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EPIPHYTIC AND ENDOPHYTIC BACTERIA FOR THE CONTROL OF *Botrytis cinerea* IN VITRO AND IN GRAPE BERRIES OF CV. SAUVIGNON BLANC IN SOUTHERN BRAZIL

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Abstract

Botrytis bunch rot (BBR) in grapevines caused by *Botrytis cinerea* is a pre- and post-harvest disease that leads to significant losses in southern Brazil. This study aimed to assess the potential of epiphytic and endophytic bacteria to control *B. cinerea in vitro* and *in vivo* in the cv. Sauvignon Blanc (SB). *B. cinerea* and epiphytic and endophytic bacterial isolates were obtained from the grape clusters and leaf phyllosphere (epiphytic and endophytic) of the SB variety, respectively. Four epiphytic and two endophytic isolates that showed the highest antibiosis indices *in vitro* and *in vivo* were identified using phenotyping and DNA sequencing. Treatments were carried out in a completely randomized experimental design with five replications, including (a) control, (b) *B. cinerea*, (c) commercial product (Ecoshot®) with *Bacillus amyloliquefaciens*, (d) epiphytic bacteria, and (e) endophytic bacteria. Among the 52 isolates, 4 epiphytic and 2 endophytic isolates showed the highest degree of inhibition and significantly inhibited the growth of *B. cinerea*. The UEP40, UEP43, UEN13, and UEN14 isolates yielded positive reactions for urea and indole. The epiphytic isolates UEP43 (identified as *Serratia marcescens*) and UEP51 (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*) and the endophytic isolate UEN13 (*Staphylococcus equorum*) reduced the severity of BBR on detached grape berries by ~55% and 49% between 72 and 168 h after inoculation, respectively. These bacterial isolates are potential biocontrol agents against *B. cinerea*. However, more research is needed to confirm their efficacy in vineyards in southern Brazil.

Keywords: Biocontrol. Botrytis bunch rot. Phyllosphere. Vitis vinifera L.

1. Introduction

The culture of grapevines (*Vitis vinifera* L.) in many regions of southern Brazil has been challenged by several diseases, which is facilitated by favorable environmental conditions (Calonnec et al. 2020). Botrytis bunch rot (BBR) is one of the most severe grapevine diseases, reducing fruit yield and quality by ~50% to 80% through direct infection of berries or by reducing plant vigor (De Bem et al. 2015). BBR is caused by the fungus *Botrytis cinerea* Pers.: Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] and can lead to ~100% losses, particularly in highly susceptible grape varieties such as Cabernet Sauvignon (CS), Chardonnay, and Sauvignon Blanc (SB) (De Bem et al. 2015). Primary infections occur during the flowering, fruit-set, and post-veraison stages of the berries, with losses resulting from the rotting of berries in the

field or during storage. Although the berries of almost all grape varieties are susceptible to BBR, losses are more prevalent in tight-clustered varieties of *V. vinifera* and French hybrids (Fedele et al. 2020).

BBR management has traditionally relied on sequential applications of synthetic chemical fungicides during the phenological stages of flowering, fruit-set, and pre/post-harvest (González–Fernández et al. 2020). However, this control method raises production costs, increases human health risks, and has a significant environmental impact, including the contamination of soil and water resources and harm to nontarget organisms (Avenot et al. 2018). Due to environmental selection pressure, high genetic variability, and a short life cycle, *B. cinerea* is considered a high-risk pathogen for the development of fungicide resistance, as seen with cyprodinil and iprodione (Shao et al. 2021). The development of complementary and alternative methods to chemical control, such as using nonpathogenic microorganisms as biological control agents before and/or after harvest, is increasingly considered a promising and attractive alternative (Fedele et al. 2020).

Plants are densely colonized by various microbes, some of which are epiphytes residing on the surface of plant organs, whereas others are endophytes located inside the plants (Haidar et al. 2016). Biocontrol by beneficial bacteria may be achieved by direct antibiosis, competition for niches and nutrients, interference with pathogen signaling, or inducing plant resistance (Compant et al. 2013). Moreover, biocontrol might be achieved by degrading virulence factors or phytotoxins of pathogens, thereby reducing disease symptoms (Hardoim et al. 2015; Nagrale et al. 2023). Yeasts and bacteria can also secrete antimicrobial compounds, such as toxins, including acidic proteins (Zaheer et al. 2016; Fedele et al. 2020; Nagrale et al. 2023). Established, thriving, and stable microbial endophytic plant communities may induce disease resistance through several mechanisms. These include the synthesis of structural compounds and inhibition of fungal penetration (Haidar et al. 2016; Nagrale et al. 2023), the induction and expression of general molecular-based plant immunity (Hardoim et al. 2015), the production of volatile organic compounds with antifungal activity (Compant et al. 2013; Haidar et al. 2016), or the simple exclusion of other organisms (phytopathogens and colonists) by niche competition (Sturz et al. 2000).

