Heritage grapevines as sources of biological control agents for Botryosphaeria dieback pathogens

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Summary. Grapevine trunk diseases cause severe damage in grapevines. Management strategies focus on protection of grapevine pruning wounds using chemical fungicides or biological control agents. Botryosphaeria dieback, caused mainly by Lasiodiplodia spp., is one of the main trunk diseases in northwest Mexico. This study obtained endophytic bacteria and fungi from the heritage grapevine Vitis vinifera cv. ‘Mission’ for potential biological control of Botryosphaeria dieback. A collection of 135 bacterial and 37 fungal isolates were obtained and initially tested for antagonistic activity against Lasiodiplodia brasiliensis. The most promising isolates belonging to Trichoderma and Bacillus spp. were selected and characterized to determine their modes of action. Bacillus isolates produced volatile organic compounds that inhibited growth of Neofusicoccum parvum, and diffusible organic compounds with antifungal effects against L. brasiliensis and N. parvum. Trichoderma isolates produced diffusible organic compounds and were mycoparasites. In greenhouse assays, plants inoculated with three Trichoderma asperellum isolates (T20BCMX, EF09BCMX, and EF11BCMX), B. amyloliquefaciens (BEVP26BCMX) or Bacillus sp. (rbES015), applied preventively in soil, gave up to 50% smaller necrotic lesions when compared with the plants inoculated only with L. brasiliensis. In the field, plants inoculated with three Bacillus isolates (BEVP02BCMX, BEVP26BCMX, BEVP31BCMX) or five Trichoderma (T11BCMX, T15BCMX, T17BCMX, T20BCMX, and EF11BCMX) had lesions up to four times smaller than control plants inoculated only with L. brasiliensis. This study has demonstrated the potential of heritage grapevines to provide biological control agents for Botryosphaeria dieback.

Keywords. Grapevine trunk diseases, Botryosphaeriaceae, Bacillus, Trichoderma.
Grapevine trunk diseases (GTDs) cause severe problems in vineyards. These are a complex of diseases that include black foot, Esca, Eutypa dieback, Petri disease, and Botryosphaeria dieback (Úrbez-Torres et al., 2012). Approx. 133 fungal species in 34 genera have been reported as causal agents of GTDs (Gramaje et al., 2018). Among them, Botryosphaeria dieback is considered to be the most important (Billones-Baajens and Savocchia, 2019). Almost 30 species of Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia, Neofusicoccum, Neoascytalidium, Phaeobotryosphaeria and Spencermartinsia have been identified as causal agents of Botryosphaeria dieback (Gramaje et al., 2018).

Among the fungi associated with Botryosphaeria dieback, Lasiodiplodia and Neofusicoccum contain the most virulent species identified in different countries, including the United States of America (Úrbez-Torres and Gubler, 2009), Iraq (Abdullah et al., 2012) and Mexico (Rangel-Montoya et al., 2021). Lasiodiplodia spp. have white colonies in culture, that later become dark gray with abundant mycelium, and their conidia can be aseptate hyaline or sepatate pigmented, with longitudinal striations. Neofusicoccum spp. have initially white colonies, and some species as N. luteum and N. australe produce yellow pigments. Their abundant aerial mycelium later turns gray, and conidia are hyaline, unicellular, and aseptate, with a subtruncate bases (Zhang et al., 2021).

Botryosphaeria dieback symptoms usually appear in grapevines several years after pathogen infections, and the symptoms include wedge-shaped perennial cankers, wood discolorations, brown streaking on the wood under the bark, and premature plant death (Bertsch et al., 2013; Spagnolo et al., 2014; Gramaje et al., 2018; Niem et al., 2020). The pathogen enters host plants through wounds, made mostly during pruning (Gramaje et al., 2018).

Management of GTDs is complex, and no curative treatments are known to date. Therefore, strategies commonly focus on implementation of cultural and preventive treatment measures, with each depending on plant damage, the pathogens involved, and the geographic region (Gramaje et al., 2018). These include the protection of pruning wounds with fungicides or biological control agents (BCAs), and curative surgery (Mondello et al., 2018).

The search for endophytic microorganisms for biological control of GTDs pathogens has increased, due to their potential to have antagonistic activities against different species of fungi and, in some cases, to also promote plant growth (Dini-Andreote, 2020; Jacob et al., 2020). Different endophytic microorganisms have been reported as antagonists of GTDs pathogens. These include fungi (Aspergillus, Chaetomium, Clonostachys, Cladosporium, Epicoccum, Fusarium, and Trichoderma), which have shown antagonistic in vitro activity against Diplodia and Neofusicoccum spp. (Almeida et al., 2020; Silva-Valderrama et al., 2021). As well, bacteria (Bacillus, Burkholderia, Paenibacillus, Pseudomonas, and Streptomyces) have been recognized as antagonists of Diplodia, Lasiodiplodia, and Neofusicoccum (Haidar et al., 2016). Among the organisms with potential as BCAs, Bacillus and Trichoderma show in vitro antagonistic activity through different mechanisms of action, including competition for space, production of volatile and non-volatile compounds, or mycoparasitism. In greenhouse and field trials, these organisms have shown efficacy for control of GTDs fungi when applied to grapevine pruning wounds (Almeida et al., 2020).

In Mexico, the Spanish introduced grapevine (Vitis vinifera) in the 16th century, and Jesuit and Dominican friars disseminated its cultivation (Crowley, 1989). ’Listan Prieto’, now known as ’Mission’, was introduced in Mexico and in the United States of America (Walker et al., 2019). In the Baja California peninsula, the first grapevines were established in the San Francisco Javier mission, located in Loreto (Magoni, 2009). From then on, grapevine cultivation continued to expand. Currently in Baja California, ’Mission’ grapevines are over 40 years old, and have been propagated from the first vines introduced in the state by the Jesuit missionaries, and these plants are considered to be heritage grapevines. Approx. 38 ha of this variety are grown with minimal management in Baja California, with yields of approx. 3.5 tons ha⁻¹ (SEFOA, 2011; Andrade et al., 2013).

