al., 2011; Hickey et al., 2019). Por

outro lado, as videiras do subgênero *Muscadinia* apresentam casca bem aderida ao tronco, caules jovens com lenticelas, brotação tardia, frutos pouco açucarados, muitos cachos por planta e poucas bagas por cacho (Denega et al., 2010). Esse subgênero é constituído por três espécies nativas da região sudeste dos Estados Unidos, compreendendo as espécies *V. rotundifolia* Michx. Syn. *M. rotundifolia* (Michx.) Small, *V. munsoniana* Simpson ex Munson e *V. popenoeii* J. H. Fennel (Andersen et al., 2010). As videiras muscadínias não apresentam importância comercial e são empregadas em programas de melhoramento genético de porta-enxertos, visando à resistência a doenças e estresses abióticos (Xu et al., 2017).

A uva, fruto da videira, é um alimento com elevado potencial nutricional, devido à presença de vitaminas, sais minerais, fibras alimentares, compostos fenólicos e antioxidantes. Dentre os compostos fenólicos, destacam-se os taninos, antocianinas e resveratrol que atuam na regulação de processos inflamatórios e na prevenção do câncer, da obesidade e de doenças crônicas, vasculares e cardíacas (Zhang et al., 2018; Tolve et al., 2021). Os frutos podem ser consumidos *in natura* ou empregados na elaboração de bebidas, vinagre, geleias, doces, entre outros (Venkitasamy et al., 2019).

No Brasil são empregados cultivares de uvas americanas percententes à espécie *V. labrusca*, como Bordô, Concord, Herbemont e Jacquez, destinados à elaboração de vinhos de mesa e sucos; cultivares de uvas europeias pertencentes à espécie *V. vinifera*, como Cabernet Sauvignon, Cabernet Franc, Riesling Itálico, Pinot Noir, Moscato Giallo, Trebbiano e Chardonnay, empregados na elaboração de vinhos finos e espumantes; e cultivares híbridos, como Goethe, Niágara, Kioho, Isabel e Couderc, para consumo *in natura*. Existem, ainda, cultivares de uvas de mesa, como Niágara Rosada, Itália, Superior Seedless, Crimson Seedless e Thompson Seedless, empregados tanto para consumo *in natura* quanto para o preparo de uvas passas (Mello e Machado, 2022).

O cultivo da videira é realizado através da enxertia, que consiste na união de tecidos entre uma variedade de interesse comercial, geralmente susceptível a estresses bióticos e abióticos, e uma variedade de porta-enxerto resistente que fornece um sistema radicular à planta (Baron et al., 2019). A utilização de porta-enxertos possibilita uma maior resistência a fitopatógenos e estresses ambientais, como seca e salinidade, além da precocidade de produção, maior vigor e produtividade (Tandonnet et al., 2010). Apesar de existirem aproximadamente 80 porta-enxertos que podem ser empregados no cultivo da videira, provenientes de cruzamentos entre as espécies *Vitis riparia*, *V. rupestris* e *V. berlandieri*, apenas alguns membros da família Berlandieri-Riparia (SO4 e Kobber 5BB) e da família Berlandieri-Rupestris (110R, Paulsen 1103 e 140Ru) são utilizados (Ollat et al., 2016). Dentre esses porta-enxertos, destaca-se o SO4

(*V. berlandieri* × *V. riparia*) que foi desenvolvido em 1941 na Alemanha. Ele confere um desenvolvimento vigoroso e boa produtividade à maioria das copas destinadas à elaboração de vinhos. Entretanto, esse porta-enxerto é sensível à fusariose (Vilvert et al., 2017) e pode apresentar problemas de dessecamento do engaço, uma anomalia ocasionada pelo desequilíbrio nutricional entre potássio, cálcio e magnésio (Camargo et al., 2011).

3.3 Doenças pós-colheita

O morango e a uva são altamente suscetíveis ao ataque de patógenos, requerendo cuidados especiais durante a colheita, manuseio, seleção, embalagem, armazenamento e transporte a fim de evitar a ocorrência de ferimentos, que facilitam a infecção fúngica (Matrose et al., 2021). As condições fisiológicas do fruto, na etapa de pós-colheita, tendem a favorecer a proliferação de agentes patogênicos que infectaram a planta no campo e permaneceram em estádio de latência (El Khetabi et al., 2022). A seguir serão descritas as duas principais doenças que afetam a cultura do morango e da videira na fase pós-colheita.

3.3.1 Podridão cinzenta

A podridão cinzenta é uma doença fúngica que afeta diferentes culturas na etapa pós-colheita, sendo causada por *Botrytis* spp., com destaque para a espécie *Botrytis cinerea* Pers. & Fr. [teleomorfo: *Botryotinia fuckeliana* (de Bary) Whetzel] (Ueno e Costa, 2016; Roca-Couso et al., 2021). A doença pode causar perdas de aproximadamente 50 % na produção mundial de alimentos, ocasionando prejuízos de 10 a 100 bilhões de dólares ao ano (Fillinger e Yigal, 2016; Amarouchi et al., 2021). *B. cinerea* é um fungo necrotrófico que promove a morte de células do hospedeiro por meio da liberação de toxinas, espécies reativas de oxigênio e enzimas líticas (Kelloniemi et al., 2015).

A infecção ocorre com a formação de um tubo germinativo e penetração do fungo através de ferimentos e aberturas naturais, como lenticelas, hidatódios e estômatos, ou ainda, por meio de ação mecânica e enzimática sobre a cutícula (Holz et al., 2007). O fungo pode permanecer quiescente até o surgimento de condições favoráveis ao seu desenvolvimento, como temperaturas entre 15 e 25 °C, elevada umidade relativa, cultivos adensados, adubação nitrogenada excessiva e irrigação por aspersão (Ueno e Costa, 2016). Assim, *B. cinerea* pode atacar desde frutos verdes, em processo de amadurecimento ou completamente maduros.

Embora o desenvolvimento ótimo do patógeno ocorra a 20 °C, é possível observar sua multiplicação mesmo em morangos armazenados sob refrigeração (Ueno e Costa, 2016).

Em morangueiros, *B. cinerea* ocasiona o escurecimento de pétalas e pedicelos, ressecamento de inflorescências e o aparecimento de lesões amarronzadas próximas ao cálice, culminando no apodrecimento e mumificação dos morangos. Em tecidos necrosados, pode-se observar o desenvolvimento de uma massa conidial de coloração acinzentada (Ueno e Costa, 2016). Similarmente, em videiras, são observadas lesões escurecidas em ramos, pecíolos, ráquis e próximo às nervuras foliares. O ataque às inflorescências pode resultar no secamento e abscisão das flores, enquanto as bagas podem exibir manchas circulares roxas ou pardacentas, sobre as quais ocorre a frutificação do fungo, levando ao aparecimento de um mofo acinzentado (Garrido e Gava, 2014).

O manejo da podridão cinzenta consiste no controle cultural com retirada de folhas e ramos em excesso, uso de porta-enxerto pouco vigorosos, adubação equilibrada, remoção de restos culturais infectados, aplicação de fungicidas, como calda sulfocálcica (Cavalcanti e Garrido, 2015), carbendazin, imazalil e thiabendazole (Khol et al., 2019), uso de óleos essenciais (Pedrotti et al., 2019) e de micro-organismos antagonistas (Hassan et al., 2021; Nikafos et al., 2021).

3.3.2 Antracnose, flor-preta do morangueiro e podridão da uva madura

A antracnose é uma doença complexa que pode afetar diferentes tecidos vegetais e ser causada por diversas espécies fúngicas (Ueno e Costa, 2016; Dowling et al., 2020). Em videira, as espécies *Elsinoë ampelina* (de Bary) Shear (anamorfo *Sphaceloma ampelinum*) e *Glomerella cingulata* (anamorfos *Colletotrichum gloeosporioides* e *C. acutatum*) têm sido relacionadas à antracnose (Garrido e Gava, 2014; Echeverrigaray et al., 2019). No entanto, o fungo *Glomerella cingulata* e suas formas anamórficas também podem causar a podridão da uva madura (Garrido e Gava, 2014). Por outro lado, a antracnose ou flor-preta do morangueiro é causada principalmente por espécies fúngicas como *Colletotrichum gloeosporioides* e *C. acutatum* (Ueno e Costa, 2016).

Em relação ao ciclo das relações patógeno-hospedeiro, verificam-se algumas diferenças entre as principais espécies envolvidas na ocorrência dessas doenças (*C. gloeosporioides* e *C. acutatum*), conforme pode ser observado na Tabela 1. Dessa forma, *C. gloeosporioides* é responsável pelo ataque preferencial a tecidos vegetativos, apresentando temperatura ótima de crescimento a 30 ºC e ciclos reprodutivos sexual e assexual. Diferentemente, *C. acutatum* se

caracteriza por colonizar principalmente órgãos reprodutivos, crescer em temperaturas amenas e apresentar ciclo de reprodução predominantemente assexual (Dowling et al., 2020).

Tabela 1. Comparativo entre os fungos *Colletotrichum gloeosporioides* e *C. acutatum*.

Características	*C. gloeosporioides*	*C. acutatum*
Disseminação	Chuva e vento	Chuva
Temperatura ótima	30 ºC	25 ºC
Tecidos infectados	Principalmente tecidos vegetativos, mas pode infectar frutos	Principalmente frutos, mas pode infectar tecidos vegetativos
Reprodução	Sexual e assexual	Assexual
Sobrevivência	Restos culturais e hospedeiros alternativos	Frutos mumificados, tecidos infectados e plantas assintomáticas

Fonte: adaptado de Dowling et al. (2020).

A antracnose do morangueiro se caracteriza pela ocorrência de lesões marrom-claras e encharcadas em frutos que progridem para lesões firmes e escurecidas. Sob condições de alta umidade, verifica-se a formação de uma massa de esporos com coloração alaranjada sobre as lesões, a qual pode cobrir totalmente os pseudofrutos com a evolução da doença (Ueno e Costa, 2016). Por outro lado, a flor-preta apresenta sintomas como lesões em pedúnculos, folhas, rizomas e escurecimento de flores, que pode ocasionar o secamento e a morte delas. Os frutos apresentam lesões necróticas e podem sofrer mumificação (Almeida et al., 2009). Similarmente à antracnose, são observadas as estruturas reprodutivas do fungo em condições de alta temperatura e umidade relativa. Ambas as doenças ocorrem principalmente em cultivos abertos ou que fazem uso de sistemas de irrigação por aspersão (Ueno e Costa, 2016). Essas doenças são de difícil manejo, uma vez que os tratamentos químicos não têm apresentado um controle satisfatório e existem poucos produtos registrados para a cultura do morangueiro (Almeida et al., 2009).

Na videira, a antracnose causa sintomas como manchas pequenas escurecidas em folhas, ramos e pecíolos. Essas manchas tendem a coalescer formando cancros ou lesões aprofundadas. Quando o ataque do patógeno ocorre em inflorescências pode ocorrer o secamento e abscisão dessas estruturas. Em frutos, verificam-se lesões escuras e arredondadas com halo pardacento (Cavalcanti e Garrido, 2015). Em contraste, na podridão da uva madura são observadas

manchas arredondadas e de coloração castanho-avermelhadas nas bagas, podendo ocorrer o aparecimento de sinais do patógeno na forma de pontuações escurecidas ou massa conidial alaranjada (Garrido e Gava, 2014; Cavalcanti e Garrido, 2015). O manejo dessas doenças é realizado mediante o uso de adubação equilibrada, retirada de cachos mumificados e realização de tratamento de inverno, com calda sulfocálcica. Somado a isso, pode-se realizar a aplicação preventiva de fungicidas sistêmicos e de contato, como carbendazin, imazalil e thiabendazole (Cavalcanti e Garrido, 2015; Khol et al., 2019).

3.4 Pé-preto da videira

O pé-preto é uma doença fúngica que afeta a videira, cujos agentes causais incluem aproximadamente 32 espécies de fungos habitantes do solo pertencentes aos gêneros *Campylocarpon*, *Cylindrocladiella*, *Cylindrocarpon*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, *Pleiocarpon* e *Thelonectria* (Agustí-Brisach e Armengol, 2013; Martínez-Diniz et al., 2021). Essa doença causa severos danos em viveiros e vinhedos jovens, levando ao definhamento e morte precoce de plantas (Martínez-Diniz et al., 2021).

No Brasil, o agente patogênico *Cylindrocarpon destructans* (Zinnsm.) Scholten foi primeiramente observado no ano de 1999, infectando vinhedos de uvas americanas na Serra Gaúcha (Garrido e Sônego, 2003). Posteriormente, análises moleculares possibilitaram a identificação de gêneros com ação patogênica, como *Campylocarpon, Ilyonectria, Rugonectria* e *Thelonectria* (Chaverri et al., 2011). Estudos de filogenia multigênica desenvolvidos por Lombard et al. (2014) demonstraram que outros gêneros estão associados com essa doença, assim como a origem parafilética de *Ilyonectria*, levando à inclusão do gênero *Dactylonectria* para englobar as espécies de *Ilyonectria* de videira.

Esses patógenos podem sobreviver no solo, como saprófitas facultativos ou na forma de clamidósporos (Petit et al., 2011; Holland et al., 2019). Somado a isso, mudas assintomáticas podem apresentar infecção latente, levando à disseminação da doença em vinhedos. Em condições ambientais favoráveis e de suscetibilidade do hospedeiro, o patógeno infecta o sistema radicular da videira através de aberturas naturais ou ferimentos, alojando-se em tecidos vasculares. Dessa forma, ocorre um decréscimo no fluxo de água e nutrientes, levando a uma redução generalizada do vigor da planta (Holland et al., 2019).

Nas plantas infectadas são observados sintomas como lesões necróticas e amolecidas em raízes, descoloração, redução da biomassa radicular e necrose na base do porta-enxerto. Na parte aérea da planta, verificam-se redução no crescimento vegetativo, atraso na brotação,

entrenós curtos, ramos e folhas esparsos, necrose e clorose internerval em folhas, murchamento e morte (Cavalcanti e Garrido, 2015; Martínez-Diniz et al., 2021). A realização de cortes transversais em estacas e ramos infectados revela a presença de necroses circulares e pontuações de coloração escura com exsudação de goma. Também pode-se visualizar a formação de estrias e o escurecimento do xilema por meio de cortes longitudinais (Alaniz et al., 2011; Correia et al., 2013).

O manejo da doença consiste em medidas culturais, como uso de mudas livres de doenças, plantio em áreas bem drenadas, prevenção de danos radiculares e desinfestação de utensílios empregados na enxertia e poda (Cavalcanti e Garrido, 2015). Pode-se, ainda, empregar o controle físico mediante o tratamento térmico de estacas previamente ao plantio (Lerin et al., 2017). Plantas infectadas no campo devem ser eliminadas visando reduzir a fonte de inóculo (Cavalcanti e Garrido, 2015). Somado a isso, pode-se adotar o controle biológico, com o uso de micro-organismos antagonistas e promotores do crescimento vegetal (Santos et al., 2016; Holland et al., 2019; Russi et al., 2020).

3.5 Solos contaminados com cobre e indução de estresse em videira

O cobre, na forma de íons mono (Cu^{+}) e divalente (Cu^{2+}), é um mineral naturalmente presente em solos (Cornu et al., 2017), que se encontra associado à matéria orgânica ou adsorvido a carbonatos, silicatos e óxidos de ferro, alumínio e manganês (Rusjan, 2012). Enquanto o íon Cu^{+} forma complexos com moléculas contendo enxofre, como os grupos tiol e tioéter, o cátion Cu^{2+} reage preferencialmente com o oxigênio ou grupos imidazólicos (Mir et al., 2021). Solos com baixo potencial hidrogeniônico, predominantes em regiões tropicais e subtropicais, apresentam níveis significativos deste mineral. Entretanto, a biodisponibilidade do cobre pode ser reduzida pela interação com outros nutrientes, como fósforo, nitrogênio e molibdênio, que favorecem sua complexação (Rusjan, 2012). A microbiota do solo também desempenha um importante papel na imobilização do cobre por meio da biossorção, bioacumulação e biomineralização (Fomina e Gadd, 2014; Cornu et al., 2017).

O cobre é um micronutriente essencial para o desenvolvimento vegetal. Assim, as plantas absorvem o cobre presente no solo de forma ativa, translocando-o via xilema a diferentes tecidos da planta. Esse nutriente está envolvido em uma ampla diversidade de processos bioquímicos e fisiológicos, tais como síntese de ácidos nucleicos, fotossíntese, respiração, distribuição e armazenamento de carboidratos em tecidos vegetais (Garrido, 2017; Shabbir et al., 2020; Mir et al., 2021).

Contudo, o controle químico de doenças com o uso intensivo de fungicidas à base de cobre pode desencadear o acúmulo desse mineral no solo (Garrido, 2017), em níveis potencialmente perigosos às plantas, microbiota e microfauna do solo (Lastochkina et al., 2019; Hassan et al., 2021). Na viticultura, a utilização frequente da calda bordalesa, hidróxido de cobre e oxicloreto de cobre para o manejo de doenças como antracnose, míldio e manchas foliares tem levado à contaminação do solo (Rusjan, 2012; Garrido, 2017).

O excesso de cobre afeta diversos processos fisiológicos na videira (Figura 1), resultando na diminuição do desenvolvimento vegetal e da biomassa radicular. O cobre também interfere em rotas fotossintéticas, ocasionando clorose em folhas e diminuição na biossíntese de carboidratos. Somado a isso, níveis elevados desse mineral promovem alterações na síntese de lignina, na sinalização hormonal e nas relações hídricas, levando à diminuição da condutância estomática e da transpiração, bem como ao aumento das espécies reativas de oxigênio (EROs). As EROs podem ocasionar peroxidação lipídica, disfunção da membrana, danos celulares e estresse oxidativo em videiras (Shangguan et al., 2018; Mir et al., 2021).

Figura 1. Efeito do excesso e da deficiência de cobre em plantas.

Fonte: adaptado de Shabbir et al. (2020).

Sabe-se que plantas submetidas a condições de estresse decorrentes de práticas de manejo, desequilíbrio nutricional, fitotoxicidade por metais pesados e pesticidas sintéticos apresentam maior susceptibilidade a doenças em geral (Menezes-Netto et al., 2016; Gramaje et al., 2018; Martínez-Diz et al., 2021). Além disso, altas concentrações de cobre podem afetar características sensoriais e a estabilidade de bebidas como vinhos e espumantes (Durguti et al., 2020). O consumo de frutos e bebidas contendo altos níveis de cobre também pode apresentar efeitos nocivos à saúde humana, desencadeando danos renais e neurológicos, cirrose hepática e anemia crônica (Dixit et al., 2015).

Diferentes estratégias podem ser adotadas para minimizar os impactos negativos do excesso de cobre no solo. Práticas de manejo, como a aragem, devem ser evitadas, uma vez que promovem a mineralização da matéria orgânica e, com isso, aumentam a disponibilidade do cobre às plantas (Cunha et al., 2015). Por outro lado, a calagem dos solos favorece o aumento na capacidade de troca catiônica (CTC) e, consequentemente, a imobilização do Cu em formas não assimiláveis pelos vegetais (Trentin et al., 2019). O emprego de plantas de cobertura também contribui para aumentar a complexação e remoção do cobre presente no solo (Brunetto et al., 2016; Trentin et al., 2019). Adicionalmente, o uso de bioestimulantes, como resíduos orgânicos, extratos de algas marinhas, quitina, derivados de quitosana, aminoácidos livres, bem como rizobactérias promotoras do crescimento vegetal e fungos micorrízicos arbusculares, contribuem para minimizar os efeitos tóxicos do cobre à videira (Monteiro et al., 2022).

3.6 *Bacillus* spp. como agentes de promoção de crescimento e biocontrole

O gênero *Bacillus* pertencente à família Bacillaceae, ordem Bacillales, filo Firmicutes (Christie e Setlow, 2020) é constituído por bactérias Gram-positivas, aeróbias estritas ou anaeróbias facultativas e formadoras de endósporos (Rabbee et al., 2019). Essas bactérias produzem uma ampla variedade de enzimas líticas (celulases, lipases, amilases e proteases), possibilitando o uso de diferentes substratos como meio de cultura (Etesami et al., 2023). Assim, elas podem se desenvolver em diversos ambientes, incluindo a rizosfera das plantas, sendo então denominadas de rizobactérias (Legein et al., 2020). Essas bactérias podem residir tanto epifiticamente, na parte aérea de plantas, quanto endofiticamente, no interior de vegetais (Eljounaidi et al., 2016).

Dessa forma, as bactérias endofíticas e rizobactérias são constituintes do ecossistema natural das plantas, permanecendo em equilíbrio com os demais micro-organismos. No entanto, a microbiota desses ambientes é influenciada por compostos exsudados pelas plantas, relações

ecológicas com demais organismos e por fatores abióticos, como condições climáticas, características do solo, práticas de manejo e aplicação de pesticidas (Legein et al., 2020). Esses micro-organismos são capazes de colonizar diferentes tecidos vegetais, formando uma barreira protetora que confere resistência a estresses biótico e abiótico (Pfeilmeier et al., 2016; Zhang et al., 2017).

Dentre os membros do gênero *Bacillus,* destaca-se a espécie *Bacillus velezensis* devido ao seu papel como agente de controle biológico e de promoção de crescimento vegetal (El Khetabi et al., 2022). A taxonomia dessa bactéria passou por diversas alterações em decorrência de análises moleculares e filogenéticas e, atualmente, engloba as espécies *Bacillus amyloliquefaciens, B. amyloliquefaciens* subsp. *plantarum* e *B. methylotrophicus* (Rabbee et al., 2019). *B. velezensis* apresenta uma grande diversidade de mecanismos de supressão de fitopatógenos (El Khetabi et al., 2022). Esses mecanismos podem ser diretos como competição por espaço e nutrientes, micoparasitismo, síntese de compostos antimicrobianos, produção de sideróforos, cianamida hidrogenada, biossurfactantes, compostos orgânicos voláteis, ou ainda, mecanismos indiretos por meio da indução de resistência em vegetais (Rabbee et al., 2019; Toral et al., 2020; De Moura et al., 2021). Somado a isso, essas bactérias podem formar biofilmes que auxiliam na adesão, proliferação e colonização do hospedeiro (Dukare et al., 2018; Dimkic et al., 2022).

Os compostos bioativos ou metabólitos secundários sintetizados por cepas de *Bacillus* pertencem a diferentes classes químicas, incluindo bacteriocinas, lipopeptídeos cíclicos, lipopeptídeos lineares e policetídeos (Ayed et al., 2017; Helfrich e Piel, 2016), conforme pode ser visualizado na Tabela 2.

Tabela 2. Principais compostos antimicrobianos sintetizados por *Bacillus* spp. e suas respectivas propriedades.

Tipo de composto	Composto	Ação	Referência
Bacteriocinas	Amilolisina, coagulina mersacidina, paenibacilina, subtilina e subtilolisina	Principalmente antibacteriana	Kumariya et al. (2019), Dimkic et al. (2022) e Zhang et al. (2022)
Lipopetídeos cíclicos	Bacilomicina, daitocidina, fengicina, halobacilina, iturina, lichenisina, micosubtilina, plipastatina e surfactina	Antibacteriana, antifúngica e antiviral	Ongena et al. (2007) e Legein et al. (2020)
Lipopetídeos lineares	Gageostatina, gageotetrina e gageopeptideo	Antifúngica	Tareq et al. (2014)
Policetídeos	Bacilaena, dificidina e macrolactina	Antibacteriana	Curran et al. (2018) e Tan et al. (2020)

As bacteriocinas são peptídeos de baixa massa molecular sintetizados nos ribossomos (Kumariya et al., 2019). Essas moléculas se caracterizam pela estabilidade térmica e resistência a diferentes condições de pH. Existem dois grupos principais de bacteriocinas: as lantibióticas e as não-lantibióticas. As bacteriocinas lantibióticas contêm aminoácidos comuns, tais como alanina, valina, serina e leucina e aminoácidos modificados, incluindo ácido β-metillântico, ácido α-aminobutírico e ácido β-hidroxifenilglicina. Muitas bacteriocinas lantibióticas têm a capacidade de formar poros na membrana da bactéria-alvo, levando à morte celular (Wang et al., 2020). As bacteriocinas não-lantibióticas apresentam grande diversidade na composição de aminoácidos, estrutura e massa molecular. Os mecanismos de ação das bacteriocinas não-lantibióticas incluem a inibição da síntese de proteica e de peptideoglicano (Zhang et al., 2022).

Por outro lado, os lipopeptídeos são moléculas anfifílicas e com propriedades tensoativas produzidos por bactérias pertencentes aos gêneros *Bacillus, Pseudomonas* e *Streptomyces*, por meio de enzimas peptídeo sintetases não-ribossomais (NRPS - *Nonribosomal Peptide Synthetases*) (Ayed et al., 2017). Essas moléculas são constituídas por uma cadeia longa de lipídeos associada a um peptídeo cíclico ou linear (Chauhan et al., 2022). Enquanto a porção peptídica é responsável pela atividade antimicrobiana, a parte lipídica facilita a interação com a membrana celular do micro-organismo alvo. Assim, os lipopeptídeos apresentam propriedades antibacterianas, antifúngicas e antivirais, em decorrência de múltiplos mecanismos ação. Esses mecanismos incluem degradação da parede celular e desestabilização de membranas em bactérias, bem como a inibição da enzima β 1,3-glucana sintetase em fungos (Segovia et al., 2021).

Estudos conduzidos recentemente indicaram que a cepa *B. velezensis* S26, foco de investigação neste estudo, apresenta capacidade de sintetizar lipopeptídeos cíclicos, como fengicina e surfactina. Essas moléculas desempenham papel crucial na supressão de fitopatógenos. A surfactina é reconhecida por sua atividade antimicrobiana, agindo principalmente por meio da alteração da integridade da membrana celular em diversos micro-organismos patogênicos. Ela também possui propriedades emulsificantes e atua na formação de biofilmes bacterianos. Por outro lado, a fengicina exibe atividade antifúngica, inibindo a formação da parede celular em fungos filamentosos ou, alternativamente, desestabilizando essas estruturas celulares (Legein et al., 2020).

Os policetídeos são metabólitos secundários com diferentes estruturas químicas, sintetizados por enzimas denominadas policetídeo sintetases (PKS – *Polyketide synthetases*) (Curran et al., 2018). Essas moléculas são conhecidas pela complexidade estrutural e diversidade de atividades biológicas, que incluem desde propriedades antimicrobianas até antitumorais. Elas podem ser sintetizadas por bactérias, fungos, vegetais e animais, e incluem compostos como tetraciclina, eritromicina, entre outros (Tan et al., 2020). Os principais policetídeos produzidos por cepas de *B. velezensis* incluem a bacilaena, a macrolactina e a dificidina que estão envolvidas, principalmente, na inibição de enzimas bacterianas (Rabbee e Baek, 2020).

Assim, a aplicação de agentes de controle biológico também conhecidos como bioagentes, contribui para reduzir tanto a incidência quanto a severidade de doenças em plantas. De acordo com Droby et al. (2009), bactérias antagonistas atuam promovendo mudanças nas comunidades microbianas e nas propriedades físicas do ambiente, intensificando os mecanismos de resistência do hospedeiro e suprimindo o patógeno por meio de estratégias

como competição, hiperparasitismo e antibiose. As restrições impostas ao patógeno podem ser alcançadas pela inviabilização do inóculo ou restrição ao processo infeccioso (Dowling et al., 2020). Da mesma forma, pode haver modificação do microambiente por meio da aplicação de bioagentes associados a nutrientes e adjuvantes, os quais podem interferir no potencial hidrogeniônico e na composição de nutrientes nas superfícies vegetais (Zhang et al., 2018). Adicionalmente, os bioagentes podem diminuir a suscetibilidade do hospedeiro ao estresse biótico, com a alteração da taxa respiratória e da síntese de etileno (Lastochkina et al., 2019).

Ademais, muitas espécies do gênero *Bacillus* são conhecidas como bactérias promotoras do crescimento de plantas (PGPB – *Plant Growth-Promoting Bacteria*), uma vez que auxiliam na mobilização e solubilização de nutrientes (Lastochkina et al., 2019; Morales-Cedeño et al., 2021) e sintetizam reguladores de crescimento, tais como auxinas, citocininas e ácido giberélico (Legein et al., 2020; De Moura et al., 2021). Essas biomoléculas atuam no desenvolvimento e na morfogênese vegetal, além de promoverem a ativação de mecanismos de resistência em plantas (Legein et al., 2020; Toral et al*.,* 2020). Enquanto as auxinas estimulam o crescimento e a diferenciação celular, as citocininas estão envolvidas na quebra da dominância apical, divisão e proliferação celular (Sharma et al., 2019). As giberelinas agem conjuntamente com as auxinas na promoção do alongamento celular, desenvolvimento de frutos e germinação de sementes (Anfang e Shani, 2021). Por outro lado, o ácido abscísico está envolvido em processos de dormência e fechamento estomático (Anfang e Shani, 2021). O etileno desempenha um papel fundamental no amadurecimento de frutos e na tolerância a estresses (Sharma et al., 2019). Assim como o etileno e o ácido abscísico, outros fitohormônios, incluindo os ácidos salicílico e jasmônico, também estão envolvidos na supressão de fungos e na ativação de mecanismos de defesa vegetal (Legein et al., 2020; Toral et al., 2020).

Contudo, a eficácia do controle biológico a campo depende de diversos fatores, como a capacidade de estabelecimento e colonização da planta, adaptabilidade a condições ambientais adversas e potencial de inibição dos fitopatógenos (Legein et al., 2020 Morales-Cedeño et al., 2021). Assim, a rápida proliferação de bactérias antagonistas em tecidos vegetais representa uma vantagem em relação à microbiota local (Yang et al., 2017). A capacidade de formação de biofilmes, aliada à presença de um amplo aparato para solubilização de nutrientes, também contribui para a sobrevivência de agentes de controle biológico em ambientes naturais (Legein et al., 2020).

3.6.1 Esporulação em bactérias do gênero *Bacillus*

Em situações ambientais desfavoráveis, com privação de nutrientes e alta densidade populacional, bactérias dos gêneros *Anoxybacillus*, *Bacillus, Geobacillus*, *Sporolactobacillus* e *Clostridium* podem formar estruturas de resistência denominadas endósporos (Mohsin et al., 2021). Esses esporos se caracterizam pela ausência de atividade metabólica e pela tolerância a condições adversas, como dessecação, solventes orgânicos, altas temperaturas e pressões, condições extremas de pH e radiação ultravioleta (Posada-Uribe et al., 2016; Cristiano-Fajardo et al., 2019; Lin et al., 2020). Tais atributos contribuem para o desenvolvimento de formulações estáveis ao armazenamento e resistentes a processos de secagem (Posada-Uribe et al., 2016; Cristiano-Fajardo et al., 2019).

Os endósporos bacterianos são estruturas complexas constituídas por três unidades principais denominadas revestimento, córtex e núcleo (Lin et al., 2020). O revestimento auxilia na proteção do endósporo contra substâncias químicas e lisozimas, sendo formado por duas camadas: revestimento interno e revestimento externo. Enquanto o revestimento interno atua como uma barreira de permeabilidade seletiva que protege o DNA contra agentes químicos, o revestimento externo é essencial para a formação do esporo, embora sua função ainda seja desconhecida. O córtex do esporo é formado por peptideoglicano, sendo responsável pela resistência e dormência dos esporos (McKenney et al., 2013; Lin et al., 2020). Finalmente, o núcleo contém enzimas, DNA cromossômico, ribossomos, tRNA e ácido dipicolínico (ADP) quelado com Ca^{2+}, que está envolvido na resistência a temperaturas elevadas e desidratação do esporo (McKenney et al., 2013). A estrutura compactada do material genético é mantida por meio de pequenas proteínas solúveis em ácido (SASP – *Small Acid Soluble Proteins*), que atuam promovendo resistência contra condições ambientais adversas (Liu et al., 2016).

A esporulação geralmente ocorre no final da fase de crescimento exponencial bacteriano e apresenta alta demanda energética, uma vez que envolve diferentes mecanismos de diferenciação celular. A indução e o progresso da esporulação são controlados por meio de interações proteína-proteína e proteína-DNA, além de diferentes mecanismos de retroalimentação, sinalização e regulação pós-traducional (Hoon et al., 2010). De acordo com Lin et al. (2020), a esporulação bacteriana compreende as seguintes etapas: replicação do material genético, divisão assimétrica do citoplasma bacteriano, formação de septo que compartimentaliza a célula-mãe do pré-esporo, engolfamento do pré-esporo, formação do exósporo, deposição do córtex, inclusão de componentes externos, e finalmente, lise da célula-mãe e liberação do esporo (Figura 2).

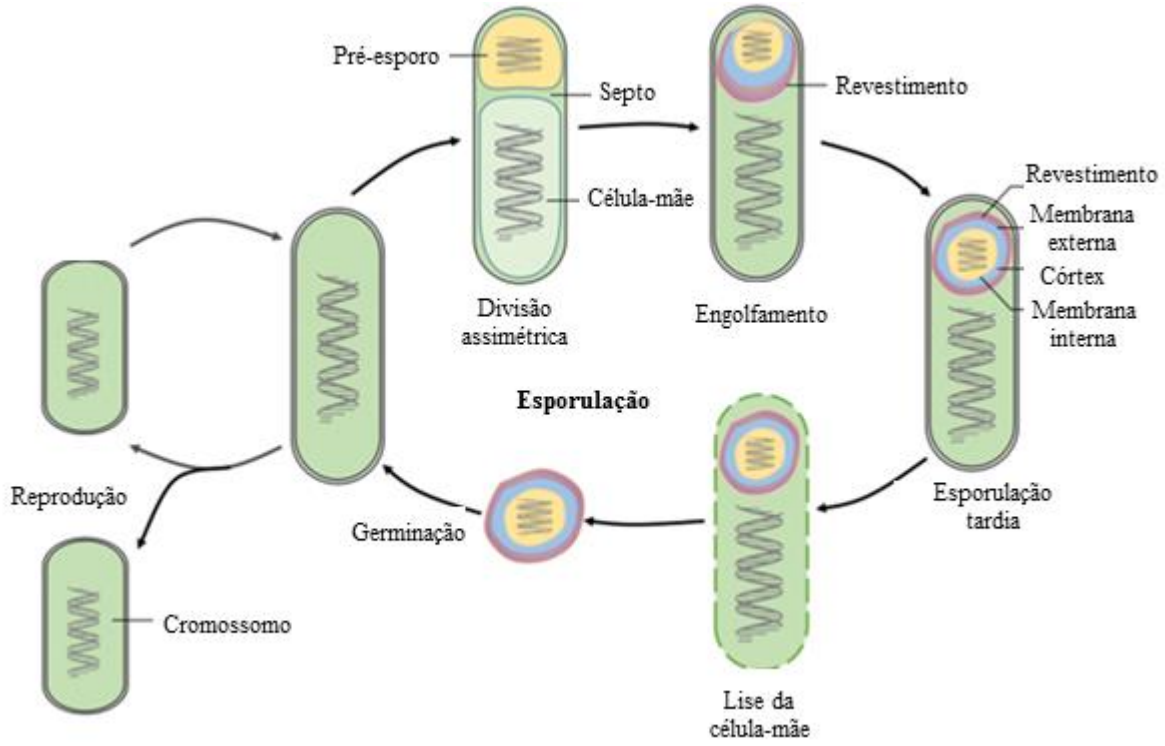

Figura 2. Etapas da esporulação em *Bacillus* spp.
Fonte: adaptado de Lin et al. (2020).

Os esporos inertes podem retomar o crescimento em condições ambientais favoráveis. Assim, na presença de L-aminoácidos e D-carboidratos específicos, o processo de germinação é iniciado e, dentro de poucos minutos, os esporos retornam ao estado vegetativo (Castillo et al., 2013; Christie e Setlow, 2020). Primeiramente, a germinação de endósporos requer a presença de receptores germinativos em seu revestimento interno que respondem a moléculas de baixa massa molecular como L-aminoácidos ou D-açúcares. Essa interação desencadeia a abertura de canais de membrana, com a liberação de H^+, K^+, Na^+ e baixos níveis de CaADP (Ca^{2+} quelado ao ácido dipicolínico). Em seguida, ocorre a abertura de um canal específico (SpoVA), com a completa liberação do CaADP e início da reidratação do esporo bacteriano. Finalmente, ocorre a ativação de enzimas córtex-líticas, que degradam o peptideoglicano presente no córtex, levando ao aumento nos níveis de água no núcleo do esporo, de 45 % para 80 %, e, assim, à retomada do metabolismo celular (Christie e Setlow, 2020).

3.6.2 Fatores envolvidos na esporulação bacteriana

A esporulação bacteriana em condições controladas depende de diferentes fatores, incluindo pH, temperatura, composição do meio de cultura, tempo de incubação, concentração

do inóculo e suplementação com nutrientes específicos (Elisashvili et al., 2019; Grauvy et al., 2021). Contudo, as condições ótimas para crescimento e esporulação variam entre as diferentes cepas bacterianas. De forma geral, meios de cultura com pH neutro favorecem o crescimento bacteriano, enquanto meios ligeiramente alcalinos desencadeiam o processo de esporulação (Elisashvili et al., 2019). O controle do pH durante o período de cultivo também pode afetar a produção de endósporos (Elisashvili et al., 2019). No entanto, Posada-Uribe et al. (2016) verificaram que tanto a densidade de esporos quanto a eficiência de esporulação de *B. subtilis* EA-CB00575 em biorreator não foram influenciadas pelo pH, quando esse foi mantido na faixa entre 5,5 e 7,0.

A temperatura de incubação também exerce influência significativa no processo de esporulação. Enquanto temperaturas mais elevadas podem acelerar a esporulação, temperaturas amenas tendem a retardar esse processo (Grauvy et al., 2021). De acordo com esses autores, a produção de esporos por *B. subtilis* BSB1 iniciou após 14 horas quando a temperatura de cultivo foi mantida a 37 °C. Entretanto, foram necessárias 51 horas para que os primeiros esporos fossem detectados quando a temperatura foi reduzida para 25 °C. O uso de temperaturas mais elevadas também leva à formação de esporos com maior resistência térmica (Isticato et al., 2020).

O meio de cultura utilizado é outro fator determinante, visto que fornece os nutrientes, necessários para o crescimento e esporulação bacteriana (Elisashvili et al., 2019). Assim, Velloso et al. (2023) avaliaram a esporulação de *B. megaterium* B119 em cinco meios de cultura distintos: meio de esporulação Difco (DSM), meio de esporulação para *Bacillus* (SBM), meio mínimo (MM), meio Luria Bertani + sais (LB) e solução salina mineral de Winogradsky (WSG). O cultivo bacteriano foi realizado em frascos de cultivo mantidos sob agitação a uma temperatura de 30 °C por 72 horas. Os autores verificaram que o uso do meio SBM levou a uma maior eficiência de esporulação no menor tempo de cultivo, ao passo que o meio DSM demonstrou boa taxa de produção de esporos ao longo de um maior período de incubação.

De forma similar, a concentração inicial do inóculo também pode ser controlada para maximizar o rendimento do processo de esporulação. O cultivo bacteriano em altas concentrações de inóculo resulta na rápida depleção de nutrientes, um fator essencial para a ativação de estratégias de sobrevivência, como a produção de endósporos (Klausmann et al., 2021; Reginatto et al., 2022). Adicionalmente, a suplementação do meio de cultura com cátions divalentes também contribui para a esporulação bacteriana. Esses íons atuam como cofatores de enzimas e formam quelatos com o ácido dipicolínico, um componente essencial da estrutura do endósporo bacteriano (Ren et al., 2018; Elisashvili et al., 2019). Assim, Ren et al. (2018)

verificaram que a suplementação combinada de Mn^{2+}, Fe^{2+} e Ca^{2+} ao meio LB promoveu um aumento na taxa de esporulação de *B. amyloliquefaciens* BS-20 em frascos de cultivo mantidos sob agitação.

3.7 Bioformulações

Bioformulações são produtos biológicos elaborados a partir de micro-organismos ou seus derivados, como enzimas e compostos metabólicos bioativos, com potencial de suprimir o desenvolvimento de fitopatógenos ou estimular o crescimento vegetal (Sahai et al., 2019; Stamenkovic et al., 2019; Kumari et al., 2021). As bioformulações são enquadradas como bioinsumos, uma vez que são produtos, processos ou tecnologias de origem microbiana, destinados ao uso na produção, armazenamento e beneficiamento de produtos agropecuários, de acordo com a Política Nacional de Bioinsumos, estabelecida pelo Decreto n°. 10.375 de 26 de maio de 2020 (BRASIL, 2020). Como essas formulações se caracterizam por promover o crescimento e os mecanismos de defesa vegetal, elas representam uma alternativa ao uso de pesticidas e fertilizantes químicos (Kumari et al., 2021).

Assim, os bioinsumos podem ser classificados em produtos fitossanitários ou bioestimulantes, conforme definido pela legislação brasileira (BRASIL, 2020). Produtos fitossanitários englobam produtos, processos e tecnologias desenvolvidos visando ao controle de pragas e doenças, incluindo feromônios, aleloquímicos, bioacaricidas, biofungicidas, produtos à base de cobre, boro, enxofre, óleo mineral, derivados de origem vegetal, animal e mineral e agentes de controle biológico. Por outro lado, os biofertilizantes ou bioestimulantes são produtos com princípios ativos de origem biológica, que promovem o desenvolvimento vegetal mediante o aumento de sua produtividade.

A composição de formulações biológicas engloba micro-organismos benéficos ou seus derivados, acrescidos de compostos carreadores e adjuvantes (Omer, 2010). Os carreadores ou veículos são empregados como suporte para os micro-organismos (Sahai e al., 2019), com a finalidade de facilitar o manuseio, aplicação e armazenamento das formulações (Omer, 2010). Esses carreadores podem ser substâncias inertes e inorgânicas, como argila, talco, bentonita, perlita, caulinita, alginato; ou substâncias orgânicas, como vermicomposto, esterco, turfa, farelos, lodos, biochares, entre outros (Berninger et al., 2018; Ajeng et al., 2020). Por outro lado, os aditivos ou adjuvantes são substâncias adicionadas à formulação para melhorar sua ação, aplicabilidade, funcionalidade, durabilidade, estabilidade, entre outros (Omer, 2010; Klein et al., 2017), conforme pode ser visualizado na Tabela 3.

Tabela 3. Principais classes de aditivos e exemplos.

Classe de aditivos	Exemplos
Acidificantes	Ácidos cítrico, acético, tartárico, fosfórico, sulfúrico, clorídrico
Aderentes	Cera, amiláceos, óleo de linhaça, silicone
Aglutinantes ou agregantes	Bentonita, amiláceos, lignosulfonato
Alcalinizantes	Hidróxido de amônio, potássio, sódio, monoetanolamina, dietanolamina
Antiempedrantes	Carvão ativado, cera, óleo, talco
Dessecantes	Sílica gel, talco, terra de diatomácea
Emulsificantes ou surfactantes	Bentonita, carboximetilcelulose, polissorbato, polietilenoglicol, silicone
Estabilizantes ou conservantes	Ácido cítrico, extrato pirolenhoso, maltodextrina, polióis, poliaminas, propilenoglicol
Tamponantes	Citrato de sódio, carbonato de potássio
Umectantes	Manitol, polietilenoglicol, polisorbato, sorbitol

Fonte: adaptado da Instrução Normativa Nº 61, de 08 de julho de 2020 do Serviço Público Federal - SPF - Ministério da Agricultura, Pecuária e Abastecimento - MAPA - e Secretaria de Defesa Agropecuária - SDA (BRASIL, 2020).

O emprego de bactérias no desenvolvimento de bioformulações apresenta algumas vantagens como: uso de diferentes mecanismos de controle de patógenos, rápida taxa de multiplicação, capacidade de crescimento em uma ampla diversidade de meios de cultura, ausência de toxicidade a animais e humanos e habilidade de produção de endósporos (Legein et al., 2020; Miljaković et al., 2020; Dimkic et al., 2022). Adicionalmente, os esporos bacterianos são resistentes às condições empregadas na fabricação de formulações e, ainda, apresentam resistência a longos períodos de armazenamento (Carmona-Hernandez et al., 2019; Lastochkina et al., 2019).

A fim de garantir sua eficácia, uma bioformulação deve apresentar características como atividade antimicrobiana em baixas concentrações, tolerância a condições ambientais adversas, ausência de compostos nocivos, efeitos neutros ou positivos sobre o hospedeiro, baixo custo de

fabricação, estabilidade e vida de prateleira satisfatórias (Sharma et al., 2009; Gotor-Vila et al., 2017; Miljaković et al., 2020). Também é necessário superar algumas limitações como a baixa reprodutibilidade dos resultados obtidos sob condições de campo, interações desfavoráveis com a microbiota natural e dificuldades na etapa de *scale-up* da produção (Matrose et al., 2021; Roca-Couso et al., 2021; Teixidó et al., 2022).

O desenvolvimento de formulações pode ocorrer em biorreatores em fase aquosa ou em fase sólida (Reginatto et al., 2022). Processos fermentativos em fase líquida empregam reatores agitados de forma contínua e são utilizados, principalmente, para o crescimento de bactérias e leveduras. Dentre as principais vantagens desses biorreatores, destacam-se as altas concentrações de bioprodutos obtidas e a facilidade na adição de nutrientes e no controle das condições de cultivo. Entretanto, esses processos geram grandes quantidades de resíduos e demandam etapas adicionais para a recuperação dos produtos finais (Teixidó et al., 2022). Diferentemente, fermentações em fase sólida costumam ser empregadas no cultivo de fungos filamentosos devido à menor atividade de água e maior semelhança com as condições existentes em seus ambientes naturais. Esse tipo de fermentação é mais simples e requer um menor volume de substrato, levando a uma baixa geração de resíduos ao longo do processo (Li e Wang, 2021; Teixidó et al., 2022).

As bioformulações podem se apresentar sob diferentes formas que variam desde formulações líquidas até preparados secos, como pós, grânulos, micro e nanocápsulas, dependendo do tamanho da partícula (Dukare et al., 2018; Mshari et al., 2019). Nesse contexto, estudos têm demonstrado que as bactérias do gênero *Bacillus* podem ser empregadas na produção comercial de formulações líquidas ou secas, com alto potencial de biocontrole e capacidade de promoção do crescimento vegetal (Stamenkovic et al., 2019).

3.7.1 Bioformulações secas

Os bioinsumos sólidos ou formulações secas apresentam ampla utilização em decorrência do baixo custo de fabricação e baixo volume final, facilitando o transporte e o armazenamento. No entanto, o processo de secagem e desidratação pode ocasionar danos às células bacterianas, reduzindo sua viabilidade (Gotor-Vila et al., 2017; Berninger et al., 2018; Teixidó et al., 2022). Assim, as bactérias formadoras de endósporos apresentam uma maior tolerância ao processo de secagem, a qual pode ser aperfeiçoada pela adição de compostos estabilizantes ou protetores, como carboidratos, polióis, albumina, caseína, lactoglobulina e

aminoácidos, contribuindo para preservar a integridade celular durante a elaboração de biopesticidas sólidos (Gotor-Vila et al., 2017).

As formulações secas podem ser desenvolvidas por meio de processos como:

a) Secagem a frio ou liofilização: método empregado para secagem de materiais termossensíveis. Consiste no resfriamento da amostra até seu completo congelamento, levando à cristalização de solutos e à formação de uma estrutura amorfa pelos solutos não cristalizados (Teixidó et al., 2022). Em seguida, ocorre a sublimação da água sob baixa pressão, resultando em um produto final de volume reduzido. No entanto, essa técnica pode ocasionar danos à membrana celular, desnaturação proteica e redução da viabilidade celular, além de apresentar altos custos em decorrência da demanda energética durante o processo (Vignesh et al., 2022);

b) Secagem por pulverização ou atomização: baseia-se na pulverização de um líquido com a formação de gotículas (10-200 μm), seguida da evaporação da água sob altas temperaturas (150-170 ºC). Em contato com o ar quente, as minúsculas gotas se transformam em partículas sólidas, as quais são coletadas por um ciclone ou filtro específico (Teixidó et al., 2022). As condições de secagem, como a temperatura de entrada, temperatura de saída, pressão durante a atomização, bem como o uso de substâncias adjuvantes podem ser otimizados para evitar a redução da viabilidade dos micro-organismos (Huang et al., 2017; Vignesh et al., 2022);

c) Secagem em leito fluidizado: é uma técnica utilizada para secagem, granulação e revestimento de pós, grânulos e esferas. Inicialmente, a amostra é transportada em uma corrente de ar quente (35-45 ºC) através de uma placa perfurada, promovendo sua fluidização. Em seguida, ela é direcionada a um granulador, sendo extrusada e cortada em forma de *pellets* (Carbó et al., 2017). O revestimento da amostra é realizado na câmara de Wurster por meio da pulverização de uma suspensão sobre os *pellets*. Esse processo é realizado em regime de transporte vertical dentro de um tubo de sucção, enquanto o fluxo do ar promove a secagem do revestimento depositado (Dreu et al., 2012);

d) Secagem por pulverização em leito fluido: essa técnica consiste na combinação entre a secagem por pulverização com o fluxo de biomassa através de um leito fluidizado submetido a baixas temperaturas. Isso possibilita a obtenção de grandes volumes de bioproduto, com bom potencial de dispersão e tamanho uniforme. Inicialmente, a amostra em associação com o adjuvante é atomizada por meio de uma bomba peristáltica em uma câmara de secagem. Posteriormente, as gotículas pulverizadas se combinam com uma substância carreadora fluidizada por um fluxo de ar quente, levando à granulação do material (Teixidó et al., 2022);

e) Encapsulamento: baseia-se na incorporação de um micro-organismo de interesse em microcápsulas constituídas por amido, quitosana, goma arábica, alginato de sódio, pectinas,

poliacrilamidas, entre outros. Essas cápsulas podem ser armazenadas por longos períodos e possibilitam uma liberação gradual do bioagente (Riseh et al., 2022; Teixidó et al., 2022). O encapsulamento pode ser feito por extrusão, secagem por pulverização e emulsão. No processo de extrusão, o bioagente associado a materiais encapsulantes é extrudado através de um bocal. Na secagem por pulverização, ocorre a dispersão do micro-organismo em uma solução de encapsulantes, formando uma emulsão que é submetida à atomização e evaporação do solvente. Por fim, a emulsificação consiste na dispersão de líquidos imiscíveis a partir de emulsificantes empregados para uniformizar a suspensão (Riseh et al., 2022).

Uma outra estratégia empregada na obtenção de formulações secas consiste na incorporação de micro-organismos a carreadores sólidos após prévia secagem ou redução do volume da suspensão microbiana. Essa abordagem tem sido adotada no desenvolvimento de biochares enriquecidos com agentes de biocontrole, promotores de crescimento vegetal ou envolvidos em processos de biorremediação (Ajeng et al., 2020). Biochar ou carvão biológico é o produto obtido da decomposição térmica de materiais orgânicos, sob condições controladas de temperatura, na ausência ou presença de baixas concentrações de oxigênio (Ajeng et al., 2020; Vanvuka et al., 2020; Da Silva et al., 2022).