Biological control using *Trichoderma*, *Serratia marcescens*, *Gliocladium roseum*, *Penicillium* sp., and *Pythium radiosum* has been attempted as a treatment to prevent infections of gray mold caused by *B. cinerea* (Fedele et al. 2020; Thome et al. 2020). These microorganisms do not present a health risk and can be found on the fruit's surface or internally (Bruisson et al. 2019). Bioagents are ideal candidates for the biocontrol of fruit phytopathogens because they are not nutritionally demanding, multiply quickly, and generally do not produce metabolites or mycotoxins that affect human health (Gonzalez–Fernandez et al. 2020; Thome et al. 2020).

This study aimed to assess and determine the potential of epiphytic and endophytic bacteria to control BBR *in vitro* and *in vivo* using detached grape berries of the CS variety in southern Brazil.

2. Material and Methods

B. cinerea isolation and pathogenicity

The BC1 isolate of *B. cinerea* was selected from a group of 12 isolates collected from different commercial vineyards in the highland region of Curitibanos County, Santa Catarina State, southern Brazil (27°28'54"S, 50°53'51"W) with an altitude of 1100 m. This isolate caused severe symptoms of BBR on SB berries during the 2021 growing season. The climate in this region is Cfb-humid exothermic according to the Köppen–Geiger climate classification (Peel et al. 2007).

A pure culture of the BC1 isolate was obtained with hyphal tips from the edges of the mycelial growth, and monosomic cultures were obtained from the reproductive structures. The monosomic cultures were stored in cryogenic tubes at −10°C, following the procedure described by Bruisson et al. (2019). The *B. cinerea* isolate was identified by morphological analysis of the reproductive structures (conidia and conidiophores) using methylene blue staining with bright-field optical microscopy (OM) (Haidar et al. 2016) and molecular analysis based on polymerase chain reaction (PCR) amplification of the 5.8S rRNA gene. The universal primers for fungi, including ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC GCT TAT TGA TAT GC-3′), were used as described by White et al. (1990). The amplification

reaction was performed in a final volume of 25 µL containing 50 pmol of each primer (ITS-1 and ITS-4), 200 µM of each dNTP, 0.5 units Taq DNA polymerase and 3 µL DNA sample in 1× Taq polymerase buffer (Invitrogen) (White et al. 1990). The mixture was denatured at 94°C for 7 min. Thirty-five cycles of PCR were performed by denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min. At the end of the last cycle, the mixture was incubated at 72°C for 10 min.

A spore suspension of the BC1 isolate was prepared for a pathogenicity assay by collecting spores from 5-day-old colonies [grown on potato dextrose agar (PDA) at 25°C] in sterile distilled water, with the addition of 0.02% Tween 20 to assist the dispersal of conidia. The spore concentration (1×10^5 cells/mL) was calculated with a hemocytometer. A 15 µL conidial suspension was deposited onto wounds (3 mm deep and 2 mm wide) of freshly detached berries using a histological needle. The inoculated berries were incubated in a biochemical oxygen demand (BOD) chamber at 25°C with a 12 h photoperiod for 7 days or until symptoms developed. *B. cinerea* was reisolated to fulfill the Koch postulate. The confirmation of BBR symptoms was made through microscopic observation of the morphological and reproductive structures of *B. cinerea* using an OM with observation at 40× objective.

Bacterial isolation from the grapevine leaf Phyllosphere

Ten leaves were collected from the top, middle, and bottom parts of five SB variety plants at the phenological stage of veraison (BBCH-81), located in the central part of the unsprayed commercial vineyards in the highland region of Curitibanos County, Santa Catarina State, southern Brazil. Bacteria were isolated from a mixture of 10 fresh leaves from different parts of the SB variety, which showed no signs of infection. The leaves were transferred to a flask containing 200 mL sterile distilled water with 0.02% Tween 20 to remove the bacteria (Vargas et al. 2012). The flasks were placed on an orbital shaker (model TE-145) at 150 rpm at room temperature (24–25°C) for 1 h (Bruisson et al. 2019). Serial dilutions of the resulting suspension were prepared by transferring 1 mL of the initial suspension into 9 mL sterile distilled water in successive steps, obtaining dilutions ranging from 10^{-1} to 10^{-6}. Aliquots of 100 µL from the 10^{-4} and 10^{-5} dilutions were plated onto 10% tryptone soybean agar (TSA) medium containing 100 µg/mL cycloheximide to inhibit fungal growth (Vionnet et al. 2018). The plates were incubated at 26°C in a BOD chamber for 48 h, after which bacterial colonies were observed and stored for further analysis.

Epiphytic bacterial isolation

Epiphytic bacterial isolation was performed by spreading 100 µL of the 10^{-4} and 10^{-5} dilutions from the prepared serial dilutions onto 10% TSA medium (Difco) supplemented with 100 µg/mL cycloheximide. Three replicates were prepared for each selected dilution. The plates were incubated in a BOD chamber at 26°C for 48 h until well-separated colonies developed. These bacterial colonies were subsequently selected and stored in cryogenic tubes containing sterile 20% glycerol (v/v) at −20°C for long-term preservation (Bruisson et al. 2019).