Different reports have shown that plants growing in arid conditions associate with microorganisms (bacteria and fungi). These can enhance plant drought resistance through various mechanisms, including improved water and nutrient absorption by inducing changes in root morphology, protecting against oxidative damage, regulating phytohormone levels, and suppressing phytopathogens such as those responsible of GTDs (Poudel et al., 2021; Riseh et al., 2021).

Considering the time since they were established and the complex climatic conditions in which the ’Mission’ grapevines have been planted (dry to very dry climate with average rainfall of 200 mm p.a. (INEGI, 2017), it is likely that these plants maintain associations with beneficial microorganisms which allow them to survive in the adverse conditions and resist plant pathogens. Therefore, the objective of the present study was to obtain endophytic bacteria and fungi from the cultivar ’Mission’ with the potential as BCAs of Botryosphaeria dieback fungi,
thus providing sustainable alternatives for the control of this disease in commercial vineyards of Baja California.

MATERIALS AND METHODS

Sampling and isolation of microorganisms from heritage grapevines

Microorganisms were isolated from lignified 1-year-old branches of heritage grapevines cultivar 'Mission', growing in local vineyards in the Guadalupe valley (31.994722, -116.683896) and Ejido Uruapan (31.628436, -116.434295), Baja California. Small tissue fragments were cut from each branch sample, and after bark removal, these were surface sterilized by flaming (Rangel-Montoya et al., 2021). Subsequently, for bacteria isolations, branch fragments were transferred to plates containing LB Agar (ATCC media No.1065), YPD Agar (1245) or King's medium B Agar (1213), or PY medium (tryptone 5.0 g, yeast extract 3.0 g, CaCl₂ 0.9 g, pH 6.8), supplemented with cycloheximide (final concentration 100 µg mL⁻¹). For fungal isolations, tissue fragments were inserted in plates containing Potato Dextrose Agar (PDA), or water agar supplemented with chloramphenicol (final concentration 25 µg mL⁻¹). The isolation plates were incubated at 30°C until microorganism growth was observed, and the resulting bacterial and fungal colonies were recovered and subcultured to obtain pure cultures. Fungal strains were preserved at 4°C in 20% glycerol, and bacteria strains in 35% glycerol solution at -20°C. Additionally, Bacillus sp. rBE015 and 35 strains of Trichoderma were obtained from the collection of the Phytopathology Laboratory of CICESE.

Screening for antifungal activity

The GTD fungi Lasiodiplodia brasiliensis MXBCL28 (Rangel-Montoya et al., 2021) and Neofusicoccum parvum 14P4MX (Rangel-Montoya, 2021) were used to test the biological control potential of the obtained fungal and bacterial isolates. Using a flame-sterilized 7 mm cork borer, an agar plug with mycelium was obtained from a 4-day-old culture, from each fungus grown on PDA. The plug was then placed on the centre of a fresh PDA plate, and incubated at 25°C. When the fungus colony reached 1 cm diam. 5 µL of four different potential BCA bacterial cultures or one mycelium plug from potential fungal BCAs, were inoculated at the edges, as described by Guevara-Avendaño et al. (2018). Plates with only GTD fungi were used as experimental controls. These assays were each carried out in triplicate. After 7 d incubation at 30°C, the inhibition of radial growth of L. brasiliensis mycelium was assessed. In total, 135 bacterial and 39 fungal isolates were screened in these assays.

After discarding isolates with low or no inhibition activity, quantitative fungal inhibition assays were carried out for 58 strains (21 bacteria and 37 fungi), as described for the qualitative assays (above). For each of these quantitative assays, the L. brasiliensis mycelium plug was placed at the edge of the Petri plate, and only one bacterial or fungi strain was inoculated directly opposed to it. The following formula (Méndez-Bravo et al., 2018) was used to calculate the percentage of inhibition of mycelial growth: %inhibition = [(R-r)/R] × 100, where R is the colony radius of the pathogenic fungus growing alone in the control plates, and r is the colony radius of the fungus growing in the plate in confrontation with a tested isolate. All in vitro antagonistic assays were performed in triplicate.

These procedures allowed selection of the most promising BCAs for used in the experiments described below.

Evaluation of antagonistic effects by volatile organic compounds

Antagonistic effects of volatile organic compounds produced by eleven selected strains (four bacteria and seven fungi) were evaluated against L. brasiliensis and N. parvum, using the two sealed base plate method of Rangel-Montoya et al. (2022). For each potential fungal BCA isolate, a mycelial plug was placed in the centre of a PDA plate, and for each bacterium, 20 µL of culture was spread in a PY plate. The lid of each plate was the replaced by a second PDA plate with a mycelial plug of the pathogen at the centre. The two plates were sealed with tape and incubated at 30°C for 4 d, with mycelium growth assessed every 24 h. As experimental controls, non-inoculated PDA or PY plates were used as the covering plate.

Evaluation of the antagonistic effects by diffusible organic compounds

The antagonistic activity of diffusible organic compounds produced by eleven selected BCAs strains was evaluated against L. brasiliensis and N. parvum. For bacteria, 5 mL of liquid PY medium was inoculated with a single colony, and then incubated at 30°C and 110 rpm in a shaker incubator. After 7 d, cultures were each centrifuged at 10,000 rpm for 20 min, and the resulting supernatant was filter-sterilized using a 20 µm syringe filter. PDA plates containing 15% (v/v) of sterile bacterial
supernatant were then prepared, and a mycelial plug of each pathogen was placed in the centre of each test plate (Salvatierra-Martínez et al., 2018). The plates were then incubated at 30°C for 4 d, registering mycelium growth every 24 h. For evaluation of fungal isolates, cellulose membrane assays were used (Mayo-Prieto et al., 2020). A mycelial plug disc of each fungus was inoculated in the centre of each PDA plate. The plug was then covered with a sterile cellulose membrane, and the plates were incubated at 30°C for 48 h. The membrane with the mycelial growth was then removed, and a mycelial plug of the pathogen was placed in the centre of the plate. Plates were incubated at 30°C for 4 d, and mycelium growth was assessed every 24 h.

