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## 12th Special issue on Grapevine Trunk Diseases

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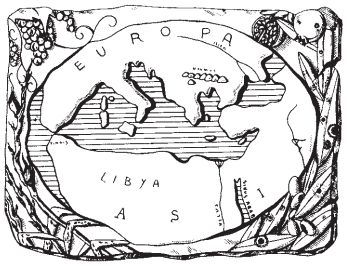


# PHYTOPATHOLOGIA MEDITERRANEA

*Plant health and food safety*

The international journal edited by the Mediterranean Phytopathological Union
founded by A. Ciccarone and G. Goidànich

*Phytopathologia Mediterranea* is an international journal edited by the Mediterranean Phytopathological Union The journal's mission is the promotion of plant health for Mediterranean crops, climate and regions, safe food production, and the transfer of knowledge on diseases and their sustainable management.
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The journal includes three issues each year, publishing Reviews, Original research papers, Short notes, New or unusual disease reports, News and opinion, Current topics, Commentaries, and Letters to the Editor.

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Research Papers – 12th Special issue on Grapevine Trunk Diseases

# 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*.

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## INTRODUCTION

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-Baaijens and Savocchia, 2019). Almost 30 species of *Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia, Neofusicoccum, Neoscytalidium, 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 septate 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 discolourations, 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 *Neofusiccocum* (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, $CaCl_2$ 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. rbES015 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 = $[(R\text{-}r)/R] \times 100$, 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-Martinez *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*

Coluorimetric 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, Coluorimetric 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 $L^{-1}$ $Ca_3(PO_4)_2$, 0.5 g $L^{-1}$ $(NH_4)_2SO_4$, 0.2 g $L^{-1}$ KCl, 0.1 g $L^{-1}$ $MgSO_4$, 0.1 mg $L^{-1}$ $MnSO_4$, 0.1 mg $L^{-1}$ $FeSO_4$, 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 $KNO_3$ 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 (AIA) 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% $HClO_4$, 1 mL 0.5 M $FeCl_3$) (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 subsequently sealed with parafilm and incubated in dark at 30°C for 4 d. Development of orange-red colour indicated positive hydrogen cyanide producer 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 the dark for 96 h at 30°C. Colour changes from blue to yellow indicated siderophore producing 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 $L^{-1}$ $MgSO_4{\cdot}7H_2O$, 3 g $L^{-1}$ $NH_4$ $(SO_4)$, 2 g $L^{-1}$ $KH_2PO_4$), 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 $\mu L^{-1}$), 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 $\mu L^{-1}$) (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 EF1and 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 $\mu L^{-1}$), 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 $\mu L^{-1}$) (Thermo Fisher) and 19.6 µL of ultrapure water to complete the volume. Amplification was 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 rbES015 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 ($1 \times 10^6$ 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.

| Species | Isolate | Isolate source | Origin | GenBank accession number 16S rRNA |
|---|---|---|---|---|
| *Bacillus amyloliquefaciens* | NBRC 15535 | Soil | Japan | NR_112685 |
| *B. amyloliquefaciens* | W9 | Marine water sample | India | MH188056 |
| *B. amyloliquefaciens* | AB-525 | Rice cake | China | KJ879953 |
| *B. amyloliquefaciens* | BsA3MX | Strawberry rhizosphere | Mexico | MW651769 |
| *B. amyloliquefaciens* | BsC11MX | Strawberry rhizosphere | Mexico | MW651770 |
| ***B. amyloliquefaciens*** | **BEVP26BCMX** | **Grapevine** | **Mexico** | **OQ073757** |
| ***B. amyloliquefaciens*** | **BEVP31BCMX** | **Grapevine** | **Mexico** | **OQ073762** |
| *B. axarquiensis* | CIP 108772 | River-mouth sediments | Spain | DQ993670 |
| ***B. axarquiensis*** | **BEVP02BCMX** | **Grapevine** | **Mexico** | **OQ073758** |
| *B. cereus* | ATCC 14579 | Unknown | Unknown | AE016877 |
| *B. circulans* | IAMI 12462 | Soil | Unknown | D78312 |
| *B. coagulans* | NBRC 12583 | Evaporated milk | Unknown | AB271752 |
| *B. licheniformis* | ATCC 14580 | Unknown | Unknown | CP000002 |
| *B. mojavensis* | IFO 15718 | Soil | USA | AB021191 |
| ***B. mojavensis*** | **BEVP01BCMX** | **Grapevine** | **Mexico** | **OQ073759** |
| *B. mycoides* | ATCC 6462 | Soil | Unknown | AB021192 |
| *B. siamensis* | PD-A10 | Poo-dong | Thailand | GQ281299 |
| *B. siamensis* | RET2912 | Landfill soil | India | MN530054 |
| *B. siamensis* | LFS1715 | Landfill soil | India | MN519261 |
| *B. subtilis* | DSM10 | Unknown | Unknown | AJ276351 |
| *B. subtilis* subsp. *spizizenii* | NBRL B-23049 | Tunisian desert | Tunisia | AF074970 |
| *B. thuringiensis* | IAM 12077 | Mediterranean flour moth | Unknown | D16281 |
| *B. vallismortis* | DSM 11031 | Soil | USA | AB021198 |
| *B. velezensis* | CR-502 | Brackish water | Spain | AY603658 |
| *Alicyclobacillus acidocaldarius* | DSM 446 | Acid hot spring | USA | AJ496806 |

Isolates from this study are highlighted in bold font.

**Table 2.** List of GenBank and culture accession numbers of fungal species used in the present study for phylogenetic analyses.

| Species | Isolate | Isolate source | Origin | GenBank accession number tef-1α |
|---|---|---|---|---|
| *Trichoderma asperellum* | Th047 | Soil | Colombia | AB568381.1 |
| *T. asperellum* | cds | Not available | Brazil | KP696459.1 |
| *T. asperellum* | ST1 | Not available | Spain | KJ677260.1 |
| ***T. asperellum*** | **T11BCMX** | **Carnation** | **Mexico** | **OQ161180** |
| ***T. asperellum*** | **T15BCMX** | **Grapevine** | **Mexico** | **OQ161181** |
| ***T. asperellum*** | **T20BCMX** | **Grapevine** | **Mexico** | **OQ161182** |
| ***T. asperellum*** | **EF09BCMX** | **Grapevine** | **Mexico** | **OQ161183** |
| ***T. asperellum*** | **EF11BCMX** | **Grapevine** | **Mexico** | **OQ161184** |
| *Trichoderma atroviride* | DAOM 238037 | Not available | Thailand | KJ871093 |
| *T. atroviride* | PARC1011 | Not available | Italy | MT454114 |
| *Trichoderma guizhouense* | DAOM 231412 | Not available | Not available | AY605764 |
| *T. guizhouense* | DAOM 231435 | Not available | Not available | EF191321 |
| *T. guizhouense* | PARC1022 | Prunus persica | Italy | MT454125 |
| *Trichoderma harzianum* | DAOM 233986 | Not available | Not available | EF392749 |
| *T. harzianum* | DAOM 242937 | Not available | Not available | KX463434 |
| *T. harzianum* | PARC1019 | Prunus persica | Italy | MT454122 |
| ***T. harzianum*** | **T06BCMX** | **Grapevine** | **Mexico** | **OQ161179** |
| *Trichoderma koningiopsis* | Arak-96 | Soil | Iran | KP985652 |
| *T. koningiopsis* | ITCC 7291 | Soil | India | LN897322 |
| *T. koningiopsis* | PARC1024 | Prunus persica | Italy | MT454127 |
| *Trichoderma longibrachiatum* | DAOM 234103 | Not available | Not available | DQ125467 |
| *T. longibrachiatum* | CIB T13 | Not available | Colombia | EU280033 |
| *T. longibrachiatum* | PARC1015 | Not available | Italy | MT454118 |
| ***T. longibrachiatum*** | **T17BCMX** | **Grapevine** | **Mexico** | **OQ161184** |
| *Trichoderma paraviridescens* | BMCC:LU786 | Not available | New Zealand | KJ871271 |
| *T. paraviridescens* | KX098484 | Not available | New Zealand | KX098484 |
| *T. paraviridescens* | PARC1016b | Not available | Italy | MT454119 |

Isolates from this study are highlighted in bold font.

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 $1 \times 10^6$ 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 $\alpha < 0.05$ test for statistical significance, using the STATISTICA 8.0 package.

## RESULTS

### *Isolation, screening and molecular identification of microorganisms 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 *Diplodia* 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

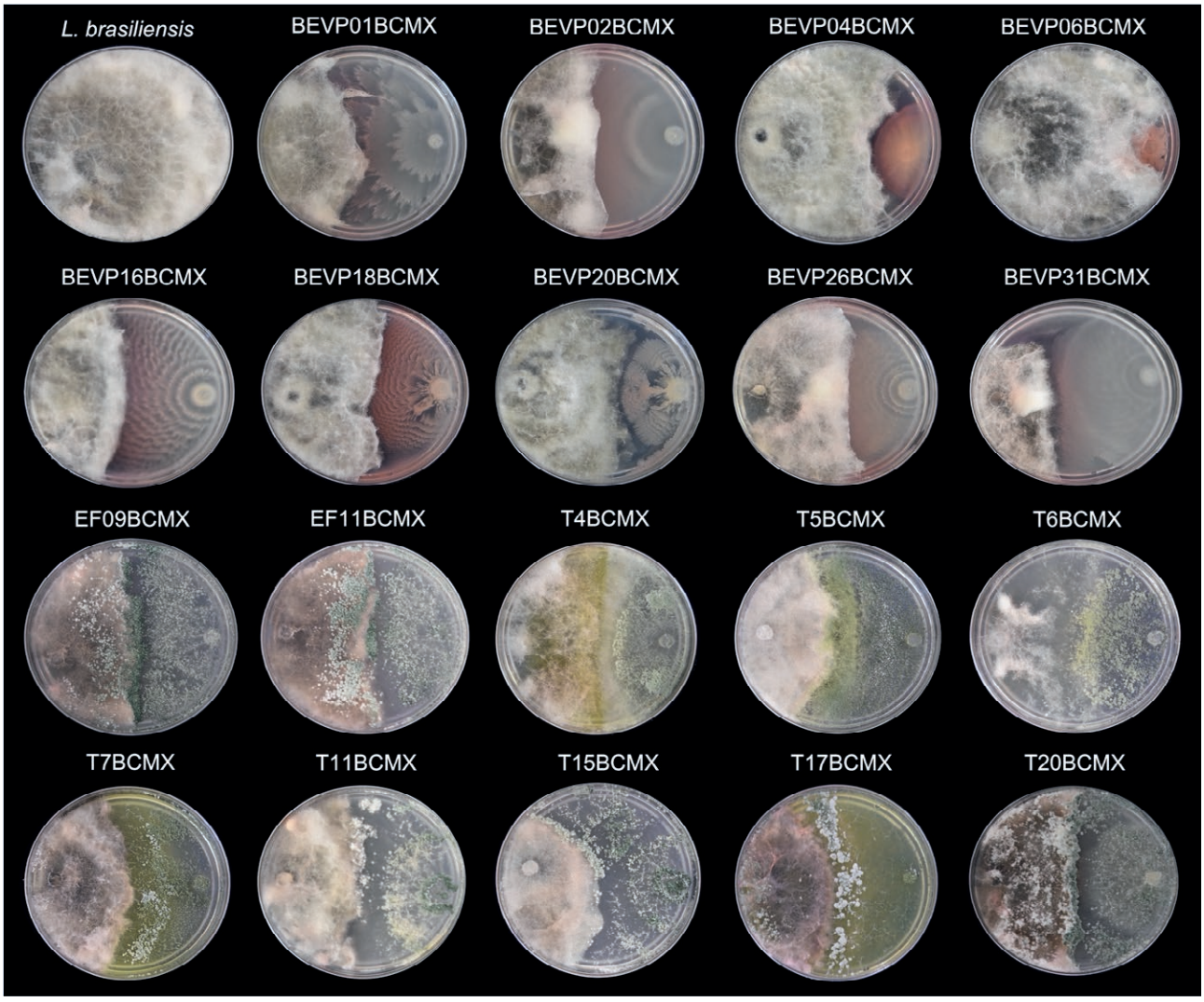


**Figure 1.** Representative images of dual culture assays of selected *Bacillus* and *Trichoderma* isolates against *Lasiodiplodia brasiliensis.*

**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.

| Isolate | Mean inhibition % | Production | | | | Solubilization | | |
|---|---|---|---|---|---|---|---|---|
| | | SID | CHI | HCN | IAA | P | K | ZN |
| *B. mojavensis* BEVP01BCMX | 51.3 | + | + | - | + | - | - | - |
| *B. axarquiensis* BEVP02BCMX | 17.1 | - | + | - | + | + | + | - |
| *B. amyloliquefaciens* BEVP26BCMX | 38.0 | + | + | - | + | + | - | - |
| *B. amyloliquefaciens* BEVP31BCMX | 50.6 | - | - | - | + | - | - | - |
| *T. asperellum* EF09BCMX | 51.8 | + | + | - | + | + | - | - |
| *T. asperellum* EF11BCMX | 51.7 | + | + | - | + | + | - | - |
| *T. harzianum* T06BCMX | 41.0 | + | + | - | + | - | - | - |
| *T. asperellum* T11BCMX | 29.1 | + | + | - | + | - | - | - |
| *T. asperellum* T15BCMX | 25.1 | + | + | - | + | - | - | - |
| *T. longibrachiatum* T17BCMX | 52.8 | + | + | - | + | - | - | - |
| *T. asperellum* T20BCMX | 39.3 | + | + | - | - | - | - | - |

+ 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.

*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).

### *Evaluation of antifungal effect of volatile and diffusible organic compounds*

The eleven isolates were further screened for the antifungal activity from diffusible and volatile organic compounds. None of the assessed *Trichoderma* or *Bacillus* isolates produced volatile organic compounds with suppressive effects on *L. brasiliensis* (Figure 2; Table 4). However, all the *Bacillus* isolates affected growth of *N. parvum*, with mean inhibition percentages ranging from 22.6% to 34.0%. Isolate BEVP31BCMX gave the greatest inhibition (Figure 2; Table 4). In contrast, the 11 isolates affected the growth of both pathogenic fungi by the production of diffusible organic compounds. The *Bacillus* isolates gave mean inhibition percentages from 40.4% to 62.1% against *L. brasiliensis*, and from 66% to 78% against *N. parvum,* while the *Trichoderma* strains gave 61.2% to 81.1% inhibition of *L. brasiliensis* and close to 100% inhibition of *N. parvum* (Figure 3; Table 4). While *T. harzianum* T06BCMX did not affect radial colony growth of either of the pathogenic fungi, this isolate caused a significant decrease in aerial mycelium (Figure 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

**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.

| Strain | Mean inhibition (%) from volatile organic compounds (%) | | Mean inhibition (%) from diffusible organic compounds | |
|---|---|---|---|---|
| | *L. brasiliensis* | *N. parvum* | *L. brasiliensis* | *N. parvum* |
| *Bacillus mojavensis* BEVP01BCMX | 0 | 31.2 | 62.1 | 78.2 |
| *B. axarquiensis* BEVP02BCMX | 0 | 23.6 | 31.6 | 76.4 |
| *B. amyloliquefaciens* BEVP26BCMX | 0 | 26.2 | 40.4 | 73.8 |
| *B. amyloliquefaciens* BEVP31BCMX | 0 | 34.0 | 49.3 | 66.0 |
| *Trichoderma asperellum* EF09BCMX | 0 | 0 | 63.5 | 100 |
| *T. asperellum* EF11BCMX | 0 | 0 | 61.2 | 98 |
| *T. harzianum* T06BCMX | 0 | 0 | 0 | 0 |
| *T. asperellum* T11BCMX | 0 | 0 | 67.1 | 100 |
| *T. asperellum* T15BCMX | 0 | 0 | 81.1 | 100 |
| *T. longibrachiatum* T17BCMX | 0 | 0 | 66.8 | 98 |
| *T. asperellum* T20BCMX | 0 | 0 | 63.5 | 100 |

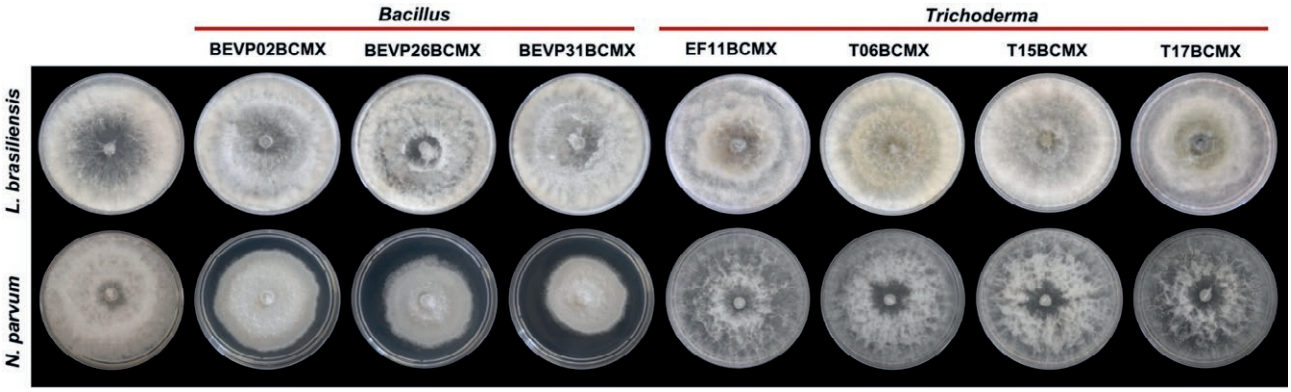


**Figure 2.** Representative images of the antifungal effects of volatile organic compounds produced by *Bacillus* and *Trichoderma* isolates against *Lasiodiplodia brasiliensis* and *Neofusioccum parvum.*

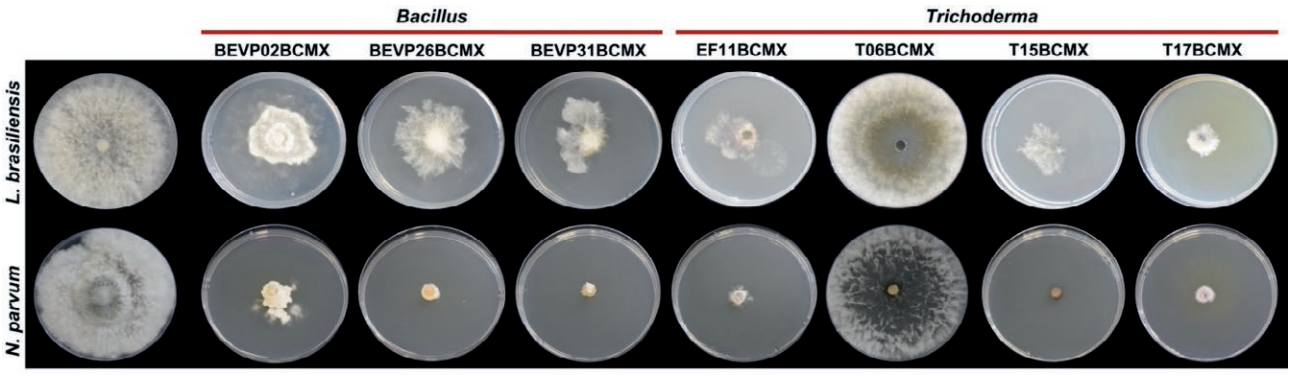


**Figure 3.** Representative images of the antifungal effect of diffusible organic compounds produced by *Bacillus* and *Trichoderma* isolates against *Lasiodiplodia brasili*ensis and *Neofusicoccum parvum*.

**Table 5.** Mean colonization percentages from pre-colonized plate assays, and microscope observations, indicating mycoparasitism activity of six *Trichoderma* isolates against *Lasiodiplodia brasiliensis*.

| Isolate | Mean colonization percentage | Type of effect of mycoparasitism | | |
|---|---|---|---|---|
| | | Coiling | Hyphae deformation | Cell lysis |
| *T. asperellum* EF09CMX | 100 | + | + | - |
| *T. asperellum* EF11BCMX | 70 | + | + | - |
| *T. harzianum* T06BCMX | 80 | + | + | - |
| *T. asperellum* T11BCMX | 90 | + | + | - |
| *T. asperellum* T15BCMX | 100 | + | + | + |
| *T. longibrachiatum* T17BCMX | 90 | + | + | + |
| *T. asperellum* T20BCMX | 86 | + | + | - |

+ positive result, - negative result.

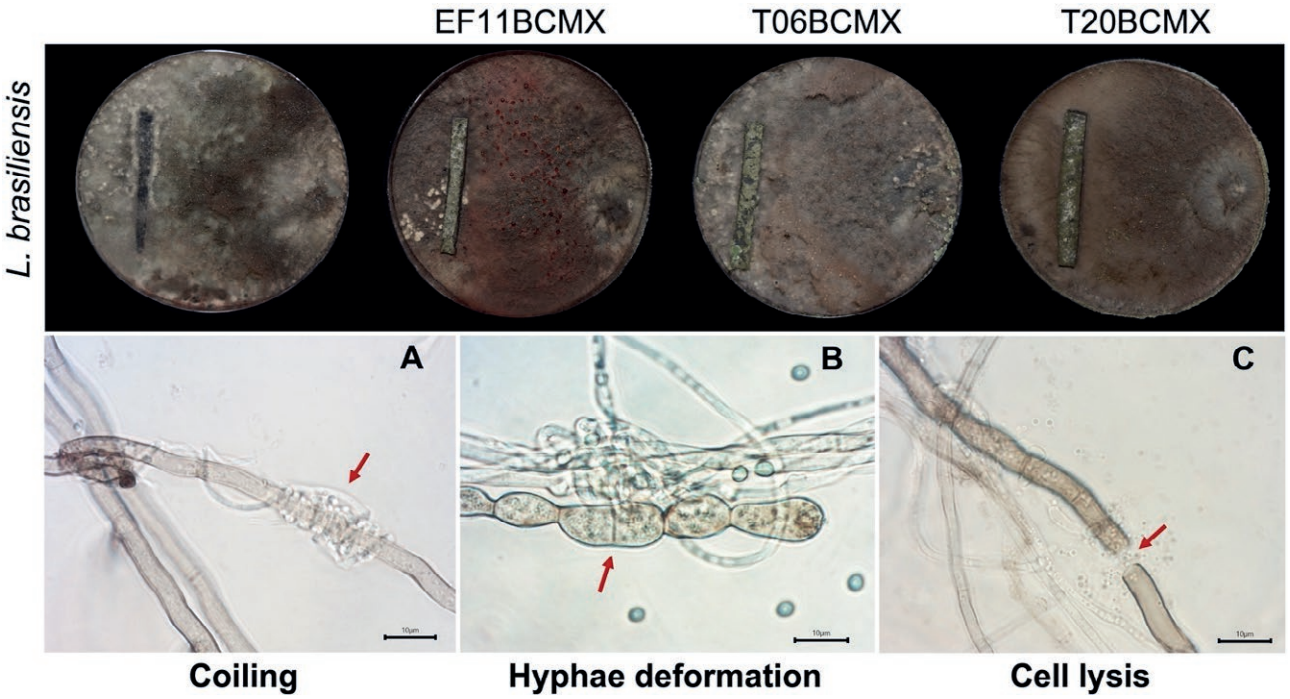


**Figure 4.** Representative microscope images (captured after 28 d incubation at 30ºC) from pre-colonization assays of *Trichoderma* isolates against *Lasiodiplodia brasiliensis* showing mycoparasitism activity of the *Trichoderma* isolates (red arrows indicate effects caused by *Trichoderma*). A, Hyphal 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.

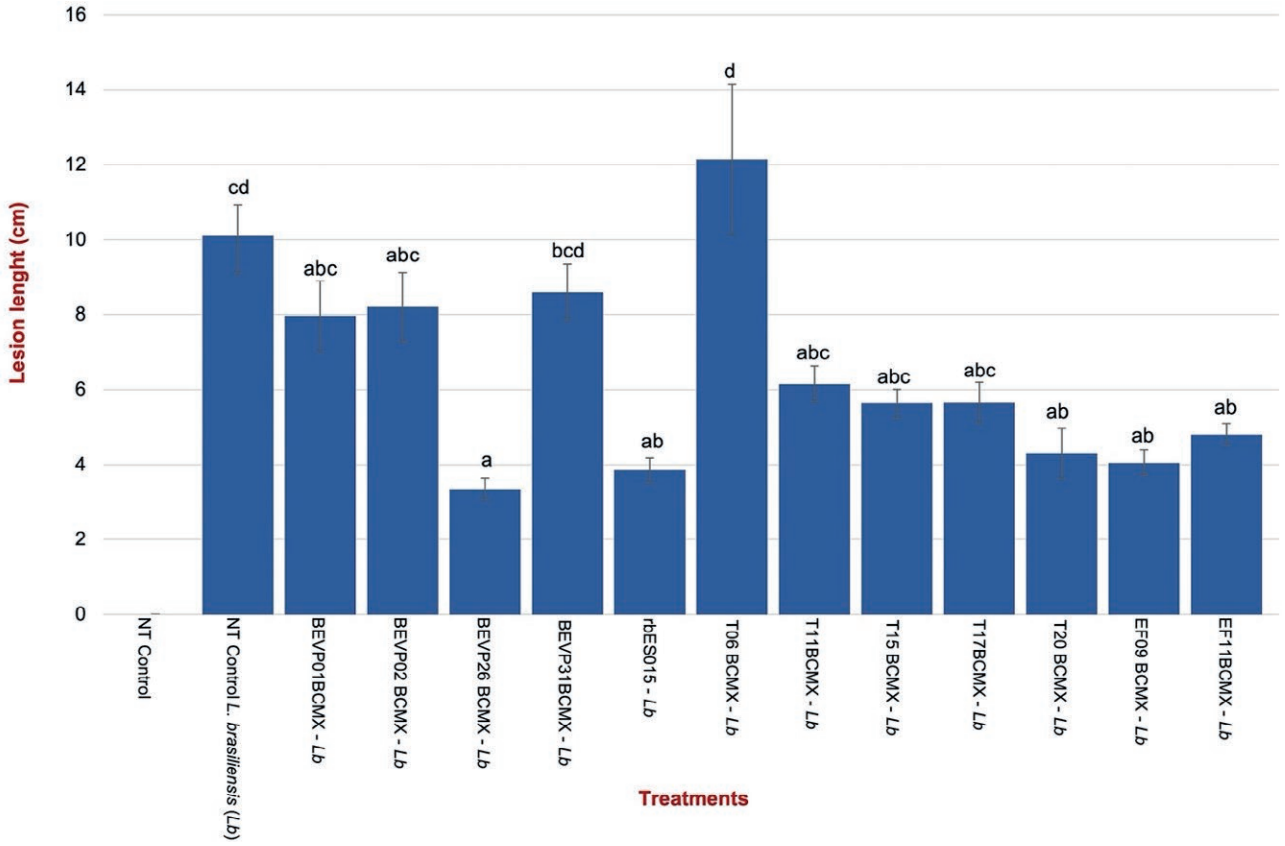


**Figure 5.** Mean internal necrotic lesion lengths caused by *Lasiodiplodia brasiliensis (Lb)* in grapevine plants, after treatments with different *Bacillus* (BEVP02BCMX, BEVP26BCMX and BEVP31BCMX) or *Trichoderma* isolates (T11BCMX, T15BCMX, T17BCMX, T20BCMX, and EF11BCMX). NT**,** Non-treated Control. 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.

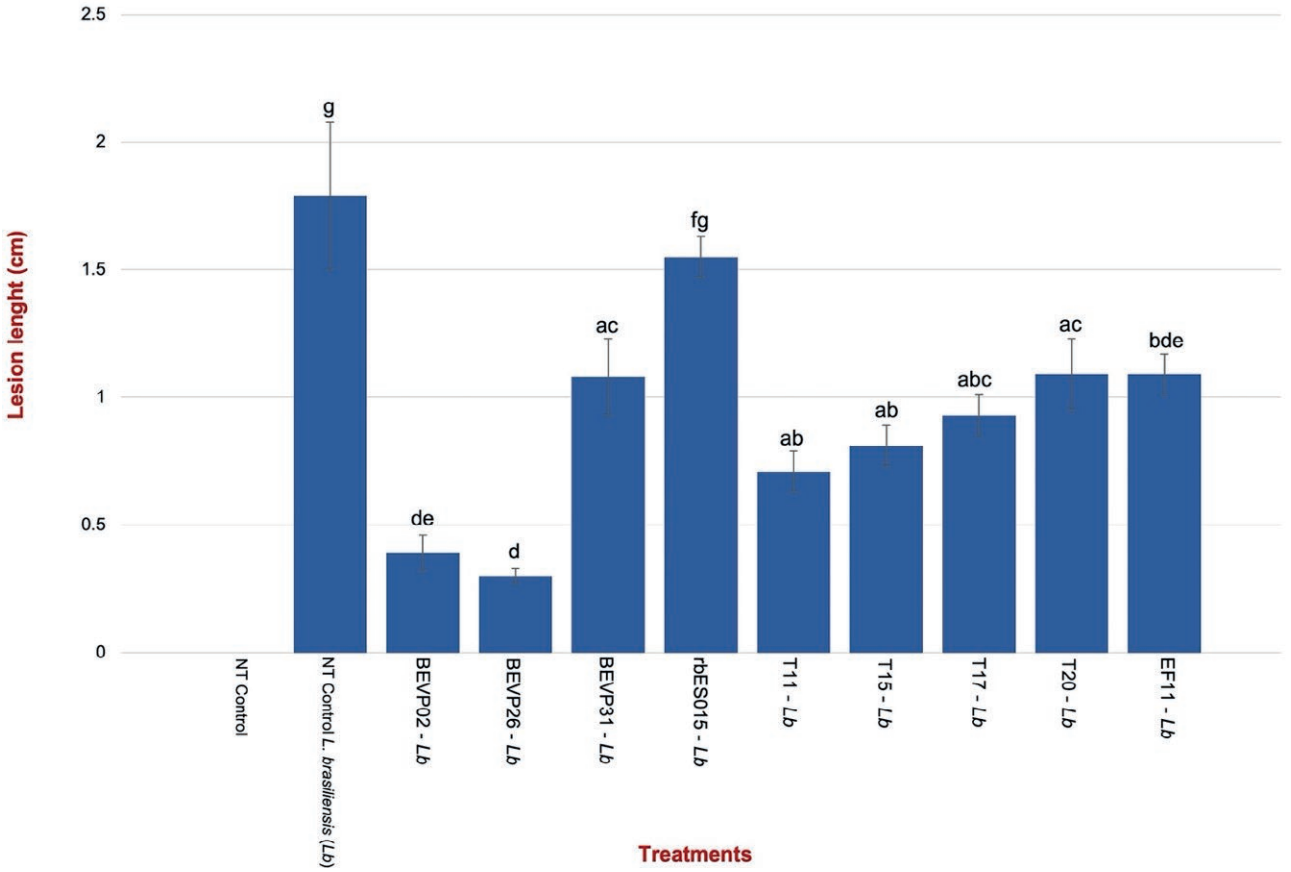


**Figure 7.** Mean internal necrotic lesion lengths in grapevines, caused by *Lasiodiplodia brasiliensis* after treatments with isolates of *Bacillus* (BEVP02BCMX, BEVP26BCMX, and BEVP31BCMX) or *Trichoderma* (T11BCMX, T15BCMX, T17BCMX, T20BCMX, and EF11BCMX). NT, Non-treated control. Each bar represents the mean for ten plants (± SE). Different letters over bars indicate differences ($P < 0.05$), according to LSD tests after ANOVA.

*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. amyloliquefacines* BEVP26BCMX gave significant differences from *B. amyloliquefacines* 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. brasilien-*

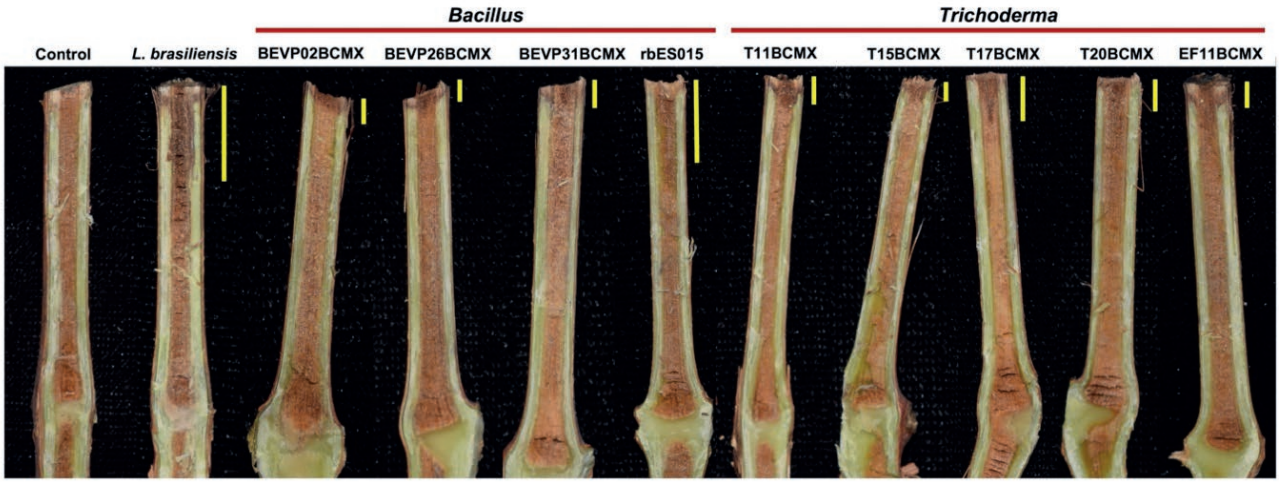


**Figure 8.** Images field-grown grapevine stems after treatments of pruning wounds with different *Bacillus* or *Trichoderma* isolates against *Lasiodiplodia brasiliensis* infections. The images are of ten replicates representative plants, taken of 30 d after *L. brasiliensis* inoculations.

*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, Diplodia 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* 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 *Moniliophthora roreri,* 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* (Díaz-Gutiérrez *et al.,* 2021), *T. harzianum* KMISO2-2-19A around *Fusarium virguliforme* hyphae (Pimentel *et al.,* 2020), and *T. koningiopsis* around hyphe of *Phytophthora xcambivora* (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 (Finkel *et al.,* 2017; Afzal *et al.,* 2019). For example, isolate *B. axarquiensis* 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 preventatively 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. Martínez-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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# Unravelling the colonization mechanism of *Lasiodiplodia brasiliensis* in grapevine plants

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**Summary.** *Botryosphaeriaceae* cause the degenerative disease Botryosphaeria dieback in many woody hosts, including grapevine. These pathogens penetrate host plants through pruning wounds, and colonize vascular tissues causing necrotic lesions, cankers, and eventually plant death. Colonization processes by *Botryosphaeriaceae* and their interactions with their hosts has been understudied. The colonization mechanisms were examined for *Lasiodiplodia brasiliensis*, a common pathogen causing Botryosphaeria dieback in Mexican vineyards. *Lasiodiplodia brasiliensis* MXBCL28 was inoculated onto grapevine 'Cabernet Sauvignon' plants, and after 2 months, infected tissues were observed with microscopy using histological techniques. *Lasiodiplodia brasiliensis* was also cultured on different carbon sources representing cell walls and non-structural plant components, to complement histology data. The host responded to wounding by developing xylem vessel occlusions with tyloses and deposition of suberin in cambium and ray parenchyma. Infection response also included deposition of suberin in pith tissues, reinforcement of cell walls with phenolic compounds, and lignin deposition in xylem vessels and ray parenchyma. The pathogen could overcome host compartmentalization mechanisms and colonize wood tissue causing extensive necrosis. The fungus was visualized in host cambium, vascular bundles, xylem vessels, and pith, and infected tissues were depleted in starch in the ray parenchyma. Cellulose, hemicellulose, and lignin in cell walls were also degraded, supporting *in vitro* data.

**Keywords.** Botryosphaeria dieback, plant defense, carbohydrate metabolism.

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## INTRODUCTION

Grapevine is threatened by many pathogenic microorganisms that reduce yields and fruit quality (Armijo *et al*., 2016). Grapevine Trunk Diseases (GTDs) are considered the most destructive diseases causing significant economic losses to the grape industry (Gubler *et al*., 2005; Kaplan *et al*., 2016; Gispert *et al*., 2020). These diseases are caused by a complex of wood-inhabiting fungi in *Lasiodiplodia, Neofusicoccum*, *Diplodia*, *Eutypa, Eutypella*, *Phaeomoniella*, and *Phaeoacremonium*, among others (Bertsch *et et al*., 2013; Fon-

taine *et al*., 2016; Claverie *et al*., 2020). These fungi form sexual and/or asexual fruiting bodies on infected woody tissues. Spores produced within these structures are released and spread in vineyards by wind and water, and enter plants through pruning wounds. Management of GTDs is mostly achieved by preventative measures that consist of protecting pruning wounds against infections (Rolshausen *et al*., 2010; Kaplan *et al*., 2016; Martínez-Diz *et al*., 2021; Blundell and Eskalen, 2022).

Following wound infection, the GTD pathogens colonize host vascular tissues after spore germination. Most fungi causing GTDs are necrotrophs, degrading structural and non-structural host cell wall components (lignin, cellulose, hemicellulose, starch) for metabolic processes. Imaging data has indicated that GTDs initially invade host xylem vessels before spreading to neighbouring tissues (Amponsah *et al*., 2012; Obrador-Sánchez and Hernandez-Martinez, 2020). The qualitative and quantitative virulence traits (cell wall degrading enzymes, toxins, degradation of anti-fungal plant compounds) are key determinants of the wood colonizing capabilities, symptom types, and aggressiveness of the pathogen (Morales-Cruz *et al*., 2015; Garcia *et al*., 2021). For example, *Eutypa lata* and *Neofusicoccum parvum* develop characteristic wedge-shaped cankers in host woody tissues, and both pathogens share similar hydrolytic enzyme profiles that can break down glucose-rich polymers in secondary cell walls. In contrast, *Phaeomoniella chlamydospora* has limited enzymatic capabilities primarily targeting pectin-rich components, and infections are restricted to xylem elements leading to symptoms concentric rings of dark spots in wood (Rolshausen *et al*., 2008; Morales-Cruz *et al*., 2015; Pouzoulet *et al*., 2017). In addition, the most aggressive *Botryosphaeriaceae* (*Lasiodiplodia* and *Neofusicoccum*) associated with the largest plant lesions also possessed the greatest numbers of protein-coding genes involved in wood degradation and host colonization (Garcia *et al*., 2021).