Endophytic bacterial isolation

The same plant materials and procedures for obtaining epiphytic bacteria were also used to isolate endophytic bacteria. However, the leaves were disinfected with 70% ethanol for 1 min, followed by a 2 min immersion in a 1.5% sodium hypochlorite solution. The leaves were rinsed five times with sterile distilled water and cut into 5 × 5 cm fragments. The leaf fragments were macerated using porcelain mortars and pestles containing 9 mL phosphate-buffered saline solution (Vionnet et al. 2018). The serial dilutions, incubation, and storage followed the same procedure as those used for epiphytic bacteria.

In vitro* antibiosis test of bacterial isolates against *B. cinerea

All bacterial isolates were tested in an *in vitro* preliminary screening to select isolates showing antagonism against *B. cinerea* mycelial growth. Liquid cultures of the bacterial isolates were prepared by

suspending two to three colonies from fresh liquid Luria-Bertani (LB) agar cultures in 3 to 5 mL sterile LB broth, followed by overnight incubation at 26°C under shaking at 180 rpm for 58 h. The cultures were centrifuged at 5000 rpm for 5 min to sediment the bacterial cells, and the supernatant was discarded. The bacterial cell pellet was washed once by suspending it in 0.9% (w/v) NaCl solution, followed by centrifugation at 5000 rpm for 5 min to remove the residual growth medium. After the washing step, the bacterial pellet was resuspended in 0.9% NaCl solution to achieve a final optical density OD_{595} of 1, ensuring consistent cell concentrations for subsequent *in vitro* assays (Bruisson et al. 2019).

A 5 mm disc of *B. cinerea* mycelium, previously grown on PDA medium for 7 days at 25°C under a 12 h photoperiod, was placed in the center of the PDA medium Petri dishes. Then, a 50 µL OD_{595} bacterial isolate suspension was inoculated at four equidistant points on the PDA medium Petri dishes containing the *B. cinerea* discs. The PDA plates were incubated in a BOD chamber at 25°C under a 12 h photoperiod and evaluated 24 and 72 h after inoculation. Each treatment with bacterial isolates was conducted in triplicate, and four replicates were used. As a control, a 50 µL sterile LB medium without a bacterial inoculum was used.

The antibiosis test was conducted by assessing the degree of mycelial growth inhibition, according to Maia (2021). The inhibition index (II) was calculated using the mycelial growth diameter and the *B. cinerea* inhibition halo (Ih), which is the area around a bacterial colony where the mycelial growth of *B. cinerea* is inhibited, according to the following formula:

$$II\ (cm) = [Dtf - Dcf / (Dft / Ih)]$$

where Dtf is the difference (cm) in *B. cinerea* growth in the presence of the bacterial isolate, Dcf is the difference (cm) in *B. cinerea* growth without the presence of the bacterial isolate (control), and Ih is *B. cinerea* Ih (cm).

Biochemical compounds assay

The bacterial isolates with the highest II values were chosen for phenotypic characterization, *in vivo* antibiosis assays, and genetic identification through 16S rDNA sequencing. These isolates underwent analysis of cellular morphology using OM and biochemical tests, including fermentation (glucose and lactose), oxidation/fermentation, lysine utilization, citrate utilization, urea utilization, indole compound production, and catalase activity (Vermelho et al. 2019).

***In vivo* epiphytic and endophytic bacterial pathogenicity**

To determine if epiphytic and endophytic bacteria selected in the previous assay were pathogenic on the grape berries, 15 µL of suspended OD_{595} bacteria [1×10^5 colony-forming units (CFU)/mL] were inoculated in a micro-wound made on the grape berry SB variety that was homogeneous in size. These berries were previously disinfected with a commercial sodium hypochlorite solution at 1% (v/v) for 1 min and placed in plastic boxes at 25°C for 7 days. It was determined that epiphytic and endophytic bacteria were pathogenic when an alteration of the berry tissue was observed (Vargas et al. 2012).