Evaluation of mycoparasitism activity

Mycoparasitism activity of seven selected Trichoderma isolates was assessed against L. brasiliensis and N. parvum, using the pre-colonized plate method described by Bailey et al., (2008). A mycelial plug of each pathogenic fungus was inoculated at the edge of a PDA plate, and after 5 d incubation at 30°C, an agar strip (4.0 × 0.5 cm) from a colony of a Trichoderma isolate was placed at the opposite side. The plates were incubated for 28 d at 30°C in darkness. Ten mycelial plugs were then collected from each plate in a straight line beginning near the agar strip and extending towards the opposite edge of the plate. The mycelial plugs were then inoculated into PDA plates and incubated for 24 h at 30°C in darkness, followed by 5 d incubation under white light at room temperature. As experimental controls, cultures of L. brasiliensis and N. parvum were used, grown without Trichoderma and maintained under the same conditions. Mycoparasitism was determined by assessing the presence of Trichoderma and the mycoparasitic fungi in the ten mycelial plugs collected. Micoparasitic activity was also assessed under a microscope. For each of these assessments, a dual culture assay was performed in water agar plates, and after 3 d incubation at 30°C, a fragment of agar was cut from the centre of the plate. The obtained samples were observed with inverted microscope (Zeiss Axiovert 200), and the obtained images were analyzed using Zeiss AxioVision SE64, Rel. 4.9.1 software.

In vitro screening for plant growth promoting traits

Coloorimetric tests were carried out to determine plant growth promotion by the potential BCA bacteria and fungi strains. For these assays, the strains were recovered from glycerol stock cultures, and bacteria were inoculated into PY liquid medium, and fungi onto PDA medium. Bacterial cultures were incubated for 2 d at 30°C and 100 rpm in a shaker incubator. Fungi were incubated at 30°C for 7 d, Colourimetric tests were carried out in triplicate, in 35 mm diam. Petri dishes for solid media or 10 mL capacity tubes for liquid media, as described in the sections below. Petri plates or tubes with the corresponding media but without BCAs were used as experimental controls.

Mineral solubilization assays

Some microorganisms are capable of hydrolyzing organic and inorganic insoluble mineral compounds to soluble forms, that can be assimilated by plants, acting as biofertilizers or plant grow-promoters. Phosphate, potassium, and zinc solubilization assays were performed for the potential BCAs. In each case, 5 µL of bacterium culture or a 7 mm diam. mycelial plug of fungus were inoculated at the centre of each assay plate.

Inorganic phosphate solubilization was evaluated on modified Pikovskaya agar (0.5 g L⁻¹ yeast extract, 10 g L⁻¹ glucose, 5 g L⁻¹ Ca₃(PO₄)₂, 0.5 g L⁻¹ (NH₄)₂SO₄, 0.2 g L⁻¹ KCl, 0.1 g L⁻¹ MgSO₄, 0.1 mg L⁻¹ MnSO₄, 0.1 mg L⁻¹ FeSO₄, 10 mg L⁻¹ of bromocresol purple, 15 g L⁻¹ agar, pH 7.2). After inoculation, Petri dishes were incubated for 72 h at 30°C. Colour change from purple to yellow indicated a positive phosphate solubilizing strain (Gupta et al., 1994; Zheng et al., 2018).

Inorganic potassium solubilization was determined on modified Pikovskaya agar (Pikovskaya, 1948), using KNO₃ and bromocresol green. After inoculation, Petri plates were incubated for 72 h at 30°C. Colour change from blue to yellow indicated a positive potassium solubilizing strain.

Zinc solubilization was assessed using zinc-supplemented Pikovskaya medium complemented with 1.2 g L⁻¹ ZnO and bromothymol blue. Inoculated Petri dishes were incubated in the dark for 72 h at 30°C. Colour change from blue to yellow indicated a positive zinc solubilizing strain (Bapiri et al., 2012).

Indole Acetic Acid (IAA) production assays

Indole acetic acid (IAA) is one of the most physiologically active auxins. It induces production of long roots and root hairs, and lateral roots, which are involved in nutrient uptake by plants (Datta and Basu, 2000). For determination of IAA production by bacterial strains, 5 µL of 1-d-old cultures were reinoculated in 96 well micro-
plates, with 200 µL PY liquid medium supplemented with tryptophan to a final concentration of 500 µg mL⁻¹. Microplates were incubated for 48 h at 30°C and 110 rpm in a shaker incubator. Subsequently, 100 µL of Salkowski reagent (50 mL, 35% HClO₄, 1 mL 0.5 M FeCl₃) (Ahmad et al., 2008) were then added per well, and the microplates were each covered with aluminum foil and incubated for 30 min. For each fungal strain, three 7 mm diam. mycelial plugs were inoculated into 5 mL of PDB medium, and these cultures were incubated for 7 d at 30°C and 110 rpm in a shaker incubator. Two hundred µL of the culture were then placed in 96 well microplates, and 100 µL of Salkowski reagent were immediately added. Microplates were each covered with aluminum foil and incubated for 30 min. For the bacteria and fungi, change of colour to pink indicated a positive result.

**Hydrogen cyanide production assays**

Hydrogen cyanide (HCN) is produced by some BCAs, and its toxicity to phytopathogens makes the BCAs suitable for biocontrol. After inoculating the bacteria onto solid PY and the fungi onto PDA in Petri plates, a filter paper moistened with a solution of 0.5% sodium carbonate in 0.5% picric acid (Ahmad et al., 2008) was fixed to each Petri plate cover. The plates were inoculated 5 µL of bacterial culture or a 7 mm diam. mycelial plug of fungus. The Petri dishes were incubated for 48 h at 30°C. Colour changes from yellow to purple indicated siderophore producing strains.

