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RESEARCH ARTICLE

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

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ABSTRACT

Grape powdery mildew (GPM) control is based on the preventive use of sulfur and curative application of synthetic fungicides, increasing the risk for producers' health and environmental and fruit contamination. This work aimed to select *Bacillus* strains isolated from the Brazilian tropical semi-arid region that are effective antagonists to GPM. In an initial screening performed by spraying bacterial suspensions in detached grape leaves, six strains of *Bacillus* spp. showed disease symptom reduction higher than 70.0%. Two greenhouse experiments showed that the bacterial strains LCB03, LCB28, and LCB30 showed control efficiency >80%, statistically similar to a commercial formulation with *Bacillus amyloliquefaciens* QST713. 16s rDNA sequencing showed that strain LCB03 showed 100.0% homology to *B. velezensis*, while LCB28 has high homology to *B. tequillensis* (99.93%), and LCB30 had 99.71% homology with *B. siamensis*. As an average for both greenhouse experiments, weekly application of *Bacillus* sp LCB03, *Bacillus* sp LCB28, and *Bacillus* sp. LCB30 reduced the average incidence by around 50% and more than 80% for GPM severity.

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Grape powdery mildew; *Bacillus* sp; biocontrol; antagonism

Introduction

Table grape (*Vitis vinifera* L.) is a prominent crop grown in the São Francisco River valley located in the Northeastern region of Brazil. The tropical semi-arid climate in this region enables continuous plant growth, allowing scheduling production throughout the year. This production system is further supported by crop management technology based on pruning, irrigation, and plant growth regulators (Camargo et al., 2008). However, grape powdery mildew (GPM) and downy mildew are the primary grape diseases in the tropics. The co-existence of grape plants in different developmental stages throughout the year complicates disease management (Buffara et al., 2014). GPM is caused by the obligate biotrophic heterothallic fungus *Erysiphe necator* (Schw.) Burr. [syn. *Uncinula necator* (Schw.) Burr., anamorph *Oidium tuckeri* Berk.] (Braun & Takamatsu, 2013). In temperate regions, GPM epidemics are triggered by rains at the beginning of the

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growing season, causing ascospore dispersal and germination (Jarvis et al., 2002). However, in tropical regions, the sexual stages of *E. necator* do not occur (Bettiga et al., 2013). Primary inocula originate from the latent infections in the prophylls within the bud, branches from previous years, and conidia transported from neighbouring fields (Bettiga et al., 2013; Buffara et al., 2014). In semi-arid tropical regions, GPM epidemic outbreaks are favoured by mild temperatures at night, higher relative humidity, and cloudy weather without rain (Bettiga et al., 2013; Jarvis et al., 2002).

The continuous grape production in the tropical regions presents a complex epidemiological environment that requires intensive use of synthetic fungicides to control GPM, mainly those in the chemical groups of triazoles, strobilurins, and sulfur (Gadoury et al., 2012; Sawant et al., 2017). However, the constant use of these fungicides poses a significant risk of contamination for both the fruit and the environment and the development of pathogen resistance. As a result, grape producers have been exploring healthier and more environmentally friendly approaches to managing GPM. Using antagonist microorganisms is one promising alternative to synthetic fungicides.

Bacillus spp. strains have been extensively studied as biocontrol agents for shoot and root plant pathogens, and they have become the most important biological control agents (BCA) currently commercialised (Shafi et al., 2017). The genus comprises a group of rhizosphere and phyllosphere-competent strains able to establish epiphytic and endophytic colonisation of plant tissues and potentially become efficient biocontrol agents against GPM (Silva et al., 2021; Zeigler & Perkins, 2021). Numerous studies have demonstrated that *Bacillus*-based biofungicides can effectively control grapevine diseases (Pertot et al., 2017), including *E. necator*, in various grape production regions (Sawant et al., 2016, 2017). It is worth noting, however, that the efficacy of *Bacillus*-based biofungicides can be affected by climate and cultural practices. Despite these challenges, using *Bacillus*-based biofungicides remains a promising strategy for sustainable and environmentally friendly grape production. Thus, continuous efforts are being made to identify and develop more efficient *Bacillus* strains for GPM management (Fira et al., 2018). This work aimed to select *Bacillus* strains isolated from different sources to be applied against the obliged biotrophic pathogen *E. necator*, the grape powdery mildew causative agent.