***In vivo* antibiosis test: inhibition of *B. cinerea* by epiphytic and endophytic bacteria on wounded berries**

Clusters of the SB variety were divided into small clusters with five BBR asymptomatic mature (~20°Brix) berries freshly excised from the rachis. The grape berries were homogenous in size and color, without visible damage or mold. The berries were surface disinfected using 1% (v/v) sodium hypochlorite for 1 min, followed by five successive sterile distilled water rinses. After air-drying, 20 grape berries were stored in each polystyrene box (Gerbox®) containing sterile Whatman no. 3 filter paper, with three replicates. The grape berries were aseptically punctured using a sterile, thin-walled, and sharp-edged cork borer, thereby creating standardized lesions measuring 3 mm in both depth and width. A 15 µL OD_{595} bacterial suspension in water containing 1×10^5 CFU/mL for each selected isolate from the *in vitro* tests

was inoculated onto the grape berry lesions and left to dry for 1 to 2 h. The identical procedure was replicated for the commercial product (Ecoshot® 5×10^{10} CFU/g of the active ingredient; isolate D-747) of *Bacillus amyloliquefaciens* as an additional control. For the negative control group, 15 µL sterile distilled water was used. After 1 to 2 h air-drying, 15 µL *B. cinerea* conidial suspension at 1×10^5 conidia/mL concentration was inoculated onto the grape berry lesions in each treatment, except for the control group. The inoculated grape berries were air-dried for an additional 2 h within a laminar flow chamber. The Gerboxes® containing the grape berries were stored in a BOD chamber with a 12 h photoperiod and at 25°C ± 1°C for 7 days. To maintain the Gerboxes® at 90% to 100% humidity, 2 mL sterile distilled water was added daily onto the filter paper. Each treatment consisted of three replicates of four Gerboxes®. The results were the mean of three independent experiments. A positive control was performed with berries inoculated only with sterile water and *B. cinerea* suspension.

II was evaluated based on *B. cinerea* growth inhibition determined 7 days after *B. cinerea* inoculation using the formula adapted from Pantelides et al. (2015):

$$II = \left(\frac{\text{diameter of } B.\ cinerea \text{ colony growth on berry treated with bacteria}}{\text{diameter of } B.\ cinerea \text{ colony growth on berry without bacteria}} \right)$$

Taxonomic identification of grapevine epiphytic and endophytic bacteria

For bacterial identification, the 16S rRNA gene sequence of the bacterial isolates with the higher biocontrol effect against *B. cinerea* in the *in vivo* antibiosis test was obtained and analyzed. Two to three colonies of each bacterial isolate were lysed in 50 µL distilled water by boiling at 100°C for 10 min. PCR was performed in a total volume of 25 µL, with 5 µL lysed bacterial solution as the template. The primers BactF (5'-AGA GTT TGA TYM TGG CTC-3') and BactR (5'-CAK AAA GGA GGT GAT CC-3') were used at a final concentration of 0.5 µM (Vargas et al. 2012). The gel loading track and the Accustart II PCR toughie (VWR-Qiagen, Hilden, Germany) polymerase were added according to the manufacturer's protocol. The PCR program was performed as follows: an initial denaturation step at 94°C for 3 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1.5 min. This was concluded with a final extension step at 72°C for 10 min. Then, a 3 µL PCR product was loaded for electrophoresis on a 1% agarose gel to confirm the correct size of the amplified product. The remaining PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. Then, a 12 µL sample was mixed with 3 µL of either BactF or BactR primer at 10 µM and sent to Macrogen, Inc. (Seoul, Korea) for sequencing. The obtained chromatograms were visually inspected, and the sequences were manually processed when necessary. The sequence analysis results of epiphytic and endophytic bacteria were aligned with the published full-length sequences in the Basic Local Alignment Search Tool database [National Center for Biotechnology Information (NCBI), US National Library of Medicine, Bethesda, MD, USA].

Experimental design and statistical analysis

The experiments were conducted in a complete randomized design with four replicates and repetitions. All data obtained from the antagonistic activity experiments were analyzed using analysis of variance. When the F-test was significant, the means were compared using the Scott-Knott method at a 5% significance level, employing SISVAR version 5.3 (Ferreira 2011).

3. Results

Effect of epiphytic and endophytic bacteria on mycelial growth of *B. cinerea in vitro*

Fifty-two bacterial isolates, 31 epiphytic and 21 endophytic, were obtained from the leaves of the SB variety. Among them, four epiphytic isolates (UEP-38, UEP-40, UEP-43, and EEP-51) and two endophytic isolates (UEN-13 and UEN-14) showed the highest II (Table 1). UEP38, UEP40, UEP43, UEP51, UEN13, and

UEN14 significantly inhibited the mycelial growth of *B. cinerea* at 24 and 72 h after inoculation (Table 1). In all culture treatments, the formation of inhibitory zones between colonies of epiphytic and endophytic bacteria with *B. cinerea* was observed after 7 days of incubation. These bacterial isolates were selected for subsequent *in vivo* antibiosis assessment on detached grape berries. The highest II values were observed among the epiphytic isolates compared to the endophytic isolates, with means of 1.39 cm at 24 h and 1.07 cm at 72 h for the epiphytic isolates and 0.99 cm at 24 h and 0.25 cm at 72 h for the endophytic isolates (Table 1). There were some antagonistic bacteria among the microbial community associated with grape leaves that were able to control *B. cinerea*.

Table 1. Inhibition Index (II) (cm) of the epiphytic (UEP) and endophytic (UEN) bacterial isolates from leaves of grape cv. Sauvignon Blanc to control the *in vitro* mycelial growth of *Botrytis cinerea* at 24 and 72 h after the inoculation.