Diferentes matérias-primas podem ser empregadas no desenvolvimento de biochares, como biomassa lenhosa, resíduos agrícolas, florestais e agroindustriais, algas marinhas, cama de frango, lodos de esgoto, resíduos domésticos orgânicos, entre outros (Sun et al., 2015; Ferjani et al., 2019; Yuan et al., 2019; Ajeng et al., 2020). As principais técnicas empregadas na decomposição térmica da biomassa são pirólise, carbonização hidrotérmica, gaseificação e torrefação (Yuan et al., 2019), conforme Tabela 4. Esses processos geram três tipos de produtos: resíduos sólidos (biochar), resíduos líquidos (extrato pirolenhoso e bio-óleo) e resíduos gasosos (CO, CO_2, H_2, CH_4, C_2H_2, C_2H_4, C_2H_6 e C_6H_6) (Magdziarz et al., 2020; Da Silva et al., 2022).

A pirólise é principal técnica empregada na obtenção de biochares e pode ser classificada em pirólise lenta (< 600 ºC), pirólise rápida (> 600 ºC), *flash* pirólise (800 a 1200 ºC) e pirólise assistida por radiação solar ou micro-ondas, dependendo do tempo de retenção da biomassa e da temperatura utilizada (Yuan et al., 2019; Ajeng et al., 2020). Diferentemente, o processo de carbonização hidrotérmica emprega temperaturas mais baixas que a pirólise, podendo utilizar biomassa úmida como matéria-prima. Por outro lado, a torrefação utiliza temperaturas intermediárias e pode ser classificada em seca ou úmida dependendo do conteúdo de água presente na amostra. Finalmente, a gaseificação usa temperaturas elevadas para converter a biomassa em uma mistura de gases. O processo ocorre em um reator de leito

fluidizado para facilitar as transferências de calor e massa, gerando biochar, cinzas inorgânicas e hidrocarbonetos aromáticos denominados alcatrões (Ajeng et al., 2020).

Tabela 4. Principais técnicas empregadas na termodecomposição da biomassa.

Características	Pirólise	Carbonização hidrotérmica	Gaseificação	Torrefação
Temperatura	~ 600 ºC	150-300 °C	> 750 ºC	200-300º C
Biomassa	Seca	Seca ou úmida	Seca	Seca ou úmida
Rendimento	10 %–20 %	Baixo rendimento		
Vantagens		Baixo consumo de energia	Biochar com propriedades mais estáveis	Baixo consumo de energia
Desvantagens	Alto consumo de energia		Alto consumo de energia	

Fonte: adaptado de Ajeng et al. (2020).

Do ponto de vista físico-químico, os biochares se caracterizam por apresentar elevada área superficial, alta porosidade, alta capacidade de troca catiônica e alta capacidade de adsorção (Yuan et al., 2019; Ajeng et al., 2020). Assim, a adição de biochar ao solo contribui para melhorar suas propriedades físicas e estruturais, como pH, condutividade elétrica, capacidade de retenção de água, porosidade, agregação e aeração (Vanvuka et al., 2020). Os biochares desempenham um importante papel do ponto de vista ambiental, contribuindo para a destinação final de resíduos, reduzindo volume, demanda bioquímica de oxigênio e presença de micro-organismos patogênicos. Esses materiais auxiliam, ainda, no sequestro de carbono no solo, diminuindo as emissões de dióxido de carbono e contribuindo para a mitigação do aquecimento global (Ferjani et al., 2019).

Diversos estudos têm relatado a incorporação de bactérias em biochares, uma vez que esses carreadores facilitam a estabilização da população microbiana, fornecendo proteção e nutrientes e evitando a lixiviação dos micro-organismos (Ajema, 2018; Azeem et al., 2021). A adesão é facilitada pela grande área superficial do biochar, assim como pela presença de grupos químicos, que interagem com a superfície desses micro-organismos por meio de processos

como floculação, adsorção, reticulação, ligação covalente, encapsulamento e aprisionamento (Rawat et al., 2019).

3.7.2 Bioformulações líquidas

Formulações líquidas consistem em suspensões de micro-organismos em caldos de cultivo, suspensões poliméricas, emulsões, solventes polares, óleos e/ou combinações destes (Gopal e Baby, 2016; Dukare et al., 2018; Allouzi et al., 2022). Apesar de as proporções dos seus constituintes serem variáveis, os biofertilizantes costumam apresentar entre 10-40 % de micro-organismos benéficos, 1-3 % de agentes suspensores, 1-5 % de dispersantes, 3-8 % de surfactantes e 35-65 % de líquidos carreadores (Mishra e Arora, 2016). Enquanto as formulações oleosas aplicadas na forma de pulverização foliar são menos susceptíveis ao escorrimento ocasionado pelas chuvas (Teixidó et al., 2022), formulações líquidas polares podem conter aditivos, como estabilizadores, protetores, emulsificantes, surfactantes, que melhoram suas propriedades físico-químicas e auxiliam na adesão superficial e colonização do hospedeiro (Allouzi et al., 2022; Teixidó et al., 2022).

De acordo com Allouzi et al. (2022), os bioinsumos líquidos são classificados em quatro classes principais: suspensões concentradas, suspensões de volume ultrabaixo, fluidos miscíveis em óleo concentrado e dispersões em óleo. As suspensões concentradas são constituídas por componentes sólidos suspensos, necessitando de diluição prévia ao uso. Diferentemente, as suspensões de volume ultrabaixo não necessitam de diluição e podem ser diretamente aplicadas. Por outro lado, fluidos miscíveis em óleo concentrado são formados pela dispersão de micro-organismos e adjuvantes em um líquido apolar, devendo ser diluídos antes do uso. Finalmente, as dispersões em óleo apresentam seus componentes principais dispersos diretamente em óleo ou solvente apolar, apresentando uma maior hidrofobicidade e, consequentemente, uma maior cobertura e menor lixiviação quando aplicadas em plantas (Mishra e Arora, 2016).

Bioformulações na forma líquida apresentam vantagens como baixos níveis contaminação, proteção contra estresses ambientais devido à adição de substâncias adjuvantes, alta eficácia a campo, fácil manuseio e podem ser aplicados por meio de fertirrigação ou pulverização tradicional (Gopal e Baby, 2016; Berninger et al., 2018; Jayasudha et al., 2018). Diversos estudos têm relatado que os bioinsumos líquidos tendem a apresentar maior viabilidade celular e vida de prateleira em comparação aos biopesticidas e biofertilizantes em pó ou grânulos (Brar et al., 2012; Jayasudha et al., 2018; Allouzi et al., 2022).

Um aspecto importante a ser considerado no desenvolvimento de bioprodutos líquidos é o emprego de meios de cultura e agentes carreadores atóxicos, de baixo custo, fácil manuseio e obtenção (Yánez-Mendizábal et al., 2012; Allouzi et al., 2022). Nesse contexto, a utilização de resíduos ou subprodutos de agroindústrias possibilita o crescimento microbiano em meios ricos em nutrientes e com baixo custo de aquisição (Yánez-Mendizábal et al., 2012). Além disso, é necessário o emprego de carreadores que forneçam condições adequadas de oxigenação, pH, equilíbrio osmótico e nutrientes para a manutenção de processos fisiológicos basais durante o armazenamento (Alouzi et al., 2022).

O uso de meios de cultura com custo reduzido para a produção de *Bacillus subtilis* CPA-8 foi avaliado por Yánez-Mendizábal et al. (2012). Foram testadas duas fontes de carbono (melaço e sacarose) e cinco fontes de nitrogênio (extrato de levedura, peptona, farinha de soja integral, casca de soja moída e farinha de soja sem gordura). De acordo com os autores, o uso de meio contendo farinha de soja sem gordura combinado com sacarose ou melaço possibilitou a obtenção de concentrações bacterianas superiores aos demais meios avaliados. De forma similar, Jayasudha et al. (2018) verificaram uma maior viabilidade de *B. subtilis* KK-9A após três meses de armazenamento em uma formulação contendo 8,5 % de óleo de pongamia (*Millettia pinnata*) em comparação ao biopesticida contendo a mesma concentração de óleo de girassol, óleo de amendoim, caldo nutriente ou água estéril.

Da mesma forma, Mshari et al. (2019) realizaram um estudo comparativo para avaliar a vida de prateleira de formulações líquidas e secas contendo *B. subtilis* Bs3. Os resultados indicaram que a formulação seca, com 50 % de talco, 0,5 % de quitosana e 3 % de carboximetilcelulose, proporcionou um aumento significativo na viabilidade celular após armazenamento por 180 dias à temperatura ambiente. Por outro lado, formulações líquidas, contendo 50 % de óleo de girassol, 50 % de óleo de parafina ou 50 % óleo de linhaça, isoladamente ou em associação com 1 % de glicerol e 3 % de carboximetilcelulose, apresentaram uma redução no número de colônias viáveis.

3.7.3 Reaproveitamento de resíduos para elaboração de bioformulações

A produção agroindustrial gera uma grande variedade e quantidade de resíduos e subprodutos ao longo do processo produtivo (Ferjani et al., 2019; Yao et al., 2021). No entanto, a disposição inadequada desses resíduos pode ocasionar danos à biodiversidade e ao meio ambiente, tais como poluição do ar, solo, corpos d´água e emissão de gases, contribuindo para as mudanças climáticas (Ferjani et al., 2019; Ayilara et al., 2020). Na indústria vitivinícola,

cerca de 25 % da massa total de matéria-prima é perdida na forma de engaço (ráquis) e bagaço, sendo este último constituído pelas cascas, sementes e restos da polpa da uva (Ferjani et al., 2019; Da Silva et al., 2022). As agroindústrias de laticínios também são responsáveis pela geração de um grande volume de subprodutos, que podem representar até 27 % do volume de leite processado (Sebastián-Nicolás et al., 2020).

Resíduos agroindustriais são excelentes fontes de nutrientes e compostos bioativos (Ferjani et al., 2019; Sebastián-Nicolás et al., 2020). O soro, por exemplo, é um subproduto rico em proteínas, cálcio, lactose e ácidos graxos (Sebastián-Nicolás et al., 2020), enquanto o bagaço da uva possui altos níveis de compostos fenólicos, vitaminas, taninos e ácidos orgânicos (Ahmad et al., 2020). No entanto, o descarte inadequado desses resíduos representa um sério problema ambiental, uma vez que eles apresentam baixo pH, elevada condutividade elétrica e elevadas demanda bioquímica de oxigênio (DBO) e demanda química de oxigênio (DQO). Devido a essas características, a disposição incorreta de resíduos pode levar à redução na fertilidade do solo, eutrofização de águas, depleção do oxigênio dissolvido e emissão de gases relacionados ao efeito estufa (Ferjani et al., 2019; Ahmad et al., 2020; Da Silva et al., 2022).

A obrigatoriedade da destinação correta dos resíduos está prevista na Lei nº 12.305/10 (BRASIL, 2010), que instituiu a Política Nacional de Resíduos Sólidos (PNRS). Nesse contexto, a produção de bioinsumos empregando resíduos biodegradáveis não perigosos como meios de cultura e agentes carreadores ou adjuvantes pode contribuir para minimizar os impactos decorrentes da geração e disposição inadequada desses resíduos (Balasubramanian et al., 2017). Dessa forma, os resíduos biodegradáveis provenientes de atividades agropecuárias, florestais, industriais ou domésticas podem ser empregados no processo de compostagem.

Assim, a compostagem consiste na decomposição aeróbica da matéria orgânica por micro-organismos, levando à mineralização e humificação dos resíduos (Ayilara et al., 2020; Vamvuka et al., 2020). A compostagem ocorre em três etapas principais que envolvem diferentes comunidades microbianas. Inicialmente, fungos, bactérias e actinomicetos realizam a decomposição de substâncias orgânicas complexas em monômeros e moléculas simples, sob condições mesofílicas (20-40 ºC). Em seguida, ocorre a fase termofílica (40-70 °C), com a degradação de ácidos graxos, celulose, hemicelulose e lignina. Essas duas etapas se caracterizam pela intensa atividade metabólica dos micro-organismos, necessitando de um elevado suprimento de oxigênio. Finalmente, ocorre o retorno à fase mesofílica, com diminuição na degradação componentes orgânicos, aumento na taxa de humificação e estabilização da matéria orgânica decomposta (Rastogi et al., 2020).

A eficiência e o tempo de duração da compostagem dependem de fatores como pH, tamanho das partículas, teor de umidade, relação C/N (carbono/nitrogênio), presença de compostos recalcitrantes e revolvimento das pilhas visando ao arejamento das mesmas (Ayilara et al., 2020). Devido às altas temperaturas atingidas durante o processo de compostagem, ocorre a eliminação de muitos agentes patogênicos e de sementes de plantas indesejadas, contribuindo para a qualidade do composto obtido (Hafeez et al., 2018).

O principal produto gerado nesse processo é o composto que contribui para melhorar as propriedades físico-químicas e biológicas do solo, estimulando o desenvolvimento das plantas e reduzindo o uso de fertilizantes químicos (Ayilara et al., 2020). Também, ocorre a liberação de gases como CO_2, PO_4, SO_2 e NO_2 e a geração de um efluente denominado chorume ou lixiviado (Ayilara et al., 2020). O chorume é um líquido proveniente da umidade natural da biomassa, da água das chuvas e da água adicionada para a manutenção da umidade nas pilhas de compostagem (Roy et al., 2018; Cruz et al., 2019). A composição do lixiviado depende da matéria orgânica empregada no processo de compostagem. Caso esse material apresente compostos tóxicos, metais pesados, xenobióticos ou micro-organismos patogênicos, essas substâncias serão incorporadas ao chorume (He et al., 2015; Roy et al., 2018; Cruz et al., 2019).

O chorume tende a apresentar altas concentrações de compostos orgânicos recalcitrantes como ácidos húmicos e fúlvicos gerados durante a decomposição microbiológica da matéria orgânica (He et al., 2015; Roy et al., 2018). A ocorrência de condições anaeróbias durante a compostagem leva à formação de ácidos graxos voláteis a partir de ácidos orgânicos, promovendo a redução do pH e afetando a ocorrência de reações de nitrificação. Assim, os principais compostos nitrogenados presentes no chorume são sais de amônio e aminas adsorvidas ao húmus (Roy et al., 2018).

Diferentes tratamentos podem ser efetuados visando reduzir a carga orgânica e as substâncias tóxicas presentes no chorume. Esses processos envolvem tratamentos físico-químicos como filtração, coagulação/floculação, eletrocoagulação, e biológicos como biofiltração, decomposição em biorreatores anaeróbios ou em reatores de membrana e deposição em *wetlands* (Roy et al., 2018). Após a realização desses tratamentos e da estabilização, o chorume ou lixiviado de compostagem pode ser empregado como fertilizante agrícola (Kim et al., 2015; Cruz et al., 2019), na bioprodução de hidrogênio por meio de processos fermentativos (Liu et al., 2015), entre outros usos.

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4 RESULTADOS E DISCUSSÃO

Ao longo deste capítulo, serão apresentados os principais resultados obtidos neste estudo na forma de artigos científicos:

4.1 Bacterial agents for controlling anthracnose and soft rot in strawberries: present status and perspectives

Publicado na revista Biocontrol Science and Technology

https://doi.org/10.1080/09583157.2024.2307455.

Essa publicação abordou duas importantes doenças fúngicas que afetam a cultura do morangueiro, a antracnose e a podridão mole. Além disso, foram discutidos os diferentes mecanismos de ação, bem como os métodos de aplicação de bactérias antagonistas para o controle biológico dessas doenças. O manejo da antracnose e da podridão mole pode ser realizado tanto no campo quanto na fase de pós-colheita. Adicionalmente, a utilização de diferentes espécies bacterianas contribuir para a redução de impactos ambientais, minimização da contaminação de frutos por resíduos químicos e a prevenção da seleção de patógenos resistentes.

4.2 Optimization of *Bacillus velezensis* S26 sporulation for enhanced biocontrol of gray mold and anthracnose in postharvest strawberries

Publicado na revista Postharvest Biology and Technology.

https://doi.org/10.1016/j.postharvbio.2023.112737

Neste artigo foram avaliadas diferentes condições de cultivo visando otimizar a esporulação de *B. velezensis* S26 em frascos contendo meio Luria-Bertani mantidos sob agitação. Em seguida, a viabilidade dos endósporos foi determinada ao longo do armazenamento a temperaturas de 4 ºC e 25 ºC por 365 dias. Também foi avaliada a eficácia de células vegetativas e endósporos de *B. velezensis* S26 na supressão do crescimento micelial e na inibição da germinação de conídios dos fungos *Botrytis* spp. e *Colletotrichum* spp. Após confirmação do potencial inibitório dos endósporos de *B. velezensis* S26, foi realizado um ensaio em morangos, empregando uma suspensão de endósporos fresca e outra armazenada durante seis meses à temperatura ambiente.

4.3 Suppression of *Colletotrichum* spp. on grape berries, vine leaves, and plants using *Bacillus velezensis* S26 endospores

Publicado na revista Scientia Horticulturae.

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Neste estudo foi investigada a atividade inibitória de uma suspensão de endósporos de *B. velezensis* S26 contra sete isolados de *Colletotrichum* spp. Os ensaios foram conduzidos em frutos, discos foliares e plantas micropropagadas de videira pertencentes a duas espécies distintas: *Vitis labrusca* e *V. vinfera*. Os endósporos bacterianos, suspensos em caldo LB, foram aplicados tanto na forma de uma suspensão fresca quanto de uma suspensão armazenada durante seis meses à temperatura ambiente.

4.4 Antagonistic potential of *Bacillus velezensis* S26 endospores against gray mold in grapevines

Este item aborda a verificação da ação antifúngica de uma suspensão de endósporos de *B. velezensis* S26 em caldo LB, na forma fresca e após armazenamento por um período de seis meses, sobre o fungo *B. cinerea* em videiras. Os ensaios foram conduzidos em bagas de uva, discos foliares e plantas micropropagadas de duas variedades de videira: Niágara branca (*V. labrusca*) e Moscato giallo (*V. vinifera*). Além disso, avaliou-se a colonização de bagas de uva por células vegetativas de *B. velezensis* S26, submetidas ou não à inoculação com o patógeno. Posteriormente, foi avaliado o efeito da aplicação de uma suspensão bacteriana na qualidade das uvas durante o armazenamento sob refrigeração.

4.5 Innovative formulations enriched with *Bacillus velezensis* S26 endospores using whey and composting leachate to enhance plant growth and mitigate strawberry diseases

Essa publicação avaliou a eficácia de diferentes formulações, contendo endósporos de *B. velezensis* S26, soro e lixiviado de compostagem do bagaço de uva, no controle dos fungos *Colletotrichum fructicola* e *Botrytis* sp. em morangueiros cv. San Andreas. Também foi determinada a ação dos endósporos de *B. velezensis* S26 na promoção do crescimento de morangueiros. Finalmente, foi conduzido um ensaio em uma estufa comercial de morangos para comparar a atividade antifúngica de quatro formulações distintas, contendo endósporos bacterianos, com o produto comercial Duravel WP® (*Bacillus amyloliquefaciens* MBI600).

4.6 *Bacillus velezensis* S26 - loaded biochar boosts plant growth, alleviates copper stress, and suppresses black foot disease in SO4 vine rootstock

Neste artigo, foi avaliado o emprego de diferentes técnicas para a incorporação de endósporos de *B. velezensis* S26 em biochar de bagaço de uva fresco e biochar de bagaço de uva compostado. Os ensaios de viabilidade celular foram realizados ao longo de seis meses de armazenamento, sob refrigeração e à temperatura ambiente. A eficácia desses biochares na promoção do crescimento, na supressão dos efeitos nocivos do excesso de cobre e no biocontrole do pé-preto foi avaliada em vários ensaios utilizando o porta-enxerto SO4 micropropagado (*Vitis berlandieri* × *V. riparia*).

4.1 Bacterial agents for controlling anthracnose and soft rot in strawberries: present status and perspectives

Abstract

Anthracnose and soft rot, caused by the fungal pathogens *Colletotrichum* spp. and *Rhizopus* spp., respectively, stand as prominent diseases impacting strawberries during the postharvest phase, resulting in significant economic losses. The use of conventional treatments involving chemical fungicides has been discouraged since they negatively impact the environment and human health. In this context, alternative strategies, such as biological control, have been required for organic and sustainable agriculture. While bacterial agents, such as *Bacillus* spp., are successfully used in commercial formulations to control postharvest diseases, there are other bacteria exhibiting antagonistic activities that hold potential in plant disease management. Hence, the current review aims to investigate studies developed over the past decade describing the primary bacterial genera with the potential to inhibit the causal agents of soft rot and anthracnose. Additionally, it aims to provide an overview of biocontrol mechanisms for suppressing these pathogens, detailing methods of application in both pre and postharvest phases, along with strategies for enhancing the efficacy of biocontrol agents. The findings of this review demonstrate that numerous bacterial species possess the ability to effectively control anthracnose and soft rot, both in growing plants and harvested strawberries. This expands the available forms of control and minimises the occurrence of resistant pathogens. Furthermore, the application of postharvest techniques such as edible coatings and bacterial encapsulation proves advantageous, extending fruit shelf life and reducing strawberry decay.

Keywords: biological control, *Colletotrichum* spp., *Rhizopus stolonifer*, postharvest diseases

1. Introduction

Strawberries (*Fragaria* × *ananassa* Duchesne) are cultivated worldwide, being appreciated for their nutritional value, flavor, and aromatic characteristics (Shen et al., 2019; Hassan et al., 2021). A growing emphasis on a healthier lifestyle has motivated the consumption of fruit produced using environmentally friendly methods and avoiding synthetic pesticides (Alijani et al., 2019; Hassan et al., 2021). Thereby, strawberry cultivation has faced several challenges as both the plant and fruit are susceptible to various fungal pathogens, including *Botrytis cinerea*, *Colletotrichum* spp., *Mucor* spp., *Penicillium* spp., and *Rhizopus* spp. (Feliziani & Romanazzi, 2016; Wang et al., 2018; Zhang et al., 2019; Chen et al., 2020). Among these plant pathogens, various species of *Colletotrichum*, *Mucor*, and *Rhizopus* contribute to the development of severe postharvest diseases, resulting in losses of approximately 30% in global fruit and vegetable production (Hodges et al., 2011; Oregel-Zamudio et al., 2017).

The fungal pathogens may attack the strawberry plant and fruit either in the field or after harvest (Fig. 1). Various abiotic stresses, including salinity, water deficiency, solar radiation,

nutritional imbalance, and improper management practices, can render the host more susceptible to diseases (Tyagi et al., 2017; El Khetabi et al., 2022). Infection by *Colletotrichum* spp. can occur through natural openings, epidermis, or wounds (Chen et al., 2020; Kumari et al., 2021; El Khetabi et al., 2022), while *Mucor* spp. and *Rhizopus* spp. enter through tissue injuries. These pathogens may persist in a dormant form, waiting for the optimal environmental conditions to initiate the infectious process (Saleh & Abu-Dieyeh, 2022). Harvested strawberries are also highly susceptible to infections due to natural deterioration, physiological changes, firmness loss, and mechanical tissue damage, all of which can compromise the anatomical barriers against pathogens (Chen et al., 2020; El Khetabi et al., 2022). Additionally, during handling, storage, and transportation, fruits may suffer wounds and mechanical injuries, increasing the proliferation of latent phytopathogens (Romanazzi et al., 2016; Hassan et al., 2021; Matrose et al., 2021).

Anthracnose is a fungal disease that causes significant losses and economic impacts (Reyes-Perez et al., 2019; Alijani et al., 2021a; Shi et al., 2021). The causal agents of anthracnose in strawberries include various species of *Colletotrichum*, including *Colletotrichum gloeosporioides* Penzig & Saccardo (teleomorph: *Glomerella cingulata* [Stoneman] Spaulding & Schrenk), *C. acutatum* JH Simmonds (teleomorph: *G. acutata* JC Guerber & JC Correll), and *C. fragariae* Brooks (Li et al., 2021b; Wu et al., 2021). The pathogen can persist as cultural remains from the previous production cycle, and under favorable environmental conditions, the spores germinate (Dowling et al., 2020). These hemibiotrophic fungi usually initiate the infection through biotrophic colonisation and, subsequently, proceed in a necrotrophic way (Tian et al., 2016; Sudheeran et al., 2020; Shi et al., 2021). Anthracnose affects several strawberry tissues and organs, causing root necrosis, irregular and black spots on leaves and fruits, bud crown rot, blossom blight, and reduced fruit yield and quality (Zhang et al., 2016).

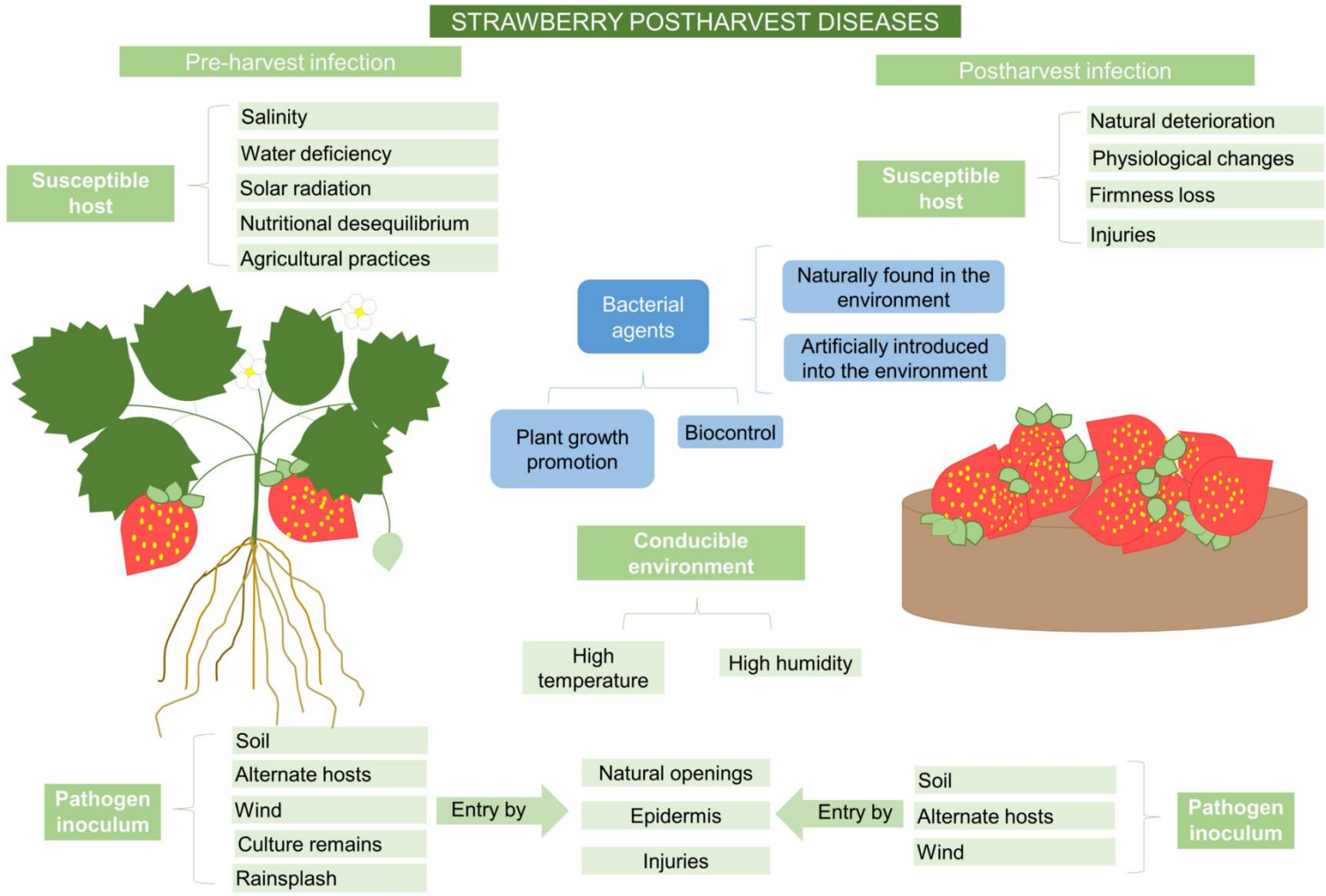

Fig. 1. Conditions for fungal infection in strawberries during preharvest (plants in the field) and postharvest (stored pseudofruit) stages, and the impact of bacterial agents on the environment.

Soft rot, also known as *Rhizopus* rot and *Mucor* rot, is another devastating disease that affects strawberry cultivation. The disease is primarily caused by *Rhizopus stolonifer* (Ehrenberg) Vuillemin, along with other species from the *Rhizopus* and *Mucor* genera (Zhang et al., 2010; Oliveira et al., 2019a). The pathogen can exist in various forms: acting as a parasite, a saprophyte, or a resistance spore known as zygospore (Feliziani & Romanazzi, 2016). Following the infection in the field, *R. stolonifer* remains latent during fruit development. Later, the pathogen shifts to a necrotrophic phase during storage, leading to significant economic losses. The symptoms of this disease include discoloration and water-soaked lesions on the fruit, which subsequently expand into dark and mushy areas (Cloutier et al., 2020).

Typically, the management of these phytopathogens involves the use of chemical fungicides. Boscalid, fludioxinol, and fenhexamid are commonly utilised for controlling *Rhizopus* rot (Feliziani & Romanazzi, 2016), while mancozeb, carbendazim, prochloraz, and Tecto 60 are employed in the management of anthracnose (Chechi et al., 2019). However, excessive use of these synthetic products can have detrimental effects on the environment, leading to pollution of soil, water, and the ecosystems. Additionally, they can contribute to pathogen resistance, persist as chemical residues on fruits, and negatively impact the health of both producers and consumers (Kumari et al., 2021; Li et al., 2021a; Matrose et al., 2021; Wang et al., 2021). In light of concerns regarding food safety, some countries have imposed restrictions on the application of fungicides on harvest fruits (Wisniewski et al., 2016; Wang et al., 2021).

Alternative approaches, including biological control, have emerged as a promising solution to replace chemical pesticides, contributing to the advancement of sustainable agriculture involving agroecological principles and preventive measures (Tyagi et al., 2017; Matrose et al., 2021). Biological products are less harmful to ecosystems compared to chemical pesticides (Zhang et al., 2017; De Moura et al., 2021) since they exhibit selective activity against pathogens and, at the same time, can enhance mechanisms of plant defense (Lastochkina et al., 2019).

Consequently, biocontrol aims to maintain microbial communities in plants, thereby regulating pathogen population to manageable levels (Carmona-Hernandez et al., 2019). Bacterial agents are the main focus of this study due to their prominence in managing postharvest diseases in organic farming (Carmona-Hernandez et al., 2019) and their importance in formulating biological products for industrial applications (Compant et al., 2019; Lastochkina et al., 2019; Roca-Couso et al., 2021). Additionally, certain bacterial strains can establish endophytic colonisation in plant tissues and employ diverse strategies for inhibiting

pathogens and inducing systemic resistance in plants (Droby et al., 2009; Legein et al., 2020; Dimkic et al., 2022).

In light of this, the present review aims to investigate relevant studies published in the past decade employing various bacterial agents to control anthracnose and soft rot in strawberries. We also intend to identify the primary bacterial species responsible for the biocontrol of these diseases, elucidate their inhibitory mechanisms, compare application strategies, and evaluate their benefits and constraints. Additionally, we will delve into recent technologies employed to enhance their antagonistic activity and discuss the perspectives and challenges encountered in the biological control of these diseases.

2. Biological control: antagonistic bacteria

Biocontrol strategies use living organisms including bacteria, yeasts, filamentous fungi, and fungi-like organisms, or their metabolites, to control the proliferation of phytopathogenic organisms, thereby improving plant resistance to diseases (Legein et al., 2020). Another equally effective strategy involves utilising natural products, such as plant and algae extracts and essential oils, to inhibit pathogens and extend fruit shelf life (Zhang et al., 2017). Specifically, biological control agents (BCAs) encompass epiphytic or endophytic microorganisms involved in controlling plant pathogens, either independently or as part of a microbial community (Zhang et al., 2017; Legein et al., 2020). These microorganisms can naturally inhabit the environment and their population can be augmented through external stimuli or artificially introduced onto plant surfaces to proliferate, and subsequently suppress the growth of pathogens (Sharma et al., 2009; Zhang et al., 2017).

In the management of postharvest diseases in strawberries, the adoption of yeasts and bacterial agents has emerged as a prominent strategy (Dukare et al., 2018; Mukherjee et al., 2020). Yeasts are distinguished for their capability to colonise environments with limited water availability. They demonstrate rapid proliferation and possess the capacity to release extracellular polysaccharides, a trait that aids in their own survival while simultaneously suppressing pathogens (Sharma et al., 2009; Dukare et al., 2018; Roca-Couso et al., 2021). Additionally, various antagonistic fungi, particularly species within the *Trichoderma* genus, have also found application in disease control during the postharvest stage (Dukare et al., 2018).

Bacterial agents exert control over pathogens through various mechanisms: competing for nutrient sources and space, colonising surfaces through biofilm formation, synthesising inhibitory substances such as antibiotics and volatile compounds, releasing hydrolytic enzymes,

and producing biosurfactants, hydrogen cyanide, and siderophores (Zhang et al., 2017; Carmona-Hernandez et al., 2019; De Moura et al., 2021; El Khetabi et al., 2022). In addition, they indirectly induce systemic resistance in plants (Lastochkina et al., 2019; Morales-Cedeño et al., 2021). Antagonistic bacteria have also demonstrated rapid growth, thereby restricting nutrient availability to pathogens (Dukare et al., 2018). Furthermore, their ability to form biofilms contributes to bioagent adhesion and proliferation on strawberry plants and fruits, providing protection against phytopathogen infections.

In this context, antagonistic bacteria or their derivatives can be combined with adjuvant compounds or carriers to create bioformulations (Arora et al., 2010; Prakash & Arora, 2020). The effectiveness of these formulations relies on the successful establishment of bacteria on the host tissues and their antagonistic activity against plant pathogens (Salvatierra-Martinez et al., 2018; Legein et al., 2020). Moreover, some specific strains exhibit various mechanisms of inhibition (Droby et al., 2009) and the ability to form endospores that are resistant to adverse environmental conditions (Lastochkina et al., 2019; Roca-Couso et al., 2021; Dimkic et al., 2022). Considering these crucial attributes, we have compiled a list of various bioformulations containing bacterial agents that are commercially available for the management of diseases in strawberries (Table 1).

Table 1. Commercial products applied in the biocontrol of strawberry diseases.

Commercial product	Bacterial strain	Target diseases	Bioagent concentration	Application method	Manufacturer
Actinovate®	*Streptomyces lydicus* WYEC108	Root decay, powdery mildew, downy mildew, gray mold, greasy spot, anthracnose, and others	1×10^7 CFU g^{-1}	Seed, soil, and foliar application	Natural Industries Inc., USA
Amylo-X® WG	*Bacillus amyloliquefaciens* subsp. *plantarum* D747 (*B. velezensis* D747)*	Gray mold and powdery mildew	5×10^{10} CFU g^{-1}	Foliar application	Certis Europe BV, Netherlands
Cease®	*Bacillus subtilis* QST713 (*B. velezensis* QST713)*	Angular leaf spot, anthracnose, gray mold, and powdery mildew	1×10^9 CFU mL^{-1}	Irrigation (drip and sprinkler) and foliar application	BioWorks, Inc., USA
Rhapsody®	*Bacillus subtilis* QST713 (*B. velezensis* QST713)*	Angular leaf spot, anthracnose, gray mold, and powdery mildew	1×10^9 CFU g^{-1}	Foliar application	AgraQuest Inc., USA
RhizoPlus® (FZB24)	*Bacillus subtilis* FZB24 (*B. velezensis* FZB24)*	Soil pathogens	2.5×10^{10} spores g^{-1}	Root zone and soil application	ABiTEP GmbH, Germany

Commercial product	Bacterial strain	Target diseases	Bioagent concentration	Application method	Manufacturer
RhizoVital®	*Bacillus amyloliquefaciens* FZB42 (*B. velezensis* FZB42)*	Soil pathogens	2.5×10^{10} spores mL^{-1}	Root zone and soil application	ABiTEP GmbH, Germany
Serenade® ASO	*Bacillus subtilis* QST713 (*B. velezensis* QST713)*	Angular leaf spot, anthracnose, common leaf spot, and powdery mildew	1×10^{9} CFU g^{-1}	Soil and foliar application	Bayer, USA
Sonata® AS	*Bacillus pumilus* QST2808 (*B. altitudinis* QST2808)*	Powdery mildew	1×10^{9} CFU mL^{-1}	Foliar application	Bayer, USA
Taegro®	*Bacillus amyloliquefaciens* FZB24 (*B. velezensis* FZB24)*	Crown rot, black root rot, and wilt	1×10^{10} CFU g^{-1}	Irrigation (drip and basal sprays), seeds, soil, and foliar application	Novozymes Biologicals, Inc., USA

CFU = Colony-forming unit.
* Identification of bacterial strains originally registered under a different species nomenclature than they are currently recognized (Dunlap, 2019).

These commercial products are offered in both liquid and dry forms. While liquid formulations may pose some restrictions concerning storage, transportation, and distribution, they can effectively colonise plant surfaces and preserve cell viability for extended periods of storage (Gotor-Vila et al., 2017; Prakash & Arora, 2020). Additionally, liquid products demonstrate the capacity to suppress a broad spectrum of postharvest diseases through various application methods, such as soil drenching, foliar spraying, and even through irrigation systems. Notably, our observations revealed that eight out of nine bioformulations encompass the utilisation of antagonistic bacteria from the *Bacillus* genus (Amylo-X®, Cease®, Rhapsody®, RhizoPlus®, RhizoVital®, Serenade®, Sonata® AS, and Taegro®), while only one commercial product (Actinovate®) employs a bioagent from a different genus (*Streptomyces lydicus* WYEC 108). Furthermore, these bioproducts were prepared using either vegetative cells or spores. Bacterial spores ensure stability and a prolonged shelf life for the formulations (Cristiano-Fajardo et al., 2019; Legein et al., 2020).

3. Bacterial agents against anthracnose and soft rot in strawberries

Given the limited range of antagonistic bacteria in commercial formulations and the growing demand for biological solutions in postharvest disease management, we conducted an extensive literature review, identifying recent studies utilising various bacterial agents for controlling soft rot and anthracnose in strawberries. Our investigation covered disease management in strawberry plants, known as preharvest treatment, as well as postharvest application on harvested strawberries, with the aim of reducing the incidence and severity of these devastating diseases (Tables 2 and 3).

The preharvest treatments involve the prophylactic application of bacterial agents in the field or greenhouses, aiming to establish beneficial microorganisms and promote their proliferation on the surface of strawberry plants (Solanki et al., 2019; Morales-Cedeño et al., 2021). In contrast, the postharvest approach involves applying antagonists to harvested strawberries through spraying or soaking in a bioagent suspension (Sharma et al., 2009; Carmona-Hernandez et al., 2019).

Applying antagonistic bacteria, either as preharvest or postharvest treatments, plays a crucial role in suppressing strawberry diseases, including anthracnose and soft rot. Furthermore, the preharvest utilisation of bacterial agents or their metabolites not only suppresses fungal pathogens but also enhances plant development and triggers plant defense mechanisms (Morales-Cedeño et al., 2021). In alignment with this, Alijani et al. (2019) found that the

preharvest application of *Staphylococcus sciuri* MarR44 in strawberry plants resulted in a higher suppression of *Colletotrichum nymphaeae* than postharvest treatments. This bacterium also demonstrated the capacity to synthesise various compounds involved in promoting plant growth, such as siderophores, indole-3-acetic acid, and gibberellins. Similarly, Wu et al. (2021) employed *B. amyloliquefaciens strains* PMB04 and PMB05 to control anthracnose in strawberry plant and fruit. The authors observed similar performance in biocontrol effectiveness between preharvest and postharvest treatments. In addition, preharvest application led to an increase in callose deposition in leaves and the generation of reactive oxygen species, demonstrating the induction of plant immune responses. Field treatments can also contribute to preventing pathogenic infections by forming a protective barrier against pathogens (Ippolito & Nigro, 2000) and inhibiting latent pathogens in the earlier stages of infection (Dukare et al., 2018). However, the preharvest application of bacterial agents faces limitations, primarily concerning their susceptibility to adverse environmental conditions (Dukare et al., 2018; Carmona-Hernandez et al., 2019). As a result, supplementary treatments with bacterial agents after harvest become necessary (Morales-Cedeño et al., 2021).

Postharvest approaches have also proven effective in inhibiting anthracnose (Alijani et al., 2019, 2020; Li et al., 2021ac) and soft rot (Oregel-Zamudio et al., 2016; Nayak et al., 2019; Thao et al., 2022). Bacterial treatments applied after harvest can extend fruit shelf life and increase overall quality. This hypothesis is supported by Li et al. (2021), who noted that *Bacillus safensis* QN1NO-4 extract reduced the incidence of anthracnose and preserved fruit mass and total soluble solids in harvested strawberries. Moreover, postharvest treatments with bacterial agents, essential oils, and plant extracts can also induce systemic acquired resistance in strawberry fruit. In a study by De Corato et al. (2018), raw extracts from brown seaweed (*Laminaria digitata*), in a preventive treatment, effectively suppressed soft rot disease in strawberries and increased peroxidase activity. Similarly, Paiva et al. (2020) reported the successful application of a commercially available seaweed extract derived from *Ascophyllum nodosum*, leading to a decrease in the incidence of *R. stolonifer* in postharvest strawberries. These seaweed extracts hold promise in stimulating plant growth, improving productivity (De Saeger et al., 2020), and inducing plant resistance (De Corato, 2019).

Table 2. Bacterial agents employed in the biocontrol of anthracnose in strawberry plant and fruit.

Bacterial agent	Strategies for application	Inhibition	References
Bacillus amyloliquefaciens Bc2	Bacterial cells in soil, both in growth chamber and field (preharvest)	100% all treatments (DI*)	Es-Soufi et al. (2020)
Bacillus amyloliquefaciens (PMB04 and PMB05)	Bacterial cells in seedlings (preharvest) and in fruit (postharvest)	41-64% in seedlings (DS***) and 40-50% in fruit (DS)	Wu et al. (2021)
Bacillus atrophaeus DM6120	Bacterial cells through soil drenching and foliar spraying in plant (preharvest) and in fruit (postharvest)	94.4% soil drenching (DS), 88.9% foliar spray (DS), and 71.4% in fruit (DS)	Alijani et al. (2021b)
Bacillus safensis QN1NO-4	Bacterial extract in fruit (postharvest)	13-67% (DI)	Li et al. (2021a)
Bacillus velezensis (IALR308, IALR585, and IALR619)	Plants soaked in bacterial culture before planting in a greenhouse (preharvest)	50% (PM**)	Mei et al. (2021)
Bacillus spp. (YN8, LN57, and MN17) and *Pseudomonas aeruginosa EN18*	Bacterial cells applied alone, through foliar and soil application in plant (preharvest) and in fruit (postharvest)	~ 90% in plants maintained in a greenhouse (DI) and 98.3-99.1% in fruit (DI)	Alijani et al. (2021a)

Bacterial agent	Strategies for application	Inhibition	References
Staphylococcus sciuri MarR44	Bacterial cells through soil and aerial application in plant (preharvest), bacterial cells and VOCs**** in fruit (postharvest)	77.8% soil drenching (DS), 72.2% aerial spraying (DS), 52.4% bacterial cells in fruit (DS), and 72.2% VOCs in fruit (DS)	Alijani et al. (2019)
Stenotrophomonas maltophilia UN1512	Cell-free filtrate in plant (preharvest) and in fruit (postharvest)	66.7% in plants maintained in a greenhouse (DS) and 86.7% in fruit (DS)	Alijani et al. (2020)
Streptomyces sp. (MBFA-172 and MBFA-227)	Spore suspension through foliar application in plant (preharvest)	26.2-33.2% using MBFA-172 (DS) and 6.4-8.9% using MBFA-227 (DS)	Marian et al. (2020)
Streptomyces sp. H4	Bacterial extracts in fruit (postharvest)	66.4-76.4% (DI)	Li et al. (2021c)

*DI = disease incidence, **PM = plant mortality, ***DS = disease severity, ****VOCs = volatile organic compounds.

In the management of anthracnose, preharvest application of bacterial agents effectively reduced both the incidence and severity of the disease. Studies have reported a significant disease inhibition, with rates exceeding 88.9% when employing strains of the *Bacillus* genus (Es-Soufi et al., 2020; Alijani et al., 2021ab). Additionally, bacterial cells of *Staphylococcus sciuri* MarR44 (Alijani et al., 2019) and metabolites released by *Stenotrophomonas maltophilia* UN1512 (Alijani et al., 2020) exhibited a substantial reduction in anthracnose severity, ranging from 66.7% to 86.7%. Postharvest treatments have also proven successful in controlling *Colletotrichum* spp. in strawberries. The use of *Bacillus* spp. suspension resulted in a 98-99% reduction in anthracnose incidence (Alijani et al., 2021a), while compounds secreted by *S.*

maltophilia UN1512 reduced symptom severity by 86.7% (Alijani et al., 2020). Moreover, the bacterial agent *S. sciuri* MarR44 led to a reduction in anthracnose severity by 52.4% when using bacterial cells and 72.2% when applying volatile compounds.

Table 3. Bacterial agents employed in the biocontrol of soft rot in strawberry plant and fruit.

Bacterial agent	Strategies for application	Inhibition	References
Bacillus megaterium (Bio-Arc®)	Bacterial cell spray to crops cultivated in the field four weeks prior to harvest (preharvest)	82.3-85.9% (DI*)	Rashid et al. (2022)
Bacillus siamensis VK199	Bacterial cells in fruit (postharvest)	22.6% (DS**)	Thao et al. (2022)
Bacillus subtilis	Bacterial cell spray to crops cultivated in the field four weeks prior to harvest (preharvest)	92.9-100% (DI)	Rashid et al. (2022)
Bacillus subtilis HFC103	Bacterial cells in fruit (postharvest) Bacterial cells + edible films of candelilla wax in fruit (postharvest)	78% (DS) 56% (DS)	Oregel-Zamudio et al. (2016)
Bacillus subtilis DTBS-5	Bacterial cells in fruit (postharvest)	Did not inhibit	Nayak et al. (2019)
Bacillus subtilis F9-8, *Pseudomonas moraviensis* F9-6, *P. arsenicoxydans* F9-7, *P. koreensis* F9-9, and *P. brenneri* F9-10	Bacterial cells in fruit (postharvest)	67% (DI) and 91% (DS) after 4 days of incubation	Cloutier et al. (2020)

Bacterial agent	Strategies for application	Inhibition	References
Pseudomonas fluorescens DTPF-3	Bacterial cells in fruit (postharvest)	Similar to the control during storage at 25 °C for 3 days or at 5 °C for 8 days	Nayak et al. (2019)

*DI = disease incidence and **DS = disease severity.

We found a limited number of studies addressing the management of soft rot in strawberries. The biocontrol of this disease has primarily been investigated utilising bacterial suspensions of *Bacillus* and *Pseudomonas* species. In a study conducted by Rashid et al. (2022), the application of a *Bacillus* spp. suspension to strawberry plants resulted in remarkable disease suppression, ranging from 82.3% to 100%. Conversely, postharvest treatments exhibited lower disease inhibition compared to field applications. Nayak et al. (2019) noted that inoculating a suspension of *Bacillus subtilis* DTBS-5 in harvested strawberries did not lead to a reduction in soft rot incidence. In contrast, Oregel-Zamudio et al. (2017) reported significant inhibition of *R. stolonifer* using *B. subtilis* HFC103 in association with candelilla wax edible coatings.

These studies demonstrated that various bacterial genera exhibit promising attributes for the biocontrol of anthracnose and soft rot in strawberries (Table 4). Nonetheless, it is crucial to acknowledge their specificities, antagonistic properties, and limitations, as outlined below.

Table 4. Comparison of the main bacterial genera employed in the biocontrol of anthracnose and soft rot in strawberries.

Bacterial genera	Endospore production	Endophytic capacity	Growth promotion	Other advantages	Main commercial products	Main commercial products for strawberries
Bacillus	x	x	x	Most studied and used in the development of bioformulations	Avogreen®, Amylo-X®, Ballad®, Biosubtilin®, Botrybell®, Cease®, Companion/Kodiak®, EcoGuard®, Ecoshot®, GB34®, Histick N/T®, Subtilex/Pro-Mix®, Nacillus®, Rhapsody®, RhizoPlus®, RhizoVital®, Serenade®, Sonata®, Sublic® Taegro®, and Yield Shield®	Amylo-X®, Biosubtilin®, Cease®, Rhapsody®, RhizoPlus®, RhizoVital®, Serenade®, Sonata®, and Taegro®
Pseudomonas	No	x	x	Presence of flagella for plant colonization and formation of biofilms	AtEze®, Bio-Save 10LP®, Bio-Ject Spot-Less®, BlighBan A506®, Cerall®, Frostban®, and Proradix®	No commercial product
Staphylococcus	No	x	x	Resistance to salinity	No commercial product	No commercial product

Bacterial genera	Endospore production	Endophytic capacity	Growth promotion	Other advantages	Main commercial products	Main commercial products for strawberries
Stenotrophomonas	No	x	x	Resistance to salinity and alkali environments, nitrogen fixing bacteria, and bioremediation	No commercial product	No commercial product
Streptomyces	x	x	x	Fungal structures facilitate root colonisation	Actinovate®, Biotrust®, Mycostop®, and Rhizovit®	Actinovate®

3.1 *Bacillus* spp.

Bacillus is comprised of a highly diverse group of non-pathogenic, strictly aerobic or facultatively anaerobic, and Gram-positive bacteria (Roca-Couso et al., 2021; Dimkic et al., 2022). They are the most common and effective bacteria used in developing bioformulations (Roca-Couso et al., 2021) due to their specific characteristics such as the ability to form spores that contribute to increasing cellular viability during extended storage periods (Legein et al., 2020). Many strains of *B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis* are recognised for their ability to control plant diseases (Legein et al., 2020; Toral et al., 2020).

Besides, several studies have reported their capacity of producing a wide range of bioactive compounds (Maksimov et al., 2015; Lastochkina et al., 2019; Dimkic et al., 2022). These antimicrobial peptides can be synthesised by both ribosomal and non-ribosomal enzymes (Dimkic et al., 2022). Ribosomally produced bacteriocins include subtilin, which is involved in phytopathogen cell death, amylocyclicin that inhibits bacteria development, and mersacidin that suppresses the formation of the fungal cell wall (Dimkic et al., 2022). Non-ribosomal lipopeptides encompass fengycin and iturin, which inhibit the formation of cell membranes in pathogenic fungi, and surfactin, which is involved in biofilm formation (Legein et al., 2020). Genes that encode volatile and non-volatile compounds can represent around 5-15% of the *Bacillus* spp. genome (Devi et al., 2019). In addition, some *Bacillus* spp. can penetrate plant tissues as endophytes and confer benefits to the host, avoiding the need to compete with local microorganisms (Sivasakthi et al., 2014). Various strains of *Bacillus* spp. have demonstrated successful application in the biocontrol of anthracnose and soft rot in both strawberry plant and fruit (Tables 2 and 3). Besides, the main commercial bioformulations employing these bacteria species can be seen in Table 4.