Host plants respond to invasions by pathogens with constitutive and induced defense mechanisms (Freeman and Beattie 2008; Mithöfer and Boland 2012). Compartmentalization is a general defense response to wounding and pathogen infection to isolate injured tissues (Shigo and Tippett, 1981; Shigo, 1984). The model of compartmentalization of decay in trees (CODIT) describes formation and host reinforcement of anatomical walls to spatially restrict the movement of pathogens. The host impedes longitudinal movement of the pathogen by producing pectin-rich tyloses and gels in the xylem vessels, whereas vertical, radial and tangential movement are restricted by depositing lignin, suberin, and phenolic compounds in ray parenchyma, growth rings and vascular cambium (Shigo and Marx, 1977; Tippett and Shigo 1981; Pearce, 1996; Pearce, 2000). Histological studies have shown reinforcement of cell walls against wood colonizing agents in several pathosystems including those in grapevine (Fleurat-Lessard *et al*., 2013; Pouzoulet *et al*., 2013; Pouzoulet *et al*., 2017; Pouzoulet *et al*., 2022).

Induced defense mechanisms are triggered by effector molecules produced by pathogens, and by host plant cell wall degradation products, and compounds include reactive oxygen species (ROS), enzymes, tannins and phytoalexins (Joshi *et al*., 2021; Kaur *et al*., 2022). Production of phytoalexins (stilbene, resveratrol, flavonoid, viniferin) plays important roles in grapevine defense mechanisms, including plant cell wall reinforcement against biotrophic and necrotrophic pathogens (Adrian *et al*., 2012), and these compounds have been reported in vines infected by GTD pathogens (Spagnolo *et al*., 2014; Magnin-Robert *et al*., 2016; Rusjan *et al*., 2017). Together, this information indicates that phenolic compounds have essential physical and chemical properties that contribute to limiting GTD pathogen colonization, and could affect disease tolerance amongst grapevine cultivars (Rolshausen *et al*., 2008; Gómez *et al*., 2016).

GTD infections lead to host decline and death, because they affect host hydraulic functions by obstructing xylem and phloem translocation of water and nutrients. Infections also affect carbohydrate metabolism by decreasing photosynthesis and depleting carbon storage (Rudelle *et al*., 2005; Fontaine *et al*., 2016; Pouzoulet *et al*., 2017; Batista *et al*., 2021). Some pathogens of the GTD complex have also been reported to live within hosts without causing symptoms (Slippers and Wingfield, 2007; Zhang *et al*., 2021). The transition from commensal to pathogenic phase has been related to stress factors such as drought or heat, which predispose plants and increase their susceptibility (Hrycan *et al*., 2020). The number of reports of diseases caused by *Botryosphaeriaceae* has increased in the past decade, possibly because of climate change (Mehl *et al*., 2017; Batista *et al*., 2021). Transcriptomic and genomic analyses have revealed gene families of cell wall degrading enzymes (CWDE), and carbohydrate metabolism, were induced under heat stress conditions (Paolinelli *et al*., 2016; Yan *et al*., 2018; Félix *et al*., 2019; Gonçalves *et al*., 2019; Garcia *et al*., 2021; Nagel *et al*., 2021).

Despite their agricultural importance, studies of *Botryosphaeriaceae* designed to increase understanding of host colonization mechanisms have been limited. *Lasiodiplodia* spp. are among the causal agents of Botryosphaeria dieback, and are aggressive pathogens of grapevine (Úrbez-Torres, 2011). A total of 749 hosts of these fungi have been reported (Batista *et al*., 2021).

In Mexico, in the states of Baja California and Sonora, *Lasiodiplodia brasiliensis* has been frequently isolated from grapevine and has been reported as highly virulent (Rangel-Montoya *et al*., 2021).

The present study aimed to increase understanding of the colonization processes of *L. brasiliensis* in grapevine, and to broaden knowledge of its pathogenesis and subsequent symptom development.

## MATERIALS AND METHODS

### *Fungus and plant materials*

*Lasiodiplodia brasiliensis* MXBCL28 was isolated from a diseased 'Cabernet Sauvignon' grapevine growing in Baja California (Rangel-Montoya *et al*., 2021). This isolate was preserved in 20% glycerol at 4°C, and has been routinely cultivated in Potato Dextrose Agar (PDA; Difco) at 30°C.

One-year-old 'Cabernet Sauvignon' grapevines (the main cultivar in Baja California) were obtained from a local nursery. Plants were inoculated with *L. brasiliensis* MXBCL28 through wounds made in the woody tissues with a drill bit (2 mm diam.) (Úrbez-Torres *et al*., 2010). For each inoculation, a mycelial plug of the fungus was placed inside the wound and was the covered with Parafilm®. Plugs of sterile PDA were used for inoculation control plants. Plants were kept in a greenhouse under semi-controlled conditions at an average temperature of 35°C day and 25°C night, for 2 months during summertime. Ten plants were used per treatment. Subsequently, samples were taken from necrotic lesions. For each sample, a 4 cm long section, including 2 cm above and 2 cm below the inoculation site, was obtained, and was then fixed in FAA solution (Formaldehyde (SIGMA), Acetic acid (Faga Lab), Ethyl Alcohol (Jalmek); 5:5:9) for 24 h at 4°C, and then rinsed and preserved in 80% ethanol at 4°C until used. Part of each sample close to the necrotic tissue was used to re-isolate the inoculated fungus onto PDA to confirm the fungus colonization.

### *Histology of grapevine plants infected with* Lasiodiplodia brasiliensis *MXBCL28*

Longitudinal and transverse sections of fixed tissues of thickness approx. 70 µm were made using a manual microtome. Sections were stained with 0.1% Toluidine B (TBO) (Mallinckrodt Chemical Works) to observe production of phenolic compounds and pectic substances. TBO is a metachromatic cationic dye that interacts with carboxylic groups, staining phenolic compounds purple to greenish-blue or bright blue and polyphenolic substances (e.g. lignin, tannins, pectic acids) purple and reddish-orange (Ling-Lee *et al*., 1977; Ruzin, 1999). For starch staining, iodine-potassium iodide (IKI; Mallinckrodt Chemical Works) was used, containing 5% iodine (Mallinckrodt Chemical Works) and 10% potassium iodide IKI intercalates into starch structure giving making it black (Ruzin, 1999). Suberin deposits were observed using 0.001% Sudan black IV (Chem-Impex) in 70% ethanol. Suberin solubilizes Sudan IV and fluoresces in red (Ruzin, 1999; Yeung, 2015). The presence of lignin in woody tissues was observed using 0.1% Phloroglucinol-HCl (Phl-HCl; Chem-Impex) and Mäule stain (Nakano and Meshitsuka, 1992). The cinnamaldehyde end groups of phenolic compounds react with Phl-HCl, making lignin pink-purple (Adler, 1977; Liljegren *et al*., 2010). The Mäule stain gives colouration to lignin due to the reaction with the syringyl lignin units (Meshitsuka and Nakano, 1977; Yamashita, *et al*., 2016). Calcofluor White M2R 0.02% (Fulka) and Congo Red 0.5% (SIGMA) were used to distinguish cellulose and hemicellulose polysaccharides. Calcofluor White M2R interacts with β-glucans and cellulosic cell walls fluoresce bright blue, while Congo Red interacts with β-glucans and β-xylans and cell walls fluoresce in red (Ruzin, 1999; Kim *et al*., 2008; Mitra and Loqué, 2014). Hyphae of *L. brasiliensis* colonizing the plant tissues were observed using Fontana-Masson stain, which is based on the ability of melanin to reduce ammoniacal silver nitrate solution to metallic silver without using an external reducing agent (Lillie, 1965; Rangel-Montoya *et al*., 2020).

All stained sections were observed using a Nikon Eclipse E200 microscope with an AxioCam HRc camera (Zeiss), and with an AxioVert200 with a RisingCam® U3CMOS camera. Epifluorescence microscopy was carried out using an AxioVert200 microscope supplied with a HBO100 100W mercury lamp with ebq100 power. Cellulose and suberin in the plant tissues were observed using a DAPI filter (excitation at 330–380 nm, emission at 420 nm). A TEXAS RED filter (excitation at 542–595 nm, emission at 644 nm) was used to analyze hemicellulose and suberin. Images were analyzed using AxioVision 4.8.2, RisingView, and ImageJ 1.49v software packages.

### *Evaluation of carbon sources for growth of* Lasiodiplodia brasiliensis *MXBCL28*

The ability of *L. brasiliensis* to use different plant components as carbon sources was evaluated. The fungus was grown in Minimal Medium 9 (MM9; 3.0 $g{\cdot}L^{-1}$ $K_2HPO_4$ (Jalmek), 3.0 $g{\cdot}L^{-1}$ $KH_2PO_4$ (JT Baker), 0.5 $g{\cdot}L^{-1}$ NaCl (Fermont), 1.0 $g{\cdot}L^{-1}$ $NH_4Cl$ (SIGMA), 15.0 $g{\cdot}L^{-1}$ agar (Agarmex)) supplemented as carbon sources with

either glucose (Fermont) 1%, xylose (SIGMA) 1%, lignin (SIGMA) 0.1%, starch (SIGMA) 1%, cellulose SIGMA 1%, xylene (Fermont) 1%, glycerol (Fermont) 1%, pectin (SIGMA) 1%, tannic acid 0.1% (Faga Lab), or ground wood 2% ('Cabernet Sauvignon'). A mycelial plug was placed on the edge of each plate, and the plates were incubated at 30°C for 7 d, during which fungal growth was marked every 24 h. Area of the mycelium covering the agar surface in each Petri dish was calculated using the Integrated density and the fraction area tools of ImageJ software. This experiment was carried out in triplicate.

Fungal biomass was measured by inoculating test tubes containing MM9-liquid supplemented with the different carbon sources listed above, each with a mycelium disk of *L. brasiliensis*. All cultures were incubated at 30°C and 120 rpm for 7 d. Subsequently, the mycelium produced for each growth condition was recovered by filtration on a previously weighed Whatman® filter grade 1, and was dried at 50°C for 3–5 d. The biomass was determined by weighing the dry mycelium obtained from each growth condition, and subtracting the weight of the filter and the mycelium disk used for inoculation. For treatments with grapevine wood, ground wood contained in each sample was carefully removed. The experiment was carried out in triplicate.

### *Statistical analyses*

Assumptions of normality of data from the biomass, growth rate, and area of the mycelium experiments from different carbon sources were confirmed using the Kolmogorov-Smirnov test ($P \geq 0.05$). One-way ANOVA followed by *post hoc* Fisher LSD analyses ($\alpha < 0.05$ for significance) were then carried out for all variables, using STATISTICA 8.0 software.

## RESULTS

### *Colonization of grapevine plants by* Lasiodiplodia brasiliensis *MXBCL28*

Two months after inoculation, grapevine plants infected with *L. brasiliensis* showed necrotic lesions along the xylem of mean length 5.5 (+/- 1.2 cm), that were not observed in the control plants (Figure 1A). Fontana-Masson stain allowed observation of melanized mycelium in the inoculation zones, in the vascular cambium of the plants, as well as in the vascular bundles, including those with occlusions (Figure 1 C, D, E and F). No hyphae were observed in the wounded areas or in the pith of the control plants (Figure 1 A).

Cross-sections of control plants stained with iodine-potassium iodide had strong dark colour, indicating the presence of starch in the ray parenchyma and in the wound areas (Figure 2 A). In contrast, cross-sections of infected plants each showed a light brown stain, indicating starch depletion in the ray parenchyma and the pith (Figure 2 B). Under epifluorescence microscopy, control plant tissues stained with Calcofluor White M2R had brownish areas at the wounds, indicating the presence of cellulose (Figure 2 C). Tissues of plants infected with *L. brasiliensis* showed dark zones, indicating depletion of cellulose in the necrotic tissues (Figure 2 D). In sections stained with Congo Red, dark zones were observed in the infected tissues (Figure 2 F) were observed, but not in the control plants (Figure 2 E), indicating the lack of hemicellulose in that area.

Staining with Toluidine Blue O allowed the visualization of phenolic compounds and tyloses in the xylem vessels in both control (Figure 3, A, B and C) and infected plants (Figure 3, D, E and F). In control plants, pectic compounds were observed in the regenerated tissue in reddish-orange and greenish-blue colors (Figure 3A), tyloses in blue-purple dark (Figure 3B), and less abundance of phenolic compounds in the pith (Figure 3C). Observed in purple and greenish-blue colors, in plants inoculated with *L. brasiliensis,* there was also pectin deposition in the occluded xylem vessels (Figure 3E), and phenolic compounds breaking down in the pith (Figure 3F) and in the vascular cambium (Figure 3D).

Infected plants showed lighter pink colour in the lesion areas and in the vascular cambium compared with non-infected tissues (Figure 4, A and B), indicating that the fungus could break down phenolic polymers. Comparison of infected and non-infected wood tissues using the Mäule stain showed that *L. brasiliensis* was able to break down lignin, as indicated by a discolouration in xylem tissues, the ray parenchyma, and the fibres surrounding the occluded vascular bundles (Figure 4 D). Staining with Sudan IV showed red suberin deposits in the control plants, in the cambium cork in the wound areas (Figure 4 E) and in the ray parenchyma (Figure 4 H). In contrast, red suberin was observed in the cambium cork (Figure 4 F), vascular cambium, ray parenchyma (Figure 4 G), and in the pith (Figure 4J), in response to fungal infection. Suberin was not observed in the necrotic rays (Figure 4 K).

### *Evaluation of carbon sources used for* Lasiodiplodia brasiliensis *MXBCL28*

Growth of *L. brasiliensis* MXBCL28 without a carbon source was weak with stunted mycelium (Figure 5

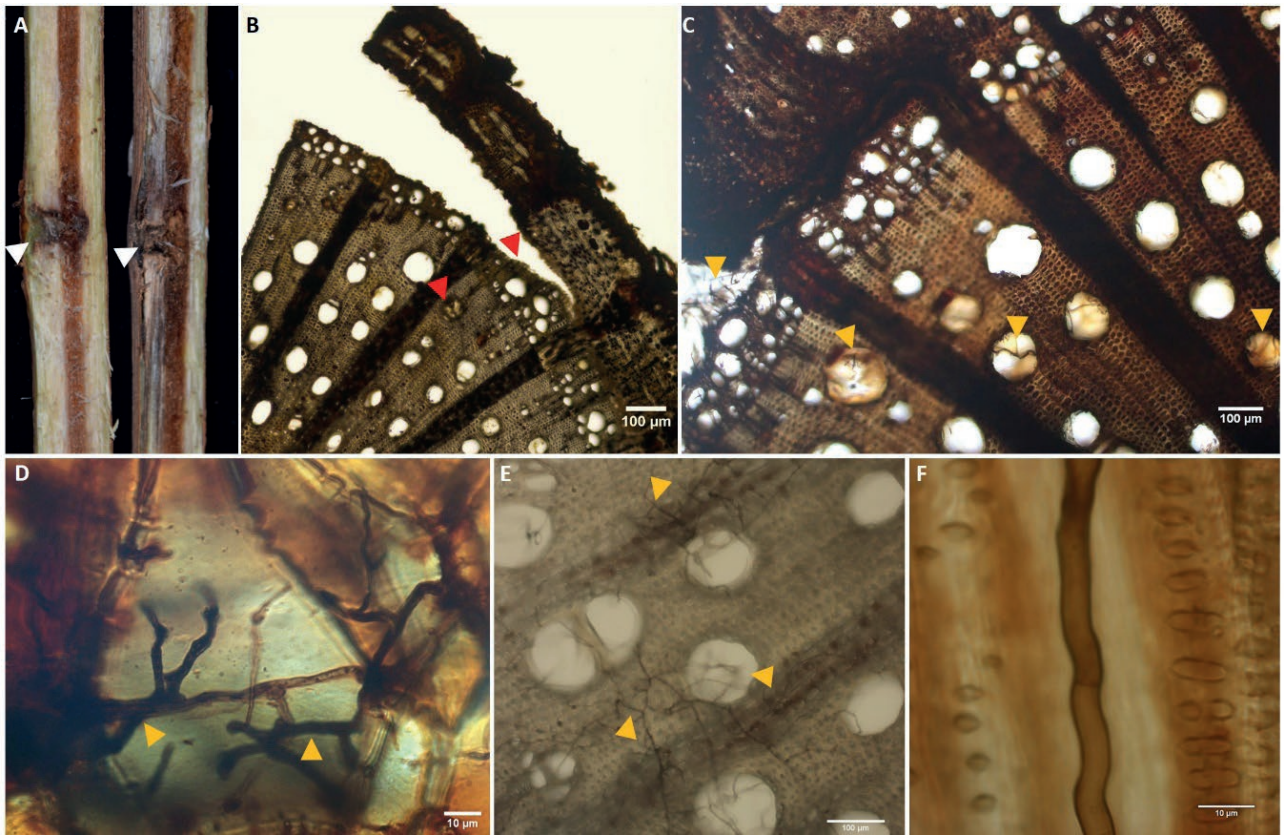


**Figure 1.** Grapevine plants 'Cabernet Sauvignon' 2 months after inoculation with *Lasiodiplodia brasiliensis* MXLBC28. A, Tissue regeneration in control plants (left), and necrotic lesion caused by *L. brasiliensis* (right); white arrows indicate the wound area. Mycelia of *Lasiodiplodia brasiliensis* growing in grapevine plants 'Cabernet Sauvignon' stained using the Masson-Fontana method. B, Control plants non-inoculated; red arrows indicate the wound area and occlusions. C, Melanized fungus colonizing the vascular cambium and vascular bundles. D, melanized fungus colonizing the plant piths. E, melanized hyphae covering the xylem and ray parenchyma. F, melanized hypha growing in a vascular bundle. Yellow arrows indicate melanized hyphae.

A), and biomass was reduced (Table 1). The fungus was able to utilize pectin, xylan, xylose, and starch as carbon sources (Figure 5 C to F), and glucose (Figure 5 B), producing dense dark gray aerial mycelium (Table 1). *Lasiodiplodia brasiliensis* had a significantly increased growth rate and biomass production using pectin as a carbon source (Figure 5C) or ground grapevine wood (Figure 5 K), since the fungus completely covered the media in Petri dishes in 4 d for these carbon sources. The fungus produced less aerial mycelium with cellulose, lignin and tannic acid as carbon sources, as indicated by the calculations of mycelium area (Figure 5, H to J and Table 1). The reduced biomass and mycelium areas were observed with lignin and tannic acid (Figure 5 H and J) as carbon sources. For tannic acid, although production of aerial mycelium and biomass were low, the fungus covered almost the entire medium surfaces in Petri dishes, with sparse mycelium after 7 days.

## DISCUSSION

This study used histology to outline the pathogenesis of *L. brasiliensis* in grapevine, by comparing mechanically wounded plants with those wounded and infected with *L. brasiliensis*. The data obtained have indicated that the physical, constitutive and induced defense responses enacted by the host did not successfully compartmentalize the pathogen.

Wood decay in infected grapevine plants was extensive after 2 months of incubation, indicating that species of *Lasiodiplodia* are highly virulent, as previously shown by Úrbez-Torres and Gubler (2009), Garcia *et al.* (2021) and Rangel-Montoya *et al.* (2021), at least under the local environmental conditions. The high maximum temperatures encountered during the summer months in Mexico are conducive to *L. brasiliensis* pathogenesis (Rangel-Montoya *et al.*, 2021). Melanized hyphae were observed in all infected host woody tissues, including

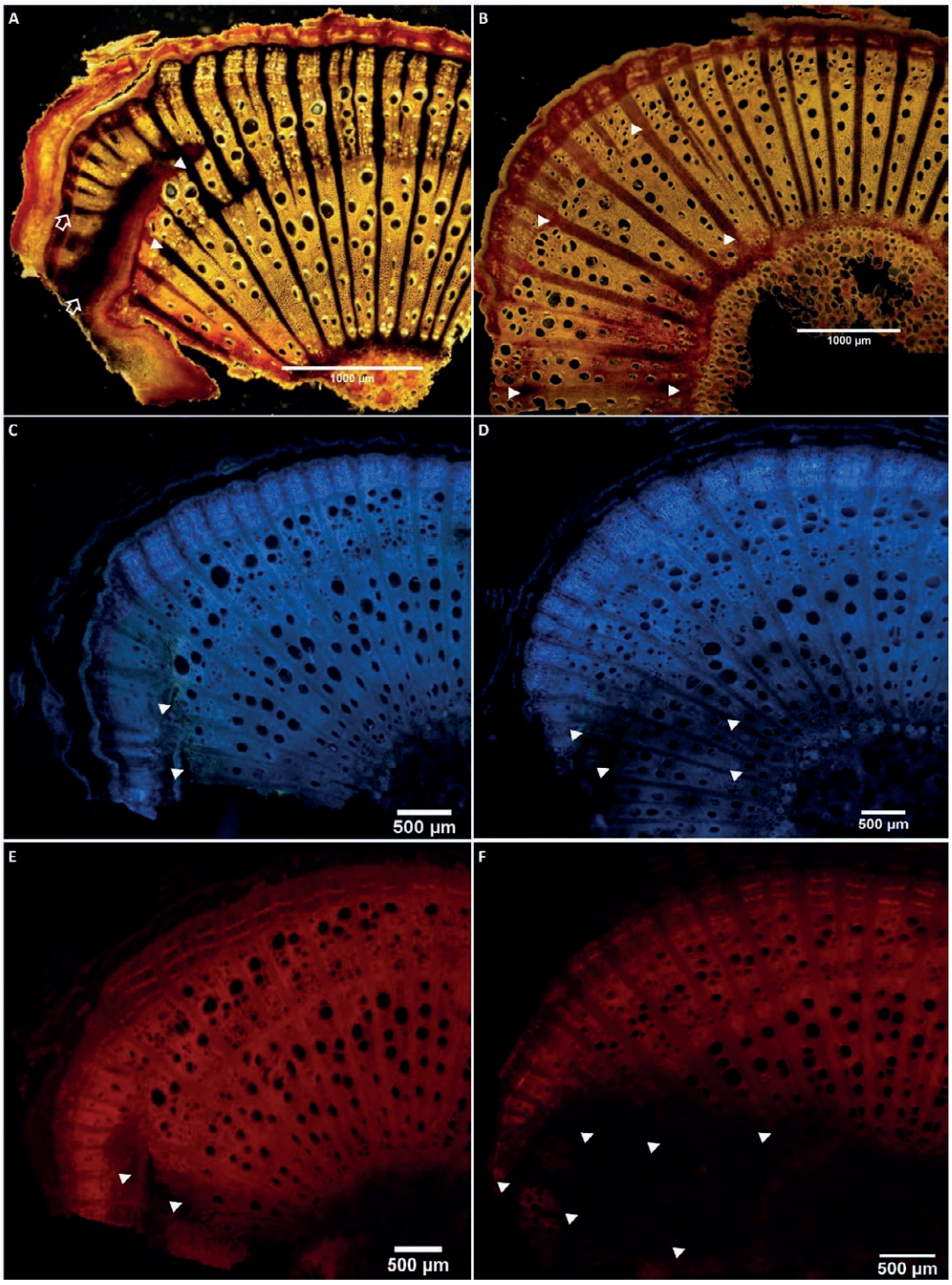


**Figure 2.** Starch and wood degradation by *Lasiodiplodia brasiliensis* in grapevine plants 'Cabernet Sauvignon' 2 months after inoculation. A, C, and E, non-inoculated plants. B, D, and F, plants inoculated with *L. brasiliensis*. A and B, cross sections stained with iodine-potassium iodide for starch content. A, starch deposition is observed as black zones in the ray parenchyma in control plants; white arrows indicate the wound area and the tissue regeneration, while hollow arrows indicate starch in the newly formed tissue. B, infected plants showing starch depletion in the ray parenchyma of the necrotic tissue; arrows indicate the ray parenchyma in the wound area and necrotic tissue. C, D, E and F, epifluorescence microscopy to observe the presence of cellulose and hemicellulose. C and D, sections stained with Calcofluor White M2R, or E and F, Congo Red. Control plants (C and E) show brownish areas in the wound areas. Plants infected with *L. brasiliensis* (D and F) show dark zones without cellulose in the necrotic tissues. White arrows indicate zones without fluorescence where there is no cellulose or hemicellulose.

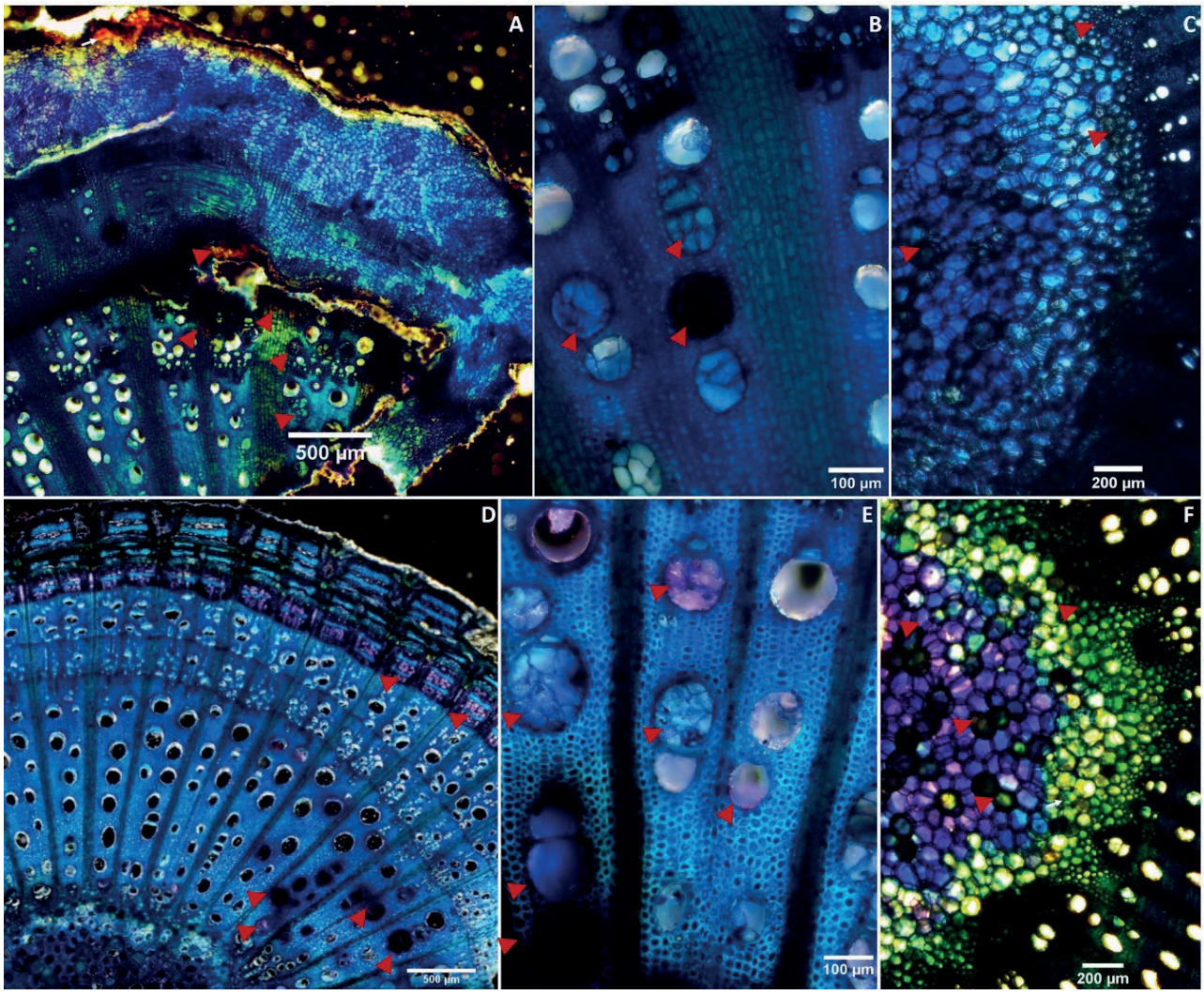


**Figure 3.** Responses of grapevine plants 'Cabernet Sauvignon' to wounding and infection, producing phenolic and pectic compounds. Cross-sections stained with toluidine blue O. A, B and C, Control plants showing production of phenolic and pectic compounds in the vascular cambium, and in the regenerated tissue. D, E and F, Plants infected with *Lasiodiplodia brasiliensis* showing phenolic compounds in the vascular cambium, phloem (D), vessel occlusions with pectic compounds (E), and the pith (F). Red arrows indicate phenolic compounds stained purple, greenish-blue and blue; and pectic compounds stained purple and reddish-orange in the regenerated tissue and occlusions.

vascular cambium, vascular bundles, and in the pith. Similar observations were made in *L. gilanensis* colonizing grapevine plants one-month post-inoculation (Rangel-Montoya *et al.*, 2020).

Melanin is considered to be a virulence factor in several phytopathogenic fungi (Eisenman *et al.*, 2020), and is commonly produced by *Botryosphaeriaceae* (Phillips *et al.*, 2013; Rangel-Montoya *et al.*, 2020). Several *L. gilanensis* genes involved in the synthesis of different melanin pathways were found differentially expressed in the presence of grapevine wood and under heat shock conditions (genes involved in DHN-melanin and pyomelanin pathways), or without heat shock (genes involved in DOPA-melanin pathway) (Paolinelli-Alfonso *et al.*, 2016). Melanin protects against UV radiation, enzymatic lysis, and ROS, and *L. gilanensis* can metabolize tyrosine as carbon and nitrogen sources, and uses this amino acid as a DOPA-melanin precursor (Rangel-Montoya *et al.*, 2020). Phenylalanine is a precursor of plant lignin and suberin syntheses (Lewis *et al.*, 1987), and could be a precursor of DOPA-melanin and pyomelanin pathways, and tyrosine catabolism (Schmaler-Ripcke *et al.*, 2009; Eisenman and Casadevall, 2012). We speculate that *Lasiodiplodia* could derail host plant

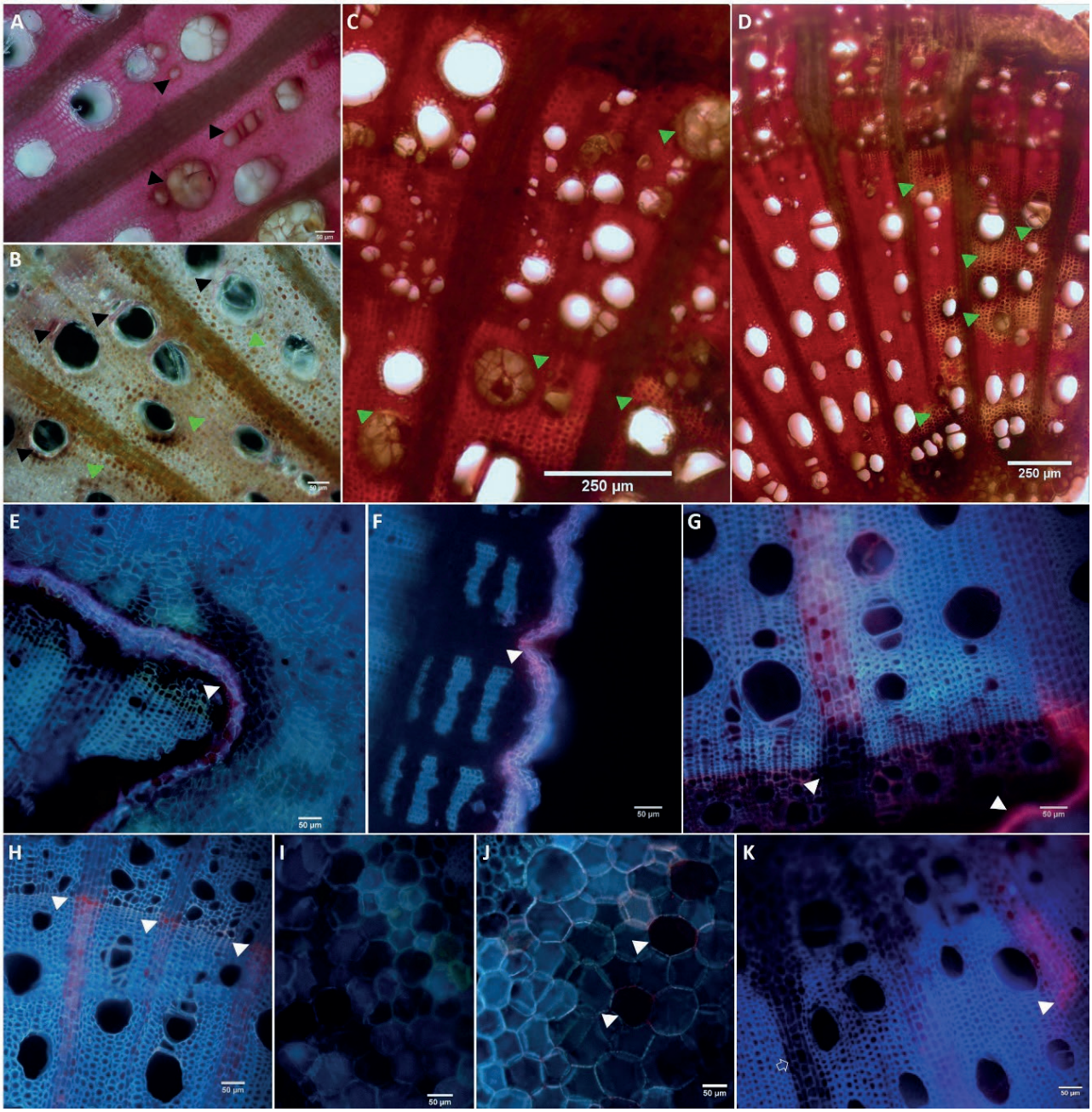


**Figure 4.** Grapevine plant 'Cabernet Sauvignon' responses of phenolic compounds, lignin, and suberin to *Lasiodiplodia brasiliensis* infections. A and B, cross sections of plant stems stained with Phloroglucinol-HCl show lignin in control plants as intense pink in all tissues (A), while in infected plants lighter pink colouration occurred in the lesion areas and vascular cambium, and phenolic deposits in the fibres surrounding the vascular bundles were observed (B). C to D, cross sections stained with the Mäule method to observe the presence of lignin (red colour). Control plants showed slight discolouration in the tissue surrounding occluded vascular bundles near the wound sites (C), while infected plants showed discolouration in the xylem, ray parenchyma, and fibres surrounding occluded vascular bundles (D). Green arrows indicate zones without lignin, and black arrows indicate lignin surrounding vessels. E to K, Epifluorescence microscopy for suberin localization of grapevine plants 'Cabernet Sauvignon'. Samples were stained with Sudan black IV, and observed under TEXAS RED and DAPI filters. Control plants presenting red suberin in cambium cork (E) and ray parenchyma (H) near the wound area, and without suberin in the pith (I). Infected plants 2 months after inoculation with *L. brasiliensis* showed red suberin deposits in the cork (F), vascular cambium, occlude vessels, and ray parenchyma (G and K), and in the pith (J). White arrows indicate the presence of suberin. The hollow arrow indicates ray parenchyma in the necrotic area without suberin.

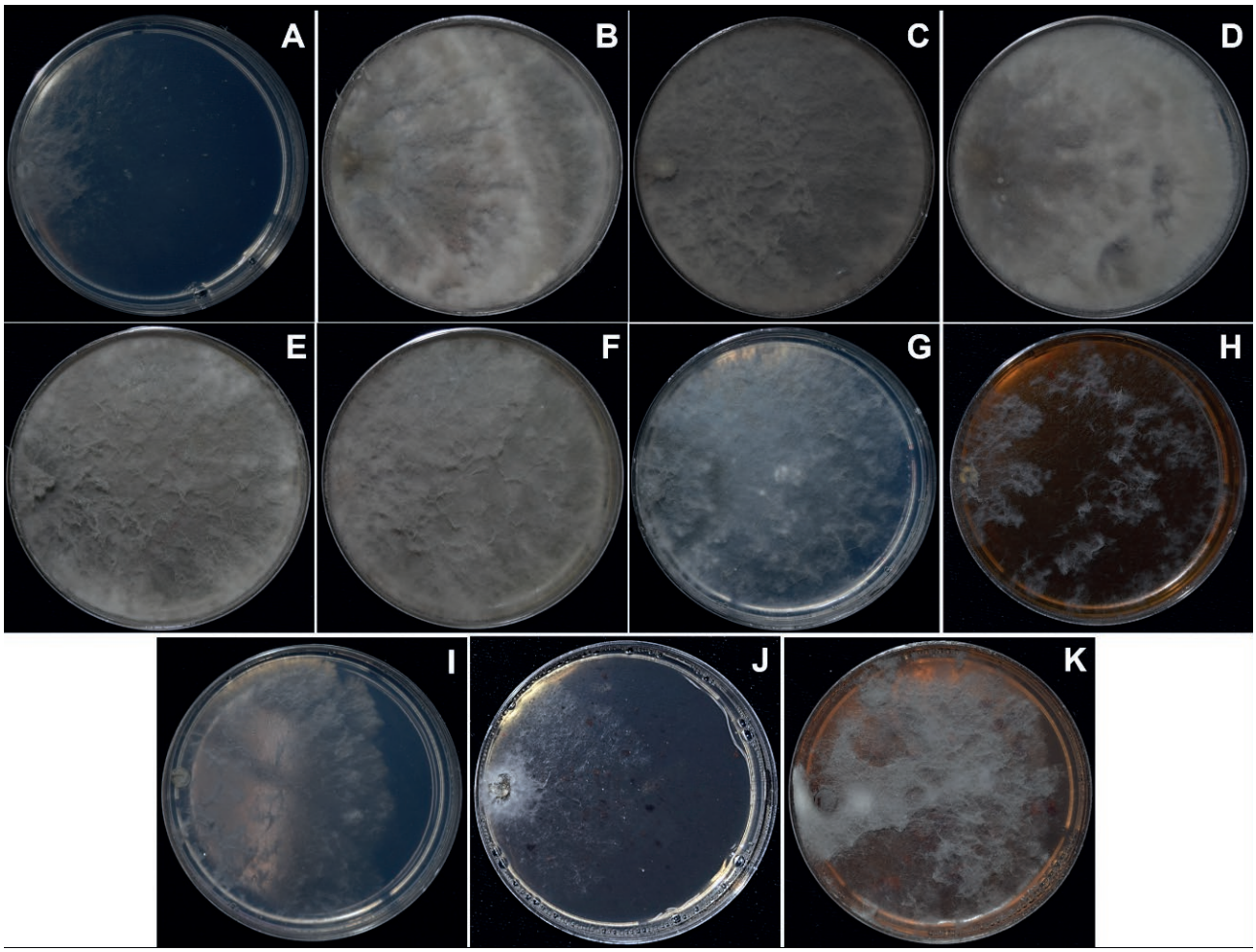


**Figure 5.** Growth of *Lasiodiplodia brasiliensis* MXBCL28 in Petri dishes containing different carbon sources. A, MM9 without a carbon source (MM9-C). B, MM9 + glucose. C, MM9 + pectin. D, MM9 + xylose. E, MM9 + xylan. F, MM9 + starch. G, MM9 + glycerol. H, MM9 + tannic acid. I, MM9 + cellulose. J, MM9 + lignin. K, MM9 + grapevine wood.