	Time after inoculation			Time after inoculation	
Bacterial isolates	24 h	72 h	Bacterial isolates	24 h	72 h
Control	3.54^{a}	4.59 ^{a}	Control	3.21^{a}	4.18^{a}
UEP13	0.21^{g}	0.00^{f}	UEN01	0.68^{d}	0.00^{d}
UEP01	0.26^{g}	0.23^{e}	UEN07	0.74^{cd}	0.00^{d}
UEP60	0.31^{g}	0.00^{f}	UEN02	0.76^{cd}	0.00^{d}
UEP02	0.37^{g}	0.26^{e}	UEN15	0.81^{c}	0.00^{d}
UEP09	0.48^{fg}	0.16^{e}	UEN04	0.86^{c}	0.00^{d}
UEP03	0.53^{f}	0.14^{e}	UEN06	0.88^{c}	0.00^{d}
UEPP20	0.66^{f}	0.14^{e}	UEN05	0.89^{c}	0.00^{d}
UEP48	0.72^{f}	0.50^{de}	UEN16	0.91^{c}	0.17^{c}
UEP26	0.78^{ef}	0.37^{e}	UEN11	0.92^{bc}	0.00^{d}
UEP45	0.83^{e}	0.62^{d}	UEN09	0.93^{bc}	0.00^{d}
UEP34	0.90^{e}	0.78^{cd}	UEN19	0.95^{b}	0.00^{d}
UEP27	0.90^{e}	0.76^{cd}	UEN17	0.96^{b}	0.00^{d}
UEP33	0.96^{e}	0.78^{cd}	UEN13	0.96^{b}	0.44^{b}
UEP 28	1.01^{de}	0.88^{c}	UEN03	0.98^{b}	0.00^{d}
UEP46	1.01^{de}	0.47^{de}	UEN14	1.00^{b}	0.47^{b}
UEP35	1.02^{de}	0.80^{c}	UEN20	1.02^{b}	0.19^{c}
UEP24	1.03^{d}	0.36^{e}	UEN08	1.05^{b}	0.16^{c}
UEP32	1.08^{d}	0.69^{d}	UEN12	1.07^{b}	0.19^{c}
UEP25	1.10^{d}	0.89^{c}	UEN10	1.07^{b}	0.14^{c}
UEP17	1.12^{c}	0.67^{d}	UEN18	1.09^{b}	0.00^{d}
UEP41	1.17^{c}	0.33^{e}	UEN28	0.26^{e}	0.00^{d}
UEP39	1.17^{c}	0.91 ^{c}			
UEP38	1.54^{b}	1.06^{b}			
UEP44	1.28^{bc}	0.48^{de}			
UEP51	1.36^{b}	0.94^{b}			
UEP43	1.34^{b}	1.04^{b}			
UEP40	1.40^{b}	1.08^{b}			
UEP37	1.42^{b}	0.83^{c}			
UEP42	1.46^{b}	0.73^{d}			
UEP49	1.47^{b}	0.53^{de}			
UEP50	1.20^{c}	0.51^{de}			
CV* (%)	27.10	36.69		15.28	33.46

Means followed by the same lowercase letter in the column are not statistically different according to the Scott-Knott method at a 5% significance level.
* Coefficient of variance.

Biochemical compounds assay

UEP38, UEP40, UEP43, UEP51, UEN13, and UEN14, which showed high levels of inhibitory effect on *B. cinerea* growth in the culture test, were used to determine the capability of producing biochemical metabolites. Table 2 shows epiphytic and endophytic bacteria with the highest II, exhibited coccoid cell morphology. and yielded negative reactions for glucose, lactose, citrate, oxidase, oxidation/fermentation,

and lysine. UEP40, UEP43, UEN13, and UEN14 yielded positive reactions for urea and indole, indicating the production of the urease enzyme and tryptophan (Table 2). Urease plays a critical role in hydrolyzing urea into ammonia and carbon dioxide, which can enhance the antagonistic effect of these isolates against *B. cinerea*, whereas indole compounds contribute to potential plant growth-promoting effects (Nagrale et al. 2023).

Table 2. Morphological and biochemical characterization of the epiphytic (UEP) and endophytic (UEN) bacterial isolates that exhibited the highest Inhibition Index (II) of mycelial growth *in vitro* against *Botrytis cinerea*

Bacteria Isolates	Celular morphology	Glicose	Lactose	Citrate	Oxidase	Oxidative-fermentation	Urea	Lysine	Indole
UEN 13	Coco	-	-	-	-	-	+	-	+
UEN 14	Coco	-	-	-	-	-	+	-	+
UEP 38	Coco	-	-	-	-	-	-	-	-
UEP 40	Coco	-	-	-	-	-	+	-	+
UEP 43	Coco	-	-	-	-	-	+	-	+
UEP 51	Coco	-	-	-	-	-	-	-	-

(+) positive; (-) Negative.