3.2 *Pseudomonas* spp.

Pseudomonas spp. constitute another crucial group of bioagents involved in plant protection against phytopathogens. These aerobic, non-sporulating, and Gram-negative bacilli are naturally present in diverse environments, including the roots and aerial parts of plants (Maignien et al., 2014; Legein et al., 2020). They are particularly abundant in the rhizosphere and serve as a model in studies concerning root colonisation (Sivasakthi et al., 2014). *Pseudomonas fluorescens*, *P. aureofaciens*, *P. chlororaphis*, *P. putida*, *P. aeruginosa*, and *P. syringae* are among the most extensively studied members of this genus (Dimkic et al., 2022).

According to the study developed by Paulsen et al. (2005), approximately 5.7% of the *P. fluorescens* Pf-5 genome encodes proteins involved in the production of antimicrobial compounds, such as phenazines. These compounds exhibit activity against phytopathogens by interfering with DNA replication. Besides, these secondary metabolites are involved in the formation of biofilms and the suppression of fungal growth (Legein et al., 2020; Dimkic et al., 2022).

Pseudomonas spp. also possess flagella used in their movement towards suitable zones for colonisation. They have the capacity to form biofilms, produce biosurfactants, and are capable of colonising various plant tissues, even as endophyte (Legein et al., 2020; Dimkic et al., 2022). The effectiveness of *P. aeruginosa* EN18 in reducing anthracnose severity in strawberries has been demonstrated (Alijani et al., 2021a). Furthermore, the application of various strains of *Pseudomonas* spp., in the postharvest phase, led to a reduction in the incidence and severity of soft rot in strawberries (Nayak et al., 2019; Cloutier et al., 2020) (Table 3). While these bacterial strains are utilised in several commercial products, none of them are currently recommended the management of postharvest diseases in strawberries (Table 4).

3.3 *Staphylococcus* spp.

Staphylococcus spp. are aerobic and Gram-positive bacteria found in diverse environments, including soil, water, plant rhizosphere, animals, and humans (Bodhankar et al., 2017; Alijani et al., 2019). While they play a pivotal role in these ecosystems, some strains can pose health risks to humans by causing infections (Gong et al., 2020). They are versatile microorganisms that can tolerate saline environments (Alijani et al., 2019). Studies have revealed that *Staphylococcus cohnii*, *S. hominis*, *S. capitis* (Dutta & Thakur, 2017), and *S. equorum* EN21 (Vega et al., 2019) can promote plant growth.

They can also be involved in the management of phytopathogens (Alijani et al., 2019; Gong et al., 2020). Vega et al. (2019) confirmed the potential of *S. equorum* EN21 to inhibit pathogen communication by silencing molecules involved in quorum sensing. They also observed that the quorum quenching approach had success in the attenuation of the pathogen virulence. The application of cellular suspension of *S. sciuri* MarR 44 in strawberry plants reduced the severity of the symptoms caused by *Colletotrichum nymphaeae* both through soil drenching and foliar spraying. Moreover, the postharvest treatment of strawberry pseudofruits with both bacterial suspension and volatile compounds synthesised by this bioagent also led to a reduction in the disease severity (Alijani et al., 2019), as seen in Table 2. At present, there is

no commercially available formulation using this bacterial agent for the management of strawberry diseases (Table 4).

3.4 *Stenotrophomonas* spp.

Stenotrophomonas spp. are aerobic and Gram-negative bacteria (Alijani et al., 2020; Pinski et al., 2020) found in various environments such as marine, soil (Reyes-Perez et al., 2019), and animal tissues, even as a pathogenic microorganism (Pinski et al., 2020). Marine microbiota exhibits suitable characteristics for biocontrol agents, including resistance to saline stress, adverse environmental conditions, and different nutritional requirements compared to microorganisms naturally found on plant surfaces (Chi et al., 2010; Rivas-Garcia et al., 2019). Besides, these bacteria can be applied in biological remediation (Pinski et al., 2020), enhance plant growth, and fix the atmospheric nitrogen (Egamberdieva et al., 2016; Reyes-Perez et al., 2019).

According to Pinski et al. (2020), *S. maltophilia* can survive in alkali lakes, oxidised metals, and saline environments. Studies have demonstrated that some strains of *S. rhizophila* and *S. maltophilia* exhibited antagonistic activity against phytopathogenic fungi and capacity to stimulate plant growth (Egamberdieva et al., 2016; Rivas-Garcia et al., 2019; Pinski et al., 2020). Additionally, *S. maltophilia* UN1512 not only decreased the severity of anthracnose caused by *Colletotrichum nymphaeae* in both strawberry plants and pseudofruits (Table 2) but also led to an augmentation in plant length and biomass production (Alijani et al., 2020). No products incorporating this bacterium were developed, as field and postharvest applications raised concerns about their potential threat to human health (Pinski et al., 2020) (Table 4).

3.5 *Streptomyces* spp.

Streptomyces spp. are ubiquitous Gram-positive bacteria whose branching filaments facilitate colonisation in the plant rhizosphere (Olanrewaju & Babalola, 2019). Due to their morphological characteristics shared with fungi, such as the presence of hyphae, mycelium, and asexual spores, these bacteria are also known as actinomycetes or actinobacteria. *Streptomyces* spp. are common endophytic microorganisms in commercial crops (Marian et al., 2020; Li et al., 2021c), and some species can be considered phytopathogens (Vurukonda et al., 2018; Olanrewaju & Babalola, 2019). However, these actinobacteria can be beneficial to plants by increasing plant biomass and yield. These microorganisms can also synthesise antifungal

metabolites and lytic enzymes (Kim et al., 2020; Li et al., 2021c; Marian et al., 2020). The effectiveness of *Streptomyces* spp. H4 against anthracnose has been demonstrated using crude extracts in postharvest strawberries (Li et al., 2021c). Moreover, Marian et al. (2020) observed a reduction in anthracnose severity when a spore suspension of *Streptomyces* sp. MBFA-172 and MBFA-227 was applied to strawberry plants. Commercial products, containing *Streptomyces* spp., such as Actinovate®, Biotrust®, Mycostop®, and Rhizovit® have been utilised in the management of postharvest diseases. However, only Actinovate® is specifically indicated for use on strawberries (Table 4).

4 Advanced technologies for enhancing the efficacy of bacterial agents

The application of biopesticides containing bacterial agents has some limitations compared to synthetic formulations. These limitations include lower efficacy, susceptibility to weather conditions, difficulties in scaling up production, and variability in the potential for pathogen inhibition (Droby et al., 2009; Carmona-Hernandez et al., 2019; Roca-Couso et al., 2021). Conversely, biocontrol agents offer some advantages in terms of cost-effective production and long-term storage (Carmona-Hernandez et al., 2019; Lastochkina et al., 2019). To enhance biocontrol efficacy, bacterial agents can benefit from various approaches, such as incorporating antagonist microorganisms into edible coatings and utilising encapsulation techniques. These innovative strategies hold significant promise for effectively suppressing anthracnose and soft rot in postharvest strawberries.

4.1 Edible coatings and bacterial bioagents

Edible coatings and films involve the application of biological polymers onto the surface of fruit and vegetables (Romero et al., 2022). These coatings can be composed of various polymers, including lipids, proteins, and carbohydrates, and may incorporate additional plasticisers or bioactive compounds (Quirós-Sauceda et al., 2014; Oregel-Zamudio et al., 2017; Romero et al., 2022). The main bioactive compounds incorporated into edible coatings include secondary metabolites, antioxidant compounds, and beneficial microorganisms (Quirós-Sauceda et al., 2014; Romero et al., 2022). Edible coatings serve a multifaceted purpose, acting as a semi-permeable barrier and preventing the exchange of gases and water loss (Oregel-Zamudio et al., 2017). Furthermore, edible coatings help prevent injuries during handling, transportation, and storage, ultimately contributing to inhibiting the proliferation of undesirable

microorganisms and preserving fruit quality over extended periods of storage (Campos et al., 2011).

Numerous studies have explored the influence of edible coatings on strawberries, aiming to preserve fruit quality during storage and decrease fruit decay caused by anthracnose and soft rot (Table 5). According to the existing literature, postharvest treatments utilising edible coatings have predominantly targeted soft rot, while anthracnose is being typically controlled in the field. Various studies have investigated the use of different materials in edible coatings, including candellila wax (Oregel-Zamudio et al., 2017), carboxymethylcellulose (Oliveira et al., 2019ab; Da Silva et al., 2020), hydroxypropyl methylcellulose (Park et al., 2005), and chitosan (Park et al., 2005; Vu et al., 2011; Hafsa et al., 2016; Khalifa et al., 2016; Ventura-Aguilar et al., 2018; Melo et al., 2020; Saleh & Abu-Dieyeh, 2022).

Chitosan is a biodegradable polymer derived from chitin known for its antimicrobial properties, which proves to be a suitable material for edible coatings (Romero et al., 2022). In a study conducted by Melo et al. (2020), coating solutions comprising chitosan nanocomposite, chitosan gel, and chitosan nanoparticles were applied to strawberries. The researchers observed that the nanocomposite influenced fungal morphology and effectively suppressed *Botrytis cinerea*, *Rhizopus stolonifer*, and *Aspergillus niger* in artificially infected strawberries. Similarly, Saleh and Abu-Dieyeh (2022) noted that strawberries treated with *Prosopis juliflora* extract, either individually or embedded in 1% chitosan, successfully maintained fruit quality parameters and delayed spoilage by reducing bacterial, yeast, and mould counts on the fruit surface during refrigerated storage.

Notably, the only study employing edible coatings in combination with a bacterial agent for controlling soft rot was conducted by Oregel-Zamudio et al. (2017). The authors assessed the inhibitory potential of edible coatings derived from candelilla wax and *Bacillus subtilis* HFC103, applied individually and in combination. Their findings demonstrated that all treatments effectively mitigated strawberry decay, reduced water loss, and also suppressed infections caused by *R. stolonifer*.

However, further investigations can be performed to associate edible coatings with beneficial bacteria, enhancing their biocontrol efficacy against anthracnose and soft rot in harvested strawberries. Likewise, plant extracts and essential oils have been successfully incorporated into edible coatings for alleviating postharvest diseases (Vu et al., 2011; Hafsa et al., 2016; Oliveira et al., 2019ab; Saleh & Abu-Dieyeh, 2022) (Table 5). Consequently, the effectiveness of edible coatings can be synergistically improved through their association with resistance inducers (Freimoser et al., 2019) and biocontrol agents (Romero et al., 2022), thereby

contributing to the control of strawberry spoilage and the maintenance of the viability of bacterial agents (Romero et al., 2022).

Table 5. Edible coatings applied to harvested strawberries targeting the suppression of anthracnose and soft rot.

Coating material + bioactive compound/bacterial agent	Disease	Positive effects	References
Chitosan and hydroxypropyl methylcellulose	Soft rot	Reduced fruit decay and mass loss	Park et al. (2005)
Chitosan and limonene oil	Soft rot	Reduced fruit decay and extended shelf life	Vu et al. (2011)
Chitosan and cinnamon essential oil	Soft rot	Reduced fruit decay and increased antioxidant activity	Hafsa et al. (2016)
Chitosan and olive oil residues	Soft rot	Reduced fruit decay and decline in total phenolic, flavonoid, and antioxidant contents	Khalifa et al. (2016)
Candelilla wax alone or combined with *Bacillus subtilis* HFC103	Soft rot	Reduced fruit decay, diminished mass loss, and extended shelf life	Oregel-Zamudio et al. (2017)
Chitosan and cinnamon essential oil or *Roselle* aqueous extract	Anthracnose	Reduced fruit decay, increased antioxidant capacity, and extended shelf life	Ventura-Aguilar et al. (2018)

Coating material + bioactive compound/bacterial agent	Disease	Positive effects	References
Cinnamon essential oil, in both emulsion and nanoemulsion	Soft rot	Reduced fruit decay	Naserzadeh et al. (2019)
Carboxymethylcellulose and *Lippia sidoides* essential oil	Soft rot	Reduced disease severity	Oliveira et al. (2019a)
Carboxymethylcellulose and *Lippia sidoides* essential oil	Anthracnose	Reduced disease severity	Oliveira et al. (2019b)
Chitosan	Soft rot	Reduced disease severity	Melo et al. (2020)
Carboxymethylcellulose and *Eucalyptus* essential oil	Soft rot	Reduced disease severity	Da Silva et al. (2020)
Chitosan and *Prosopis juliflora* ethanolic extract	Soft rot	Reduced fruit decay, extended shelf life, and maintenance of quality parameters	Saleh and Abu-Dieyeh (2022)

4.2 Encapsulation of bacterial agents

Microbial encapsulation is a valuable strategy for safeguarding beneficial microorganisms (Vassilev et al., 2020; Moradi Pour et al., 2022). It involves using biodegradable and edible materials, such as alginate, carboxymethylcellulose, chitosan, gelatin, xanthan gum, Arabic gum, starch, and whey protein for coating biocontrol agents (Moradi Pour et al., 2022). These encapsulating materials must maintain stability under various environmental conditions, not alter the sensory characteristics of fruits, and have a low production cost (Saberi Riseh et al., 2023). Moreover, it is imperative for these products to ensure food safety by not containing any harmful substances for human health (Saberi Riseh et al., 2023).

On the other hand, nanoencapsulation involves enclosing biocontrol agents in nanoparticles (Moradi Pour et al., 2019). This technique provides a controlled and sustained release of bioactive compounds and bioagents, thereby enhancing their effectiveness in disease

management and reducing the quantity required for efficacy (Moradi Pour et al., 2019). As a result, these encapsulation methods facilitate the precise delivery of these agents to the infection sites, optimising their antagonistic activity against the target pathogens. Furthermore, nanoencapsulation protects biocontrol agents from adverse environmental conditions, ensuring their viability and functionality (Martau et al., 2019; Vassilev et al., 2020).

Until now, the majority of studies in nanoencapsulation aiming to decrease strawberry decay have primarily focused on the encapsulation of essential oils. In a study by Naserzadeh et al. (2019), the suppressive potential of cinnamon essential oil, in both emulsion and nanoemulsion forms, was investigated against *Rhizopus stolonifer* and *Botrytis cinerea* in harvested strawberries. The research revealed that the nanoemulsion effectively reduced fungal infections to levels comparable to thiabendazole. Likewise, Ansarifar and Moradinezhad (2022) demonstrated the effectiveness of encapsulating thyme essential oil into a zein electrospun fiber film in preserving the overall quality of strawberries. This was achieved by reducing mass loss, maintaining anthocyanin levels, and preserving fruit firmness and colour during a 15-day storage period.

5 Conclusion, challenges, and perspectives

Biological control has proven to be a successful alternative for managing anthracnose and soft rot in strawberries, offering a viable alternative for synthetic pesticides. This approach involves the use of commercial products or the development of new formulations from a diverse range of antagonistic bacteria, each possessing distinct attributes, environmental requirements, and antagonistic properties. Furthermore, bacterial agents stand as a valuable tool in preventively controlling postharvest diseases, both in field conditions and in harvested strawberries. In addition, the adoption of edible coatings and nanoencapsulation techniques represents a transformative advancement, and their application can contribute to enhancing the inhibitory potential of these bioagents. However, bioproducts encounter several challenges that have hindered their widespread adoption, which should be addressed to ensure the sustainability of agriculture.

Although biological management of postharvest diseases has received considerable attention for its promising outcomes, there are also numerous challenges encompassing aspects, such as commercial development, food safety, bureaucratic constraints, and agricultural acceptance (Barratt et al., 2018). Moreover, replacing synthetic fungicides with biological products requires demonstrating equivalent effectiveness, prolonged shelf life, and similar

performance under field conditions (Zhang et al., 2020; Matrose et al., 2021; Roca-Couso et al., 2021). Given these considerations, it is unrealistic to expect biopesticides alone will completely manage postharvest diseases in strawberries. As a result, biological control agents are primarily employed in organic crop systems or integrated disease management programs (Dukare et al., 2018; Dwiastuti et al., 2021). Currently, bioproducts represent approximately 5% of the worldwide market but it is expected to reach 20% in the next three decades (Isman, 2020). Consequently, efforts should be directed towards researching more effective agents, improving the scale-up capabilities, reducing production costs, and developing stable and safe bioformulations.

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4.2 Optimization of *Bacillus velezensis* S26 sporulation for enhanced biocontrol of gray mold and anthracnose in postharvest strawberries

Abstract

The growing demand for organic and pesticide-free food has prompt the adoption of biological strategies for controlling postharvest diseases. While strawberries are appreciated for their sensory characteristics and nutritional value, the pseudofruit are highly susceptible to fungal infections, leading to potential contamination with chemical residues. *Bacillus* spp. are endospore-forming bacteria that offer an alternative for managing various plant diseases. The present study aimed to investigate the effectiveness of both *Bacillus velezensis* S26 endospores and endospore-rich inoculant in controlling anthracnose and gray mold in strawberries. Initially, the sporulation process was fine-tuned, followed by the utilization of vegetative cells and endospores for *in vitro* assays. Subsequently, *in vivo* assays were performed using an endospore-rich inoculant on strawberries. The optimized sporulation conditions led to the production of 10.3 $\log_{10}$ spores mL^{-1} after 16 h of cultivation (sporulation yield of 96 %). Both the vegetative cells and endospores of *B. velezensis* S26 inhibited mycelial growth and conidial germination of *Botrytis cinerea* and *Colletotrichum* spp., which are the causal agents of gray mold and anthracnose, respectively. While the cell viability of endospores decreased after storage, fresh and stored inoculants proved effective in controlling anthracnose and gray mold during storage. Consequently, the endospores of *B. velezensis* S26 demonstrated their viability and antagonistic potential against postharvest diseases in strawberries, thereby contributing to an extended bacterial shelf life.

Keywords: antagonism, bacterial endospores, cell viability, *Fragaria* × *ananassa*, postharvest diseases

1. Introduction

Strawberries (*Fragaria* × *ananassa* Duch.) are highly perishable pseudofruit (Rico et al., 2019; Wang et al., 2021) susceptible to damage in the field and during harvesting, handling, transportation, and storage (Rosero-Hernandez et al., 2019; Hassan et al., 2021). As a result, these injuries can lead to fruit decay and facilitate fungal infections (Hassan et al., 2021; Sare et al., 2021). Zhang et al. (2017) proposed that preventing post-harvest losses has a comparable impact to increasing cultivated area or improving crop productivity. Therefore, employing sustainable tools, as the biological control, is imperative for effectively managing postharvest diseases in strawberries, including gray mold and anthracnose (De Moura et al., 2021; Mei et al., 2021; Shi et al., 2021).

Bacillus species are recognized as effective promoters of plant growth and biocontrol agents (Lastochkina et al., 2019; Dimkíc et al., 2022). Numerous studies have demonstrated their antagonistic properties against gray mold (Calvo et al., 2019; Chen et al., 2019; De Moura et al., 2021; Hassan et al., 2021; Wang et al., 2021) and anthracnose (Alijani et al., 2021a, 2021b; Li et al., 2021; Mei et al., 2021; Wu et al., 2021) in strawberries. However, biopesticides containing vegetative cells face certain limitations with regard to product stability and cellular viability after storage (Chumthong et al., 2008).

Sporulation is crucial for bacterial survival in harsh environments, such as extreme temperatures and desiccation. Endospores are primarily produced at the end of the logarithmic phase of bacterial growth in response to glucose and nutrient scarcity (Driks, 2002; Ren et al., 2018). However, only a few genera of bacteria, including *Bacillus*, *Clostridium*, and some *Streptomyces* species, possess the capability to develop these resistant structures (Higgins and Dworkin, 2012). This characteristic allows for the production of formulations with high stability and extended shelf life (Posada-Uribe et al., 2016; Cristiano-Fajardo et al., 2019). In conducive conditions, bacterial endospores can germinate and suppress pathogenic microorganisms (Galindo et al., 2013; Cristiano-Fajardo et al., 2019). Thus, the effectiveness of biocontrol depends on maintaining cell viability of bacterial endospores after dormancy (Omer, 2010; Bernier et al., 2022).

Consequently, several strategies can be employed to stimulate and improve bacterial sporulation. This includes identifying the optimal culture medium, pH, water activity, and incubation temperature (Elisashvili et al., 2019; Grauvy et al., 2021), as well as mineral concentration (Ren et al., 2018; Elisashvili et al., 2019), cultivation method (Elisashvili et al., 2019), and cell density (Cristiano-Fajardo et al., 2019; Elisashvili et al., 2019). Nevertheless, culturing conditions may differ depending on the bacterial species and strain (Khardziani et al., 2017; Ren et al., 2018; Elisashvili et al., 2019).

The aims of this study were to optimize bacterial sporulation under shaking-flask conditions, and to compare the *in vitro* antagonism of *Bacillus velezensis* S26 endospores and vegetative cells against *Colletotrichum* spp. and *Botrytis cinerea.* Furthermore, we aimed to evaluate the viability of bacterial endospores and their inhibitory potential by employing fresh and stored inoculants on harvested strawberries.

2. Material and methods

2.1. Bacterial growth and sporulation

The rhizobacterium *Bacillus velezensis* strain S26 was isolated from the soil of an organic vineyard situated in Caxias do Sul, Rio Grande do Sul State, Brazil (latitude 29°15'94'' S and longitude 51°21'45'' W), by Debastiani et al. (2023). This particular strain was selected for its tolerance to high copper concentration, absence of virulence genes, and its lack of resistance to common antibiotics. Molecular identification involved partial sequencing of the *16S rDNA* gene. This sequence was deposited in the GenBank database under the accession number OP938800. Subsequently, bacterial identification was confirmed through whole-genome sequencing. The bacterium was preserved in Luria-Bertani (LB) medium supplemented with 40 % (v/v) glycerol at -80 °C.

The bacterial pre-inoculum was prepared by adding a loop of a single colony-forming unit (CFU) into a 50 mL plastic flask containing 10 mL of LB liquid medium (1 % tryptone, 0.5 % yeast extract, and 0.5 % sodium chloride, at pH 7.2). The culture was incubated on a rotary shaker (Ethik Technology, Vargem Grande Paulista, SP, Brazil) at 130 rpm and 28 °C. After 24 h, this pre-inoculum was transferred to a 250 mL Erlenmeyer flask containing 90 mL of LB liquid medium. The culture was further incubated under the same aforementioned conditions for 24 h.

2.2. Endospore production optimization

The experiments were performed using a non-complete factorial design, where one condition was held constant while the others were systematically varied. The evaluation of bacterial sporulation in shaking-flasks encompassed different conditions of pH (5.0, 6.0, 7.0, and 8.0), temperature (35, 37, 40, 42, and 45 °C), inoculum concentration (4 $\log_{10}$, 5 $\log_{10}$, and 6 $\log_{10}$ CFU mL^{-1}), and incubation time (0, 4, 8, 12, 16, 20, 24, 40, and 48 h). Additionally, the effect of nutrient supplementation (2 mM $MnCl_2.4H_2O$, 2 mM $CaCl_2$, 3 mM $FeSO_4.7H_2O$-EDTA, 3 mM $MgSO_4.7H_2O$, and 44 mM glucose) was also assessed in LB liquid medium.

Sporulation assays were performed using 250 mL Erlenmeyer flasks containing 100 mL of LB liquid medium. The bacterial inoculum was added to the sporulation medium at a ratio of 1: 10 (v/v). The cultures were placed on a rotary shaker at 130 rpm for 12 h. Subsequently, the vegetative cells were inactivated at 80 °C for 10 min. The resulting endospores underwent

serial dilution and were spread onto LB medium plates. Following a 24-hour incubation at 37 °C, the count of germinated endospores was recorded and expressed as spores per milliliter (spores mL^{-1}). The optimal conditions for *B. velezensis* S26 sporulation were also evaluated in YPG liquid medium (1 % yeast extract, 1 % peptone, and 1 % glucose). These assays were performed in triplicates.

2.3. Viability of bacterial endospores during storage

The viability of *B. velezensis* S26 endospores was evaluated during storage in two distinct forms: suspended in a saline solution and suspended in a culture broth as an inoculant. The inoculant was prepared in LB liquid medium employing the optimal conditions for bacterial sporulation previously determined (pH 8.0, temperature of 37 °C, inoculum concentration of 6 $\log_{10}$ CFU mL^{-1}, and 2 mM $CaCl_2$). The sporulation was conducted in a rotary shaker at 130 rpm for 48 h. Subsequently, a portion of the inoculant underwent heat inactivation (80 °C for 10 min) and centrifugation (Thermo Scientific, Massachusetts, USA) at 10,000 rpm for 15 min. The resulting pellet was washed three times and resuspended in a 0.85 % NaCl solution to the same concentration of the original inoculant (9.5 $\log_{10}$ spores mL^{-1}).

Both the inoculant (I) and the saline solution (S) containing *B. velezensis* S26 endospores were stored at 4 °C and 25 °C for 365 d. Bacterial viability was assessed every 30 d over the initial six months and at the experiment's conclusion by counting the unit forming-colonies on LB medium, after incubation at 37 °C for 24 h. Additionally, once a month, a loop of each suspension was spread onto a slide and stained using the method developed by Schaeffer and Fulton (1933) to differentiate endospores from vegetative cells. The slides were examined under an optical microscope using oil immersion to evaluate any morphological changes that occurred during the storage period. The experiment was conducted using a completely randomized design, with three replicates for each treatment.

2.4. Bacterial cells, endospores, and cell-free supernatant

The rhizobacterium *B. velezensis* S26 was employed in three different forms for *in vitro* assays: as a bacterial cell suspension (BC), a bacterial endospore suspension (BE), and as a cell-free supernatant (CFS). Following bacterial growth and sporulation, as described in Section 2.2, the culture broth underwent centrifugation at 10,000 rpm for 15 min. The resulting pellet was washed three times with a 0.85 % NaCl solution. This pellet was then suspended in saline

solution and split into two portions. One portion, subjected to heat inactivation at 80 °C for 10 min to eliminate vegetative cells, was used as a bacterial endospore suspension (BE). Another portion was utilized as a bacterial cell suspension (BC). The concentration of both these bacterial suspensions (BE and BC) was determined through serial dilution, followed by incubation on LB medium plates at 37 °C for 24 h. Subsequently, the final concentration of each suspension was adjusted to 6 $\log_{10}$ CFU mL^{-1} or 6 $\log_{10}$ spores mL^{-1}, using a 0.85 % NaCl solution. Lastly, the cell-free supernatant (CFS) was obtained by filtering the bacterial culture broth through a 0.22 μm cellulose acetate membrane (Millipore Corporation, Massachusetts, USA).

2.5. Pathogen strains

Isolates of *Colletotrichum* spp. and *Botrytis cinerea* were sourced from the University of Caxias do Sul and Embrapa Grape and Wine, both located in Rio Grande do Sul State, Brazil (Supplementary Material 1). These pathogens were isolated from visibly symptomatic grapevines and strawberries. Following isolation, the pathogens underwent molecular characterization and were employed in pathogenicity assays on strawberry plants.

Mycelial plugs (5 mm in diameter) of each pathogen were inoculated onto plates containing PDA medium (2 % dextrose, 1.5 % agar, and potato broth, at pH 5.6). The plates were incubated in a dark chamber (Eletrolab, São Paulo, Brazil) at 28 °C for 14 d. Subsequently, conidia were collected by scraping the fungal mycelium with 0.85 % NaCl solution, under aseptic conditions. This suspension was then filtered through three layers of sterile gauze to remove debris. The conidial concentration was adjusted to 6 $\log_{10}$ conidia mL^{-1} with saline solution supplemented with a few drops of Tween 80, using a hemocytometer (Precicolor, Germany).

2.6. Inhibition of mycelial growth by co-culture and volatile compounds

Co-culture assay was performed using PDA medium plates (90 mm in diameter), as described by Castillo et al. (2013). A 7-d-old mycelial plug (5 mm diameter) from each pathogen was centrally placed on the plate. After 24 h, 25 μL of *B. velezensis* S26 cells (6 $\log_{10}$ CFU mL^{-1}) or endospores (6 $\log_{10}$ spores mL^{-1}) were inoculated at four equally spaced points around the fungal mycelium. Antagonism through volatile compounds was assessed following the method developed by Mokhtar and Dehimat (2012), with some modifications. This assay

was carried out using two PDA plates. In one plate, a 7-d-old mycelium of the pathogen (5 mm diameter) was inoculated, while in the other plate, 100 μL of bacterial or endospores were spread. The plate containing the bacteria was then inverted and sealed to the other plate with plastic wrap. Plates inoculated with a mycelium plug and saline solution were used as controls. Incubation was performed in a chamber at 25 °C in the dark.

The diameter of the colonies in both assays was assessed daily with a digital caliper (Marberg, Moscow, Russia) until all replicates of the control completely covered the plate. The mycelial growth rate (MGR, mm/d) was determined using the following formula:

$$MGR = \sum \left[\frac{(md1 - md2)}{d}\right] \qquad \text{Eq. 1}$$

Where $md2$ and $md1$ denote the mycelial diameter on the current day and the day before, respectively, and d represents the number of days of incubation. Moreover, the percentage of inhibition (PI) for each treatment in comparison to the control was determined on the last day of the assay, employing the formula developed by Oliveira et al. (2016):

$$PI\ (\%) = \left[\frac{(dc - dt)}{dc}\right] \times 100 \qquad \text{Eq. 2}$$

Where dc and dt represent the mean colony diameters of the control and treated groups. The experiment followed a completely randomized design, with seven replicates per treatment.

2.7. Inhibition of conidial germination

Conidial germination was assessed in 10 mL plastic tubes containing 5 $\log_{10}$ conidia mL^{-1} of each pathogen isolate, along with *B. velezensis* S26 cells (5 $\log_{10}$ CFU mL^{-1}), endospores (5 $\log_{10}$ spores mL^{-1}), or cell-free supernatant (4 mL). The final volume was adjusted to 5 mL with LB liquid medium, and tubes were incubated on a rotary shaker at 130 rpm and 28 °C for 24 h. The quantification of germinated conidia was conducted by observing 100 randomly selected conidia from each treatment using an optical microscope. The conidium was considered germinated when the germ tube exceeded its diameter. The experiment was performed using a completely randomized design in triplicates.

2.8. Application of *B. velezensis* S26 in postharvest strawberries

Asymptomatic and uniform-sized strawberries cv. Albion were surface disinfected with 70 % (v/v) ethanol for 1 min, immersed in 2 % (v/v) sodium hypochlorite solution for 3 min, and washed three times with sterile water. Subsequently, the strawberries were immersed in a fresh suspension (FS) containing *B. velezensis* S26 endospores suspended in a saline solution (9.5 $\log_{10}$ spores mL^{-1}). The pseudofruit were subsequently arranged in plastic boxes. After a 2-h drying under aseptic conditions, 10 μL of the pathogen suspension (6 $\log_{10}$ conidia mL^{-1}) was applied to the equatorial zone of each pseudofruit. The negative control was treated with saline solution, whereas the positive control was soaked in saline solution and inoculated with the pathogen suspension. The plastic boxes were placed in a dark chamber at 25 ± 2 °C and 85-95 % of relative humidity for 5 d. The experiment was repeated using the stored suspension (SS), which had a concentration of 8.4 $\log_{10}$ spores mL^{-1} after being stored at room temperature for 180 d.

Following a 5-day storage period, we recorded both the incidence and severity of the diseases. Disease incidence (DI) was determined using the formula:

$$DI\ (\%) = \left(\frac{\text{number of infected pseudofruit}}{\text{total number of pseudofruit}}\right) \times 100 \qquad \text{Eq. 3}$$

Disease severity (DS) was measured based on the following scale (De Moura et al., 2021): 0 = absence of symptoms, 1 = affected area < 25 %, 2 = affected area varying from 26 to 50 %, 3 = affected area varying from 51 to 75 %, and 4 = affected area > 76 %. Disease severity index (DSI) was calculated as follows:

$$DSI\ (\%) = \sum\left[\frac{(\text{disease severity score} \times \text{number of strawberries at disease severity score})}{(\text{total number of strawberries} \times \text{maximum disease severity score})}\right] \times 100 \quad \text{Eq. 4}$$

The assays were carried out in three independent replicates, each consisting of 20 pseudofruit per treatment.

2.9. Statistical analysis

The normality of data and homogeneity of variances were determined using the Kolmogorov-Smirnov and Levene's tests, respectively. Parametric data were analyzed using one-way ANOVA, followed by Tukey's test to determine optimal sporulation conditions (pH, temperature, inoculum concentration, incubation time, and nutrient supplementation), viability of stored endospores, inhibition of mycelial growth, and inhibition of conidial germination. Additionally, the paired t-test, was employed after one-way ANOVA to differentiate the effect of the pH on nutrient supplementation.

In the assay employing postharvest strawberry, parametric data were evaluated using one-way ANOVA and Tukey's test, while non-parametric data were compared using the Kruskal-Wallis test, followed by the Dunn-Bonferroni test ($P < 0.05$). All analyses were performed using SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Endospore production optimization

Defining the optimal conditions for endospore production and enhancing the spore yield are pivotal for the development of biopesticides, as these characteristics significantly contribute to biocontrol effectiveness. As a result, several experiments were performed to optimize the sporulation of *B. velezensis* S26 in shaking-flasks. We observed that elevating pH positively influenced the sporulation of *B. velezensis* S26 (Fig. 1), with the highest concentration of endospores achieved at pH 8.0 (7.5 $\log_{10}$ spores mL^{-1}).

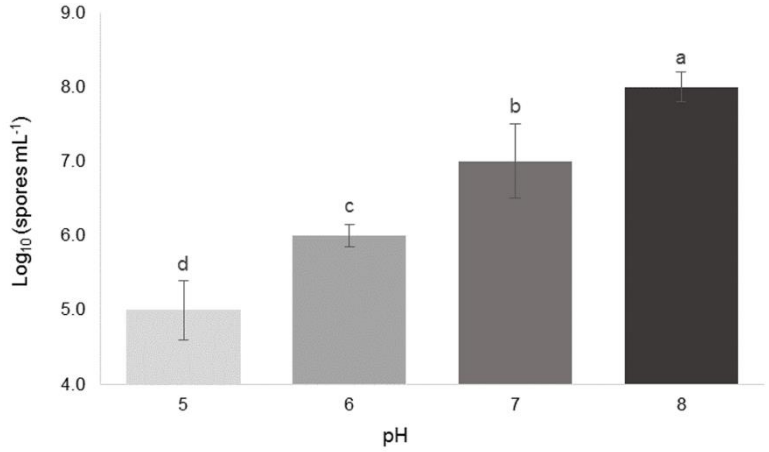

Fig. 1. Effect of pH on the concentration of *Bacillus velezensis* S26 endospores in LB liquid medium after incubation in a rotary shaker at 37 °C for 12 h. Statistical analyses were conducted using ANOVA and Tukey's test ($P < 0.05$). The error bars represent standard deviation of three replicates.

The experiments revealed that the ideal conditions for cultivating *B. velezensis* S26 in shaking-flasks involved incubation at 28 °C for bacterial growth and 37 °C for endospore production, resulting in a concentration of 8.9 $\log_{10}$ spores mL^{-1}. Moreover, the spore yield reached 90 % when incubated at 37 °C (Fig. 2). In contrast, a temperature of 35 °C led to the lowest endospore concentration (6.8 $\log_{10}$ spores mL^{-1}) and an 84 % sporulation yield.

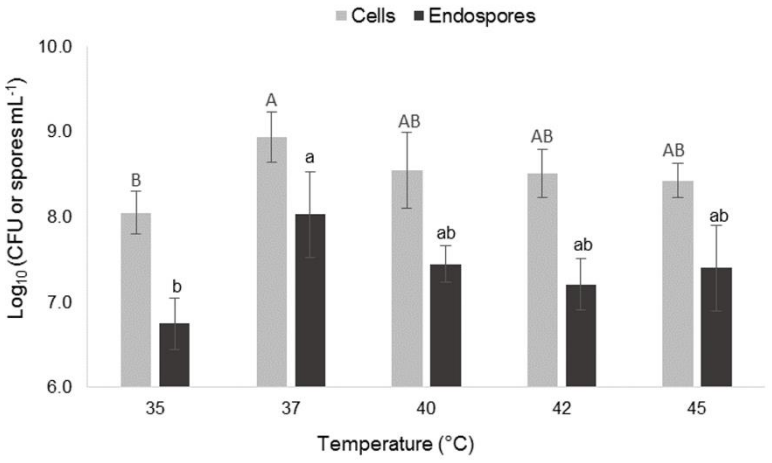

Fig. 2. Effect of temperature on the concentration of *Bacillus velezensis* S26 cells and endospores after a 12-h incubation in LB liquid medium, pH 8.0. Separate statistical analyses were performed for bacterial cells and endospores, using ANOVA followed by the Tukey's test ($P < 0.05$). The error bars indicate standard deviation of three replicates.

Following this, we investigated the effect of inoculum concentration and incubation period on bacterial growth and endospore production (Fig. 3). The highest endospore concentration was observed when employing an inoculum with 6 $\log_{10}$ CFU mL^{-1} of *B. velezensis* cells (Fig. 3C). Under these conditions, the endospore concentration reached 7.7 $\log_{10}$ spores mL^{-1}, and a spore yield of 91.3 % was achieved after 12 h of cultivation. Furthermore, cultivating vegetative cells for 48 h resulted in a concentration of 9.1 $\log_{10}$ spores mL^{-1}, yielding a sporulation efficiency of 96 % (Fig. 3C).

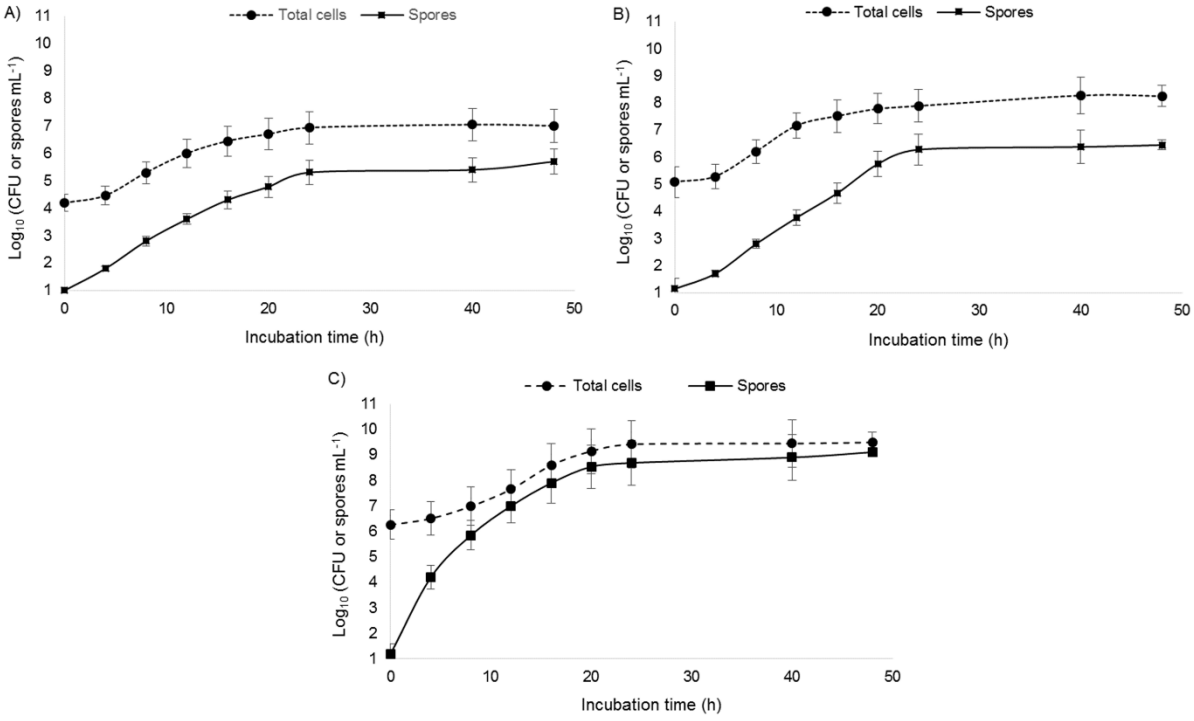

Fig. 3. Concentration of *Bacillus velezensis* S26 cells and endospores in LB liquid medium, pH 8.0, at 37 °C, during 50 h, using different inoculum concentrations: A) 4 $\log_{10}$ CFU mL^{-1}, B) 5 $\log_{10}$ CFU mL^{-1}, and C) 6 $\log_{10}$ CFU mL^{-1}. The error bars indicate standard deviation of three replicates.

Hence, we selected 6 $\log_{10}$ CFU mL^{-1} as the optimal pre-inoculum concentration and proceeded to investigate other factors for further enhancement of bacterial sporulation. Initially, we examined nutrient supplementation in LB liquid medium, and subsequently, we assessed the most promising conditions in YPG liquid medium (Table 1). While the amendment of Ca^{2+}, Fe^{2+}, Mg^{2+}, and $Mg^{2+}/Fe^{2+}/Ca^{2+}$ to both LB and YPG media led to an increase in endospore concentration compared to the control, only the addition of Ca^{2+} resulted in a significant enhancement. The highest endospore concentration was achieved in LB liquid medium at pH

8.0 supplemented with 2 mM Ca^{2+} (10.3 $\log_{10}$ spores mL^{-1}). Nevertheless, no statistical difference was observed between this treatment and the one with pH adjusted to 7.0 (9.7 $\log_{10}$ spores mL^{-1}).

Table 1. Concentration of *Bacillus velezensis* S26 endospores (9 $\log_{10}$ spores mL^{-1}) after a 12-h incubation in LB and YPG liquid media, at two distinct pH conditions, amended with nutrients.

Nutrients	pH 7.0	pH 8.0
LB liquid medium		
Control	0.43 ± 0.05 bB	2.63 ± 0.86 bA
Ca^{2+}	4.50 ± 0.80 aA	20.0 ± 11.7 aA
Fe^{2+}	3.43 ± 1.10 abA	1.33 ± 0.31 bB
Mg^{2+}	3.03 ± 0.96 abA	2.50 ± 1.17 bA
Mn^{2+}	0.27 ± 0.07 bA	0.39 ± 0.17 bA
Mg^{2+}/Fe^{2+}	0.44 ± 0.13 bB	4.00 ±0.85 bA
$Mg^{2+}/Mn^{2+}/ Fe^{2+}$	0.29 ± 0.03 bA	0.36 ± 0.07 bA
$Mg^{2+}/Fe^{2+}/Ca^{2+}$	2.87 ± 1.25 abA	0.45 ± 0.06 bB
Glucose	0.93 ± 0.02 bA	0.40 ± 0.18 bB
Ca^{2+}/Glucose	0.03 ± 0.00 bB	0.32 ± 0.11 bA
YPG liquid medium		
Control	0.61 ± 0.05 bA	0.68 ± 0.15 bA
Ca^{2+}	1.14 ± 0.14 bA	0.68 ± 0.03 bB
Fe^{2+}	1.02 ± 0.09 bA	0.82 ± 0.13 bA
Mg^{2+}	1.03 ± 0.16 bA	0.76 ± 0.15 bA
$Mg^{2+}/Fe^{2+}/Ca^{2+}$	1.08 ± 0.10 bA	0.65 ± 0.16 bB

Equal uppercase letters indicate no statistical difference between the pH, according the t-test ($P < 0.05$) and equal lowercase letters indicate no statistically significant difference among the treatments, as determined by ANOVA, followed by Tukey's test ($P < 0.05$). Each value represents the mean ± standard deviation of three replicates.

Consequently, the optimal conditions for *B. velezensis* S26 sporulation, as determined by the shaking-flask experiment, involved using a pre-inoculum containing 1×10^6 CFU mL^{-1} of bacterial cells in a slightly alkaline LB liquid medium (pH 8.0), supplemented with 2 mM Ca^{2+}. Additionally, incubation on a rotary shaker at 37 °C for 16 h resulted in the highest endospore concentration of 10.3 $\log_{10}$ spores mL^{-1}, with a spore yield of 96 %.

3.2. Viability of stored endospores

The viability of *B. velezensis* S26 endospores decreased over the storage period, whether stored as an inoculant or suspended in saline solution (Fig. 4 and Supplementary Material 2). Storing endospores as an inoculant at 25 °C and as a saline suspension at 4 °C resulted in a thousand-fold reduction in endospore viability after a 365-d storage. The highest spore concentration was achieved when *B. velezensis* S26 endospores were stored in saline solution at 25 °C, resulting in a ten-fold decrease compared to the initial state (ranging from 9.5 $\log_{10}$ to 7.7 $\log_{10}$).

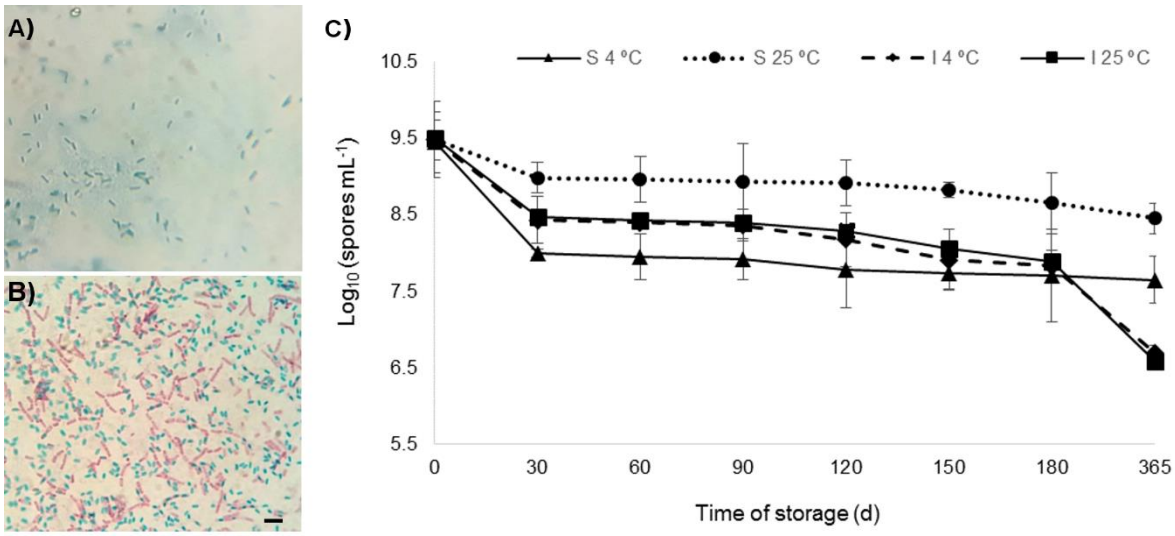

Fig. 4. Photographs illustrating the staining process of *Bacillus velezensis* S26 and showcasing the differences between fresh suspension (A) and suspension after 365 days of storage (B) under 1,000 X magnification. Bacterial cells are highlighted in red, and endospores are distinguished in green. Endospore concentration in the inoculant (I) and saline suspension (S) was evaluated during storage at 4 °C and 25 °C (C). Scale bar: 5 μm. The error bars indicate the standard deviation of three replicates.

3.3. Inhibition of fungal growth by co-culture and volatile compounds

The co-culture assay was performed to evaluate the antagonistic effects of *B. velezensis* S26, through antibiosis and competition for space and nutrients. This experiment demonstrated the efficacy of *B. velezensis* S26 against the causal agents of gray mold (*B. cinerea*) and anthracnose (*Colletotrichum* spp.). Treatments using bacterial cells (BC) and bacterial

endospores (BE) led to a significant reduction in *Colletotrichum* spp. growth compared to the control (Table 2). BC exhibited inhibition against *Colletotrichum* spp. isolates ranging from 18.4 % (Ci015) to 72.1 % (Cact), while BE inhibited mycelial growth of *Colletotrichum* spp. ranging from 16.6 % (Ci015) to 68.9 % (Cact). BC also demonstrated notable suppression of the mycelial growth of *Botrytis cinerea* isolates when compared to BE (Table 2). The inhibition of fungal growth through BC varied from 28.8 % (CNPUV53) to 78.8 % (A21/17A), while BE exhibited suppression ranging from 25.3 % (CNPUV53 and CNPUV80) to 49.4 % (CNPUV61).

Table 2. Mycelial growth rate (MGR, mm/d) in the co-culture assay: pathogen (control), pathogen + *Bacillus velezensis* S26 cells (BC), pathogen + *B. velezensis* S26 endospores (BE). Percentage of inhibition (PI, %) was determined on the last day of the experiment.

Pathogen isolates	MGR		PI	MGR	PI
	Control	BC		BE	
		Colletotrichum spp.			
Cg4A	12.1 ± 0.0 a	4.6 ± 0.3 b	62.0	5.2 ± 0.7 ab	57.0
Cg3D	12.1 ± 0.0 a	5.2 ± 0.7 b	57.0	5.5 ± 0.5 b	54.5
Ci020	8.5 ± 0.0 a	3.1 ± 0.5 b	63.5	2.8 ± 0.2 b	67.1
Ci029	12.1 ± 0.0 a	4.5 ± 0.3 b	62.8	4.6 ± 0.3 b	62.0
Ci015	28.3 ± 0.0 a	23.1 ± 0.5 b	18.4	23.6 ± 0.7 b	16.6
Ca017	12.1 ± 0.0 a	5.4 ± 0.5 b	55.4	6.0 ± 0.5 b	50.4
Cact	6.1 ± 0.0 a	1.7 ± 0.6 b	72.1	1.9 ± 0.1 ab	68.9
Mean	**13.07 ± 6.7 a**	**6.8 ± 6.8 b**	**55.9**	**7.1 ± 7.0 b**	**53.8**
		Botrytis cinerea			
001/11	8.5 ± 0.0 a	3.9 ± 0.4 b	54.1	5.1 ± 0.5 ab	40.0
A3717	17.0 ± 0.0 a	10.4 ± 0.5 b	38.8	12.1 ± 0.2 ab	28.8
A21/17A	8.5 ± 0.0 a	1.8 ± 0.6 b	78.8	4.6 ± 0.8 ab	45.9
CNPUV53	17.0 ± 0.0 a	12.1 ± 0.3 c	28.8	12.7 ± 0.6 b	25.3
CNPUV61	8.5 ± 0.0 a	4.7 ± 0.9 b	44.7	4.3 ± 0.7 b	49.4
CNPUV62	17.0 ± 0.0 a	11.0 ± 1.0 b	35.3	12.7 ± 0.2 ab	28.8
CNPUV80	17.0 ± 0.0 a	11.5 ± 1.1 b	32.4	12.1 ± 0.6 b	25.3
Mean	**13.4 ± 4.3 a**	**7.9 ± 4.1 b**	**44.7**	**9.1 ± 3.9 ab**	**34.8**

Equal lowercase letters indicate no statistically significant difference among the treatments (control, BC, and BE) for each pathogen isolate, based on ANOVA followed by Tukey's test ($P < 0.05$). The analyses were performed separately for *Colletotrichum* spp. and *B. cinerea* isolates.