Materials and methods

Inoculum of E. necator

Powdery mildew inoculum was obtained from naturally infected leaves of *Vitis vinifera* cv. 'Sugraone' and cv. 'Thompson Seedless' collected in a vineyard at the Experimental Farm of Embrapa (Petrolina, Brazil). A conidial suspension was extracted from heavily sporulating lesions using Triton X-100 0.05% (v/v). The suspensions were standardised at 10^7 conidia mL^{-1} and inoculated onto plantlets of grape cv. Sugraone. The plants were kept at 26 (±1)° C and 70% relative humidity (RH) in a growth chamber with 12 h of photoperiod. After the initial symptoms were observed, infected plants were transferred to a greenhouse.

Bacillus *strains and production of a technical-grade formulation*

Forty-five *Bacillus* strains maintained in the Collection of Microorganisms of Agriculture Interest of the Embrapa Semiárido (CMISA) were evaluated for their ability to reduce the

incidence and severity of lesions caused by *E. necator*. The bacterial strains were maintained at −80°C, subcultured after thawing in nutrient agar (Himedia, Mumbai, India), and kept at 27°C for 48 h. The bacterial strains were grown in Luria broth (LB) media (Himedia, Mumbai, India) for 24 h in an orbital shaker (120 rpm) and standardised to an optical density (OD) of 0.5 at 595 nm.

Technical grade formulations (TGF) of the *Bacillus* strains were prepared by adding cell suspensions to a preparation containing previously autoclaved natural polymer solution at 1.2% (patent pending).

Prescreening Bacillus *strains against GPM*

A fast-throughput experiment using detached grape leaves was conducted to screen antagonist strains. Fully developed grape leaves were collected from grape plants cv. 'Sugraone' grown in Embrapa experimental farm (Petrolina, Brazil; −9.134949, −40.307601). Immediately, their petioles were inserted into microtubes containing sterilised distilled water (SDW) and transported to the laboratory. The leaves were superficially sterilised using cotton soaked in sodium hypochlorite solution (1.0% v/v) and placed in an aseptic chamber with UV-C light on each side for 5 min.

Three treatments were applied in addition to the *Bacillus* strains: control (autoclaved distilled water); micronized sulfur (Kumulus® DF, BASF, São Paulo, Brazil) 1.0 g L^{-1}; *B. amyloliquefaciens* QST713 (Serenade, Bayer CropScience, Tlaxcala, Mexico) 10^7 endospores L^{-1}. Bacterial suspensions were sprayed on ten leaves per treatment using a bench atomiser. After evaporating the excess liquid, they were inoculated with 20 µL of a conidia suspension of *E. necator* with 10^7 conidia mL^{-1} at three points on the abaxial leaf surface (two at the base and one at the apex of the leaf). After applying the treatments, the leaves were kept in a growth chamber (25°C; 70% RH) with 12 h of photoperiod.

The diameter of the lesions caused by GPM was measured seven days after inoculation using a caliper in two perpendicular axes, one parallel to the main nerve of the leaves. The average of the measures was used to calculate lesioned areas for each leaf, considering it a perfect circle. Leaf area was calculated using ImageJ software V. 1.5 h (Schneider et al., 2012), available at https://imagej.net/ij/index.html. Disease severity was expressed as the percentage of leaf area colonised by *E. necator* mycelia. The complete experiment was repeated twice using independent leaf groups.

Control efficiency in greenhouse experiments

Healthy grape seedlings cv. 'Sugraone' were planted in pots containing 5 kg Yellow Argisol soil collected from the experimental farm of Embrapa Semiárido (Petrolina, Brazil; – 9.134949, – 40.307601) and mixed with 10% (w/w) manure. A second experiment was conducted using potted plants of grapes cv. 'Redglobe'.