Pathogenicity of the epiphytic and endophytic bacteria on grape berries

Among the selected epiphytic and endophytic bacteria isolates used in all experiments, none exhibited a damaging effect when they were inoculated on wounded grape berries of the SB variety.

Effect of epiphytic and endophytic bacteria isolates on mycelial growth of *B. cinerea* on wounded berries

All grape berries in the *in vivo* experiments showed some degree of BBR, with at least one lesion per berry. The six bacterial isolates (UEN13, UEN14, UEP38, UEP40, UEP43, and UEP51) significantly reduced BBR on grape berries over the time intervals of 72, 120, and 168 h after inoculation (Table 3), representing 11.5% of the total bacterial isolates selected. Among these isolates, 66.6% were epiphytic and 33.3% were endophytic.

Table 3. Inhibition Index (II) of *Botrytis cinerea* mycelial growth in detached and wounded berries of cv. Sauvignon Blanc (SB) inoculated with epiphytic and endophytic bacterial isolates that exhibited the highest II *in vitro* assessed up to 7 days (72, 120, and 168 h) after inoculation.

	Hours after inoculation		
Isolates	72 h	120 h	168 h
Control	$0^{Fa \pm 0.14}$	$1^{Ea \pm 0.08}$	$1^{Ea \pm 0.22}$
Botrytis cinerea	$56^{Aa \pm 0.05}$	$60^{Aa \pm 0.06}$	$65^{Aa \pm 0.11}$
Bacillus amyloquefaciens	$15^{Eb \pm 0.03}$	$22^{Da \pm 0.09}$	$26^{Da \pm 0.04}$
UEN13	$23^{DEb \pm 0.03}$	$31^{CDa \pm 0.07}$	$36^{CDa \pm 0.09}$
UEN14	$30^{Cb \pm 0.05}$	$46^{Ba \pm 0.21}$	$47^{Ba \pm 0.18}$
UEP38	$38^{Cb \pm 0.09}$	$43^{Ba \pm 0.08}$	$50^{Ba \pm 0.17}$
UEP40	$48^{Ba \pm 0.31}$	$49^{Ba \pm 0.16}$	$48^{Ba \pm 0.19}$
UEP43	$24^{Db \pm 0.10}$	$37^{Ca \pm 0.06}$	$45^{BCa \pm 0.05}$
UEP51	$25^{Db \pm 0.07}$	$38^{Ca \pm 0.09}$	$38^{Ca \pm 0.11}$
CV* (%)	37,4	29,3	36,7

Means followed by the same uppercase letter in the column and lowercase in the line are not statistically different according to the Scott-Knott method at a 5% significance level.
* Coefficient of variance.

UEP43, UEP51, and UEN13 showed the lowest BBR severity and significantly differed from the other bacterial isolates. Among these isolates, only UEN13 showed the lowest BBR severity for 72, 120, and 168 h

after inoculation. However, UEN13 did not differ significantly from UEP43 and UEP51 at the early time point of 72 h (Table 3). Remarkably, UEN13 showed the same BBR disease reduction for 72, 120, and 168 h as the commercial product Ecoshot® with *B. amyloliquefaciens*.

The six antagonistic bacteria selected for further study, which showed a significant inhibitory effect on pathogen growth *in vitro,* were efficient in reducing BBR caused by *B. cinerea* on wounded berries that were artificially inoculated after application of the antagonists. All six isolates did not completely prevent infection of wounded berries but decreased the pathogen mycelial growth and BBR (Table 3).

Molecular identification of epiphytic and endophytic bacteria with greater biocontrol activity

The six epiphytic and endophytic bacterial isolates assessed in the *in vivo* assays and identified through genetic sequencing of the 16S rRNA gene are presented in Table 4. Molecular analysis demonstrated high levels of sequence similarity between the isolates and closely related species in the nucleotide sequence databases of the NCBI.

UEP38, UEP43, and UEN14 were identified as *S. marcescens,* and UEP51 and UEN13 were identified as *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* and *Staphylococcus equorum*, respectively (Table 4). Among these six most efficient bacteria, UEP43, UEP5, and UEN13 showed the highest value of *B. cinerea* control. To the authors' knowledge, this is the first study in which epiphytic and endophytic bacteria were isolated from grape leaves in southern Brazil and assessed for their potential antagonistic ability against *B. cinerea*. UEN13, which significantly inhibited >54% of *B. cinerea* development over a 168 h evaluation, revealed identity and homology of 81% with *S. equorum*.

Table 4. Molecular identification of 16S rRNA gene of the epiphytic and endophytic bacterial isolates with *in vitro* and *in vivo* antagonistic trails.