Volatile organic compounds (VOCs) exhibited reduced effectiveness in suppressing the radial growth of pathogens (Table 3 and Supplementary Material 2). VOCs synthesized by both BC and BE demonstrated an average percentage inhibition of 3.6 % against *Colletotrichum* spp. isolates, with percentage of inhibition ranging from 0 % (Cg4A and Ci015) to 10.7 % (Ca017). Similarly, the volatile compounds released by BC and BE against *B. cinerea* isolates resulted in mean inhibition percentages of 5.1 % and 6.6 %, respectively. The inhibition percentage from BC was notably higher against *B. cinerea* isolate A21/17A (15.3 %), while volatiles from BE induced maximal inhibition against *B. cinerea* isolate 001/11 (38.8 %). Nevertheless, the mean radial growth rate (MGR) of treatments with BC and BE did not exhibit a statistically significant difference compared to the control.

Table 3. Mycelial growth rate (MGR, mm/d) in the antagonism through volatile compounds: pathogen (control), pathogen + *Bacillus velezensis* S26 cells (BC), pathogen + *B. velezensis* S26 endospores (BE). Percentage of inhibition (PI, %) was determined on the last day of the experiment.

Pathogen isolates	MGR		PI	MGR	PI
	Control	BC		BE	
		Colletotrichum spp.			
Cg4A	12.1 ± 0.0 a	12.1 ± 0.8 a	0	11.9 ± 0.2 a	1.7
Cg3D	12.1 ± 0.0 a	11.2 ± 1.3 a	7.4	11.6 ± 1.0 a	4.1
Ci020	8.5 ± 0.0 a	8.5 ± 0.0 a	0	8.5 ± 0.1 a	0
Ci029	12.1 ± 0.0 a	12.0 ± 0.1 a	0.8	12.1 ± 0.0 a	0
Ci015	28.3 ± 0.0 a	28.3 ± 0.0 a	0	28.1 ± 0.3 a	0.7
Ca017	12.1± 0.0 a	10.8 ± 1.1 b	10.7	11.3 ± 1.0 ab	6.6
Cact	8.3 ± 0.2 a	7.8 ± 0.2 b	6.0	7.8 ± 0.2 b	6.0
Mean	**13.4 ± 6.4 a**	**13.0 ± 6.6 a**	**3.6**	**13.0 ± 6.4 a**	**3.6**
		Botrytis cinerea			
001/11	8.5 ± 0.0 a	8.0 ± 0.4 a	5.9	5.2 ± 1.6 b	38.8
A3717	17.0 ± 0.0 a	15.3 ± 1.6 b	10.0	17.0 ± 0.0 a	0
A21/17A	8.5 ± 0.0 a	7.2 ± 1.1 b	15.3	8.5 ± 0.0 a	0
CNPUV53	17.0 ± 0.0 a	16.9 ± 0.2 a	0.6	16.7 ± 0.8 a	1.8
CNPUV61	10.9± 0.9 a	11.6 ± 0.4 a	6.4*	10.9 ± 1.6 a	0
CNPUV62	17.0 ± 0.0 a	16.2 ± 1.3 a	4.7	16.1 ± 1.2 a	5.3
CNPUV80	17.0 ± 0.0 a	16.1 ± 1.2 a	5.3	16.9 ± 0.2 a	0.6
Mean	**13.7 ± 3.9 a**	**13.1 ± 4.0 a**	**5.1**	**13.0 ± 4.6 a**	**6.6**

Equal lowercase letters indicate no statistically significant difference among the treatments (control, BC, and BE) for each pathogen isolate, based on ANOVA followed by Tukey's test ($P < 0.05$). The analyses were performed separately for *Colletotrichum* spp. and *B. cinerea* isolates. Each value represents the mean ± standard deviation of three replicates.

* Growth induced in comparison to the control.

3.4. Inhibition of conidial germination

The treatment with BC reduced conidial germination in all *Colletotrichum* spp. isolates (averaging 77.9 %) and six *B. cinerea* isolates (001/11, A3717, CNPUV53, CNPUV61, CNPUV62, and CNPUV80) (averaging of 53.7 %). Similarly, BE led to a decrease in

germinated conidia in six *Colletotrichum* spp. isolates (averaging 64.9 %) (Cg3D, Cg4A, Ci020, Ci029, Ci015, and Cact), as well as in five *B. cinerea* isolates (A3717, CNPUV53, CNPUV61, CNPUV62, and CNPUV80) (averaging 57.7 %). The cell-free supernatant (CFS) exhibited the highest inhibition of conidial germination in all *B. cinerea* isolates (averaging 80.8 %) and significantly controlled conidial germination in *Colletotrichum* spp. isolates (averaging 72.0 %) (Table 4).

Table 4. Number of germinated conidia in the following treatments: pathogen (control), pathogen + *Bacillus velezensis* S26 cells (BC), pathogen + *B. velezensis* S26 endospores (BE), and pathogen + cell-free supernatant (CFS). The percentage of inhibition (PI, %) for each treatment (BC, BE, and CFS) was determined in comparison to the control.

Pathogen	Control	BC	PI*	BE	PI*	CFS	PI*
			Colletotrichum spp.				
Cg4A	46.0 ± 4.6 a	10.0 ± 4.6 b	78.3	8.0 ± 1.7 b	82.6	12.0 ± 3.0 b	73.9
Cg3D	54.7 ± 5.0 a	5.3 ± 2.3 c	90.2	14.0 ± 5.3 bc	74.4	20.7 ± 3.1 b	62.2
Ci020	55.3 ± 6.1 a	5.3 ± 3.1 b	90.4	4.0 ± 2.0 b	92.8	7.3 ± 3.1 b	86.7
Ci029	47.0 ± 9.2 a	5.0 ± 1.7 b	89.4	6.0 ± 3.0 b	87.2	18.0 ± 6.0 b	61.7
Ci015	53.0 ± 7.6 a	19.0 ± 3.5 b	64.2	30.0 ± 6.0 b	43.4	29.0 ± 7.5 b	45.3
Ca017	61.0 ± 6.6 a	30.7 ± 3.1 b	49.7	61.7 ± 3.5 a	1.1**	7.7 ± 2.5 c	87.4
Cact	53.0 ± 10.5 a	9.0 ± 3.0 b	83.0	13.3 ± 3.8 b	74.8	7.0 ± 1.7 b	86.8
Mean	**52.9 ± 7.8 a**	**12.0 ± 9.4 b**	**77.9**	**19.6 ± 19.7 b**	**64.9**	**14.5 ± 8.7 b**	**72.0**
			Botrytis cinerea				
001/11	44.0 ± 14.4 a	17.3 ± 4.6 ab	60.6	21.3 ± 6.1 ab	51.5	10.0 ± 5.3 b	77.3
A3717	38.7 ± 2.3 a	17.3 ± 6.1 b	55.2	21.3 ± 6.1 b	44.8	13.3 ± 6.1 b	65.5
A21/17A	55.3 ± 2.1 a	55.0 ± 4.0 a	1.2	43.7 ± 9.1 ab	21.1	28.7 ± 6.4 b	48.2
CNPUV53	45.7 ± 4.7 a	3.7 ± 3.8 c	92.0	13.7 ± 1.5 b	70.1	2.3 ± 0.6 c	94.9
CNPUV61	49.0 ± 6.2 a	18.7 ± 7.1 b	61.9	12.7 ± 2.5 bc	74.1	3.3 ± 2.1 c	93.2
CNPUV62	56.0 ± 6.2 a	43.3 ± 1.5 b	22.6	20.7 ± 5.9 c	63.1	3.7 ± 2.1 d	93.5
CNPUV80	41.7 ± 2.5 a	6.3 ± 0.6 bc	84.8	8.7 ± 2.5 b	79.2	3.0 ± 1.0 c	92.8
Mean	**47.2 ± 8.5 a**	**23.2 ± 18.7 b**	**53.7**	**20.3 ± 11.8 b**	**57.7**	**9.2 ± 9.7 b**	**80.8**

Equal lowercase letters indicate no statistically difference among the treatments (control, BC, BE, and CFS) for each pathogen isolate, based on ANOVA followed by Tukey's test ($P < 0.05$). The analyses were performed separately for *Colletotrichum* spp. and *B. cinerea* isolates. Each value represents the mean ± standard deviation of three replicates.

3.5. Application of *B. velezensis* S26 in postharvest strawberries

The effectiveness of *B. velezensis* S26 endospore-rich suspension in managing gray mold and anthracnose in strawberries was determined during the postharvest phase, using both the fresh and stored suspensions. The application of the fresh suspension (FS) containing *B. velezensis* S26 endospores led to a reduction in the incidence of gray mold compared to the control (Fig. 5). This reduction was observed in the *B. cinerea* isolates A3717 (23.4 %), CNPUV53 (41.1 %), and CNPUV62 (23.9 %). The stored suspension (SS) also decreased the incidence of the disease caused by the *B. cinerea* isolates A3717 (42 %) and CNPUV53 (24.5 %). Furthermore, both the fresh and stored suspensions reduced gray mold severity in the *B. cinerea* isolates 001/11, A3717, and CNPUV53. As a result, these suspensions containing *B. velezensis* S26 endospores proved to be effective in controlling the incidence and severity of gray mold in harvested strawberries, showing efficacy against six out of seven *B. cinerea* strains.

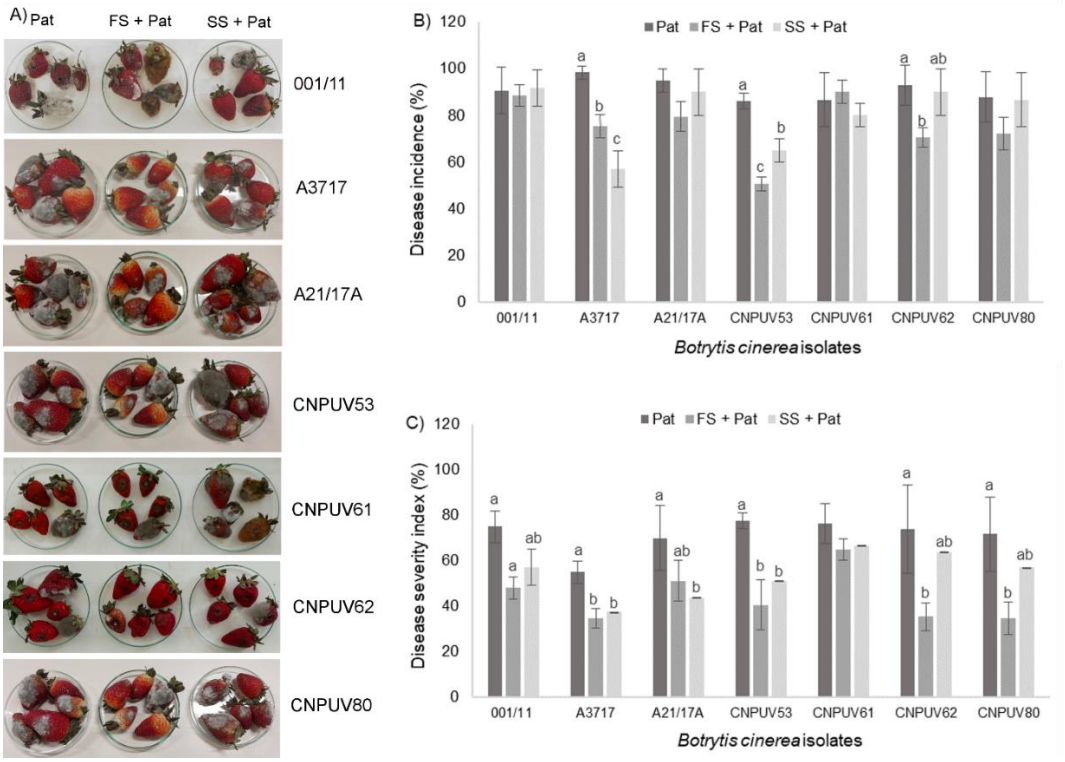

Fig. 5. *Bacillus velezensis* S26 antagonism against *Botrytis cinerea* (Pat) in strawberries, using fresh suspension (Pat + FS) and stored suspension (Pat + SS). (A) Pseudofruit challenged with seven pathogen isolates. (B) Disease incidence. (C) Disease severity index. The error bars indicate standard deviation of three replicates. Equal lowercase letters indicate no statistically significant difference among the treatments (Pat, FS + Pat, and SS + Pat) for each pathogen isolate, based on ANOVA followed by Tukey's test ($P < 0.05$).

Similarly, treating strawberries with both FS and SS decreased the incidence of anthracnose in strawberries (Fig. 6). FS notably lowered the incidence of the disease caused by the *Colletotrichum* spp. isolates Cg3D (38.9 %), Ci029 (24 %), and Cact (68.3 %) in comparison to the control. Likewise, SS exhibited inhibitory effects on the *Colletotrichum* spp. isolates Ci029 (26.8 %) and Cact (57.5 %). In terms of the anthracnose severity, FS demonstrated effective control over *Colletotrichum* spp. isolates Cg4A (41.3 %), Ci015 (40 %), Ca017 (34.4 %), and Cact (53.4 %) when compared to the control. Despite a slight decrease in the inhibitory capacity of *B. velezensis* S26 after storage, the endospore suspension still significantly reduced the severity of anthracnose caused by *Colletotrichum* spp. Cact (54 %) (Fig. 6).

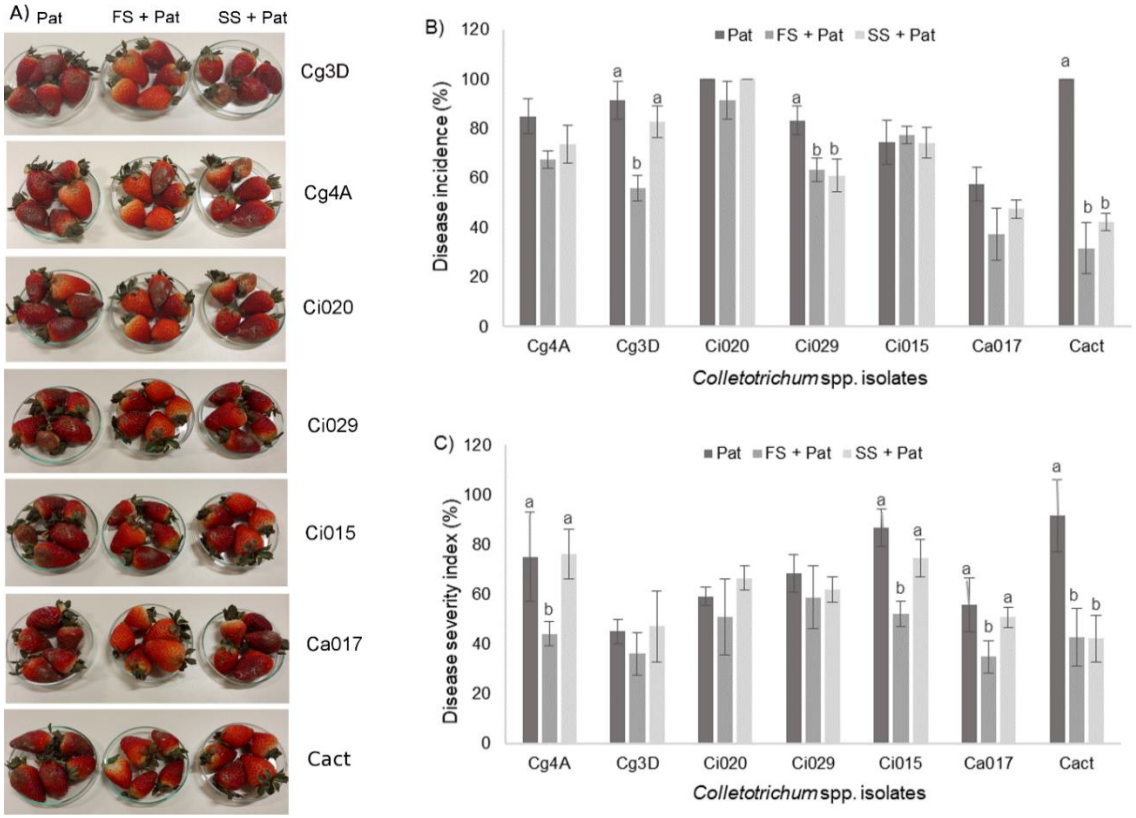

Fig. 6. *Bacillus velezensis* S26 antagonism against *Colletotrichum* spp. (Pat) in strawberries, using fresh suspension (Pat + FS) and stored suspension (Pat + SS). (A) Pseudofruit challenged with seven pathogen isolates. (B) Disease incidence. (C) Disease severity index. The error bars indicate standard deviation of three replicates. Equal lowercase letters indicate no statistically significant difference among the treatments (Pat, FS + Pat, and SS + Pat) for each pathogen isolate, based on ANOVA followed by Tukey's test ($P < 0.05$).

4. Discussion

Bacterial sporulation plays a crucial role in the survival of biocontrol agents under adverse environmental conditions and nutrient scarcity. Consequently, endospores ensure the development of bioproducts with an extended shelf life. By refining culturing conditions, we enhanced bacterial density, thus facilitating their conversion into resilient endospores (Cristiano-Fajardo et al., 2019; Elisashvili et al., 2019). In this study, we assessed the impact of various environmental conditions on bacterial growth and sporulation. Through optimization assays, we successfully increased the spore production of *B. velezensis* S26 achieving concentrations comparable to those observed in studies involving *B. subtilis* MB24 (Monteiro et al., 2005), *B. amyloliquefaciens* B128 (Tzeng et al., 2008), *B. amyloliquefaciens* CPA-8 (Gotor-Vila et al., 2017), *B. amyloliquefaciens* BS-20 (Ren et al., 2018), and *B. subtilis* BSB1 (Grauvy et al., 2021).

In our experiments, the sporulation of *B. velezensis* S26 was improved by using a medium with a slightly alkaline pH, whereas low pH values significantly decreased the spore concentration. Differently, Monteiro et al. (2005) observed that the growth and sporulation of *B. subtilis* MB24 cultivated on Difco Sporulation Medium (DSM) was not significantly affected by the medium pH, although lower values (pH 5.0) reduced the spore yield. In agreement with these findings, Tzeng et al. (2008) also noted that using an acidic culture medium decreased bacterial sporulation. In the same way, bacterial cultivation in bioreactors under non-controlled pH conditions had no impact on the concentration of bacterial cells and endospores (Tzeng et al., 2008; Posada-Uribe et al., 2015). Interestingly, Grauvy et al. (2021) designed a dynamic experiment by changing pH values during bacterial growth and observed a decrease in the sporulation rate under unfavorable conditions, followed by a reestablishment in spore production under conducive conditions.

We also noted that utilization of a high cell density inoculum containing 6 $\log_{10}$ CFU mL^{-1} of *B. velezensis* S26 resulted in the highest spore concentration and yield. This finding is supported by Collins and Jacobsen (2003), who observed maximal spore levels when employing a concentrated inoculum of *B. subtilis* BacB. Conversely, Ren et al. (2018) achieved similar spore production with a higher cell density (7 $\log_{10}$ CFU mL^{-1}), while Gotor-Vila et al. (2017) attained comparable results with a less concentrated inoculum (5.3 $\log_{10}$ CFU mL^{-1}). We propose that various factors can influence bacterial growth, and a highly concentrated inoculum accelerates nutrient depletion, thereby initiating the sporulation process.

The optimal temperature for *B. velezensis* S26 sporulation was determined based on achieving the highest spore concentration and maximal spore yield. Consequently, we found that incubation at 37 °C for 48 hours resulted in the maximal spore concentration. This aligns with the findings of Calvo et al. (2017), who reported that growing *B. amyloliquefaciens* BUZ-14 at 37 °C for 16 hours led to maximal endospore production. In contrast, Tzeng et al. (2008) observed significant sporulation when cultivating *B. amyloliquefaciens* B128 at lower temperatures for approximately 50 hours. Gotor-Vila et al. (2017) also reported high levels of sporulation in *B. amyloliquefaciens* CPA-8 grown in a 5-L bioreactor at 30 °C for 68-72 hours.

The chemical composition of the culture medium also influences bacterial growth and sporulation (Elisashvili et al., 2019). As a result, spore production can be either enhanced or suppressed depending on the nutrient source and concentration (Monteiro et al., 2005; Chen et al., 2010; Elisashvili et al., 2019). It is well known that bacteria require nitrogen and carbon sources to grow and reach a high density of vegetative cells. Subsequently, nutrient depletion triggers sporulation as an adaptive response to adverse environments (Cristiano-Fajardo et al., 2019). Our findings revealed that increasing the glucose concentration in the culture medium did not lead to an improvement in spore production, and the combination of glucose and calcium in LB liquid medium did not stimulate sporulation in *B. velezensis* S26 compared to the control. Cristiano-Fajardo et al. (2019) also found that a sharp decrease in glucose was correlated with a peak in bacterial cell concentration, demonstrating that a longer period of growth would be needed for glucose exhaustion and the initiation of spore production.

Divalent cations, including Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Zn^{2+}, are essential components of spore structure in association with dipicolinic acid and play a role in activating enzymes involved in various metabolic pathways (Ren et al., 2018; Elisashvili et al., 2019). As a result, an assay was performed to improve spore yield by adding metal ions to the culture medium. However, our results did not show a significant improvement in spore concentration. Only Ca^{2+} had a positive influence on bacterial sporulation. In contrast, Omer (2010) reported maximal spore concentration in Bacillus megaterium when adding a mixture of $MgSO_4$, $CaCl_2$, $ZnSO_4$, and KCl to an agar nutrient medium. Ren et al. (2018) achieved a 3.4-fold increase in spore yield while growing *Bacillus amyloliquefaciens* BS-20 in shaking-flasks containing LB-based liquid medium supplemented with Mn^{2+}, Fe^{2+}, and Ca^{2+}.

Subsequently, we conducted *in vitro* experiments to compare the antagonistic effects of vegetative cells and endospores of *B. velezensis* S26. Our findings demonstrated that both forms effectively reduced the mycelial growth of *Colletotrichum* spp. isolates. Additionally, vegetative cells exhibited higher inhibition compared to bacterial endospores against *B. cinerea*

isolates. These results align with the observations of Collins and Jacobsen (2003), who noted that vegetative cells of *B. subtilis* BacB were more effective than spores in inhibiting *Cercospora beticola* Sacc. in a growth chamber experiment. Interestingly, germinated spores exhibited biocontrol efficacy under field conditions, comparable to that of vegetative cells. Furthermore, Tzeng et al. (2008) reported that the endospores of *Bacillis amyloliquefaciens* B128 reduced the radial growth of *Botrytis elliptica* in a concentration-dependent manner in a co-culture assay.

Additionally, the antagonistic activity of *B. velezensis* S26 against *Colletotrichum* spp. and *B. cinerea* isolates, through both bacterial cells and bacterial endospores, was more pronounced in co-culture assays compared to antagonism through volatile compounds. According to Luo et al. (2023), co-culture assay facilitates short-distance interactions, aiding the diffusion of secondary metabolites and stimulating competition for niche and nutrients.

B. velezensis S26 also demonstrated effective suppression of conidial germination against *Colletotrichum* spp. and *B. cinerea* isolates. Notably, cell-free supernatant reduced conidial germination in all *Botrytis cinerea* isolates, while it exhibited a less pronounced inhibition against *Colletotrichum* spp. As a result, vegetative cells, endospores, and cell-free supernatant suppressed the germination of *Colletotrichum* spp. conidia. Wu et al. (2021) also confirmed that applying the filtrate of *B. amyloliquefaciens* PMB04 suppressed conidial germination of *Colletotrichum gloeosporioides* SC01, while the filtrate of *B. amyloliquefaciens* PMB005 did not inhibit pathogen germination. The authors hypothesized that these contrasting results are attributed to the concentration of secreted lipopeptides. According to our previous studies, *B. velezensis* S26 can synthesize secondary metabolites such as fengycin and surfactin during growth. While fengycin possesses antifungal properties, surfactin is involved in pathogen-host interactions and the formation of biofilms (Li et al., 2019; Legein et al., 2020).

In terms of storage effects, *B. velezensis* S26 endospores demonstrated significant microbial survival over a 365-day storage period. Bazilah et al. (2011) suggested that the decline in cell viability over time may be attributed to the accumulation of toxic compounds and modification in pH conditions. Similarly, Wong et al. (2019) assessed the cell viability of *Pseudomonas aeruginosa* in three dry formulations (pesta granules, talc powder, and alginate beads) and one liquid formulation subjected to six months of storage. The results revealed that the liquid formulation stored under refrigeration ensured the stability of bacterial population and maintained a proper biocontrol efficacy. Likewise, Jayasudha et al. (2018) examined the viability of various antagonistic bacteria after three months of storage in a liquid formulation enriched with natural oils. They observed that pongamia oil resulted in the highest cell count,

with survival rates ranging from 31.1 % to 60.8 %. These studies underscore the importance of carriers and additives in improving bacterial survival during prolonged storage. Moreover, liquid suspensions tend to preserve cell viability during storage due to their high moisture content (Manikandan et al. 2010).

Our findings also demonstrated that storing the fresh suspension in a saline solution at 25 °C resulted in the highest cell viability. Similarly, Chumthong et al. (2008) reported a high survival rate of *Bacillus megaterium* endospores, prepared as water-soluble granules stored at room temperature for 24 months. Omer (2010) also observed remarkable survival of *B. megaterium* over six months of storage at room temperature. The authors reported 80% cell viability using cellulose-clay as an inert carrier in combination with carboxymethylcellulose, sodium benzoate, and calcium carbonate in a powder formulation. Nevertheless, high temperatures have been associated with accelerated metabolic activity, potentially resulting in an accumulation of toxic residues (Bazilah et al., 2011).

The experiment conducted on strawberries demonstrated that applying *B. velezensis* S26 suspension effectively reduced the incidence and severity of gray mold. Consistent with our findings, Chen et al. (2019) observed a significant reduction in gray mold incidence when treating strawberries with a suspension of *B. subtilis* Z4 cells. Similarly, De Moura et al. (2021) achieved a notable decrease in gray mold incidence by applying *Pantoea* sp. MQT16M1, *B. subtilis* strains, and *B. cereus* strains on harvested strawberries. Their results indicate that the effectiveness of gray mold suppression depended on the bioagent strain, and the degree of disease inhibition was influenced by the specific strawberry cultivar challenged. Additionally, they noted that strawberries cv. Albion exhibited lower disease suppression compared to 'San Andreas' and 'Monte Rey', suggesting a greater susceptibility of the former to *B. cinerea* infection. Furthermore, Wang et al. (2021) reported a 93.3 % reduction in gray mold incidence and a 24.8 % decrease in disease severity in strawberry fruit after a 2-day storage when using *B. halotolerans* KLBC XJ-5. However, after a longer incubation period, no statistical difference was observed between the control and the bacterial treatment.

The suspension of *B. velezensis* S26 effectively inhibited *Colletotrichum* spp. in strawberries, resulting in reduced incidence and severity of anthracnose. This aligns with the findings of Li et al. (2021), who used an extract from *Bacillus safensis* QN1NO-4 culture to suppress *Colletotrichum fragariae* in strawberries cv. Zhang Ji. In addition, Wu et al. (2021) noted a decline in the incidence of *C. gloeosporioides* SC01 in strawberry fruit when applying a suspension of *B. amyloliquefaciens* PMB04. Similarly, Alijani et al. (2021b) successfully controlled *C. nymphaeae* on strawberries by employing various *Bacillus* spp. strains (YN8,

LN57, MN17) and *Pseudomonas aeruginosa* EN18. In the same study, *B. atrophaeus* DM6120 achieved a remarkable 71.4% reduction in gray mold severity.

The application of a freshly prepared suspension containing *B. velezensis* S26 cells, endospores, and metabolites can contribute to controlling anthracnose and gray mold in harvested strawberries. While we observed a decrease in biocontrol efficacy during storage, the bioproduct still maintained its inhibitory potential. Similarly, Guo et al. (2004) investigated the suppressive capacity of each biocontrol agent separately (*Serratia* sp. J2, *Pseudomonas* sp. J3, and *Bacillus* sp. BB11) in a water-based formulation stored for 1-2 years against *Ralstonia solanacearum*. The authors noted remarkable biocontrol efficacy of the three formulations in field assays, using applied fresh and stored bioproducts.

In terms of food safety, *B. velezensis* strains have being successfully employed as probiotics in animal feed (Khalid et al., 2021), and their secreted lipopeptides can remarkably contribute for controlling animal diseases (Gao et al., 2017). Even though various strains of *Bacillus* spp. can be considered safe (Khalid et al., 2021), it is essential to assess their pathogenicity, allergenic potential, and cytotoxic properties. Additionally, the horizontal transfer of antimicrobial resistance genes and their environmental impact must be taken into consideration (Fung et al., 2018; Lee et al., 2019). Stringent quality control measures, including the testing for contaminants and ensuring the absence of harmful substances, should be implemented throughout the production of bioformulations. Moreover, products enriched with bacteria must adhere to the rigorous regulatory standards established by food safety authorities in different countries (Fung et al., 2018), as the toxic effects can be inherent properties of each bacterial strain (Khalid et al., 2021).

5. Conclusion

The suspension of *B. velezensis* S26 vegetative cells and endospores effectively controlled *Colletotrichum* spp. and *Botrytis cinerea* isolates *in vitro*, inhibiting mycelial growth and conidial germination. Furthermore, the optimization assays performed to enhance bacterial sporulation resulted in an endospore-rich suspension, with preserved cell viability during storage. This ensured effective biocontrol against anthracnose and gray mold in strawberries during the postharvest phase. In summary, *B. velezensis* S26 proves to be a promising bioagent for managing postharvest diseases in strawberries, and the ability to store it as an endospore suspension allows for maintaining its characteristics and antagonistic properties. Nevertheless,

further investigations are imperative to assess the toxigenic potential of the bacterial suspension, thereby safeguarding both public health and the environment.

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Supplementary Material 1

Table S1. Pathogen isolates used in the experiments.

Isolates	Species	Isolated from	Origin (city/country)	Institution
Cg4A	*Colletotrichum gloeosporioides*	Grapevine	Caxias do Sul, Brazil	University of Caxias do Sul
Cg3D	*Colletotrichum gloeosporioides*	Grapevine	Caxias do Sul, Brazil	University of Caxias do Sul
Ci020	*Colletotrichum fructicola*	Grapevine	Bento Gonçalves, Brazil	University of Caxias do Sul
Ci029	*Colletotrichum fructicola*	Grapevine	São Valentin do Sul, Brazil	University of Caxias do Sul
Ci015	*Colletotrichum nymphaeae*	Grapevine	Bento Gonçalves, Brazil	University of Caxias do Sul
Ca017	*Colletotrichum nymphaeae*	Grapevine	Bento Gonçalves, Brazil	University of Caxias do Sul
Cact	*Colletotrichum acutatum*	Strawberry	Caxias do Sul, Brazil	University of Caxias do Sul
001/11	*Botrytis cinerea*	Grapevine	Caxias do Sul, Brazil	University of Caxias do Sul
A3717	*Botrytis cinerea*	Strawberry	Caxias do Sul, Brazil	University of Caxias do Sul
A21/17A	*Botrytis cinerea*	Grapevine	Bento Gonçalves, Brazil	University of Caxias do Sul
CNPUV53	*Botrytis cinerea*	Grapevine	Garibaldi, Brazil	Embrapa Grape and Wine
CNPUV61	*Botrytis cinerea*	Grapevine	Caxias do Sul, Brazil	Embrapa Grape and Wine
CNPUV62	*Botrytis cinerea*	Grapevine	Caxias do Sul, Brazil	Embrapa Grape and Wine
CNPUV80	*Botrytis cinerea*	Grapevine	Bento Gonçalves, Brazil	Embrapa Grape and Wine

Supplementary Material 2

Table S2. Cell viability of four suspensions containing *Bacillus velezensis* S26 endospores. Equal letters indicate no statistically significant difference among the formulations, as determined by one-way ANOVA, followed by Tukey's test ($P < 0.05$). Each value represents the mean ± standard deviation of three replicates.

Treatments	Days of storage							
	0	30	60	90	120	150	180	365
S 4 ºC	9.5 ± 0.4 a	8.0 ± 0.1 b	8.0 ± 0.3 b	7.9 ± 0.3 b	7.8 ± 0.5 b	7.7 ± 0.2 b	7.7 ± 0.6 b	7.7 ± 0.3 b
S 25 ºC	9.5 ± 0.5 a	9.0 ± 0.2 a	9.0 ± 0.3 a	8.9 ± 0.5 a	8.9 ± 0.3 a	8.8 ± 0.1 a	8.7 ± 0.4 a	8.5 ± 0.2 a
I 4 ºC	9.5 ± 0.4 a	8.4 ± 0.5 ab	8.4 ± 0.2 b	8.4 ± 0.2 ab	8.2 ± 0.5 ab	7.9 ± 0.2 b	7.8 ± 0.3 b	6.7 ± 0.5 c
I 25 ºC	9.5 ± 0.3 a	8.5 ± 0.3 ab	8.4 ± 0.0 b	8.4 ± 0.2 ab	8.3 ± 0.4 ab	8.1 ± 0.4 b	7.9 ± 0.2 b	6.6 ± 0.1 c

4.3 Suppression of *Colletotrichum* spp. on grape berries, vine leaves, and plants using *Bacillus velezensis* S26 endospores

Abstract

Grape ripe rot and anthracnose are fungal diseases caused by *Colletotrichum* spp., which negatively impact viticulture. Disease management often involves the use of synthetic fungicides, whose toxicity poses risks to the environment and human health. As a result, biopesticides have been developed as an alternative approach for suppressing plant diseases in the field or postharvest. The present study aimed to evaluate the inhibitory potential of a bacterial inoculant containing endospores of *Bacillus velezensis* strain S26 against seven isolates of *Colletotrichum* spp. in grapevines (*Vitis vinifera* and *V. labrusca*). The bioassays were performed on grape berries, leaf discs, and micropropagated plants using both a fresh inoculant and an inoculant stored for six months at room temperature. The findings revealed that the biocontrol activity was more pronounced in fruit when compared to vine plants and leaves. Besides, the bacterial inoculant demonstrated a strain-dependent capacity to suppress the pathogen, with higher effectiveness observed in controlling the disease in *V. vinifera* compared to *V. labrusca*. Regarding the effect of storage on bacterial antagonism, the stored inoculant maintained its inhibitory ability against the pathogen. In summary, *B. velezensis* S26 demonstrates potential as a biocontrol agent against *Colletotrichum* spp. in grapevines, making it suitable for use in the development of bioproducts.

Keywords: Antagonism, Anthracnose, Biocontrol, Ripe rot, *Vitis* spp.

1 Introduction

Brazil is among the world's largest grape producers (FAOSTAT, 2021). In 2021, the country held the seventh position in the ranking of mayor table grape producers (OIV, 2021). Besides, winemaking using grape species such as *Vitis vinifera*, *V. labrusca*, and hybrids has gained prominence (Echeverrigaray et al., 2019). However, tropical and subtropical viticulture faces significant challenges in controlling fungal diseases due to the warm climate and high humidity, which increase infections like ripe rot and other grape bunch rots (Sawant et al., 2016; Echeverrigaray et al., 2020). Furthermore, the incidence of anthracnose and ripe rot has risen in grapevines, possibly due to breeding programs focusing on resistance to powdery and downy mildew (Nigar et al., 2023).

Glomerella cingulata (Stonemam) Spauld & Schrenk anamorph *Colletotrichum* spp. is the causal agent of ripe rot in grapevines. Infection can occur in the field or after harvest, leading to reduced crop yields (Hamaoka et al., 2021; Dou et al., 2022) and diminished grape quality (Echeverrigaray et al., 2019; Poveda et al., 2020). Although, anthracnose in grapevines is primarily caused by *Elsinoë ampelina* (Santos et al., 2018; Modesto et al., 2020), certain species

of the genus *Colletotrichum* have also been associated with this disease (Yan et al., 2015; Sawant et al., 2016; Echeverrigaray et al., 2020; Fan et al., 2023; Nigar et al., 2023). Unlike *E. ampelina*, symptoms caused by *Colletotrichum gloeosporioides* include necrotic and water-soaked lesions (Ellis and Erincik 2008; Yan et al., 2015). Furthermore, *Colletotrichum* spp. can also affect vine stems, foliage, and fruit (Nigar et al., 2023).

Biocontrol strategies are gaining interest as alternative to conventional disease management. Chemical fungicides applied close to harvest can persist on fruit as residues, impacting human health (Wang et al., 2021). In addition, pesticides are harmful to the environment and can contribute to the emergence of resistant pathogens (Hamaoka et al., 2021; Rodrigues et al., 2021). Thus, antagonistic bacteria such as *Bacillus* spp. have been effective in controlling *Colletotrichum* spp. in various crops, including strawberries (Es-Soufi et al., 2020; Alijani et al., 2021a, 2021b; Li et al., 2021; Wu et al., 2021), papaya (Chiquito-Contreras et al., 2019; Silva-Jara et al., 2019; Murakami et al., 2020), grapevines (Furuya et al., 2011; Mochizuki et al., 2012; Aoki et al., 2017; Sawant et al., 2016), among others.

The present study aimed to investigate the biocontrol activity of *Bacillus velezensis* strain S26 against anthracnose and ripe rot in two grapevines species. For this purpose, the grape varieties Moscato giallo (*V. vinifera* L.) and Niagara branca (*V. labrusca* L.) were selected. Moscato giallo is characterized by its floral and fruity flavor and is used in the production of aromatic wines (Marcon et al., 2019). On the other hand, Niagara branca is consumed as table grape and utilized in the production of white wines (Souza and Fochesato, 2007). Thus, the antagonistic effect of *B. velezensis* S26 was assessed against seven strains of *Colletotrichum* spp. on grape berries, leaf discs, and vine plantlets.

2 Material and methods

2.1 Pathogen e bacterial inoculant

The fungal pathogen strains (*Colletotrichum gloeosporioides* 3D, *C. gloeosporioides* 4A, *C. nymphaeae* Ci015, *C. nymphaeae* Ca017, *C. fructicola* Ci020, *C. fructicola* Ci029, and *C. acutatum*) were obtained from the Laboratory of Plant Disease Biological Control collection at the University of Caxias do Sul, Rio Grande do Sul, Brazil (Supplementary Material 1). Fungal cultures were reactivated on Potato Dextrose Agar (PDA) medium and then incubated in a chamber at 25 ºC with a 12-h light/12-h dark cycle for 10 days. Conidia were obtained by flooding the fungal mycelium with a 0.85 % NaCl solution containing 4 drops of Tween 80.

After filtration through four layers of sterile gauze, the conidia concentration was adjusted to 1 × 10^6 conidia mL^{-1}.

The bacterium *Bacillus velezensis* strain S26, which was isolated from copper-contaminated soil under organic crop system and molecularly characterized by Debastiani et al. (2023), was used in the development of the bioinoculant. A fresh culture was obtained by inoculating an individual colony into 10 mL of Luria-Bertani (LB) broth at pH 7.0. After incubating on a rotary shaker at 130 rpm and 28 °C for 24 h, cells were subcultured in 90 mL of LB broth under the same growth conditions to increase the bacterial population. Subsequently, endospore production was induced in shaking-flasks containing 100 mL of LB broth with the pH adjusted to 8.0 before autoclaving. Incubation was performed at 130 rpm and 37 °C for 16 h. The fresh inoculant (FI) consisted of the culture broth containing 2.1 × 10^8 spores mL^{-1}, while the stored inoculant (SI) contained 7.9 × 10^6 spores mL^{-1} after storage at room temperature for 180 days (Supplementary Material 2).

2.2 Plant material

Biological tissues (leaves and stems) were obtained from 2-year-old vines of *Vitis vinifera* (cv. Moscato giallo) and *V. labrusca* (cv. Niagara branca) grown in a greenhouse. Grape bunches of these same grapevine species were collected from vineyards located at Embrapa Grape and Wine, Bento Gonçalves, Rio Grande do Sul, Brazil (latitude 29°09’51” S, longitude 51°31’53” W, elevation 623 m).

The samples included: a) leaves in the third and fourth positions on the vine branches, b) uniform berries detached from grape bunches maintaining the pedicel, and c) stems without leaves, divided into nodal segments (2-3 cm in length). The biological materials were surface disinfested in a laminar flow cabinet. Initially, the samples were immersed in 70 % (v/v) ethanol for 1 min, and then treated with 2 % (v/v) NaOCl and 0.02 % (w/v) Tween 40 for 5 min. Subsequently, the grapevine tissues were rinsed three times with sterile water.

2.3 Biocontrol activity in leaf discs

Previously disinfested leaf discs, measuring 25 mm in diameter, were soaked in fresh or stored inoculants containing *B. velezensis* S26 endospores for 3 min. The control group was immersed in LB broth. Subsequently, leaf discs with the abaxial side facing upwards were inoculated onto plates with agar-water medium (15 g L^{-1} agar). After 24 h, 10 μL of 0.85 %

NaCl solution or pathogen suspension (1×10^6 conidia mL^{-1}) was applied to each leaf disc. The following treatments were performed: control, fresh inoculant (FI), stored inoculant (SI), pathogen suspension (Col), fresh inoculant + pathogen suspension (FI + Col), and stored inoculant + pathogen suspension (SI + Col). Each treatment consisted of four plates, each inoculated with seven leaf discs (n = 28). The plates were placed in a growth chamber at 25 ± 2 °C with a photoperiod of 12-h light/12-h dark for 7 days. Disease incidence (DI) was calculated using the formula: DI (%) = (number of leaf discs with lesions or pathogen growth / total number of discs) × 100. Disease severity (DS) was determined by averaging three measurements of the lesion diameter on each leaf disc. In cases where the discs had more than one lesion, the total affected area was considered.

2.4 Biocontrol activity in micropropagated plants

Following the external disinfestation procedure described in Section 2.2, nodal segments were cultured on MS medium (Murashige and Skoog) supplemented with 30 g L^{-1} sucrose, 6 g L^{-1} agar, and 0.5 mg L^{-1} 6-benzyl aminopurine (BAP), with the pH adjusted to 5.8 prior to autoclaving. The explants were subcultured every 4 weeks and then rooted in half-strength MS medium at pH 5.8, supplemented with 15 g L^{-1} sucrose, 6 g L^{-1} agar and 0.1 μg L^{-1} NAA (α-naphthalene acetic acid). Cultures were maintained at 25 ± 2 °C under a 16-h light/8-h dark photoperiod (light intensity of 80 μmol m^{-2} s^{-1}). Subsequently, rooted plantlets were transferred to plastic pots containing 180 mL of autoclaved substrate (90 % sphagnum and 10 % vermiculite) at pH 5.5. Acclimatization was carried out at 25 °C, 70 % relative humidity, under a 16-h light/8-h dark cycle (irradiance of 350 μmol m^{-2} s^{-1}).

The biocontrol potential of *B. velezensis* S26 endospores was evaluated in 30-day-old plants using a completely randomized design. Then, 10 mL of fresh inoculant (2.1×10^8 spore mL^{-1}) was drenched onto soil around each plant. The control group was treated with full-strength LB broth. After 3 days, plants were foliar sprayed with 5 mL of a mixture of *Colletotrichum* spp. strains (1×10^6 conidia mL^{-1}). The experiment was maintained in a greenhouse and plants were watered every 1-2 days. Disease incidence, disease severity, and pathogen re-isolation were assessed after 14 days. Disease incidence (DI) was calculated as follows: DI (%) = (number of infected plants / total number of plants) × 100. Disease severity (DS) was determined according to the scale developed by Horsfall and Heuberger (1942), where: 0 = no symptoms, 1 = affected area between 1-10 %, 2 = affected area between 11-25

%, 3 = affected area between 26-50 %, 4 = affected area between 51-75 %, and 5 = affected area between 76-100 %. Afterward, disease severity index (DSI) was determined using the formula: DSI (%) = [$\sum$ ($N_i \times DS_i$) / ($N \times DS$)] × 100, where N_i = number of plants showing the same disease severity score, DS_i = disease severity score (0 to 5), N = total number of plants, and DS = maximum disease score obtained in each treatment. The pathogen was re-isolated from symptomatic lesions and identified based on its morphological characteristics. The experiment was performed in triplicates with 20 plants per treatment.

2.5 Biocontrol activity in fruit

The inhibitory potential of *B. velezensis* S26 against *Colletotrichum* spp. was assessed on healthy grape berries. Surface-disinfested fruit were wounded using a sterile pin and then immersed in bacterial inoculant or LB broth for 5 min. After 3 h, 10 µL of *Colletotrichum* spp. suspension (1×10^6 conidia mL^{-1}) or a 0.85 % NaCl solution was inoculated into the wounds.

The following treatments were conducted: control, fresh inoculant (FI), stored inoculant (SI), pathogen suspension (Col), fresh inoculant + pathogen suspension (FI + Col), and stored inoculant + pathogen suspension (SI + Col). Fruits were placed in plastic trays and maintained at 25 ± 2 °C with 80-90 % of relative humidity for 5 days. Disease severity (DS, %) was scored according to the grading scale adapted from De Moura et al. (2021): 0 = without symptoms, 1 = injured area < 25 %, 2 = injured area between 26-50 %, 3 = injured area between 51-75 %, and 4 = injured area > 76 %. The results were expressed as disease severity index (DSI) using the formula: DSI (%) = [$\sum$ ($N_i \times DS_i$) / ($N \times DS$)] × 100, where N_i = number of berries with the same disease severity score, DS_i = disease severity score (0 to 4), N = total number of berries, and DS = maximum disease score obtained in each treatment. The experiment was carried out using a completely randomized design, in triplicates, with 20 grape berries per treatment.

2.6 Statistical analysis

Biocontrol assays were evaluated using analysis of variance (ANOVA) and the means were compared using the Tukey's test. Data that did not meet the criteria for normality and homoscedasticity, determined respectively by the Shapiro-Wilk and Levene's tests, were compared using the Kruskal-Wallis test and differentiated using the Dunn-Bonferroni test. Treatments were evaluated at a significance level of 5 %. The statistical analysis was performed using SPSS 22.0 software for Windows.

3 Results

3.1 Biocontrol activity in leaf discs

The disease incidence in leaf discs of Niagara branca after 7 days of incubation ranged from 42.9 % to 100 %, with a mean of 80.3 % (Fig. 1A). Although none of the bacterial inoculants reduced the disease incidence, the necrotic lesions caused by *C. fructicola* Ci20 were controlled through the preventive application of the fresh inoculant (Fig. 1B). In contrast to the results observed in *V. labrusca*, the variety Moscato giallo exhibited a lower disease incidence (ranging from 33.3 % to 76.2 %, with a mean of 60.5 %) and a more favorable response to the treatment with *B. velezensis* S26 endospores (Fig. 1C and 1D). Both the fresh and stored inoculants reduced the disease incidence caused by *C. gloeosporioides* 3D, *C. nymphaeae* Ci015, *C. nymphaeae* Ca017, *C. fructicola* Ci20, and *C. fructicola* Ci029 (Fig. 1C). On the other hand, infection by *C. acutatum* was only controlled by the fresh inoculant. The severity of the symptoms in Moscato giallo (Fig. 1D) was diminished by the application of both fresh (*C. gloeosporioides* 3D, *C. fructicola* Ci20, and *C. acutatum*) and stored inoculants (*C. gloeosporioides* 3D, *C. nymphaeae* Ca017, *C. fructicola* Ci20, and *C. acutatum*).

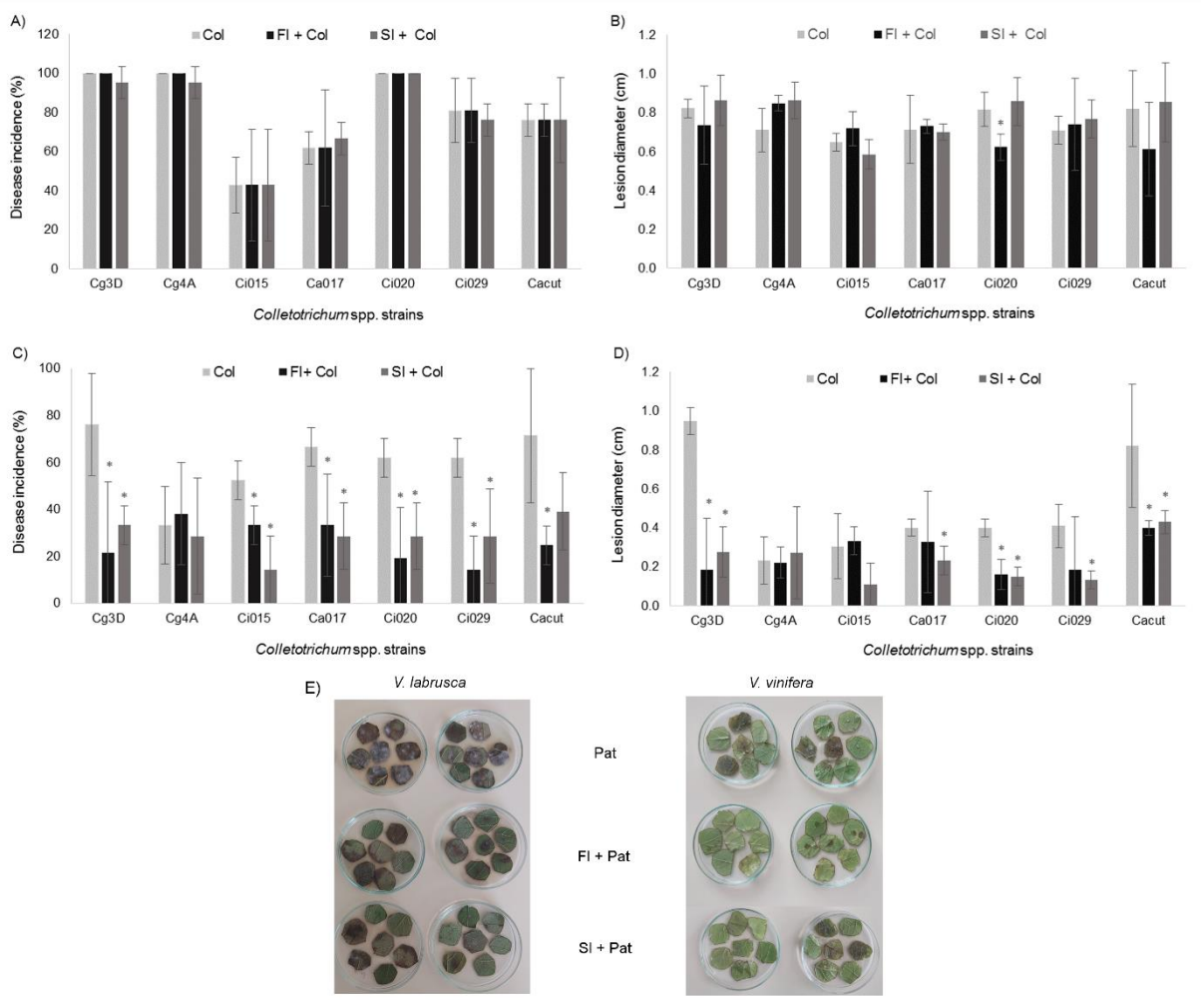

Fig. 1. Leaf discs treated with *Colletotrichum* spp. (Col), fresh inoculant containing *Bacillus velezensis* S26 and *Colletotrichum* spp. (FI + Col), and stored inoculant containing *B. velezensis* S26 and *Colletotrichum* spp. (SI + Col). A) Disease incidence (DI) and B) Lesion diameter in *Vitis labrusca*. C) DI and D) Lesion diameter in *V. vinifera*. E) Photographs showing the experiment in leaf discs. Pat = pathogen or *Colletrotrichum* spp. Means were compared using ANOVA followed by Tukey's test or Kruskal-Wallis followed by Dunn-Bonferroni test for each isolate. Asterisks indicate a significant difference ($P < 0.05$) and the error bars represent the standard deviation of three replicates.