The experiments were performed in greenhouse conditions (26.5 ±2.0°C, RH 60 ±10%) with forced ventilation. Soil water content was monitored by weighing pots every two days, and irrigation was conducted using a drip irrigation system (flow rate 4.1 L h^{-1}). The treatments were: (1) a control treatment sprayed only with SDW; (2) micronized sulfur at 1.0 g L^{-1}; 3. *B. amyloliquefaciens* QST713 10^7 endospores mL^{-1};

4–9. Suspensions containing 10^7 cells mL^{-1} of *Bacillus* sp. LCB03, LCB05, LCB28, LCB30, LCB42, and LCB45. The experiments were repeated twice using a different set of plants and carried out in a completely randomised design with three repetitions and ten plants per replicate. The treatments started when plants reached five leaves with an average transect larger than 5.0 cm.

Treatment spraying was performed using an electric handheld sprayer with a standard hollow cone nozzle (flow rate 120 mL min^{-1}). After spraying, the plants were divided into groups containing all treatments and evenly distributed in the greenhouse. The inoculation of the pathogen occurred naturally, distributing two plants with high incidence and severity of GPM to each group (Punja et al., 2019). The position of the inoculum-producing plants was changed daily throughout the experiment to ensure the homogeneous distribution of GPM inoculum. The treatments were applied weekly for four weeks. GPM's incidence (number of symptomatic leaves) and severity (injured area) were evaluated weekly using the diagramatic scale developed by Buffara et al. (2014).

Identification of bacterial strains

Three selected bacterial strains were identified by 16S-rRNA gene sequencing. PCR amplification was performed using a universal primer 27F (5′-AGAGTTT-GATCCTGGCTCAG-3′) and 1492R (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al., 1989). PCR products were purified and sequenced using the Sanger sequencing method by the service of Macrogen Inc. (Seoul, Korea). The degree of similarity of reported sequences was analyzed using EzBioCloud database (https://www.ezbiocloud.net/identify database v. 08 2023) (Yoon et al., 2017). Sequences with the highest similarity were selected and aligned using Clustal-W (Chenna et al., 2003). The neighbor-joining tree was determined using a maximum-likelihood method based on model testing using MEGA11 (Kumar et al., 2016). Measures of bootstrap support for internal branches were obtained from 500 pseudoreplicates (Felsenstein, 1985). Sequences of the identified strains were submitted to GenBank.

Data analysis

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

In the greenhouse experiments, disease severity data were used to calculate the area under the disease progress curve (AUDPC), according to Madden et al. (2007). Disease severity was obtained using the equation $DI\% = \frac{(D \times n)}{N} \times 100$, adapted from the McKinney index (Madden et al., 2007), where D is the value conferred using the diagrammatic scale, n is the number of leaves to each the value for each plant, and N is the number of leaves for each plant. The apparent infection growth rate (r) was estimated

using the procedure defined by Kushalappa and Ludwig (1982). The control efficiency (E %) was calculated based on the percentage reduction of the injured leaf area at the end of the experiments.

Data from the greenhouse experiments were subjected to analysis of normality (Kolmogorov–Smirnov test) and homogeneity of variance ('Levenes' test) before ANOVA. An initial ANOVA was conducted using a factorial design, in which the experiments were defined as a factor. This procedure showed a significant interaction between the two experiments, and the data were analyzed separately. Percentage data were arcsine transformed while AUDPC was square root transformed for ANOVA, but the results were presented as the original unities. Incidence data for control treatment in the first experiment reached 100.0% in all repetitions, resulting in a variance equal to zero, and they were removed from the data set for ANOVA in this experiment (Gelman et al., 2005). ANOVA was followed by Tukeys' multiple comparison test ($p < 0.05$). Statistical analysis was performed using Statistica for Windows v. 12 (StatSoft Inc., Tulsa, USA).