**Table 1.** Mean weights of biomass, growth rates, and mycelium areas of *Lasiodiplodia brasiliensis* MXBCL28 colonies growing in Petri plates containing PDA amended with different carbon sources.

| Carbon source | Mean biomass* (mg) | Mean growth rate* (mm $d^{-1}$) | Mean mycelium area ($mm^2$) |
|---|---|---|---|
| Grapevine wood | 70.3 ± 12.6 a | 18.7 ± 1.764 a | 5248.8 ± 11.8 ab |
| Pectin | 57.6 ± 3.9 b | 17.7 ± 0.8 a | 5391.5 ± 62.0 ab |
| Xylan | 47.6 ± 7.7 bc | 11.7 ± 0.6 c | 5422.6 ± 35.3 a |
| Glucose | 42.3 ± 11.2 cd | 14.7 ± 0.5 b | 5425.4 ± 39.3 a |
| Xylose | 40.4 ± 2.4 cd | 9.3 ± 0.8 d | 5424.6 ± 40.7 a |
| Starch | 33.7 ± 6.9 de | 9.7 ± 0.2 d | 5425.0 ± 39.4 a |
| Cellulose | 28.4 ± 1.6 ef | 7.0 ± 0.9 e | 4678.6 ± 44.0 c |
| Glycerol | 27.9 ± 9.0 ef | 8.4 ± 0.7 de | 5273.5 ± 71.1 b |
| Lignin | 21.9 ± 5.4 ef | 4.8 ± 0.2 f | 3289.3 ± 15.1 d |
| Tannic acid | 19.9 ± 5.7 f | 12.6 ± 0.5 c | 1998.3 ± 60.8 e |
| No carbon source | 7.2 ± 1.0 g | N/A** | 1426.4 ± 10.4 f |

metabolic pathways and use phenylalanine or tyrosine as precursors for melanin biosynthesis.

Grapevine responded to *L. brasiliensis* infection by occluding xylem vessels with tyloses and gels, and reinforcing cell walls with phenolic compounds in tyloses, vascular bundles, cambium, and pith. Infections were also accompanied by starch depletion in the ray parenchyma. These results support previous observations from histological studies conducted on several pathogens causing GTDs (Fleurat-Lessard *et al*., 2013; Pouzoulet *et al*., 2013; Gómez *et al*., 2016; Pouzoulet *et al*., 2017; Obrador-Sánchez and Hernandez-Martinez, 2020), indicating that these are constitutive responses that are enacted by host plants regardless of the type of GTD fungal infection.

In woody plants, non-structural carbohydrate reserves stored mainly as starch in ray parenchyma are

remobilized to fulfill several biological processes, including refilling embolized xylem vessels caused by freeze-thaw cycles or drought, supporting plant vegetative growth after dormancy, and activating host defense systems (Nardini *et al*., 2011; Fontaine *et al*., 2016; Noronha *et al*., 2018; Călugăr *et al*., 2019). Depletion of starch in wood has been observed in many pathogen and grapevine interactions (Rudelle *et al*., 2005; Rolshausen *et al*., 2008; Pouzoulet *et al*., 2017). The present study *in vitro* data also indicated that *L. brasiliensis* can use starch as a carbon source. Genomic and transcriptomic analyses indicated that a putative amylase was induced by *L. gilanensis* in the presence of grapevine wood (Paolinelli-Alfonso *et al*., 2016), and that genes involved in starch metabolism were induced in *L. theobromae* during infection (Yan *et al*., 2018). Together, these results suggest that starch plays a pivotal role as an energy pool accessible for GTD fungi for wood colonization and for grapevine to activate its defense system.

Several fungi causing GTDs can also metabolize structural carbohydrates located in plant cell walls. The hemicellulosic fraction has been shown to be especially degraded during infection by *E. lata*, and aggressive fungi such as *Neofusicoccum* can break down cellulosic material (Rolshausen *et al*., 2008; Stempien *et al*., 2017). Results from the present study showed that cellulose and hemicellulose were depleted in necrotic tissues, and were metabolized *in vitro* by *L. brasiliensis*. Cellulose and hemicellulose are carbohydrate-rich compounds that each constitute 30% of grapevine cells (Rolshausen *et al*., 2008). Genomic information from virulent *Botryosphaeriaceae* (*Neofusicoccum* and *Lasiodiplodia*) demonstrated the high numbers of genes encoding carbohydrate-active enzymes (Garcia *et al*., 2021), indicating that these compounds embedded in cell walls are primary targets for fungal metabolism.

Vessel occlusion was observed in control and inoculated (infected) plants, but the response was much stronger in *L. brasiliensis* infected plants, as was shown in grapevine responses to *P. chlamydospora* inoculations (Pouzoulet *et al*., 2017). Vessel occlusions, such as tyloses, gums, and gels, are formed in vascular bundles to limit the longitudinal spread of the fungus (De Micco *et al*., 2016; Pouzoulet *et al*., 2017). Several *Botryosphaeriaceae* have been shown to first invade xylem vessels before spreading to neighbouring woody tissues (Amponsah *et al*., 2012; Obrador-Sánchez and Hernandez-Martinez, 2020). Tyloses and gels are pectin-rich, and the present study results indicated that *L. brasiliensis* growth was stimulated in the presence of pectin in comparison to other substrates. Expression of a pectate lyase was up-regulated during the first stages of *L. gilanensis* infection in grapevine (Paolinelli-Alfonso, *et al*., 2016). Occlusion of xylem vessels results in loss of hydraulic conductivity, which eventually leads to decreased host physiological functions (Zhao *et al*., 2014).

The present study has shown that suberin accumulated in tyloses walls, and that lignin was deposited near ray parenchyma next to occluded xylem vessels, probably as responses to pathogen tracheid invasion. Suberin deposition was observed in other tissues, including vascular cambium, vascular cork, xylem fibre of vascular bundles, and ray parenchyma. Pouzoulet *et al*. (2013) also noted that grapevine accumulates suberin over lignin, in response to *P. chlamydospora* infections in those tissues. Qualitative and quantitative accumulation of phenolic compounds as physical and chemical barriers has been shown to be a common compartmentalizing mechanism for wood-decay fungi in other pathosystems (Skyba *et al*., 2013; Mounguengui *et al*., 2016). In grapevine, increased cell wall phenolics were found in the wood of the *Eutypa lata* tolerant cultivar Merlot compared with susceptible Cabernet Sauvignon (Rolshausen *et al*., 2008). Increased resveratrol induction was also measured in *N. parvum* tolerant *Vitis sylvestris* compared to *V. vinifera* (Labois *et al*., 2020).

Phenolic compounds in grapevine have also been shown to inhibit the growth of several GTD fungi (Lambert *et al*., 2012; Gómez *et al*., 2016). However, accumulation of phenolics (flavonoids, and stilbenoids) did not effectively wall-off aggressive pathogens, and these compounds are fungistatic rather than fungicidal (Lambert *et al*., 2012; Galarneau *et al*., 2021). The present study did not measure accumulation of phytoalexins. *Lasiodiplodia*, like *Neofusicoccum*, has been reported to display similar virulence factor patterns with respect to detoxification of antimicrobial compounds (Garcia *et al*.,2021). These fungi can degrade phenolic compounds that restrict *in planta* movement. Several genes involved in phenolic metabolism have been found in *Lasiodiplodia* spp. (Paolinelli-Alfonso *et al*., 2016; Yan *et al*., 2018; Gonçalves *et al*., 2019; Garcia *et al*, 2021). These could assist metabolization of phenolic compounds such as salicylic acid and phenylpropanoids produced by plants, to avoid the host defense responses. In addition, results from the present *in vitro* studies showed that *L. brasiliensis* was able to grow in culture media supplemented with glycerol, tannic acid, and lignin, supporting its ability to use these substrates as carbon sources.

Based on the information obtained in this study, and from previously published reports, we propose that once *Lasiodiplodia* conidia penetrate grapevines through wounds, the fungus initially colonizes xylem vessels following germination. The proposed model

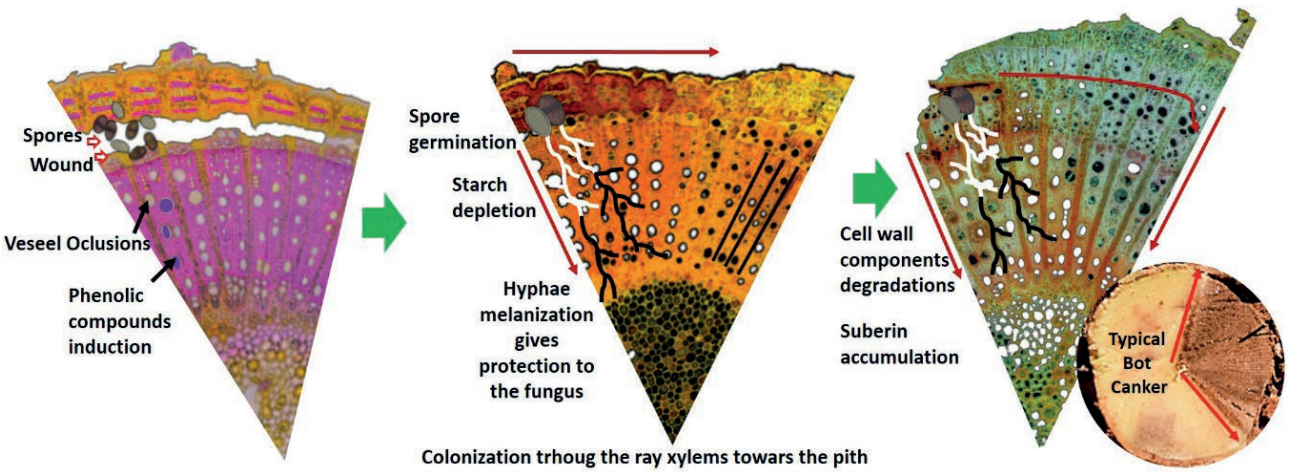


**Figure 6.** Proposed model of the colonization of grapevine by *Lasiodiplodia brasiliensis*. Spores of *L. brasiliensis* enter a plant wound, germinate and penetrate through xylem vessels. When growing within the host, the melanized mycelium protects the fungus from the host defenses. At the same time, the fungus produces cell wall degrading enzymes, and continues the colonization process using hemicellulose, starch and pectin as carbon sources. This induces vessel occlusions and the production of phenolic compounds. Over time, the fungus degrades lignin and suberin, moving to further xylem rays. This movement induces formation of typical Botryosphaeria cankers.

of the colonization process is illustrated in Figure 6. Upon recognition of the pathogen, the host remobilizes starch resources to trigger defense responses, with rapid xylem occlusion. The pathogen initially metabolizes the readily available starch stored in parenchyma cells and pectin-rich tyloses walls to weaken the intensity of host response and further colonize xylem vessels. Subsequently, the pathogen spreads to neighbouring tissues degrading cell wall structures to gain access to more complex sugars for metabolic processes. Host plant production of suberin, lignin and phytoalexins to compartmentalize the pathogen is probably inadequate against *Lasiodiplodia*, as the pathogen melanin can deactivate their antimicrobial effects and further breaks down phenolics. The pathogen then colonizes host cambium to initiate development of canker symptoms.

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Research Papers – 12th Special issue on Grapevine Trunk Diseases

# Current status of grapevine trunk disease pathogens on asymptomatic nursery-produced grapevines in Türkiye

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**Summary.** Good health of grapevine plants is important for productivity and sustainability of newly established vineyards, and accurate detection of bacterial and fungal pathogens is a prerequisite for managing the diseases they cause in nurseries. This study screened marketable, bare-rooted grapevine plants, obtained from different geographical regions of Türkiye, for fungal pathogens associated with grapevine trunk diseases (GTDs). In 2021, 43 grapevine nurseries located in eight provinces were surveyed to reveal the status of GTD pathogens on asymptomatic marketable plants. Fungal pathogens isolated from the roots and basal parts of asymptomatic dormant grapevines were identified using with morphological characteristics and molecular markers, and were subjected to pathogenicity tests. Six species; *Cytospora viticola, Diaporthe ampelina, Diplodia seriata, Lasiodiplodia brasiliensis, Neofusicoccum parvum,* and *Truncatella angustata* (associated with dieback), and six species; *Cadophora ferruginea, Cadophora luteo-olivacea, Cadophora malorum, Phaeoacremonium minimum, Phaeoacremonium tuscanicum* and *Phaeomoniella chlamydospora* (associated with Petri disease) were identified based on DNA sequencing of ITS and TEF1-α genes. GTD pathogens were detected in 12 and 14 of the 43 nurseries, respectively. Pathogenicity tests on 1103P vines revealed that all species were pathogenic (*N. parvum* and *C. luteo-olivacea* being the most virulent), and caused significant wood necroses when compared to non-inoculated experimental controls. This is the first report of *C. ferruginea, C. malorum, L. brasiliensis,* and *P. tuscanicum* associated with GTDs in Türkiye.

**Keywords.** *Cadophora, Cytospora, Lasiodiplodia, Phaeoacremonium,* trunk pathogens, *Vitis vinifera*.

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## INTRODUCTION

Grapevine nurseries became important early in the 20 Century, when grafted vines began to be planted in many regions. According to data from

the Turkish Ministry of Agriculture and Forestry, more than 60 establishments currently produce grapevine saplings, and over three million standard grafted grapevine plants (registered) are produced annually (Anonymous, 2019). Grafted vines are often in demand for establishment of each new vineyard, for planting to replace dying grapevines, or for small scale retail sales. Although vineyard areas in Türkiye decreased by 9.8% between 2012 and 2017, demand remains high for grafted grapevine saplings (Söylemezoğlu *et al.,* 2020).

In newly established vineyards, plants may die due to factors related to physiological issues and cultivation techniques, such as unfavourable climatic conditions, poor planting practices, nutritional disorders, and the quality of propagation material. Nematodes, insects, soil-borne fungi, and grapevine trunk pathogens also cause serious plant losses in nurseries (Gramaje and Armengol, 2011).

Among fungal pathogens, *Cylindrocarpon*-like fungi are associated with black foot (Agusti-Brisach and Armengol, 2013); *Cadophora*, *Pleurostoma, Phaeoacremonium* spp., and *P. chlamydospora* are associated with Petri Disease (PD) (Travadon *et al*., 2015); *Botryosphaeriaceae, Cytospora, Diaporthe, Eutypa*, and *Truncatella* spp. are associated with other GTDs (Essakhi *et al*., 2008; Arzanlou *et al*., 2013; Billones-Baaijens *et al*., 2013); and *Fusarium* spp. are associated with root rots (Halleen *et al*., 2003). Young plants infected by these fungi may show various symptoms, such as reduced vigour or growth, delayed sprouting, chlorotic foliage, failure of grafting, reduced root biomass, necrotic roots, and dieback (Pintos *et al*., 2018). Fungal grapevine trunk pathogens can affect grapevine quality, reduce marketable-seedling yields and eventually cause plant death in grapevine nurseries. Most of these fungi can spread latently over large areas with propagation material, dormant plants, or through their reproductive structures such as pycnidia or asexual conidia. Some pathogens (*Cylindrocarpon*-like fungi, *P. chlamydospora*, and *Fusarium* spp.) can also survive in soil for many years as chlamydospores (Retief *et al*., 2006). The members of *Botryosphaeriaceae, Diatrypaceae*, *Cytospora, Diaporthe*, and Pestalotioid fungi (*Neopestalotiopsis, Pestalotiopsis, Seimatosporium,* and *Truncatella*) have opportunistic natures, strong saprophytic abilities and broad host ranges. These organisms can survive in plant residues for many years and threaten many host species in nurseries (Úrbez-Torres, 2011; Lawrence and Travadon, 2018).

Botryosphaeria dieback, Petri disease, and black foot have been shown as the most common diseases in grapevine nurseries in many countries, including South Africa (Fourie and Halleen, 2004), Italy (Pollastro *et al.,* 2009), Portugal (Rego *et al.,* 2009), Spain (Aroca *et al.,* 2010), Australia (Whitelaw-Weckert *et al.,* 2013), France (Lecomte *et al.,* 2018; Pintos *et al.,* 2018), Canada (Hrycan *et al.,* 2022), California (Todd *et al.,* 2022), and Uruguay (Carbone *et al.,* 2022).

Most of the previously described GTD pathogens were also identified in Türkiye in mature vineyards, regional grapevine nurseries and young vineyards. Poyraz and Onoğur (2013) carried out a survey targeting just Petri Disease and Esca pathogens in the nurseries and mature vineyards in the Aegean Region, where Akgül *et al.,* (2015) identified fungal trunk pathogens in 10-30-year-old Sultana Seedless plants. Akgül and Ahioğlu (2019) screened GTD pathogens associated with young grapevine decline in 1-3-year-old vineyards in Southern Türkiye. A survey 3 years later revealed occurrence and diversity of black foot pathogens in nurseries (Akgül *et al.,* 2022). However, no substantial information is available on the current status of other GTD pathogens in marketable dormant plants in Turkish grapevine nurseries. Determining the latent fungal pathogens in each nursery may help to develop appropriate plant protection technology for producing healthy plants.

The objectives of the present study were: (i) to assess the presence of GTD pathogens on asymptomatic marketable plants produced in Turkish grapevine nurseries; (ii) to identify the associated fungal species based on molecular characterization; and (iii) to determine the virulence of representative isolates using pathogenicity tests on dormant grapevine cuttings.

## MATERIALS AND METHODS

### *Survey and isolation of dieback and Petri Disease pathogens*

During January 2021, a total of 450 dormant grapevine plants were sampled from the nurseries (10–12 plants from each nursery), located in eight provinces Türkiye (Adıyaman, Bursa, Denizli, Manisa, Mersin, Şanlıurfa, Tekirdağ, and Tokat) (Figure 1). Roots and rootstocks were washed under running tap water, and were then superficially disinfected with sodium hypochlorite solution (>5% active chlorine, and diluted in sterile distilled water (1:1 v/v)) for 3 min. The tissues were then rinsed with sterile distilled water, briefly blotted on sterile paper towels, and allowed to dry under a sterile cabinet. Root hairs and inner tissues of the rootstocks (3–4 mm long) were then placed (six to seven fragments per Petri dish) onto PDA (Potato Dextrose Agar, Conda Lab) amended with streptomycin sulfate

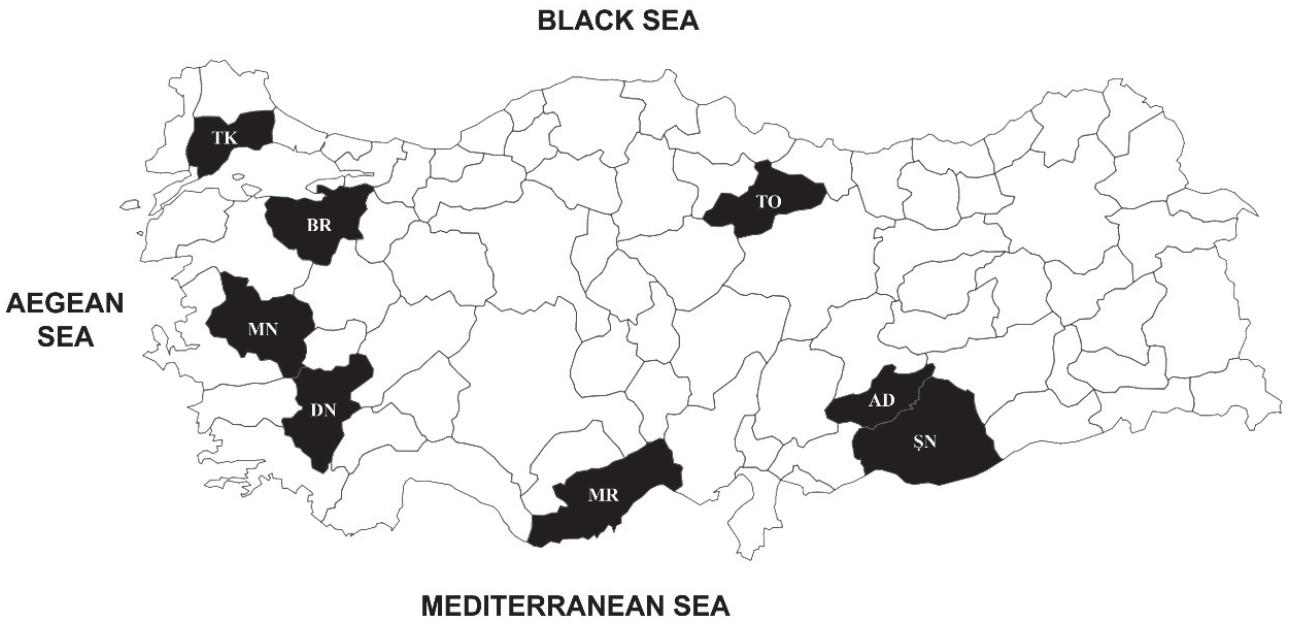


**Figure 1.** Provinces in Türkiye from which grapevine nurseries were sampled in this study. AD: Adıyaman, BR: Bursa, DN: Denizli, MN: Manisa, MR: Mersin, ŞN: Şanlıurfa, TK: Tekirdağ, TO: Tokat.

(250 mg×$L^{-1}$). Petri dishes (10–12 dishes per nursery) were incubated at 24°C in the dark for 7–10 d. Resulting fungal colonies were examined under a light microscope (Olympus BX51), and were sub-cultured to fresh PDA plates for further studies. Altogether, 285 fungal colonies were isolated from the samples. Based on microscopic features and colony morphologies specified in relevant publications (Barnett and Hunter, 2003; Essakhi *et al*., 2008; Trouillas and Gubler, 2010; Phillips *et al*., 2013; Lawrence *et al*., 2017; Maciá-Vicente *et al*., 2020), *Botryosphaeriaceae, Cadophora, Cytospora, Diaporthe, Phaeomoniella, Phaeoacremonium,* and *Truncatella* spp. were considered as probable GTDs pathogens. As well, *Acremonium, Alternaria, Aspergillus, Aureobasidium, Cladosporium, Clonostachys, Entoleuca, Epicoccum, Fusarium, Mortierella, Petriella, Penicillium* and *Trichoderma* spp. were also determined, but they were considered as endophytic species. Isolation frequencies (%) of Petri disease and other trunk pathogen fungi was calculated as proportions of 70 tissue fragments from 10–12 vines in each nursery (Table 1). The overall isolation frequency (%) of each fungus was calculated from isolation frequencies in each nursery. The prevalence of each species (%) was calculated as the proportion of the nursery numbers (pathogen detected) to the total nursery number. The overall disease prevalence (%) was calculated as the proportion of nursery numbers (pathogen detected) to the total nursery number.

MOLECULAR IDENTIFICATION OF FUNGI

To obtain pure cultures and provide genetic purity, single spores or hyphal tips of the fungi were isolated under a light microscope. Twenty-six representative isolates were used for identification with molecular markers. Approx. 50 mg of aerial mycelia was harvested from each pure culture (grown on PDA, incubated at 25°C, in the dark, for 7–10 d). Total DNA was extracted following the CTAB (2%) protocol (O'Donnell *et al*., 1998). Genomic DNA from each isolate was diluted with 80 µL of PCR grade water, then stored at -18°C for further use. For sequencing, ITS1, 5.8S, and ITS2 regions of the rDNA, and partial TEF 1-α (translation elongation factor) genes, were amplified with PCR reactions using the ITS4/ITS5 and EF688F-EF1251R primers (White *et al.,* 1990; Alves *et al.,* 2008). The PCR reaction mixtures each contained 5 µL of buffer (10× Green Buffer, DreamTaq Green DNA Polymerase; Thermo Scientific), 2 µL of the dNTPs mixture (10 mM each, Thermo Scientific), 1 µL of forward and reverse primers (stock concentration, 10 pmol·$µL^{-1}$), 0.25 µL of Taq polymerase (DreamTaq Green DNA Polymerase; Thermo Scientific), 39.75 µL PCR grade water, and 1 µL genomic DNA (approx. 100 ng·$µL^{-1}$). PCR amplifications were using a Simpli-Amp A24811™ Thermal Cycler (Applied Biosystems), with the following conditions; 95°C for 3 min. (initial denaturation), followed by 35 cycles each of denaturation at 95°C for 1 min, annealing at 52°C (for ITS) or 53°C (for TEF1-α) for

**Table 1.** Locations of surveyed grapevine nurseries, cultivars, isolation frequency (%) and prevalence (%) of Petri disease and other grapevine trunk disease (GTD) fungi.

| Nursery | Location | Rootstock / Cultivar | Isolation Frequency (%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Trunk Pathogen Fungi | | | | Petri Disease Fungi | |
| | | | *Botryosphaeriaceae* | *Cytospora* | *Diaporthe* | *Truncatella* | *Cadophora* | *Phaeomoniella chlamydospora* *Phaeoacremonium* spp. |
| 1 | Bursa | 1103P-Trakya İlkeren | - | - | - | - | - | - |
| 2 | Mersin | 1103P- Victoria | 15.7 | - | - | - | - | - |
| 3 | Salihli, Manisa | Thompson Seedless | - | 2.9 | 11.4 | - | - | - |
| 4 | Salihli, Manisa | Sultana Seedless | - | - | - | - | - | - |
| 5 | Salihli, Manisa | Sultana Seedless | - | - | - | - | - | 7.1 |
| 6 | Salihli, Manisa | Sultana Seedless | - | - | - | - | - | 1.4 |
| 7 | Salihli, Manisa | 1103P / Sultana Seedless | - | - | 7.1 | - | 11.4 | 1.4 |
| 8 | Alaşehir, Manisa | Sultana Seedless | - | - | - | - | - | - |
| 9 | Alaşehir, Manisa | Sultana Seedless | - | - | - | - | - | - |
| 10 | Alaşehir, Manisa | Sultana Seedless | - | - | - | - | - | - |
| 11 | Sarıgöl, Manisa | Sultana Seedless | 7.1 | - | - | - | - | - |
| 12 | Salihli, Manisa | Sultana Seedless | - | - | - | - | - | - |
| 13 | Tekirdağ | Kober 5BB / Sultan 1 | 1.4 | - | - | - | 7.1 | 15.7 |
| 14 | Tekirdağ | Kober 5BB / Bozbey | - | - | - | - | - | 1.4 |
| 15 | Tekirdağ | 1103P-Tekirdağ Seedless | 11.4 | - | - | - | - | - |
| 16 | Tekirdağ | 110R-Yapıncak | - | - | - | - | - | - |
| 17 | Denizli | 41B / Sultana Seedless | - | - | - | - | - | - |
| 18 | Denizli | 41B / Sultana Seedless | - | - | - | - | - | - |
| 19 | Denizli | 41B / Sultana Seedless | - | - | - | - | - | - |
| 20 | Denizli | 41B / Sultana Seedless | - | - | - | - | - | - |
| 21 | Denizli | 41B / Michele Palieri | - | - | - | - | - | - |
| 22 | Şanlıurfa | 1103P - Ergin Seedless | - | - | - | 21.8 | - | - |
| 23 | Şanlıurfa | 110R - Horozkarası | - | - | - | 4.2 | - | - |
| 24 | Şanlıurfa | 99R - Çiloreş | - | - | - | - | - | - |
| 25 | Şanlıurfa | 1103P - Victoria | - | - | - | - | - | 21.4 |
| 26 | Manisa | 41B / Red Globe | - | - | - | - | - | 2.9 |
| 27 | Manisa | Kober 5BB / Royal | - | - | - | - | - | - |
| 28 | Manisa | 1103P - Sultana Seedless | - | - | - | - | 3.8 | - |
| 29 | Manisa | Kober 5BB - Sultana Seedless | - | - | - | - | - | 1.4 |
| 30 | Manisa | 1103P - Crimson Seedless | - | - | - | - | - | 2.9 |
| 31 | Manisa | 110R / Alicante Bouschet | - | - | - | - | - | - |
| 32 | Alaşehir, Manisa | 1103P - Thompson Seedless | - | - | - | - | - | - |
| 33 | Manisa | Kober 5BB / Ata Sarısı | - | - | - | - | - | - |
| 34 | Turgutlu, Manisa | Kober 5BB /Sultana Seedless | - | - | - | - | - | - |
| 35 | Manisa | Kober 5BB / Trakya İlkeren | - | - | - | - | - | - |
| 36 | Tokat | 1103P - Narince | 4.3 | - | - | - | - | - |
| 37 | Tokat | 1103P/Narince | - | - | - | - | - | - |
| 38 | Tokat | 1103P/Narince | - | - | - | - | 1.4 | - |
| 39 | Tokat | 1103P/Sultan7 | 28.6 | - | - | - | - | - |
| 40 | Tokat | 1103P/Narince | - | - | - | - | 6.7 | - |
| 41 | Tokat | Du Lot / Narince | - | - | - | - | - | 12.8 |
| 42 | Adıyaman | Kober 5BB / Hatun Parmağı | - | - | - | 14.3 | - | 0.9 |
| 43 | Mersin | 1103P / Victoria | - | - | 1.8 | - | - | - |
| | | Average (%) | 11.4 | 2.9 | 6.8 | 13.4 | 6.1 | 6.3 |
| | | Prevalence of each species (%) | 13.9 | 2.3 | 7.0 | 7.0 | 11.6 | 25.9 |
| | | Prevalence of all species (%) | | | 27.9 | | | 32.5 |

1 min, and extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were sequenced by Macrogen Co. The electronic sequence files of the isolates were extracted with Chromas Lite (Technelsiyum™) software, and sequences were compared with those deposited in the National Center for Biotechnology Information (NCBI) USA National Institute of Health database, using nucleotide BLAST® (Basic Local Alignment Search Tool) software. The ITS and TEF1-α sequences were submitted to the NCBI GenBank, and accession numbers were obtained (Table 2).

*Pathogenicity tests*

All the 26 isolates were subjected to pathogenicity tests (two replicates in a year) on potted 1103 Paulsen rootstock plants in a controlled climate room (26°C, 80% relative humidity, and 12 h illumination). Dormant cuttings (each 30 cm long, with three buds) were superficially disinfected with sodium hypochlorite solution (2.5%) for 3 min. They were then rinsed once with sterile distilled water and blotted on sterile paper towels. The ends of the cuttings were then cut with a sterile pruning shear, and their bases were dipped in gibberellic acid solution (2000 $\mu g \cdot mL^{-1}$) for 10 sec, to induce root formation. The apical ends were inoculated with mycelium agar discs (5 mm diam.) of fungal isolates and wrapped with parafilm (Curwood®) to allow colonization. For non-inoculated controls, sterile agar discs were placed on the apical ends of the cuttings (Ayres *et al.,* 2011). After inoculation, the cuttings were planted in plastic bags (one cutting per bag) each containing 1 L of the potting mix (peat moss, perlite, and sawdust in equal volumes) and watered. Twelve plants per isolate (one plant per pot and four replicates with three plants

**Table 2.** Petri disease and other grapevine trunk disease pathogens identified in this study, their locations, hosts, and GenBank accession numbers.

| Nursery | Isolate code | Fungal species of trunk pathogens | Location | Rootstock / Cultivar | GenBank Accession Numbers | |
|---|---|---|---|---|---|---|
| | | | | | ITS | TEF1 |
| 1 | AFP21 | *Cytospora viticola* | Mezitli, Mersin | 1103P - Victoria | OP412792 | OP508220 |
| 2 | AFP26 | *Diaporthe ampelina* | Salihli, Manisa | Sultana Seedless | OP412793 | OP508221 |
| 3 | AFP121 | *Diaporthe ampelina* | Mezitli, Mersin | 1103P - Victoria | OP412794 | OP508222 |
| 4 | AFP282 | *Diaporthe ampelina* | Salihli, Manisa | 1103P - Sultana Seedless | OP412795 | OP508223 |
| 5 | AFP11 | *Diplodia seriata* | Mezitli, Mersin | 1103P - Victoria | OP412796 | OP508224 |
| 6 | AFP301 | *Lasiodiplodia brasiliensis* | Sarıgöl, Manisa | Sultana Seedless | OP412797 | OP508225 |
| 7 | AFP312 | *Lasiodiplodia brasiliensis* | Tekirdağ | 5BB - Sultan 1 | OP412798 | OP508226 |
| 8 | AFP315 | *Lasiodiplodia brasiliensis* | Tekirdağ | 5BB - Bozbey 1 | OP412799 | OP508227 |
| 9 | AFP317 | *Lasiodiplodia brasiliensis* | Tekirdağ | 1103P - Yapıncak | OP412800 | OP508228 |
| 10 | AFP22 | *Neofusicoccum parvum* | Tekirdağ | 1103P - Tekirdağ Seedless | OP412801 | OP508229 |
| 11 | AFP91 | *Neofusicoccum parvum* | Tokat | 1103P - Narince | OP412802 | OP508230 |
| 12 | AFP92 | *Neofusicoccum parvum* | Tokat | 1103P - Sultan 7 | OP412803 | OP508231 |
| 13 | AFP145 | *Neofusicoccum parvum* | Tokat | 1103P - Narince | OP412804 | OP508232 |
| 14 | AFP152 | *Neofusicoccum parvum* | Tokat | 1103P - Sultan 7 | OP412805 | OP508233 |
| 15 | AFP83 | *Truncatella angustata* | Şanlıurfa | 1103P - Ergin Seedless | OP412806 | OP550034 |
| 16 | AFP134 | *Truncatella angustata* | Adıyaman | 5BB - Hatun Parmağı | OP412807 | OP550035 |
| 17 | AFP217 | *Truncatella angustata* | Şanlıurfa | 99R - Çiloreş | OP412808 | OP550036 |
| | Petri disease | | | | | |
| 18 | AFP159 | *Cadophora ferruginea* | Tokat | 1103P - Narince | OP412809 | OP961938 |
| 19 | AFP23 | *Cadophora luteo-olivacea* | Salihli, Manisa | 1103P - Sultana Seedless | OP412810 | OP550037 |
| 20 | AFP24 | *Cadophora luteo-olivacea* | Tekirdağ | 5BB - Sultan 1 | OP412811 | OP550038 |
| 21 | AFP53 | *Cadophora luteo-olivacea* | Şanlıurfa | Victoria | OP412812 | OP550039 |
| 22 | AFP119 | *Cadophora luteo-olivacea* | Manisa | 110R - Sultan 7 | OP412813 | OP550040 |
| 23 | AFP143 | *Cadophora malorum* | Tokat | 1103P - Narince | OP412814 | OP550041 |
| 24 | AFP57 | *Phaeoacremonium minimum* | Şanlıurfa | 1103P - Victoria | OP412815 | OP550042 |
| 25 | AFP56 | *Phaeoacremonium tuscanicum* | Şanlıurfa | 1103P - Ergin Seedless | OP412816 | OP550043 |
| 26 | AFP203 | *Phaeomoniella chlamydospora* | Adıyaman | 5BB - Hatun Parmağı | OP412817 | OP550044 |

per replicate) were inoculated with each of the isolates. The plants were supplied with Hoagland solution twice each month for 4 months to provide balanced nutrition.

At the end of the period, the plants were uprooted, and their shoots and roots were removed with a pruning shear. The inoculation points were split with a knife, and the lengths of necrotic wood tissues were measured with a caliper and recorded. The pathogenicity of the isolates was confirmed by following Koch's postulates. The inoculated pathogens were re-isolated from the symptomatic wood chips but not from the non-inoculated controls. Mean lesion lengths were analyzed separately as Petri diseases or other trunk pathogens, because the growth rates of the fungi causing the diseases were not the same either in PDA cultures or in wood tissues.

*Statistical analyses*

Analysis of variance (ANOVA) was carried out on data of lengths of wood necrosis (mean lengths of two experiments), and the data were checked for normality. Means were compared using Fisher's least significant difference (LSD) test (at $P = 0.05$) (Gomez and Gomez, 1984).

## RESULTS

*Isolation of the fungi involved in disease prevalence in the nurseries*

According to morphological/microscopic examination, some of the fungi related to Petri diseases and other GTD pathogens, referred to here as trunk pathogens, were detected in marketable plants in the surveyed grapevine nurseries (Table 1). These pathogens were isolated from 23 (54%) of the 43 surveyed nurseries. No pathogens were detected in 20 nurseries. One or more fungal species associated with both diseases were isolated from the roots and rootstocks of asymptomatic dormant vines. When the fungal pathogens were grouped separately, the isolation frequencies of Petri diseases were 27.9%, and other trunk pathogens 32.5%. *Phaeoacremonium* spp. and *P. chlamydospora* were the most prevalent fungi isolated (25.9%), followed by *Botryosphaeriaceae* (13.9%), *Cadophora* (11.6%), *Diaporthe* and *Truncatella* spp. (7.0%).