**Siderophore production assays**

Siderophores are competitive traits used for BCAs to sequester iron, depriving pathogens of this element required for their growth and pathogenesis. To test for siderophore production, chrome azurol S agar (CAS) medium (Schwyn and Neilands, 1987) was prepared as described by Lynne et al. (2011). In the centre of each assay plate was inoculated 5 µL of bacterial culture or a 7 mm diam. mycelial plug of fungus. The Petri dishes were incubated in dark at 30°C for 4 d. Development of orange-red colour indicated positive hydrogen cyanide producer strains.

**Chitinase production assays**

Chitin is an important component of the cell walls of fungi, and chitinolytic microorganisms are likely to act as biocontrol agents and pathogen antagonists. Chitinase determination basal medium (0.3 g L⁻¹ MgSO₄·7H₂O, 3 g L⁻¹ NH₄(SO₄), 2 g L⁻¹ KH₂PO₄, 1 g L⁻¹ citric acid, 15 g L⁻¹ agar, 0.2 g L⁻¹ Tween-80, pH 4.7) was supplemented with 4.5 g L⁻¹ colloidal chitin and 0.15 g L⁻¹ bromocresol purple (Agrawal and Kotasthane, 2012). After inoculation, the Petri dishes were incubated for 48 h at 30°C. Colour changes from yellow to purple indicated chitinase producer strains.

**Molecular identification of selected isolates**

Bacterial isolates were identified by sequencing of the 16S rRNA genes. Genomic DNA was purified using the Gentra Puregen kit (Qiagen). The 16S rRNA gene was amplified using the 27F and 1492R primers (Frank et al., 2008). PCR reactions were each prepared in a final volume of 25 µL, containing 1 µL of genomic DNA (25 ng µL⁻¹), 2.5 µL of Taq Buffer 10×, 0.5 µL of dNTP mix (10 mM), 0.5 µL of primer 27F (10mM), 0.5 µL of primer 1492R (10mM), 0.2 µL of Taq DNA polymerase (5 units µL⁻¹) (Thermo Fisher), and 19.8 µL of ultrapure water to complete the volume. Amplifications were each carried out in a MiniAmp Plus Thermal Cycler (Thermofisher), under the following conditions: a 3 min initial denaturation step of 95°C, followed by 30 cycles of 95°C for 30 sec, 48°C for 30 sec, 72°C for 1 min, and a final cycle 72°C for 10 min.

Fungal isolates were identified by sequencing of the elongation factor tef-1α gene. Total genomic DNA was extracted from mycelia using cetyltrimethylammonium bromide (CTAB), as described by Wagner et al. (1987). The tef-1α gene was amplified using EF1α and EF2 primers (O’Donnell et al., 1998). PCR reactions were each prepared in a final volume of 25 µL, containing 1 µL of genomic DNA (25 ng µL⁻¹), 2.5 µL of Taq Buffer 10×, 0.5 µL of dNTP mix (10 mM), 0.62 µL of primer EF1 (10 mM), 0.62 µL of primer EF2 (10 mM), 0.125 µL of Taq DNA polymerase (5 units µL⁻¹) (Thermo Fisher) and 19.6 µL of ultrapure water to complete the volume. Amplifications were each carried out in a MJ Mini Gradient Thermal Cycler (BioRad) under the following conditions: a 3 min initial denaturation step of 95°C, followed by 35 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final cycle 72°C for 10 min.

All obtained PCR products were verified by electrophoresis on 1% agarose gels, purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich), and sent to Eton Bioscience Inc. for sequencing.

The resulting sequences were aligned using MEGA XI (Kumar et al., 2018), with the multiple alignment program MUSCLE. The bacteria sequences were blasted against the GenBank 16S Ribosomal RNA sequences database (Table 1), and the Trichoderma spp. sequences were compared with the GenBank elongation factor 1α gene sequences database (Table 2), and the clos-
est matches were used to construct each alignment. A
Maximum-Parsimony method was used with Bootstrap
values based on 1,000 replicates. New sequences were
deposited in the GenBank (Tables 1 and 2).

Greenhouse biocontrol assays of Lasiodiplodia brasiliensis

Grapevine plants (Vitis vinifera ‘Cabernet Sauvignon’) obtained from 1-year-old cuttings were used
to determine the biocontrol activity of selected bacterial and fungal strains and a rhizosphere strain rBE015 obtained in a previous study (Delgado-Ramírez et al., 2021). Grapevine shoots were submerged in a 3 g L⁻¹ solution of rooting agent ROOTEX (Cosmocel SA), and were then planted in tubs containing Cosmopeat substrate (Cosmocel SA). After 45 d, the plants were transplanted into 3.78 L plastic pots. Two weeks after transplanting, 50 mL of a solution (1 × 10⁶ CFU) of each potential beneficial microorganism was applied at the base of the plant stem, followed by a second application 7 d later. Control treatments were inoculated with sterile water. For each tested isolate, ten replicates were used. Immediately after the second application of potential BCA, inoculations of the plants with L. brasiliensis were carried out through mechanical wounds in the woody tissues, each made with a drill bit (2 mm diam.), followed by insertion of a mycelium plug inside each hole. After inoculation, the wounds were each covered with parafilm. Plugs of sterile PDA were used as experimental control inoculations. The plants were then kept under greenhouse conditions for 60 d, and necrotic lesions generated in the stems were measured. Attempts were also made to recover the inoculated microorganisms.

Vineyard biocontrol assays of Lasiodiplodia brasiliensis

A field biocontrol trial was carried out in a 2-year-old ‘Chenin Blanc’ vineyard, in Ejido el Porvenir, Baja

Table 1. GenBank and culture accession numbers of bacterium species used in the present study for phylogenetic analyses.