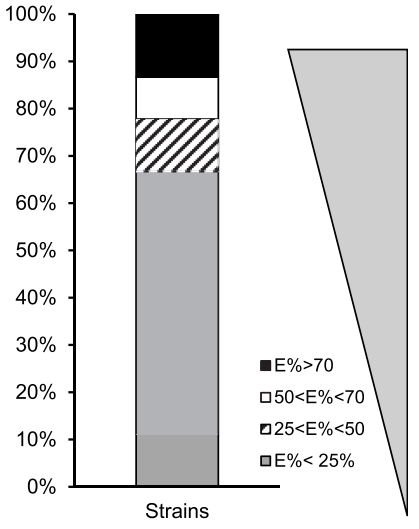
Results

Prescreening of Bacillus *strains against GPM*

Despite being kept in growth chambers with controlled RH, some detached leaves quickly dehydrated and had to be removed from the experiment. However, inserting their petioles into microtubes containing SDW immediately after cutting reduced the leaves' dehydration. All detached grape leaves showed GPM symptoms after inoculation, with the control treatment having an average 75.28% injured area (Figure 1). Among the tested strains, 66.8% did not exhibit more than a 25% reduction in the injured area compared to the control treatment. Only the six *Bacillus* strains (13.3%) highlighted in the table embedded in Figure 1 demonstrated a relative control efficiency of 70% or higher on detached grape leaves. These strains were selected for further testing as potential BCA against the GPM. It's worth noting that *Bacillus* strains LCB03 and LCB45 had average severity values similar to those of the reference treatments, resulting in relative efficiency of 77.28% and 76.41%, respectively.

Control efficiency in greenhouse experiments

In the first experiment, we evaluated the effect of weekly spraying of a technical grade formulation containing six experimental strains over the occurrence of GPM in the grape cultivar Sugraone. Applying the antagonists significantly reduced GPM incidence ($F_{8;\ 27} = 16.8385$; $p < 0.001$) and severity ($F_{8;\ 27} = 13.0813$; $p < 0.001$). The control treatment showed 68.8% disease incidence on the fifth day, reaching 100% on the 12th after introducing the inoculum source. Thus, control data from the experiment were excluded from ANOVA and post hoc tests. The treatments also significantly affected the AUDPC ($F_{8;\ 27} = 3{,}661$; $p = 0{,}023$). The sulfur treatment delayed the development of symptoms until the eighth day, resulting in a lower disease growth ratio, severity, and AUDPC. All *Bacillus* strains except for LCB42 significantly reduced GPM incidence by the Tukeys' test ($p< 0.05$). According to Tukeys' test, treatments with LCB03, LCB28, LCB30, and LCB45 showed control efficiency similar to QST713 and sulfur (Table 1).



Treatments	Severity (%)	Efficiency (E%)
Control	75.22 (9.48)	0.00
Sulfur	3.31 (0.57)	95.56
QST713	12.97 (2.16)	82.57
LCB03	16.95 (2.92)	77.28
LCB45	17.58 (2.65)	76.41
LCB30	22.09 (3.67)	73.66
LCB05	21.40 (3.46)	71.50
LCB28	22.05(3.97)	70.56
LCB42	22.59 (3.70)	69.74

Figure 1. Distribution of strains into the different classes of reduction in symptomatic leaf area produced by co-inoculation of *E. necator* conidial suspension and *Bacillus* strains. The table on the right shows the GPM severity (means ± SD) in detached leaves and the average reduction observed for the most efficient strains.

However, treatments exhibited different disease growth curves. Control efficiency curves showed a decay in BCAs' ability to interfere with disease incidence increase (Supplementary Figure (SF) 1) and severity (SF 2) increase over time. All strains showed reduced control efficiency around the eighth day when the incidence and severity of GPM rapidly increased. Nevertheless, plants treated with LCB05 showed an incidence lower than 50% on day 12, and LCB05 and LCB45 showed a control efficiency curve close to the sulfur treatment.

The application of the TGF containing the BCAs during the second greenhouse experiment had a significant impact on GPM incidence ($F_{8;\ 27} = 22.2608$; $p < 0.001$) and severity ($F_{8;\ 27} = 28.8802$; $p < 0.001$). The control treatment showed a disease incidence of 98.9%

Table 1. Effect of weekly application of *Bacillus* strains on the disease incidence, severity (McKinney index), area under the disease progress curve (AUDPC), apparent disease growth rate (*r*), and control efficiency of powdery mildew in plants of grape cv 'Sugraone' in greenhouse conditions.