*Molecular identification of the isolates*

Identification of 26 isolates was performed using partial sequencing of ITS and TEF 1-α genes, and nucleotide sequences were deposited in the GenBank with accession numbers OP412792 to OP412817, OP508220 to OP508233, and OP550034 to OP550044 (Table 2). According to nucleotide BLAST searches (with ≥99 similarities), nine isolates were associated with Petri disease, and 17 isolates were detected as trunk pathogens. *Botryosphaeriaceae* species (*D. seriata* De Not, *L. brasiliensis* M.S.B. Netto, M.W. Marques & A.J.L. Phillips, and *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips) constituted 58.8% (ten isolates) of all the dieback-related fungi, followed by *D. ampelina* (Berk. & M.A. Curtis) R.R. Gomes, Glienke & Crous (three isolates; 17.6%), *Truncatella angustata* (Pers.) S. Hughes (three isolates; 17.6%), and *Cytospora viticola* D.P. Lawr., Travadon & Pouzoulet (one isolate; 5.9%). *Cadophora* spp. (*C. ferruginea* Koukol & Maciá-Vicente, *C. luteo-olivacea* (J.F.H. Beyma) T.C. Harr. & McNew, and *C. malorum* (Kidd & Beaumont) W. Gams)) predominated (six isolates; 66.7%) the fungi related to Petri disease. Only one isolate each (5.9%) of *Phaeoacremonium minimum, P. tuscanicum,* and *P. chlamydospora* belonged to *Togniniaceae* and *Phaeomoniellaceae*. Among these, *C. ferruginea, C. malorum, L. brasiliensis,* and *P. tuscanicum* had not been previously recorded in vineyards in Türkiye.

*Pathogenicity tests*

After 4 months in of 1103 Paulsen cuttings, all isolates caused blackish-brown vascular discolourations (of mean lengths from 8.2 to 40.0 mm) below inoculation points (Figure 2). No visible symptoms were observed on the green shoots or leaves of the plants. When the inoculated fungal groups (Petri disease pathogens and other GTD pathogens) are examined separately, wood lesions caused by Petri disease pathogens were less extended than those caused by other GTD pathogens. Among the dieback fungi, *N. parvum* produced the most extended lesions (from 36.8 to 40.0 mm), and was the most virulent pathogen (Table 3). It was followed by *L. brasiliensis* (mean lesion length 24.7 to 32.6 mm), *T. angustata* (22.5 to 29.9 mm), and *D. ampelina* (9.7 to 13.2 mm) (Table 3). Average lesion lengths produced by *C. viticola* were slightly longer (8.2 mm) than non-inoculated control (6.2 mm). Similarly, the isolates associated with Petri disease did not cause shoot or leaf symptoms, but their mean internal lesion lengths were longer ($P \leq 0.05$) than that for the control. *Cadophora* isolates were more virulent (mean lesion length 9.4 to 9.7 mm), compared to *Phaeoacremonium* spp. and *P. chlamydospora* (8.5 to 8.9 mm), but no statistical difference was found between mean lesion lengths of *Cadophora* spp. (Table 4). The re-isolation experiment also confirmed severe colonization of the plant wood tissues. Both dieback and Petri disease fungi were recovered, with isolation

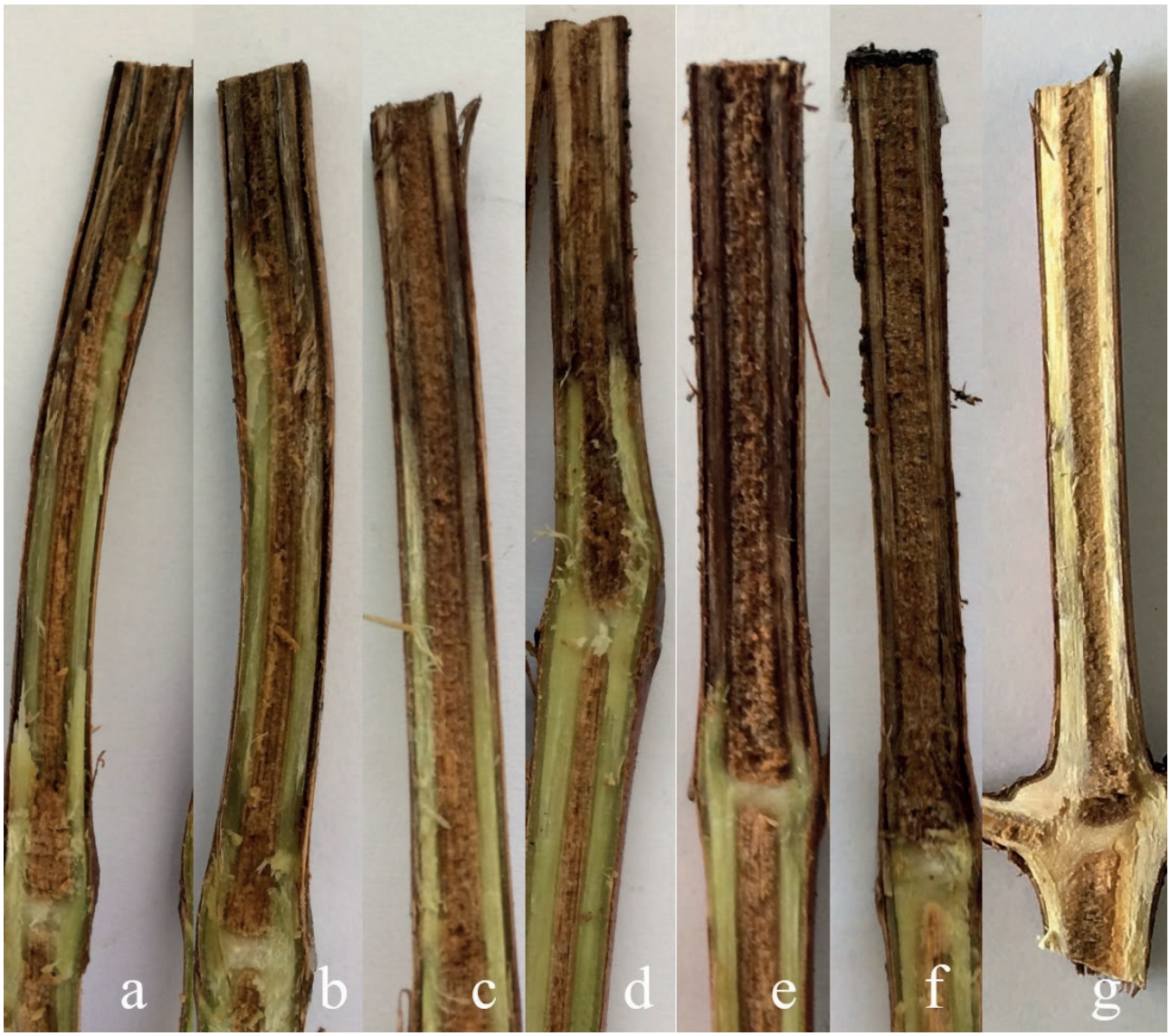


**Figure 2.** Internal wood symptoms caused by grapevine trunk disease pathogens on potted 1103P rootstock plants, 4 months inoculations with: a, *Cadophora luteo-olivacea*; b, *C. malorum*; c, *Diaporthe ampelina*; d, *Truncatella angustata*; e, *Lasiodiplodia brasiliensis*; f, *Neofusicoccum parvum*; and g, non-inoculated control.

rates from 18.6 to 72.4%. The virulence variability between the species was found by averaging the lesion lengths produced by isolates within each species (Figures 3 and 4). According to this evaluation, *Cadophora luteo-olivacea* was the most virulent fungus causing Petri disease and *N. parvum* was the most virulent fungus causing other GTDs.

## DISCUSSION

Grafted grapevine production is a critical sector of Turkish viticulture. Screening the plants for health and determining related pathogens are important steps for preventing the distribution of diseases. The present study was conducted in Turkish grapevine nurseries to determine prevalence of GTD pathogens on asymptomatic marketable plants. The results showed that dieback (in 30% of surveyed nurseries) and Petri disease pathogens (33% of nurseries) were moderately prevalent. Previously, black foot pathogens were found to be very common in most of surveyed nurseries (Akgül *et al.*, 2022). Nine of 43 nurseries producing non-grafted Sultana Seedless grapevines were examined in this survey, and dieback and Petri disease pathogens were identi-

**Table 3.** Mean lesion lengths and proportions (%) of re-isolations for species of fungi associated with grapevine trunk diseases (except Petri disease) on 1103 Paulsen rootstock plants.

| Fungal species | Isolate | Lesion length | Re-isolation |
|---|---|---|---|
| | | (mm) | (%) |
| *Neofusicoccum parvum* | AFP91 | 40.0 ± 0.3 l* | 72.4 |
| *N. parvum* | AFP152 | 39.3 ± 1.0 kl | 65.6 |
| *N. parvum* | AFP145 | 38.8 ± 0.7 k | 68.1 |
| *N. parvum* | AFP92 | 37.1 ± 0.3 j | 59.4 |
| *N. parvum* | AFP22 | 36.8 ± 0.6 j | 60.2 |
| *Lasiodiplodia brasiliensis* | AFP315 | 32.6 ± 0.3 i | 63.9 |
| *L. brasiliensis* | AFP301 | 30.8 ± 0.5 h | 55.3 |
| *L. brasiliensis* | AFP312 | 30.5 ± 0.1 h | 70.1 |
| *Truncatella angustata* | AFP217 | 29.9 ± 0.9 h | 65.9 |
| *L. brasiliensis* | AFP317 | 24.7 ± 0.6 g | 50.6 |
| *T. angustata* | AFP134 | 23.7 ± 0.3 f | 52.8 |
| *T. angustata* | AFP83 | 22.5 ± 0.5 e | 54.7 |
| *Diaporthe ampelina* | AFP282 | 13.2 ± 0.6 d | 38.6 |
| *Diplodia seriata* | AFP11 | 10.4 ± 0.4 c | 63.4 |
| *D. ampelina* | AFP121 | 9.8 ± 0.3 c | 44.1 |
| *D. ampelina* | AFP26 | 9.7 ± 0.2 c | 50.3 |
| *Cytospora viticola* | AFP21 | 8.2 ± 0.2 b | 36.1 |
| Non-inoculated control | - | 6.2 ± 0.1 a | - |

*Means accompanied by the same letter are not significantly different ($P$ = 0.05), according to LSD tests. Each mean is the average for 24 cuttings (12 per experiment).

**Table 4.** Mean lesion lengths resulting from inoculations of grapevine plants with different fungal species associated with Petri disease on 1103 Paulsen grapevine rootstock plants.

| Fungal species | Isolate | Lesion length | Re-isolation |
|---|---|---|---|
| | | (mm) | (%) |
| *Cadophora luteo-olivacea* | AFP23 | 9.7 ± 0.4 d* | 28.6 |
| *C. luteo-olivacea* | AFP24 | 9.7 ± 0.4 d | 22.9 |
| *C. luteo-olivacea* | AFP119 | 9.6 ± 0.1 d | 30.5 |
| *C. luteo-olivacea* | AFP53 | 9.4 ± 0.7 d | 38.9 |
| *C. ferruginea* | AFP159 | 9.4 ± 1.0 d | 22.1 |
| *C. malorum* | AFP143 | 9.4 ± 0.4 d | 45.1 |
| *Phaeoacremonium minimum* | AFP57 | 8.9 ± 0.2 c | 18.6 |
| *Phaeoacremonium tuscanicum* | AFP56 | 8.6 ± 0.1 bc | 30.4 |
| *Phaeomoniella chlamydospora* | AFP203 | 8.5 ± 0.3 b | 27.6 |
| Non-inoculated control | - | 6.2 ± 0.1 a | - |

*Means accompanied by same letter are not significantly different ($P$ = 0.05) according to LSD tests. Each mean is the average for 24 grapevine cuttings (12 per experiment).

fied only in three nurseries, from asymptomatic plants. These results indicate that most of the rootstock mother

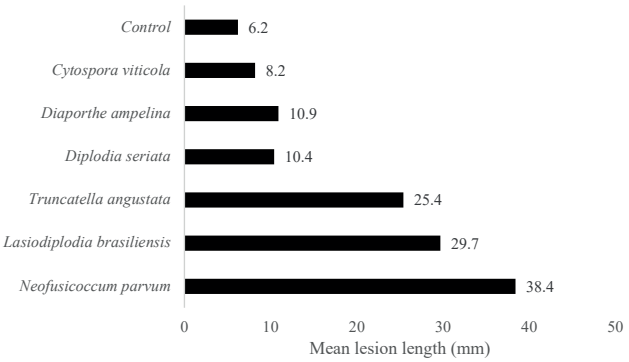


**Figure 3.** Mean lesion lengths (mm) from inoculations with different fungi associated with grapevine trunk diseases (except Petri disease), in a pathogenicity test.

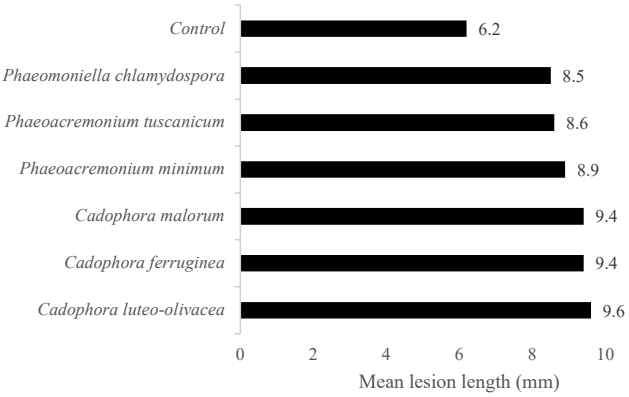


**Figure 4.** Mean lesion lengths (mm) from inoculations with different fungi associated with Petri disease, in pathogenicity test.

plant plots obtained latent infections during vegetative or propagation stages in the infested nurseries. Almost all grapevine rootstock mother fields in Türkiye are irrigated by flooding or furrow irrigation. Plants are also not grown on trellises, so the shoots of rootstock mother grapevines are sprawled on the soil surfaces. Soil-borne inocula of *Cadophora, Phaeomoniella,* and *Phaeoacremonium* may have caused shoot infections of the rootstock mother vines, resulting in greater prevalence of Petri disease than GTDs in the surveyed nurseries.

Rootstock/scion mother plants used for grapevine propagation may harbour trunk pathogens without showing disease symptoms. Therefore, these pathogens can be disseminated over large areas within young infected vines, and disease symptoms may appear 8-10 years later (Gramaje and Armengol, 2011). This has been confirmed in several studies using conventional fungal isolation and next-generation sequencing methods. Aroca *et al.* (2010) evaluated plant health in grapevine nurs-

ery propagation processes in five commercial nurseries in Spain. They found that apparently healthy dormant cuttings were contaminated with *Botryosphaeria dothidea, Lasiodiplodia theobromae, Neofusicoccum australe, N. mediterraneum, N. parvum, N. vitifusiforme P. minimum, P. parasiticum, P. chlamydospora,* and *D. ampelina*. Hofstetter *et al*. (2012) compared the fungal microbiota in adult grapevines with or without Esca symptoms, using conventional isolation methods, and found 158 fungal species. Along with many endophytic and saprobic fungi, *B. dothidea*, *Cadophora luteo-olivacea, P. minimum, P. chlamydospora*, *D. ampelina,* and *T. angustata* were also detected in symptomless and symptomed plants. Eichmeier *et al*. (2018) identified 189 fungal genera (including *C. luteo-olivacea, C. malorum, D. ampelina, Diplodia seriata, Pm. chlamydospora, Ph. minimum, T. angustata* species) from dormant marketable grapevines, using conventional isolation and high-throughput amplicon sequencing methods in Spanish and Czech grapevine nurseries. Billones-Baaijens *et al.* (2013) determined the presence of *Botryosphaeriaceae* fungi in marketable young plants in New Zealand grapevine nurseries. In Türkiye, we have also detected many of the species listed above in some of the surveyed nurseries, and these fungi were moderately prevalent. Incidence of Botryosphaeriaceous fungi was calculated as 19% in rootstocks and 17% in scion cuttings. These rates are greater than those (11.4%) determined in the present study.

*Cadophora ferruginea*, *C. malorum, L. brasiliensis* and *P. tuscanicum* were detected for the first time in Türkiye. Some *Cadophora* spp. (including *C. luteo-olivacea, C. malorum, C. melinii, C. orientoamericana, C. novi-eboraci*, and *C. spadicis*) have been previously detected in grapevine nurseries, in California, Canada, Germany, South Africa, and Uruguay (Rooney-Latham, 2005; Halleen *et al.,* 2007; Navarrete *et al.,* 2011; Gramaje *et al.,* 2011; Úrbez-Torres *et al.,* 2014; Gierl and Fischer, 2017; Travadon *et al.,* 2015). These fungi have been suggested as having important roles in the occurrence of Petri disease in young grapevines. The thesis of Özben (2020) reported *C. luteo-olivacea* and *T. angustata* (identified morphologically and microscopically) in 12 grapevine nurseries in Türkiye, but no pathogenicity or molecular identification data were reported. *Cadophora ferruginea* has been reported on grapevine in the United States of America (Travadon *et al*., 2022), so our isolate (AFP159) would be the second of this fungus. *Lasodiplodia brasiliensis* was first reported on table grapes in Brazil (Correia *et al*., 2016), then in Mexico (Rangel-Montoya *et al*., 2021). *Phaeoacremonium tuscanicum* was first reported in Spain (Essakhi *et al*., 2008), and then in the other countries, including New Zealand (Graham *et al*., 2009), Algeria (Berraf-Tebbal, 2011), Iran (Mohammadi, 2012), and Canada (Úrbez-Torres *et al*., 2014). We carried out an additional search to find the occurrence of these fungi in scientific journals and the Mycobank databases, but no record was found aside from the above countries.

Regarding to virulence of the isolates, fungal pathogens associated with Petri disease were found to be more virulent than the other GTD pathogens on 1103P rootstock plants (4 months after incubations) in pathogenicity tests. In previous studies, *Lasiodiplodia* and *Neofusicoccum* were shown to be highly virulent on grapevines when compared to other Botryosphaeriaceous fungi (Luque *et al*., 2009; Úrbez-Torres and Gubler, 2011). *Neofusicoccum parvum* was the most virulent species among 17 dieback-related isolates evaluated, followed by *L. brasiliensis, T. angustata,* and *D. ampelina* in the present study. Correia *et al*. (2016) studied the phylogeny, distribution, and pathogenicity of *Lasiodiplodia* species on table grapes in Brazil, and *L. brasiliensis* was the most virulent species, followed by *L. theobromae, L. pseudotheobromae* and *L. hormozganensis* on detached-green shoots of the Isabel grape cultivar, after 10 d incubation. Similarly, Rangel-Montoya *et al.* (2021) tested the pathogenicity of *L. brasiliensis, L. gilanensis, L. exigua,* and *L. crassispora*, and *L. brasiliensis* was the most virulent, causing average lesion lengths of 5.1-5.5 mm on Cabernet Sauvignon cuttings after 2 months incubation. Akgül *et al*. (2015) tested pathogenicity of several grapevine trunk disease pathogens, obtained in the Aegean Region of Türkiye. These tests were for approx. 4 months on potted 1-year old Sultana Seedless plants. Among these fungi, *N. parvum* was the most virulent, producing lesions of average length 79.1 mm, followed by *L. theobromae* (59.8 mm), *D. ampelina* (34.6 mm), *Phaeomoniella chlamydospora* (27.5 mm), *Togninia minima* (25.5 mm), *B. dothidea* (24.8 mm) and *D. seriata* (21.4 mm).

The pathogenicity results from some studies were different, however, from the those outlined above. Mondello *et al*. (2020) indicated that Sicilian isolates of *P. chlamydospora* produced larger lesions than *N. parvum* on excised grapevine canes (cv. Grecanio) 15 days after inoculation. Raimondo *et al*., (2019) investigated the current status of newly detected fungal GTD pathogens (*C. luteo-olivacea, Colletotrichum fioriniae, Seimatosporium vitis-vinifera* and, *T. angustata*) from Apulia and Molise regions of Italy. These fungi produced characteristic wood necroses (for *C. luteo-olivacea*: 12.9-14.1 cm, *C. fioriniae*: 8.4-8.8 cm, *S. vitis-vinifera*: 16.9-23.6 cm, *T. angustata*: 15.7-18.0 cm), when compared to non-inoculated controls (0.6 cm), after 8 months on two different grapevine cultivars in field conditions. Halleen *et al.* (2007) in a study in South Africa, showed lesion lengths

caused by *C. luteo-olivacea* were 17.1 cm, by *P. chlamydospora* were 9.6 cm, and by *P. minimum* were 8.9 mm, on 101-14 Mgt/Shiraz plants after 3 months incubation in a greenhouse.

The pathogenicity test results from the present study were similar to the previous studies but not to all. Some inconsistencies can also be seen among previous pathogenicity test results, probably due to differences in cultivar susceptibility, incubation conditions, the virulence of fungal isolates, and the inoculation methods used.

This research has demonstrated the current status of GTD pathogens on asymptomatic, marketable, nursery-produced grapevines in Türkiye. Although these pathogens were rare, the results indicate that appropriate disease control methods, such as screening of pathogens, hot water treatments, and use of biological pesticides, should be adopted in rootstock plots of grapevine nurseries.

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## AUTHORS CONTRIBUTIONS

Davut Soner AKGÜL designed this study, identified-archived the fungal isolates, performed statistical analyses, molecular studies and pathogenicity tests, wrote the paper. Nurdan GÜNGÖR SAVAŞ and Murat YILDIZ surveyed the grapevine nurseries, obtained-archived the fungal isolates. İzzet BÜLBÜL surveyed the grapevine nurseries, obtained-archived the fungal isolates, did molecular identification studies. Mümine ÖZARSLANDAN surveyed the grapevine nurseries, obtained-archived the fungal isolates.

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# Black foot in nursery grapevines in Uruguay caused by *Dactylonectria* and *Ilyonectria*

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**Summary.** Black foot is a serious soilborne fungal disease causing decline of young grapevines. Affected plants show brown to dark streaks developing from the rootstock bases, wood necroses at trunk bases, sunken necrotic lesions on roots, and reduced root biomass. Several fungi, commonly known as *Cylindrocarpon*-like asexual morphs, have been associated with black foot. Nursery vines are infected during rooting in propagation processes, which is important for dissemination of the pathogens. Species associated with black foot in nursery vines produced in Uruguay were characterized by molecular, phenotypical and pathogenicity studies. From 2017 to 2019, 181 rooted vines grafted onto '1103P', 'SO4', '101-14', '3309C' or 'Gravesac' rootstocks were sampled, and 71 *Cylindrocarpon*-like fungal isolates were recovered from rootstock tissues (basal ends and roots). Based on multi-gene phylogenetic analyses of HIS3, TEF and TUB2, and supported by phenotypical characterization, five species of *Dactylonectria* and *Ilyonoectria* were identified, with *D. macrodidyma* being the most prevalent followed by *D. novozelandica*, *D. torresensis*, *D. palmicola* and *I. liriodendri*. Four *Ilyonectria* isolates could not be identified to species level. Isolate pathogenicity was assessed using healthy rooted 'Gravesac' plants. After three months, isolates of all species infected the plants, causing necrotic lesions on roots and reducing root biomass. On average, 39% of ready-to-plant nursery vines were affected by black foot, emphasizing the need to develop integrated management to reduce black foot incidence in Uruguayan grapevine nurseries, based on studies under local conditions.

**Keywords.** *Cylindrocarpon*-like fungi, grapevine trunk disease, *Vitis vinifera*.



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## INTRODUCTION

Black foot is a serious soilborne fungal disease affecting nursery grapevine plants and young vineyards (Halleen *et al*., 2006a; Alaniz *et al*., 2007; Gramaje and Armengol, 2011; Agustí-Brisach and Armengol, 2013). This disease, considered a major cause of young vine decline (Gramaje and Armengol, 2011), occurs in the main grape-growing countries worldwide, including Portugal (Rego *et al*., 2000), South Africa (Fourie and Halleen, 2001), New Zealand (Halleen *et al*., 2004), France (Halleen *et al*., 2004), United States (Petit and Gubler, 2005), Spain (Alaniz *et al*., 2007), Australia (Whitelaw-Weckert *et*

*al*., 2007), Uruguay (Abreo *et al*., 2010), Canada (Petit *et al*., 2011), Turkey (Özben *et al*., 2012), Iran (Mohammadi *et al*., 2013), Brazil (dos Santos *et al*., 2014), Italy (Carlucci *et al*., 2017), Czech Republic (Pečenka *et al*., 2018), Algeria (Aigoun-Mouhous *et al*., 2019), China (Ye *et al*., 2021), and Argentina (Longone *et al*., 2022).

Grapevines affected by black foot pathogens show reduced root biomass, sunken necrotic root lesions, dark brown to black streaks that develop from rootstock bases, and wood necroses at trunk bases (Rego *et al*., 2000; Halleen *et al*., 2006a; Alaniz *et al*., 2007; Agustí-Brisach and Armengol, 2013). Foliar symptoms usually appear during the first 3 to 5 years after planting, and involve delayed and failed bud-break, reduced vigour, shortened internodes, chlorotic foliage with necrotic margins, wilting and usually plant death (Halleen *et al*., 2006a; Agustí-Brisach and Armengol, 2013). Death occurs quickly when young vines are infected, while as vines age, a more gradual decline occurs (Gubler *et al*., 2004).

Black foot was first known to be caused by "*Cylindrocarpon*" species, but in the last decade, this genus has undergone extensive taxonomic revision (Chaverri *et al*., 2011; Cabral *et al*., 2012a, 2012b; Lombard *et al*., 2014). Currently, more than 30 fungal species of *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, *Pleiocarpon,* and *Thelonectria,* commonly known as *Cylindrocarpon*-like asexual morphs, are associated with black foot (Agustí-Brisach and Armengol, 2013; Lombard *et al*., 2014; Carlucci *et al*., 2017; Aigoun-Mouhous *et al*., 2019). Among these, *D. torresensis* has been reported as the prevalent species in several countries (Reis *et al*., 2013; Berlanas *et al*., 2017; Carlucci *et al*., 2017; Aigoun-Mouhous *et al*., 2019; Akgül *et al*., 2022).

Although the epidemiology of black foot has not been completely clarified, it is well known that black foot pathogens can produce abundant conidia which are dispersed by free water in the soil (Petit *et al*., 2011) and can infect grapevines through natural openings or wounds in trunk bases and roots (Agustí-Brisach and Armengol, 2013). Some species are also able to produce chlamydospores which allow long-term survival in soil (Halleen *et al*., 2004). In addition, several weeds are hosts of black foot pathogens, and these hosts can be inoculum sources for grapevine infections (Agustí-Brisach *et al*., 2011).

Black foot pathogens have been frequently isolated from nursery grapevine plants, indicating that these plants play important roles in the spread of this disease (Halleen *et al*., 2006a; Abreo *et al*., 2010; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013; Carlucci *et al*., 2017; Pintos *et al*., 2018; Aigoun-Mouhous *et al*., 2019; Berlanas *et al*., 2020; Akgül *et al*., 2022). Several studies have focused on determining when infection occurs during propagation processes, and there is consensus that the nursery rooting phase is where black foot pathogen infections increase significantly (Halleen *et al*., 2003; Agustí-Brisach *et al*., 2013; Carbone *et al*., 2022). These pathogens can infect nursery plants from the incomplete callus zones or from wounds on roots during rooting in soil, as demonstrated by Probst *et al*. (2019).

A recent study in Uruguay revealed that many plants produced at a local nursery were infected with black foot pathogens (Carbone *et al*., 2022). The present study focused on molecular and phenotypic characterization of the pathogens associated with black foot in nursery vines and assessed the pathogenicity of identified species after inoculation of rooted grapevine rootstocks.

## MATERIALS AND METHODS

### *Sampling material and fungal isolations*

During 2017, 2018 and 2019, a total of 181 1-year-old ready-to-plant grapevine plants, grafted onto rootstocks of '1103P' (*Vitis berlandieri* × *V. rupestris*), 'SO4' (*V. riparia* × *V. berlandieri*), '101-14' (*V. riparia* × *V. rupestris*), '3309C' (*V. riparia* × *V. rupestris*), or 'Gravesac' ('161-49' (*V. berlandieri* × *V. riparia*) × '3309C'), were randomly selected from the main commercial grapevine nursery in Uruguay. Fifty-two plants were sampled in 2017, 83 in 2018, and 46 were sampled in 2019. The nursery is located in Canelones (34°34'48.45"S; 56°17'50.17"W), the traditional grape-growing region of Uruguay. To isolate black foot pathogens, the basal part and roots of each plant were separated and then surface sterilized by soaking each portion in 95% ethanol for 1 s followed by flaming (Delgado *et al*., 2016). Cross and longitudinal cuts were then made at the basal portion of the rootstock to reveal internal black foot symptoms. Seven pieces of wood, approx. 5 mm in length, were taken from the margin between necrotic and apparently healthy tissues, using a sterile scalpel, including the basal part of the rootstock and roots. In 2017 and 2019, the wood pieces were selected equally from the basal parts of the rootstocks and roots, while in 2018, the pieces were selected predominantly from the basal parts of the rootstocks. The small pieces of wood were plated onto potato dextrose agar (PDA) (Oxoid Ltd.) supplemented with 0.4 g $L^{-1}$ of streptomycin sulphate (PDAS) (Sigma-Aldrich). The plates were incubated for 5 to 21 d at 25°C in the dark and examined daily for fungal growth. Fungal colonies resembling black foot pathogens, i.e., with aerial and cottony mycelia ranging from white to yellow or light to dark brown, and with mac-

roconidia and microconidia (Halleen *et al*., 2004; 2006b; Cabral *et al*., 2012a), were subculture onto fresh PDA plates to obtain pure cultures. Single conidium isolates were obtained (Carlucci *et al*., 2017), and were stored in colonized sterile filter papers at -20°C. Representative isolates were deposited in the fungal culture collection of the Department of Plant Protection, Faculty of Agronomy, University of the Republic, Uruguay.

*Molecular identification of isolates*

Total genomic DNA was extracted from 1-week old pure cultures grown on PDA at 25°C in the dark, using the commercial Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZymoResearch), following the manufacturer´s instructions. Primary identification of black foot pathogens was conducted by sequencing part of the histone H3 gene (HIS3) and comparing the sequences with those deposited in the GenBank, using the BLAST source (https://blast.ncbi.nlm.nih.gov/Blast.cgihttps://blast.ncbi.nlm.nih.gov/Blast.cgihttps://blast.ncbi.nlm.nih.gov/Blast.cgi). To confirm the isolate identity, partial regions of the translation elongation factor 1-α (TEF) and the beta-tubulin (TUB2) genes were also sequenced, and a multilocus phylogenetic analysis was performed on the three combined gene regions (Cabral *et al*., 2012a; 2012b; Berlanas *et al*., 2020). The primers used were CYLH3F and CYLH3R for HIS3 (Crous *et al*., 2004), CylEF-1 (5'-ATGGGTAAGGAVGAVAAG AC-3'; J. Z. Groenewald, unpublished) and CylEF-R2 (Crous *et al*., 2004) for TEF, and T1 (O'Donnell and Cigelnik, 1997) and BT2b (Glass and Donaldson, 1995) for TUB2.

Polymerase chain reaction (PCR) amplifications were performed on a MultiGene™ Mini (Labnet International Inc.). Each PCR reaction contained 1× PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.4 µM of each primer, 1 U of DNA polymerase (Bioron), and 1 µL of template DNA. The PCR reaction was adjusted to a final volume of 20 µL with MQ water. The PCR conditions consist of an initial step at 94°C for 3 min followed by 34 cycles for TUB2 and TEF regions, and 40 cycles for HIS3 gene, of denaturation at 94°C for 30 s, annealing at 58°C for TUB2 and TEF and 55°C for HIS3, for 30 s, and elongation at 72°C for 45 s. A final extension was performed at 72°C for 10 min. PCR products were visualized in 1.5% agarose gels stained with GelRedTM, through a transilluminator under UV light. A GeneRuler 100-bp DNA ladder plus (Thermo) was used as a molecular weight marker. PCR products were purified and sequenced by Macrogen Inc., Seoul, Korea.

For each fungus genus, sequences of each gene region were aligned using the ClustalW program, available within MEGA 11.0.11 (https://www.megasoftware.net/), and were manually edited when necessary. Related sequences and sequences of the phylogenetically closest species obtained from GenBank, including ex-type isolates, were incorporated to the alignments (Supplementary Table 1). Multilocus alignments were carried out using Sequence Matrix v.1.8 (http://www.ggvaidya.com/taxondna/). Multilocus phylogenetic analyses were constructed using Bayesian inference (BI) and Maximum likelihood (ML) methods. BI and ML analyses were inferred with, respectively, MrBayes v3.2.7a and RAxML v8.2.12 programs, implemented in CIPRES Science Gateway v3.3 (http://www.phylo.org/). For BI analysis, best-fit models of nucleotide substitution were selected for each gene according to the Akaike information criterion (AIC), using the jModelTest2 v2.1.6 tool (Darriba *et al*., 2012) implemented in CIPRES Science Gateway v3.3. Four Markov chain Monte Carlo (MCMC) chains were run simultaneously, starting from a random tree to 10 million of generations. Trees were sampled every 1000 generations, and the first 2500 were discarded as the burn-in phase of each analysis. Posterior probabilities were determined from a majority-rule consensus tree generated from the remaining 7500 trees. For the ML analysis, the Generalized Time Reversible (GTR) model, with gamma correction (G) nucleotide substitution, and 1000 bootstrap iterations, were indicated. The other parameters were used as default settings. Sequences obtained in this study were submitted to the GenBank database (Supplementary Table 2).

*Morphological characterization of isolates*

A sub-sample of nine representative isolates belonging to *Dactylonectria* and *Ilyonectria*, identified in this study using molecular analyses, was selected for phenotypical characterization (Supplementary Table 2). Cultures were grown on PDA and incubated at 25°C in darkness. Ten days later, colony morphological characteristics were observed, and colony colour (Rayner, 1970) was determined. Lengths and widths of 50 conidia per isolate, including macro- and microconidia, were measured at 400× magnification, using a digital camera (Microscope eye-piece camera, AM-4023X, Taiwan) incorporated into the microscope. Conidium colour, shape, and number of septate, and presence of chlamydospores, were recorded.

*Pathogenicity tests*

The nine isolates selected for morphological characterization were used to determine their pathogenicity on

rooted grapevine plants. Dormant cuttings (0.3 m long) of 'Gravesac' rootstock were surface disinfected according to Akgül *et al.* (2022), placed in a plastic box containing sterilized growth substrate, irrigated, and kept in an acclimatized room (25°C, 85% relative humidity, 12 h photoperiod) for 1 month to induce root formation. The isolates were grown on PDA at 25°C in the dark for 2 weeks and were then liquefied in distilled water (one plate in 150 mL of distilled water). Root tips of the rooted cuttings were slightly cut and were then inoculated by immersing the roots in the culture suspension for 30 s (one liquified plate of one isolate per plant). The inoculated plants were then individually planted in a 2 L capacity pot containing commercial plant growth substrate. Six plants per isolate were inoculated, and six plants were treated with distilled water as controls. The plants were irrigated with tap water and maintained in greenhouse conditions (at 20±2°C), in a completely randomized experimental design.

Three months after inoculation the plants were uprooted, and their roots were carefully washed with tap water and dried in an air-circulated oven at 65°C for 48 h to constant weight, and root dry weights were recorded. Root dry weight data were analysed for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. Data were subjected to statistical analyses by performing analysis of variance (ANOVA) and means comparison by Duncan test at $P = 0.05$, using InfoStat/E version 2020 (http://www.infostat.com.ar). In addition, Koch´s postulates were completed by re-isolation of the inoculated fungi. For this, roots were separated, washed with tap water, and surface sterilized (as above). Small pieces of the roots were then cut, plated onto PDAS, and incubated in the same conditions as indicated above. Isolates identity was determined by morphological characteristics.

## RESULTS

### *Fungal isolations*

A total of 71 isolates resembling *Cylindrocarpon*-like asexual morphs were obtained from ready-to-plant grafted vines with characteristic black foot vascular symptoms. The symptoms consisted of wood necroses at the trunk bases, dark brown streaks developing from the bases of the plants, and sunken necrotic lesions on the roots (Figure 1). Based on the isolation frequency, incidence of black foot was 60% in 2017, 15% in 2018 and 61% in 2019.


**Figure 1.** Internal symptoms of black foot in ready-to-plant nursery grapevine plants. Wood necrosis and dark brown streaks developing from the base of the plant (a) and necrosis in roots (b).

*Molecular identification of isolates*

BLAST search of the partial HIS3 gene region placed 58 isolates in *Dactylonectria* and 13 within *Ilyonectria*. Subsequently, phylogeny of the individual data sets from the HIS3, TUB2 and TEF gene regions showed no significant conflicts in tree topology, so the trees were combined. The *Dactylonectria* dataset contained 96 taxa (58 from this study and two outgroups) and 1856 characters including gaps (TUB2 = 1-579; TEF = 580-1387; HIS3 = 1388-1856), of which 564 were parsimony informative. The *Ilyonectria* dataset consisted of 52 taxa (13 from this study and two outgroups) and 1882 characters including gaps (TUB2 = 1-567; HIS3 = 568-1038; TEF = 1039-1882), of which 526 were parsimony informative.

The AIC best-fit evolutionary models of nucleotide substitution used for the Bayesian Inference analysis for the *Dactylonectria* dataset were GTR with gamma distributed with invariant sites rates (G+I) for HIS3, and GTR+G for TUB2 and TEF. For *Ilyonectria*, the best-fit nucleotide substitution models were GTR+G+I for HIS3, Hasegawa-Kishino-Yano (HKY) model +G for TUB2, and GTR+G for TEF. In both genera, the topologies of the BI and ML consensus trees were similar, so only the BI trees with posterior probability values and bootstrap support values are presented.

Phylogenetic analyses allowed identification of four species among the 58 *Dactylonectria* isolates, with *D. macrodidyma* being the prevalent species (n = 31), followed by *D. novozelandica* (n = 14), *D. torresensis* (n = 10), and *D. palmicola* (n = 3) (Figure 2). Within the *Ilyonectria* group, the analyses identified nine isolates as *I. liriodendri,* and the remaining four isolates were grouped in a separate clade with the unidentified *Ilyonectria* strain STEU 8918 from South Africa. The node support value for this clade was 0.75 according to the BI method, while with ML method this node was not formed (Figure 3).