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Isolates from this study are highlighted in bold font.
Biological control of Botryosphaeria dieback

California. Fifty plants, which did not show symptoms associated with wood diseases, were chosen per row on five vineyard rows, leaving an interval of three to five plants between each selected vine. The experimental design was completely randomized with ten grapevines per treatment. Putative BCAs evaluated included five bacteria (BEVP01BCMX, BEVP02BCMX, BEVP26BCMX, BEVP31BCMX, and rbES015) and six fungi (T06BCMX, T11BCMX, T15BCMX, T17BCMX, T20BCMX, and EF11BCMX). In each selected plant, a pruning cut was made in a woody branch, and 10 µL of a 1 × 10^6 CFU suspension of the selected biocontrol organism were inoculated, and 20 plants were treated with each isolate. Five days later, a second inoculation of the biocontrol agent was made in the same wound. One hour later, 10 µL of a 1x10^5 suspension of fragmented mycelium of L. brasiliensis was applied to ten of the plants. Negative controls were inoculated only with sterile distilled water. The inoculated branches were each sealed with parafilm and then covered with a paper bag (Figure S1). One month later, the treated branches were cut, the length of the lesions produced by L. brasiliensis was measured, and a tissue fragment from each branch was inoculated onto PDA to assess if the pathogen and the inoculated BCA was present.

**Statistical analyses**

Data obtained from the greenhouse and vineyard biocontrol experiments were analyzed using one-way ANOVA, with post-hoc LSD analysis, and an α < 0.05 test for statistical significance, using the STATISTICA 8.0 package.
RESULTS

Isolation, screening and molecular identification of micro-organisms from heritage grapevines

A total of 135 isolates of bacteria from the heritage grapevine tissues were characterized by morphological characteristics as Bacillus, Paenibacillus and Pseudomonas. Isolates of fungi included two Trichoderma spp., and Alternaria, Chaetomium, Sordaria and Dipsidia spp. Given the small number of potential beneficial fungal isolates recovered, 35 uncharacterized Trichoderma strains from our laboratory collection were included in this study.

From the 172 evaluated strains, 37 fungal and 21 bacterial isolates showed antagonistic activity in the qualitative antagonism assays (Figure 1). Quantitative dual culture assays and screening for plant growth promotion traits were performed only for those 58 isolates. Results showed that mean inhibition proportions for these BCAs against L. brasiliensis were between 3.4% to 52.8%, and that the isolates had different plant growth promotion characteristics (Table S1).

Based on inhibition proportions, and possession of at least one growth promoting trait, four bacteria (BEVP01BCMX, BEVP02BCMX, BEVP26BCMX, and BEVP31BCMX) and seven fungi (EF09BCMX, EF11BCMX, T06BCMX, T11BCMX, T15BCMX, T17BCMX, and T20BCMX) were selected. Molecular identification of these isolates confirmed that all the bacteria were Bacillus spp. (Figure S2), and all the fungi were...
Biological control of Botryosphaeria dieback

*Trichoderma* spp. (Figure S3). Mean inhibition proportions ranged from 17.1% to 51.8%. Almost all the selected isolates (except *T. asperellum* T20BCMX) produced AIA, and (except *B. amyloliquefaciens* BEVP31BCMX) produced chitinase. Most of the isolates (except *B. axarquiensis* BEVP02BCMX and *B. amyloliquefaciens* BEVP31BCMX) produce siderophores. Four isolates (*B. axarquiensis* BEVP02BCMX, *B. amyloliquefaciens* BEVP26BCMX, *T. asperellum* EF09BCMX and *T. asperellum* EF11BCMX) solubilized phosphate, and one isolate (*B. axarquiensis* BEVP02BCMX) solubilized potassium. None of the isolates solubilized zinc, or produced HCN (Table 3).

**Characterization of mycoparasitic activity of Trichoderma strains**

The pre-colonized plate experiments showed that all the assessed *Trichoderma* isolates had vigorous mycoparasitic activity, with colonization percentages ranging from 70% to 100% (Table 5). When the colonization percentage was 100%, the inoculated phytopathogenic fungus could not be recovered, indicating total suppression. Microscope observations from dual culture assays indicated that all the *Trichoderma* isolates coiled around, and cause morphological deformations, of *L. brasiliensis* hyphae, while the isolates *T. asperellum* T15BCMX and *T. longibrachiatum* T17BCMX also induced lysis of mycelium walls (Table 5; Figure 4).

**Evaluation of biocontrol activity of selected bacterial and fungal isolates in greenhouse trials**

The preventative application to soil of the *Trichoderma* and *Bacillus* isolates, for suppression of *L. brasiliensis* infection revealed the following. While the untreated

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**Table 3. Mean percent inhibition of *Lasiodiplodia brasiliensis* by different potential biocontrol microorganisms, and their respective production of plant growth promotion compounds, for selected *Bacillus* and *Trichoderma* isolates.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mean inhibition %</th>
<th>Production</th>
<th>Solubilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SID</td>
<td>CHI</td>
</tr>
<tr>
<td><em>B. mojavensis</em> BEVP01BCMX</td>
<td>51.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. axarquiensis</em> BEVP02BCMX</td>
<td>17.1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>B. amyloliquefaciens</em> BEVP26BCMX</td>
<td>38.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. amyloliquefaciens</em> BEVP31BCMX</td>
<td>50.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>T. asperellum</em> EF09BCMX</td>
<td>51.8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>T. asperellum</em> EF11BCMX</td>
<td>51.7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>T. harzianum</em> T06BCMX</td>
<td>41.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>T. asperellum</em> T11BCMX</td>
<td>29.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>T. asperellum</em> T15BCMX</td>
<td>25.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>T. longibrachiatum</em> T17BCMX</td>
<td>52.8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>T. asperellum</em> T20BCMX</td>
<td>39.3</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ positive result, - negative result. SID, siderophore production; CHI, chitinase production; HCN, HCN production; IAA, indole acetic acid production; P, phosphate solubilization; K, potassium solubilization; Zn, zinc solubilization.
Table 4. Mean percent inhibition of *Lasiodiplodia brasiliensis* and *Neofusicoccum parvum* from volatile organic compounds and diffusible organic compounds by different *Bacillus* and *Trichoderma* isolates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean inhibition (%) from volatile organic compounds</th>
<th>Mean inhibition (%) from diffusible organic compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. brasiliensis</em></td>
<td><em>N. parvum</em></td>
</tr>
<tr>
<td><em>Bacillus mojavensis</em> BEVP01BCMX</td>
<td>0</td>
<td>31.2</td>
</tr>
<tr>
<td><em>B. axarquiensis</em> BEVP02BCMX</td>
<td>0</td>
<td>23.6</td>
</tr>
<tr>
<td><em>B. amyloliquefaciens</em> BEVP26BCMX</td>
<td>0</td>
<td>26.2</td>
</tr>
<tr>
<td><em>B. amyloliquefaciens</em> BEVP31BCMX</td>
<td>0</td>
<td>34.0</td>
</tr>
<tr>
<td><em>Trichoderma asperellum</em> EF09BCMX</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. asperellum</em> EF11BCMX</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. harzianum</em> T06BCMX</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. asperellum</em> T11BCMX</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. asperellum</em> T15BCMX</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. longibrachiatum</em> T17BCMX</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. asperellum</em> T20BCMX</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2. Representative images of the antifungal effects of volatile organic compounds produced by *Bacillus* and *Trichoderma* isolates against *Lasiodiplodia brasiliensis* and *Neofusicoccum parvum*.