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

*Average incidence in control treatment was 100.0% in all repetitions, showing variance equal to zero. Therefore, these data could not be applied to ANOVA and Tukeys' test (Gelman, 2005). †Treatments with the same letters in the columns did not differ by Tukeys' test ($p < 0.05$).

and a severity of 32.5% (Table 2). The statistical analysis showed that all treatments effectively reduced the GPM apparent growth rate (*r*) in grape plants compared to the control treatment, significantly decreasing disease severity and AUDPC. While treatments with LCB03 and LCB28 showed similar disease incidence to the sulfur and QST713 treatments, LCB30 and LCB45 treatments had a lower AUDPC, which was similar to the sulfur treatment based on Tukeys' test results ($p < 0.05$).

In the second experiment, the disease incidence curves revealed that sulfur and LCB28 treatments prolonged the onset of initial symptoms until day 13 (SF 3). The incidence curves also indicated that LCB05 was ineffective in preventing *E. necator* infection after the initial exposure to the inoculum source, as disease symptoms were observed on the fifth day and rapidly increased. In contrast, LCB03 and LCB30 consistently delayed symptom development and demonstrated a reduced infection rate of new leaves (SF 3). However, the most noteworthy findings were observed in the disease severity evolution curves, which demonstrated a significant reduction in leaf area damage caused by GPM throughout the experiment due to applying the BCAs (SF4).

Identification of the bacteria strains

The strain LCB03 was isolated from a grape rhizosphere sample collected from a grapevine cultivated in a sandy Ultisol, and LCB28 and LCB30 were isolated from the rhizosphere and root tissue, respectively, of *Passiflora edulis* Sims (Passifloraceae: Malpighiales) grown in the same soil in the experimental farm of Embrapa (Petrolina, Brazil). They are gram-positive and spore-forming bacteria with rod-shaped cells. BLAST and EzTaxon-edatabase–EzBioCloud (https://www.ezbiocloud.net/identify) analysis were used to compare the 1500-bp 16S rRNA gene sequence. The results showed that all three antagonic bacteria are closely related to *Bacillus* (Firmicutes; Bacilli; Bacillales; Bacillaceae). Contig blasting showed that LCB03 was 100.0% related to the type strain of *B. velezensis* CR-502 (AY603658) Ruiz-García et al. (2005), while LCB28 showed 99.93% nucleotides identity shared with *B. tequilensis* KCTC13622 (AYTO01000043) Gatson et al. (2006), and LCB30 shared 99.71% homology with *B. siamensis* KCTC13613 Sumpavapol et al. (2010).

Table 2. Suppression of GPM by the weekly application of *Bacillus* strains on cv 'Sugraone' plants in greenhouse conditions. The table shows the average (± standard deviation) of disease incidence, severity (''McKinney's' index), area under the disease progress curve (AUDPC), apparent disease growth rate (*r*), and control efficiency of GPM.