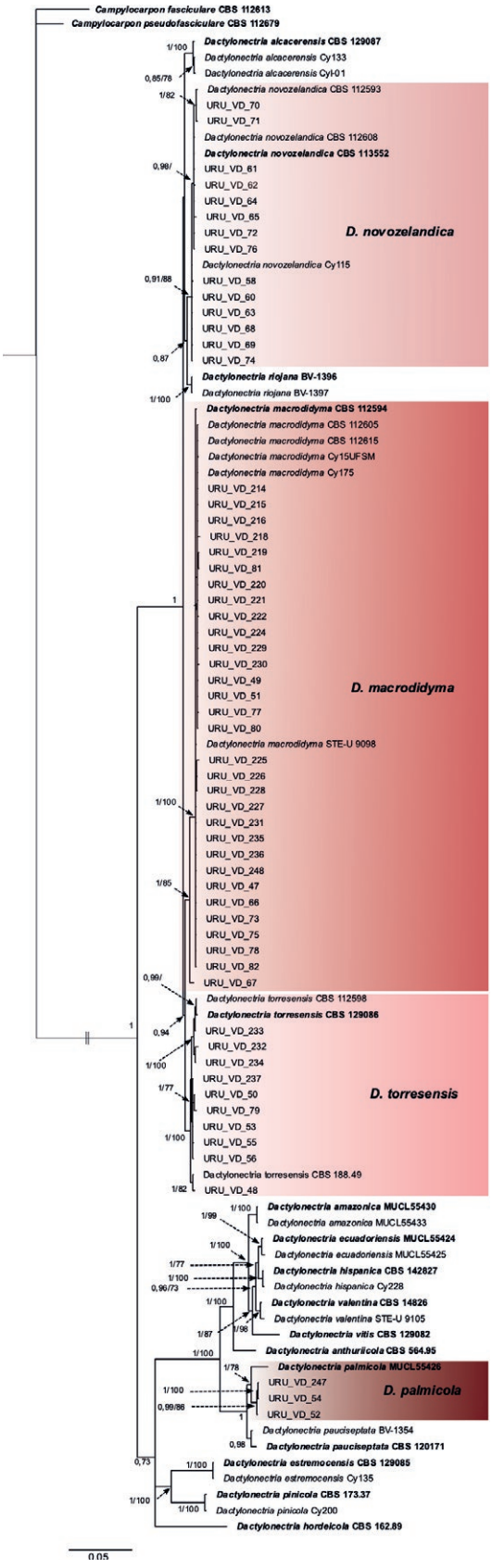


**Figure 2.** Bayesian inference phylogenetic tree built using the concatenated sequences of the HIS3, TEF and TUB2 genomic regions of 58 *Dactylonectria* isolates from Uruguayan nursery grapevines, and sequences retrieved from the GenBank (ex-type indicated in bold font). *Campylocarpon fasciculare* CBS 112613 and *Campylocarpon pseudofasciculare* CBS 112679 were used as the outgroups. Posterior probability and maximum likelihood bootstrap support values greater than, respectively, 0.70 and 70 are shown at the nodes before and after each branch. The scripts indicate that the nodes do not exist in the maximum likelihood tree. Double hash marks indicate branch lengths shortened at least 2-fold to facilitate visualization. The scale bar represents the estimated number of substitutions per site.

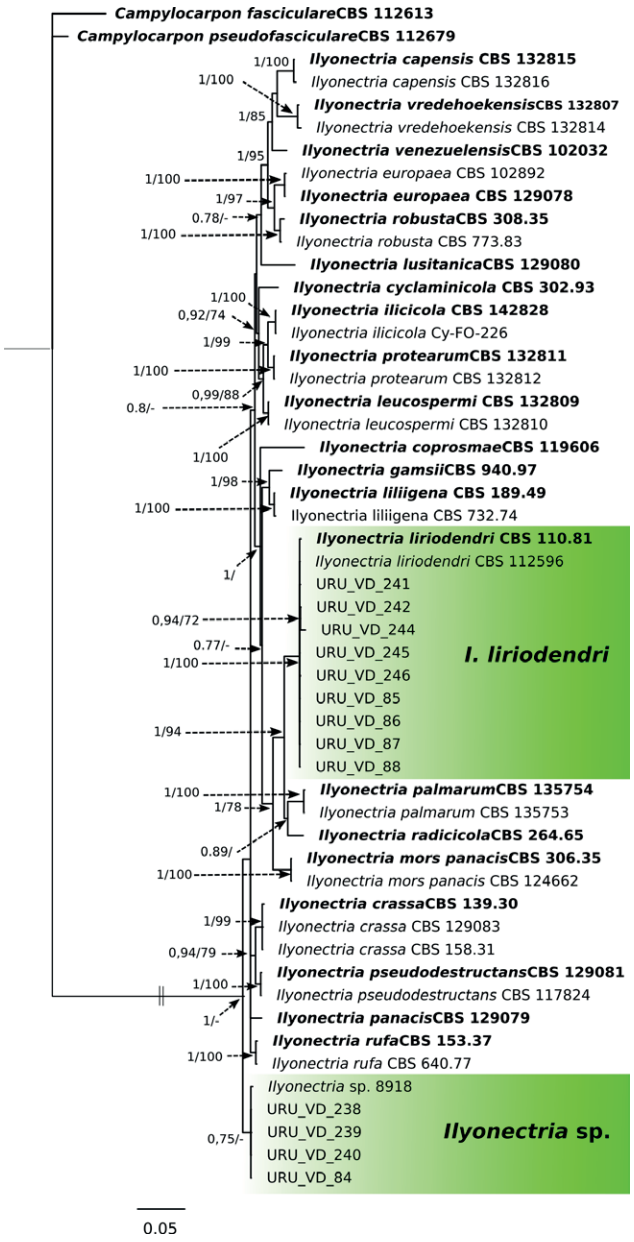


**Figure 3.** Bayesian inference phylogenetic tree built using the concatenated sequences of the HIS3, TEF and TUB2 genomic regions of 13 *Ilyonectria* isolates obtained from Uruguayan nursery grapevines, and sequences retrieved from the GenBank (ex-type indicated in bold font). *Campylocarpon fasciculare* CBS 112613 and *Campylocarpon pseudofasciculare* CBS 112679 were used as outgroups. Posterior probability and maximum likelihood bootstrap support values greater than 0.70 and 70 are shown at the nodes, respectively, before and after each bar. The scripts indicate that the nodes do not exist in the maximum likelihood tree. Double hash marks indicate branch lengths shortened at least 2-fold to facilitate visualization. The scale bar represents the estimated number of substitutions per site.

*Morphological characterization of isolates*

All the *Dactylonectria* and *Ilyonectria* isolates had aerial and cottony mycelia, and their colonies were white to yellow or light to dark brown on PDA. Conidiophores were simple or complex, sporodochial, and produced microconidia and macroconidia (Figure 4, Table 1). Macroconidia were predominantly straight, occasionally slightly curved and typically cylindrical, for the *D. macrodidyma*, *D. novozelandica*, *D. torresensis, D. palmicola,* and *I. liriodendri* isolates. Microconidia were generally ellipsoidal to ovoidal and straight. In addition, the isolates URU-VD-80 and URU-VD-242 produced chlamydospores, which is consistent with the characteristics expected for *D. macrodidyma* and *I. liriodendri* (Halleen *et al*., 2006b). The isolate URU-VD-84, identified as *Ilyonectria* sp., also had simple or complex conidiophores, produced sporodochia, and microconidia that were 0-1 septate, ellipsoidal to ovoidal and slightly curved, and macroconidia that were 1-3 septate (predominantly 1-septate), cylindrical and usually slightly curved, and produced chlamydospores (Figure 4, Table 1).

*Pathogenicity tests*

All the evaluated isolates were pathogenic on 'Gravesac' rootstock cuttings. At 3 months after inoculation, significant reductions ($P$ = 0.0193) of mean root biomass were recorded from the inoculation treatments compared with the control treatment (Table 2). The inoculated plants had sunken necrotic lesions on roots and more brownish roots than the non-inoculated plants. Mean root dry weights ranged from 1.14 g to 1.76 g in inoculated plants and was 2.03 g for the non-inoculated controls. According to root dry weight reduction, both *D. macrodidyma* isolates, URU-VD-80 and URU-VD-231, were the most virulent, causing, respectively, 44% and 42% reductions compared with the non-inoculated control. The isolates of *I. liriodendri* (URU-VD-242), *D. torresensis* (URU-VD-234) and *D. novozelandica* (URU-VD-71) were the least virulent (causing, respectively, 20%, 18% and 13% reductions in root dry weight), but the mean root weights from these isolates were not significantly different ($P$ > 0.05) from the control treatment. The other evaluated isolates showed intermediate behaviour. In addition, all the inoculated fungi were re-isolated from inoculated plants, with re-isolation rates ranging from 22% to 100%, whereas no pathogens were re-isolated from the non-inoculated controls (Table 2).

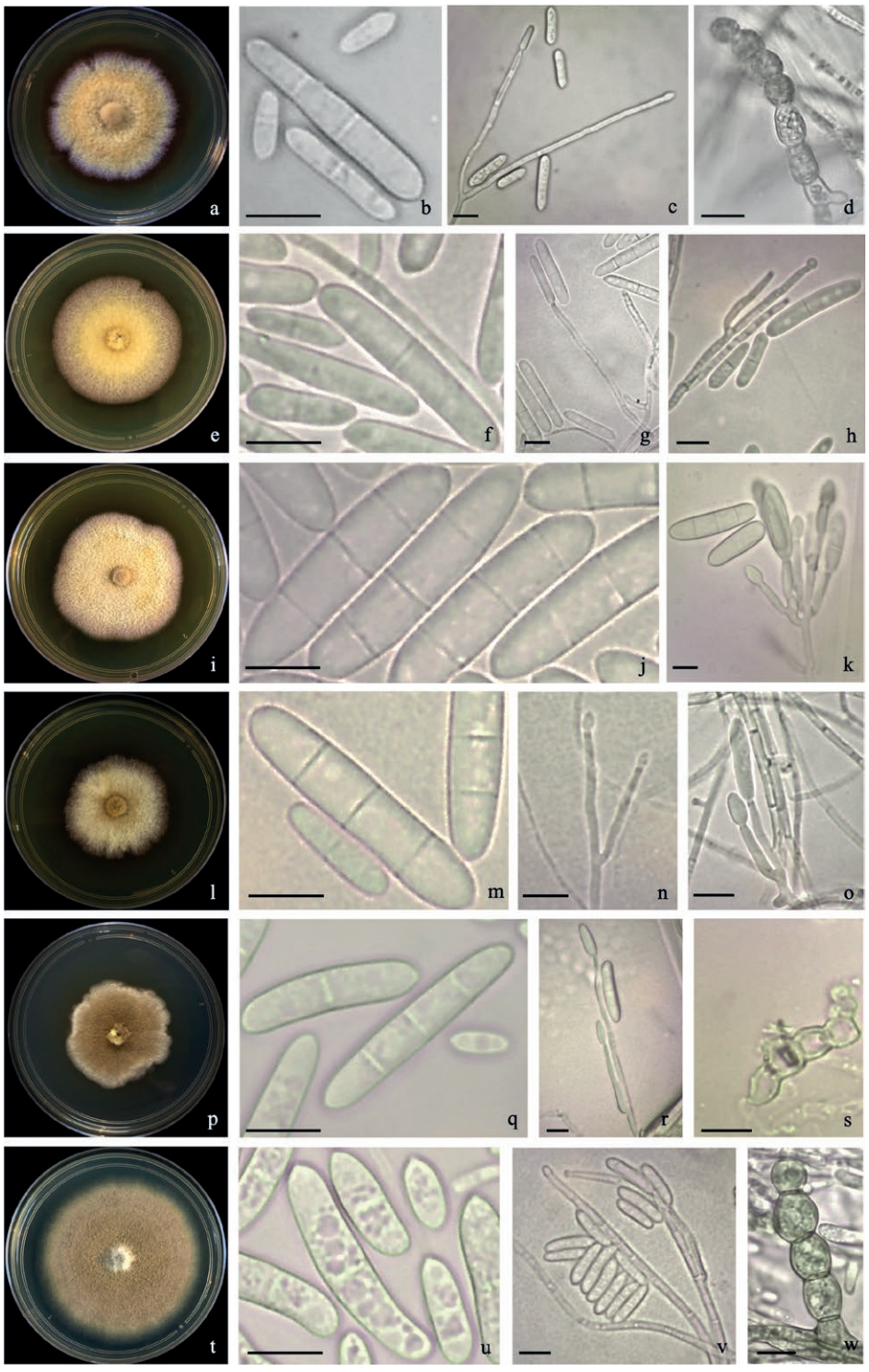


**Figure 4.** Morphological characteristics of *Dactylonectria* and *Ilyonectria* species isolated from grapevine nursery plants in Uruguay. Ten-d-old colonies on PDA at 25°C, of *D. macrodidyma* (a), *D. novozelandica* (**e**), *D. palmicola* (**i**), *D. torresensis* (l), *I. liriodendri* (p), and *Ilyonectria* sp. (t). Macro- and microconidia (b), simple conidiophore (**c**) and a chain of chlamydospores (d) of *D. macrodidyma*. Macro- and microconidia (f) and simple conidiophores (g and h) of *D. novozelandica*. Macroconidia (**j**) and a complex sporodochial conidiophore (k) of *D. palmicola*. Macro- and microconidia (m) and simple conidiophores (n and o) of *D. torresensis*. Macro- and microconidia (q), simple conidiophore (r) and a chain of chlamydospores (**s**) of *I. liriodendri*. Macro- and microconidia (u), simple conidiophore (v) and chain of chlamydospores (w) of *Ilyonectria* sp. Bars = 10 µm.

**Table 1.** Mean dimensions of aseptate and septate conidia of nine representative *Dactylonectria* and *Ilyonectria* isolates obtained in this study.

| Species | Isolate | Microconidia | | | | Macroconidia | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Aseptate | | One-septate | | One-septate | | Two-septate | | Three-septate | |
| | | Length | Width | Length | Width | Length | Width | Length | Width | Length | Width |
| *Dactylonectria macrodidyma* | URU-VD-80 | 10.32 ± 1.08 | 3.38 ± 0.34 | 14.11 ± 1.43 | 3.89 ± 0.27 | 22.08 ± 1.99 | 4.29 ± 0.28 | 24.17 ± 2.34 | 4.35 ± 0.40 | - | - |
| *D. macrodidyma* | URU-VD-231 | 10.37 ± 1.38 | 3.42 ± 0.37 | 13.62 ± 1.89 | 3.71 ± 0.32 | 23.53 ± 2.69 | 5.05 ± 0.69 | 28.77 ± 1.76 | 5.98 ± 0.74 | 31.48 ± 0.82 | 5.11 ± 0.74 |
| *D. novozelandica* | URU-VD-64 | 10.64 ± 0.61 | 3.29 ± 0.22 | 14.61 ± 2.22 | 3.56 ± 0.39 | 27.52 ±1.65 | 4.74 ± 0.37 | 31.17 ± 2.94 | 5.41 ± 0.77 | 33.25 ± 2.01 | 5.56 ± 0.43 |
| *D. novozelandica* | URU-VD-71 | 10.95 ± 1.18 | 3.01 ±0.47 | 14.89 ±2.03 | 3.66 ± 0.33 | 27.07 ±1.67 | 4.62 ± 0.42 | 29.02 ± 1.66 | 5.07 ± 0.43 | 31.45 ± 2.50 | 5.19 ± 0.48 |
| *D. torresensis* | URU-VD-79 | 11.30 ± 1.37 | 3.84 ± 0.55 | 15.01 ± 2.14 | 4.26 ± 0.57 | 26.76 ± 3.00 | 5.92 ± 0.47 | 29.56 ± 2.10 | 5.79 ± 0.38 | 30.04 ± 0.04 | 5.87 ± 0.55 |
| *D. torresensis* | URU-VD-234 | 12.00 ± 1.87 | 4.05 ± 0.49 | 14.65 ± 2.04 | 4.65 ± 0.30 | 29.35 ± 4.04 | 6.23 ± 0.58 | 32.42 ± 2.45 | 7.03 ± 0.81 | 34.47 ± 4.12 | 7.18 ± 0.70 |
| *D. palmicola* | URU-VD-54 | 12.79 ± 3.13 | 5.30 ± 1.46 | - | - | 37.22 ± 2.14 | 8.30 ± 0.82 | 36.44 ± 2.09 | 8.15 ± 0.60 | 37.37 ± 2.27 | 8.30 ± 0.70 |
| *Ilyonectria liriodendri* | URU-VD-242 | 9.39 ± 1.60 | 3.75 ± 0.43 | 13.02 ± 2.53 | 3.66 ± 0.48 | 22.70 ± 1.92 | 4.42 ± 0.75 | 27.01 ± 2.35 | 4.59 ± 0.35 | 29.13 ± 3.04 | 4.95 ± 0.50 |
| *Ilyonectria* sp. | URU-VD-84 | 10.79 ± 1.65 | 4.07 ± 0.40 | 17.41 ± 2.35 | 4.59 ± 0.45 | 24.88 ± 2.24 | 5.03 ± 0.45 | 27.59 ± 1.88 | 5.60 ± 0.81 | 29.11 | 4.84 |

**Table 2.** Mean root dry weights and proportions of inoculated fungus re-isolations for selected *Dactylonectria* and *Ilyonectria* isolates inoculated on rooted grapevine cuttings of 'Gravesac' rootstock.

| Fungal species | Isolate | Dry root weights (g)[a] | Re-isolation (%) |
|---|---|---|---|
| *Dactylonectria macrodidyma* | URU-VD-80 | 1.14 a | 80 |
| *D. macrodidyma* | URU-VD-231 | 1.17 a | 100 |
| *D. torresensis* | URU-VD-79 | 1.21 ab | 86 |
| *Ilyonectria sp.* | URU-VD-84 | 1.32 ab | 22 |
| *D. palmicola* | URU-VD-54 | 1.37 ab | 57 |
| *D. novozelandica* | URU-VD-64 | 1.47 ab | 100 |
| *I. liriodendri* | URU-VD-242 | 1.62 abc | 83 |
| *D. torresensis* | URU-VD-234 | 1.66 abc | 25 |
| *D. novozelandica* | URU-VD-71 | 1.76 bc | 83 |
| Non-inoculated control | | 2.03 c | 0 |

[a] Data are the mean of six replicates for each isolate. Means with same letter are not significantly different ($P$ = 0.05) according to Duncan test.

## DISCUSSION

Based on phylogenetic analyses, morphological studies and pathogenicity tests, the present study has identified five species of *Dactylonectria* and *Ilyonectria* causing black foot in locally produced nursery grapevines in Uruguay. The species identified were *D. macrodidyma* (31 isolates), *D. novozelandica* (14 isolates), *D. torresensis* (ten isolates), *I. liriodendri* (nine isolates) and *D. palmicola* (three isolates), while four *Ilyonectria* isolates could not be identified to species level.

All isolates were recovered from nursery grapevine plants showing typical black foot symptoms (wood necrosis at the trunk bases, dark brown streaks developing from the bases of plants, and sunken necrotic lesions on roots), as previously described by Halleen *et al*. (2006a) and Agustí-Brisach and Armengol (2013). Incidence of black foot based on proportions of pathogen isolations, was approx. 60% in 2017 and 2019, but substantially less (15%) in 2018. This difference was probably because isolations in 2018 were predominantly from the basal parts of the rootstocks. Probst *et al*. (2019) demonstrated that both *D. macrodidyma* and *I. liriodendri* can infect grapevines through wounded roots and callused basal ends. Results obtained in the present study indicate that under Uruguayan nursery production conditions, wounded roots are the main pathway of infection for these pathogens, rather than the basal callus tissues of the plants.

The multilocus phylogenetic approach allowed identification of black foot pathogens at species level, and morphological characterization supported these results. Col-

ony morphology and characteristics (shape and size) of macro- and microconidia, as well as production of chlamydospores by some isolates, were consistent with those expected for the identified species (Halleen *et al*., 2004; 2006b; Cabral *et al*., 2012a; Gordillo and Decock, 2017).

*Dactylonectria macrodidyma* was the prevalent species found causing black foot in the nursery plants analysed. This species was first described associated with grapevine in South Africa as *C. macrodidyma* by Halleen *et al*. (2004), and subsequently named as *D. macrodidyma* by Lombard *et al*. (2014). This species has been associated with black foot disease in several other countries, including New Zealand (Halleen *et al*., 2004; Probst *et al*., 2019), Chile (Auger *et al*., 2007), Spain (Alaniz *et al*., 2007), Switzerland (Hofstetter *et al*., 2009), Canada (Petit *et al*., 2011; Úrbez-Torres *et al*., 2014), Portugal (Cabral *et al*., 2012a; Reis *et al*., 2013), Turkey (Özben *et al*., 2012; Akgül *et al*., 2022), Brazil (dos Santos *et al*., 2014), Algeria (Aigoun-Mouhous *et al*., 2019), China (Ye *et al*., 2021) and Argentina (Longone *et al*., 2022). In a previous study in Uruguay, Abreo *et al*. (2010) found *D. macrodidyma* as the prevalent species causing black foot on symptomatic plants collected from commercial vineyards.

The second most common pathogen found was *D. novozelandica*, which was first described in grapevine as *I. novozelandica* by Cabral *et al*. (2012a), and then renamed *D. novozelandica* by Lombard *et al*. (2014). This species has been associated with black foot in New Zealand (Cabral *et al*., 2012a), Peru (Alvarez *et al*., 2012), South Africa (Cabral *et al*., 2012a), United States (Cabral *et al*., 2012a), Portugal (Reis *et al*., 2013), Spain (Agustí-Brisach *et al*., 2013), Algeria (Aigoun-Mouhous *et al*., 2019) and Turkey (Akgül *et al*., 2022). In Uruguay, *D. novozelandica* has been found causing crown and root necrosis on strawberry (Vigliecca *et al*., 2022).

*Dactylonectria torresensis* was first described by Cabral *et al*. (2012a) as *I. torresensis* in grapevine in Portugal and was then reclassified as *D. torresensis* by Lombard *et al*. (2014). This species has been reported as the prevalent cause of black foot in Portugal (Reis *et al*., 2013), Italy (Carlucci *et al*., 2017), Spain (Berlanas *et al*., 2017), Algeria (Aigoun-Mouhous *et al*., 2019) and Turkey (Akgül *et al*., 2022). In addition, the fungus has been associated with black foot in Australia, New Zealand, South Africa, United States (Cabral *et al*., 2012a), Canada (Úrbez-Torres *et al*., 2014), and China (Ye *et al*., 2021).

*Dactylonectria palmicola* was the fourth *Dactylonectria* species found associated with black foot in this study. This species was described by Gordillo and Decock (2017) in *Euterpe precatoria* in the Amazon rainforest of Ecuador. The present study is the first record of *D. palmicola* causing black foot on grapevine. The isolates URU-VD-52 and URU-VD-54 identified as *D. palmicola* in the present study, were previously misidentified as *D. pauciseptata* when phylogenetic analysis was performed using only the HIS3 gene region (Carbone *et al*., 2022). Although the HIS3 region has been demonstrated to be the most robust locus for identification of black foot pathogens (Cabral *et al*., 2012a), the present results suggest that multilocus sequence analysis, including HIS3, TUB2 and TEF, is essential to ensure correct identification of closely related fungi causing black foot.

Within *Ilyonectria, I. liriodendri* was the prevalent species found in the present study. This pathogen was first described in grapevine by Halleen *et al*. (2006b) as *C. liriodendri*, and subsequently classified as *I. liriodendri* by Chaverri *et al*. (2011). This species has been reported on grapevine in South Africa (Halleen *et al*., 2006b), Australia (Whitelaw-Weckert *et al*., 2007), Spain (Alaniz *et al*., 2007), Switzerland (Casieri *et al*., 2009), Iran (Mohammadi *et al*., 2009), Brazil (Russi *et al*., 2010), United States (Petit *et al*., 2011), Canada (Petit *et al*., 2011; Úrbez-Torres *et al*., 2014), Portugal (Reis *et al*., 2013), New Zealand (Pathrose *et al*., 2014), Italy (Carlucci *et al*., 2017), Argentina (Longone *et al*., 2022) and Turkey (Akgül *et al*., 2022). In Uruguay, *I. liriodendri* was first reported affecting symptomatic plants in commercial vineyards by Abreo *et al*. (2010).

Isolates classified as *Ilyonectria* sp. in the present study were grouped with the unidentified South African *Ilyonectria* strain STEU 8918, but with a low support value (0.75) in the BI analysis, and absence of this clade in the ML analysis. For this South African isolate, which was obtained by van der Merwe (2019) from the crown of a nectarine nursery tree, only the HIS3 gene region is available on the GenBank database. This probably explains the low support of the clade in the BI phylogenetic tree and absence of this clade in the ML tree. The pathogenicity tests conducted in the present study confirmed pathogenicity of this species to grapevine, which emphasizes the importance of prescriptive description of this new species.

Results obtained here have shown that all inoculated *Dactylonectria* and *Ilyonectria* isolates infected rooted 'Gravesac' rootstocks, but virulence varied among species and between isolates within species, which is in accordance with previous studies (Probst *et al*., 2019; Aigoun-Mouhous *et al*., 2019; Berlanas *et al*., 2020; Akgül *et al*., 2022). After 3 months from inoculation, the maximum reduction in root dry weight was 44%, and no plant death was recorded. A longer post inoculation period may have resulted in greater reductions of root biomass, because development of black foot symptoms

is usually slow (Whitelaw-Weckert, *et al*. 2007), and may have allowed all isolates to significantly reduce root dry weights compared to the control treatment. Whitelaw-Weckert *et al*. (2007) were unable to detect consistent black foot symptoms after 18 months from inoculation with *C. liriodendri* of 1-year-old rooted *V. vinifera* plants, although they confirmed pathogenicity by re-isolation of this fungus, as occurred in the present study.

*Dactylonectria macrodidyma* was the most aggressive species in the present study. Both isolates of this fungus caused the lowest root dry weights. This result agrees with those of Ye *et al*. (2021) in China, where *D. macrodidyma* was the most aggressive pathogen compared with *D. torresensis*, *D. alcacerensis*, *Cylindrocladiella lageniformis* and *Neonectria* sp. In contrast, in research conducted in Algeria by Aigoun-Mouhous *et al*. (2019), an isolate of *D. torresensis* was the most virulent, followed by isolates of *D. novozelandica* and *D. macrodidyma*. Pathogenicity tests recently conducted in Turkey on '1103P' rootstock cuttings showed that *D. novozelandica* was the most virulent species compared with *Cylindrodendrum alicantinum*, *Cylindrocladiella peruviana*, *D. macrodidyma*, *D. torresensis*, *I. liriodendri* and *I. robusta* (Akgül *et al*., 2022). This is in accordance with the study by Berlanas *et al*. (2020) in Spain, where a strain of *D. novozelandica* was found to be the most virulent compared with several black foot fungi, including *D. macrodidyma*, *D. torresensis* and *I. liriodendri*, inoculated on *V. vinifera* 'Tempranillo'. In contrast, Probst *et al*. (2019) testing different inoculation methods and propagule types in New Zealand, observed that *I. liriodendri* was generally more pathogenic than *D. macrodidyma*.

In conclusion, the present study has shown that about 39% of analysed ready-to-plant nursery vines were infected by black foot pathogens. This high proportion is likely to compromise the longevity of new vineyards. This result emphasizes the need to implement integrated management strategies to reduce black foot incidence in Uruguayan grapevine nurseries. Physical practices such as hot-water treatments have shown promising results for controlling black foot in several countries, but with unacceptable levels of disease control (Gramaje and Armengol, 2011). Nevertheless, this technology should be evaluated in each grapevine region. Use of antagonist microorganisms is currently a major objective of the research to prevent grapevine trunk diseases, but the results remain unconvincing (Martínez-Diz *et al*., 2021). Other practices, such as biofumigation with *Brassica* spp., have been shown to reduce soilborne pathogen inoculum levels and help prevent infection by black foot pathogens in young plants (Berlanas *et al*., 2018). Appropriate and environmentally-friendly chemical controls can also be considered, while local regulations allow the use of promising chemical active ingredients. Taking this into account, future studies should focus on evaluating different practices to avoid or reduce fungal infections by black foot pathogens in nursery grapevines under local conditions.

## AUTHOR CONTRIBUTIONS

MJC was responsible for performing the assays, data analyses and drafted the manuscript of this work. RR assisted in experimental assays, data analyses, and made contributions to the manuscript. PM contributed to the interpretation of the results and made critical revisions to the manuscript. SA supervised the assays, analysis and interpretation of the results and performed critical revisions of the manuscript. All the authors approved the final manuscript.

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# Activity of biocontrol agents against the grapevine pathogen *Fomitiporia mediterranea*

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**Summary.** Biological control agents (BCAs) have shown efficacy against several pathogens associated with Esca of grapevines, but their effects on the white rot pathogen *Fomitiporia mediterranea* (*Fmed*) have not been extensively studied. An assessment of several potential BCAs evaluated activity against *Fmed*. This included isolates of *Trichoderma simmonsii*, *T. citrinoviride*, *T. atroviride*, *Bacillus subtilis, B. amyloliquefaciens/velezensis* and *Pseudomonas koreensis*, all obtained from grapevines in Austria. Effects of the BCAs on *Fmed* growth were assessed in dual culture assays and in assays with fresh and autoclaved grapevine wood disks. In the dual culture assays, all the BCAs reduced growth of *Fmed* compared to experimental controls. In the *Trichoderma* experiments, *Fmed* growth only marginally exceeded the size of the initial mycelium plugs, and growth inhibition for all *Fmed* isolates and strains was 91 to 97%. Growth of *Fmed* was inhibited by 55 to 66% by *B. amyloliquefaciens/velezensis* isolates, by 41 to 49% by *B. subtilis* isolates, and by 55 to 66% by *P. koreensis*. In the wood disc assays, *Fmed* colonized fresh and autoclaved wood. All the *Trichoderma* isolates almost completely suppressed *Fmed* growth on fresh and autoclaved wood. Less but statistically significant inhibition was recorded for an isolate of *B. amyloliquefaciens/velezensis* and one of *P. koreensis*.

**Keywords.** *Trichoderma* spp., *Bacillus amyloliquefaciens/velezensis*, *Bacillus subtilis*, *Pseudomonas koreensis*, dual culture assays, wood disk assays.

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## INTRODUCTION

Grapevine trunk diseases (GTDs) cause serious problems for viticulture. This disease complex is associated with many fungi affecting grapevine trunks, leading to wood decay and death of the plants. Esca in the GTD complex is present in vineyards in both world hemispheres (Fontaine *et al.,* 2016; Claverie *et al.,* 2020), and several disease symptoms have been included in the 'Esca' designation. Vascular symptoms, which likely result from blocking of host vessels by colonizing fungi combined with water stress, include longitudinal browning and necrosis of the young vessels below bark tissues.

More or less extensive white rot in the trunks of mostly old vines may impede their vital functions. Yellowing or drying of the leaf zones between the main veins results in striped appearance ("tiger stripes") of leaves. Most vines showing tiger stripes die some years after the first appearance of leaf symptoms. Esca is also associated with apoplexy, the sudden wilting of vines followed by a rapid death (Mugnai *et al.,* 1999; Lecomte *et al.,* 2012; Fontaine *et al.,* 2016; Ouadi *et al.,* 2019; Claverie *et al.,* 2020; Moretti *et al.,* 2021; Kassemayer *et al.,* 2022).

Generally, it is assumed that the fungal species involved in this disease are endophytic, but have the potential of becoming pathogenic during the lives of infected vines. Several studies have provided evidence that the non-necrotic wood of grapevines showing "tiger stripe symptoms" and visually healthy grapevines hosted a more or less similar mycoflora (Bruez *et al.,* 2014, Elena *et al.,* 2018, Del Frari *et al.,* 2019). Under suitable conditions, potentially pathogenic fungi already colonizing the plants could become prevalent and lead to disease symptoms. Factors such as plant age, cultivar or pedo-climatic conditions probably influence the fungal communities within host plants (Bruez *et al.,* 2014; Bettenfeld *et al.,* 2021), and several field studies corroborate these assumptions. Climatic conditions, including high rainfall and cool temperatures in summer, have been shown to favour leaf symptoms (Calzarano *et al.,* 2018), whereas drought inhibited symptom development (Bortolami *et al.,* 2021). Vineyard soils, application of macro- and micronutrients, and plant age can also affect the disease (Kovács *et al.,* 2017; Calzarano *et al.,* 2023). No completely resistant host variety is known, but as reviewed by Beris *et al*., (2023) grapevine cultivars have different levels of tolerance or susceptibility to Esca.

Several reports indicate that the Ascomycetes *Phaeomoniella chlamydospora* (*Pch*) and *Phaeoacremonium minimum* (*Pmin*), and the Basidiomycetes *Fomitiporia mediterranea* (*Fmed*) and other *Fomitiporia* spp., are the main pathogens associated with Esca development. *Pch* and *Pmin* have predominantly been related to vascular disease symptoms, while *Fmed* and other *Fomitiporia* spp. are involved in wood decay (Mugnai *et al.,* 1999; Fischer and Garcia, 2015; Fontaine *et al.,* 2016; Claverie *et al.,* 2020; Moretti *et al.,* 2021; Kassemayer *et al.,* 2022). These conclusions are strengthened by studies showing that *Pch* and *Fmed* prevail in the microbiome of Esca affected grapevines (Del Frari *et al.,* 2019; Bruez *et al.,* 2020). A wide range of other fungi have also been found in symptomatic grapevines, including *Stereum hirsutum, Eutypa lata*, *Cadophora luteo-olivacea*, and members of the *Botryosphaeriaceae*, but their roles in disease development are considered to be less relevant (Fischer, 2002; Fontaine *et al.,* 2016; Gramaje *et al.,* 2018; Fischer and Peighami-Ashnaei, 2019; Claverie *et al.,* 2020). Co-occurrence with *Fmed* and *Pch* of some bacterial species, such as *Sphingomonas, Mycobacterium* and *Paenibacillus,* possibly indicate their roles in disease development and wood degradation (Bruez *et al.,* 2020; Haidar *et al.,* 2021).

Eradication of the pathogens involved in Esca development is not possible. Therefore, control practices rely on disease prevention, or, if already present, mitigation of its effects. Vine training and pruning options considering an undisturbed sap flux may influence the Esca severity. Plants trained with long cordons were generally less affected by the disease than those with short or no cordons (Lecomte *et al.,* 2018). Surgery of infected vines to remove white rot affected wood has been shown to be effective for trunk remediation (Pacetti *et al.,* 2021).

Protection of pruning wounds, aiming to prevent infections by airborne pathogen spores, is likely one of the most effective GTD management practices. Treatments can include liquid or paste products forming barriers over pruning wounds, fungicides alone, fungicides in combinations with mechanical barriers, or biological control agents (BCAs). Fungicides, however, have the disadvantage, that the compounds remain effective for short periods, but pruning wounds remain susceptible to pathogens for several weeks to months. BCAs colonizing the pruning wounds may therefore be alternatives to chemical control methods or control by mechanical barriers. In addition, BCAs can increase resistance of host plants to biotic or abiotic stresses, and have potential to elicit systemic induced resistance. Regarding Esca management, BCAs have been evaluated as pruning wound protectants, for effects on pathogen spread during nursery processes, and for their general effects on plant growth, health, and resistance to the disease (for reviews see Gramaje *et al.,* 2018 and Mondello *et al.,* 2018).

*Trichoderma* spp. have long been recognized as potential biocontrol agents for plant diseases. Their effects have been linked to production of antimicrobial compounds, induction of host resistance, mycoparasitism, and/or competition for nutrients and space (for review see Harman *et al.,* 2004). Numerous reports have indicated abilities of *Trichoderma* spp. to control several pathogens involved in the Esca complex, such as *Pch*, *Pmin* and *Botryospaeriaceae* (citations found within Mondello *et al.,* 2018). Promising results have led to the homologation of biopesticide products based on *Trichoderma* spp. for pruning wound protection in several European countries, e.g. for *T. atroviride* in Austria (BAES, 2023). Field studies on the effect of these pesti-

cides, however, have shown inconsistent efficacy. Experiments in Spain, including artificial inoculation of pruning wounds, detected no effects of *T. atroviride*-based treatments on infections by *Diplodia seriata* or *Pch* (Martínez-Diz *et al.,* 2021). In contrast, in recent studies in Italy *T. asperellum* and *T. gamsii* treatments reduced the ability of artificially inoculated *Pch* to colonize the vines (Di Marco et al., 2022). Under practical conditions in four vineyards in Northern Italy preventive *Trichoderma* applications over 9 years gave 66 to 90% reductions in Esca incidence (Di Marco *et al.,* 2022). These results were similar to those from another experiment (Bigot *et al.,* 2020), in which *T. asperellum* and *T. gamsii* applications over 7 years reduced incidence of infected grapevines by 22% in three 'Sauvignon blanc' vineyards in the Friuli Venezia Giulia region of North-eastern Italy.

Apart from antagonistic activity of *Trichoderma* spp. against GTD associated pathogens, other fungi, including the Ascomycetes *Clonostachys rosea* and *Epicoccum layuense* (Del Frari *et al.,* 2019; Silva-Valderrama *et al.,* 2021), and *Fusarium oxysporum* (Gkikas *et al.,* 2021), and the Oomycete *Pythium oligandrum* (Yacoub *et al.,* 2016), may also have antagonistic effects against GTD pathogens.

So far, bacterial BCAs have been less tested and research has predominantly focused on isolates from the *B. subtilis* group. *In vitro* studies indicated effects of *B. subtilis* against *Pch, Pmin* and *Lasiodiplodia theobromae* (Compant *et al.,* 2013). In the field, a *B. subtilis* isolate inoculated on pruning wounds reduced incidence of *Pch* (Kotze *et al.,* 2011). *Pseudomonas* spp. isolated from grapevine were effective against *Pch* and *Pmin* in dual culture assays (Niem *et al.,* 2020), and *Paenibacillus alvei* showed antagonistic activity against *Pch* (Gkikas *et al.,* 2021).

There are many reports of effects of BCAs on the Esca associated vascular pathogens, *Eutypa* and *Botryosphaeriaceae*. In contrast, studies on control of *Fmed* by BCAs have been few, although *Fmed* is considered to be the main white rot inducer in the Esca disease complex (Moretti *et al.,* 2021). The aim of the present study was to assess a range of BCAs for their inhibitory effects against *Fmed*. BCAs isolated from grapevines and a commercial BCA product were included in this research.