Isolate Code [a]	Specie	Assess n°[b]	% of identity
UEN13	*Staphylococcus equorum*	MH493712	81%
UEN14	*Serratia marcescens*	CP041233	99%
UEP38	*Serratia marcescens*	MW265916	99%
UEP40	*Stenotrophomonas rhizophila*	KX588618	100%
UEP43	*Serratia marcescens*	OP317557	99%
UEP51	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	HE716917	100%

[a]Code of selected isolate with antagonistic trails. [b]GenBank sequence accession numbers of selected isolates.

4. Discussion

Effect of epiphytic and endophytic bacteria on mycelial growth of *B. cinerea in vitro*

BBR, caused by *B. cinerea*, is considered the main post-harvest decay, particularly in southern Brazil, due to the significant damage that it causes during the harvest season and storage (De Bem et al. 2015; Tome et al. 2020). The natural presence of antagonistic microorganisms on grapes supports the application of selected antagonistic bacteria as a viable strategy for manipulating these populations to achieve biological control of pathogens (Kasfi et al. 2018). Currently, biological control is considered a promising alternative to synthetic fungicides in controlling the post-harvest decay of fruits and vegetables, with a particular emphasis on grapes (Yu et al. 2008; Vargas et al. 2012; Vionnet et al. 2018; Nagrale et al. 2023).

The isolation and identification of antagonistic organisms have been extensively studied for decades. Vargas et al. (2012), Bruisson et al. (2019), Chávez-Arteaga et al. (2022), and Nagrale et al. (2023) reported the isolation of plant growth-promoting bacteria, including Azotobacter spp., *Azospirillum* spp., *Pseudomonas fluorescens*, *Bacillus megaterium, Bacillus subtilis,* and *Bacillus cereus,* from the roots, leaves, and berries of different grapevine varieties. These findings were consistent with this study and reinforced the understanding that the microbial community in the epiphytic habitat is larger than the endophytic community, as reported by Zaheer et al. (2016), Fedele et al. (2020), Gao et al. (2023), and

Nagrale et al. (2023). In this study, UEP43, UEP51, and UEN13 demonstrated significant inhibition of *B. cinerea* on grape berries, with reductions in disease severity of ~55% and 49% for UEP43 and UEN13, respectively, over a 168 h period. These inhibition levels aligned with or surpassed some previously reported findings. For example, *Actinobacter iwoffii* PTA-113 and *P. fluorescens* PTA-CT2 isolates demonstrated 20% to 50% inhibition of *B. cinerea* infection on grape leaves, whereas *A. iwoffii* PTA-113 and *Pantoea agglomerans* PTA-AF1 showed efficiencies of 45% to 70% on grape berries (Gao et al. 2023). In addition, Bruisson et al. (2019) reported that *Bacillus* isolates typically reduce *B. cinerea* severity by 40% to 70%, a range comparable to or slightly higher than the reduction observed with our isolates. This comparison highlighted that the inhibition levels achieved by the epiphytic and endophytic isolates in this study are competitive with or, in some cases, exceed those of previously reported biocontrol agents. These results underscored the potential of *S. marcescens* (UEP43), *C. flaccumfaciens* (UEP51), and *S. equorum* (UEN13) as effective biocontrol agents against *B. cinerea* on grape berries.

Biochemical compounds assay

This study indicated that antagonistic isolates apparently produced substances capable of reducing pathogen growth. The effect of these antagonists on fungal growth was considered indicative of the pathogen's sensitivity to the action of bacteria obtained from the same biological niche. Inhibition zones in the cultures could be due to the production of antibiotics, siderophores, and toxic or antifungal metabolites used by these organisms as biological control mechanisms (Haidar et al. 2016; Nagrale et al. 2023). However, as these substances were not specifically evaluated in this study, their exact role in the observed inhibition cannot be confirmed. The size of the observed inhibition zones likely represents the concentration and diffusivity of the inhibitory compounds secreted by each isolate (Kasfi et al. 2018). However, the production of these compounds in the culture medium does not necessarily indicate their production at action sites on berries (Dal Bello et al. 2008). Significant differences were observed among the assayed endophytic and epiphytic bacteria in terms of inhibitory effects, with four epiphytic isolates (UEP-38, UEP-40, UEP-43, and EEP-51) and two endophytic isolates (UEN-13 and UEN-14) being the most effective in inhibiting *B. cinerea* growth *in vitro* (Table 1).

The urease enzyme and tryptophan can degrade urea and utilize it as a carbon and nitrogen source (Nagrale et al. 2023). Urease production by the isolates suggests a role in their antagonistic activity against *B. cinerea*. By metabolizing the available urea, the isolates could influence the surrounding environment, potentially limiting the resources for the pathogen. This activity, combined with the ability to inhibit fungal growth, highlights their effectiveness as biocontrol agents against *B. cinerea*.