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

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

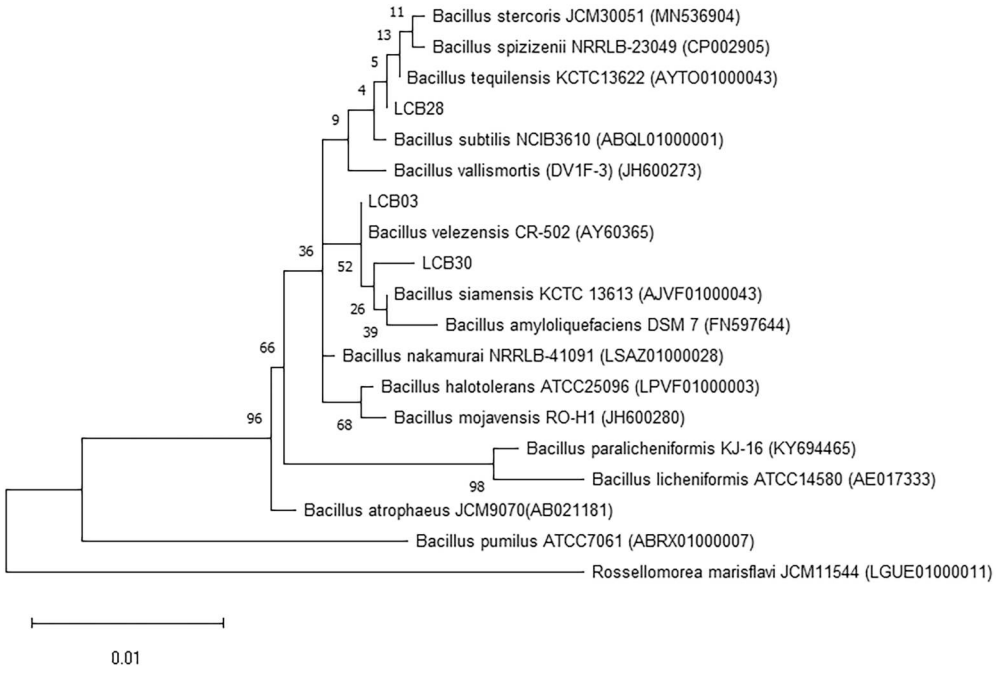


Figure 2. Phylogenetic tree of the *Bacillus* strains based on the sequence of 16S rDNA gene sequence analysis constructed using the neighbor-joining method. The tree was rooted using the 16S rDNA sequence of *Rosselomorea marisflavi* (Bacillaceae; Rossellomorea), and the level of bootstrap support (1000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

Phylogenetic analysis of the 16S rRNA sequences strain using maximum-likelihood relatedness suggested that LCB03 clustered in one group with *B. velezensis,* showing 100.0% homology (Figure 2), while LCB30 grouped within a subcluster together with *B. siamensis* KCTC13613 (99.71% homology) and *B. amyloliquefasciens* DSM7 ((99.64% homology). LCB28 clustered with three species: *B. tequilensis* KCTC13622 (99.93% homology), *B. stercoris* JCM30051 (99.78% homology) (Adelskov & Patel, 2017; Dunlap et al., 2020) and *B. spizizenii* (99.78% homology) JCM30051 (Dunlap et al., 2020; Nakamura et al., 1999). The 16S-rRNA sequences of the strains were deposited in GenBank with accession numbers *B. velezensis* LCB03 OP453366, *B. tequilensis* LCB28 OP453367, and *B. siamensis* LCB30 OP454462.

Discussion

This work aimed to select antagonistic *Bacillus* strains against *E. necator* in a collection obtained from different plants and soils in the Brazilian semi-arid region. These strains have already shown *in vitro* and *in vivo* antagonism against pathogens such as *Fusarium* spp. and *Sclerotium rolfsii* (Sá et al., 2019b, 2019a), *Lasiodiplodia theobromae* (non-published data), and nematode (Carvalho-Júnior et al., 2021). Since the experiments should use living tissue, testing potential biocontrol agents against obligatory biotrophic plant pathogens like *E. necator* was challenging. The duration of the experiments in the pre-screening assays, for example, was limited to the period before the grape leaves

showed senescence signals. However, applying detached leaves assays, six strains reduced more than 70% of the lesioned area caused by the inoculation of conidia of *E. necator,* reaching control efficiency similar to that obtained by the application of the commercial strain *B. amyloliquefaciens* QST713.

When preventively applied to the potted grape plants before their exposition to the inocula, three of the *Bacillus* strains selected in the prescreening reduced GPM development in the greenhouse experiments. Considering the average of the two greenhouse experiments, the strains LCB03, LCB28, and LCB30 showed incidence values half of the control treatment and reduced GPM severity by nearly 90%. Their GPM control efficiency was similar to those obtained with weekly spraying sulfur and the commercial strain QST713. Previous studies with preventive spraying of antagonist *Bacillus* strains presented effective control, such as the results obtained for *B. subtilis* GLB191 and GLB197 against grape downy mildew (Zhang et al., 2017) and *B. subtilis* B27 and B29 applied against GPM (Maachia et al., 2015). In these studies, the control efficiency also decayed rapidly on the eighth day after exposing the grape plants to the inoculum source. In fact, even sulfur treatments showed a reduction of control efficiency throughout the experiment duration.