## MATERIALS AND METHODS

### *Bacterial and fungal isolates*

Potential bacterial and fungal antagonists included in this study are listed in Table 1. The isolated strains had been recognized as potentially antagonistic in several multiannual Esca experiments. They were recovered from trunks or dormant canes of old asymptomatic grapevines by placing wood pieces on malt extract agar (MEA, Roth), containing 20 g $L^{-1}$ malt extract and 16 g $L^{-1}$ agar (pH 6.8–7.2). The commercial BCA product *T. atroviride* (Vintec, Belchim, Schwechat, Austria) was also included in the experiments. The isolates *Fmed*_133 and *Fmed*_2395 were obtained from symptomatic grapevine trunks (Table 1).

For identification of bacterial isolates, suspensions of the bacteria in 0.01% Triton-X100 (Roth) were prepared and heated to 95°C for 7 min. Dilutions of the suspensions were then used directly for PCR. Fungal DNA was isolated with the DNeasy Plant Mini Kit as specified by the manufacturer (Qiagen). GoTaq G2 Green Master Mix (Promega) was used for all amplifications. Annealing temperature (Tm; Table 2) and elongation time at 72 °C were adjusted according to the target genes. All programs were run for 35 cycles. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), and were sent to LGC (Berlin, Germany) for sequencing. The following markers were amplified and sequenced: small subunit rRNA (SSU), large subunit rRNA (LSU), intergenic region (IGS), internal transcribed spacer (ITS), DNA gyrase subunit B (gyrB), and translation elongation factor EF1α. As the isolates originated from different test series, different sets of markers were used for molecular identifications. Primers for amplification and sequencing of markers are summarized in Table 2.

### *Dual culture assays*

Freshly growing cultures of the BCAs and *Fmed* were used for experiments. Three inoculation loops of each bacterial culture or *Trichoderma* conidium masses were suspended in 5 mL of sterilized PBS. Suspensions (each of 0.5 mL) were plated onto MEA plates and incubated at 28°C for 1 week. Cultures of *Fmed* were obtained by transferring three small pieces of a *Fmed* culture on MEA to each MEA plate, and the plates were then incubated for 10 d at 28°C. For dual culture assays, mycelial discs (12 mm diam.) were taken out from the *Fmed* cultures and placed in the centres of MEA culture plates (10 cm diam.). Identical discs were taken from potential antagonist cultures, cut into four quarters, and then were placed at the edge of each plate at regular intervals. The plates were incubated in the dark at 28°C. After 10 d the diameters of the *Fmed* cultures were measured and the radii less initial mycelium plugs were calculated. Petri dishes including *Fmed* cultures in the centres and MEA quarters at the edges served as experimental controls. Experiments including *Fmed*_133 were repeated 8 times.

**Table 1.** Bacterial and fungal isolates included in this study.

| Species and isolate | Abbreviation | Source | SSU[a] | IGS/ITS | LSU | gyrB | EF1α |
|---|---|---|---|---|---|---|---|
| *Bacillus amyloliquefaciens/ velezensis*_624 | *B. amylo_velez*_624 | Unnamed grapevine cross, Langenzersdorf, A | OQ533503 | | | | |
| *B. amyloliquefaciens/ velezensis*_2143 | *B. amylo_velez*_2143 | 'Grüner Veltliner', Langenzersdorf, A | OQ533504 | | | | |
| *B. amyloliquefaciens/ velezensis*_2277 | *B. amylo_velez*_2277 | 'Pinot Noir', Langenzersdorf, A | OQ534377 | OQ534377 | OQ534377 | | |
| *B. subtilis*_224 | *B. subtilis*_224 | Unnamed grapevine cross, Langenzersdorf, A | OQ534529 | OQ534529 | OQ565287 | | |
| *B. subtilis*_230 | *B. subtilis*_230 | Unnamed grapevine cross, Langenzersdorf, A | OQ534530 | OQ534530 | OQ565288 | | |
| *Pseudomonas koreensis* subgroup 2273 | *P. koreensis*_2273 | 'Pinot Noir', Langenzersdorf, A | OQ565286 | OQ565286 | OQ565289 | OQ541843 | |
| *Trichoderma citrinoviride*_232 | *T. citrino*_232 | Unnamed grapevine cross, Langenzersdorf, A | | OQ534541 | OQ534541 | | OQ541844 |
| *T. simmonsii*_804 | *T. simmonsii*_1056 | Unnamed grapevine cross, Langenzersdorf, A | | OQ534542 | OQ534542 | | OQ541845 |
| *T. simmonsii*_1056 | *T. simmonsii*_804 | 'Saint Laurent', Langenzersdorf, A | | OQ534543 | OQ534543 | | OQ541846 |
| *T. atroviride* SC1 | *T. atro*_Vintec | Vintec, Belchim (Schwechat, A) | | | | | |
| *Fomitiporia mediterranea*_133 | *Fmed*_133 | 'Roesler', Langenzersdorf, A | | OQ534544 | OQ534544 | | |
| *F. mediterranea*_2395 | *Fmed*_2395 | 'Sauvignon blanc', Eppan, IT | | OQ534545 | OQ534545 | | OQ541847 |

[a] GenBank accession numbers for phylogenetic markers: SSU = small subunit rRNA gene; LSU = large subunit rRNA gene; IGS = intergenic spacer; ITS = internally transcribed spacer; gyrB = DNA gyrase subunit B; EF1α = translation elongation factor EF1α.

**Table 2.** Primers for amplification and sequencing of phylogenetic markers for bacteria and fungi.

| Primer | Marker[a] | Direction | Sequence | Temperature | References |
|---|---|---|---|---|---|
| *Bacteria* | | | | | |
| 16S0008F-YM | SSU | fwd | AGAGTTTGATYMTGGCTCAG | 55°C | Frank *et al.,* 2008 |
| 16S0968F | SSU/IGS | fwd | AACGCGAAGAACCTTAC | 55°C | Felske *et al.,* 1996 |
| 16S1512R | SSU | rev | ACGGTTACCTTGTTACGAC | 55°C | Lane, 1991 |
| pHr | IGS/LSU | fwd | TGCGGCTGGATCACCTCCTT | 55°C | Massol-Deya *et al.,* 1995 |
| p23SR01 | LSU | rev | GGCTGCTTCTAAGCCAAC | 55°C | Massol-Deya *et al.,* 1995 |
| UP-1 | gyrB | fwd | GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA | 60°C | Yamamoto and Harayama, 1995 |
| UP-2r | gyrB | rev | AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCA | 60°C | Yamamoto and Harayama, 1995 |
| *Fungi* | | | | | |
| ITS1F | ITS/LSU | fwd | CTTGGTCATTTAGAGGAAGTAA | 54°C | Gardes and Bruns, 1993 |
| TW14 | ITS/LSU | rev | GCTATCCTGAGGGAAACTTC | 54°C | Setaro *et al,.* 2006 |
| EF1-0728F | EF1α | fwd | CATCGAGAAGTTCGAGAAGG | 50°C | Carbone and Kohn, 1999 |
| EF1-1620R | EF1α | rev | GACGTTGAADCCRACRTTGTC | 50°C | Stielow *et al.,* 2015 |

[a] SSU = small subunit rRNA gene; LSU = large subunit rRNA gene, IGS = intergenic spacer; ITS= internally transcribed spacer; gyrB DNA gyrase subunit B; EF1α= translation elongation factor EF1α.

To confirm the outcome of these experiments, a second isolate, *Fmed*_2395, was included and experiments with *Fmed*_2395 were repeated 4 times. Inhibition of mycelium growth (%) was calculated as follows:

$$C\text{-}T/C*100$$

where: C = radius of the fungal colony less radius (mm) of the initial mycelium plug in the control plates, and
T = radius of the fungal colony less initial mycelium plug in the BCA treatment.

*Wood disc model*

To confirm antagonistic effects observed in the dual culture assays a protocol was developed using grapevine wood sections. Young grapevine plants or rooted cuttings in pots were excluded because *Fmed* is a coloniser and degrader of (older) wood. Most research has cultivated *Fmed* only in agar plates, cultivation on dried and sterilized sawdust of grapevine trunks has been published recently (Schilling *et al.,* 2022). Cross sections of grapevine trunks placed on water agar were used in the present study. *Fmed* does not sporulate on agar plates (Fischer, 2002), so as with the dual culture assays (above), *Fmed* mycelium discs were used for *Fmed* inoculations. Healthy 10- to 15-year-old grapevines 'Rotburger' ('Zweigelt') in a vineyard in Langenzersdorf (Austria) were uprooted and their trunks were cut into approx. 4 cm thick cross sections. Initially and to keep the experiment similar to plant situations, the experiments used freshly cut trunk cross sections that were immediately used. However, sizes of *Fmed* colonies within the treatments, particularly from the treatments with bacterial BCAs, gave variable results (experiments in October and December 2021; Figures 1 and 2). In consequence, the experiments were enlarged using autoclaved wood discs. For autoclaving, all wood pieces required

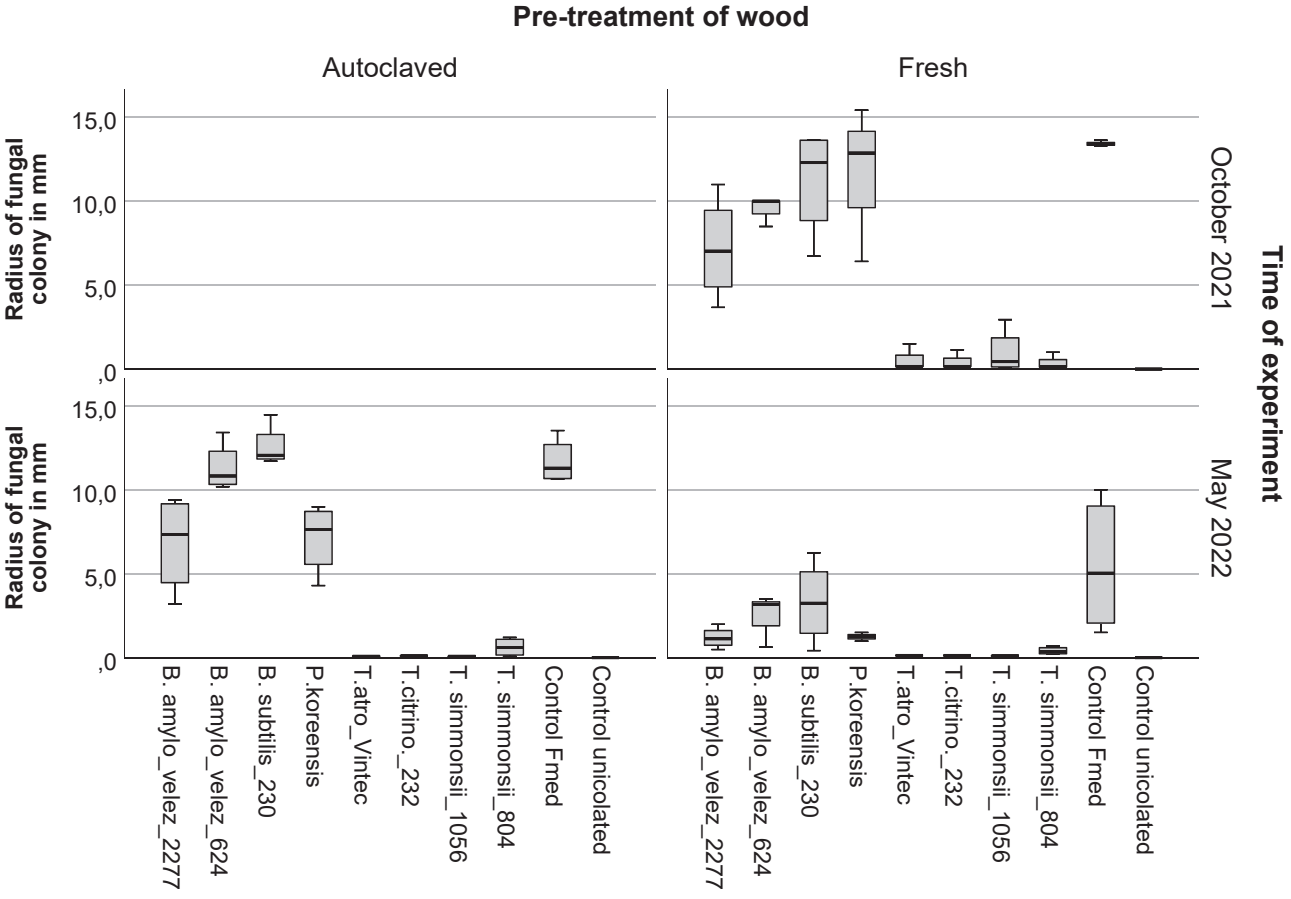


**Figure 1.** Data of radii of *Fomitiporia mediterranea* colonies on grapevine wood discs receiving different pre-treatments, in experiments carried out at different times of the year. Each boxplot shows a median value, and the box boundaries indicate the 25th and 75th percentiles of each distribution.

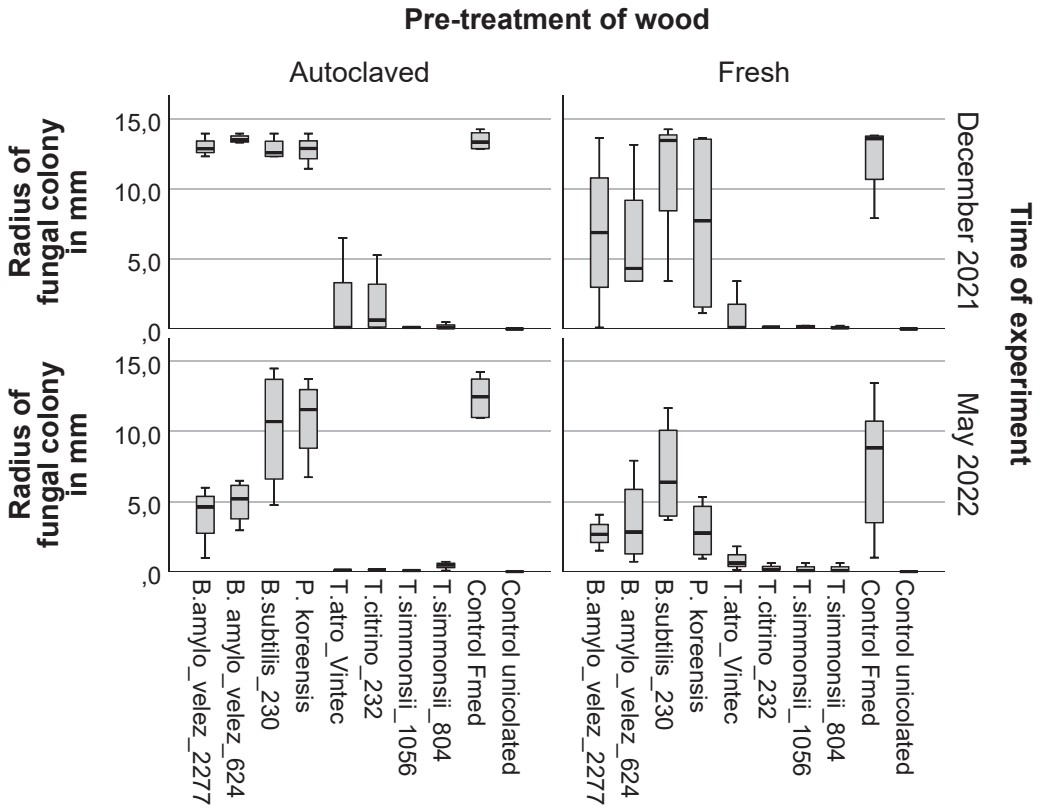


**Figure 2.** Data of radii of *Fomitiporia mediterranea* colonies on grapevine wood discs receiving different pre-treatments, in experiments carried out at different times of the year. Each boxplot shows a median value, and the box boundaries indicate the 25th and 75th percentiles of each distribution.

for one repetition of the experiment were together immersed in 500 mL of PBS, were autoclaved and then allowed to stand in PBS for approx. 1 week.

Bacterial BCA isolates were cultivated on MEA at 28°C. After 1 week, three inoculation loops of bacterial colonies were transferred to 40 mL of liquid tryptic soy medium (trypticase peptone, 17 g $L^{-1}$; soy peptone, 3 g $L^{-1}$; NaCl, 5 g $L^{-1}$, $K_2HPO_4$, 2.5 g $L^{-1}$; glucose, 2.5 g $L^{-1}$, pH 7.2: chemicals supplied by Roth; US Food and Drug Administration, 2023), and the cultures were incubated at room temperature on a shaker for 18 h. The cultures were then centrifuged at 5400 g for 10 min. Resulting pellets were washed twice in PBS, resuspended in PBS, and the OD600 was adjusted to 0.15 0.2. *Trichoderma* species isolated during previous experiments were cultivated on MEA as described (above) for the dual culture assays. After 7 d, the Petri dishes were each covered with 10 mL of sterile PBS. *Trichoderma* conidia were released from the culture plates by aid of a Drigalski spatula. The resulting conidium suspensions were each filtered through a cheese cloth. Conidium counts were determined in duplicate using a Neubauer's chamber, and the inoculation suspensions were adjusted to $1 \times 10^8$ CFU $mL^{-1}$. *T. atroviride*_Vintec inoculation suspension was prepared according to the manufacturer's instructions (2 g $L^{-1}$ (w/v) in tap water).

The tests followed two temporal sequences: *A. Preventative treatment – BCA inoculation of stem cross sections before inoculation with* Fmed. The wood pieces were each submersed in BCA suspension for a few seconds, run-off was allowed, and they were placed onto water agar. After incubation at 28°C for 7 d, a *Fmed* mycelium plug produced as described (above) for dual culture experiments was placed on the centre of each wood piece. After further incubation for 4 weeks, two perpendicular diameters of each developing *Fmed* colony were measured, and the colony radius (less the radius of the initial mycelium plug) was calculated. In rare cases, especially when fresh wood discs were used, two *Fmed* colonies grew on the wood discs. In these cases, the radii of both colonies were summed. The experiment in October 2021 (harvest of grapevine trunk on 6 October) comprised fresh wood discs only, while the experiment in May 2022 (harvest of trunk 31 May) included fresh

and autoclaved wood discs. Each experiment comprised four repetitions.

*B. Curative treatment* – Fmed *inoculation of trunk cross sections before antagonist application. Fmed* mycelium plugs were placed in the centres of the wood discs on water agar. After 7 days of incubation (as above), the *Fmed* agar plugs were removed. The wood discs were then immersed in antagonist conidium/spore suspensions for a few seconds, and placed on fresh water agar plates. After further incubation for 4 weeks, the sizes of the developed *Fmed* colonies were determined (as described above). Each experiment was repeated four times in December 2021 (trunk harvest 9 December) and in May 2022 (trunk harvest 24 May). Trunk cross sections treated with PBS only ("Control uninoculated") and cross sections inoculated with *Fmed* but not with BCAs ("Control *Fmed*") served as experimental controls. For each repetition of the experiments, wood from one grapevine was used.

### *Statistical analyses*

Statistical analyses were carried out using the program SPSS 26.0 (SPSS, IBM, Vienna, Austria). All data were processed using generalised linear models, including the distribution and link function "gamma with log link" and the dependent variable "size of the *Fmed* colony".

For analysis of the dual culture assays, the model included the explanatory variables "treatment". For each *Fmed* isolate, an individual model was calculated (Table 3).

Data obtained for the wood discs treated according to the "preventative treatment" (BCA inoculation of trunk cross sections before inoculation with *Fmed*) in October 2021 or May 2022 were included in two separate models. The model for data from October 2021 comprised the explanatory variable "BCA treatment" only, and the model for data from May 2022 comprised the explanatory variables "BCA treatment" and "pre-treatment of wood" (autoclaved or fresh wood) (Table 4). The model for the wood discs from the "curative treatment" (*Fmed* inoculation before BCA application) included the explanatory variables "BCA treatment", "pre-treatment of wood" and "time of experiment" (December 2021 or May 2022) (Table 5). Main effects were assessed, as offered by the program. Differences in efficacy of the treatments were identified using Least significant difference (LSD) tests. The data sets for the wood disk models are illustrated as boxplots, each illustrating the median and the 25th and 75th percentiles of the distribution. Median *Fmed* colony radii are presented in the text.

## RESULTS

### *Dual culture assays*

As illustrated in Figure 1, all of the potential BCAs included in this study reduced ($P \leq 0.05$) growth of *Fmed* as compared to the untreated controls. The three *B. amyloliquefaciens/velezensis* isolates inhibited fungal growth by 55 to 66%, and the two *B. subtilis* isolates reduced growth by 41 to 49%. *P. koreensis*_2273 was more effective in experiments with *Fmed*_133, inhibiting growth of the pathogen by 66%, while for *Fmed*_2395 growth inhibition was 55%. In the *Trichoderma* experiments, *Fmed* growth only marginally exceeded the size of the initial mycelium plugs, giving growth inhibition of 91 to 97% for both *Fmed* strains from all the *Trichoderma* isolates. The generalised linear model indicated statistically significant effects of the factor "BCA treatment" for both *Fmed* strains (*Fmed*_133: Wald $\chi^2$ = 378.99, df = 10; $P$ = 0.000; *Fmed*_2395: Wald $\chi^2$ = 251.60, df = 10, $P$ = 0.000). The *Trichoderma* isolates were more effective ($P \leq 0.05$) against *Fmed* than the bacterial BCAs (Table 3).

### *Wood disc model*

Placement of *Fmed* mycelium discs on the wood discs for 7 d allowed fungal colonisation of fresh and autoclaved wood. *Fmed* formed at the beginning white mycelia on the discs, which gradually turned yellow as the fungus developed.

### *Preventive treatment. BCA inoculation of the wood cross sections before inoculation with* Fmed

In October 2021, the median radius of the *Fmed* cultures on the fresh control discs was 12.9 mm, and in the *Trichoderma* experiments the *Fmed* colonies measured between 0.1 and 0.45 mm. Median colony radii for the bacterial BCAs were from 7.0 mm (*B. amlyo_velez*_2277) to 12.9 mm (*P. koreensis*_2273). No fungal growth was observed on uninoculated wood discs (Figure 2). The generalised linear model indicated significant effects of the factor "BCA treatment" on *Fmed* colony size (Wald $\chi^2$ = 148.97, $P$ = 0.000, df = 8). All the *Trichoderma* isolates but none of the bacterial BCAs reduced ($P \leq 0.05$) fungal growth compared to the *Fmed* controls. (Table 4).

In May 2022, *Fmed* median colony radius on autoclaved wood in the control treatments was 11.3 mm, and for treatments with the *Trichoderma* isolates was from 0.1 to 0.6 mm. In treatments with the bacterial BCAs mean

**Table 3.** Activity of BCAs in reducing growth of two *Fomitiporia mediterranea* isolates (*Fmed*_133 or *Fmed*_2395) in dual culture assays. Outcomes of the generalised linear models calculated for each isolate and mean colony dimensions of *F. mediterranea*. Means accompanied by different letters are different ($P \leq 0.05$).

| Dependant variable: Radius (mm) of fungal colony less radius of mycelium plug. | | | | | | |
|---|---|---|---|---|---|---|
| Factor: BCA treatment | *Fmed*_133 | | | *Fmed*_2395 | | |
| | Wald $\chi^2$ = 378.99, $P$ = 0.000, df = 10 | | | Wald $\chi^2$ = 251.60, $P$ = 0.000, df = 10 | | |
| | Mean radius | SD | % Inhibition | Mean radius | SD | % Inhibition |
| *B. amylo_velez*_2143 | 6.1bc | 0.7 | 58.6 | 7.5b | 0.5 | 60.5 |
| *B. amylo_velez*_2277 | 5.4b | 0.2 | 63.2 | 6.4b | 1.6 | 66.5 |
| *B. amylo_velez*_624 | 5.8b | 0.6 | 60.7 | 8.5b | 1.0 | 55.3 |
| *B. subtilis*_224 | 8.7d | 2.8 | 40.6 | 9.6b | 1.1 | 49.3 |
| *B. subtilis*_230 | 8.4cd | 1.1 | 42.7 | 9.7b | 1.0 | 48.7 |
| *P. koreensis*_2273 | 5.0b | 0.8 | 66.0 | 8.4b | 0.6 | 55.9 |
| *T. citrino*_232 | 1.3a | 2.3 | 91.0 | 0.6a | 0.4 | 96.7 |
| *T. atro*_Vintec | 0.8a | 0.7 | 94.9 | 0.9a | 0.3 | 95.4 |
| *T. simmonsii*_1056 | 0.9a | 0.4 | 93.7 | 1.0a | 0.6 | 94.7 |
| *T. simmonsii*_804 | 0.5a | 0.5 | 96.6 | 1.3a | 0.7 | 93.4 |
| Control *Fmed* | 14.6e | 2.0 | | 19.0c | 0.3 | |

colony radii were 7.3 mm from *B. amlyo_velez*_2277, and 12.1 mm from *B. subtilis*_230. On fresh wood, in May 2022, median size of *Fmed* colonies in the controls was 5.0 mm, from all *Trichoderma* treatments was 0.1 to 0.35 mm, and from the bacterial BCAs was from 1.1 mm (*B. amlyo_velez*_2277) to 3.2 mm (*B. subtilis*_230) (Figure 1). No *Fmed* growth was detected on the uninoculated wood discs Statistical analyses confirmed effects ($P \leq 0.05$) of the factors "BCA treatment" (Wald $\chi^2$ = 592.18, $P$ = 0.000, df = 8) and "pre-treatment of wood" (Wald $\chi^2$ = 20.92, $P$ = 0.000, df = 1) on *Fmed* growth. All the *Trichoderma* isolates and *B. amylo_velez*_2277 and *P. koreensis*_2273 reduced ($P \leq 0.05$) *Fmed* growth, compared to the *Fmed* control. *Fmed* grew more rapidly on autoclaved than on unautoclaved wood pieces (Table 4).

*Curative treatment. Inoculation of wood discs by* Fmed *before BCA application.*

In December 2021, the median size of *Fmed* colonies in the control treatment on autoclaved wood was 13.3 mm, and on fresh wood 13.6 mm. Median colony sizes for the *Trichoderma* treated wood discs were between 0.1 and 0.6 mm on autoclaved wood, and were 0.1 mm for all isolates on fresh wood. On autoclaved wood, median

**Table 4.** Preventive activity of biocontrol agents on growth of *Fomitiporia mediterranea* (*Fmed*_133) on grapevine wood disks. Outcomes of the generalised linear models and estimated marginal mean colony dimensions of *F. mediterranea*. Values accompanied by different letters are different ($P \leq 0.05$).

| Dependant variable: Radius (mm) of fungal colony less radius of mycelium plug | | |
|---|---|---|
| Factor | Variant | Estimated marginal mean radius |
| October 2021 | | |
| BCA treatment Wald $\chi^2$ = 148.97, $P$ = 0.000, df= 8. | *B. amylo_velez*_2277 | 7.17b |
| | *B. amylo_velez*_624 | 9.49b |
| | *B. subtilis*_230 | 11.23b |
| | *P. koreensis*_2273 | 11.88b |
| | *T. atro*_Vintec | 0.49a |
| | *T. citrino*_232 | 0.40a |
| | *T. simmonsii*_1056 | 0.99a |
| | *T. simmonsii*_804 | 0.35a |
| | Control *Fmed* | 13.43b |
| May 2022 | | |
| BCA treatment Wald $\chi^2$ = 592.18, $P$ = 0.000, df = 8. | *B. amylo_velez*_2277 | 3.24cd |
| | *B. amylo_velez*_624 | 6.03de |
| | *B. subtilis*_230 | 6.76de |
| | *P. koreensis*_2273 | 3.62cd |
| | *T. atro*_Vintec | 0.12a |
| | *T. citrino*_232 | 0.13a |
| | *T. simmonsii*_1056 | 0.10a |
| | *T. simmonsii*_804 | 0.51b |
| | Control *Fmed* | 7.93e |
| Pre-treatment of wood Wald $\chi^2$ = 20.92, $P$ = 0.000, df = 1. | Autoclaved wood | 1.61a |
| | Fresh wood | 0.08b |

*Fmed* colony sizes on wood discs treated with bacterial BCAs ranged from 12.6 to 13.5 mm, and on fresh wood, from 4.3 mm (*B. amylo_velez*_624) to 13.4 mm (*B. subtilis*_230). In May 2022, median *Fmed* colony radii on autoclaved wood discs were 12.5 mm and on fresh control discs 8.8 mm. Median radii of *Fmed* colonies on *Trichoderma* treated discs in no case exceeded 0.6 mm, on autoclaved and on fresh wood. For the bacterial BCAs, *Fmed* colony radii on autoclaved wood varied from 4.6 mm (*B. amylo_velez*_2277) to 11.6 mm (*P. koreensis*_2273), and on fresh wood from 2.7 mm (*B. amylo_velez*_2277) to 6.4 mm (*B. subtilis*_230). No *Fmed* growth was observed on uninoculated control discs (Figure 2). Statistical analyses showed significant effects of the factors "BCA treatment" (Wald $\chi^2$ = 500.24, $P$ = 0.000, df = 8), "pre-treatment of wood" (Wald $\chi^2$ = 8.14, $P$ = 0.004, df = 1), and "time of the experiment"

**Table 5.** Curative activity of biocontrol agents on growth of *Fomitiporia mediterranea* (*Fmed*_133) on grapevine wood disks. Outcomes of the generalised linear models and estimated marginal mean colony dimensions of *F. mediterranea*. Values accompanied by different letters are different ($P \leq 0.05$).

| Dependant variable: Radius (mm) of fungal colony less radius of mycelium plug | | |
|---|---|---|
| Factors | Variant | Estimated marginal mean radius |
| BCA treatment Wald $\chi^2 = 500.24$, $P = 0.000$, df = 8. | *B. amylo_velez*_2277 | 6.38b |
| | *B. amylo_velez*_624 | 5.88bc |
| | *B. subtilis*_230 | 9.89bc |
| | *P. koreensis*_2273 | 7.81bc |
| | *T.atro*_Vintec | 0.84a |
| | *T.citrino*_232 | 0.47a |
| | *T.simmonsii*_1056 | 0.16a |
| | *T.simmonsii*_804 | 0.23a |
| | Control *Fmed* | 11.45c |
| Pre-treatment of wood Wald $\chi^2 = 8.14$, $P = 0.004$, df = 1. | Autoclaved wood | 2.47a |
| | Fresh wood | 1.61b |
| Time of experiment Wald $\chi^2 = 8.52$, $P = 0.004$, df = 1. | December 2021 | 2.48a |
| | May 2022 | 1.60b |

(Wald $\chi^2 = 8.53$, $P = 0.004$, df = 1). A significant effect on *Fmed* growth as compared to the control was proven for all *Trichoderma* isolates and for the bacterial BCA *B. amylo_velez*_2277. *Fmed* grew faster on autoclaved wood and in the experiment in December 2021 (Table 5).

## DISCUSSION

In the first step of our experiments, the dual culture assays on MEA, all of the *Trichoderma* isolates were highly effective. They reduced mycelium growth of the *Fmed* isolates by more than 90%, and in all cases overgrew the pathogen colonies (data not shown). Effects on growth of *Fmed* in dual culture assays were previously reported for *T. asperellum* and *T. gamsii* where growth inhibition up to 65% was reported. *T. asperellum* completely overgrew *Fmed* at 18 and 23°C (Di Marco *et al.,* 2022). Reasons for the higher *Fmed* growth inhibition in the present study are unclear, but may be due to differences in efficacy of the tested BCA strains, the sensitivity of the *Fmed* isolates, or in the experimental methods (e.g. different temperatures and/or culture media).

The dual culture experiments recorded biocontrol activity for all the bacterial BCAs assessed. Compared to previous reports, growth inhibition rates of approx. 60% recorded in the present study for *B. amyloliquefaciens/velezensis* and *P. koreensis* appeared promising. Haidar *et al.* (2021) tested 59 bacterial species from various taxa, isolated from grapevines, for interactions with *Fmed* in co-cultures on agar plates. Only six of the tested isolates inhibited fungal growth at rates greater than 50%. Of these, two *Bacillus* sp. isolates gave mean inhibition of 55.7%, and one for one *Pseudomonas* sp. isolate was 52.7%. Efficacy of bacterial BCAs in laboratory assays has also been reported against other Esca associated pathogens. Several strains of *Pseudomonas poae* and *P. moraviensis* induced growth inhibition of up to 70% for *Pch*, but only up to 26% for *Pmin* (Niem *et al.,* 2020). In a study including potential bacterial BCAs isolated from Bordeaux vineyards, 46 isolates were screened for effects against *Pch* using dual inoculations of grapevine stem cuttings. Reductions of stem necroses between 31% and 39% were recorded for *Paenibacillus, Enterobacter, Pantoea* and *Bacillus* isolates (Haidar *et al.,* 2016).

Developmental conditions on MEA strongly differ from the situation in grapevines, so a protocol closer to the situation *in planta* was developed to test the antifungal potential of BCA strains beyond dual culture assays. This included autoclaved and fresh grapevine wood, and *Fmed* mycelium discs, which colonised the wood within 7 d. The observed developing *Fmed* colonies were white and later yellow, as previously reported for *Fmed* cultivation on sawdust (Schilling *et al.,* 2022). At the beginning of the present experiments, fresh wood discs were used to keep experimental conditions as close as possible to the situation in grapevines. However, as illustrated in Figures 1 and 2, the size of *Fmed* colonies in control experiments and treatments with bacterial BCAs showed high variability, although each experiment contained wood discs from the same grapevine trunk and an identical procedure was used.

Pathogens and other microorganisms occurring in natural ecosystems (including host plants) are parts of complex microbial communities. Members of each community interact with one another and with host plants. Likewise, host plants depend on their microbiomes for survival and defence from pathogen attack. Development of pathogens or BCA agents depends on several factors, such as host genotype and nutrient status, abiotic and other environmental stresses, and microbial interactions (Brader *et al.,* 2017). Grapevine wood microbiota is particularly in rich in species, interactions within the microbial community and between the microbiota and vine physiology can strongly affect the pathogen devel-

opment (Hofstetter *et al.,* 2012; for review see Claverie *et al.,* 2020).

The current data for fresh grapevine wood discs indicated that growth of the *Fmed* colonies and establishment of bacterial BCAs were both strongly influenced by the natural microbiome in the wood. In consequence, the test design was expanded to include autoclaved wood discs. The results of these subsequent tests confirmed the presumption that the grapevine microbiome reduced growth of *Fmed*. Over all experiments, the fungus developed more rapidly on autoclaved than on non-autoclaved wood (Tables 4 and 5), and use of autoclaved wood discs to some extent reduced variability of colony growth in experimental controls and on wood discs treated with bacterial BCAs (Figures 1 and 2). Therefore, the methods used gave conditions to assess interactions between BCAs and *Fmed* and effects of natural grapevine microbiomes on interaction between BCAs and *Fmed*.

Apart from pre-treatment of wood (fresh or autoclaved), *Fmed* growth on the wood discs depended on the factor "time of the experiment". In both experimental types (models for preventive or curative treatments), *Fmed* grew more rapidly on trunk cross sections harvested in autumn or early winter than on trunks harvested in May (data for preventive treatment not presented, data for curative treatment Table 5). Previous reports have indicated declines in macro nutrients in grapevine perennial structures from bud-burst to flowering, and increases during post-harvest periods when nutrients are stored for the next growing season (Holzapfel *et al.,* 2019). Bruez *et al.,* (2014) attributed changes within fungal communities in grapevine trunks to seasonal nutrient dynamics. It therefore seems possible that fluctuating nutrient dynamics in the grapevine trunks accounted (at least partly) for the seasonal differences in *Fmed* growth observed in the current study.

The observed variability of *Fmed* growth data on wood discs within identical experiments, in several cases lacking normal distributions and the significant impacts of experimental season and pre-treatment of wood, led to the decision to waive calculation of growth inhibition rates. Instead, the results were presented as box plots allowing insights into the data sets. Aiming to consider the interlinked factors, statistical analyses of the data were carried out using multifactor generalised models.

Despite the *Fmed* growth variations, the wood disc models improved insight into the multitrophic interactions between *Fmed*, the tested BCAs, the trunk microbial community and the host plant physiology. For all of the T*richoderma* treatments, similar results were obtained, regardless of the mode of treatment (preventive or curative), the time of the experiment, or pre-treatment of the wood discs. *Fmed* growth hardly exceeded the size of the initial mycelial plugs (Figures 1 and 2). Development of the *Trichoderma* isolates was not affected by the microbiome within the fresh wood discs, and was, at maximum, slightly affected by the season in which the trunks had been harvested. Comparable to our dual culture experiments and previous assays on agar plates (Di Marco *et al.,* 2022), all the *Trichoderma* isolates completely overgrew the *Fmed* mycelia on the initial mycelial plugs (data not presented). Overall, present and previous laboratory data indicate that that the *Trichoderma* spp. efficiently supressed *Fmed*.

Field studies (Bigot *et al.,* 2020, Di Marco *et al.,* 2022) have indicated prominent roles of *Trichoderma* pruning wound protection for reducing Esca disease indices. In addition to *Fmed*, *Pch* and *Pmin* are important pathogens involved in Esca (Mugnai *et al.,* 1999; Fischer and Garcia, 2015; Fontaine *et al.,* 2016; Claverie *et al.,* 2020; Moretti *et al.,* 2021; Kassemayer *et al.,* 2022). *Pch* and *Pmin* penetrate grapevine wood in several ways. They colonize (pruning) wounds (Mugnai *et al.,* 1999), and frequently spread during plant propagation processes (Aroca *et al.,* 2010; Gramaje and Armengol, 2011). In addition, vineyard or nursery soils might be inoculum sources for *Pch* and *Pmin* (Agusti-Brisach *et al,* 2013; Saccà *et al.,* 2018). In contrast, for *Fmed*, infections of (pruning) wounds by basidiospores or transmitted inoculum from pruning tools, are considered to be the only modes of pathogen entry to host plants (Mugnai *et al.,* 1999; Moretti *et al.,* 2021). The results of previous and the present *in vitro* studies indicate a high efficacy of *Trichoderma* for protecting pruning wounds from *Fmed* infections. In the field *Trichoderma* pruning wound protection might largely prevent *Fmed* infections and this way strongly contribute to the reduction of Esca incidence in treated vineyards.

Beyond protection of pruning wounds, the curative effects of treatments against *Fmed* observed in the present study, and the ability of *Trichoderma* spp. to overgrow *Fmed* (Di Marco *et al.,* 2022), indicate that *Trichoderma* spp. could also have curative efficacy against *Fmed* infections in grapevines. *Trichoderma* conidia could be infiltrated into white rot zones of diseased vines or applied in nanomaterials (Spasova *et al.,* 2022). However, *Trichoderma* spp. have not only been isolated from asymptomatic grapevines but to an even higher extent from diseased vines. In a 10-year-old 'Cabernet Sauvignon' vineyard, *Trichoderma* spp. were isolated from 75% of asymptomatic plants and 93% of Esca symptomatic plants (Bruez *et al.,* 2014). Future field experiments are required to determine if *Trichoderma* treatments can be successful curative Esca treatments.