3.2 Biocontrol activity in micropropagated plants

Aiming to corroborate the results obtained from the bioassay on leaf discs, an experiment was carried out using 30-day-old vines of *V. vinifera* and *V. labrusca* (Table 1). Records taken after 14 days post-inoculation showed no statistically significant differences in disease incidence and severity of symptoms between the two species infected with the

pathogenic agent. Pathogen re-isolation from symptomatic tissues was employed to confirm the infection. Additionally, the fresh inoculant was able to mitigate the intensity and area of lesions in *V. vinifera* treated with the *Colletotrichum* spp. suspension.

Table 1. Evaluation of disease incidence (DI) and disease severity index (DSI) in micropropagated plants (*Vitis vinifera* and *V. labrusca*) subjected to the treatments: *Colletotrichum* spp. suspension (Col), fresh inoculant containing *Bacillus velezensis* S26 and *Colletotrichum* spp. suspension (FI + Col).

	DI (%)		DSI (%)	
	V. labrusca	*V. vinifera*	*V. labrusca*	*V. vinifera*
Col	80.0 ± 5.6 aA	74.0 ± 5.6 aA	76.7 ± 8.0 aA	79.2 ± 6.7 aA
FI + Col	76.3 ± 8.0 aA	62.3 ± 7.0 aA	70.2 ± 2.8 aA	52.0 ± 8.0 bB

Equal lowercase letters indicate no statistically significant difference between the treatments (Col and FI + Col), and equal uppercase letters indicate no statistical difference between the vine species (*V. vinifera* and *V. labrusca).* The analyses were performed using the t-test ($P < 0.05$). Each value represents the mean ± standard deviation of three replicates.

3.3 Biocontrol activity in grape berries

Grape berries of Niagara branca (*V. labrusca)* infected with the pathogen showed disease incidence ranging from 45 % to 95 %, with a mean of 58.6 % (Fig. 2A). Interestingly, the stored inoculant reduced pathogenic infection caused by *C. nymphaeae* Ca017 and *C. fructicola* Ci020, while the fresh inoculant did not inhibit the occurrence of the disease in comparison to the control treated with the pathogen alone. Disease severity caused by *C. gloeosporioides* 3D, *C. nymphaeae* Ci015, *C. nymphaeae* Ca017, and *C. fructicola* Ci20 was effectively reduced by the fresh inoculant, whereas the stored inoculant reduced the intensity of the symptoms in *C. nymphaeae* Ca017 and *C. acutatum* (Fig. 2B).

Disease incidence was higher in berries of Moscato giallo (ranging from 54 % to 100 %, with a mean of 90.1 %) compared to Niagara branca (Fig. 2C). The treatment with the fresh inoculant effectively reduced disease incidence and severity in berries inoculated with *C. gloeosporioides* 3D, *C. gloeosporioides* 4A, *C. nymphaeae* Ca017, *C. fructicola* Ci020, *C. fructicola* Ci029, and *C. acutatum* (Fig. 2C and 2D). Although the infection was not suppressed by the fresh inoculant, necrotic lesions caused by *C. nymphaeae* Ci015 were reduced. Furthermore, the inoculant stored for 180 days preserved its inhibitory potential towards the

pathogen and decreased the disease incidence caused by six *Colletotrichum* spp. strains (*C. gloeosporioides* 3D, *C. gloeosporioides* 4A, *C. nymphaeae* Ca017, *C. fructicola* Ci020, *C. fructicola* Ci029, and *C. acutatum*). The severity of the symptoms caused by *C. gloeosporioides* 3D, *C. gloeosporioides* 4A, and *C. nymphaeae* Ci015 was also suppressed by the stored inoculant (Fig. 2D).

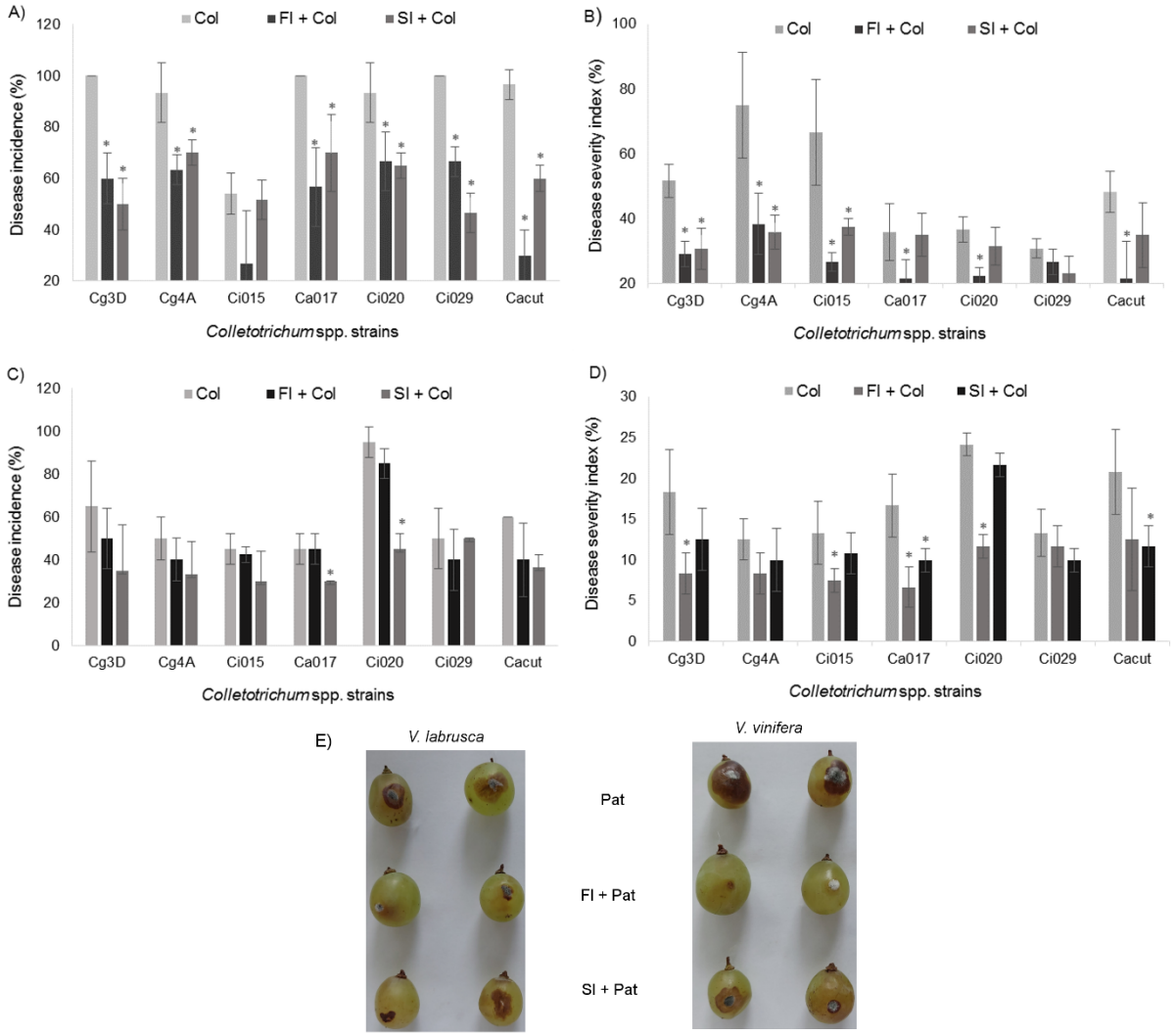

Fig. 2. Grape berries treated with *Colletotrichum* spp. (Col), fresh inoculant containing *Bacillus velezensis* S26 and *Colletotrichum* spp. (FI + Col), and stored inoculant of *B. velezensis* S26 and *Colletotrichum* spp. (SI + Col). A) Disease incidence (DI) and B) Disease severity index (DSI) in *Vitis labrusca*. C) DI and D) DSI in *V. vinifera*. E) Photographs showing the experiment in grape berries. Pat = pathogen or *Colletrotrichum* spp. Means were compared using ANOVA followed by Tukey's test or Kruskal-Wallis followed by Dunn-Bonferroni test for each isolate. Asterisks indicate a significant difference ($P < 0.05$) and the error bars represent the standard deviation of three replicates.

4 Discussion

The members of the genus *Bacillus* are known for their biocontrol capacity against several fungal diseases. In the current study, the antagonistic potential of *Bacillus velezensis* strain S26, isolated from the soil of organic vineyards, was tested against seven strains of *Colletotrichum* spp. Although *B. velezensis* S26 was originally adapted to the rhizospheric region, it demonstrated successful colonization as an inhabitant of the grapevine phyllosphere and internal tissues, defending its ecological niche and competing with the pathogen for space and nutrients. Our findings are consistent with the results obtained by Nifakos et al. (2021), who successfully used an endophytic bacterium *B. velezensis* Bvel1 isolated from the roots of olive trees to control gray mold in detached grape berries.

Biocontrol assays demonstrated that the inoculant with *B. velezensis* S26 endospores can effectively reduce the development of anthracnose and minimize the severity of symptoms in *V. vinifera* cv. Moscato giallo. On the other hand, the bacterial antagonism in micropropagated vines was less pronounced. Corroborating our results, Sawant et al. (2016) reported the control of the anthracnose in grape berries of *V. vinifera* (cv. Thompson seedless) treated with seven promising strains of *Bacillus* spp. Necrotic lesions were reduced by more than 80 %, and an effective biocontrol was also observed in vine cuttings. Additionally, the authors confirmed the biocontrol potential of five bacterial strains in vineyards with a performance similar to the systemic fungicide carbendazim.

The application of *Bacillus cereus* NRKT to grape bunches also led to a reduction in the infection caused by *Colletotrichum gloeosporioides*, with a biocontrol efficacy higher than 35 %, in vineyards of *V. vinifera* cv. Pinot Noir in Japan (Aoki et al., 2017). Similarly, the filtrate of *Bacillus subtilis* KS1 suspension was effective in controlling bunch rots caused by *Botrytis cinerea* and *C. gloeosporioides* in vineyards of *V. vinifera* cv. Koshu. After four months of treatment, the disease incidence was 20.3 %, while the control group showed 38.5 % of rotten bunches (Furuya et al., 2011). Furthermore, *Bacillus amyloliquefaciens* S13-3 demonstrated inhibition against *C. gloeosporioides* in another study conducted by Mochizuki et al. (2012). In this research, the bacteria also reduced ripe rot incidence by 30.7 % in vineyards *V. vinifera* cv. Semillon.

In agreement with our findings, several studies have reported that *Bacillus* spp. can effectively control anthracnose in fruit and plants. For instance, the extract of *Bacillus safensis* sp. QN1NO-4 and its metabolites inhibited *Colletotrichum fragariae* in strawberry pseudofruit and seedlings (Li et al., 2021). Similarly, Alijani et al. (2021b) observed a reduction in the

development of *C. nymphaeae* in fruit and plants treated by foliar spraying or soil drenching with a suspension of *Bacillus atrophaeus* JCM 6120. Es-Soufi et al. (2020) also reported that *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR suppressed anthracnose in strawberry leaflets and plants grown in greenhouse or field.

Regarding the vine species evaluated in this study, varying degrees of susceptibility to *Colletotrichum* spp. were observed. Resistance was found to be a tissue-dependent characteristic influenced by the grapevine species. Meanwhile, the berries of Moscato giallo (*V. vinifera*) exhibited higher susceptibility to the infection, leaf discs of this vine species demonstrated lower susceptibility compared to the Niagara branca (*V. labrusca*). Therefore, even though *V. vinifera* tends to exhibit less resistance to fungal diseases compared to other vine species, it can also serve as a valuable source of resistance genes for genetic breeding programs (Goyal et al., 2020; Villano and Aversano, 2020; Fedorina et al., 2022). Biocontrol activity in grape berries, lealf discs, and plants was higher in *V. vinifera* (cv. Moscato giallo) compared to *V. labrusca* (cv. Niagara branca). Additionally, *Colletotrichum* spp. strains exhibited varying levels of virulence, and their pathogenicity depended on the vine tissue infected. Likewise, Yan et al. (2015) studied the pathogenicity of 34 isolates of *Colletotrichum* spp. and found both inter and intraspecific variation in the virulence of the pathogen. Echeverrigaray et al. (2019) and He et al. (2019) also noted that *Colletotrichum* spp. demonstrate specificities concerning pathogenicity, host colonization, aggressiveness, and susceptibility to pesticides.

Furthermore, our findings demonstrated that both fresh and stored inoculants were effective in controlling the infection in grape berries, vine leaves, and plants. Although the count of endospores decreased, the formulation preserved its biocontrol capacity throughout storage at room temperature. This suggest that the concentration of viable cells after storage was sufficient to suppress the severity of the infection. Besides, liquid formulations tend to have a long shelf life due to the addition of nutrient and protective compounds (Gopi et al., 2019). Sriram et al. (2011) reported that a talc formulation of *Trichoderma harzianum* supplemented with 3 % and 6 % glycerol preserved its antagonistic potential against *Fusarium oxysporum* f. sp. *lycopersici* after 12 months of storage. The control of Fusarium wilt in tomato seeds was similar to the fresh formulation. On the other hand, Wong et al. (2019) evaluated two dry and two liquid formulations enriched with *Pseudomonas aeruginosa* DRB1 and *T. harzianum* CBF2 against Fusarium wilt in bananas. The bacterial count in the liquid formulation decreased by ten thousand times during six months of storage at room temperature, while storage under refrigeration drastically reduced the *P. aeruginosa* DRB1 population to zero. Biocontrol assays

revealed that all fresh products contributed to reducing the severity of symptoms in a greenhouse experiment. Yánez-Mendizábal et al. (2012) developed a liquid formulation with *Bacillus subtilis* CPA-8 using low-cost products such as molasses, maltose, peptone, soy, sucrose, and yeast extract. They verified its antagonistic efficacy against *Monilinia fructicola* on peaches, resulting in a 95 % inhibition of brown rot incidence.

5. Conclusion

The rhizobacterium *Bacillus velezensis* strain S26 demonstrated the ability to suppress *Colletotrichum* spp. infections in vine tissues, especially when applied as a fresh inoculant. Furthermore, the inhibitory potential against the disease was a pathogen- and tissue-dependent characteristic, with higher antagonism observed in grape berries, leaf discs, and plants of *V. vinifera* cv. Moscato giallo. Therefore, *B. velezensis* S26 is a promising biological control agent for controlling grape ripe rot and anthracnose, and it can be used in the development of effective formulations. However, further studies are necessary to optimize bacterial survival and extend the shelf life of biopesticides.

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Supplementary Material 1

Table S1. Pathogen isolates used in the experiments.

Isolates	Species	Isolated from	Origin (city/country)	Institution
4A	*Colletotrichum gloeosporioides*	Grapevine	Caxias do Sul, Brazil	University of Caxias do Sul
3D	*Colletotrichum gloeosporioides*	Grapevine	Caxias do Sul, Brazil	University of Caxias do Sul
Ci020	*Colletotrichum fructicola*	Grapevine	Bento Gonçalves, Brazil	University of Caxias do Sul
Ci029	*Colletotrichum fructicola*	Grapevine	São Valentin do Sul, Brazil	University of Caxias do Sul
Ci015	*Colletotrichum nymphaeae*	Grapevine	Bento Gonçalves, Brazil	University of Caxias do Sul
Ca017	*Colletotrichum nymphaeae*	Grapevine	Bento Gonçalves, Brazil	University of Caxias do Sul
Cact	*Colletotrichum acutatum*	Strawberry	Caxias do Sul, Brazil	University of Caxias do Sul

Supplementary Material 2

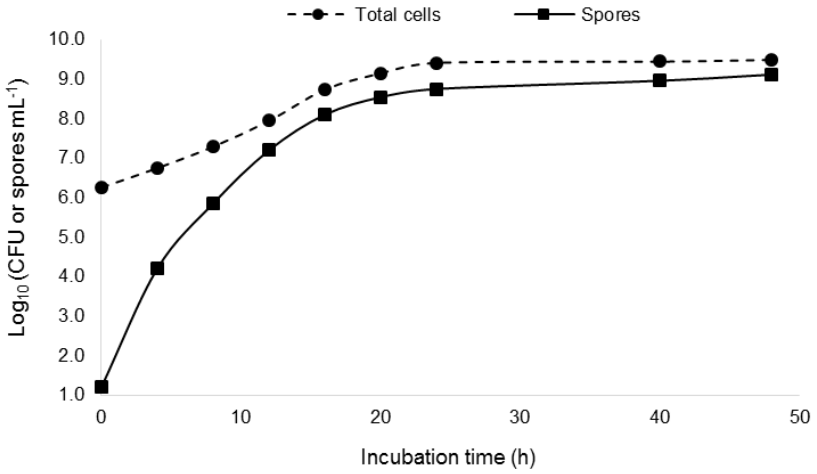

Fig S1. Growth and sporulation dynamics of *Bacillus velezensis* S26 when cultivated in LB liquid medium under shaking flask conditions at 130 rpm, 37°C, and pH 8.0.

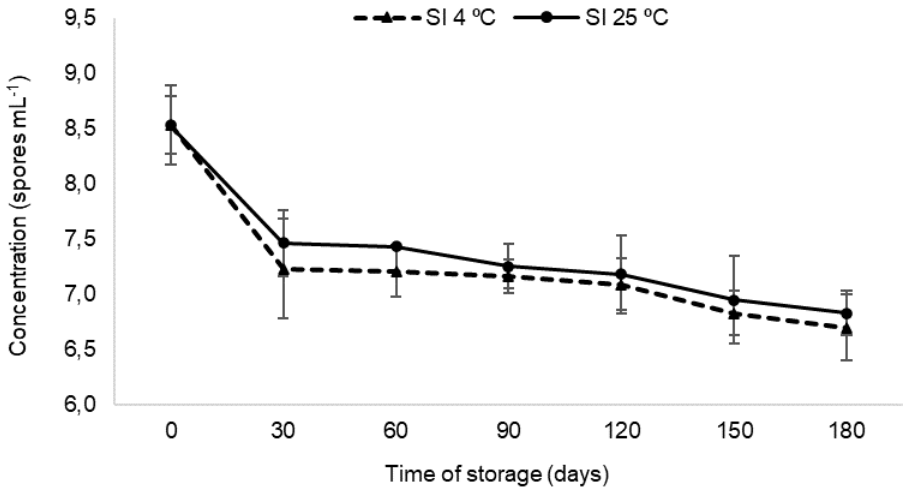

Fig. S2. Viability of *Bacillus velezensis* S26 endospores in stored inoculant (SI) over a 180-day storage at 4 °C and 25 °C. No statistically significant difference was observed between the two storage temperatures. The bars represent the standard deviation of three replicates.

4.4 Antagonistic potential of *Bacillus velezensis* S26 endospores against gray mold in grapevines

Abstract

Botrytis cinerea is a cosmopolitan pathogen that affects several crops worldwide. Gray mold, or *Botrytis* bunch rot, has caused significant economic losses in viticulture, both in the field and during the postharvest phase. Traditional strategies for managing fungal diseases, such as chemical control, have negative effects on the environment and human health, prompting the development of eco-friendly products and biopesticides. The purpose of this study was to evaluate the inhibitory potential of *Bacillus velezensis* strain S26 endospores against *B. cinerea* in grapevines. Thus, bacterial antagonism was assessed in leaf discs, grape berries, and vine plantlets (*Vitis vinifera* and *V. labrusca*). In addition, an experiment was performed to evaluate bacterial colonization and its impact on the quality of grape berries. Our findings demonstrated that fresh and stored inoculants containing *B. velezensis* S26 endospores can effectively suppress the pathogen in an isolate- and tissue-specific manner. In the colonization assay, the bacterium was able to growth in grape berries in the presence of the pathogen and the application of the bacterial inoculant did not affect grape quality. Therefore, *B. velezensis* S26 represents an important biotechnological tool for controlling gray mold in the field or harvested grapes.

Keywords: bacterial inoculant, biological control, biopesticide, *Botrytis cinerea*, bacterial endospores

1 Introduction

Grapevine (*Vitis* spp.) is a perennial crop cultivated worldwide for fresh consumption and the production of wine products (Esmaeel et al., 2020; Poveda et al., 2020). According to the International Organisation of Vine and Wine (OIV, 2021), 74.8 million tons of grapes were produced in 2021, with 50 % destined for wine and juice production, and 40 % commercialized as table grapes. However, fungal diseases such as gray mold have a devastating economic impact on viticulture (Poveda et al., 2020). *Botrytis* spp., including *B. cinerea* Pers., are the major pathogens causing gray mold or *Botrytis* bunch rot and primarily affecting harvested grapes (Nifakos et al., 2021). Global losses from gray mold can reach USD 10-100 billion per year (Fillinger and Yigal, 2016; Amarouchi et al., 2021). In addition, these pathogens impact wine quality by altering the profile of volatile organic compounds (Santos et al., 2022).

Conventionally, chemical pesticides have been employed to manage fungal diseases (Bruisson et al., 2019; Amarouchi et al., 2021). Nevertheless, biological control has gained increasing attention as an alternative to the adverse effects caused by synthetic fungicides on natural resources and human health (Poveda et al., 2020; Wang et al., 2021). This approach also aligns with UN Sustainable Development Goal 2, which aims to “end hunger, achieve food

security and improved nutrition, and promote sustainable agriculture" (United Nations - General Assembly, 2015).

The *Bacillus* genus encompasses a group of endospore-forming bacteria renowned for their capacity to enhance plant growth, control plant diseases, and induce systemic resistance in plants (Nifakos et al., 2021). Among the members of this genus, *Bacillus velezensis* stands out for its remarkable ability to synthesize antibiotic molecules such as bacillaene, bacillibactin, bacillomycin-D, bacilysin, difficidin, fengycin, iturin, macrolactin, and surfactin (Nifakos et al., 2021). Numerous studies have demonstrated the inhibition of *Botrytis cinerea* by *B. velezensis* XT1 CECT 8661 in strawberries (Toral et al., 2018, 2020), *B. velezensis* strains 5YN8 and DSN012 in pepper (Jiang et al., 2018), *B. velezensis* strain S23 (Amarouchi et al., 2021), and *B. velezensis* Bvel1 (Nifakos et al., 2021) in grapevines.

The present study aimed: a) to evaluate the antagonistic activity of *Bacillus velezensis* strain S26 endospores, applied as both fresh and stored inoculants, against seven isolates of *Botrytis* spp. on leaf discs, grape berries, and micropropagated plants of two grapevines species (*Vitis vinifera* and *V. labrusca*); b) to analyze the colonization of grape berries (*V. labrusca*) by a *B. velezensis* S26 suspension; and c) to determine the effects of bacterial inoculation on grape quality in *V. labrusca*.

2 Materials and methods

2.1 Bacterial inoculants

The bacterium *Bacillus velezensis* strain S26, used in both *in vitro* and *in vivo* assays, was isolated from soil in an organic vineyard situated in Caxias do Sul, Rio Grande do Sul State, Brazil (latitude 29°15'94'' S, longitude 51°21'45'' W), as reported by Debastiani et al. (2023). Initially, the rhizospheric bacterial strain was identified using molecular techniques through partial sequencing of the *16S rDNA* gene. The resulting sequence was deposited in the GenBank database under the accession number OP938800. The bacterial culture was maintained in Luria-Bertani (LB) medium supplemented with 40 % (v/v) glycerol at -80 °C.

A pre-inoculum was prepared by introducing a loop of a single colony-forming unit into a plastic tube containing 10 mL of Luria-Bertani (LB) broth at pH 7.0. The culture was then placed on a rotary shaker (Ethik Technology, Brazil) at 130 rpm and 28 °C. After incubation for 24 h, this culture was transferred to a 250 mL Erlenmeyer flask containing 90 mL of LB broth at pH 7.0, maintaining the same growth conditions. Following this, an aliquot of this

culture (1×10^6 CFU mL^{-1}) was utilized as inoculum for bacterial sporulation at a ratio of 1: 10. The endospore production was performed in shaking flasks containing LB broth at pH 8.0, supplemented with 2 mM $CaCl_2$. Subsequently, samples underwent heat inactivation (80 °C for 10 min), and the concentration of bacterial endospores was determined through serial dilution on LB medium plates. These plates were then incubated at 37 °C for 24 h.

The bacterium was utilized in two different forms for the experiments: as a fresh inoculant and as a stored inoculant. The fresh inoculant (FI) comprised the sporulation broth containing *B. velezensis* S26 endospores, along with any remaining vegetative cells and metabolites released during the sporulation, as detailed in Supplementary Material 1. The stored inoculant (SI) was the same suspension maintained at 25 °C for 180 days.

2.2 Pathogen suspension

Three isolates of *Botrytis* spp. (001/11, A3717, and A021/17A) were provided by the Laboratory of Biological Plant Disease Control at the University of Caxias do Sul, Rio Grande do Sul State, Brazil. Additionally, four isolates of *Botrytis* spp. (CNPUV53, CNPUV61, CNPUV62, and CNPUV80) were supplied by the Laboratory of Phytopathology at Embrapa Grape and Wine, Rio Grande do Sul State, Brazil (Supplementary Material 2).

In a previous *in vitro* assay, the mycelial growth of *Botrytis* spp. isolates was inhibited in a dual-culture using *B. velezensis* S26 endospores (Supplementary Material 3). Fungi were cultivated on Potato Dextrose Agar (PDA) medium in a growth chamber (Eletrolab, São Paulo, SP, Brazil) at 25 °C, with 12 h of light for 14 days. Conidia were harvested by scraping the fungal mycelium with a 0.85 % NaCl solution containing 0.05 % Tween 80. The suspension was then filtered through sterile gauze to remove fungal hyphae. The final concentration was adjusted to 1×10^6 conidia mL^{-1} using a hemocytometer (Precicolor, Germany).

2.3 Grapevine materials

Grapevine materials (bunches, leaves, and shoots) were collected from grapevine plants: cv. Moscato giallo (*Vitis vinifera* L.) and cv. Niagara branca (*V. labrusca* L.), which were grown in a greenhouse at Embrapa Grape and Wine, Rio Grande do Sul State, Brazil.

2.4 Antagonistic activity of *B. velezensis* S26 endospores on vine leaf discs

Young leaves from grapevine cuttings were harvested using the method described by Haidar et al. (2016). The leaves underwent surface sterilization in a laminar flow cabinet (FilterFlux, Piracicaba, SP, Brazil) utilizing 70 % (v/v) ethanol for 1 min, followed by immersion in a 2 % (v/v) sodium hypochlorite solution for 3 min. Subsequently, the leaves were rinsed three times with sterilized distilled water. Leaf discs (25 mm in diameter) were collected using a cork borer and immersed in either fresh inoculant, stored inoculant, or LB broth for 3 min. Following this, seven leaf discs were arranged on each plate with agar-water medium (15 g L^{-1} agar), ensuring the adaxial surface of the leaves made contact with the culture medium. After 24 h, 10 µL of each pathogen suspension (1×10^6 conidia mL^{-1}) or 0.85 % NaCl solution containing 0.05 % Tween 80 were inoculated at the center of each leaf disc. The bioassay involved the use of seven *Botrytis* spp. isolates.

The experiment comprised six treatments: LB broth + NaCl solution (control), fresh inoculant + NaCl solution (FI), stored inoculant + NaCl solution (SI), LB broth + pathogen suspension (Pat), fresh inoculant + pathogen suspension (FI + Pat), and stored inoculant + pathogen suspension (SI + Pat). The plates were then incubated at 25 °C with a 12-h light / 12-h dark cycle for 7 days. Disease incidence (DI) was determined using the formula: DI (%) = (number of discs with necrosis / total number of discs) × 100. Disease severity was assessed by averaging two perpendicular measurements of each lesion (Haidar et al., 2016). The experiment was performed using a completely randomized design. Each treatment was replicated on four plates, resulting in 28 leaf discs per treatment. The experiment was repeated twice.

2.5 Grapevine micropropagation

Grapevine shoots (20 cm in length) were cut into small pieces (approximately 1 cm in length), each containing a single bud. These nodal segments were then surface-disinfected in a laminar flow cabinet. Initially, they were immersed in 70 % (v/v) ethanol for 1 min, followed by treatment with a 2 % (v/v) sodium hypochlorite solution containing 0.02 % (w/v) Tween 40 for 20 min. The explants were rinsed three times with sterilized distilled water and then inoculated into culture tubes containing 15 mL of Murashige and Skoog (MS) medium. This medium was supplemented with 3 % (w/v) sucrose, 0.6 % (w/v) agar, and 0.5 mg L^{-1} 6-benzyl aminopurine (BAP), with the pH adjusted to 5.8 prior to autoclaving.

After three subcultures, the explants were rooted in half-strength MS medium supplemented with 1.5 % (w/v) sucrose, 0.6 % (w/v) agar, and 0.1 μg L^{-1} NAA (α-naphthalene acetic acid), pH adjusted to 5.8. The culture tubes were incubated in a growth chamber at 25 °C, with relative humidity of 80 %, in 16-h light / 8-h dark cycle with light intensity of 80 μmol m^{-2} s^{-1}. Subsequently, plantlets were acclimatized in 180 mL plastic cups filled with autoclaved substrate (90 % peat and 10 % vermiculite) and maintained under the same environmental conditions.

2.6 Antagonistic activity of *B. velezensis* S26 endospores on micropropagated grapevines

Thirty days after transplantation, the substrate around the plants was drenched with 10 mL of fresh inoculant (FI) containing *B. velezensis* S26 endospores (1×10^8 spores mL^{-1}) or LB broth. Three days later, the plants were sprayed with a *Botrytis* sp. CNPUV62 suspension (1×10^6 conidia mL^{-1}) or 0.85 % NaCl solution containing 0.05 % Tween 80 until runoff. The treatments were as follows: LB broth and NaCl solution (control), LB broth and suspension of *Botrytis* sp. CNPUV62 (Pat), *B. velezensis* S26 endospores and suspension of *Botrytis* sp. CNPUV62 (FI + Pat).

The experiment was maintained at 25 ± 2 °C, with relative humidity of 70 %, and light intensity of 500 μmol m^{-2} s^{-1}. After 14 days, disease incidence, disease severity, and pathogen re-isolation were assessed. Disease incidence (DI) was determined using the formula: DI (%) = (number of infected plants / total number of plants) × 100. Disease severity (DS) was assessed using the rating scale developed by South et al. (2020) with some modifications: 0 = no symptoms, 1 = 10 small lesions, 2 = more than 10 small lesions, 3 = medium-sized lesions, 4 = large-sized lesions, 5 = lesions covering half or more of the leaf.

Subsequently, the disease severity index (DSI) was calculated using the formula (Camili et al., 2007): DSI (%) = $[\sum (N_i \times DS_i) / (N \times DS)] \times 100$, where N_i represents the number of plants with the same disease score, DS_i is the disease severity (ranging from 0 to 5), N is the total number of plants, and DS is the maximal severity score. The experiment followed a completely randomized design, with 15 plants per treatment, and was conducted in triplicate.

2.7 Antagonistic activity of *B. velezensis* S26 endospores on grape berries

Uniformly colored and sized berries were detached from the bunches while preserving the pedicel (0.3 mm in length). The fruits were surface sterilized as described in section 2.4.

Subsequently, the grapes were washed three times with sterile water and dried under sterile airflow for 10 min. The bacterial inoculant containing *B. velezensis* S26 endospores (1×10^8 spores mL^{-1}) or LB broth were sprayed onto the previously wounded fruit. All wounds were caused using a sterilized pin (3 mm width × 3 mm depth). After a 2-h period, 10 μL of pathogen suspension (1×10^6 conidia mL^{-1}) or 0.85 % NaCl solution containing 0.05 % Tween 80 were inoculated into the wounds. The bioassay involved the use of seven *Botrytis* spp. isolates.

The treatments consisted of: LB broth + NaCl solution (control), fresh inoculant + NaCl solution (FI), stored inoculant + NaCl solution (SI), LB broth + pathogen suspension (Pat), fresh inoculant + pathogen suspension (FI + Pat), and stored inoculant + pathogen suspension (SI + Pat). Following these treatments, the grapes were placed in plastic boxes and incubated at 25 ± 2 °C, with relative humidity of 85-95 % for 5 days.

Disease incidence (DI) was determined 5 days after inoculation using the formula: DI (%) = (number of symptomatic berries / total number of berries) × 100. Disease severity (DS) was scored according to the scale suggested by Calvo et al. (2017): 0 = no symptoms, 1 = symptomatic area < 25 %, 2 = symptomatic area between 25-50 %, 3 = symptomatic area between 50-75 %, and 4 = symptomatic area > 75 %. The disease severity index (DSI) was calculated as described in section 2.6. The experiment was conducted using a completely randomized design with three independent replicates and 20 grape berries per treatment.

2.8 Bacterial colonization of wounded grape berries

The assessment of bacterial colonization on Niagara branca berries followed the method described by Chen et al. (2020) with some modifications. Initially, 10 μL of *B. velezensis* S26 cells, suspended in 0.85 % saline solution (1×10^8 CFU mL^{-1}), were inoculated into a wound made with a sterile pin (3 mm width × 3 mm depth). After 2 h, half of the berries were challenged by applying 10 μL of *Botrytis* sp. CNPUV62 suspension (1×10^6 conidia mL^{-1}) at the same wound site. The grape berries were then arranged in plastic trays (10 fruits per box) and incubated in a dark chamber at 25 ± 2 °C, with relative humidity of 85-95 %.

Tissue samples around the wounds were harvested after 0, 24, 48, 72, 96, and 120 h using a cork borer (5 mm diameter × 5 mm depth). The harvested tissue was ground using a mortar and pestle. After serial dilution, the samples were spread on LB medium plates and incubated at 37 °C for 24 h. The colony-forming units per wound were determined and expressed as $\log_{10}$ CFU $wound^{-1}$. The experiment followed a completely randomized design with three replicates of 10 fruits per treatment. The assay was repeated three times.

2.9 Impact of bacterial treatment on grape quality

Uniform-sized clusters of Niagara branca were selected for the experiment. The grape bunches were immersed in a bacterial suspension (1×10^8 CFU mL^{-1}) for 3 min. The control was treated with 0.85 % NaCl solution. Subsequently, the bunches were placed on open plastic trays to air-dry under sterile conditions for 10 min. The incubation was carried out under refrigeration at 4 °C for 21 days. Each treatment group consisted of 30 bunches.

Samples were harvested at intervals of 0, 7, 10, 14, and 21 days, and the following parameters were assessed: fresh mass, total soluble solids, pH, and total titratable acidity. Fresh mass (FM) was measured using a semi-analytical balance (Marte Científica, Santa Rita do Sapucaí, MG, Brazil). Total soluble solids (TSS) were determined using a portable refractometer (Akso, São Leopoldo, RS, Brazil), with two drops of a homogenized suspension obtained by crushing the sample. An aliquot of this suspension was diluted in Erlenmeyer flasks containing 100 mL of distilled water and used to determine pH at 23 °C using a pH meter (PHOX Suprimentos Científicos, Colombo, PR, Brazil). Total titratable acidity (TTA) was measured through neutralization titration of 2 mL of grape juice with 0.1 M NaOH and 1 % phenolphthalein solution. TTA was expressed as grams of tartaric acid per liter. Five berries from each bunch were mixed and utilized for the analysis of physicochemical parameters. The experiment was performed with three independent replicates, using a completely randomized design.

2.10 Statistical analysis

Data normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was examined through the Levene's test. In the assays evaluating the antagonistic activity of *B. velezensis* S26 on leaf discs and grape berries, parametric data were analyzed using one-way ANOVA followed by the Tukey's test. On the other hand, non-parametric data were compared using the Kruskal-Wallis test, and differences were determined using the Dunn-Bonferroni test. These statistical analyses were performed separately for each pathogen isolate.

For the experiment on bacterial antagonism on micropropagated grapevines, bacterial colonization of grape berries, and bacterial impact on fruit quality, parametric data were analyzed using the t-test, while non-parametric data were evaluated using the Mann-Whitney test. The significance threshold was set at $P < 0.05$. All statistical analyses were performed using SPSS version 22.0 software for Windows (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Antagonistic activity of *B. velezensis* S26 endospores on vine leaf discs

Grapevine leaf discs demonstrated lower susceptibility to *Botrytis* spp. infection compared to grape berries. In Moscato Giallo (*V. vinifera*) leaf discs, disease incidence exceeded 40 % (Fig. 1). Among the pathogenic isolates, *Botrytis* sp. CNPUV80 caused the highest disease incidence at 95.2 %, while the isolates A3717 and CNPUV53 induced the lowest incidence, each at 42.9 %.

The application of both fresh and stored inoculants led to reduced disease incidence in *Botrytis* spp. isolates 001/11 and CNPUV80, respectively. Moreover, the isolate CNPUV62 was effectively controlled by both bacterial inoculants. Disease severity, determined by lesion diameter measurement, decreased in *Botrytis* spp. isolates CNPUV62 and CNPUV80 when treated with either fresh or stored suspensions. The fresh inoculant also decreased gray mold severity caused by isolate CNPUV53. However, despite the fresh suspension reduced the severity of *Botrytis* sp. isolate CNPUV61, no statistical difference was observed compared to the control group infected with the pathogens.

Similarly, in leaf discs of cv. Niagara Branca (*V. labrusca*), disease incidence ranged from 47.6 % (CNPUV53) to 95.2 % (CNPUV62) (Fig. 1). While the bacterial inoculant did not lead to a reduction in gray mold incidence, the fresh inoculant effectively decreased the lesion diameter in *Botrytis* spp. isolates A3717, CNPUV61, and CNPUV80 compared to the control. In contrast, the stored inoculant did not reduce gray mold severity in vine leaves.

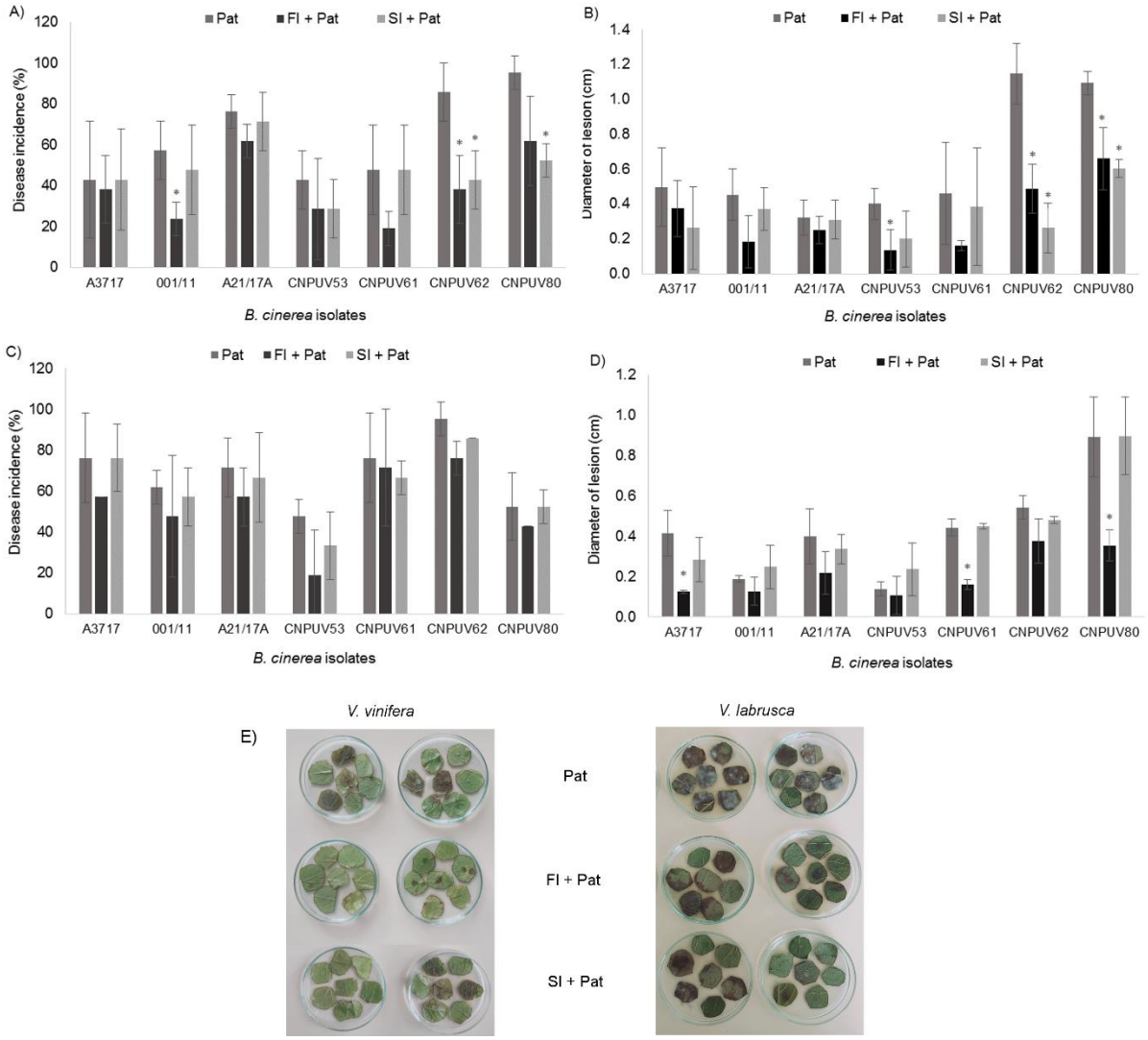

Fig. 1. Grapevine leaf discs inoculated with *Botrytis* spp. isolates (Pat), fresh inoculant + *Botrytis* spp. (FI + Pat), and stored inoculant + *Botrytis* spp. (SI + Pat) in *Vitis vinifera*: A) Disease incidence, B) Diameter of lesion; and in *V. labrusca:* C) Disease incidence, D) Diameter of lesion. E) Photographs showing the experiment with *Botrytis* sp. CNPUV53. Means were compared using ANOVA followed by Tukey's test or Kruskal-Wallis followed by Dunn-Bonferroni test for each isolate. Asterisks indicate a significant difference ($P < 0.05$) and the error bars represent the standard deviation of three replicates.

3.2 Antagonistic activity of *B. velezensis* S26 endospores on micropropagated grapevines

The selection of the *Botrytis* sp. isolate CNPUV62 for the *in vivo* experiment was based on its high pathogenicity in the leaf disc assay. As a result, inoculating the pathogen in *V. vinifera* plants resulted in a gray mold incidence of 84.3 %. However, treating the plants with the fresh inoculant before pathogen inoculation led to a 79.0 % decrease in disease incidence

(DI) (Table 1). Conversely, the bacterial application resulted in a 22.2 % reduction in the disease severity index (DSI), with values ranging from 61.5 % to 79.0 % in vines subjected to Bac + Pat and Pat treatments, respectively.

Similarly, *V. labrusca* plants exhibited comparable susceptibility to *Botrytis* spp. as *V. vinifera*. However, treatment with *B. velezensis* S26 did not reduce DS and DSI when compared to the control group solely inoculated with *Botrytis* sp. CNPUV62 (Table 1). The presence of the disease was confirmed through the re-isolation of the pathogen from the lesions in all treatments.

Table 1. Disease incidence (DI) and disease severity index (DSI) in grapevine plants (*Vitis vinifera* and *V. labrusca*) treated with *Botrytis* sp. CNPUV62 (Pat) or fresh inoculant and *Botrytis* sp. CNPUV62 (FI + Pat). Each value represents the mean ± standard deviation of three replicates.

	V. vinifera		*V. labrusca*	
	DI (%)	DSI (%)	DI (%)	DSI (%)
Pat	84.3 ± 5.7 a	79.0 ± 6.2 a	76.3 ± 6.7 a	76.5 ± 8.0 a
FI + Pat	79.0 ± 6.2 a	61.5 ± 3.1 b	73.0 ± 8.0 a	67.5 ± 4.8 a

Equal lowercase letters indicate no statistically significant difference between the treatments (Pat and FI + Pat) according to the t-test ($P < 0.05$). Additionally, there was no statistically significant difference between the grapevine species according to the t-test ($P < 0.05$).

3.3 Antagonistic activity of *B. velezensis* S26 endospores on grape berries

Preventive treatment of wounded grape berries with *B. velezensis* S26 led to a decrease in both the incidence and severity of gray mold (Fig. 2). In *V. vinifera* berries, the DI exceeded 80 % across six isolates (A3717, 001/11, CNPUV53, CNPUV61, CNPUV62, and CNPUV80). The application of the stored inoculant was found to reduce gray mold incidence in berries infected with *Botrytis* spp. isolates A021/17A, CNPUV53, and CNPUV62. Similarly, the fresh inoculant proved effective in decreasing disease incidence in A021/17A and CNPUV62 isolates. Moreover, gray mold severity was diminished with the application of both the fresh (isolates 001/11, A021/17A, and CNPUV62) and stored inoculants (isolates A021/17A, CNPUV53, and CNPUV62).

Among the *Botrytis* spp. isolates, five strains infected over 90 % of *V. labrusca* plants, while isolates 001/11 and A021/17A exhibited lower infection percentages. In addition, both

bacterial inoculants significantly suppressed *Botrytis* sp. isolate CNPUV80. This experiment clearly demonstrated the efficacy of *B. velezensis* S26 endospores, whether applied as fresh or stored inoculants, in controlling the severity of gray mold. The fresh endospore suspension reduced the DSI in all *Botrytis* spp. isolates. Likewise, the suspension stored for six months also diminished gray mold severity in six pathogen isolates (A3717, 001/11, A021/17A, CNPUV53, CNPUV61, and CNPUV80).

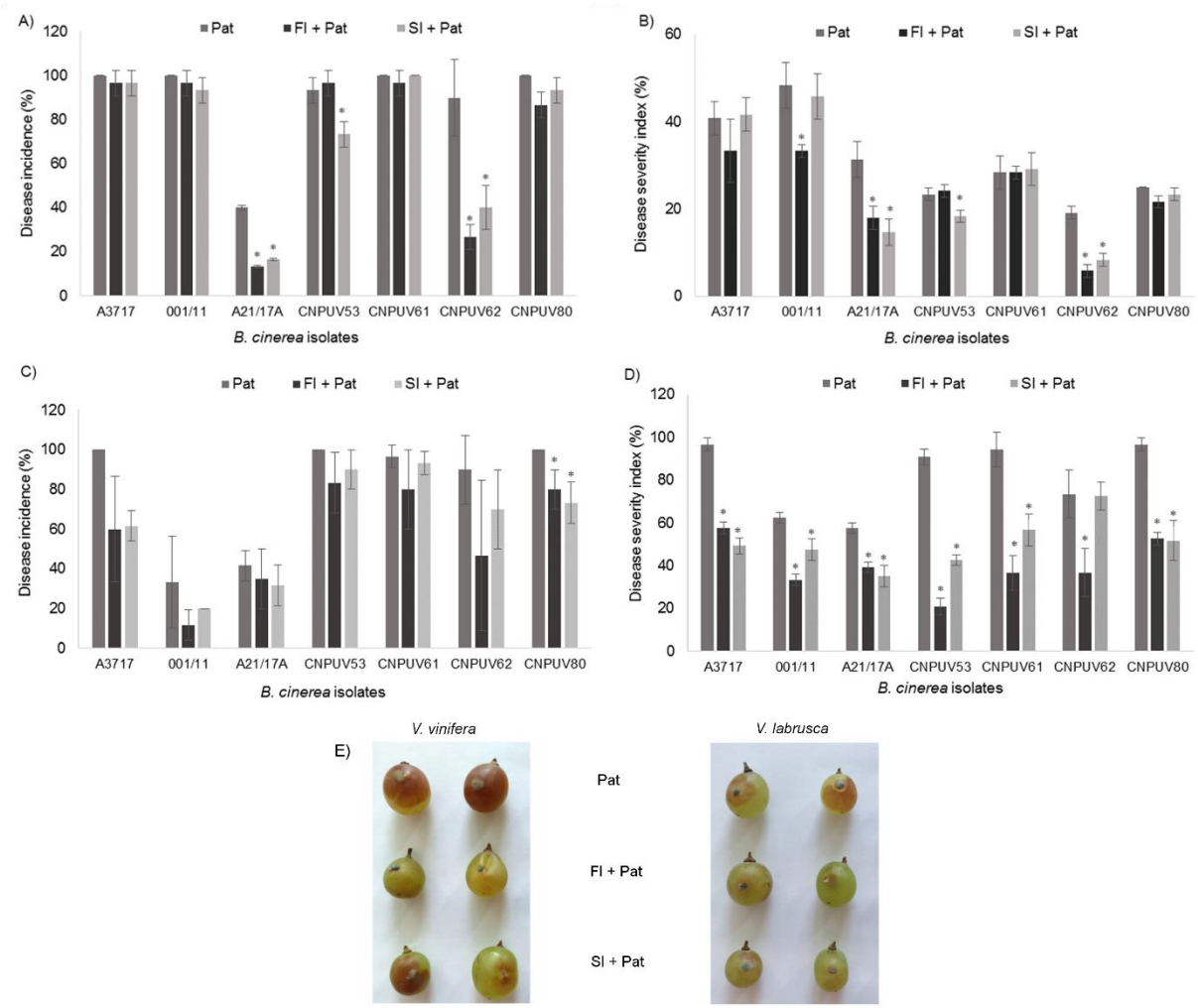

Fig. 2. Grape berries inoculated with *Botrytis* spp. isolates (Pat), fresh inoculant + *Botrytis* spp. (FI + Pat), and stored inoculant + *Botrytis* spp. (SI + Pat) in *Vitis vinifera*: A) Disease incidence, B) Disease severity index; and in *V. labrusca:* C) Disease incidence, D) Disease severity index. E) Photograph showing the experiment with *Botrytis* sp. CNPUV62. Means were compared using ANOVA followed by Tukey's test or Kruskal-Wallis followed by Dunn-Bonferroni test for each isolate. Asterisks indicate a significant difference ($P < 0.05$). The error bars indicate the standard deviation of three replicates.

3.4 Bacterial colonization of wounded grape berries

The progression of colonization by *B. velezensis* S26 was evaluated in wounded grape berries (Fig. 3). In the Bac treatment, the bacterial concentration rose from $\log_{10}$ 6.11 to $\log_{10}$ 6.66 CFU wound^{-1}, while in the Bac + Pat treatment, it decreased from $\log_{10}$ 6.02 to $\log_{10}$ 5.86 CFU wound^{-1}. Despite the decline in *B. velezensis* S26 concentration in the Bac + Pat treatment, it peaked at $\log_{10}$ 6.74 CFU wound^{-1} at 24 h, followed by a sharp decrease. Throughout the experiment, there was a statistically significant difference in bacterial concentration between the treatments.