Effect of epiphytic and endophytic bacterial isolates on mycelial growth of *B. cinerea* on wounded berries

The inhibition or delay of the initial *B. cinerea* infection process can significantly reduce losses in terms of grape productivity and wine quality due to BBR. These data were consistent with those of Bruisson et al. (2019), who obtained a similar percentage, with 6 of 78 bacterial isolates closely related to *Bacillus* isolates inducing strong inhibition (>60%) of *B. cinerea* mycelial growth in grapes. UEN13, UEP43, and UEP51 showed an average reduction of 54% in BBR severity in the grape berries of the SB variety compared to the *B. amyloliquefaciens* treatment, which reduced BBR severity by ~73%. Although this reduction in BBR severity was slightly lower than that achieved by the commercial Ecoshot® with *B. amyloliquefaciens*, it still produced above-average results and could be considered a potential biocontrol agent. The *Bacillus* genus is well known for its efficiency, typically achieving 40% to 70% inhibition of *B. cinerea*.

Although none of the six isolates completely prevented infection of wounded berries, they significantly decreased the pathogen's mycelial growth and reduced berry rot (Table 3). These findings were consistent with those of Thome et al. (2020), who studied the effect of yeast against *B. cinerea*. These results aligned with previous studies where various isolates of rhizosphere and plant growth-promoting bacteria were documented to be effective against several fungal pathogens (Liu et al. 2022). Similar results

were reported by González-Fernández et al. (2020) and Nagrale et al. (2023), who observed a significant decrease in the degree of infection by *B. cinerea* on grapes treated with yeasts and bacteria before inoculation with the pathogen compared to the grapes treated with yeasts and immediately inoculated with the pathogen.

Molecular identification of epiphytic and endophytic bacteria with greater biocontrol activity

The molecular results indicated the presence of antagonistic bacteria within the microbial community from the leaves of the SB variety capable of controlling *B. cinerea*. These bacteria were identified through 16S rRNA gene sequencing, which revealed high sequence similarity levels between our isolates and closely related species in the nucleotide sequence databases of the NCBI.

Martins et al. (2013) and Hardoim et al. (2015) demonstrated that the EN-21 isolate of *S. equorum*, isolated from the rhizosphere of the *Salicornia hispanica* plant, enhanced disease resistance by ~45% to 65% against *Dickeya solani*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *Erwinia amylovora* on potatoes, carrots, and pears, respectively. Hardoim et al. (2015) also reported a variation in efficiency between 30% and 50% for the EN-21 isolate of *S. equorum* on tomato growth under controlled conditions. They found that this isolate exhibited 60% to 80% capability to degrade molecules involved in phytopathogenic bacterial cell-cell signaling (N-acyl homoserine lactones). Several *S. marcescens* isolates have been described as plant growth promoters and/or agents for the biological control of diseases (Chávez-Arteaga et al. 2022; Liu et al. 2022), supporting the results of this study. UEN14 and UEP43, identified as belonging to this species, showed a positive reaction for indole production, which is a precursor of IAA and is effective in reducing BBR in grapes.

UEP51 from *C. flaccumfaciens* pv. *flaccumfaciens* has been previously isolated from grapevine leaves (Martins et al. 2013). Its antifungal activity on spore germination has been reported in some studies (Nagrale et al. 2023). Evseen et al. (2022) demonstrated that the application of the ME-1 *C. flaccumfaciens* isolate significantly reduced (≥60%) the severity of anthracnose and angular leaf spot in cucurbits under field conditions. The reduction in the disease index increased by >60% when *C. flaccumfaciens* was applied in combination with the INR-7 and GB-03 isolates of *Bacillus pumilus* and *B. subtilis*, respectively. These findings confirm that *C. flaccumfaciens*, *S. marcescens,* and *S. equorum* possess significant potential as biological control agents, supporting the results of this study and highlighting their efficacy in reducing the severity of *B. cinerea.*

Results demonstrated the presence of a bacterial community associated with the phyllosphere of the SB grape variety that has the potential for biological control of BBR in detached and wounded grape berries caused by *B. cinerea.* However, additional studies are necessary to investigate the synergistic action between these bacterial isolates and commercial biocontrol products, aiming to enhance the biocontrol efficacy against *B. cinerea* in grapevines cultivated in the highland region of southern Brazil.

5. Conclusions

Among the 52 isolates, 4 epiphytic and 2 endophytic isolates exhibited the highest degree of mycelial inhibition of *B. cinerea in vitro* and detached and wounded berries of the SB variety. Four of six bacterial isolates tested positive for urea and indole production, which may contribute to the inhibition of *B. cinerea* mycelial growth *in vitro*. The bacterial epiphytic isolates UEP43 and UEP51 and the endophytic isolate UEN13 demonstrated the highest II of *B. cinerea* mycelial growth, reducing BBR severity by 20% to 60% in detached and wounded grape berries of the SB variety. These isolates were identified using 16S rRNA gene sequencing as *S. marcescens* (UEP43)*, C. flaccumfaciens pv. flaccumfaciens* (UEP51), and *S. equorum* (UEN13)*.*

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