Figure 3. Representative images of the antifungal effect of diffusible organic compounds produced by *Bacillus* and *Trichoderma* isolates against *Lasiodiplodia brasiliensis* and *Neofusicoccum parvum*. 
Table 5. Mean colonization percentages from pre-colonized plate assays, and microscope observations, indicating mycoparasitism activity of six Trichoderma isolates against Lasiodiplodia brasiliensis.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mean colonization percentage</th>
<th>Type of effect of mycoparasitism</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. asperellum EF09CMX</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>T. asperellum EF11BCMX</td>
<td>70</td>
<td>+</td>
</tr>
<tr>
<td>T. harzianum T06BCMX</td>
<td>80</td>
<td>+</td>
</tr>
<tr>
<td>T. asperellum T11BCMX</td>
<td>90</td>
<td>+</td>
</tr>
<tr>
<td>T. asperellum T15BCMX</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>T. longibrachiatum T17BCMX</td>
<td>90</td>
<td>+</td>
</tr>
<tr>
<td>T. asperellum T20BCMX</td>
<td>86</td>
<td>+</td>
</tr>
</tbody>
</table>

+ positive result, - negative result.

Figure 4. Representative microscope images (captured after 28 d incubation at 30°C) from pre-colonization assays of Trichoderma isolates against Lasiodiplodia brasiliensis showing mycoparasitism activity of the Trichoderma isolates (red arrows indicate effects caused by Trichoderma). A, Hyphal coiling of T. asperellum EF11BCMX against L. brasiliensis. B, T. harzianum T06BCMX causing deformation in L. brasiliensis hyphae. C, Lysis of the hyphal wall of L. brasiliensis induced by T. longibrachiatum T17BCMX.

Grapevine plants inoculated with L. brasiliensis developed wounds of mean length up to 10.0 cm, the plants treated with three isolates of T. asperellum (T20BCMX, EF09BCMX, and EF11BCMX), B. amyloliquefaciens BEVP26BCMX and Bacillus sp. rbES015 showed significantly shorter necrotic lesions (Figures 5 and 6). Plants inoculated with L. brasiliensis and treated with T. harzianum T6BCMX developed larger lesions than plants inoculated only with L. brasiliensis (Figures 5 and 6). This isolate was the only Trichoderma isolate that gave no effect in the diffusible compounds assays (Figure 3). In the greenhouse tests, all the Bacillus and Trichoderma isolates were recovered from the root tissues of the inoculated plants, and L. brasiliensis was re-isolated from the stems of the plants.
Figure 5. Mean internal necrotic lesion lengths caused by *Lasiodiplodia brasiliensis* (*Lb*) in grapevine plants, after treatments with different *Bacillus* (BEVP02BCMX, BEVP26BCMX and BEVP31BCMX) or *Trichoderma* isolates (T11BCMX, T15BCMX, T17BCMX, T20BCMX, and EF11BCMX). NT, Non-treated Control. Each bar represents the mean for ten (± SE). Different letters indicate differences (*P* < 0.05), according to LSD tests after ANOVA.

Figure 6. Images of grapevine stems after preventive soil inoculations with *Bacillus* or *Trichoderma* isolates and *Lasiodiplodia brasiliensis*. The yellow lines indicate the lengths of necrotic lesions caused by *L. brasiliensis* in ‘Cabernet Sauvignon’ stems.
Evaluation of biocontrol activity of selected isolates under vineyard conditions

In the field assessments, grapevine branches preventively treated with most of the evaluated *Bacillus* and *Trichoderma* isolates showed two to five times shorter lesions than the untreated branches inoculated only with *L. brasiliensis* (Figure 7). Between the *Trichoderma* isolates no statistically significant differences were detected, while among *Bacillus* isolates, *B. axarquiensis* BEVP02BCMX and *B. amyloliquefaciens* BEVP26BCMX gave significant differences from *B. amyloliquefaciens* BEVP31BCMX. Isolate *B. amyloliquefaciens* BEVP26BCMX gave the strongest effect, with a 5-fold reduction on the length of the necrotic lesions (Figures 7 and 8). All the beneficial organisms were re-isolated from the respective treated branches, while *L. brasiliensis* was only re-isolated from the experimental controls and the *Bacillus*-treated vines but not from the *Trichoderma*-treated branches.

**DISCUSSION**

This study has identified endophytic microorganisms associated with heritage grapevines that biocontrol *L. brasiliensis* and *N. parvum*, which are two of the most virulent fungi associated with Botryosphaeria dieback (Gramaje et al., 2018; Rangel-Montoya et al., 2021). The isolates used in this research were originally obtained from GTD symptomatic grapevines growing in the Guadalupe Valley in Baja California, Mexico (Rangel-Montoya et al., 2021). Considering the conditions in which heritage vines grow in this region, with no irrigation and with little cultural management, the study has identified beneficial microorganisms that could be useful in commercial vineyards. This is the first study focusing on biological control potential of microorganisms associated with heritage grapevines.