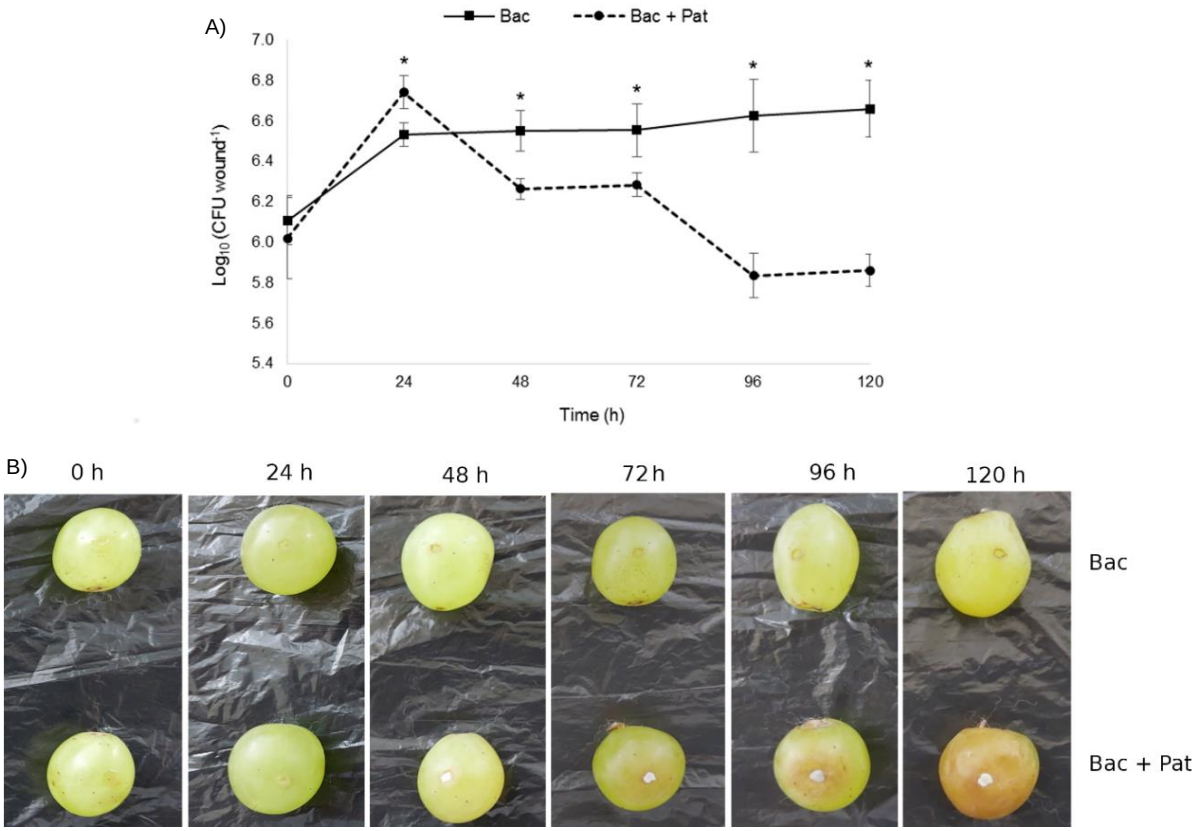

Fig. 3. Concentration of *Bacillus velezensis* S26 (Bac), and *B. velezensis* S26 + *Botrytis* sp. CNPUV62 (Bac + Pat) in wounded grape berries cv. Niagara branca (A). Photographs showing the colonization progress in the Bac and Bac + Pat treatments (B). Means were compared using t-test (parametric data) or Mann-Whitney test (non-parametric data). Asterisks indicate a significant difference ($P < 0.05$). The error bars indicate the standard deviation of three replicates.

3.5 Impact of bacterial treatment on grape quality

The analysis of physicochemical parameters in grape berries revealed that the inoculation of *B. velezensis* S26 cells had no impact on grape quality compared to the untreated fruit over the 21-day storage at 4 ºC (Supplementary Material 4). Although a slight reduction in total soluble solids (TSS), total titratable acidity (TTA), and fresh mass (FM) was observed in all evaluated grapes, pH exhibited a contrasting trend, ranging from 2.61 to 2.96 in the control treatment and from 2.69 to 2.97 in grapes inoculated with the *B. velezensis* S26 suspension. Additionally, TSS decreased by 7.9 % and 8.1 %, TTA diminished from 43.1 % to 39.7%, and FM varied from 5.4 % to 4.0 % in the untreated and treated grapes, respectively. Furthermore, there was a 7.9 % and 8.1 % decrease in total soluble solids (TSS), a reduction in total titratable acidity (TTA) from 43.1 % to 39.7 %, and a variation in fresh mass (FM) from 5.4 % to 4.0 % in the untreated and treated grapes, respectively. However, the statistical analysis indicated no significant difference between the bacterized and non-bacterized grapes.

4 Discussion

The current study emphasizes the growing interest in antagonistic microorganisms for their efficacy in suppressing various plant pathogens. We specifically investigated the inhibitory potential of *Bacillus velezensis* S26 against diverse *Botrytis* spp. isolates, focusing on *Vitis vinifera* and *V. labrusca* species, both *in vitro* and *in vivo* assays. Grapevine species from East Asia and America, including *V. labrusca*, have demonstrated moderate to high resistance against several pathogenic agents due to their co-evolution. In contrast, European species, such as *V. vinifera*, are known for their higher susceptibility to fungal diseases (Naegele, 2018; Rukavtsova et al., 2022).

Our leaf disc experiments revealed similar susceptibility of the vine species to seven *Botrytis* spp. isolates. However, the severity of gray mold, measured by the lesion diameter, was more pronounced in *V. vinifera.* The resistance of both *V. vinifera* and *V. labrusca* species also varied depending on the specific pathogen isolate. Naegele (2018) noted that moderately resistant grapevines exhibited isolate-specific resistance to *B. cinerea*. In agreement with our findings, in a study by Wan et al. (2021), *B. cinerea* exhibited lower germination and infection rates in detached leaves of the resistant variety *V. amurensis* compared to *V. vinifera* cv. Red

Globe. After ninety-six hours of inoculation, not only the gray mold severity but also the incidence was lower in *V. amurensis* leaves.

In terms of the antagonism of *B. velezensis* S26 on leaf discs, the application of bacterial inoculants resulted in a slight reduction in gray mold incidence in *V. labrusca* and a significant decrease in lesion size in both grapevine species. In contrast to our observations, Maachia et al. (2015) reported successful control of *B. cinerea* using *Bacillus* spp. strains B27 and B29 on detached leaves (*V. vinifera* L. cv. Cot Noir). However, the higher severity of gray mold observed in our study may be attributed to the inoculation of the pathogen on leaf discs rather than detached leaves, where the absence of cut-induced lesions hindered pathogen infection. Similarly, Haidar et al. (2016) examined the effect of 46 bacterial strains on leaves of *V. vinifera* cv. Cabernet Sauvignon against *B. cinerea transposa* 213T and *vacuma* 357. Their experiments revealed that while some bacteria inhibited gray mold by approximately 90 %, others increased the lesion size compared to the control.

Moreover, our experiments demonstrated that applying *B. velezensis* S26 endospores as a fresh inoculant resulted in a more effective control of gray mold compared to the stored inoculant, possibly due to the decline in cell viability during storage. Nevertheless, in certain instances, the stored suspension exhibited greater antagonistic ability than the fresh formulation, suggesting that LB broth acted as a resistance inducer in plants or a protective agent (Yaguchi et al., 2017). In line with our observations, Sahai et al. (2019) proposed that protective compounds can be incorporated into bacterial suspensions to enhance cell viability and preserve bacterial metabolites. Wang et al. (2024) also noted that secondary metabolites released by *Bacillus velezensis* wr8 during storage preserved their antimicrobial properties in citrus fruit infected with *Penicillium* sp. Additionally, Gotor-Vila et al. (2017) suggested that the culture broth provides essential nutrients for bacterial survival during storage. Therefore, we hypothesize that LB broth may have preserved bacterial metabolites or even triggered their production upon inoculation onto grapevine leaves.

Following this, the inhibitory potential of *B. velezensis* S26 was evaluated in grapevine plants obtained through micropropagation. The bacterial endospores mitigated disease severity in plants of *V. vinifera* cv. Moscato giallo, while no statistically significant effect was observed in *V. labrusca*. In a similar vein, Amarouchi et al. (2021) reported low disease severity in vine plants of cv. Chardonnay clone 7535 (*V. vinifera*) and highlighted the antagonistic capacity of *B. velezensis* S3 against gray mold. Esmaeel et al. (2019) also achieved effective control of *B. cinerea* in grapevine plantlets (*V. vinifera* cv. Chardonnay) through inoculation with *Burkholderia* strains BE10, BE15, and BE22.

In the biocontrol assay involving grape berries, we observed a similar incidence of gray mold between the grapevine varieties, although the disease severity was more pronounced in *V. labrusca*. This susceptibility can be attributed to the genetic background of Niagara branca, which is an interspecific hybrid of Cassady (*V. labrusca*) and Concord, believed to be a cross between a wild *V. labrusca* vine and an unidentified *V. vinifera* variety. Our findings also demonstrated that *B. velezensis* S26 exhibited higher inhibitory ability in grape berries compared to vine leaves. Similarly, Haidar et al. (2016) noted varying levels of biocontrol efficacy depending on the infected grapevine tissue. The authors emphasized that bacterial antagonism is influenced by the characteristics of the pathogen and environmental factors. Furthermore, injuries facilitated the penetration of the pathogen into the berries of Niagara branca, thereby compromising anatomical barriers associated with resistance to gray mold, such as low number of natural openings, increased thickness, a high number of cell layers, and elevated wax concentration, as described by Gabler et al. (2003).

The application of *B. velezensis* S26 suspension led to a reduction in gray mold incidence in both grapevine species. Additionally, there was a significant decrease in lesion diameter in numerous *Botrytis* spp. isolates compared to the control. Nifakos et al. (2021) also reported a significant inhibitory capacity of *B. velezensis* Bvel1 against *B. cinerea* in grape berries, using a low-concentration bacterial suspension. Likewise, *B. velezensis* GSBZ09 effectively controlled *Coniella vitis*, the causal agent of grape white rot, in *V. vinifera* cv. Red Globe. As a result, the cell-free supernatant was found to reduce both incidence and disease severity index in grape berries (Yin et al., 2022).

Subsequently, the bacterial colonization assay demonstrated that the concentration of *B. velezensis* S26 was higher in the absence of the pathogen. However, it increased 24 hours after pathogen inoculation, followed by a gradual slowdown as the necrotic area expanded. In contrast to our findings, Chen et al. (2020) reported that the concentration of *Lactobacillus plantarum* CM-3 in strawberries exhibited a slow increase, regardless of the presence or absence of *B. cinerea*, and reached its highest population after 120 hours of pathogen inoculation. *B. velezensis* Bvel1 also exhibited restrained growth when applied to wounded grape berries, whether in the presence or absence of *B. cinerea* (Nifakos et al., 2021). According to these authors, the bacterial populations displayed a similar growth pattern, achieving their maximum concentration after 96 hours. In another study, Chen et al. (2019) compared the colonization patterns of four *Bacillus* spp. strains in tomatoes, strawberries, and grapes. They concluded that colonization is influenced by both the plant species and the bacterial strains. In

grape berries, the population of two bacterial strains increased, while one remained stable, and the other decreased over the course of the experiment.

The impact of postharvest application of *B. velezensis* S26 was assessed on Niagara Branca grapes. Although no improvement in grape quality was observed, the bacterization did not adversely affect physicochemical parameters. Our findings indicated a decline in fresh mass throughout the experiment, which could be attributed to water loss through transpiration and respiration processes (Gregory et al., 2006). Moreover, there was an increase in pH, coupled with a reduction in total titratable acidity. Tumbarski et al. (2019) also observed a comparable pattern in strawberries treated with carboxymethylcellulose (CMC) and CMC embedded with bacteriocin. They proposed that these physiological changes may result from carbohydrate degradation and fruit deterioration during storage.

In contrast to our results, Zhang et al. (2019) reported that inoculating the *Bacillus subtilis* supernatant onto table grapes (*Vitis vinifera* cv. Kyoho) preserved high concentrations of organic acids compared to the control, even though total titratable acidity exhibited a decrease trend during storage. Since total titratable acidity primarily comprises tartaric acid in grapevines, controlling this parameter helps to prevent fruit decay and flavor loss (Jia et al., 2016; Zhang et al., 2019). Consequently, establishing a protective microbial community on the grape surface generates a hostile environment for decay-causing organisms. This can be particularly beneficial when bacterial agents are incorporated into edible coatings, contributing to the prevention of spoilage, extending shelf life, and simultaneously maintaining fruit quality (Marín et al., 2019).

Conclusion

The application of a suspension of *B. velezensis* S26 endospores led to a reduction in the incidence and severity of gray mold on leaf discs, grape berries, and micropropagated plants, demonstrating an isolate- and tissue-specific antagonism. Both freshly prepared and stored inoculants containing *B. velezensis* S26 endospores effectively suppressed specific isolates of *Botrytis* spp. and diminished lesion size in various tissues. Furthermore, the colonization of grape berries by the bioagent was influenced by the presence of the pathogen, with no detrimental effects on fruit quality during the postharvest phase.

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Supplementary Material 1

Table S1. Concentration of *Bacillus velezensis* S26 cells and endospores in the inoculants.

	Bacterial cells (CFU mL^{-1})	Bacterial endospores (spores mL^{-1})
Fresh inoculant (FI)	5.3×10^8	2.1×10^8
Stored inoculant (SI)	4.4×10^7	7.9×10^6

Supplementary Material 2

Table S2. Pathogen isolates used in the experiments.

Isolates	Species	Isolated from	Origin (city/country)	Institution
001/11	*Botrytis* sp.	Grapevine cv. Isabella	Caxias do Sul, Brazil	University of Caxias do Sul
A3717	*Botrytis* sp.	Strawberry cv. San Andreas	Caxias do Sul, Brazil	University of Caxias do Sul
A021/17A	*Botrytis* sp.	Grapevine cv. Isabella	Bento Gonçalves, Brazil	University of Caxias do Sul
CNPUV53	*Botrytis* sp.	Grapevine cv. Glera	Garibaldi, Brazil	Embrapa Grape and Wine
CNPUV61*	*Botrytis* sp.	Grapevine cv. Niagara branca Plant 1	Caxias do Sul, Brazil	Embrapa Grape and Wine
CNPUV62*	*Botrytis* sp.	Grapevine cv. Niagara branca Plant 2	Caxias do Sul, Brazil	Embrapa Grape and Wine
CNPUV80	*Botrytis* sp.	Grapevine cv. Cabernet Sauvignon	Bento Gonçalves, Brazil	Embrapa Grape and Wine

*The *Botrytis* spp. isolates CNPUV61 and CNPUV62 were obtained from grapevine plants in different vineyards belonging to the same winegrower.

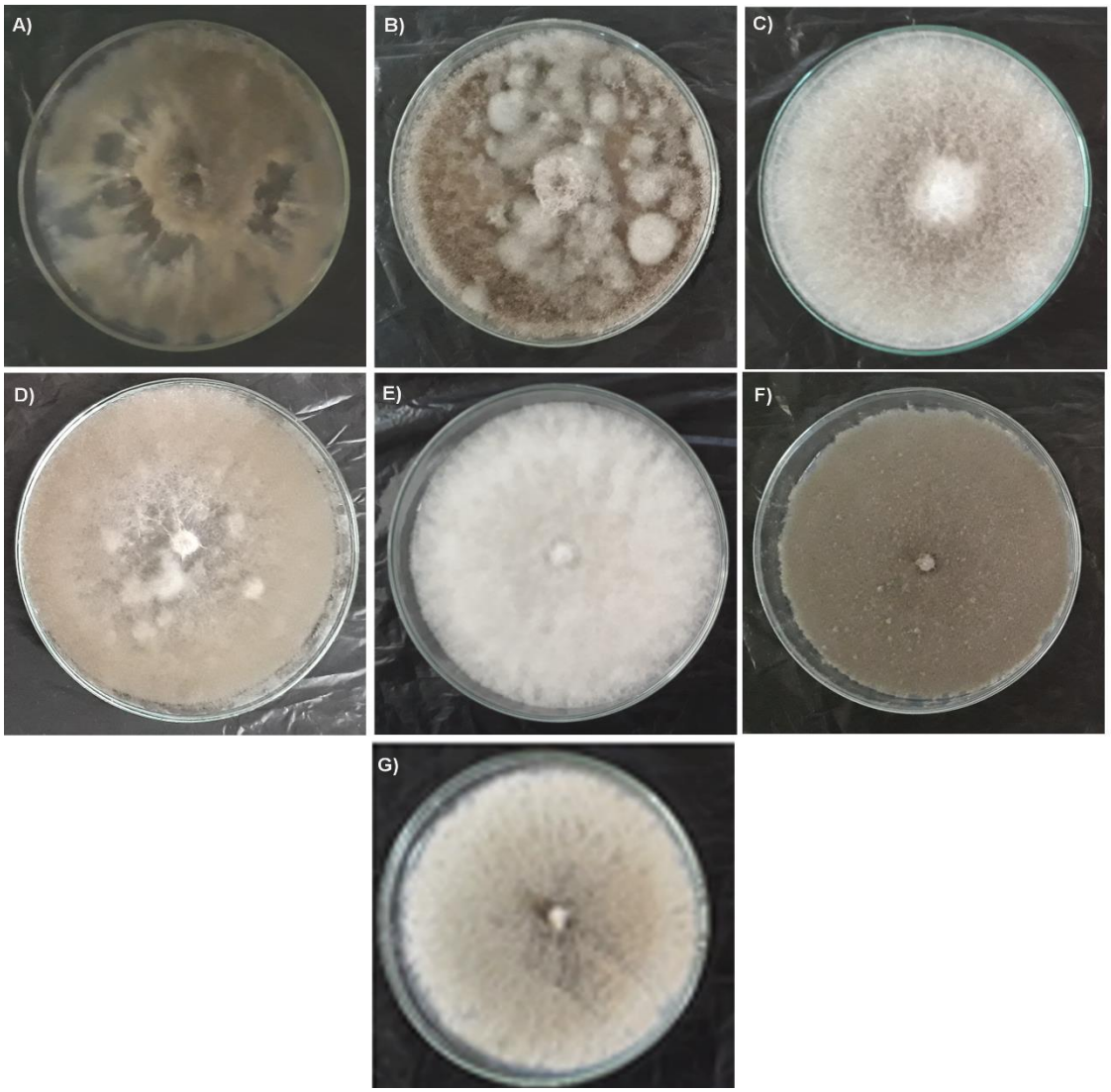

Fig. S2. Morphological aspect of fungal colonies cultivated on Potato Dextrose Agar (PDA) medium in a growth chamber at 25 °C with 12 h of light for 14 days. A) A3717, B) 001/11, C) A021/17A, D) CNPUV53, E) CNPUV61, F) CNPUV62, and G) CNPUV80.

Supplementary Material 3

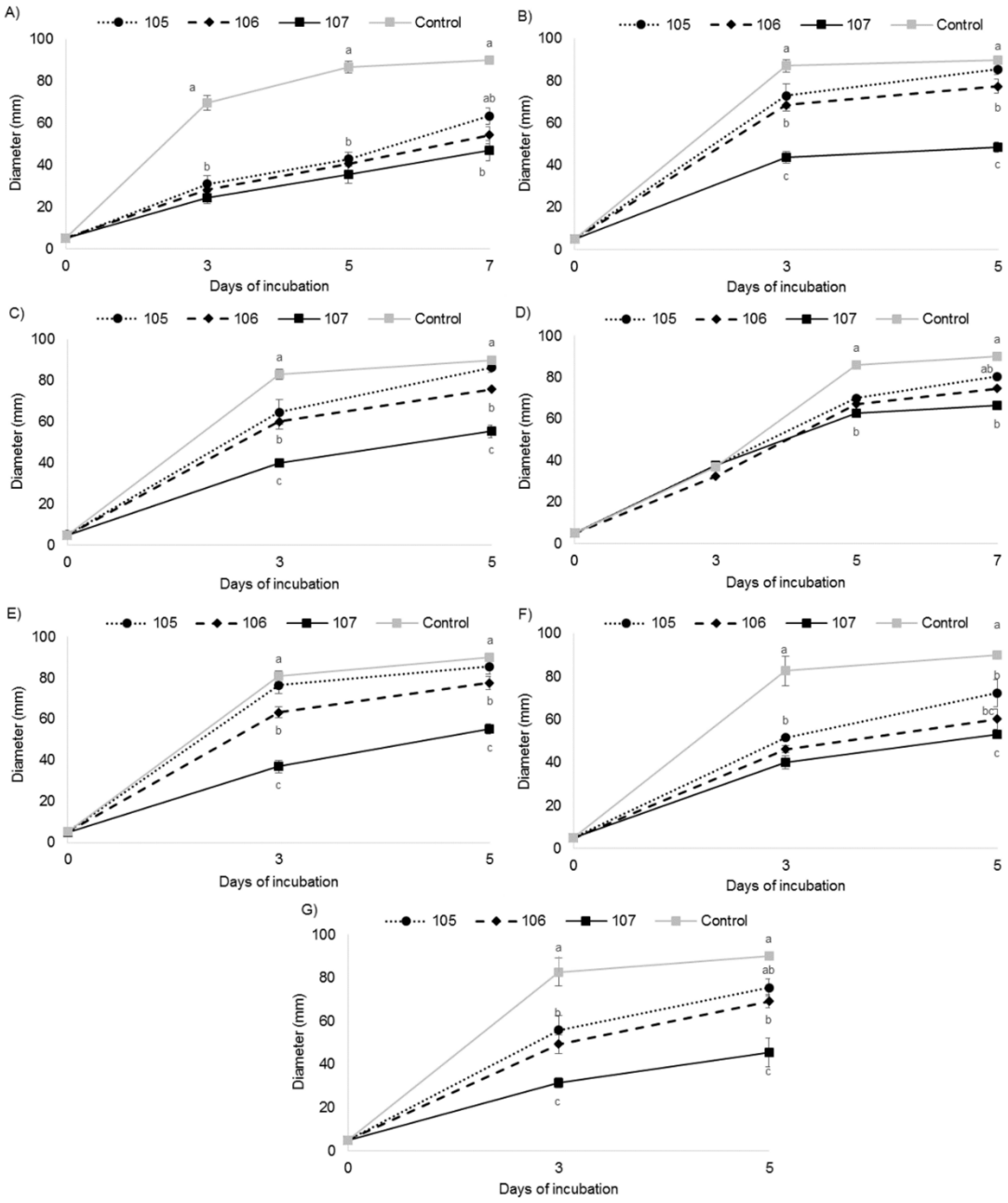

Fig. S3. Radial growth of seven isolates of *Botrytis* spp.: A) A3717, B) 001/11, C) A021/17A, D) CNPUV53, E) CNPUV61, F) CNPUV62, and G) CNPUV80. A dual-culture assay was conducted using a fungal colony disc and three concentrations of *Bacillus velezensis* S26 endospores: 10^5, 10^6, and 10^7 spores mL^{-1}. The control was inoculated with the pathogen and saline solution. Fungal growth was assessed continued until the control group reached plates' border (90 mm in diameter). Each value represents the mean of seven replicates, with error bars denoting the standard deviations. Means were compared using ANOVA followed by the Tukey's test ($P < 0.05$)

Supplementary Material 4

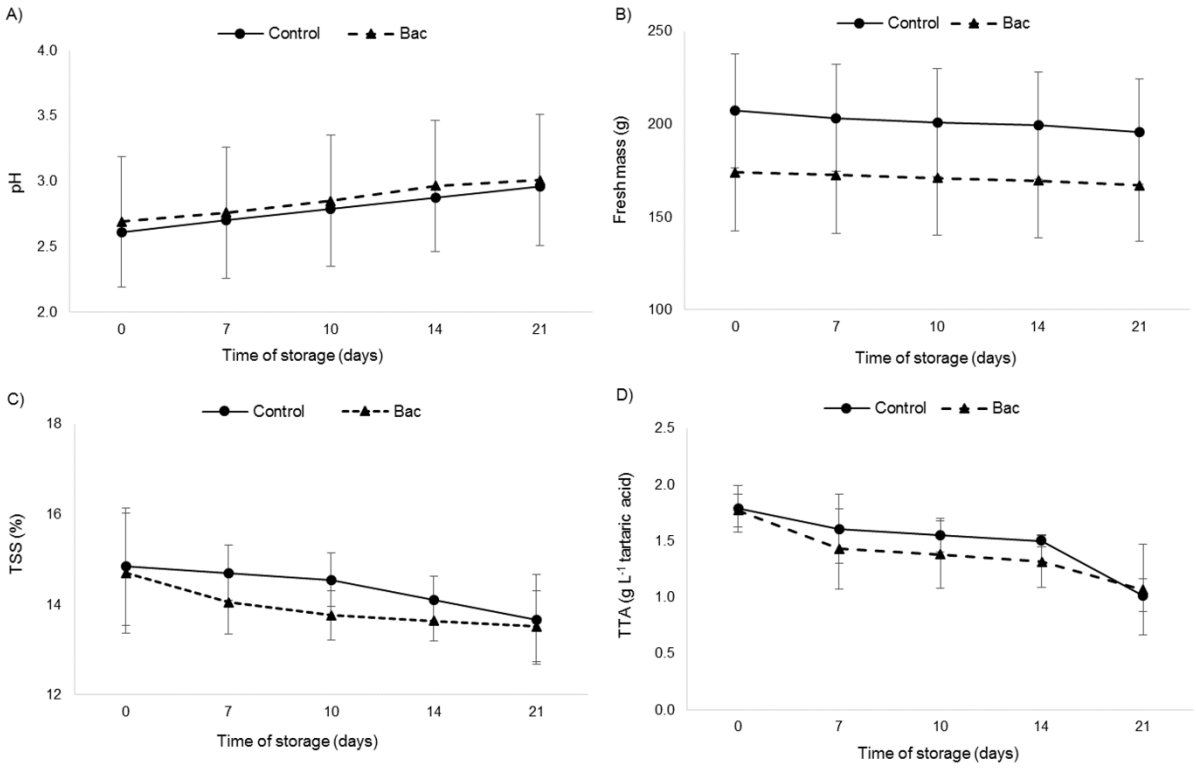

Fig. S4. Physicochemical parameters in Niagara branca stored at 4 ºC for 21 days. A) pH, B) Fresh mass, C) Total soluble solids (TSS), and D) Total titratable acidity (TTA). The error bar represents standard deviation of three replicates. Means were compared using t-test (parametric data) or Mann-Whitney test (non-parametric data). Asterisks indicate a significant difference ($P < 0.05$).

4.5 Innovative formulations enriched with *Bacillus velezensis* S26 endospores using whey and composting leachate to enhance plant growth and mitigate strawberry diseases

Abstract

Bacillus velezensis is a beneficial bacterium renowned for enhancing plant growth, inhibiting pathogenic agents, and triggering plant defense mechanisms. Notably, these endospore-forming bacteria can be employed in developing stable formulations, ensuring cell viability over extended storage periods. This study aims to harness the potential of *B. velezensis* S26, creating a cost-effective formulation with endospores using biological wastes and focusing on evaluating its efficacy in disease management and fostering growth in strawberry plants. The viability of bacterial endospores during six months of storage was assessed in four bioformulations containing saline solution, whey, composting leachate, and a combination of whey with leachate. The potency of *B. velezensis* S26 in suppressing anthracnose and gray mold and improving plant development was examined in a pot experiment and a commercial production greenhouse. Our findings revealed that the bioformulation BF4, containing whey, exhibited the highest endospore concentration after storage at room temperature. Regarding biocontrol, the bioformulation BF1 effectively reduced the incidence of anthracnose by 33 % and gray mold by 18.6 % in strawberry plants. Moreover, all formulations demonstrated effectiveness similar to Duravel® against strawberry diseases. Furthermore, treating strawberry plants with an endospore-rich bioformulation (BF4) led to increased vegetative measurements in a pot experiment. Therefore, valorizing whey as a carrier for *B. velezensis* S26 endospores showcases the potential to preserve cell viability during prolonged storage periods, increase plant growth, and suppress anthracnose and gray mold in strawberry plants.

Keywords: anthracnose, bioformulation, *Fragaria* x *ananassa*, gray mold, sustainable agriculture, waste valorization

1 Introduction

The escalating global population and increased consumption trends have propelled waste management to the forefront of contemporary environmental concerns (Roy et al., 2018). This surge in human activity has led to a substantial increase in the production of various forms of waste, ranging from household refuse to industrial byproducts. For instance, in wine production, approximately 25 % of a grape's mass ends up as residual products, resulting in five million tons of grape marc produced globally each year, leading to significant environmental impacts and challenges regarding its final disposal (Martinez et al., 2016; Muhlack et al., 2018; Da Silva et al., 2022). Similarly, dairy industries contribute significantly to waste generation, with residual whey alone accounting for about one-fourth of the total volume of milk and its derivatives produced (Sebastián-Nicolás et al., 2020).

Improper waste disposal poses a serious concern due to the characteristics of byproducts, which include low pH, high electrical conductivity, elevated biochemical oxygen

demand (BOD), and chemical oxygen demand (COD). As a result, this can lead to various detrimental effects such as reduced soil fertility, water eutrophication, depletion of dissolved oxygen, and greenhouse gas release (Ahmad et al., 2020; Da Silva et al., 2022). On the other hand, agroindustrial byproducts offer a cost-effective source of nutrients for cultivating beneficial microorganisms (Yánez-Mendizábal et al., 2012; Sawatphanit et al., 2022). Furthermore, biological wastes can serve as a culture medium and carriers in formulations, providing a suitable environment for the survival of microorganisms (Teixidó et al., 2022). Consequently, materials including whey and composting leachate, often deemed waste, can be repurposed and utilized to produce valuable biological products.

Bacillus velezensis is a Gram-positive, endospore-forming bacterium that has garnered attention for its versatile characteristics and beneficial applications, initially identified as *B. amyloliquefaciens* subsp. *plantarum* and *B. methylotrophicus*, its classification underwent reevaluation through molecular analyses (Rabbee et al., 2019). These bacteria possess remarkable abilities to suppress plant pathogens, induce systemic resistance, and enhance plant growth by solubilizing phosphate and potassium, fixing nitrogen, producing siderophores, and synthesizing plant growth regulators (Mshari et al., 2019; Morales-Cedeño et al., 2021).

Applying plant growth-promoting bacteria, such as *B. velezensis*, has the potential to alleviate the demand for fertilizers, which excessive use can lead to nutrient runoff and contamination of water bodies and impact soil quality and microbial diversity (Wang et al., 2019; Sutton et al., 2021). Additionally, *B. velezensis* is recognized as an eco-friendly strategy for managing plant diseases, minimizing the need for synthetic pesticides (Maciag et al., 2020). These fungicides have adverse effects on the environment, impacting non-target organisms, leading to the emergence of resistant strains, disrupting ecosystems and biodiversity, and causing harmful effects on human health (Wang et al., 2021).

Regarding the global importance of strawberry cultivation, *Bacillus velezensis* strains hold promise for managing diseases and improving plant growth. In light of this, several studies have demonstrated the inhibitory potential of *B. velezensis* strains against strawberry diseases caused by *Botrytis cinerea* (Toral et al., 2020; Bertê et al., 2022; Russi et al., 2024), as well as *Colletotrichum* spp. (Feng et al., 2020; Mei et al., 2021; Debastiani et al., 2023; Russi et al., 2024). However, the development of effective bioformulations employing these beneficial bacteria must meet specific requirements, including resistance to environmental conditions, low variability in biocontrol activity, low toxicity, cost-effectiveness, long shelf life, ease of handling, and scalable production (Gotor-Vila et al., 2017; Allouzi et al., 2022).

The aims of this research were as follows: a) to induce *Bacillus velezensis* S26 sporulation utilizing agroindustrial byproducts, such as whey and leachate from grape marc composting; b) to develop bioformulations using bacterial endospores and assess their viability over a six-month storage period; c) to evaluate the biocontrol effectiveness of the selected bioformulation against anthracnose and gray mold in strawberry plants in a pot experiment; d) to determine the biocontrol efficacy of the formulations against strawberry diseases in a commercial production greenhouse; and e) to test the capacity of the formulation in enhancing the growth of strawberry plants.

2 Materials and methods

2.1 Microorganisms

The rhizobacterium *Bacillus velezensis* S26 (OP938800) was sourced from the soil of an organic vineyard situated in Caxias do Sul, Rio Grande do Sul State, Brazil, with geographical coordinates at latitude 29°15'94" S and longitude 51°21'45" W, as reported by Debastiani et al. (2023).

Botrytis sp. A021/17A (MW646016) and *Colletotrichum fructicola* Ci020 (MN758913), which are causal agents of gray mold and anthracnose, respectively, were obtained from the Laboratory of Biological Plant Disease Control collection at the University of Caxias do Sul, Rio Grande do Sul State, Brazil.

2.2 Agroindustrial byproducts

The acid whey resulting from the production of ricotta cheese was provided by Santa Clara Ltda., a dairy company located in Carlos Barbosa, Rio Grande do Sul State, Brazil. The composting leachate, derived from the biofertilizer industry, was supplied by Beifiur Ltda., located in Garibaldi, Rio Grande do Sul State, Brazil. The physicochemicals of composting leachate are detailed in a study conducted by Ferrari et al. (2019). The wastes were collected and transported to the laboratory under refrigeration. These byproducts had their pH adjusted to 7.0 and were placed in 500 mL bottles. The bottles were autoclaved three times at 121 ºC for 45 min.

2.3 Bacterial growth and sporulation

The inoculum of *B. velezensis* S26 was prepared by transferring a loop of a colony-forming unit into 10 mL of Luria-Bertani (LB) broth at pH 7.0. The flask was placed on a rotary shaker set at 130 rpm and 28 °C for 24 h. After, the bacterial population was increased by inoculating this pre-inoculum into a 250 mL Erlenmeyer flask containing 90 mL of culture broth. Fourteen distinct culture broths were utilized, comprising composting leachate diluted in water (at the concentrations 10 %, 25 %, 50 %, 75 %, and 100 % cl/wa), composting leachate diluted in whey (10 %, 25 %, 50 %, and 75 % cl/wh), and whey diluted in water (10 %, 25 %, 50 %, 75 %, and 100 % wh/wa). The incubation was carried out under the same conditions mentioned earlier for an additional 24 h.

Bacterial sporulation was then induced in 100 mL of the four most promising media for bacterial growth (50-50 % cl/wa, 50-50 % cl/wh, 25-75 % cl/wa, and 25-75 % wh/wa) with the pH adjusted to 8.0. The inoculum was added to the sporulation medium at a ratio of 1:10 (v/v), and the cultivation was conducted at 130 rpm and 37 °C for 48 h.

2.4 Optimizing sporulation in shaking-flasks

The results from the previous assay were used to identify the two most promising culture broths (CB1 and CB2) for *B. velezensis* S26 growth (BG) and sporulation (BS). The CB1 broth for bacterial growth (CB1-BG) consisted of 10 % composting leachate and 90 % whey (10-90 % cl/wh). The CB1 broth for bacterial sporulation (CB1-BS) was formulated with 25 % composting leachate and 75 % water (25-75 % cl/wa), supplemented with Ca^{2+} (0.4 and 0.8 g L^{-1}), Mg^{2+} (0.3 and 0.6 g L^{-1}), and Fe^{3+} (0.5 and 1 g L^{-1}), applied as $CaCO_3$, $MgCl_2$, and Fe_2O_3, respectively.

The CB2-BG comprised 25 % whey and 75 % water (25-75 % wh/wa), while CB2-BS consisted of 75 % whey and 25 % water (75-25 % wh/wa), supplemented with sucrose (2, 5, 6 and 10 g L^{-1}), starch (2.5, 5, 10, and 15 g L^{-1}), glycerol (9, 20, and 40 g L^{-1}), and glucose (2.5, 5, 7.5, and 15 g L^{-1}).

2.5 Bacterial growth and sporulation in bioreactor

Batch cultivation was conducted in 7-L bench bioreactor BioFlo/Celligen 115 (New Brunswick, USA), which was equipped to control fermentation parameters. The pre-inoculum

was prepared by cultivating a colony-forming unit of *B. velezensis* S26 in four Erlenmeyer flasks, each containing 100 mL of LB broth at pH 7.0, using a rotary shaker at 130 rpm and 28 °C for 24 h. Further, bacterial growth and sporulation were performed in CB1 and CB2 culture broths. An optimized LB broth served as a control, following the cultivation conditions established by Russi et al. (2024).

After autoclaving 3.6 L of the culture broth (LB; CB1: 10-90 % cl/wh; and CB2: 25-75 % wh/wa) with pH adjusted to 7.0, the bioreactor was inoculated with 400 mL of pre-inoculum. Fermentation conditions were set at 28 ºC, 200 rev min^{-1}, and 0.2 vvm air feeding for 24 h. Subsequently, 2 L of the culture broth was removed, and 2 L of fresh autoclaved liquid medium for bacterial sporulation was added (LB: LB supplemented with 0.2 g L^{-1} of $CaCl_2$; CB1: 25-75% cl/wa supplemented with 0.8 g L^{-1} of $CaCO_3$ and 0.5 g L^{-1} of $MgCl_2$, and CB2: 75-25 % wh/wa supplemented with 6 g L^{-1} of sucrose). The pH was adjusted to 8.0 and controlled using 5 N NaOH or 5 N H_2SO_4 through computer-controlled peristaltic pumps. The cultures were maintained at 37 °C, 200 rev min^{-1}, and 0.2 vvm airflow for 48 h. The fermentation experiment was conducted twice.

Following this, samples of each culture (LB, CB1, and CB2) were harvested at 24 h and 48 h and subjected to heat inactivation at 80 °C for 10 min to eliminate vegetative cells. Subsequently, these samples underwent centrifugation at 10,000 rpm for 15 min, and the resulting pellet was washed three times before being resuspended in 0.85 % NaCl solution. The concentration of endospores was adjusted to 1.0×10^{12} spores mL^{-1}.

2.6 Bioformulation storage and endospores viability

Four bioformulations were elaborated: BF1 (0.85 % NaCl solution), BF2 (25 % composting leachate + 75 % water), BF3 (50 % composting leachate + 50 % whey), and BF4 (50 % composting leachate + 50 % whey). These formulations were supplemented with 10 % (v/v) glycerol and 10 % (v/v) *B. velezensis* S26 endospores in saline solution (1×10^{12} spores mL^{-1}). The formulations were stored at 4 °C and 25 °C, and the viability of *B. velezensis* S26 endospores was recorded at intervals of 0, 30, 60, 90, 120, 150, and 180 days. For this purpose, a 1 mL sample of each bioformulation was heat-inactivated at 80 °C for 10 min. Subsequently, the endospore suspension was serially diluted and spread onto LB medium plates. Following incubation at 37 °C for 24 h, the concentration of endospores was determined and converted into $\log_{10}$ (spores mL^{-1}). The experiment was performed in triplicate.

2.7 Plant material

Explants were obtained from runners of one-year-old strawberry plants cv. San Andreas (*Fragaria* × *ananassa* Duch.), which were maintained in a greenhouse in Antônio Prado, Rio Grande do Sul State, Brazil. The micropropagation and acclimatization of plantlets was conducted following the procedures described by Russi et al. (2024).

2.8 Pot experiment: biocontrol

The inhibitory potential of the bioformulation BF1, containing *B. velezensis* S26 endospores in a saline solution, was evaluated against anthracnose (*C. fructicola* Ci020) and gray mold (*Botrytis* sp. A021/17A). Fungal conidia were harvested by flooding and rubbing the fungal mycelium of 14-day-old colonies with 0.85% NaCl solution. After filtration through a sterile cheesecloth, the conidia were counted using a hemacytometer, and the suspension was adjusted to a concentration of 1×10^6 conidia mL^{-1} with saline solution and a few drops of Tween 80.

Plants were foliar sprayed with bioformulation BF1 (2.4×10^{10} spores mL^{-1}) until runoff. After a two-day interval, a pathogen suspension (1×10^6 conidia mL^{-1}) was spray-inoculated onto the plants. The positive control was treated with pathogen suspension, while the negative control was sprayed with saline solution. The experiment was maintained in a moist chamber at 25 ± 2 °C, with a relative humidity of 90-95 %, and a 16-h photoperiod. After three days, the plants were transferred to a greenhouse and watered every two or three days, maintaining soil moisture at approximately 70 % of the maximum water-holding capacity.

Leaf number and plant height for each strawberry plant were recorded on day 0 for subsequent analyses. After 30 and 45 days, the following responses were assessed: increase in leaf number (ΔLeaf 0-30 and ΔLeaf 30-45), increase in plant height (ΔHeight 0-30 and ΔHeight 30-45), disease incidence (DI), and disease severity index (DSI). DI and DSI were determined as described by Russi et al. (2024). Disease severity on strawberry leaves was scored based on the rating scale developed by Marian et al. (2020), where 0 = no lesions, 1 = one leaf with symptoms, 2 = two to three leaves with symptoms, 3 = four to five leaves with symptoms, 4 = the entire plant with symptoms, and 5 = plant dead. The assay was carried out in a completely randomized design, with three replicates of 20 plants, and the experiment was repeated twice.

2.9 Commercial production experiment

The experiment was performed in a commercial greenhouse in Antônio Prado, Rio Grande do Sul State, Brazil (longitude 51°11'25.3'' W, latitude 28°54'25.6'' S, altitude 692 m). This greenhouse is covered with a 150 µm polyethylene film, accommodating 2,100 strawberry plants, cv. San Andreas spaced 15 cm apart in hanging flats. The plants were cultivated using a semi-hydroponic and organic system with drip irrigation. The local climate is categorized as a humid subtropical climate (Cfa) according to the Köppen classification. Meteorological data was obtained from the weather station located at Embrapa Grape and Wine, in Bento Gonçalves, Rio Grande do Sul State, Brazil (latitude 29°09'52.4'' S, longitude 51°32'02.8'' W, and altitude 691 m).

The trial was conducted from May 2023 to October 2023, involving six-month-old strawberry plants. The experiment employed a randomized block design with five treatments and three replicates, yielding 15 plots, each containing 20 strawberry plants. The plants were treated with the following formulations: Duravel WP® (*Bacillus amyloliquefaciens* MBI600), bioformulation BF1, bioformulation BF2, bioformulation BF3, and bioformulation BF4. All products were applied at a concentration of 9.1×10^8 spores mL^{-1}, as recommended by the manufacturer of Duravel WP® (BASF Inc., Florham Park, NJ). A negative control was not included to avoid financial losses for the farmer. Each treatment group received a weekly hand-spraying of 1.2 L of the bioformulation, resulting in 24 foliar applications during the experiment.

The incidence and severity of anthracnose and gray mold were assessed at 15-day intervals, following the procedures outlined by Russi et al. (2024). The assessment of disease severity utilized a diagrammatic scale developed by Pedrotti et al. (2021) (Supplementary Material 1). Moreover, each treatment assessed fruit yield by measuring the number and mass of pseudofruits. Pseudofruit area and volume were evaluated, considering strawberries with a conical shape, as suggested by Alijani et al. (2019).

2.10 Pot experiment: growth promotion

The effectiveness of bioformulation BF4 in enhancing plant growth was assessed in one-month-old strawberry plants obtained through micropropagation. At this stage, the number of leaves and height of each plant were recorded for subsequent assessments (day 0). The plants underwent the following treatments through soil drenching (SD) or foliar spraying (FS): control

(sterile water), bioformulation 4 without spores (B4ws), and bioformulation 4 (BF4). Bioformulation BF4, stored for six months at 25 °C, was applied with a final concentration of 5.0×10^7 spores mL^{-1} of *B. velezensis* S26. The treatments were inoculated weekly for one month. The strawberry plants were maintained in a greenhouse at 23 ± 2 °C for 45 days. Irrigation was carried out daily, maintaining the soil moisture at approximately 50 % of its maximum capacity.

The increase in leaf number (ΔLeaf 0-30 and ΔLeaf 30-45) and increase in plant height (ΔHeight 0-30 and ΔHeight 30-45) were evaluated at 30 and 45-day intervals. Additionally, the plants were removed from the substrate and rinsed with running tap water. Subsequently, root length, root dry mass, and shoot dry mass were measured. The dry mass assessments were conducted by drying the plant material at 60 °C until a constant mass was achieved. The experiment was completely randomized, with three replicates of 20 plants per treatment. The assay was repeated twice.

2.11 Data analysis

Shapiro-Wilk test was employed to assess the normality of the data set and Levene's test was used to evaluate the homogeneity of variances. For parametric data, means were compared using one-way ANOVA, followed by Dunnett's T3 test for the commercial production experiment and Tukey's test for the other assays ($P < 0.05$). Non-parametric data were assessed using the Kruskal-Wallis test, and means were compared using the Dunn-Bonferroni test ($P < 0.05$). All statistical analyses were conducted using SPSS version 22.0 for Windows.

3 Results

3.1 Bacterial sporulation

Culturing of *B. velezensis* S26 using raw composting leachate as a culture medium did not induce endospore production. In light of this, we explored using diluted composting leachate with whey as culture media for *B. velezensis* S26 growth and sporulation (Fig. 1).

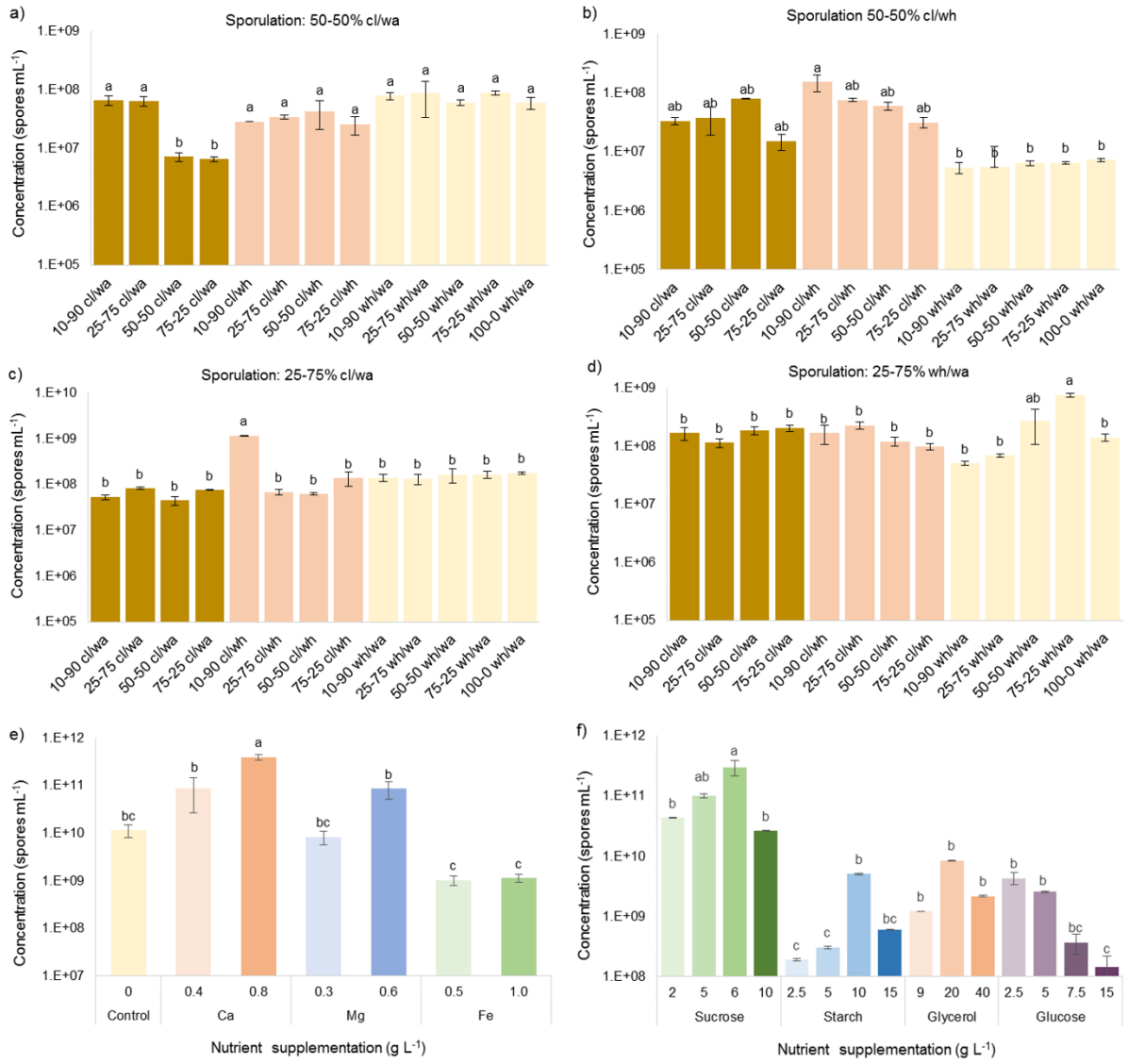

Fig. 1. Concentration of *Bacillus velezensis* S26 endospores after a 48-h cultivation in shaking flasks using the following media for bacterial sporulation: a) 50-50% cl/wa, b) 50-50% cl/wh, c) 25-75% cl/wa, and d) 25-75% wh/wa. Where: cl = composting leachate, wa = water, and wh = whey. Effect of nutrient supplementation in: e) CB1: 25-75% cl/wa, and f) CB2: 75-25% wh/wa. The error bars indicate the standard deviation of three replicates. The means were compared using ANOVA, followed by the Tukey's test for parametric data or Kruskal-Wallis followed by the Dunn-Bonferroni test for non-parametric data ($P < 0.05$).

The optimum conditions for sporulation were achieved using 10-90 % cl/wh for bacterial growth, coupled with 25-75 % cl/wa for bacterial sporulation, resulting in 1.1×10^9 spores mL^{-1} (Fig. 1c). This combination of culture broths for bacterial growth and sporulation was designated as CB1. Similarly, culture broth CB2, encompassing 75-25 % wh/wa for bacterial growth, along with 25-75 % wh/wa for bacterial sporulation, yielded 7.5×10^8 spores

mL^{-1} (Fig. 1d). Subsequently, the culture broths CB1 and CB2 were optimized through nutrient supplementation. In CB1, the addition of 0.8 g L^{-1} of Ca^{2+} led to an endospore concentration of 3.9×10^{11} spores mL^{-1}, while the amendment of 0.6 g L^{-1} of Mg^{2+} increased it to 8.4×10^{10} spores mL^{-1} (Fig. 1e). Moreover, the combination of Ca^{2+} and Mg^{2+} in CB1 culture broth resulted in 9.8×10^{11} spores mL^{-1}. On the other hand, adding 6 g L^{-1} sucrose to the CB2 culture broth led to 9.2×10^{12} spores mL^{-1}.

Additionally, we validated our findings by cultivating *B. velezensis* S26 in bioreactors using LB, CB1, and CB2 as culture broths and achieving comparable endospore concentrations to those observed in the shaking-flasks experiment (Fig. 2). LB yielded 1.9×10^{11} spores mL^{-1} (with a spore yield of 91.6 %), while CB1 culture broth achieved a concentration of 2.4×10^{11} spores mL^{-1} (with a spore yield of 93.5 %), respectively. Differently, the CB2 broth exhibited higher sporulation reaching a final concentration of 1.3×10^{12} spores mL^{-1}, and a spore yield of 90.1 % after 48 h of fermentation.