Four successive reapplications of the strains in the second greenhouse experiment showed a lower disease growth ratio, incidence, and AUDPC through six weeks. Most treatments showed a U-shaped curve for the control efficiency estimated with incidence data throughout the experiment, except for sulfur, LCB03, and LCB28, which showed a slow decay of control efficiency. A similar reduction in disease control efficiency during the experiment was observed from the disease incidence data in a study by Ghule et al. (2019), applying mycoparasites for the biological control of GPM in India. Analyzing the incidence curve of cucumber PM caused by *Podosphaera xanthii* Sarhan et al. (2020) also showed that while a synthetic fungicide showed a linear decay of control efficiency, the application of BCA treatments, including *Bacillus subtilis*, also showed an inverted parabola. A U-shaped curve would indicate a recovery in the control efficiency over time, which would not be accurate. This pattern is likely due to the rapid increase in the GPM growth rate in the control treatment in the late days of the experimental period.

A linear loss of efficiency over time is commonly observed, even when applying synthetic fungicides, given the increase in the inoculum pressure. Meanwhile, U-shaped efficiency curves may be caused by climate events that could originate in highly favourable periods for inoculum production and tissue infection, spiking epidemic outbursts. Conversely, efficiency loss, or a low residual effect, could be caused by poor colonisation of grape leaves, maintaining tissue spots open for infections. Compared to the rhizosphere, the phyllosphere is an oligotrophic environment where nutrients are scarce and show a heterogeneous nature, requiring the BCA to adapt to the habitat (Vorholt, 2012). On the other hand, a limited competence in colonising plants' phyllosphere and infection entries (hydathodes and stomata) could cause the limited effectiveness of a BCA. Since GPM does not require entry sites, infection of non-protected tissue spots is still more probable. Besides, fast-growing young leaves can double their area in a few days, allowing the formation of unprotected spots. Therefore, new studies are required to evaluate the phyllosphere colonisation competence and adjust an efficient spraying strategy.

All three species selected in this study belong to the Subtilis Clade, gathering *Bacillus* species recognised to stimulate plant growth, biocontrol of plant disease, increase soil nutrient availability and induce plant tolerance to abiotic stress (Luo et al., 2022). In this study, 16S rRNA sequence analysis showed that LCB03 was 100.0% related to the type strain of *B. velezensis*, a species with diverse strains with the potential to control plant pathogens already used in commercial formulations (Wang et al., 2023). Strain LCB28 was closely related to *B. spizizenii*, *B. stercoris,* and *B. tequilensis.* All these species showed a close relationship with *B. subtilis,* sharing a common ancestor (Gatson et al., 2006), and only recently were identified as independent species (Dunlap et al., 2020). Meanwhile, the strain LCB30 was closely related to *B. siamensis,* a species phylogenetically close to *B. amyloliquefasciens* (Sumpavapol et al., 2010), known to produce volatile and cyclic polypeptides with a broad antifungal spectrum (Xu et al., 2018). Additional studies, including multilocus sequencing, shall be conducted to provide a clear species identification.

Achieving efficient control using bacteria-based biofungicide as a sole strategy in open field experiments is complex because field reinfection can occur by inoculum entry from neighbouring plots. Nevertheless, more importantly, the antagonists would face adverse climate conditions (Ghule et al., 2019). This study did not evaluate the mechanisms of action of the *Bacillus* strains. Still, previous works have already shown that *Bacillus* species produce hydrosoluble and volatile antifungal compounds and lytic enzymes and elicit plant defense responses (Wang et al., 2023). Nevertheless, new studies are necessary to define formulations and application strategies for inserting the strains in an integrated GPM management programme.

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

Disclosure statement

No potential conflict of interest was reported by the author(s).

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