Several *Trichoderma* isolates from the trunks in the experimental vineyard at Langenzersdorf were identified as *T. citrinoviride*, which can grow at human body temperature (Jaklitsch, 2011) and is classified as risk group 2 fungus (TRBA 460, 2016). Grapevine trunk temperatures can regularly reach temperatures above 30°C, which selects for fungi that are adapted to these temperatures such as *Fmed* (Fischer, 2002) or possibly also *T. citrinoviride*. As an opportunistic human pathogen, *T. citrinoviride* cannot be applied as a BCA, but the results from the present study demonstrate that other *Trichoderma* spp. have potential to control growth of *Fmed*.

For the bacterial BCAs, the tests using grapevine wood discs confirmed the results obtained in the dual culture assays, but only to some extent. Figures 1 and 2 show the varied results between the different experimental protocols, and also within each protocol. Efficacy of the treatments was probably influenced by the multilateral interactions between the BCAs, grapevine microbiomes, trunk nutrient status, and pathogen development (Brader *et al*., 2017*)*. Over all the experiments, the isolate *B. amylo_velez*_2277 was the most effective in reducing *Fmed* growth on wood discs, and *P. koreensis* had some effects. *B. subtilis*_230 and *B. amylo_velez*_624 gave results that were not significantly different from the control treatments in any of the experiments (Tables 4 and 5). In previous field experiments including artificial infections of pruning wounds by *Pch*, a *B. subtilis* isolate showed some effect against the pathogen, but efficacy of *Trichoderma* sp. was superior (Kotze *et al.,* 2011). Studies in Greece, including several grapevine cultivars and different vineyards, showed positive correlations of *Bacillus* and *Streptomyces* with asymptomatic vines and negative co-occurrence of these bacteria with *Pch* and *Pmin* (Beris *et al.,* 2022). Protective effects of a *B. velezensis* isolate added to pruning wounds, from infections with *Neofusicoccum parvum* and *Diplodia seriata* were recorded by Langa-Lomba *et al.* (2023).

Experiments on the effects of BCAs on *Fmed* have rarely been reported (Moretti *et al.,* 2021), and previous studies have been carried out using agar plate assessments. Therefore, the present study adds new knowledge on the potential of BCAs for control of diseases caused by the main white rot pathogen in the Esca disease complex. Inconsistent results of BCA treatments in the field have been linked to the fact that many commercial products do not originate from the plant species or plant part they are applied to (Bruisson *et al.,* 2019). In the present study, all the BCAs except *T. atroviridae*_Vintec were isolated from grapevine wood. This and the observed efficacy against *Fmed* encourage further evaluation of the two *T. simmonsii* isolates _804 and _1056 for preventive Esca control in the field. Future efforts towards the development of suitable BCA applications within trunk tissues could allow assessment of their efficacy as curative *Fmed* treatments. The present study has also demonstrated significant efficacy of bacterial BCAs for control of *Fmed* infections, though their activity was weaker than observed for *Trichoderma* spp. Nevertheless, particularly the isolate *B. amylo_velez*_2277, may be worth further examination, in a first step for practical protection of grapevine pruning wounds.

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# *In vitro* evaluation of drying supports and adhesive polymers as adjuvants for biocontrol of *Diplodia seriata* by *Trichoderma harzianum* and *Clonostachys rosea*

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**Summary.** Grapevine trunk diseases (GTDs) can cause large losses in vineyards. *Diplodia seriata* is an important GTD pathogen in Chile. Development and use of bioprotectors is a complementary alternative to the use of agrochemicals for disease management. To produce bioformulations for management of *D. seriata*, additives could be used to maintain viability and survival of biocontrol agents, such as *Trichoderma harzianum* and *Clonostachys rosea*. Effects of drying supports (inulin, maltodextrin, lactose, or talc) and adhesive polymers (carboxymethylcellulose, Aloe vera gel, or chitosan) were assessed on *D. seriata* conidium viability and mycelium development of *T. harzianum* and *C. rosea*, and for their biocontrol capacity against *D. seriata*. *T. harzianum* and *C. rosea* cultured in Potato Dextrose Agar containing inulin (at 10% w/v) maltodextrin (10% w/v), lactose (6% w/v), or talc (4% w/v), or the adhesive polymers carboxymethylcellulose (0.5% w/v), Aloe vera gel (0.5% w/v), or chitosan (1.5% w/v), maintained their biocontrol activity against *D. seriata*. These additives did not enhance *D. seriata* development. Therefore, these preparations, at the respective indicated concentrations, can be included in bioformulations for management of disease caused by this pathogen.

**Keywords.** Adjuvants, bioformulation, *Botryosphaeria*, drying supports, sticky polymers.

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## INTRODUCTION

Grapevine trunk diseases (GTDs) can cause severe damage to grapevine productivity (OIV, 2016). These diseases are caused by a complex of pathogenic fungi, and there are no satisfactory methods for their management (Matei *et al*., 2017; Besoain, 2018). Botryosphaeria dieback, an important GTD, causes yield losses between 36 to 48% in Chilean 'Cabernet Sauvignon' vineyards located in O'Higgins and Maule regions, with average yield losses estimated to be 5,800 kg ha$^{-1}$ (Torres *et al*., 2017; Larach *et al*., 2020). *Dip-*

*lodia seriata* has the greatest prevalence as the cause of Botryosphaeria dieback in Chile (Morales *et al*., 2012; Díaz *et al*., 2013; Torres *et al*., 2017).

Botryosphaeria dieback pathogens enter grapevine plants mainly through pruning wounds, in the absence of any chemical and/or biological control organisms (Gramaje *et al*., 2018; Mondello *et al*., 2018). Chemical fungicides may induce occurrence of resistant pathogen strains and may cause environmental damage. Biological controls can be alternatives to prevent plant infections by these pathogens, due to the different mechanisms of the biocontrol agents (BCAs). In addition, BCAs could be included in rotations with chemical pesticides (Fravel, 2005; Khamna and Yokota, 2008; Gramaje *et al*., 2018).

*Trichoderma* spp. and *Clonostachys rosea* (Link: Fr.) could be BCAs against several pathogens, such as *Rhizoctonia solani* (Kühn) (Kakvan *et al.,* 2013), *Phytophthora* spp. (Bae *et al.,* 2016), *Alternaria* spp. (Jensen *et al*., 2004), *Sclerotinia sclerotiorum* (Lib.) de Bary (Rodríguez *et al*., 2011), and others (Jensen *et al*., 2000; Morandi *et al*., 2003; Morandi *et al*., 2007). *Trichoderma harzianum* Rifai and *C rosea* have also been tested individually and together, as conidium suspensions, for the control of *Diplodia seriata* and *Neofusiccocum australe* in greenhouse and field assays (Arriagada, 2015).

To overcome instability of conidium suspensions, it is probably important to incorporate BCAs into appropriate formulations, that could be used alone or combined with chemical pesticides to achieve effective pathogen control (Papavizas, 1985; Arriagada, 2015). However, formulations can additionally contain adjuvants that are used to formulate, facilitate application, and maintain microbial viability in harmful field and/or storage conditions (Gašić and Tanović, 2013). These adjuvants may modify the biocontrol capacity of the active ingredients or favor development of target pathogens (Bernhard *et al*., 1998). Thus, it is important that candidate adjuvants are assessed for effects on biocontrol capacity of the active principals before they are incorporated into formulations. It is also important to consider formulation types and methods of field application (Chammem *et al*., 2022). Potential adjuvants include drying supports for the preparing powder formulations, and polymers to ensure adherence to host plants.

Drying supports are adjuvants used for microencapsulation of microorganisms, when spray drying technology is used. These include compounds that do not affect the environment due to their short or null persistence, and include lactose, inulin, talc, or maltodextrin, among others (Wilkins, 1990). Use of lactose can improve survival of *T. harzianum* during storage (Kumar *et al*., 2016), and inulin protects *T. harzianum* viability against freezing and freeze drying (Mensink *et al*., 2015; Nunes *et al.,* 2018). Maltodextrin has been used to extend bioformulation shelf-life after production using the spray drying technology (Leslie *et al*., 1995; Agudelo *et al.,* 2017), and talc has been used as a carrier additive for solid formulations containing *Trichoderma* spp. (Kakvan *et al.,* 2013).

Polymers have been used to promote adherence of bioformulations to plant tissues (Gašić and Tanović, 2013). Carboxymethylcellulose (CMC) is commonly used for this purpose because in addition to its function as adherent (Bernhard *et al*., 1998), it provides a carbon source for microbial development. This compound has been tested in formulations containing *C. rosea* (Musiet, 2015) and *T. harzianum* (Samolski, 2014). Chitosan could also trigger host defense mechanisms, and has been tested for control of *D. seriata* (Meng and Tian, 2009; Cobos *et al*., 2015). Extracts from *Aloe vera* that contain a viscous gel with antimicrobial activity have also been tested against bacteria (Pereira *et al*., 2013) and fungi (Sitara *et al*., 2011; Navarro, 2013).

The mixture of *T. harzianum* and *C. rosea* that controls *D. seriata* (UChile, 2021) could be used in a formulation to which adjuvants could be added to aid biocontrol activity. The objective of the present study was to assess drying supports and polymers for efficacy as biocontrol formulation additives to *T. harzianum* and *C. rosea* for control of disease caused by *D. seriata*.

## MATERIALS AND METHODS

### *Fungus strains and culturing*

*Trichoderma harzianum* (strain RGM2218) and *C. rosea* (strain RGM2217) were used, from the laboratory fungal collection at Laboratorio de Fitopatología y Control Biológico de Enfermedades, Departamento de Sanidad Vegetal, Facultad de Ciencias Agronómicas, Universidad de Chile. These strains had previously shown good biocontrol activity against *D. seriata* (strain 1009), whether alone or in mixture (Arriagada, 2015). The isolates were activated on potato dextrose agar (PDA; Difco), and were grown on PDA plates in darkness at 30°C. *Diplodia seriata* was activated and grown on PDA in darkness at 25°C. Cultures were stored at 4°C and sub cultured weekly. All microbiological procedures were carried out under sterile conditions.

### *Chemicals*

All reagents were technical grade, and were obtained from the following providers: a) drying supports; inu-

lin (Reutter S.A.), maltodextrin (Quimatic S.A.), lactose (Reutter S.A) and talc (Reutter S.A); b) polymers carboxymethyl cellulose (CMC, Winkler Ltd.), Aloe vera gel (liquid extract; Proaltec) and chitosan (Reutter SA); c) Culture media; potato dextrose agar and agar (DIFCO), glucose (Merck).

*Biocontrol agent conidium viability*

Conidium suspensions of *T. harzianum* and *C. rosea* were prepared, respectively, from 7- or 14-d-old cultures, in 9 g·L$^{-1}$ sterile NaCl solution, and were then filtered through two layers of sterile gauze to remove mycelia. Conidium suspension (100 mL containing 1 × 10$^5$ conidia·mL$^{-1}$ (*T. harzianum* or *C. rosea*) was placed in a Petri dish containing glucose agar (GA; 10 g L$^{-1}$ glucose and 5 g L$^{-1}$ agar). The inoculum was spread over the entire agar surface in the dish using a Drigalski's spatula. All plates were incubated for 24 h at 30°C. One hundred conidia were assessed for germination, with conidia classified as germinated when germ tubes were ≥ twice the diameter of the conidia (Latorre and Rioja, 2002).

To test effects of different compounds on conidium viability, the compounds were added to culture medium before autoclaving. Drying supports were added at 0, 2.0, 4.0, 6.0, 8.0, or 10.0% (w/v) and polymers were added at 0, 0.5, 1.0, 1.5 or 2.0 % (w/v).

*Mycelium growth from biocontrol agents*

Mycelium discs (0.5 cm diam.) were taken from a 5-d-old PDA culture of *T. harzianum* or a 10-d-old culture of *C. rosea,* and were each placed in the middle of a Petri dish containing PDA. Plates were then incubated at 30°C for 2 d for *T. harzianum*, and for 6 d for *C. rosea*. Colony growth in each dish was then measured two perpendicular axes, then averaged, and expressed as diameter in mm.

To test the effects of the different compounds on mycelium growth, the compounds were added to culture medium (PDA) before autoclaving. Drying supports were added at 0, 2.0, 4.0, 6.0, 8.0 or 10.0% (w/v), and polymers were added at 0, 0.5, 1.0, 1.5 or 2.0 % (w/v).

*Antagonistic activity against* Diplodia seriata

The antagonistic activities of *T. harzianum* or *C. rosea* against *D. seriata* were assessed in the absence or presence of each adjuvant that did not affect growth of *T. harzianum* or *C. rosea* growth, in dual cultures. Ten µL of conidium suspension (1 × 10$^5$ conidia mL$^{-1}$) of each biocontrol agent was applied to a 0.5 cm diam. mycelium disc from a 5-d-old *D. seriata* culture (Figure 1). Conidium suspensions and PDA medium containing specific adjuvants were prepared as described above. Experimental controls were included, replacing conidium suspensions with sterile water. All plates were incubated for 5 d at 25°C. Radii of *D. seriata* colonies were measured for each treatment (Figure 1), and percent inhibition was determined.

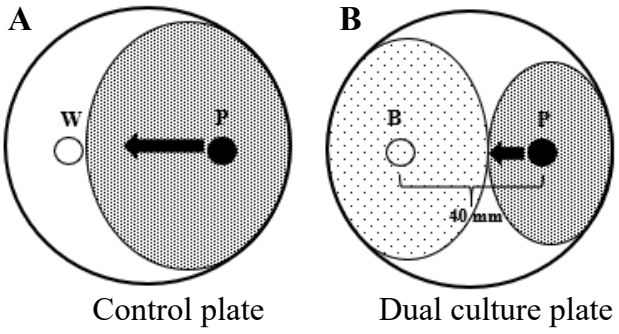


**Figure 1.** Measurement of radial colony growth of *Diplodia seriata* in dual cultures A: W, sterile water and P, pathogen (*D. seriata*); B: B, biocontrol agent (*Trichoderma harzianum* or *Clonostachys rosea* conidium suspension) + adjuvant and P, pathogen (*D. seriata*). The culture medium was PDA.

*Experimental design and statistical analyses*

Completely randomized designs (CRDs) with unifactorial structures were used to determine effects of the different concentrations of the drying supports and the polymers, and to establish the greatest concentration of these where no negative effects were detected on viability of conidia or mycelium growth of the of the antagonists. The factors assessed were type of drying support (inulin, maltodextrin, lactose, or talc) or polymers (CMC, Aloe vera gel, or chitosan), each independently, at their different % w/v concentrations.

The experimental units were four Petri dishes (each as a subsample), with three repetitions for each treatment. Infostat software was used for the statistical analyses with interface of R, and Wald Tests were applied, through a general and mixed linear model. If statistically significant differences ($P \leq 0.05$) were determined, LSD Fisher LSD Multiple Comparison Tests was carried out.

A CRD with a factorial structure was used to determine the biocontrol capacity of the antagonists on *D. seriata* (antagonism), using type of drying support (inulin, maltodextrin, lactose, or talc) or polymer (CMC, Aloe vera gel, or chitosan), and type of antagonist (*T. harzianum*, or *C. rosea*). Drying supports and polymers were analyzed independently.

## RESULTS

### *Viability of conidia in the presence of different adjuvants: drying supports or polymers*

The drying supports inulin and maltodextrin did not affect viability of *T. harzianum* conidia at any of the concentrations tested, while lactose decreased mean viability by 3.02%, and talc by 15.67% (Table 1A). Viability of *C. rosea* conidia was not affected by inulin, lactose or talc (Table 1B), but maltodextrin at 8% (w/v) in the culture medium increased viability of *C. rosea*.

The sticky polymers Aloe vera gel and chitosan did not affect viability of *T. harzianum* conidia, while CMC decreased viability by 5% at concentrations of 1% (w/v) and greater (Table 2A). Viability of *C. rosea* conidia was not affected by CMC or by chitosan, while concentrations of Aloe vera gel of 1.5% (w/v) and greater decreased viability (Table 2B).

### *Growth of biocontrol agents in the presence of different adjuvants*

Mycelium growth of *T. harzianum* was not modified by the presence of the drying supports inulin, maltodextrin, or talc in the culture media (Table 3A). Lactose concentrations of 8% w/v or greater decreased growth of this fungus. Growth of *C. rosea* was not affected by inulin, maltodextrin, or talc, but lactose at 6% w/v or greater significantly increased growth of this BCA (Table 3B).

Growth of *T. harzianum* was not modified by the sticky polymer CMC in culture medium at the different concentrations tested (Table 4A). Aloe vera gel concentrations of 1% w/v and greater and chitosan at 2% w/v, significantly decreased growth of this BCA. None of the adjuvants at any of the assessed concentrations modified growth of *C. rosea* (Table 4B).

### *Antagonistic activity against* Diplodia seriata

The antagonistic activity of the BCAs against *D. seriata* was tested with the highest concentrations of the drying support and sticky polymers adjuvants that did not modify either their viability or growth. These were 10% inulin, 10% maltodextrin, 6% lactose and 4% talc for the drying supports; and 0.5% CMC, 0.5% Aloe vera and 1.5% chitosan for the sticky polymers.

Results showed that none of the drying supports tested (Table 5A) or the sticky polymers tested (Table 5B), at the concentrations already mentioned, modified the ability of *T. harzianum* or of *C. rosea* to inhibit *D. seriata* growth, although significant differences were observed between the two BCAs in % inhibition of the pathogen. Controls performed in the absence of the BCAs showed that none of the adjuvants modified the growth of *D. seriata*.

**Table 1.** Mean conidium viability (%) of *Trichoderma harzianum* (A) and *Clonostachys rosea* (B) after culture in Petri dishes containing glucose agar amended with different drying supports adjuvants (inulin, maltodextrin, lactose, or talc) at different concentrations (0, 2.0, 4.0, 6.0 ,8.0 or 10.0 % w/v).

A. *Trichoderma harzianum*

| Drying supports | Concentrations (% w/v) | | | | | | Wald Test *P* value |
|---|---|---|---|---|---|---|---|
| | 0 | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | |
| Inulin | 91.17a | 94.83a | 96.25a | 94.00a | 95.17a | 96.08a | 0.3502 |
| Maltodextrin | 95.25a | 94.92a | 96.83a | 96.00a | 95.42a | 97.42a | 0.4895 |
| Lactose | 97.67a | 93.75b | 95.58b | 95.83b | 94.33b | 93.75b | 0.0154 |
| Talc[a] | 97.00a | 81.67b | 81.00b | - | - | - | 0.0047 |

B. *Clonostachys rosea*

| Drying supports | Concentrations (% w/v) | | | | | | Wald Test *P* value |
|---|---|---|---|---|---|---|---|
| | 0 | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | |
| Inulin | 92.33a | 95.92a | 95.08a | 96.58a | 95.92a | 91.58a | 0.4760 |
| Maltodextrin | 94.25c | 96.42bc | 98.17ab | 96.58bc | 99.00a | 97.92ab | 0.0002 |
| Lactose | 87.42a | 87.67a | 85.58a | 89.00a | 89.42a | 86.42a | 0.6214 |
| Talc[a] | 83.33a | 86.25a | 86.58a | - | - | - | 0.8973 |

[a]Concentrations of talc greater than 4% (w/v) interfered with viability analyses.
Means for each treatment followed by different letters indicate differences ($P \leq 0.05$), according to Fisher's LSD tests.

**Table 2.** Mean conidium viability (%) of *Trichoderma harzianum* (A) and *Clonostachys rosea* (B) in Petri dishes containing glucose agar amended with polymers adjuvants (carboxymethylcellulose (CMC), Aloe vera gel, or chitosan), each at different concentrations (0, 0.5, 1.0, 1.5 or 2.0 % w/v).

A. *Trichoderma harzianum*

| Polymers | Concentration (% w/v) | | | | | Wald Test *P* value |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 1.5 | 2.0 | |
| CMC | 97.25a | 96.62a | 91.92b | 92.58b | 92.25b | 0.0001 |
| Aloe vera gel | 96.25a | 95.08a | 93.83b | 94.25b | 93.75b | 0.0001 |
| Chitosan | 92.83a | 82.42b | 83.25b | 86.00b | 84.08b | <0.0001 |

B. *Clonostachys rosea*

| Polymers | Concentration (% w/v) | | | | | Wald Test *P* value |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 1.5 | 2.0 | |
| CMC | 84.00a | 84.00a | 83.58a | 84.58a | 86.50a | 0.9943 |
| Aloe vera gel | 88.75a | 81.83ab | 82.17a | 75.17b | 58.25c | 0.0003 |
| Chitosan | 91.08a | 85.25b | 88.58ab | 88.92ab | 89.63ab | 0.0036 |

Means for each treatment followed by different letters indicate differences ($P \leq 0.05$), according to Fisher's LSD tests. CMC= carboxymethylcellulose.

## DISCUSSION

Bioformulation development requires assessment of included adjuvants for viability modification, growth and the biocontrol effects on the BCAs that are the active ingredients of the formulations. Also, each bioformulation will focus on biocontrol of specific or groups of pathogens, so it is important to consider pathogen characteristics, routes of entry to plant host, and the diseases produced. As fungi belonging to the *Botryosphaeriaceae* mainly enter hosts through pruning wounds, the biocontrol formulations should be applied directly to the damaged zone of the grapevines. The formulations should remain on damaged host surfaces to prevent infections by these fungi. Solid formulations (powders), or semi-solids (pastes), could be appropriate. Therefore, adjuvants such as the drying supports and polymers tested in the present study are likely to be these types of formulation.

Presence of the drying supports inulin, maltodextrin, lactose, or talc, or of the polymers CMC, chitosan, or Aloe vera gel, at the concentrations assessed (Table 5) did not modify the biocontrol properties of *T. harzianum* or of *C. rosea*. It has been previously shown that some components may decrease or increase the antagonistic capacity of BCAs towards the pathogen targets (Bernhard *et al.*, 1998). This was not observed with the selected adjuvants in the present study. On the other hand, assessments of viability and growth of the BCAs in the presence of different adjuvants (Tables 1 to 4) allowed selection of the adjuvants that could be used in bioformulations, including their appropriate concentrations, to ensure that the adjuvants do not harm biocontrol agent conidium viability, reproductive structures, or

**Table 3.** Mean colony diameters (mm) of *Trichoderma harzianum* (A) and *Clonostachus rosea* (B) after culture in Petri dishes containing potato dextrose agar amended with different drying support adjuvants (inulin, maltodextrin, lactose, or talc) at different concentration (0, 2.0, 4.0, 6.0, 8.0, and 10.0 % w/v)

A. *Trichoderma harzianum*

| Drying supports | Concentrations (% w/v) | | | | | | Wald Test *P* value |
|---|---|---|---|---|---|---|---|
| | 0 | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | |
| Inulin | 80.76a | 84.59a | 85.00a | 84.14a | 85.00a | 84.44a | 0.0818 |
| Maltodextrin | 80.71a | 81.23a | 81.46a | 83.72a | 84.18a | 85.00a | 0.8702 |
| Lactose | 85.00a | 84.72a | 85.00a | 83.38a | 78.77b | 75.58c | <0.0001 |
| Talc | 82.29a | 79.22a | 83.43a | 83.70a | 83.24a | 80.86a | 0.7307 |

B. *Clonostachys rosea*

| Drying supports | Concentrations (% w/v) | | | | | | Wald Test *P* value |
|---|---|---|---|---|---|---|---|
| | 0 | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | |
| Inulin | 36.78a | 37.03a | 37.56a | 36.83a | 36.55a | 37.26a | 0.0501 |
| Maltodextrin | 45.04a | 44.99a | 44.67a | 45.93a | 45.49a | 45.76a | 0.6139 |
| Lactose | 44.02b | 45.09b | 45.33b | 46.01a | 46.23a | 46.16a | 0.0004 |
| Talc | 44.69a | 46.67a | 47.61a | 47.33a | 48.95a | 47.73a | 0.1755 |

Means for each treatment followed by the different letters indicate differences ($P \leq 0.05$), according to Fisher's LSD tests.

**Table 4.** Mean colony diameters (mm) of *Trichoderma harzianum* (A) and *Clonostashys rosea* (B) after culture in Petri dishes containing potato dextrose agar amended with polymer adjuvants (carboxymethylcellulose, Aloe vera gel, or chitosan) at different concentration (0, 0.5, 1.0, 1.5 and 2.0 % w/v).

A. *Trichoderma harzianum*

| Polymers | Concentration (% w/v) | | | | | Wald Test *P* value |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 1.5 | 2.0 | |
| CMC | 85.00a | 76.26a | 64.79a | 54.33a | 67.96a | 0.3003 |
| Aloe vera gel | 85.00a | 81.81a | 74.67b | 70.18bc | 66.04c | 0.0026 |
| Chitosan | 76.41a | 68.87ab | 65.84ab | 62.40ab | 56.45b | 0.0179 |

B. *Clonostachys rosea*

| Polymers | Concentration (% w/v) | | | | | Wald Test *P* value |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 1.5 | 2.0 | |
| CMC | 44.34a | 42.22a | 41.85a | 41.17a | 42.02a | 0.5045 |
| Aloe vera gel | 45.59a | 44.51a | 45.38a | 44.33a | 42.93a | 0.5557 |
| Chitosan | 45.49a | 46.25a | 44.05a | 45.40a | 44.38a | 0.2733 |

Means for each treatment followed by the different letters indicate differences (P≤ 0.05), according to Fisher's LSD statistical. CMC = carboxymethylcellulose.

**Table 5.** Mean percent inhibition of *Diplodia seriata* growth caused by *Trichoderma harzianum* or *Clonostachys rosea* in the presence of: (A) drying supports (inulin, maltodextrin, lactose, or talc); or (B) sticky polymers (carboxymethyl cellulose, Aloe vera gel, or chitosan), in dual cultures in Petri dishes containing potato dextrose agar.

A. Drying supports

| Drying supports | % (w/v) | % Inhibition of *D. seriata* growth | | | Wald Test *P* value |
|---|---|---|---|---|---|
| | | *T. harzianum* | *C. rosea* | None | |
| Inulin | 10.0 | 61.39a | 26.85b | 0.00c | <0.0001 |
| Maltodextrin | 10.0 | 61.07a | 23.70b | 2.26c | <0.0001 |
| Lactose | 6.0 | 61.16a | 27.40b | 0.00c | <0.0001 |
| Talc | 4.0 | 59.93a | 34.43b | 2.73c | <0.0001 |
| $H_2O$ (control) | - | 60.40a | 29.57b | 0.00c | <0.0001 |

B. Polymers

| Polymers | % (w/v) | % Inhibition of *D. seriata* growth | | | Wald Test *P* value |
|---|---|---|---|---|---|
| | | *T. harzianum* | *C. rosea* | None | |
| CMC | 0.5 | 61.00a | 26.53b | 0.06c | <0.0001 |
| Aloe vera gel | 0.5 | 61.36a | 32.85b | 4.11c | <0.0001 |
| Chitosan | 1.5 | 62.99a | 24.68b | 1.92c | <0.0001 |
| $H_2O$ (control) | - | 60.40a | 29.57b | 0.00c | <0.0001 |

Means for each treatment followed by the different letters indicate differences ($P \leq 0.05$), according to Fisher's LSD tests. CMC = carboxymethylcellulose.

growth, or establishment in plants (Guijarro *et al*., 2008; Sabuquillo *et al*., 2009). The type of formulation will also depend on the disease and the phytopathogen to be controlled (Bernhard *et al*., 1998).

The different tested drying support or polymer compounds tested here did not modify the antagonistic activity of *T. harzianum* or *C. rosea* towards *D. seriata*. Thus, their use in formulations at the concentrations tested is likely to modify viability or growth of the BCAs. Nevertheless, final selection of specific adjuvants to be included in formulations should consider characteristics of each compound. For example, inulin which neither modified conidium viability of growth nor interfered with antagonistic activities of *T. harzianum* and *C. rosea* against *D. seriata*, could be used as a formulation adjuvant. Inulin is a suitable carbon source for microorganisms (Kelly, 2008), it stabilizes proteins, and protects conidia during freeze-drying (Mensink *et al*., 2015; Nunes *et al*., 2018). However, some *Trichoderma* isolates are not able to hydrolyze inulin (Cordeiro *et al*., 1997; Souza-Motta *et al*., 2003); so, this compound may not be useful unless adequately evaluated. The drying support maltodextrin, that has been widely used in formulations (Samborska *et al*., 2007; Du *et al*., 2014; Wenzel *et al*., 2017; Fernández and Sepúlveda, 2019) did not modify the parameters analyzed in the present study, except at greater than 4% w/v, an increase of *C. rosea* conidium germination was detected, indicating a protective effect. These results are like those previously described for *T. harzianum*, where maltodextrin protected from protein denaturation increased shelf-life by up to 6 months at temperatures between 15°C and 35°C (Rai and Tewari, 2016).

Lactose is a drying support used in production of bioformulations, for minimization microorganism viability losses from high temperatures during spray drying or low temperatures during freeze-drying (Tan *et al*., 2007; Higl *et al*., 2008; Cabrefiga *et al*., 2014). Results in the present study showed that addition of lactose decreased *T. harzianum* conidium germination at concentrations less than 2%, and mycelium growth at less than 8% w/v. However, results were different for *C. rosea*, where lactose favored mycelium growth and did not modify conidium viability. These results could be due to different use of lactose by the microorganisms, as has been reported for filamentous fungi which use lactose at low rates (Swartz, 1985), and in two possible ways: direct absorption of the disaccharide and subsequent intracellular hydrolysis, or extracellular hydrolysis and subsequent absorption of the resulting monosaccharides (Seiboth *et al*., 2007). The present study results could be explained by the biocontrol agents using different routes

of lactose utilization from the culture medium. It has also been reported that incorporation of lactose in bioformulations can favor stability and survival of *T. harzianum* over a wide range of storage temperatures (-20 to 30°C; Kumar *et al*., 2016), since lactose protects against desiccation, stabilizes proteins and lipids in cell membranes (Santos *et al*., 2011), or can be a prebiotic (Chávez and Ledeboer, 2007).

Talc, although showing no effects on mycelium growth of *C. rosea* or *T. harzianum*, decreased the conidium germination of *T. harzianum*, probably because this substance can form barriers surrounding conidia, reducing the water and nutrient, and germination of the conidia (IARC, 2010). However, the 80% conidium germination obtained in the presence of talc indicates that it could be used in formulations, since it favored survival and storage of CFUs for up to 150 d at temperatures 0 to 40°C (Bhat *et al*., 2009; Kumar *et al*., 2013). Similarly, Rai and Tewari (2016) used talc for moisturizing and liquid formulations containing *T. harzianum*. However, use of talc must be accompanied by other adjuvants such as CMC, since single component adjuvants cause short shelve lives (approx. 3 months) and dehydration (Jayaraj *et al*. 2006; Sallam *et al.* 2013).

The results obtained with up to 0.5% CMC, where conidium viability or mycelium growth of both BACs were not affected, are like those from other studies for bioformulations containing *T. harzianum* (Mukherjee *et al*., 2014) or *C. rosea* (Musiet, 2015; Wu *et al*., 2018), where CMC was used as a binder or adherent.

Chitosan did not adversely affect conidium viability or mycelium growth of both the biocontrol agents. However, chitosan has been reported to inhibit *T. harzianum* spp. conidium germination at 2.0% w/v (Palma-Guerrero *et al*. 2008). This could be related to chitosan prevention of *T. harzianum* conidium germination (Ruiz-de-la-Cruz *et al*. 2017) or to the presence of antibiotic activity (El-Mohamedy *et al*. 2014).

Concentrations of Aloe vera gel greater than 1% w/v decreased in conidium viability and mycelium growth of both BCAs, which agrees with the results of Sitara *et al.* (2011). This could be due to the diversity of bioactive molecules in *Aloe vera* gel that have antimicrobial and antioxidant properties (Davis 1997; Vega-Gálvez *et al*., 2011), that have been used for medicinal and therapeutic purposes (Ahlawat and Khatkar, 2011).

The greatest concentrations of drying supports or polymers that did not affect conidium germination or mycelium growth of both BCAs did not affect growth of *D. seriata* growth.

This study has shown that the maximum concentrations of the drying supports used in bioformulations containing *T. harzianum* and *C. rosea* were 10.0% w/v for inulin, 10.0% w/v for maltodextrin, 6.0% w/v for lactose, and 4% w/v for talc. Similarly, maximum concentrations for polymer additives were 0.5% w/v for carboxymethylcellulose, 0.5% w/v for Aloe vera gel, and 1.5% w/v for chitosan. These compounds did not affect *D. seriata* development in the absence of the two various BCAs assessed.

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# Phenotyping grapevine cultivars for resistance to Eutypa dieback

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**Summary.** Eutypa dieback of grapevine is a trunk disease that affects vineyard productivity. Wood symptoms of this disease develop consistently in greenhouse-grown plants, after inoculation of woody stems with the causal fungus *Eutypa lata*. Wood symptoms are a common measure of host cultivar resistance and *E. lata* isolate virulence. Leaf symptoms of the disease also develop in the greenhouse, although reports of low correlations between severity of wood and leaf symptoms (for some cultivars and isolates) indicate that a definitive procedure is required for evaluating cultivar resistance. Three 'phenotyping assays', replicated with two *E. lata* isolates (BX1-10 and M14), were assessed for quantifying resistance of a set of *Vitis vinifera* cultivars ('Black Corinth', 'Carignane', 'Husseine', 'Merlot', 'Muscat Hamburg', 'Palomino', 'Peloursin', 'Primitivo', and 'Thompson Seedless'). The methods were: Assay 1 (leaf and woody-stem symptoms measured 1 year post-inoculation on plants propagated from rooted, dormant cuttings); Assay 2 (green stem symptoms measured 4 months post-inoculation on plants propagated from rooted, green cuttings); and Assay 3 (leaf symptoms measured 6 weeks post-inoculation on plants propagated from rooted, dormant cuttings). High rates of mortality among some cultivars ('Merlot') in Assay 3 confounded results based on leaf symptoms. Results from Assays 1 and 2 were more consistent with each other, especially for the most resistant cultivars ['Merlot' and 'Primitivo' (aka 'Zinfandel')]', than they were for these cultivars in Assay 3. Compared to resistant cultivars, there was more variation in the most susceptible cultivar, including 'Black Corinth', 'Carignane', 'Husseine', and 'Thompson Seedless', regardless of the assay. Assay 1 with isolate BX1-10 was the most repeatable and provided data on wood and leaf symptoms for cultivar comparisons. Assay 2 was the most rapid, and gave results similar to those from Assay 1. Assay 2 also accommodated germplasm that can only be propagated from green cuttings.

**Keywords.** Grapevine Trunk Disease, disease resistance, *Vitis vinifera*.

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## INTRODUCTION

Eutypa dieback of grapevine (*Vitis vinifera*), caused by the fungus *Eutypa lata* (Pers: Fr.) Tul and C. Tul. (syn. *E. armeniacae* Hansf. and M.V. Carter), is a chronic disease, which negatively impacts crop yield and vineyard longevity (Munkvold *et al.*, 1994; Creaser and Wicks 2000; Siebert

2001; Kaplan *et al.,* 2016). Wind-dispersed ascospores of *E. lata* infect grapevines through pruning wounds (Carter 1991). After infection, internal wood cankers form near the infected wounds. *Eutypa lata* colonizes all host xylem tissue types, utilizing structural glucose, xylose of hemicellulose, and starch as nutrition sources (Rolshausen *et al*., 2008; Rudelle *et al*., 2005). Similar to several *Diatrypaceae* [e.g., *Eutypella parasitica*, the canker pathogen of maple (Worrall *et al.,* 1997)], the type of wood decay caused by *E. lata* has been classified as a 'soft rot' (English and Davis 1978), a term currently applied to all forms of decay caused by Ascomycota (Goodell *et al.,* 2008).

Leaf symptoms of Eutypa dieback typically appear 3 to 8 years after infection, on shoots growing from fruiting positions near wood infections (Carter 1991). Most apparent between budbreak and bloom, stunted shoots have dwarfed, deformed leaves ('cup-shaped' or flattened, with veins growing in parallel, rather than the typical fan-shape vein orientations), and leaves have brown, necrotic margins. Symptomatic shoots may die late in the growing season, and then the entire fruiting position may die during the dormant season, with no shoot growth the following growing season. This is the 'dieback' symptom. The few flower inflorescences that form on symptomatic shoots frequently become scorched and fail to develop into fruit clusters (Moller and Kasimatis 1978). Over years, symptomatic vines accumulate dead fruiting positions and produce less fruit, which is how Eutypa dieback impacts vineyard longevity. In addition to wood degradation, the metabolites produced by *E. lata* (acetylenic phenols, such as eutypinol, eulatichromene, and eutypine) (Mauro *et al.,* 1988; Tey-Rulh *et al.,* 1991; Molyneux *et al.,* 2002; Mahoney *et al.,* 2003; Lardner *et al.,* 2006) are also probably important in the infection processes, as some are phytotoxic (Mahoney *et al.,* 2003; Rudelle *et al.,* 2005b). Further characterization of compounds produced *in vitro* by *E. lata* identified polypeptide compounds, including hydrolytic enzymes (Schmidt *et al.,* 1999; Rolshausen *et al.,* 2008). Leaf symptoms probably result, in part, from translocation of *E. lata* metabolites and polypeptide compounds via host vascular systems, from mycelium (in infected wood) to shoots, as indicated by detection of some of these compounds in shoots of symptomatic grapevines (Octave *et al.,* 2006a; Octave *et al.,* 2009). Although *E. lata* causes chronic wood infections, and consistent leaf symptoms would be expected, there are annual variations in symptom presence/severity (Sosnowski *et al.,* 2007b).