Under *in vitro* conditions, several *Bacillus* isolates obtained from heritage grapevines inhibited *L. brasiliensis*...
sis and N. parvum, and showed additional characteristics associated with plant growth promotion, including production of siderophores and indole acetic acid, and solubilization of phosphate and potassium. In dual culture assays five Bacillus strains inhibited growth of L. brasiliensis by up to 51%, showing that these assays were useful for the initial screening and selection of strains with antagonistic activity. This method was previously shown to be useful for identifying two Bacillus strains with antagonistic effects against Diaporthe ampelina, Diplo dia seriata, Eutypa lata and N. parvum (Blundell et al., 2021), and two strains of Bacillus velezensis against eight different fungi, including L. theobromae, D. seriata and N. parvum (Bustamante et al., 2022).

While Bacillus spp. inhibited the growth of N. parvum by up to 34% through the production of organic volatile compounds, L. brasiliensis was not affected. However, a decrease in formation of aerial mycelium was observed, indicating a slight antagonistic effect on this fungus. However, diffusible compounds had strong antifungal effects against both pathogenic fungi, although these were less against L. brasiliensis (from 40% to 62%), indicating that diffusible compounds were the main antagonistic mechanisms of these Bacillus isolates. The production of volatile antifungal compounds, such as ketones, alcohols, esters, pyrazine, acids, hydrocarbons, heterocycles, aldehydes, phenols, thioalcohols, and thioesters, has been reported for Bacillus spp. For example, B. amyloliquefaciens CPA-8 produced 1,3 pentadiene, acetoin (3-hydroxy-2 butanone), and thiophene, that reduced in vitro mycelial growth of Monilina laxa, M. fructicola, and Botrytis cinerea (Gotor-Vila et al., 2017). Diffusible compounds with antifungal activity have also been identified from Bacillus spp., including iturin, fengycin, macrolactin, surfactin. Bacillus INECOL-6004, INECOL-6005, and INECOL-6006 showed antagonistic activity against Fusarium kuroshium by the production of iturin, fengycin, and surfactin (Guevara-Avedaño et al., 2020). Identifying the metabolites produced by the Bacillus isolates obtained in the present study, and their role in disease suppression, would be worthwhile.

All of the seven selected Trichoderma strains showed in vitro antagonistic activity. Additionally, they all produced siderophores, chitinase, and indole acetic acid. Several previous studies have identified Trichoderma spp. with activity against GTD pathogens. Úrbez-Torres et al., (2020) evaluated the antagonistic activities of sixteen Trichoderma strains against D. seriata, E. lata and N. parvum, with T. atroviride PARC1018 giving the greatest inhibition of D. seriata and E. lata, and T. koningiopsis against N. parvum. Similarly, Blundell et al., (2021) reported that T. asperellum UC8360 inhibited D. seriata, E. lata, N. parvum and D. ampelina, with D. ampelina being the most inhibitory, while a T. harzianum isolate gave high rates of inhibition of N. parvum (Langa-Lomba et al. 2022). In the present study, three strains showed inhibition proportions greater that 50%. Two of these (T. asperellum EF09BCMX and T. asperellum EF011BCMX) were isolated from heritage grapevines, indicating that the heritage grapevine cv. ‘Mission’ contains beneficial microorganisms that can be used as BCAs with additional benefit of plant growth promotion. Different Trichoderma strains inhibit growth of phytopathogenic fungi through the production of volatile compounds (Zhao et al., 2022). In the present study, however, the selected strains did not inhibit the mycelial growth of L. brasiliensis.
growth of *L. brasiliensis* or *N. parvum* by the production of volatile compounds, although decreases in the formation of aerial mycelium were observed.

Mycoparasitism is considered to be an important biocontrol mechanism of *Trichoderma* (Sood et al., 2020). However, previous studies showed that this mode of action is not always present. For example, from 50 *Trichoderma* isolates evaluated against *Moniliphthora roleri*, mycoparasitism varied between 0% and 100%, with only nine isolates reaching 100% (Reyes-Figueroa et al., 2016). Leiva et al. (2020) also found that for 199 *Trichoderma* isolates, mycoparasitism rates varied from 32% to 100%. Isolates with this capacity parasitize and colonize phytopathogens, reducing the fungal inoculum and alleviating the intensity of the diseases they cause (Nusaibah and Musa, 2019, Mukherjee et al., 2022). The pre-colonized plate assays of the present study showed a colonization rates of *L. brasiliensis* from 70% to 100%. When *T. asperellum* EF09BCMX and *T. asperellum* T15BCMX were evaluated, *L. brasiliensis* was not recovered from the plates, indicating total elimination of the pathogen, as expected by the 100% colonization obtained. In contrast, the microscopical observations showed that hyphae from seven *Trichoderma* isolates coiled around hyphae of *L. brasiliensis*. This ability has been extensively reported. For example, *T. asperellum* UDEAGIEM-H01 formed coils around hyphae of *F. oxysporum* and *Macrophomina phaseolina* (Diaz-Gutierrez et al., 2021), *T. harzianum* KMISO2-2-19A around *Fusarium virguliforme* hyphae (Pimentel et al., 2020), and *T. koningiopsis* around hyphae of *Phytophthora xcam-bivora* (Frascella et al., 2022). Coiling around hyphae is the first step of *Trichoderma* mycoparasitic activity, and is followed by production of hydrolytic enzymes that allow *Trichoderma* to penetrate the hosts and absorb their contents (Rocha-Ramirez et al., 2002). *Trichoderma* isolates produce extracellular cell wall degrading enzymes such as endochitinases, b-1,3- glucanases, and proteases, that lyse pathogen mycelium (Harman et al., 2004; Druzhinina et al., 2011). Although the production of cell wall degrading enzymes was not evaluated in the present study, *T. asperellum* T15BCMX and *T. longibrachiatum* T17BCMX caused deformation and the cell wall lysis of *L. brasiliensis* hyphae, indicating production of enzymes that damaged cell walls, and potential as BCAs.