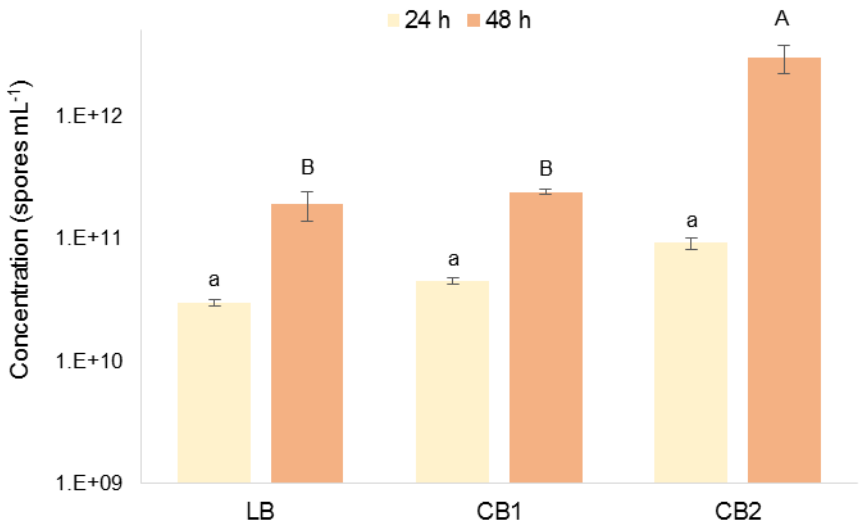

Fig 2. Concentration of *Bacillus velezensis* S26 endospores after 24 h and 48 h of cultivation in a 7 L bioreactor, using three culture broths: LB (Luria-Bertani), CB1 (composting leachate and whey), and CB2 (whey). The culture media were compared using ANOVA followed by Tukey's test ($P < 0.05$), separately for the time of cultivation (lowercase letters: 24 h and uppercase letters: 48 h). The error bars indicate the standard deviation of three replicates.

3.2 Cell viability during storage

Our findings demonstrated that liquid bioformulations containing *B. velezensis* S26 endospores effectively maintained cell viability during a six-month storage (Fig. 3 and Supplementary Material 3). The highest concentration of viable cells was observed in the bioformulation BF4, stored at room temperature (ranging from 9.7 $\log_{10}$ to 7.7 $\log_{10}$) (Fig. 3a). Conversely, storage at low temperatures had a similar effect on preserving cell viability in all evaluated formulations. Additionally, the bioformulation BF4 stored under refrigeration exhibited a higher concentration of germinated endospores after six months of storage (from 9.7 $\log_{10}$ to 7.3 $\log_{10}$) compared to the other formulations maintained at the same temperature (Fig. 3b).

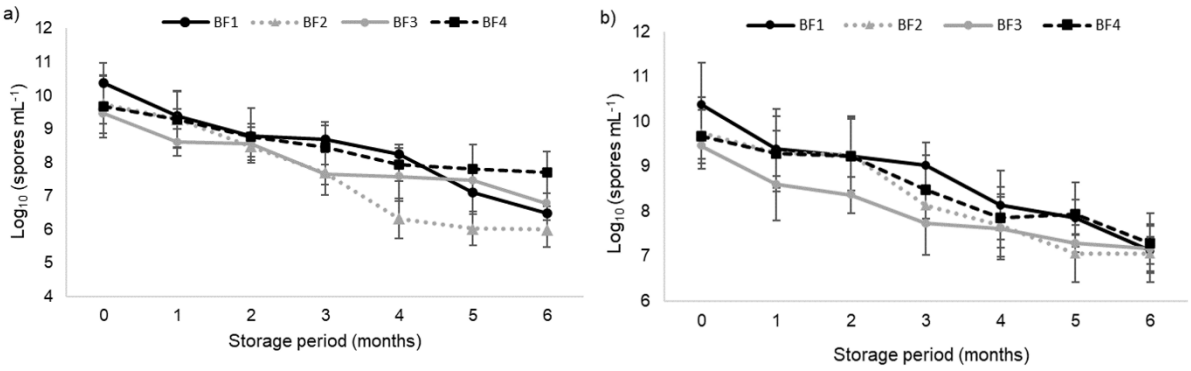

Fig 3. Viability of *Bacillus velezensis* S26 endospores incorporated into four bioformulations (BF1, BF2, BF3, and BF4) during a six-month storage at 25 °C (a) and 4 °C (b). The error bars indicate the standard deviation of three replicates.

3.3 Pot experiment: biocontrol

The bioformulation BF1 demonstrated efficacy in the control of anthracnose and gray mold in strawberry plants. Treatment with this formulation resulted in a 33% reduction in the occurrence and a 16.1% decrease in the severity of symptoms caused by *C. fructicola* Ci020 (Fig. 4a). Furthermore, bioformulation BF1 led to an 18.6% decline in the incidence of *Botrytis* sp. A021/17A compared to plants infected with the pathogen, while no statistically significant difference was observed in the severity of gray mold (Fig. 4b). Bioformulation BF1 also enhanced leaf number compared to strawberry plants inoculated only with the pathogens, with a more pronounced effect in the BF1 + Col treatment (Fig. 4c). Moreover, the BF1 + Bot

treatment contributed to an increase in the height of strawberry plants compared to the Bot treatment (Fig. 4d).

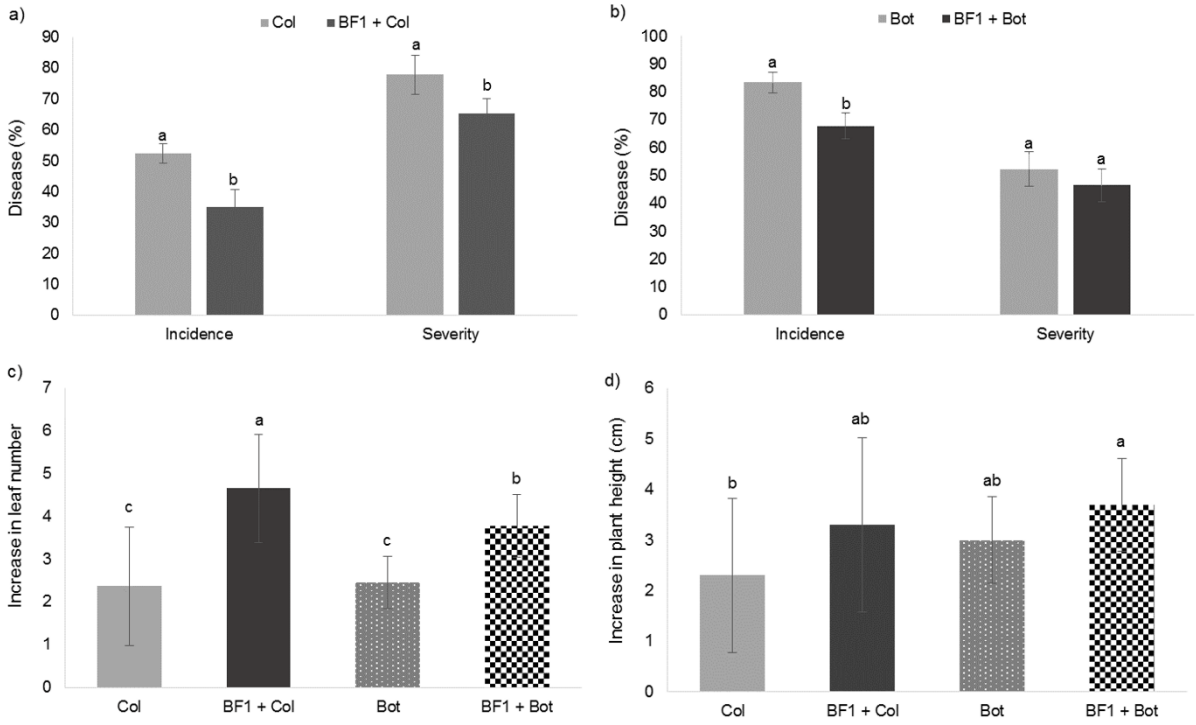

Fig. 4. Incidence and severity of anthracnose (a) and gray mold (b), and increase in leaf number (c) and plant height (d) in strawberry plants treated with *C. fructicola* Ci020 (Col), *Botrytis* sp. A021/17A (Bot), and bioformulation 1 in combination with the pathogens (BF1+ Col and BF1 + Bot). The data subjected to Kruskal-Wallis test followed by the Dunn-Bonferroni test ($P < 0.05$). The error bars indicate the standard deviation of three replicates.

3.4 Commercial production experiment

All bioformulations successfully controlled anthracnose and gray mold in strawberry plants. In addition, meteorological conditions influenced the occurrence of the strawberry diseases, even though cultivation was conducted in a covered greenhouse. Specifically, high relative humidity during the June-July period (varying between 79.8 and 81.7%) and September-October (ranging from 81.2 to 81.6%) favored the pathogenic infections (Fig. 5a).

We also observed that both the incidence and severity of anthracnose exhibited a sharp peak in June. However, there was a decline in the severity of the disease in July, followed by an increasing trend in the subsequent months (Fig. 5b-c). The application of Duravel WP® resulted in an anthracnose incidence ranging from 0% to 15.8%, and a severity of symptoms

ranging from 0% to 3.6% among the evaluated treatments (Fig. 5b-c). In contrast, the bioformulations enriched with *B. velezensis* S26 endospores demonstrated superior biocontrol potential, with anthracnose incidence ranging from 0% to 5.3%, and severity ranging from 0% to 2.4%. Nevertheless, there were no statistically significant differences among the formulations (Fig. 5b-c).

Gray mold was observed in strawberry plants all over the experiment, with higher incidence in June-July period (Fig. 5d-e). While the incidence of the disease ranged from 0% to 6.9% applying Duravel WP®, it varied from 0% to 3.6% with the other bioformulations. Similarly, gray mold severity was higher in July, reaching 4.1% by applying Duravel WP®. However, we did not observe statistically significant differences among the bioformulations.

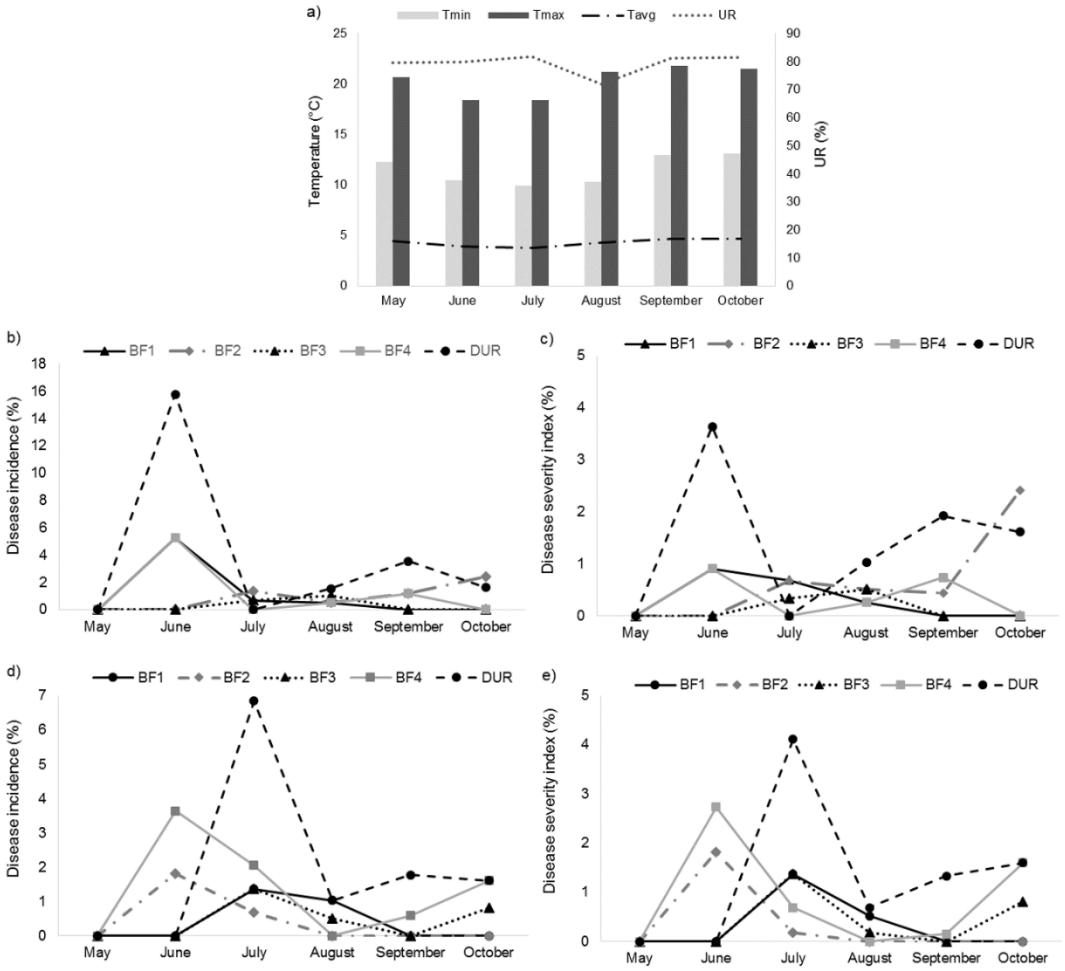

Fig. 5. Meteorological data (a) from a weather station at Embrapa Grape and Wine, Brazil (May-October 2023). Anthracnose incidence (b) and severity (c), and gray mold incidence (d) and severity (e) in strawberry plants treated with bioformulations (BF1, BF2, BF3, and BF4) and Duravel® (DUR). No statistically significant difference was observed according to ANOVA, followed by the T3 Dunnet's test ($P < 0.05$).

3.5 Pot experiment: growth promotion

While all formulations exhibited a substantial biocontrol efficacy, bioformulation BF4 selected for further experimentation for its ability to preserve cell viability during storage. Consequently, bioformulation BF4, enriched with *B. velezensis* S26 endospores, enhanced agronomic measurements, including ΔHeight 0-30, ΔLeaf 30-45, SDW, and RDW compared to the untreated control group (Table 1). In addition, applying bioformulation 4 without spores (BF4ws) also result in an improvement in ΔHeight 0-30 and RDW depending on the method of application. However, no significant effect was observed on ΔLeaf 0-30 and root length using these bioformulations (Table 1).

In terms of application method, foliar spraying (FS) exhibited a higher effect on plant growth than soil drenching (DS). As a result, employing aerial application of bioformulation 4 without spores (FS - BF4ws) resulted in higher ΔHeight 0-30 and ΔLeaf 30-45 compared to soil application (SD - BF4ws). Similarly, treating strawberry plants through foliar spraying with bioformulation (FS - BF4) led to an improvement in ΔLeaf 30-45 compared to applying this formulation through soil drenching (SD - BF4), underscoring the importance of foliar spraying for increasing strawberry plant measurements (Table 1 and Supplementary Material 5).

Although the formulations containing *B. velezensis* S26 endospores did not improve plant growth in a commercial greenhouse compared to the commercial product Duravel WP®, we noticed that strawberry plants in a pot experiment exhibited heightened vigor and biomass compared to an untreated control group. This remarkable result was evident even when using bioformulation BF4 stored for six months, demonstrating that despite a tenfold decrease in endospore concentration over storage, the product retained its beneficial properties for enhancing plant development.

Table 1. Increase in leaf number (ΔLeaf), increase in plant height (ΔHeight), root length, shoot dry mass, and root dry mass in strawberry plants treated with bioformulation 4 (BF4) and bioformulation 4 without spores (BF4ws), through soil drenching (SD) and foliar spraying (FS). Treatments with the same letter are not significantly according to ANOVA followed by Tukey's test ($P < 0.05$). Each value represents the mean ± standard deviation.

Treatments	ΔLeaf 0-30	ΔHeight 0-30 (cm)	ΔLeaf 30-45	ΔHeight 30-45 (cm)	Root length (cm)	Shoot dry mass (g)	Root dry mass (g)
Control	4.33 ± 0.65	0.33 ± 0.32 d	1.83 ± 0.39 bc	1.18 ± 0.69 ab	9.33 ± 2.42	0.66 ± 0.25 c	0.38 ± 0.14 b
SD – BF4ws	4.42 ± 0.51	0.67 ± 0.49 cd	1.25 ± 0.45 c	1.07 ± 0.61ab	8.42 ± 2.17	0.88 ± 0.14 bc	0.69 ± 0.13 a
FS – BF4ws	4.33 ± 0.49	1.50 ± 0.64 a	1.92 ± 0.51 b	1.50 ± 0.71 a	9.71 ± 1.83	0.81 ± 0.14 bc	0.62 ± 0.16 a
SD – BF4	4.33 ±0.49	1.33 ± 0.49 ab	2.42 ± 0.67 b	1.56 ± 0.48 ab	10.25 ± 0.78	1.03 ± 0.20 ab	0.70 ± 0.17 a
FS – BF4	4.83 ± 0.39	0.88 ± 0.48 bc	3.17 ± 0.58 a	0.88 ± 0.48 b	8.63 ± 1.45	1.26 ± 0.29 a	0.77 ± 0.18 a

4. Discussion

Recent research has extensively addressed biocontrol strategies to reduce or replace synthetic fungicides (Teixidó et al., 2022). However, effectively harnessing the potential of these beneficial microorganisms requires not only enhancing their antagonistic properties against the pathogens but also preserving their cell viability and effectiveness during storage (Droby et al., 2016). As a result, endospore-forming bacteria ensure the production of biopesticides resistant to adverse environmental and manufacturing conditions, supporting extended periods of storage (Posada-Uribe et al., 2016).

The initial attempts to induce *B. velezensis* S26 sporulation using raw composting leachate inhibited the endospore production. As suggested by Sanadi et al. (2019), leachate may contain inhibitory compounds, and their impact on bacterial growth and sporulation depends on the composted feedstock or modifications during the composting process. Although diluting the leachate reduced the concentration of toxic substances, it also decreased nutrient levels, requiring supplementation with nutrient-rich substrates.

Subsequently, utilizing composting leachate and whey as a culture medium, individually or in combination, resulted in significant endospore production in shaking flasks and under bioreactor conditions. Consistent with our observations, Santiago Badillo et al. (2021) successfully employed diluted leachate and whey permeate for cultivating *B. subtilis* ATCC 6633, yielding a concentration of 2.0×10^9 CFU mL^{-1} after 144 h of cultivation in a 2L bioreactor. The authors also noted that the scale-up from shaking flasks to fermenters replicated or increased the bacterial concentration.

Our findings demonstrated that liquid bioformulations containing *B. velezensis* S26 endospores maintained cell viability over a six-month storage period. The decline in the bacterial population over time is attributed to the premature germination of endospores, leading to the consumption of nutrients and the release of residual compounds, thereby altering the physicochemical properties of the formulation (Bazilah et al., 2011). Regarding the composition of the bioformulations, despite the leachate not containing any toxic compounds or heavy metals, the low carbohydrate levels associated with the presence of fulvic and humic acids may have adversely affected the survival of *B. velezensis* S26. Conversely, whey-rich formulations exhibit a significant lactose concentration, potentially acting as osmoprotectants to safeguard bacterial cells and endospores from environmental stressors (Chompa et al., 2023).

Our observations also illustrated that, although low temperature enhanced the viability of *B. velezensis* S26 endospores during storage, using a whey-rich bioformulation stored at 25

°C resulted in the highest endospore survival. Similarly, Wong et al. (2019) investigated the viability of *Pseudomonas aeruginosa* DRB1 incorporated into both liquid and dry formulations. They found that *P. aeruginosa* DRB1 stored as a liquid formulation at 25 °C for six months exhibited the highest cell viability compared to dry formulations stored at this same temperature. However, refrigerated storage resulted in complete bacterial death, and the highest cell survival was achieved by incorporating bacterial cells into pesta granules.

Additionally, our experiments revealed that applying bioformulation BF1, containing *B. velezensis* S26 endospores in saline solution, effectively suppressed the incidence and severity of anthracnose in strawberry plants. Similarly, Alijani et al. (2019) reported a significant reduction in the severity of anthracnose when treating strawberry plants with *Staphylococcus sciuri* MarR44. Es-Soufi et al. (2020) also achieved remarkable biocontrol effectiveness using *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR to suppress *Colletotrichum* sp. Ca6 in strawberry plants in a greenhouse experiment.

Furthermore, the formulation BF1 led to a decrease in the incidence of gray mold in strawberry plants. In line with our observations, Hagag et al. (2012) noted that a liquid formulation containing *Pseudomonas fluorescens* Pf-5 resulted in increased yield and higher fresh and dry mass in strawberry plants. Moreover, bacterial application significantly reduced both the incidence and intensity of gray mold, surpassing the effectiveness of the commercial fungicide Ridomil Plus.

All formulations containing *B. velezensis* S26 endospores effectively controlled strawberry diseases in a commercial greenhouse. Similar results were achieved in a field trial conducted by Es-Soufi et al. (2020). According to these researchers, *B. amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR demonstrated effective control of gray mold, anthracnose, and powdery mildew in strawberry plants compared to the control group that did not receive any chemical or biological treatment.

Regarding growth promotion, bioformulations did not enhance strawberry yield compared to Duravel WP®. This result aligns with Sylla et al. (2015), who observed that applying *B. amyloliquefaciens*, *Aureobasidium pullulans*, and *Beauveria bassiana*, individually or in combination, did not affect strawberry yield under field conditions. However, strawberry plants treated with bioformulation 4 exhibited heightened vigor and biomass compared to a control group in a pot experiment. This remarkable improvement in plant development persisted even when using bioformulation 4 stored for six months. Despite a tenfold decrease in endospore concentration over storage, the product retained its beneficial properties, underscoring its efficacy in enhancing plant growth.

We also observed a significant improvement in plant growth when applying bioformulation BF4 and bioformulation BF4 without spores (BF4ws) through foliar spraying rather than soil drenching. In contrast, Efthimiadou et al. (2020) investigated the inoculation of *Azotobacter chroococcum, Bacillus subtilis*, and *Priestia megaterium*, through soil and foliar application in maize plants. Their results indicated that the soil application of *P. megaterium* and the combination of *A. chroococcum* and *B. subtilis* increased maize yield. Furthermore, Kordatzaki et al. (2022) conducted a study comparing the effects of soil and foliar application methods. They verified that soil inoculation of *B. subtilis* resulted in increased biomass of kale plants (*Brassica oleracea* L. var. *acephala*), whereas foliar application improved photosynthetic rate.

5 Conclusion

The current investigation ensured the development of an effective and low-cost bioformulation incorporating *B. velezensis* S26 endospores. Additionally, utilizing whey as a liquid carrier guaranteed the maintenance of endospore viability during storage without the need for refrigeration. Furthermore, the bioformulation BF4 showcased comparable biocontrol effectiveness to Duravel WP® in reducing the occurrence and severity of strawberry diseases. Concerning growth promotion, bioformulation BF4 significantly improved vegetative measurements compared to the untreated control group, with greater efficacy through foliar spraying. This bioformulation retained its beneficial properties even after a six-month storage period.

In summary, by repurposing of biological waste, we address the challenges associated with waste disposal and contribute to fostering an environmentally aware and resource-efficient agricultural model. This approach aligns with the goals of promoting circular economies and minimizing the ecological impact of conventional waste disposal methods. Consequently, the successful integration of waste valorization into agricultural practices offers a promising avenue for achieving environmental sustainability and agricultural productivity.

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Supplementary Material 1

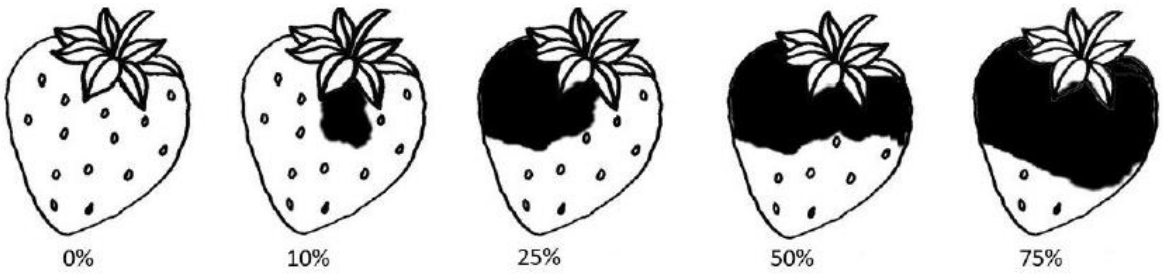

Fig S1. Diagrammatic scale for evaluating strawberry rot severity adapted from Pedrotti et al. (2021).

Supplementary Material 2

Table S2. Viability of *Bacillus velezensis* S26 endospores in four bioformulations: BF1 (0.85% NaCl solution), BF2 (25% composting leachate + 75% water), BF3 (50% composting leachate + 50% whey), and BF4 (25% whey + 75% water). Equal letters indicate no statistically significant difference among the formulations, using one-way ANOVA followed by the Tukey's test ($P < 0.05$). Each value represents the mean ± standard deviation of three replicates.

Temperature	Bioformulations	Time of storage (months)					
		0	2	3	4	5	6
25 °C	BF1	10.4 ± 0.6 a	9.4 ± 0.2 a	8.8 ± 0.8	8.2 ± 0.3 a	7.1 ± 0.6 b	6.5 ± 0.2 de
	BF2	9.7 ± 0.9 b	9.3 ± 0.9 a	8.5 ± 0.3	6.3 ± 0.6 c	6.0 ± 0.5 b	6.0 ± 0.5 e
	BF3	9.5 ± 0.3 b	8.6 ± 0.4 b	8.6 ± 0.5	7.6 ± 0.7 bc	7.5 ± 0.4 b	6.8 ± 0.3 cde
	BF4	9.7 ± 0.9 b	9.3 ± 0.8 a	8.8 ± 0.4	7.9 ± 0.5 a bc	7.8 ± 0.7 a	7.7 ± 0.6 a
4 °C	BF1	10.4 ± 0.9 a	9.4 ± 0.9 a	9.2 ± 0.9	8.1 ± 0.8 ab	7.9 ± 0.4 a	7.1 ± 0.3 bc
	BF2	9.7 ± 0.8 b	9.3 ± 0.5 a	9.3 ± 0.8	7.7 ± 0.7 abc	7.1 ± 0.6 b	7.1 ± 0.6 bcd
	BF3	9.5 ± 0.3 b	8.6 ± 0.8 b	8.4 ± 0.4	7.6 ± 0.7 bc	7.3 ± 0.2 b	7.2 ± 0.5 bc
	BF4	9.7 ± 0.6 b	9.3 ± 0.8 a	9.2 ± 0.9	7.9 ± 0.7 abc	7.9 ± 0.7 a	7.3 ± 0.7 b

4.6 *Bacillus velezensis* S26 - loaded biochar boosts plant growth, alleviates copper stress and suppresses black foot disease in SO4 vine rootstock

Abstract

The excessive use of copper-based agrochemicals poses a serious concern to viticulture worldwide. As a consequence, vineyard soil can become contaminated, leading to reduced vine growth and increased susceptibility to soilborne pathogens. Black foot, caused by various pathogenic species, including *Dactylonectria macrodidyma*, is a fungal disease that impacts the trunk and roots of vines. Enhancing plant biomass and productivity can involve various strategies, such as utilizing beneficial bacteria immobilized in biochar. This study aimed to investigate the impact of applying biochars derived from grape bagasse and enriched with *Bacillus velezensis* S26 endospores on alleviating copper stress, controlling black foot disease, and promoting the growth of the vine rootstock SO4 (*Vitis berlandieri* × *V. riparia*). Initially, we assessed various methods for immobilizing bacterial endospores into fresh bagasse biochar (FBB) and composted bagasse biochar (CBB). Subsequently, the cell viability of incorporated *B. velezensis* S26 endospores was evaluated over a 180-day storage at 4 and 25 ºC. Finally, a pot experiment was conducted using micropropagated SO4 plants to investigate the growth-promotion capacity and biocontrol potential of both biochar and biochar enriched with *B. velezensis* S26 endospores. Our findings revealed that refrigerated storage ensured the highest cell viability in CBB, while no statistically significant difference was observed among the evaluated treatments for FBB. The application of *B. velezensis* S26 endospores, FBB, and CBB, either as a single inoculation or combined treatments, resulted in increased plant growth measurements. In addition, both FBB and CBB enriched with *B. velezensis* S26 endospores enhanced the growth and biomass of SO4 rootstocks cultivated in substrates with high copper concentrations or inoculated with *D. macrodidyma* TD1110. Furthermore, the frequency of pathogen re-isolation decreased by 29 % and 31.5 % using fresh and composted grape bagasse biochars enriched with bacterial endospores, respectively. Therefore, the utilization of fresh and composted bagasse biochars not only contributed to improving rootstock development measurements and suppressing the effects of *D. macrodidyma* TD1110 in SO4 vine rootstocks but also to waste valorization and a more sustainable agriculture.

Keywords: Bacterial endospores, copper-contaminated soils, *Dactylonectria macrodidyma*, grape bagasse biochar, viticulture

1. Introduction

The extensive use of copper-based fungicides in viticulture for controlling fungal diseases has led to significant environmental impacts, including the contamination of water bodies and vineyard soil (Brunetto et al., 2016; Rehman et al., 2019). While copper is an essential micronutrient for plant development, elevated concentrations paradoxically hinder grapevine growth and render vines more susceptible to pathogen infections (Halleen and Crous, 2006; Ameh and Sayes, 2019). Consequently, there is an urgent need for more sustainable practices to address soil contamination and control soilborne pathogens.

One particular concern is the impact of black foot disease on vineyards and nurseries. Various fungal genera, such as *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, *Pleiocarpon*, and *Thelonectria*, have been associated with this disease (Carlucci et al., 2017; Aigoun-Mouhous et al., 2019). Additionally, combined with a complex of diverse pathogenic agents, it can lead to grapevine trunk diseases (Agustí-Brisach et al., 2013; Aigoun-Mouhous et al., 2019). This syndrome primarily affects young vines, causing a gradual decline, reducing plant production, and resulting in substantial economic losses (Mondello et al., 2018). Managing these pathogens is challenging since various factors can influence infection and tissue colonization, including environmental conditions, plant susceptibility, and abiotic stresses (Halleen and Crous, 2006).

Bacillus species are recognized for their efficacy in suppressing plant diseases, promoting plant growth, and alleviating toxic compounds through environmental bioremediation (Brunetto et al., 2016; El-Saadony et al., 2022). Moreover, the *Bacillus* genus can form resilient structures known as endospores, ensuring high bacterial survival and contributing to the development of bioproducts with a prolonged shelf life (Posada-Uribe et al., 2016). To enhance these beneficial properties, bacteria can be loaded into various carriers. An optimal carrier must preserve cell viability during storage, gradually release the microorganisms into the plant rhizosphere, and improve soil quality (Vanek et al., 2016; Ajeng et al., 2020).

Biological charcoal or biochar is a carbonaceous material formed during the pyrolysis of organic raw materials without or under low oxygen levels (Ajeng et al., 2020; Azeem et al., 2021). When applied to soil, biochar enhances soil aggregation, aeration, fertility, and porosity. Additionally, it increases cation exchange capacity, water-holding capacity, and nutrient retention (Husna et al., 2019). Due to waste valorization and low production costs, biochar offers advantages over traditional carriers, including alginate, peat moss, and vermiculite (Husna et al., 2019; Ajeng et al., 2020). Furthermore, its high porosity, adsorption potential, extensive surface area, and sterility make it an effective carrier for beneficial microorganisms, providing a safety niche and nutrients for the immobilized microorganisms (Batista et al., 2018; Siddiq et al., 2018; Azeem et al., 2021).

In the wine industry, 25 % of the total grape mass processed results in waste, including grape marc, stalks, seeds, and skins (Ibn Ferjani et al., 2019; Ahmad et al., 2020; da Silva et al., 2022). However, these residues are unsuitable for agricultural fertilization due to elevated salt levels, high concentrations of organic acids, and low pH values (Ahmad et al., 2020). As a result, grape waste can be valorized through sustainable approaches, such as composting,

bioenergy production, biochar production, and the recovery of feedstock for the development of value-added products (Ibn Ferjani et al., 2019; Frikha et al., 2021).

In light of these considerations, this study aimed to assess the impact of two biochars derived from grape marc and loaded with *Bacillus velezensis* S26 endospores on promoting vine growth, alleviating copper stress, and inhibiting *Dactylonectria macrodidyma*, the causal agent of black foot disease, in micropropagated plants of the rootstock SO4 (*Vitis berlandieri* × *V. riparia*).

2 Materials and methods

2.1 Bacterial growth and sporulation

Bacillus velezensis strain S26 was isolated from copper-contaminated soil (737.2 mg dm^{-3}) in an organic vineyard located in Caxias do Sul, Rio Grande do Sul State, Brazil (29°15'34" S, 51°21'45" W). Initial genus identification was performed through the partial sequencing of the *16S rDNA* gene, and the resulting sequence was deposited in the GenBank under the accession number OP938800 (Debastiani et al., 2023). Subsequently, a comprehensive identification was validated through whole-genome sequencing.

Bacterial growth involved inoculating a loop from a colony-forming unit into a plastic flask with 10 mL of Luria-Bertani (LB) broth (1 % tryptone, 0.5 % sodium chloride, and 0.5 % yeast extract, pH 7.2). This culture was maintained under rotation at 130 rpm and 28 °C for 24 h and transferred to a 250 mL Erlenmeyer flask with 90 mL of LB broth. The flask was incubated in a rotary shaker under the same growth conditions for 24 h more. Next, sporulation was induced in 100 mL of LB broth at pH 8.0, supplemented with $CaCl_2$ to a final concentration of 2 mM. The bacterial inoculum was added at a 1:10 (v/v) ratio. Endospore counts were performed after heat inactivation of vegetative cells at 80 °C for 10 min. The endospore suspension underwent serial dilution and inoculation on LB medium plates. The log-transformed results of spores per milliliter, expressed as spores mL^{-1}, were determined after incubating plates at 37 ºC for 24 h.

B. velezensis S26 endospores were preserved in LB culture broth at 12 $\log_{10}$ spores mL^{-1}. Subsequently, a portion of this suspension was centrifuged at 10,000 rpm for 15 min. The resulting pellet was washed three times and resuspended in a 0.85 % NaCl solution to achieve the same concentration as the initial endospore suspension in LB broth.

2.2 Biochar production and shelf-life evaluation

Fresh and composted grape bagasse were utilized for biochar production at Beifiur Ltda., a biofertilizer company in Garibaldi, Rio Grande do Sul State, Brazil. The biochar was produced through the pyrolysis of grapevine biomass in a muffle furnace, using the following temperature ranges: 0-100 °C for 20 min, 100-240 °C for 120 min, and 240-350 °C for 45 min. After cooling for 24 h, the biochar underwent milling and sieving to achieve a particle size of 2 mm (Fig. 1). Subsequently, 1 g of the biochar sample was blended with 10 mL of sterile water. This mixture was subjected to 1 h of stirring followed by 1 h of standing, after which pH and electrical conductivity were assessed.

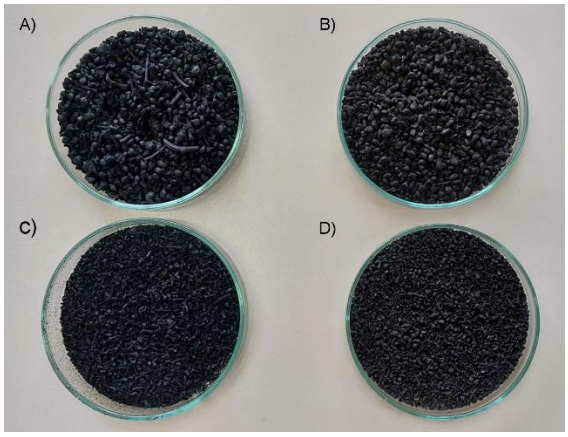

Fig. 1. Photographs depicting biochar derived from fresh grape bagasse before A) and after milling C), as well as biochar from composted grape bagasse before B) and after milling D). The milling process was conducted with a diameter set at 2 mm.

In subsequent steps, both fresh bagasse biochar (FBB) and composted bagasse biochar (CBB) were divided into 50 mL plastic flasks containing 10 g of each biochar. These flasks were autoclaved at 121 °C for 20 min. Each biochar sample was mixed with *B. velezensis* S26 endospores suspended in LB broth at three ratios: 0.5 g mL^{-1}, 1 g mL^{-1}, and 1.5 g mL^{-1}. The final concentration in each biochar composite was adjusted to contain 10 $\log_{10}$ spores g^{-1} or 1×10^{10} spores mL^{-1}. The experiment was incubated on a rotary shaker at 120 rpm and 37 °C for 48 h. After drying in a laminar flow chamber for 24 h, the concentration of *B. velezensis* S26 endospores was determined by suspending 1 g of each biochar in 5 mL of sterile water. This suspension was agitated for 10 min and then subjected to heat inactivation at 80 °C for 10 min. The endospore concentration was determined using the counting-plate method after incubating

LB medium plates at 37 °C for 24 h. The experiment was performed in three independent replicates.

Subsequently, we explored the effect of water washing and chemical treatments (H_2SO_4 and KOH) to enhance *B. velezensis* incorporation into each biochar. The treatments with H_2SO_4 and KOH followed the methods described by Liu et al. (2012) and Lin et al. (2012), respectively. Water washing included mixing biochars with sterile water at 1:5 (g mL^{-1}). This mixture was incubated in a rotary shaker (150 rpm and 30 ºC) for 2 h, as described by Boakye et al. (2019). In addition, we examined the application of *B. velezensis* endospores suspended in both LB broth and saline solution at the final concentration of 10 $\log_{10}$ spores g^{-1}. Incubation was conducted on a rotary shaker at 120 rpm and 37 °C for 48 h. The assessment of endospore concentration in each biochar followed the procedures earlier described. The assay was conducted in triplicates.

Finally, 10 g of fresh bagasse biochar and composted bagasse enriched with *B. velezensis* S26 endospores were suspended in LB broth and saline solution. These composites were placed in sterile plastic bags and stored at 4 ºC and 25 ºC. Subsequently, the viability of *B. velezensis* S26 endospores was assessed at 0, 30, 60, 90, 120, 150, and 180-day intervals. For this, a 1 g sample of each composite was mixed with 4 mL of sterile water in 15 mL plastic flasks. These flasks underwent heat inactivation at 80 °C for 10 min followed by serial dilution and plating on a LB medium. The plates were incubated in a stove at 37 °C for 24 h. The concentration of *B. velezensis* S26 endospores was log-transformed, and the results were expressed as spores per gram (spores g^{-1}).

2.3 Plant growth-promoting characteristics

B. velezensis S26 was previously identified for its capacity to produce indolic compounds and siderophores (Debastiani et al., 2023). This rhizobacterium can also synthesize cyclic lipopeptides, including fengycin and surfactin, which have antifungal properties. The current study evaluated additional characteristics, focusing on gibberellic acid production, phosphate solubilization, and pectinolytic and proteolytic activities.

Production of gibberellic acid was evaluated by culturing *B. velezensis* S26 cells in Jenson broth (20 g L^{-1} sucrose, 1 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 0.5 g L^{-1} NaCl, 0.1 g L^{-1} $FeSO_4$, 0.005 g L^{-1} $Na_2MoO_4.2H_2O$, and 2 g L^{-1} $CaCO_3$) on a rotary shaker at 26 ± 2 °C for 5 days. After centrifugation at 5,000 rpm for 3 min, the supernatant was harvested, and its pH was adjusted to 1.0-2.0 with 0.1 M HCl. Subsequently, liquid extraction was performed three

times using ethyl acetate at a ratio of 1:1 (v/v). The apolar phase was collected, and an equal volume of phosphate buffer (pH 7.0) was added. The concentration of gibberellic acid was determined at 254 nm using a spectrophotometer.

Phosphate solubilization was determined by inoculating 10 µL of *B. velezensis* S26 suspension into holes made with a cork borer in Pivoskaya medium (10 g L^{-1} glucose, 5 g L^{-1} $MgCl_2 \cdot 6H_2O$, 0.25 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1} KCl, 0.1 g L^{-1} $(NH_4)_2SO_4$, and 5 g L^{-1} $Ca_3(PO_4)_2$). Plates were then incubated in the dark at 26 ± 2 °C for 7 days. The appearance of clear areas around the colony-forming units confirmed the phosphate solubilization.

The activity of pectinase and proteolytic enzymes was also assessed. For pectinase estimation, 10 µL of *B. velezensis* S26 suspension was placed in holes made in Vincent's agar medium (10 g L^{-1} pectin, 2 g L^{-1} $NaNO_3$, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4$, 1 g L^{-1} KH_2PO_4, 0.5 g L^{-1} tryptone, and 4.5 g L^{-1} agar) and incubated at 37 ± 2 °C for 24 h. Enzyme activity was confirmed by visualizing clear zones after flooding the plates with a 50 mM iodine solution. The presence of proteolytic enzymes was evaluated by inoculating 10 µL of the rhizobacterium suspension on skim milk agar medium (5 g L^{-1} casein, 1 g L^{-1} dextrose, 2.5 g L^{-1} yeast extract, 28 g L^{-1} skim milk, and 15 g L^{-1} agar, pH 7.0). Clear zones around the bacterial colonies indicated positive results after incubation.

2.4 Plant material

Vine branches (15 cm long) were harvested from two-year-old SO4 rootstock plants cultivated in a greenhouse at Embrapa Grape and Wine, Bento Gonçalves, Rio Grande do Sul State, Brazil. These branches were cut into nodal segments (1-2 cm in length) and disinfected in a laminar flow cabinet. The disinfection process involved immersing the segments in 70 % (v/v) ethanol for 1 min, then soaking them in a 1 % sodium hypochlorite solution with two drops of Tween 20 for 20 min. Subsequently, the nodal segments were washed three times with sterile distilled water before being inoculated onto glass tubes with 12 mL of MS medium (Murashige and Skoog, 1962), amended with 6 g L^{-1} agar, 30 g L^{-1} sucrose, and 0.5 mg L^{-1} BAP (6-benzyl aminopurine). The pH was adjusted to 5.8 using a 0.1 M NaOH solution.

Cultures were maintained at 25 ± 2 °C, 80 % relative humidity, with a 16-h light/8-h dark photoperiod (72 µmol m^{-2} s^{-1}) for 45 days. Following three subcultures, plantlets were rooted in half-strength MS medium supplemented with 6 g L^{-1} agar, 15 g L^{-1} sucrose, and 0.1 µg L^{-1} NAA (α-naphthalene acetic acid). Acclimatization involved carefully transferring plantlets from culture flasks to autoclaved substrate (9:1 v/v, peat: vermiculite). The substrate

was autoclaved three times at 121 °C for 1 h to eliminate soilborne pathogens, following the method, as described by Wei et al. (2023). Plants were then maintained at 25 ± 3 °C, 60-80 % relative humidity, with a 16-h light/8-h dark photoperiod (350 μmol m^{-2} s^{-1}).

2.5 Plant growth promotion assay

The ability of *B. velezensis* S26 endospores to promote plant growth was evaluated in micropropagated SO4 plants. After a 30-day acclimatization period, each rootstock plant was individually transplanted into 1 L plastic pots filled with autoclaved substrate (9:1 v/v, peat: vermiculite). The leaf number and plant height were recorded for further analysis. The experiment comprised six treatments: control, END (*B. velezensis* S26 endospores), FBB (fresh bagasse biochar), CBB (composted bagasse biochar), FBB + END (fresh bagasse biochar loaded with *B. velezensis* S26 endospores), and CBB + END (composted bagasse biochar loaded with *B. velezensis* S26 endospores). The FBB + END and CBB + END treatments, both containing 6.3 $\log_{10}$ spores mL^{-1} of *B. velezensis* S26, were evaluated after a 4-month storage period at 4 °C.

In the FBB, CBB, FBB + END, and CBB + END treatments, biochars were uniformly integrated into the substrate at a concentration of 3 % (w/w). The END treatment involved inoculating the substrate around SO4 plants with a 10 mL suspension of *B.* ve*lezensis* S26 (10 $\log_{10}$ spores mL^{-1}). The control group received soil inoculation with a 0.85 % NaCl solution. The experiment was maintained in a greenhouse under controlled conditions (23 ± 2 °C and 60-80 % relative humidity). The moisture level was maintained at approximately 70 % of the maximum water-holding capacity, and no fertilizer was applied. After 60 days, various responses were determined, including the increase in leaf number (ΔLeaf), increase in plant height (ΔHeight), root length (Rlength), shoot dry mass, and root dry mass. The assay followed a completely randomized design, with three replicates, each consisting of 20 rootstock plants per treatment.

2.7 Copper alleviation assay

The autoclaved substrate was treated with 300 mg kg^{-1} of $CuSO_4.5H_2O$, ensuring a uniform distribution and chemical interaction through watering until saturation and allowing it to rest for 30 days. The substrate was distributed into 1 L plastic pots. Subsequently, each 30-day-old SO4 plant was individually transplanted into them. The plants underwent the following

treatments: control (without copper and biochar), Cu (copper), Cu + FBB + END (copper + fresh bagasse biochar loaded with *B. velezensis* S26 endospores), and Cu + CBB + END (copper + composted bagasse biochar loaded with *B. velezensis* S26 endospores). Both FBB + END and CBB + END treatments, containing 6.3 $\log_{10}$ spores mL^{-1} of *B. velezensis* S26, were applied at a 3 % (w/w) ratio to the copper-treated substrate after 4 months of refrigerated storage. Plant height and the number of unfolded leaves in SO4 plants were assessed at this stage.

The greenhouse conditions were maintained at 23 ± 2 °C, with a relative humidity of 60-80 %. Daily watering was administered to SO4 plants until 70 % water holding capacity was reached, and no additional fertilization occurred during the trial. Vine rootstocks were examined after 60 days to determine the increase in leaf number (ΔLeaf), increase in plant height (ΔHeight), root length (Rlength), shoot dry mass, and root dry mass. The experiment followed a completely randomized design with three replicates, each consisting of 20 plants per treatment.

2.8 Black foot biocontrol assay

The causal agent of black foot diseases on grapevines, *Dactylonectria macrodidyma* TD1110 (GenBank accession number MK421587), was obtained from the fungal collection of the Laboratory of Phytopathology at the Federal Institute of Education Science and Technology of Rio Grande do Sul, Bento Gonçalves, Brazil. The pathogen inoculum was prepared from 14-day-old colonies cultivated on potato dextrose agar (PDA) medium (2 % dextrose, 1.5 % agar, and potato broth, pH 5.6) at 28 ºC, under a 12-h light/12-h dark photoperiod. Conidia were harvested by rubbing the fungal mycelium with a 0.85% NaCl solution. The conidial suspension was filtered through sterile cheesecloth to remove mycelial fragments, and its concentration was adjusted to 6 $\log_{10}$ conidia mL^{-1} or 1×10^6 conidia mL^{-1}, using a hemocytometer.

In a greenhouse experiment, each 30-day-old micropropagated plant of SO4 was transplanted into a pot containing 1 L of autoclaved substrate with 3 % (w/w) of the biochars loaded with *B. velezensis* S26 endospores and stored for four months under refrigeration. At this point, the leaf number and plant height of each rootstock were determined. After seven days, 10 mL of the *D. macrodidyma* TD1110 suspension was applied around each plant. The experiment comprised the following treatments: control (saline solution), TD1110 (*D. macrodidyma* TD1110), FBB + END + TD1110 (fresh bagasse biochar + *D. macrodidyma* TD1110), and CBB + END + TD1110 (composted bagasse biochar + *D. macrodidyma* TD1110).

The experiment was conducted under the conditions described in section 2.6 for 60 days. Subsequently, an assessment of the increase in leaf number (ΔLeaf), increase in plant height (ΔHeight), root length (Rlength), shoot dry mass, root dry mass, and frequency of pathogen re-isolation was performed. The experiment was completely randomized, with three replicates and 20 rootstock plants per treatment.

2.9 Data analysis

Statistical analysis was performed using SPSS 22.0 software for the test data. The normality and homoscedasticity of the dataset were assessed using Shapiro-Wilk's and Levene's tests, respectively. Parametric data were subjected to one-way ANOVA, and post hoc comparisons were conducted using the Tukey test. Non-parametric data were analyzed using Kruskal-Wallis, followed by the Dunn-Bonferroni test. The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 *B. velezensis* S26 incorporation into biochar

We noted that utilizing a concentration of 1 g mL^{-1} (biochar: *B. velezensis* S26 endospore suspension) for fresh bagasse biochar (FBB) led to increased endospore incorporation (7.9 $\log_{10}$ or 8.0×10^7 spores g^{-1}). Meanwhile, using the proportion 0.5 g mL^{-1} for composted bagasse biochar (CBB) was more effective in retaining *B. velezensis* S26 endospores (8.4 $\log_{10}$ or 2.5×10^8 spores g^{-1}) within the biochar composite (Fig. 2).

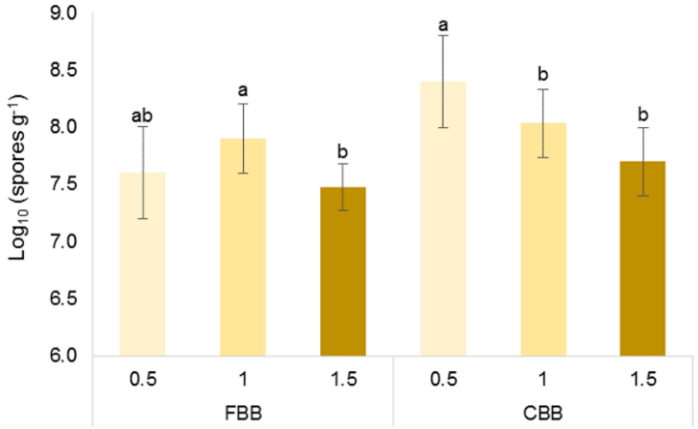

Fig. 2. Endospore concentration after treating fresh bagasse biochar (FBB) and composted bagasse biochar (CBB) with *Bacillus velezensis* S26 endospores suspended in LB broth at three ratios: 0.5, 1, and 1.5 g mL^{-1}. The means were compared using ANOVA followed by the Tukey's test ($P < 0.05$). Error bars indicate the standard deviation of three replicates.

Following this, we assessed the impact of various treatments on biochar (water washing, H_2SO_4, and KOH) and the application of *B. velezensis* S26 endospores suspended in both saline solution (SS) and Luria-Bertani broth (LB). Our findings revealed that, in fresh bagasse biochar (Fig. 3A), the highest bacterial incorporation was achieved by inoculating bacterial endospores in LB broth without any biochar treatment (9.5 $\log_{10}$ or 3.4×10^9 spores g^{-1}). Conversely, H_2SO_4 and water washing treatments resulted in lower bacterial adsorption (Fig. 3A). Similarly, for composted bagasse biochar (Fig. 3B), we observed that applying *B. velezensis* S26 endospores in LB broth without biochar modifications resulted in a higher bacterial concentration (8.8 $\log_{10}$ or 6.2×10^8 spores g^{-1}). In contrast, treating composted bagasse biochar with H_2SO_4 resulted in the lowest endospore concentration (5.9 $\log_{10}$ or 8.0×10^5 spores g^{-1}).

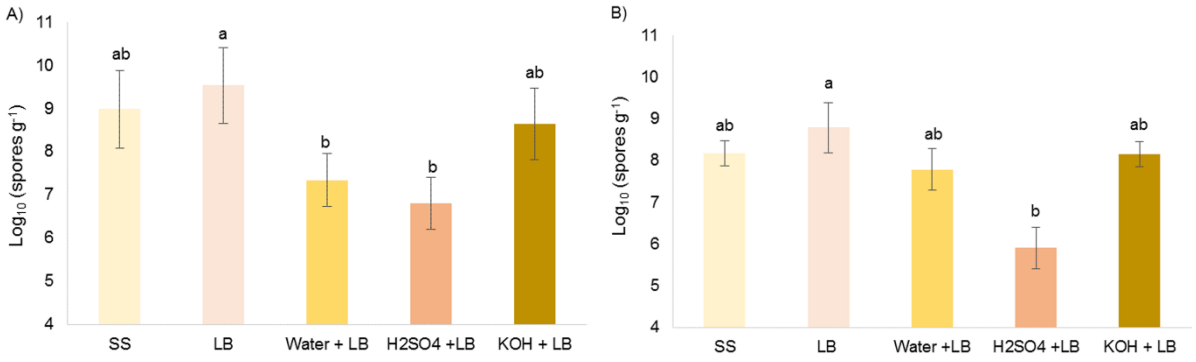

Fig. 3. Concentration of *Bacillus velezensis* S26 endospores incorporated into: A) fresh bagasse biochar and B) composted bagasse biochar. Endospores were loaded into biochar in saline solution (SS) or Luria-Bertani broth (LB). Biochars underwent water washing or chemical treatments with H_2SO_4 and KOH. The means were compared using ANOVA followed by the Tukey's test for parametric data or Kruskal-Wallis followed by the Dunn-Bonferroni test for non-parametric data. Error bars indicate the standard deviation of three replicates.

3.2 Shelf-life of endospore-loaded biochar

Regarding the physicochemical properties, fresh and composted bagasse biochar exhibited alkaline pH values, with readings of 9.7 and 9.6, respectively. The electrical conductivity ranged from 140 µS cm^{-1} for fresh bagasse biochar to 268 µS cm^{-1} for composted bagasse biochar.

The cell viability of *B. velezensis* S26 endospores immobilized into biochars derived from grape bagasse was evaluated over a 180-day storage period at 4 and 25 °C. Additionally, we assessed two different conditions for endospore immobilization in saline solution (SS) and Luria-Bertani broth (LB) (Fig. 4 and Supplementary Material 1).

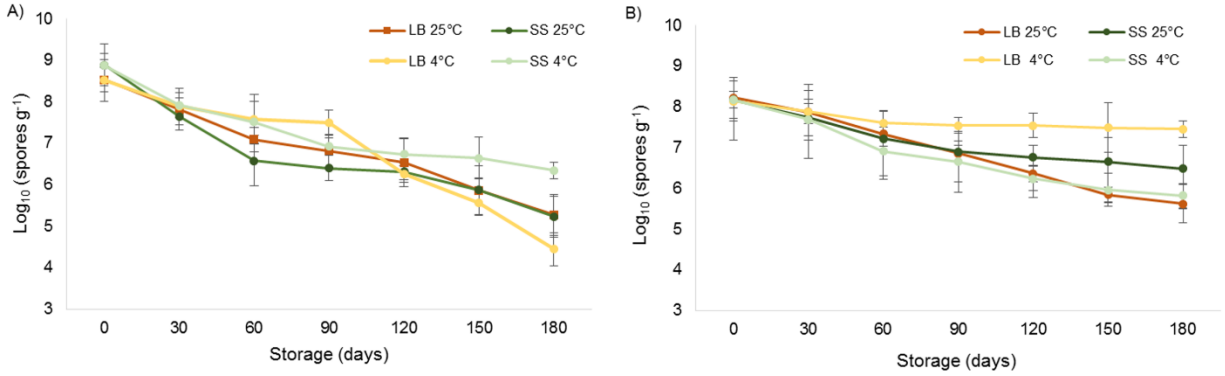

Fig. 4. Concentration of *Bacillus velezensis* S26 endospores suspended in LB broth (LB) and saline solution (SS) during 180 days of storage at 4 °C and 25 °C in: A) fresh bagasse biochar and B) composted bagasse biochar. Error bars indicate the standard deviation of three replicates.

For fresh bagasse biochar (FBB), the highest cell viability was obtained in the treatment FBB SS 4ºC (6.3 $\log_{10}$ or 2.6 × 10^6 spores g^{-1}), while the lowest bacterial survival was detected in the FBB LB 4ºC treatment (4.4 $\log_{10}$ or 2.8 × 10^4 spores $g^{-1)}$ (Fig. 4A). In contrast, for composted bagasse biochar (CBB), the highest immobilization of *B. velezensis* S26 endospores was achieved in the CBB LB 4 °C treatment (7.4 $\log_{10}$ or 2.8 × 10^7 spores g^{-1}) (Fig. 4B). On the other hand, the CBB SS 4 °C and CBB LB 25 ºC treatments demonstrated the lowest endospore survival, ranging from 5.8 $\log_{10}$ or 6.6 × 10^5 spores g^{-1} to 5.6 $\log_{10}$ or 4.2 × 10^5 spores g^{-1}.

3.3 Plant growth-promoting characteristics

The rhizobacterium *B. velezensis* S26 can produce lytic enzymes, including pectinases and proteases. Furthermore, our observations indicate its capacity to synthesize substantial amounts of gibberellic acid (13 mg L^{-1}). However, *B. velezensis* S26 did not solubilize the phosphate present in the culture medium (Supplementary material 2).

3.4 Plant growth promotion assay

The efficacy of biochar and biochar enriched with *B. velezensis* S26 endospores was validated in a potted experiment with vine rootstock SO4. The application of *B. velezensis* S26 endospores (END) increased leaf number, plant height, and shoot dry mass compared to the control (Table 1). Similarly, fresh bagasse biochar (FBB) enhanced leaf number and shoot dry

mass, while composted bagasse biochar (CBB) improved these same responses and positively influenced plant height and root dry mass.

Furthermore, our experiments revealed that soil inoculation of biochar loaded with *B. velezensis* S26 endospores (FBB + END and CBB + END) had a beneficial impact on various plant growth measurements (Table 1). The FBB + END treatment improved leaf number and shoot dry mass, while the CBB + END treatment enhanced leaf number and plant height. Although none of the treatments increased root length compared to the untreated control, composted bagasse biochar contributed to increase root dry mass. Overall, most treatments positively influenced the development of SO4 aerial parts, including leaf number, plant height, and shoot dry mass.

Table 1. Growth promotion measurements for vine rootstock SO4 subjected to the following treatments: control, END (*Bacillus velezensis* S26 endospores), FBB (fresh bagasse biochar), CBB (composted bagasse biochar), FBB + END, and CBB + END. Each value represents the mean ± standard deviation of three replicates.

Treatments	ΔLeaf number	ΔHeight (cm)	Rlength (cm)	Shoot dry mass (g)	Root dry mass (g)
Control	1.9 ± 0.7 c	16.7 ± 2.7 b	19.3 ± 1.7	4.0 ± 1.5 c	3.2 ± 1.0 b
END	3.6 ± 0.6 b	26.0 ± 3.7 a	19.6 ± 2.3	5.4 ± 0.6 ab	3.5 ± 0.9 b
FBB	3.3 ± 0.8 b	16.8 ± 5.7 b	19.0 ± 2.8	5.8 ± 1.5 a	3.6 ± 1.1 ab
CBB	3.5 ± 0.7 b	24.9 ± 6.1 a	20.9 ± 4.4	5.8 ± 1.4 a	4.7 ± 1.3 a
FBB + END	4.1 ± 0.9 ab	16.1 ± 3.2 b	20.4 ± 3.5	4.9 ± 1.0 ab	3.6 ± 0.9 ab
CBB + END	4.7 ± 1.3 a	22.3 ± 4.2 a	19.1 ± 1.9	4.4 ± 0.7 abc	3.8 ± 1.3 ab

Equal letters indicate no statistical difference among the treatments according to ANOVA, followed by the Tukey's test ($P < 0.05$).

3.5 Copper alleviation assay

Our findings demonstrated the adverse impacts of elevated copper concentrations on the root length of SO4 plants (Table 2). Applying *B. velezensis* S26 endospores immobilized in biochar enhanced agronomic measurements in SO4 rootstocks. Specifically, the Cu + FBB + END treatment increased shoot dry mass compared to rootstocks cultivated in a substrate with

high copper levels. Also, the Cu + CBB + END treatment resulted in an augmentation in leaf number, root length, and shoot dry mass (Table 2 and Fig. 5).

Table 2. Growth promotion measurements for grapevine rootstock SO4 subjected to the following treatments: control, Cu (copper), Cu + FBB + END (copper + fresh bagasse biochar with *Bacillus velezensis* S26 endospores), and Cu + CBB + END (copper + composted bagasse biochar with *B. velezensis* S26 endospores). Each value represents the mean ± standard deviation of three replicates.

Treatments	ΔLeaf number	ΔHeight (cm)	Root length (cm)	Shoot dry mass (g)	Root dry mass (g)
Control	1.6 ± 0.5 ab	11.5 ± 4.0 b	16.9 ± 2.0 a	2.7 ± 0.6 b	1.1 ± 0.4
Cu	1.2 ± 0.2 b	14.0 ± 3.4 ab	12.8 ± 1.9 b	2.0 ± 0.5 b	1.1 ± 0.4
Cu + FBB + END	1.6 ± 2.4 ab	16.1 ± 4.0 a	14.3 ± 1.9 b	4.0 ± 0.1 a	1.1 ± 0.1
Cu + CBB + END	1.9 ± 0.5 a	15.2 ± 4.4 ab	18.9 ± 2.9 a	4.2 ± 0.9 a	1.4 ± 0.5

Equal letters indicate no statistical difference among the treatments according to ANOVA, followed by the Tukey's test ($P < 0.05$).

Fig. 5. Photgraphs showing the growth measurements of SO4 rootstocks subjected to the treatments: control, copper (Cu), copper + fresh bagasse biochar loaded with *Bacillus velezensis* S26 endospores (Cu + FBB + END), and copper + composted bagasse biochar enriched with *B. velezensis* S26 endospores (Cu + CBB + END), at 60 days post-inoculation.

3.6 Black foot biocontrol assay

In the biocontrol assay of black foot disease on vine rootstocks, the inoculation of *D. macrodidyma* TD1110 (TD1110 treatment) resulted in reduced root length compared to the untreated control (Table 3). Nevertheless, applying biochar enriched with *B. velezensis* S26 endospores (FBB + END + TD1110 and CBB + END + TD1110 treatments) contributed to a decrease in the frequency of pathogen re-isolation, from 75.3 % in rootstocks infected with the pathogen to 51.6 % and 53.4 % in SO4 plants treated with FBB + END and CBB + END, respectively (Table 4).

Moreover, the FBB + END + TD1110 treatment improved leaf number, shoot dry mass, and root dry mass compared to SO4 vine rootstocks inoculated solely with *D. macrodidyma* TD1110. Likewise, the CBB + END + TD1110 treatment enhanced leaf number, root length, and root dry mass compared to the diseased rootstocks (Table 3).

Table 3. Growth promotion measurements for vine rootstock SO4 subjected to the following treatments: control, TD1110 (*Dactylonectria macrodidyma* TD1110), FBB + END + TD1110 (fresh bagasse biochar enriched with *Bacillus velezensis* S26 endospores + *D. macrodidyma* TD1110), and CBB + END + TD1110 (composted bagasse biochar with *B. velezensis* S26 endospores + *D. macrodidyma* TD1110). Each value represents the mean ± standard deviation of three replicates.

Treatments	ΔLeaf number	ΔHeight (cm)	Rlength (cm)	Shoot dry mass (g)	Root dry mass (g)	Pathogen re-isolation (%)
Control	1.6 ± 0.5 ab	11.5 ± 4.0	16.9 ± 2.0 b	2.7 ± 0.6 b	1.5 ± 0.3 b	0
TD1110	1.3 ± 0.6 b	11.0 ± 6.9	17.6 ± 2.4 b	3.3 ± 0.4 b	1.1 ± 0.4 c	75.3 ± 0.7a
FBB + END + TD1110	2.0 ± 0.5 a	14.4 ± 5.4	18.4 ± 1.7 ab	4.7 ± 1.0 a	2.2 ± 0.3 a	51.6 ± 0.4 b
CBB + END + TD1110	2.1 ± 1.0 a	15.2 ± 4.9	20.0 ± 2.5 a	3.4 ± 1.0 b	2.0 ± 0.5 a	53.4 ± 0.6 b

Equal letters indicate no statistical difference among the treatments according to ANOVA, followed by the Tukey's test ($P < 0.05$).

4. Discussion

In this study, we utilized *B. velezensis* S26, a rhizobacterium capable of thriving in highly copper-contaminated vineyards, to enhance vine growth, alleviate the adverse effects of copper, and suppress black foot disease in grapevine rootstock SO4. Aiming to optimize bacterial performance, we explored various methods to immobilize *B. velezensis* S26 endospores into biochar derived from fresh and composted grape pomace, thereby ensuring waste valorization with cost-effectiveness.

Initially, we examined the impact of various biochar and endospore suspension ratios to determine optimal conditions for bacterial incorporation. Our findings revealed that composted bagasse biochar required a lower endospore suspension volume than fresh bagasse biochar. Consistent with our findings, Azeem et al. (2021) achieved a high concentration of *Bacillus cereus* cells when incorporating them into biochar derived from tea leaves (*Camellia sinensis* L.) using a lower biochar: bacterial suspension proportion. Conversely, Jia et al. (2022) obtained an effective bacterial immobilization by loading a high volume of *Bacillus subtilis* Tpb55 culture broth into a tamarisk stem biochar. However, the effect of varying the biochar: bacterial suspension ratios on bacterial adsorption still needs to be investigated.

In our study, grape bagasse biochar was produced at a pyrolysis temperature below 350 ºC, indicating the preservation of cellulose and lignin, which require higher temperatures for thermal decomposition (Frikha et al., 2021). As a result, these biochars only undergo the loss of organic volatiles, low-mass carbohydrates, and hemicellulose. Biochars produced at low pyrolysis temperatures exhibit specific characteristics, including high cation exchange capacity, contributing to plant development and growth (Day et al., 2005; Lin et al., 2012). Nevertheless, these biochars possess hydrophobic properties and lower surface area due to the retention of various complex compounds, restricting their water storage capacity (Joseph et al., 2021).

Additionally, we assessed various methods, including water washing and treatments with KOH and H_2SO_4, to improve the adsorption potential of biochars. These approaches contribute to removing impurities and promoting modifications in surface area, chemical groups, and adsorption capacity (Liu et al., 2012). While KOH treatment can eliminate humic molecules and enhance surface area (Novtny et al., 2007; Lin et al., 2012), H_2SO_4 and HNO_3 treatments increase carboxyl groups on the biochar surface (Wang and Wang, 2019). Nevertheless, our results demonstrated that modified biochars did not increase *B. velezensis* S26 immobilization. Conversely, Liu et al. (2012) reported that alkali-modified biochar increased surface area and π- π interactions, resulting in higher adsorption of tetracycline from

aqueous wastewater. In alignment with our observations, Vanek et al. (2016) verified that washing hardwood biochar pyrolyzed at 450 °C with acetone and treating white pine, sugar cane bagasse, and grape biochars with 1 % HCl and acetone reduced *Rhizobium tropici* CIAT 899 survival compared to the untreated biochars. Despite the contribution of chemical treatments to eliminating organic compounds soluble in acetone, toxic molecules can also become more accessible, affecting bacterial cells.

The impact of employing saline solution and Luria-Bertani broth for immobilizing *B. velezensis* S26 endospores in biochar was also examined. Although no statistical difference was observed between these methods, using LB broth led to a higher concentration of bacterial endospores in both grape bagasse biochars. Bacterial immobilization in biochar involves physicochemical adsorption and biofilm formation (Wang et al., 2022). The adsorption depends on electrostatic and hydrophobic interactions and the concentration of bacterial inoculum (Ha et al., 2022). In addition, the composition of the bacterial suspension can influence cell viability, endospore germination, and release of extracellular polymeric substances for the biofilm matrix (Pinto et al., 2019).

Regarding cell viability, we observed that low temperatures maintained a high concentration of *B. velezensis* S26 endospores after storage. A study by Azeem et al. (2021) evaluated the cell viability of *Bacillus cereus* loaded in biochar derived from tea leaves (*Camellia sinensis* L.). The authors found an increase in the *B. cereus* population after a 30-day storage at 10 and 20 °C. This difference from our results can be attributed to the fact that the endospores are dormant, and their population is not expected to grow. Furthermore, the authors noted higher survival of *B. cereus* in biochar when stored at 10 °C for 90 days. Similarly, Jia et al. (2022) observed the highest cell viability of *Bacillus subtilis* Tpb55 in biochar stored at 10 °C compared to those stored at 25 °C for 100 days.

Aiming to minimize copper stress in vineyards, increase vine growth, and suppress soilborne pathogens, we employed the bacterium *B. velezensis* S26, sourced from the rhizosphere of a copper-contaminated vineyard by Debastiani et al. (2023). These authors demonstrated that *B. velezensis* S26 could synthesize siderophores and indolic compounds. Moreover, our research revealed its capacity to produce gibberellic acid and hydrolytic enzymes. Iron-chelating siderophores inhibit fungal pathogens through competition for nutrients and also enhance plant iron absorption (Etesami et al., 2023). In addition, indolic compounds and gibberellins stimulate morphogenesis and plant growth (Anfang and Shani, 2021). Our recent unpublished findings indicate that *B. velezensis* S26 can also produce fengycin and surfactin, which possess antifungal properties (Cawoy et al., 2015) and

involvement in biofilm formation (Etesami et al., 2023), respectively. Surfactin can also enhance plant defense mechanisms and exhibit synergistic activity when associated with fengycin (Crouzet et al., 2020; Etesami et al., 2023).

Our experiments demonstrated that applying *B. velezensis* S26 endospores and biochar, either as a single treatment or as loaded biochar, improved various plant growth measurements in the rootstock SO4 compared to the untreated plants. Frikha et al. (2021) also evaluated the impact of applying biochars derived from exhausted grape marc (EGM) and produced at three different temperatures (EGM450, EGM500, and EGM550). The authors observed that inoculating EGM450 biochar into the soil resulted in the highest ryegrass dry mass while inoculating EGM550 biochar led to a reduced plant mass compared to the control group. Biochars can improve plant development even when applied alone due to their high content of organic carbon, macro-, and micronutrients. Additionally, they enhance physicochemical characteristics, including soil porosity, acidity, and cation exchange capacity (Bruckman et al., 2016; Frikha et al., 2021). Nevertheless, nutrients provided by biochars may be insufficient to meet plant requirements completely, and they cannot replace synthetic fertilizers alone (Ajeng et al., 2020). In a similar way, the inoculation of a *B. velezensis* S26 endospores suspension exhibited a lower positive impact on plant growth compared to the biochar-immobilized bacteria, demonstrating that biochar can provide protection to the loaded bacterial agents against the indigenous microrganisms (Zhang et al., 2023).

We also evaluated the addition of both biochars alone and enriched with *B. velezensis* S26 endospores into the substrate at a 3 % (w/w) ratio. In line with our findings, Azeem et al. (2021) verified that applying *B. cereus* loaded-biochar in mung bean (*Vigna mungo*) at a 1 % (w/w) increased nodule number, nitrogen concentration in the shoots, nitrogen uptake, total nitrogen, and nitrogen fixation in a pot experiment under greenhouse conditions. As a result, mung bean plants exhibited increased shoot length, root length, shoot fresh mass, and shoot dry mass compared to plants treated with peat, unloaded biochar, or *B. cereus* suspension. Similarly, Egamberdieva et al. (2016) investigated the application of three biochars (maize biochar, wood biochar, and hydrochar from maize silage) to the soil at 1 %, 2 %, and 3 % (w/w) ratios. They noted a significant increase in soybean plants' root and shoot dry mass when inoculated with hydrochar at 1 % and 2 %, respectively. The authors also concluded that the type of biochar is the primary factor influencing plant growth and the microbiota community in the rhizosphere.

In our pot experiment using a copper-contaminated substrate, both grape bagasse biochars enriched with *B. velezensis* S26 endospores effectively improved the growth of SO4 rootstocks. Similarly, Debastiani et al. (2023) reported that employing *B. velezensis* S26 cells

enhanced the root dry matter of Paulsen 1103 cuttings (*Vitis berlandieri* × *V. rupestris*) when cultivated in copper-contaminated soil. Another study by Zhang et al. (2023) demonstrates that applying biochar enriched with *Sphingobium abikonense* to the soil reduced phenanthrene and copper levels. The authors also observed that even when applied alone, biochar could immobilize these toxic compounds and increase the abundance of beneficial bacteria involved in soil remediation. Likewise, Tu et al. (2020) noted that both maize straw biochar and *Pseudomonas* sp. NT-2 loaded biochar stabilized Cd and Cu levels in the soil. They reported an increase in soil pH when applying biochar enriched with *Pseudomonas* sp. NT-2 compared to the single biochar application.

In the biocontrol experiment, both fresh bagasse biochar and composted bagasse biochar loaded with *B. velezensis* S26 endospores positively influenced SO4 rootstocks by reducing *D. macrodidyma* TD1110 re-isolation and improving the root length of diseased plants. In agreement with our results, El-Hadidy (2019) assessed various formulations (peat moss, sawdust, biochar, sawdust-biochar, peat moss-biochar, and kaolin biochar) embedded with *Bacillus pseudomycoides* M3, *Brevibacillus brevis* M4, and *Stenotrophomonas maltophilia* BG4 for treating tomato seeds. They found that biochar-based formulations effectively decreased the incidence and symptoms caused by *Fusarium oxysporum* f. sp. *lycopersici*.

Similarly, Li et al. (2022) noted that applying wood biochar significantly reduced the density of ginseng root rot causal agents, *Fusarium solani* and *Cylindrocarpon destructans*, in soil by 48 % and 63 %, respectively. In a related study, Rasool et al. (2021) investigated the impact of adding wood and green waste biochar, either alone or loaded with *Bacillus subtilis*, to enhance tomato plant growth and control *Alternaria solani*. They observed reduced early blight incidence and severity when treating tomato plants with wood biochar. Furthermore, phenolic compounds and antioxidant enzymes were increased in plants treated with green waste biochar alone and enriched with *B. subtilis*, reducing disease severity.

Although both grape bagasse biochars improved rootstock development even under biotic and abiotic stress conditions, composted bagasse biohar exhibited greater performance and stability over storage when compared to fresh bagasse biochar. Moreover, the application of grape bagasse biochars loaded with *B. velezensis* S26 endospores can offer significant benefits to the plants than their utilization without the functional bacteria. Therefore, *B. velezensis* S26 stands out in suppressing soilborne disesases and acting synergistically with the biochars to stimulate plant growth. In agreement with our findings, various studies have demonstrated that the combined effect of the biochar and bacterial agent is higher than single treatments (Tu et al., 2020; Song et al., 2021; Zhang et al., 2023).

5. Conclusions

The beneficial attributes of grape bagasse biochars can be enhanced by using them as a carrier for *B. velezensis* S26 endospores. Additionally, when stored under refrigeration, we demonstrated that these composites maintained their properties, thereby contributing to addressing various challenges in vineyards. As a result, enriched biochars promote eco-friendly viticulture by increasing soil fertility and mitigating the negative impacts of synthetic pesticides and fertilizers.

Our findings underscore the remarkable capacity of fresh and composted bagasse biochars loaded with *B. velezensis* S26 endospores in enhancing the growth of SO4 plants. Furthermore, these biochar composites effectively reduce the impact of copper-contaminated soils and control black foot disease. These results support the practical implementation of biochars as a promising strategy to address the effects of copper stress and soilborne pathogens in viticulture.

Lastly, utilizing grape marc as a biofertilizer and biofungicide contributes to the value aggregation of winery residue, minimizing the environmental repercussions of improper disposal and contributing to carbon sequestration. Therefore, the valorization of grape marc for biochar production underscores the significance of a circular economy and its pivotal role in fostering sustainable viticulture practices.

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Supplementary Material 1

Table S1. Viability of *Bacillus velezensis* S26 endospores loaded in fresh bagasse biochar using Luria-Bertani broth (LB) and saline solution (SS). Equal letters indicate no statistically significant difference among the formulations, as determined by one-way ANOVA, followed by Tukey's test ($P < 0.05$). Each value represents the mean ± standard deviation of three replicates.

Treatments	Temperature	Days of storage							Reduction in endospore viability
		0	30	60	90	120	150	180	
LB	25 °C	8.5 ± 0.3 b	7.8 ± 0.5 a	7.1 ± 0.3	6.8 ± 0.4 b	6.5 ± 0.6 ab	5.9 ± 0.3 b	5.3 ± 0.5 b	37.6 %
SS	25 °C	8.9 ± 0.5 a	7.6 ± 0.2 b	6.6 ± 0.6	6.4 ± 0.3 b	6.3 ± 0.2 b	5.9 ± 0.6 b	5.2 ± 0.5 b	41.6 %
LB	4 °C	8.5 ± 0.5 b	7.9 ± 0.2 a	7.6 ± 0.6	7.5 ± 0.3 a	6.3 ± 0.2 b	5.6 ± 0.3 b	4.4 ± 0.4 c	48.2 %
SS	4 °C	8.9 ± 0.3 a	7.9 ± 0.3 a	7.5 ± 0.5	6.9 ± 0.2 b	6.7 ± 0.4 a	6.6 ± 0.5 a	6.3 ± 0.2 a	29.2 %

Table S2. Viability of *Bacillus velezensis* S26 endospores loaded in fresh bagasse biochar using Luria-Bertani broth (LB) and saline solution (SS). Equal letters indicate no statistically significant difference among the formulations, as determined by one-way ANOVA, followed by Tukey's test ($P < 0.05$). Each value represents the mean ± standard deviation of three replicates.

Treatments	Temperature	Days of storage							Reduction in endospore viability
		0	30	60	90	120	150	180	
LB	25 °C	8.2 ± 0.5	7.9 ± 0.7 ab	7.3 ± 0.3 b	6.9 ± 0.4 b	6.4 ± 0.2 b	5.8 ± 0.4 b	5.6 ± 0.5 c	31.7 %
SS	25 °C	8.2 ± 0.2	7.7 ± 0.5 b	7.2 ± 0.7 b	6.9 ± 0.5 b	6.8 ± 0.3 b	6.7 ± 0.6 b	6.5 ± 0.6 b	20.7 %
LB	4 °C	8.1 ± 0.5	7.9 ± 0.3 a	7.6 ± 0.3 a	7.5 ± 0.2 a	7.5 ± 0.3 a	7.5 ± 0.6 a	7.4 ± 0.2 a	8.6 %
SS	4 °C	8.2 ± 0.2	7.7 ± 0.4 b	6.9 ± 0.6 b	6.6 ± 0.2 c	6.2 ± 0.3 b	6.0 ± 0.4 b	5.8 ± 0.3 c	29.3 %

Supplementary Material 2

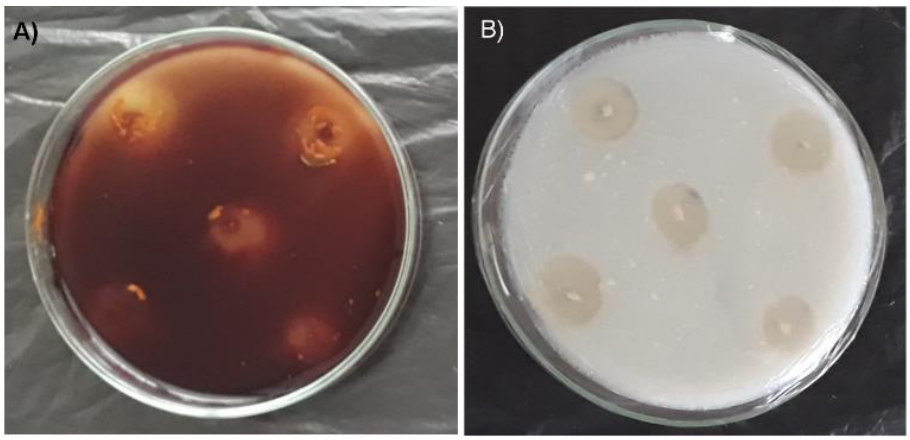

Fig. S2. Photographs depicting plant growth-promoting abilities of *Bacillus velezensis* S26: A) pectinase activity, B) proteolytic activity.

5 DISCUSSÃO GERAL

No presente estudo, utilizou-se a bactéria *Bacillus velezensis* S26, isolada da rizosfera de um vinhedo conduzido sob manejo orgânico e com solo contendo elevados níveis de cobre. Essa rizobactéria foi selecionada também por sua habilidade de produzir compostos indólicos e sideróforos, conforme previamente demonstrado por Debastiani et al. (2023). Esses autores verificaram, ainda, que a aplicação de uma suspensão de células de *B. velezensis* S26 inibiu o crescimento micelial *in vitro* de vários isolados de *Colletotrichum* spp. e auxiliou na promoção do crescimento de estacas do porta-enxerto P1103 (*Vitis berlandieri* × *V. rupestris*), cultivadas em solo contaminados com cobre.

Levando em consideração esses importantes atributos, no presente estudo, foram realizados ensaios destinados a induzir a esporulação de *B. velezensis* S26. Os endósporos são estruturas produzidas em condições ambientais adversas, que asseguram a viabilidade celular bacteriana e proporcionam o desenvolvimento de bioformulações estáveis e com boa vida de prateleira (Posada-Uribe et al., 2016; Grauvy et al., 2021). Após a definição de um protocolo para a produção de esporos de *B. velezensis* S26 em um meio de cultura comercial, demonstrou-se a ação antifúngica desses endósporos, por meio da realização de ensaios *in vitro*.

Embora os esporos necessitem germinar e retomar sua forma vegetativa para, em seguida, promover a inibição dos patógenos, não houve grande discrepância entre o potencial inibitório dessas estruturas em relação às células vegetativas de *B. velezensis* S26. Esse resultado pode ser explicado pela velocidade com que ocorre o processo de germinação de endósporos bacterianos, bem como pela presença do ácido dipicolínico na constituição desses esporos. Segundo estudos recentemente desenvolvidos (Song et al., 2020; Wang et al., 2022), esse composto apresenta propriedades antifúngicas, contribuindo para inibir o crescimento micelial e a germinação de conídios.

Posteriormente, o biocontrole da antracnose e da podridão cinzenta foi verificado por meio da inoculação preventiva de uma suspensão de endósporos de *B. velezensis* S26 em morangos. Neste ensaio, tanto a aplicação de endósporos frescos quanto armazenados em solução salina por um período de seis meses reduziram a incidência e a severidade dessas doenças fúngicas. Similarmente, o emprego de endósporos suspensos em caldo LB possibilitou um efetivo controle da antracnose e da podridão cinzenta em ensaios conduzidos em bagas, discos foliares e plantas de videira. Entretanto, o tratamento com endósporos em caldo de cultivo tende a proporcionar uma maior supressão de doenças fúngicas quando comparado ao emprego de endósporos centrifugados e suspensos em solução salina. Isso ocorre em

decorrência da preservação dos metabólitos, com propriedades antimicrobianas, liberados pelas células bacterianas durante seu crescimento (Gotor-Vila et al., 2017; Sahai et al., 2019) e da possível ação do extrato de levedura, presente na composição desse caldo, como indutor de mecanismos de resistência na planta (Yaguchi et al., 2017). No entanto, a suspensão de endósporos de *B. velezensis* S26 em caldo LB apresentou uma maior perda na viabilidade dos esporos durante o armazenamento. De acordo com Christie and Setlow (2020), nutrientes como L-aminoácidos e D-carboidratos atuam induzindo a germinação dos endósporos, e com isso, diminuindo a viabilidade dessas células.

Os endósporos de *B. velezensis* S26 suspensos em caldo LB controlaram a antracnose e a podridão cinzenta em videiras, prinicipalmente na espécie *V. vinifera*. Contudo, as espécies de videira não apresentaram diferença significativa na suscetibilidade a essas doenças, com algumas diferenças pontuais, dependendo do isolado patogênico inoculado. Espécies de origem americana, como *V. labrusca*, costumam apresentar uma maior resistência a doenças fúngicas quando comparadas às variedades europeias, como *V. vinifera*. Essa resistência se deve, entre outros fatores, à presença de características anatômicas e estruturais que dificultam a penetração do patógeno (Gabler et al., 2003; Herzog et al., 2015; Naegele, 2018). Somado a isso, Kulakiotu et al. (2004) verificaram que os compostos aromáticos voláteis produzidos pela videira cv. Isabella (*V. labrusca*) inibiram a esporulação e reduziram a virulência de *B. cinerea.* Apesar disso, a suscetibilidade a doenças não depende unicamente da espécie, mas também de características inerentes a cada variedade (Wang et al., 2021b).

De forma geral, não houve diferença na suscetibilidade à antracnose e podridão cinzenta entre os diferentes tecidos vegetais infectados com o mesmo isolado fitopatogênico. Todavia, alguns isolados apresentaram uma maior patogenicidade em bagas de uvas da espécie *V. vinifera*. Desse modo, a capacidade de inibição fúngica da suspensão de endósporos de *B. velezensis* S26 variou com a espécie de videira e com o tecido vegetal inoculado. Em concordância com nossos resultados, Krol (1998) testou diferentes agentes de controle biológico em videira e verificou que a ação antagonística desses micro-organismos foi mais efetiva em determinados tecidos vegetais. Por outro lado, Naegele (2018) não observou diferença na patogenicidade e na virulência de dois isolados de *B. cinerea,* quando inoculados em um mesmo tecido vegetal.

Frutos tendem apresentar uma maior suscetibilidade ao ataque de patógenos em virtude do processo de maturação que ocasiona significativas alterações em suas características bioquímicas e fisiológicas. Essas mudanças incluem perda da resistência mecânica, acúmulo de açúcares, sólidos solúveis dissolvidos e ácidos orgânicos, redução do potencial hidrogeniônico

e alterações hormonais (Huang et al., 2017; Fedorina et al., 2022). Entretanto, em um estudo conduzido por Naegele (2018), não houve correlação entre o acúmulo de sólidos solúveis em frutos e a suscetilidade à podridão cinzenta em videiras. Dessa forma, a resistência de bagas de uva a podridões também tem sido relacionada à ocorrência de compostos fenólicos na casca dos frutos (Gauthier et al., 2014), sendo estes presentes em maiores concentrações em uvas tintas do que em uvas brancas, como Niágara branca ou Moscato giallo.

Em termos gerais, a suspensão de endósporos de *B. velezensis* S26 inibiram, de maneira eficaz, tanto isolados de *Botrytis* spp. quanto de *Colletotrichum* spp. em videiras e morangueiros. Nesse sentido, vários estudos demontraram que as bactérias *B. amyloliquefaciens* (Hao et al., 2011; Alvindia, 2013; Alvindia e Acda, 2015), *B. subtilis* (Zhang et al., 2020) e *B. velezensis* (Reyes-Estebanez et al., 2020) apresentaram ação inibitória contra diferentes agentes fitopatopatogênicos em um mesmo hospedeiro. Essa ampla habilidade de supressão fúngica contribui para aumentar a aplicabilidade de bioformulações, garantindo proteção contra diversos patógenos por meio da realização de um único tratamento a campo (Wang et al., 2022).

Entretanto, em uma revisão da literatura realizada por Wang et al. (2022), apesar de *B. amyloliquefaciens* e *B. velezensis* terem apresentado atividade antifúngica contra diversos patógenos em um mesmo hospedeiro, essa eficácia diminuiu drasticamente quando os agentes de biocontrole foram aplicados, contra esses mesmos patógenos, em hospedeiros distintos. Dessa forma, evidências têm indicado uma maior efetividade dos micro-organismos antagonistas em hospedeiros pertencentes à mesma espécie da qual foram isolados (Wang et al., 2022).

Em conformidade com nossos resultados, Toral et al. (2020) observaram que a bactéria *B. velezensis* XT1 isolada de *Juncus effusus* controlou a podridão cinzenta em morangos e tomates. Além disso, os autores verificaram que *B. velezensis* XT1 inibiu o crescimento micelial de vários fungos, incluindo *Alternaria alternata*, *Fusarium oxysporum*, *Magnaporthe oryzae*, *Sclerotinia sclerotiorum* e *Thanatephorus cucumeris*. Similarmente, Lee et al. (2012) observaram que aplicação de *B. subtilis* S16 reduziu a severidade da infecção causada por *Penicillium expansum*, *Botrytis cinerea* e *Colletotrichum acutatum* em maçãs, pêssegos e nectarinas. Assim, esses estudos demonstram a viabilidade do emprego de uma mesma bactéria no controle biológico de patógenos presentes em diferentes culturas.

Em relação ao armazenamento das suspensões contendo endósporos de *B. velezensis* S26, verificou-se que a solução salina armazenada a temperatura de 25 ºC proporcionou uma maior viabilidade celular. Diferentemente, em outros estudos, foi verificada uma maior

viabilidade celular após o armazenamento de formulações líquidas sob refrigeração (Abadias et al., 2003; Melin et al., 2007). Corroborando nossos resultados, Gotor-Vila et al. (2017) obtiveram uma boa viabilidade celular de *B. amyloliquefaciens* CPA-8 tanto em formulações líquidas quanto em sólidas armazenadas a 22 ºC durante o período de um ano.

Uma estratégia para minimizar as perdas na viabilidade celular e compensar um potencial declínio na população desses micro-organismos ao longo do armazenamento envolve a elaboração de formulações mais concentradas. No entanto, esse processo é dispendioso, tornando mais economicamente viável minimizar a taxa de deterioração das bioformulações (Gotor-Vila et al., 2017). Isso pode ser alcançado por meio do armazenamento desses bioprodutos em condições ambientais controladas, bem como evitando a exposição ao oxigênio, temperaturas elevadas, umidade excessiva e incidência luminosa direta (Santhosh, 2015).

Verificada a eficácia da suspensão de endósporos de *B. velezensis* S26 no controle biológico da antracnose e podridão cinzenta, inclusive após armazenamento, iniciou-se o processo de desenvolvimento de bioformulações. Assim, em sintonia com a atual demanda por uma agricultura mais sustentável e seguindo os preceitos da economia circular, optou-se pela elaboração de bioinsumos utilizando subprodutos agroindustriais como uma forma de minimizar custos de produção e, simultaneamente, contribuir para a agregação de valor a resíduos e subprodutos (Yánez-Mendizábal et al., 2012; Sayara et al., 2020).

Dessa forma, o lixiviado da compostagem do bagaço de uva foi utilizado como substituto aos meios comerciais para induzir a esporulação de *B. velezensis* S26. Entretanto, esse subproduto, quando empregado de forma isolada, não conseguiu suprir os nutrientes necessários ao crescimento e esporulação de *B. velezensis* S26. Isso se deve ao fato desses efluentes apresentarem baixas concentrações de carboidratos e compostos nitrogenados, como resultado das transformações ocorridas durante o processo de compostagem (Chen et al., 2019). Somado a isso, esses lixiviados podem apresentar compostos recalcitrantes, como ácidos húmicos e fúlvicos, com ação inibitória sobre as bactérias (Mullane et al., 2015). No entanto, a diluição desse resíduo em água leva a uma redução na concentração das substâncias potencialmente nocivas, viabilizando seu emprego como caldo para cultivo de micro-organismos (Santiago Badillo et al., 2021).

Considerando essas limitações do lixiviado de compostagem, utilizou-se o soro do leite como forma de agregar uma fonte rica em compostos nutritivos. Posteriormente, os ensaios de esporulação demonstraram que tanto a combinação entre o lixiviado de compostagem com o soro de leite quanto o soro diluído em água, promoveram a esporulação em *B. velezensis* S26, em valores superiores àqueles obtidos no meio de cultura LB. De forma similar, Santiago

Badillo et al. (2021) verificaram uma baixa taxa de crescimento e esporulação das bactérias *B. subtilis* ATCC 6633 e *B. megaterium* ATCC 14581 quando cultivadas com lixiviado de compostagem em frascos sob agitação. No entanto, após suplementação com um extrato de levedura proveniente de resíduos da fermentação de cerveja ou permeado do soro de leite houve um aumento na concentração bacteriana no meio de cultivo.

Em seguida, foram elaboradas três formulações líquidas, constituídas por lixiviado de compostagem do bagaço de uva e soro de leite, de forma isolada ou em combinação. Também foi utilizada uma formulação controle constituída por endósporos de *B. velezensis* S26 em solução salina. A eficácia dessas formulações foi avaliada por meio de um ensaio realizado em uma estufa comercial de morangos, com aplicações semanais dos bioinsumos, ao longo de um período de seis meses. Os resultados demonstraram que todas as bioformulações reduziram a incidência e a severidade da antracnose e da podridão cizenta em morangos, não apresentando diferença estatisticamente significativa em relação ao produto Duravel WP®, quando aplicados na mesma concentração.

A viabilidade dos endósporos de *B. velezensis* S26 nessas formulações líquidas, foi avaliada ao longo de seis meses de armazenamento, à temperatura ambiente e sob refrigeração. Os resultados indicaram que a formulação contendo soro de leite armazenada à temperatura ambiente possibilitou uma melhor preservação dos esporos bacterianos. Essa é uma importante característica, visto que viabiliza a estocagem de bioprodutos sem demandar o uso de refrigeração. Em ensaio realizado posteriormente, verificou-se que a formulação contendo soro em sua composição também estimulou o crescimento de morangueiros micropropagados. Isso se deve ao potencial de *B. velezensis* S26 de sintetizar ácido giberélico e enzimas hidrolíticas, como pectinases e proteases, que, em associação com compostos indólicos e sideróforos auxiliaram na promoção do crescimento vegetal (De Moura et al., 2021; Morales-Cedeño et al., 2021). Estudos em andamento demonstraram que *B. velezensis* S26 pode sintetizar lipopeptídeos cíclicos, como fengicina e surfactina. Enquanto a fengicina possui ação antifúngica, a surfactina está envolvida na formação de biofilmes e na indução de mecanismos de resistência em plantas (Etesami et al., 2023).

De forma similar, as formulações secas contendo endósporos de *B. velezensis* S26 imobilizados em biochar de bagaço de uva, mantiveram sua viabilidade durante seis meses de armazenamento. A formulação contendo biochar de bagaço uva compostado apresentou maior estabilidade ao longo do armazenamento e o emprego da refrigeração assegurou uma maior sobrevivência bacteriana, ao passo que a viabilidade dos endósporos não foi influenciada pela temperatura de armazenamento no biochar do bagaço de uva fresco. Diferentemente, Gotor-

Vila et al. (2017) observaram uma acentuada redução na viabilidade de *B. amyloliquefaciens* CPA-8 em uma formulação liofilizada após apenas quatro meses de armazenamento, independentemente da temperatura empregada. Por outro lado, a concentração bacteriana permaneceu estável em uma formulação líquida constituída por caldo de cultivo armazenada ao longo de quatro meses a 4 ºC, enquanto formulações secas obtidas por meio de secagem em leito fluidizado mantiveram a viabilidade celular ao longo de 12 meses nas temperaturas de -20, 4 e 22 ºC.

A viabilidade dos endósporos bacterianos é influenciada por diversos fatores, incluindo a composição físico-química das formulações, aspectos intrínsecos de cada cepa microbiana, bem como as condições de armazenamento (Terpou et al., 2019). Assim, Sorokulova et al. (2008) verificaram que a presença de compostos protetores, como carvão ativado, tapioca e goma de acácia, em formulações secas reduziu a taxa de degradação dos esporos de *B. subtilis* ATCC 6051 durante dois meses armazenamento. Adicionalmente, em um estudo conduzido por André et al. (2021), a sobrevivência dos endósporos de diferentes linhagens de *B. coagulans* foi influenciada pela temperatura de armazenamento, ao passo que a utilização de formulações com valores distintos de pH não apresentou um efeito significativo sobre a viabilidade dos endósporos.

Nossas pesquisas também demonstraram que as formulações secas elaboradas com os biochares de bagaço de uva promoveram o crescimento de porta-enxertos micropropagados de videira. Esse resultado foi verificado tanto por meio da utilização do biochar de forma isolada quanto como suporte para os endósporos de *B. velezensis* S26. Isso se deve ao fato de que a adição do biochar ao solo contribui para melhorar suas propriedades físico-químicas, como capacidade de retenção de água, pH, porosidade, agregação e aeração (Ajeng et al., 2020). Adicionalmente, a presença de nutrientes no biochar também pode contribuir para aumentar a fertilidade do solo (Arabi et al., 2018).

No entanto, a imobilização de micro-organismos benéficos, como agentes de biocontrole e bactérias promotoras de crescimento potencializa a ação benéfica dos biochares (Ajeng et al., 2020). Dessa forma, o uso dos biochares do bagaço de uva associado com os endósporos de *B. velezensis* S26, resultou na redução da ocorrência do pé-preto no porta-enxerto de videira SO4 e no incremento do crescimento e da biomassa, inclusive de porta-enxertos cultivados em substratos contaminados com cobre.

Em síntese, bioformulações contendo endósporos de *B. velezensis* S26 promoveram o biocontrole dos fungos *Botrytis* spp. e *Colletotrichum* spp., tanto em ensaios *in vitro* quanto em experimentos realizados com frutos e plantas micropropagadas de videira e morangueiro.

Somado a isso, a viabilidade celular e o potencial inibitório de *B. velezensis* S26 foram preservados após armazenamento, tanto em formulações líquidas quanto em formulações secas, contribuindo para o controle da antracnose, podridão cinzenta e pé-preto, bem como para estimular o crescimento vegetal de morangueiros e videiras e suprimir os efeitos adversos do excesso de cobre no solo.

6 CONCLUSÕES

A partir dos resultados obtidos neste estudo foi possível concluir que:

a) A realização de ensaios em frascos de cultivo possibilitou otimizar a esporulação de *B. velezensis* S26 em meio LB, resultando em uma concentração final de $2,0 \times 10^{10}$ esporos mL^{-1}, por meio do ajuste de condições de cultivo como pH, temperatura, tempo de esporulação, concentração de inóculo e adição de nutrientes;

b) A utilização das condições ótimas para esporulação, determinadas em meio LB e adaptadas às características dos subprodutos agroindustriais, possibilitou a obtenção de concentrações de $1,2 \times 10^{12}$ esporos mL^{-1} empregando lixiviado de compostagem combinado com soro de leite e $4,3 \times 10^{12}$ esporos mL^{-1} utilizando soro isoladamente, em frascos de cultivo;

c) A indução da esporulação de *B. velezensis* S26 em biorreatores possilitou a obtenção de concentrações de endósporos similares àquelas obtidas em frascos de cultivo, tanto em meio LB quanto nos meios elaborados com lixiviado de compostagem e soro de leite;

d) Tanto a aplicação de uma suspensão de endósporos quanto de uma suspensão de células vegetativas de *B. velezensis* S26, suprimiu o crescimento fúngico micelial de *Colletotrichum* spp. e *Botrytis* spp., principalmente no antagonismo por compostos difusíveis;

e) Os tratamentos com endósporos, células vegetativas e filtrado do cultivo de *B. velezensis* S26 promoveram uma significativa inibição da germinação de conídios de *Colletotrichum* spp. e *Botrytis* spp.*;*

f) A viabilidade celular e a eficácia no biocontrole e na promoção do crescimento de uma suspensão endósporos de *B. velezensis* S26 foram preservados após seis meses de armazenamento. Embora, o armazenamento sob refrigeração do biochar de bagaço fresco de uva tenha possibilitado uma maior sobrevivência bacteriana, o armazenamento à temperatura ambiente da formulação líquida contendo soro em sua composição mostrou-se igualmente eficaz;

g) A aplicação de uma suspensão de endósporos de *B. velezensis* S26, tanto na forma fresca quanto após seis meses de armazenamento, promoveu uma redução na incidência e severidade da antracnose e da podridão cinzenta em morangos;

h) O uso de uma suspensão de endósporos de *B. velezensis* S26, tanto na forma fresca quanto após seis meses de armazenamento, controlou a antracnose e a podridão cinzenta em bagas de uva, discos foliares e plantas de videira micropropagadas, com resultados mais significativos em bagas de uvas da espécie *V. vinifera*;

i) Três formulações líquidas distintas foram elaboradas com soro de leite e lixiviado de compostagem, isoladamente ou em combinação. No entanto, a formulação contendo soro em sua composição garantiu uma maior viabilidade dos endósporos de *B. velezensis* S26 após seis meses de armazenamento;

j) As formulações líquidas promoveram o biocontrole da antracnose e da podridão cinzenta em morangueiros comercialmente produzidos, com uma eficácia similar ao produto Duravel WP®;

k) Duas formulações secas foram desenvolvidas pela incorporação dos endósporos de *B. velezensis* S26 a biochares resultantes da pirólise do bagaço de uva fresco e compostado. Enquanto a temperatura de armazenamento não teve influência na viabilidade celular dos endósporos imobilizados no biochar de bagaço compostado, o uso de refrigeração possibilitou uma maior sobrevivência bacteriana em biochar proveniente do bagaço de uva fresco;

l) Os biochares contendo endósporos de *B. velezensis* S26 foram eficazes no controle do pé-preto, no aumento da biomassa e na mitigação dos efeitos inibitórios do cobre em porta-enxertos de videira micropropagados.

7 PERSPECTIVAS

As perspectivas futuras deste trabalho incluem:

a) Avaliar o efeito do choque térmico e da adição de diferentes aditivos no aumento da viabilidade dos endósporos de *B. velezensis* S26 ao longo do armazenamento;

b) Verificar o efeito do armazenamento sob condições variáveis de temperatura;

c) Analisar quantitativa e qualitativamente os compostos antimicrobianos liberados em caldo de cultivo, bem como as alterações ocorridas durante o armazenamento das formulações líquidas produzidas nos biorreatores;

d) Determinar a eficácia das formulações líquidas obtidas por meio do cultivo em biorreator na promoção do crescimento e no biocontrole de doenças do morangueiro e da videira a campo;

e) Analisar a eficácia de formulações secas a campo, tanto no controle biológico de doenças fúngicas quanto na promoção do crescimento vegetal, em morangueiros e videiras;

f) Determinar os intervalos para reaplicação das bioformulações visando maior persistência ao longo do tempo;

g) Verificar a presença de genes de patogenicidade e de compostos toxicogênicos no genoma de *B. velezensis* S26;

h) Avaliar a aplicação dos endósporos de *B. velezensis* S26 associado a revestimentos comestíveis em frutos durante a etapa de pós-colheita;

i) Testar o efeito cumulativo de sucessivas aplicações de bioformulações, desde a produção até o armazenamento, na fase de pós-colheita;

j) Avaliar o efeito da aplicação de uma suspensão de endósporos de de *B. velezensis* S26 no aumento da vida de prateleira de morangos;

k) Determinar as condições ótimas para esporulação de *B. velezensis* S26, empregando permeado de soro;

l) Analisar a composição físico-química do soro e dos biochares, assim como, demais aspectos envolvidos na adsorção dos endósporos de *B. velezensis* S26 aos biochares;

m) Realizar ensaio a campo com morangueiros, empregando um controle negativo e comparando a eficácia das bioformulações com outros produtos comerciais.

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