Although the in vitro tests provided information on the antagonistic potential of the evaluated strains, low inhibition proportions may not indicate that isolates will perform poorly when applied as biocontrol agents under field conditions. Effectiveness of biocontrol in the field often depends on capacity to colonize plant tissues, establish compatible interactions, prevail in the hosts, and tolerate abiotic factors (Finkiel et al., 2017; Afzal et al., 2019). For example, isolate *B. axar-quiensis* BEVP02BCMX showed low inhibition in dual culture assays against *L. brasiliensis*, but when it was applied as a preventive pruning protectant, it reduced the size of the necrotic lesions. Effectiveness of this strain could be related to its ability to colonize grapevine tissues, which is possibly expected considering its endophytic nature. However, beneficial microorganisms do not always reduce damage caused by pathogens, as was observed here with *T. harzianum* T06BCMX, since plants showed longer necrotic lesions compared to those inoculated only with *L. brasiliensis*. This effect has been observed previously. Leal et al. (2021) reported that plants inoculated with *T. atroviride* SC1 and *B. subtilis* PTA-271, applied in soil against *N. parvum*, developed longer necrotic lesions than experimental controls. This is a good reason why potential biological control agents should be thoroughly assessed.

In plants, beneficial microorganisms have been evaluated using different methods, including preventative applications in substrates or directly applied to pruning wounds (Haidar et al., 2016). In the present study, selected isolates were first evaluated by direct application to soil, and then in a vineyard by applying them to pruning wounds. In the greenhouse assay, two *Bacillus* isolates (*Bacillus* sp. rbES015 and *B. amyloliquefaciens* BEVP26BCMX) and three *T. asperellum* isolates (EF09BCMX, EF11BCMX, and T20BCMX) reduced necrotic lesion lengths caused by *L. brasiliensis*. Since the beneficial microorganisms were applied in soil without direct contact with the pathogen, the observed effect could be due to activation of host systemic response. Previous studies have indicated that non-pathogenic bacteria and fungi have capabilities to reduce damage caused by pathogens through activation of induced host systemic resistance. Haidar et al. (2016) and Zehra et al. (2021) identified different bacteria isolates with biocontrol activity against *P. chlamydospora*, when applied prevenatively as drenches. Similarly, Haidar et al. (2021) identified different bacteria isolates that reduced necrotic lesions caused by *N. parvum* in grapevines when inoculated in soil. This opens the possibility for applying selected biocontrol isolates during irrigation or as drenches, diminishing the costs of biocontrol applications.

Pathogens causing GTDs enter grapevines mainly through pruning wounds (Gramaje et al., 2018), so control strategies should focus on wound protection. Few studies have been carried out in field conditions, and they generally used commercial formulations and specific strains. Martinez-Diz et al. (2020) evaluated the *Trichoderma* isolates SC1 and 1-1237 against *D. seriata*
and *P. chlamydospora*, observing low efficacy. The low effectiveness of commercial formulations based on biocontrol agents is common, and has been mainly attributed to microorganism failure to colonize plant tissues (Mutawila et al., 2016). In the present study, eight isolates applied directly to grapevine wounds reduced necrotic lesions caused by *L. brasiliensis*, and only *Bacillus* sp. rbES015 failed. This isolate was obtained from soil so may be incapable of colonizing grapevine tissues. The microorganisms obtained in the present study showed strong biocontrol activity, even though they were applied without the addition of carriers and protectants. Although comprehensive evaluation needs to be carried out, it may be possible to transfer the selected strains to a company or association to develop a formulation based on the strains to improve its usability and stability for grape producers.

In this research, a strain of *T. longibrachiatum* was shown to be a good candidate as a biological control agent. However, this species has been reported as an opportunistic human pathogen of immunosuppressed patients (Myoken et al., 2002; Lipový et al., 2021; Vasiliki et al., 2021). Therefore, the use of *T. longibrachiatum* T17BCMX as biological control agent should be restricted, though it showed excellent antagonistic activity in assays carried out under *in vitro* and *in planta* conditions. Most of the other identified *Trichoderma* strains were *T. asperellum*, which is widely used as a biological control agent, without reported human risks.

The antagonistic activity of the *Bacillus* isolates was mainly due to production of diffusible compounds, while in the *Trichoderma* spp. it resulted from production of diffusible compounds and the mycoparasitism. However, volatile compounds may also have contributed to the biological control activity of the isolates, since all the selected strains produced these compounds that inhibited *Neofusicoccum in vitro*.

In the present study strains with antagonistic activity were tested separately. Combination of isolates of *Bacillus* and *Trichoderma* have been previously shown to be successful. For example, *B. subtilis* PTA-271 and *T. atroviride* SC1 were evaluated individually and together against *N. parvum* Bt67 in two grapevine varieties (‘Tempranillo’ and ‘Chardonnay’). ‘Tempranillo’ plants inoculated with either *T. atroviride* SC1 or the consortium had fewer internal lesions caused by *N. parvum* (Leal et al., 2021). In the future, compatibility among the isolates identified in the present study could be determined for the development of a consortium that takes advantage of the strengths of different isolates.

In conclusion, this study has identified *Bacillus* and *Trichoderma* isolates with biocontrol activity against *L. brasiliensis* when applied preventatively to soil and to the pruning wounds. Therefore, heritage grapevines of Baja California have been shown to be a reservoir of beneficial microorganisms, which can be potentially utilized in commercial grapevine varieties to help reduce damage caused by grapevine trunk disease fungi.

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**LITERATURE CITED**


Biological control of Botryosphaeria dieback


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tion of fungal pathogens associated with grapevine trunk diseases in Arkansas and Missouri. *Fungal Diversity* 52: 169–189. DOI: 10.1007/s13225-11-0110-4