*Vitis* species and grapevine cultivars vary in susceptibility to Eutypa dieback (Dubos 1987; Péros and Berger 1994; Sosnowski *et al.,* 2007a; Travadon *et al.,* 2013; Moisy *et al.,* 2017; Sosnowski *et al.,* 2022). The traditional method for evaluating cultivar resistance and/or isolate virulence is based on measurements of internal wood symptoms or extent of wood colonization by the pathogen (Sosnowski *et al.,* 2007a; Travadon *et al.,* 2013). This method is repeatable, but can take up to 2 years for obtaining results, and is only applicable to host germplasm that roots from dormant cuttings. More rapid methods (also for host germplasm propagated from dormant cuttings) quantify fungal biomass in inoculated stems by quantitative real-time PCR (qPCR) (Moisy *et al.,* 2017), or simply rate leaf symptom severity (Péros and Berger 1994). However, leaf symptoms are not always correlated with the extent of wood colonization, but may be well-correlated with lengths of wood symptoms (Sosnowski *et al.,* 2007a) and/or pathogen biomass in the wood (Moisy *et al.,* 2017).

The objectives of the present study were to: (i) compare three previously used methods to evaluate grapevine cultivar resistance, as greenhouse 'phenotyping assays' for plants rooted from dormant and green cuttings, and (ii) evaluate relationships between leaf and stem symptoms for individual cultivars.

## MATERIALS AND METHODS

### *Plant material*

Nine grapevine cultivars were used, that represent the genetic diversity of *Vitis vinifera*, which was previously characterized based on a total of 366 accessions of the USDA-ARS National Clonal Germplasm Repository (Aradhya *et al.,* 2003). The cultivars 'Carignane', 'Primitivo', and 'Muscat Hamburg' represented Central European grapes, 'Thompson seedless', 'Husseine', and 'Black Corinth' represented Mediterranean table grapes, and 'Palomino', 'Merlot', and 'Peloursin' represented Western European wine grapes (Table 1, Supplementary Figure 1). These three groups correspond to eco-geographical groups (Negroul 1946) and morphological groups (Troshin *et al.,* 1990), which were previously defined, respectively, as *Pontica*, *Orientalis*, and *Occidentalis*. 'Merlot' is considered to be resistant, based on leaf symptoms of Eutypa dieback (Dubos 1987; Péros and Berger 1994), so was included as a resistant control. The cultivars with black fruit were 'Black Corinth', 'Carignane', 'Merlot', 'Muscat Hamburg', 'Peloursin', and 'Primitivo' (also known as 'Zinfandel'), while those with white fruit were 'Husseine', 'Palomino', and 'Thompson Seedless'.

**Table 1.** The nine *Vitis vinifera* cultivars phenotyped for resistance to Eutypa dieback, representing three genetic groups (Aradhya *et al.,* 2003). Accessions of each cultivar originated from the National Clonal Germplasm Repository, United States Department of Agriculture, Agricultural Research Service [Germplasm Resources Information Network (http://www.ars-grin.gov/)]. Plants for Assays 1 and 3 (see Materials and Methods) were propagated by the Baumgartner Laboratory. Plants for Assay 2 were propagated by Foundation Plant Services, University of California, Davis.

| Genetic group | Cultivar | Clone | Accession number | Country of origin |
|---|---|---|---|---|
| Central European grapes | Carignane | 3 | DVIT 1064 | Spain |
| | Muscat Hamburg | 3 | DVIT 1059 | Germany |
| | Primitivo[a] | 3 | DVIT 1342 | Croatia |
| Mediterranean table grapes | Black Corinth | 2 | DVIT 0354 | Greece |
| | Husseine | 2 | PI 171099 | Afghanistan |
| | Thompson Seedless | 02A | DVIT 0535 | Turkey |
| Western European wine grapes | Merlot | 15 | DVIT 0826 | France |
| | Palomino | 01A | DVIT 0882 | Spain |
| | Peloursin | 1 | DVIT 0710 | France |

[a] Also known as 'Zinfandel'.

*Phenotyping Assay 1 – Leaf symptoms (severity, incidence), shoot lengths, and woody-stem symptoms (lesion lengths) measured 1 year post-inoculation on plants propagated from rooted, dormant cuttings*

Two replicate experiments began 2 weeks apart. In each experiment, plants were arranged in a randomized complete block design (RCBD) with two blocks, located in two separate greenhouses (Armstrong Plant Pathology Field Station, Davis, California, United States of America). Greenhouse temperatures were 25 ± 1°C (day), 18 ± 3°C (night), with natural photoperiod, unless noted otherwise. Plants were each watered daily for 15 min using a drip-irrigation system (0.5 L $h^{-1}$). Each block consisted of ten replicate plants per cultivar per each of three inoculation treatments (ten plants per cultivar × nine cultivars × three inoculation treatments × two blocks × two experiments = 1,080 total plants). The three inoculation treatments were as follows:

1. *Eutypa lata* isolate BX1-10. This is a virulent isolate, which has previously been used in studies in France (Péros and Berger 1994, 1999; Camps *et al.,* 2010; Moisy *et al.,* 2017; Cardot *et al.,* 2019). It originates from perithecia on dead wood of 'Cabernet-Sauvignon' from Bordeaux, France (Péros and Berger 1994). Inoculum consisted of mycelium fragments from liquid Potato Dextrose Broth (PDB; Difco) cultures (Travadon *et al.,* 2013).
2. *Eutypa lata* isolate M14. This is an isolate shown to be virulent in greenhouse studies (Travadon *et al.,* 2013). It is a mass-hyphal isolate from symptomatic wood of 'Merlot' from Napa, California, United States of America (Travadon *et al.,* 2012). Inoculum consisted of mycelium fragments from liquid PDB cultures (Travadon *et al.,* 2013).
3. Non-inoculated control. These plants were 'mock-inoculated' with sterile PDB).

Plants were each propagated from dormant cuttings, the woody stem of which was inoculated after callusing and at the time of planting. One-year-old dormant canes were cut into dormant cuttings of uniform length (≈ 30 cm) containing three nodes. The cuttings were surface-sterilized in 1% sodium hypochlorite (Clorox®) solution for 15 min and then rinsed in water overnight. The cuttings were then callused for 3 weeks in a mixture of perlite and vermiculite (1:1, v/v), at 30°C and 85% relative humidity. Once root and shoot initials emerged from the callus tissues, a power drill was used to wound each woody stem (2 mm width × 3 mm depth) at approx. 3 cm below the top node. Each cutting was then inoculated by pipetting 20 µL of liquid inoculum ($1 \times 10^6$ mycelium fragments $mL^{-1}$) into the wound, which was then sealed with Vaseline® (Unilever) and Parafilm® (American National Can). Non-inoculated experimental controls were each 'mock-inoculated' with 20 µL of PDB. After inoculation, the cuttings were submerged in melted paraffin wax (Gulf Wax®, Royal Oak Enterprises) within 4 cm of the roots and were then potted in sterile potting mix ['UC mix' (Baker 1957)], amended with slow-release fertilizer (Osmocote® Pro 24-4-9, Scotts).

In a previously published assay that compared leaf symptoms and wood symptoms among *V. vinifera* cultivars (Sosnowski *et al.,* 2007a), inoculated plants were kept in an outdoor shadehouse (under more natural climate conditions than in a greenhouse) for 2 years. Leaf symptoms were visible on the new shoots that grew in spring, after a normal winter period of dormancy and winter pruning (i.e., at the start of the second 'growing season' for the potted grapevines). This assay was modified for the greenhouse and for 1 year, as follows:

1. Inoculated plants were grown for 7 months (from May to November, with natural light and at summer greenhouse temperatures of 24 to 27°C during the day and 16 to 22°C at night). Plants were watered twice per week for 15 min using a drip-irrigation system (0.5 L $h^{-1}$).
2. Shoots were pruned to two buds, and plants were forced into a winter period of dormancy for 3 months (from December to February, with natural light and at winter greenhouse temperatures of 10 to 13°C during the day and 3 to 6°C at night).

3. Plants were brought out of dormancy by returning to summer greenhouse temperatures (as 1., above). Leaf symptoms were rated on the new shoots that grew during the final 2 months of the assay (from February to April).

Following budbreak in February, the plants were monitored for the presence of leaf symptoms, and final assessments were made in April, at 1 year post-inoculation (after approx. 6 to 8 weeks of shoot growth). Severity of leaf symptoms was rated visually on a scale of 0 to 5, using an ordinal scale adapted from that of Péros and Berger (1994) (Figure 1). The lengths of the green shoots were measured at 1 year post-inoculation. Also at this time, internal lesions were measured in the woody stems. Plants were removed from the soil, roots and shoots were excised, and bark was scraped from their woody stems. The stems were surface sterilized in 1% sodium hypochlorite solution for 2 min, and then rinsed with tap water. The lengths of the woody stems were measured, and each stem was then cut longitudinally and the length of internal wood discolouration extending above and below the inoculation site (i.e., lesion length) was measured with an electronic caliper.

To evaluate the extent of wood colonization by mycelium of each isolate, attempts were made to recover the pathogen from a subset of plants in each treatment. Four small pieces of wood (each approx. 5 × 2 mm) were cut from the woody stem with a flame-sterilized scalpel at 0, 2, 4, and 6 cm below each inoculation site, and at the lower margin of the lesion if present at > 60 mm below the inoculation site. Wood pieces were then surface-sterilized for 1 min in 0.6% sodium hypochlorite solution (pH 7.2), rinsed twice (1 min each) in sterile distilled water, and then incubated on PDA amended with 0.01% tetracycline hydrochloride for 2 weeks.

*Statistical analysis*. All statistical analyses were conducted using SAS v. 9.4 (SAS Institute). ANOVAs were carried out for the main and interactive effects of each experiment (1 or 2), block (1 or 2), inoculation treatment (control, BX1-10, or M14), and cultivar ('Black Corinth', 'Carignane', 'Husseine', 'Merlot', 'Muscat Hamburg', 'Palomino', 'Peloursin', 'Primitivo', or 'Thompson Seedless'), on lesion length, shoot length, and incidence of leaf symptoms. ANOVAs were carried out using the MIXED procedure, with all effects considered as fixed, except for the main and interaction effects of experiment and block (random effects). Normality was assessed using normal probability plots and homogeneity of variances was evaluated using Levene's test. Transformations of lesion lengths ($\log_{10}$) and shoot lengths (square root) were used to meet parametric assumptions. For statistical significance ($F$ values with $P < 0.05$), means were compared using the LSMEANS procedure. $P$-values and 95% confidence limits for mean differences were adjusted for multiple comparisons using the Tukey-Kramer method ($\alpha = 0.05$).

Because the severity of leaf symptoms was rated on an ordinal scale, non-parametric analysis with PROC MIXED (Shah and Madden 2004) was used to determine the main and interaction effects on leaf-symptom ratings of experiment, block, inoculation treatment, and cultivar. Rather than comparing the mean for each cultivar (as is done for continuous data), the measure used for comparison of leaf symptom ratings in this non-parametric analysis was the relative treatment effect and its corresponding 95% confidence interval. Relative treatment effects were calculated from the PROC MIXED lsmeans, using the LD_CI macro, which uses rank

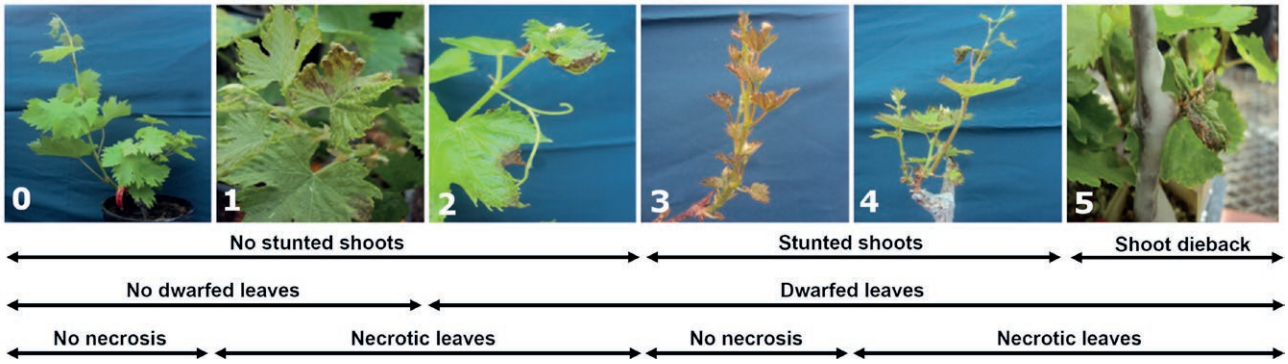


**Figure 1.** Leaf symptom severity scale used to assess grapevine shoots that were 6 to 8 weeks old, in Assays 1 and 3. 0 = no symptoms ('Muscat Hamburg'); 1 = normal-sized leaves and shoots, but some leaves have necrotic (brown) margins ('Muscat Hamburg'); 2 = normal shoot length, but some leaves dwarfed and/or with necrotic margins ('Thompson Seedless'); 3 = stunted shoots with dwarfed leaves, but no leaves with necrotic margins ('Black Corinth'); 4 = stunted shoots with dwarfed leaves, and some leaves dwarfed with necrotic margins ('Primitivo'); and 5 = no shoot growth or stunted shoot that grew and then died ('Merlot').

transformations of the medians as a basis for calculating the relative treatment effects (Brunner *et al.*, 2002). For statistically significant effects (ANOVA $F$ values with $P < 0.05$), relative treatment effects without overlapping confidence intervals were considered significantly different ($\alpha = 0.05$). Correlations among the relative treatment effects of the leaf symptom ratings, the incidence of leaf symptoms, and lesion lengths were determined for each inoculation treatment × cultivar combination, using the CORR procedure in SAS based on the Spearman rank-order correlation (i.e., a non-parametric measure of association, based on the ranks of the data values).

*Phenotyping Assay 2 – Shoot lengths and green-stem symptoms (lesion lengths, incidence of external cankers) measured 4 months post-inoculation on plants propagated from rooted, green cuttings*

The experimental design and the three inoculation treatments were the same as used in Assay 1. In contrast to Assay 1, inoculum for Assay 2 was grown on solid medium (PDA) and 3 mm diam. agar plugs were used for inoculations. Plants were propagated from green cuttings, the green stems of which were inoculated after the cuttings were rooted. After fruit set in June, green shoots were cut from grapevines in the field, with each shoot of uniform length, containing two nodes (≈ 10 cm long × 0.8 cm diam.). The basal bud was removed, and one leaf at the top node was retained (trimmed to a leaf area ≈ 2 $cm^2$). Green cuttings were then rooted for 2 to 3 weeks in a mixture of perlite and vermiculite (1:1, v/v), with natural light, at greenhouse temperatures of 24 to 27°C during the day and 16 to 22°C at night, under a mist system with mist sprayed from the top of the greenhouse for 5 sec every 2 min (during daylight hours). After roots formed (during the 2 to 3 weeks in the mist system), plants were removed from the mist system, transplanted into a mixture of peat, sand, and perlite (1:1:1, v/v/v), and grown for 1 month in the greenhouse. Then, approx. 2 months after the green cuttings were made (August), a 3 mm diam. cork borer was used to wound each plant's green stem at approx. 3 cm below the uppermost node. To inoculate the green stems, a 3 mm agar plug from the margin of a 5 d PDA culture was inserted into each wound, and sealed with Vaseline® and Parafilm®. Non-inoculated controls were 'mock-inoculated' with sterile PDA.

At 4 months post-inoculation (December), shoot lengths and lesion lengths were measured, and the presence/absence of cankers was assessed. Each green shoot that grew over the 6 months of the experiment (2 months pre-inoculation to 4 months post-inoculation) from the top of the green stem was measured. Presence of an external canker was noted, visible as an area of the stem surface that was desiccated, dry, and brown. Plants were removed from the peat/sand/Perlite medium, and their roots and shoots were excised. Stems were surface sterilized in 1% sodium hypochlorite solution for 2 min, and rinsed with tap water. The length of each stem was measured, and the stem was cut longitudinally and the length of internal discolouration extending above and below the point of inoculation was measured with an electronic caliper. In order to confirm infection, attempts were made to recover the pathogen from each inoculation site of all the inoculated plants, using the methods described above.

*Statistical analysis*. All statistical analyses were conducted in SAS v. 9.4. ANOVAs were carried out for the main and interaction effects of experiment (1 or 2), block (1 or 2), inoculation treatment (control, BX1-10, or M14), and cultivar ('Black Corinth', 'Carignane', 'Husseine', 'Merlot', 'Muscat Hamburg', 'Palomino', 'Peloursin', 'Primitivo', or 'Thompson Seedless'), for lesion length, shoot length, and incidence of cankers. ANOVAs were carried out using the MIXED procedure, with all effects considered as fixed, except for the main and interaction effects of experiment and block (random effects). Normality of data was evaluated using normal probability plots, and homogeneity of variances was assessed using Levene's test. Transformation of lesion lengths (reciprocal square root) was used to meet parametric assumptions. For significant effects ($F$ values with $P < 0.05$), means were compared using the LSMEANS procedure. $P$-values and 95% confidence limits for mean differences were adjusted for multiple comparisons using the Tukey-Kramer method ($\alpha = 0.05$). Correlations among shoot lengths, incidence of cankers, and lesion lengths were determined for each inoculation treatment × cultivar combination, using the CORR procedure in SAS, based on the Spearman rank-order correlation (i.e., a non-parametric measure of association, based on the ranks of the data values).

*Phenotyping Assay 3 – Leaf symptoms (severity, incidence) and shoot lengths measured 6 weeks post-inoculation on plants propagated from rooted, dormant cuttings*

The experimental design and the three inoculation treatments were the same as those used in Assay 2 (above). Inoculum was grown on solid medium (PDA) and 3 mm diam. agar plugs were used for inoculations. Plants were propagated from dormant cuttings, but, in contrast to Assay 1, the cuttings were inoculated and immediately planted without first callusing or rooting. The plants for Assays 1 and 3 were propagated from the same vines. However, these assays were not carried out at the same time because there was not enough cane

wood on the vines for all the cuttings required of both assays and available greenhouse space was not sufficient for both assays together.

Cuttings (≈ 15 cm long) from 1-year-old dormant canes were harvested from dormant field-grown grapevines, and the basal buds were removed. The cuttings were then surface-sterilized in 1% sodium hypochlorite for 15 min and rinsed in water overnight. The cuttings were then stored in plastic bags at 4°C for 2 months. A 3 mm diam. cork borer was then used to wound each cutting at approx. 3 cm below the top node. A 3 mm diam. agar plug from the margin of a 5 d PDA culture of either *E. lata* isolate was then inserted into the wound, and sealed with Vaseline® and Parafilm®. Non-inoculated controls were 'mock-inoculated' with sterile PDA. After inoculation, cuttings were submerged in melted paraffin wax within 4 cm of the roots and the cuttings were potted in a mix of perlite and vermiculite (1:1, v/v) in plant bands (5 × 5 × 20 cm; Monarch Manufacturing Inc.), which were held in plastic trays (35 × 35 × 15 cm; 49 plants per tray) placed on top of rooting mats to promote root growth at 24°C. At 4 to 6 weeks post-inoculation, following budbreak, severity of leaf symptoms on the new shoots of each cutting was rated visually using the 0 to 5 scale (Figure 1). The length of the green shoot emerging from the node above the inoculation site was also measured.

*Statistical analyses*. All statistical analyses were conducted in SAS v. 9.4. ANOVAs were carried out on data of shoot length and incidence of leaf symptoms, for the main and interactive effects of experiment (1 or 2), block (1 or 2), inoculation treatment (control, BX1-10, or M14), and cultivar ('Black Corinth', 'Carignane', 'Husseine', 'Merlot', 'Muscat Hamburg', 'Palomino', 'Peloursin', 'Primitivo', or 'Thompson Seedless'). ANOVAs were carried out using the MIXED procedure, with all effects considered as fixed, except for the main and interaction effects of experiment and block (random effects). Normality of data was evaluated using normal probability plots, and homogeneity of variances were was evaluated using Levene's test. Transformation of shoot lengths (square root) was used to meet parametric assumptions. For significant effects ($F$ values with $P < 0.05$), means were compared using the LSMEANS procedure. $P$-values and 95% confidence limits for mean differences were adjusted for multiple comparisons using the Tukey-Kramer method ($\alpha = 0.05$). The same methods described (above) for Assay 1 were used to calculate and analyze leaf symptom severity. Correlations among the relative treatment effects of the leaf symptom severity, incidence of leaf symptoms, and shoot lengths were determined for each inoculation treatment × cultivar combination using the CORR procedure in SAS, based on the Spearman rank-order correlation (i.e., a non-parametric measure of association, based on the ranks of the data values).

## RESULTS

*Phenotyping Assay 1 – Leaf symptoms (severity, incidence), shoot lengths, and woody-stem symptoms (lesion lengths) measured 1 year post-inoculation on plants propagated from rooted, dormant cuttings*

Mean lesion lengths for inoculated plants varied among cultivars ($P < 0.0001$). The effect of isolate on either measure of lesion length was not significant ($P = 0.5$), nor was the interaction of cultivar × inoculation treatment ($P > 0.3$). For plants inoculated with either isolate, 'Primitivo' had the smallest lesions compared to 'Thompson Seedless', which had the largest lesions. All the other cultivars had intermediate mean lesion lengths (Table 2). In addition to 'Thompson Seedless' having the longest lesions, the pathogen colonized the stems of this cultivar far beyond the lesion margins, compared to all the other cultivars. Maximum recovery distances below the inoculation sites of 'Thompson Seedless' were 219 mm for plants inoculated with BX1-10, and 215 mm from M14 inoculations.

Comparing lesion lengths of plants inoculated with each isolate, relative differences among cultivars were consistent for those with the smallest lesions ('Primitivo', 'Merlot') and for those with the largest lesions ('Thompson Seedless', 'Husseine', 'Carignane', 'Black Corinth'; Table 2). Positive recovery of each isolate was similar for the two isolates, with recovery rates ranging from 43 to 82% for BX1-10 and 25 to 73% for M14 (Table 2). Mean lesion lengths of the non-inoculated controls were < 10 mm.

Incidence of leaf symptoms (% symptomatic plants) varied among the cultivars ($P = 0.0003$) and between the two isolates ($P = 0.009$), although the interaction effect of cultivar × inoculation treatment was not significant ($P = 0.2$). Based on the results of a non-parametric analysis of the relative treatment effects (RTEs), there was a significant interaction effect of cultivar × inoculation treatment for leaf symptom severity ratings ($P = 0.01$). 'Black Corinth' and 'Husseine' had consistently high RTEs and a high incidence of leaf symptoms, regardless of pathogen isolate (Table 3). Non-inoculated control plants showed no leaf symptoms (median = 0), which amounted to an RTE of 0.39. However, for cultivar × inoculation treatment combinations with the greatest incidence of leaf symptoms (cvs 'Black Corinth' and 'Husseine'), only 45 to 54% of plants had leaf symptoms. As such, the majority of cultivar × inoculation treatment combina-

**Table 2.** Assay 1. Mean internal woody stem lesion lengths of rooted, dormant cuttings, at 1 year post-inoculation, for plants inoculated with either *Eutypa lata* isolate BX1-10 or isolate M14. Each value is the mean of 17 to 37 observations, summed across two replicate experiments, with two blocks per experiment. Means for each inoculation treatment accompanied by different letters are significantly different (Tukey's test, $P > 0.01$, $\alpha = 0.05$).

| Inoculation treatment | Cultivar | Total plants | Mean lesion length (mm) | Recovery attempts (No. plants) | Farthest recovery distance below inoculation site[a] (mm) | | | | | | | | | No. positive plants (% recovery attempts) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 0 | 10 | 20 | 30 | 40 | 50 | 60 | >60 | Max | |
| BX1-10 | Primitivo | 31 | 15.0a | 11 | 1 | 1 | 2 | 0 | 0 | 0 | 2 | 0 | 60 | 6 (55%) |
| | Merlot | 31 | 20.1ab | 10 | 0 | 0 | 2 | 0 | 2 | 0 | 1 | 0 | 60 | 5 (50%) |
| | Muscat Hamburg | 32 | 22.7ab | 11 | 1 | 0 | 3 | 0 | 3 | 0 | 1 | 1 | 117 | 9 (81%) |
| | Peloursin | 29 | 23.8ab | 12 | 0 | 0 | 1 | 1 | 3 | 0 | 1 | 2 | 76 | 8 (67%) |
| | Palomino | 26 | 31.0ab | 15 | 0 | 0 | 2 | 2 | 3 | 0 | 1 | 0 | 60 | 8 (53%) |
| | Black Corinth | 32 | 31.8ab | 17 | 0 | 0 | 1 | 2 | 1 | 0 | 2 | 4 | 121 | 10 (59%) |
| | Carignane | 22 | 37.3ab | 13 | 0 | 0 | 0 | 0 | 5 | 0 | 1 | 1 | 80 | 7 (54%) |
| | Husseine | 26 | 38.3ab | 17 | 2 | 0 | 4 | 3 | 1 | 2 | 1 | 1 | 110 | 14 (82%) |
| | Thompson Seedless | 28 | 67.9b | 23 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 5 | 219 | 10 (43%) |
| M14 | Primitivo | 31 | 17.8a | 12 | 2 | 1 | 1 | 0 | 2 | 0 | 1 | 0 | 60 | 7 (58%) |
| | Merlot | 28 | 18.4ab | 10 | 2 | 0 | 2 | 1 | 1 | 0 | 0 | 0 | 40 | 6 (60%) |
| | Peloursin | 38 | 20.6ab | 10 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 60 | 4 (40%) |
| | Palomino | 23 | 23.5ab | 11 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 2 | 74 | 5 (45%) |
| | Muscat Hamburg | 29 | 25.3ab | 9 | 0 | 0 | 1 | 1 | 3 | 0 | 1 | 0 | 60 | 6 (67%) |
| | Black Corinth | 34 | 25.9ab | 15 | 0 | 0 | 5 | 1 | 3 | 0 | 2 | 0 | 60 | 11 (73%) |
| | Carignane | 29 | 31.1ab | 19 | 0 | 0 | 1 | 1 | 3 | 0 | 2 | 0 | 60 | 7 (37%) |
| | Husseine | 28 | 39.7ab | 17 | 1 | 0 | 3 | 0 | 0 | 2 | 1 | 1 | 76 | 8 (47%) |
| | Thompson Seedless | 22 | 84.9b | 20 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 3 | 215 | 5 (25%) |

[a] Means for each distance are numbers of plants from which *E. lata* was recovered from the inoculation site (0 cm) or below.

tions gave median leaf symptom values of 0; hence the utility of RTE for statistical comparisons. Plants inoculated with isolate BX1-10 had a greater incidence than those inoculated with M14, for seven of nine evaluated cultivars (Table 3).

The cultivars 'Black Corinth' and 'Husseine' had the greatest incidence and RTEs for leaf symptoms, regardless of pathogen isolate, but this was the only consistent trend in relative resistance among cultivars between the isolates (Table 3). In contrast, cultivars with the least incidences and RTEs of leaf symptoms varied between the two isolates ('Merlot' for BX1-10 and 'Primitivo' for M14). Strength of the association between mean lesion lengths and RTEs of leaf symptoms was significant for plants inoculated with BX1-10 (Spearman correlation coefficient of $r = 0.83$, $P = 0.006$), but not for those inoculated with M14 (Spearman correlation coefficient of $r = 0.55$, $P = 0.1$; Figure 2). In spite of the consistently large lesions on plants inoculated with either isolate, 'Thompson Seedless' had among the greatest RTEs (0.65) when inoculated with isolate BX1-10, but among the least (0.49) when inoculated with isolate M14 (Table 2).

Mean shoot lengths were significantly different ($P > 0.0001$) among the cultivars (Table 3). This was the only significant effect of the treatments on shoot length. There was a trend, though not statistically significant, for shorter shoot lengths of inoculated plants compared to the non-inoculated controls for all cultivars except 'Muscat Hamburg' and 'Palomino'.

*Phenotyping Assay 2 – Shoot lengths and green-stem symptoms (lesion lengths, incidence of external cankers) measured 4 months post-inoculation on plants propagated from rooted, green cuttings*

Mean lesion lengths for inoculated plants varied among the cultivars and between the two isolates (interaction effect of cultivar × inoculation treatment, $P = 0.04$). 'Merlot', 'Muscat Hamburg', and 'Primitivo' had the smallest lesions, from both isolates (Table 4). Consistently intermediate in mean lesion lengths, regardless of isolate, were 'Black Corinth' and 'Palomino'. Depending on the isolate, 'Husseine' or 'Carignane' had larger lesions than 'Merlot'. 'Peloursin' and 'Thompson Seed-

**Table 3.** Assay 1. Mean incidence of leaf symptoms (% symptomatic plants of total inoculated), leaf symptom severity (median, relative treatment effects), and mean lengths of shoots from rooted, dormant cuttings, at 1 year post-inoculation, for grapevine plants inoculated with either *Eutypa lata* isolate BX1-10 or isolate M14. Each value is the mean of 17 to 37 observations, summed across two replicate experiments, with two blocks per experiment. Means for each inoculation treatment accompanied by different letters are significantly different [based on Tukey's test ($P > 0.01$, $\alpha = 0.05$) for mean incidences of leaf symptoms and mean shoot lengths; based on no overlap of 95% confidence intervals for relative treatment effects].

| Inoculation treatment | Cultivar | Mean incidence of leaf symptoms (% symptomatic plants) | Leaf symptom severity | | Mean shoot length (mm)[b] |
|---|---|---|---|---|---|
| | | | Median[a] | Relative treatment effect (RTE) | |
| BX1-10 | Merlot | 5.6 a | 0 | 0.43 a | 353.0 bc |
| | Muscat Hamburg | 23.7 ab | 0 | 0.49 ab | 278.7 ab |
| | Primitivo | 28.4 ab | 0 | 0.54 ab | 404.9 bc |
| | Palomino | 41.1 b | 0 | 0.61 b | 367.8abc |
| | Carignane | 46.5 b | 0 | 0.63 b | 216.1 a |
| | Peloursin | 47.3 b | 0 | 0.62 b | 494.5 c |
| | Thompson Seedless | 52.5 b | 1.5 | 0.65 b | 283.0 abc |
| | Husseine | 53.6 b | 1 | 0.65 b | 303.9 abc |
| | Black Corinth | 54.2 b | 1 | 0.66 b | 246.4 ab |
| M14 | Primitivo | 11.9 a | 0 | 0.45 a | 447.0 cd |
| | Thompson Seedless | 14.4 ab | 0 | 0.49 ab | 291.1 abc |
| | Palomino | 19.0 ab | 0 | 0.50 ab | 423.2 bcd |
| | Merlot | 19.4 ab | 0 | 0.47 ab | 294.8 abc |
| | Muscat Hamburg | 21.9 ab | 0 | 0.51 ab | 287.7 abc |
| | Peloursin | 30.0 ab | 0 | 0.53 ab | 559.2 d |
| | Carignane | 36.5 ab | 0 | 0.56 ab | 239.7 ab |
| | Husseine | 45.0 ab | 0.5 | 0.64 b | 248.1 ab |
| | Black Corinth | 53.6 b | 0.5 | 0.65 b | 193.5 a |

[a] Leaf symptom severity was assessed visually using a 0 to 5 scale, where 0 = no symptoms and 5 = no shoot growth or stunted dead shoot (See Figure 1).

[b] Mean lengths of the new green shoots that grew from the top of rooted, dormant cuttings were measured 1 year post-inoculation, although each shoot grew for 2 months.

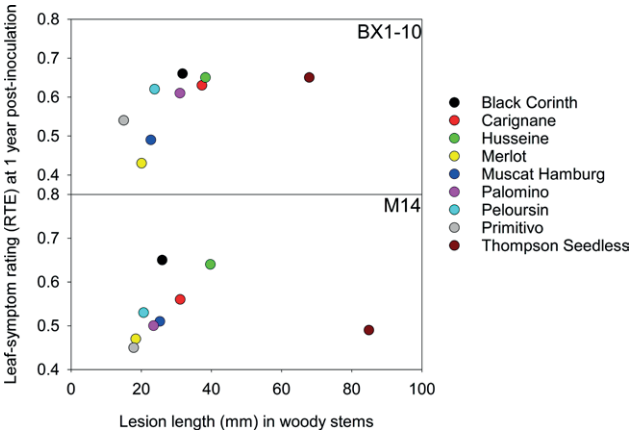


**Figure 2.** Assay 1. Relationships between mean lengths of internal lesions in woody stems of nine grapevine cultivars and mean leaf symptom severity ratings [relative treatment effect (RTE)], at 1 year post-inoculation, from plants inoculated with either *Eutypa lata* isolate BX1-10 or isolate M14.

less' had different mean lesion lengths between the two isolates, with both cultivars having lesion lengths either intermediate or large (relative to 'Merlot'), depending on the isolate. For plants inoculated with isolate BX1-10, all cultivars had larger lesions than those inoculated with M14, except for cvs 'Peloursin' and 'Husseine'. Mean lesion lengths of non-inoculated controls were < 10 mm. Cultivars with the smallest lesions in Assays 1 and 2 were 'Primitivo', 'Merlot', and 'Muscat Hamburg' (Figure 3). Mean lesion lengths were similar in both assays, in spite of the tripled incubation period for Assay 1, for all the cultivars, except 'Thompson Seedless', which developed much smaller lesions in Assay 2 (mean lesion lengths of 22.1 to 31.9 mm, depending on the isolate; Table 4), than in Assay 1 (mean lesion lengths of 67.9 to 84.9 mm, depending on the isolate; Table 2).

No leaf symptoms developed in this assay. However, external cankers were visible on the surfaces of the green stems, with mean incidence of cankers of ≥ 50%

**Table 4.** Assay 2. Mean internal green stem lesion lengths, mean incidence of external cankers on the surfaces of green stems, and mean lengths of shoots of rooted, green cuttings at 4 months post-inoculation, for grapevine plants inoculated with either *Eutypa lata* isolate BX1-10 or isolate M14. Each value is the mean of 18 to 31 observations, summed across two replicate experiments, with two blocks per experiment. Means for each inoculation treatment, in each column, accompanied by different letters are significantly different (Tukey's test, $P > 0.01$, $\alpha = 0.05$).

| Inoculation treatment | Cultivar | Mean lesion length (mm) | Mean incidence of cankers (% plants) | Mean shoot length (mm)[a] |
|---|---|---|---|---|
| BX1-10 | Merlot | 11.9 a | 0 a | 234.9 a |
| | Muscat Hamburg | 16.4 ab | 21 ab | 265.2 ab |
| | Primitivo | 22.1 bc | 43 bc | 344.4 bc |
| | Peloursin | 22.4 bc | 37 bc | 374.4 bc |
| | Black Corinth | 26.7 bc | 69 c | 318.3 b |
| | Palomino | 28.5 bc | 70 c | 291.9 ab |
| | Husseine | 29.7 c | 53 bc | 330.2 bc |
| | Thompson Seedless | 31.9 c | 59 bc | 407.3 c |
| | Carignane | 36.4 c | 76 c | 291.5 ab |
| M14 | Merlot | 11.0 a | 0 a | 273.1 ab |
| | Muscat Hamburg | 15.3 ab | 17 ab | 288.6 ab |
| | Primitivo | 19.4 bc | 27 bc | 343.1 ab |
| | Thompson Seedless | 22.1 bc | 43 bc | 481.0 c |
| | Black Corinth | 22.6 bc | 41 bc | 311.0 ab |
| | Palomino | 23.9 bc | 43 bc | 266.6 a |
| | Carignane | 24.6 bc | 50 bc | 357.7 b |
| | Peloursin | 29.4 c | 61 c | 316.1 ab |
| | Husseine | 35.1 c | 60 c | 283.9 ab |

[a] Mean lengths of the new green shoots that grew from the top of rooted, green cuttings were measured after 6 months (2 months pre-inoculation plus 4 months post-inoculation).

for five of the nine cultivars inoculated with isolate BX1-10 and three of the cultivars inoculated with M14 (Table 4). There was an interaction cultivar × inoculation effect ($P = 0.04$) on incidence of cankers (% plants). Plants inoculated with isolate BX1-10 had greater incidence of cankers than those inoculated with M14, except for cvs 'Peloursin' and 'Husseine' (Table 4). Regardless of isolate, 'Merlot' had no cankers on inoculated plants, and 'Muscat Hamburg' had the lowest incidence of cankers, but this was the only consistent trend in relative resistance among cultivars between isolates. For plants inoculated with isolate BX1-10, 'Carignane', 'Palomino', and 'Black Corinth' had greater incidence of cankers than 'Merlot'. For plants inoculated with M14, 'Peloursin', 'Husseine'', and 'Carignane' had greater incidence of cankers than 'Merlot'. The relationships between lesion lengths (mm) and incidence of cankers was significant and positive for

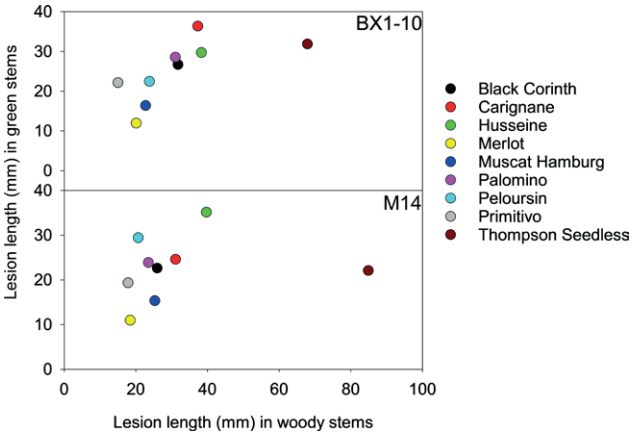


**Figure 3.** Relationships for nine grapevine cultivars, between mean lengths of internal lesions in the woody stems at 1 year post-inoculation (Assay 1), versus mean lengths of internal lesions in their green stems, at 6 weeks post-inoculation (Assay 2). Plants in both assays were inoculated with either *Eutypa lata* isolate BX1-10 or isolate M14.

plants inoculated with BX1-10 (Spearman correlation coefficient of $r = 0.85$, $P = 0.004$) or M14 (Spearman correlation coefficient of $r = 0.93$, $P = 0.0002$), so there was a trend for cultivars with long lesions to also have a high incidence of cankers.

There was a significant interaction cultivar × inoculation treatment effect ($P = 0.007$) on shoot lengths. In comparison to shoot lengths of the non-inoculated controls, the only consistency between isolates was for 'Muscat Hamburg', which had shorter shoots (though not statistically significant) than those of the non-inoculated controls. There was no significant association between shoot lengths and lesion lengths (for both pathogen isolates; Spearman correlation coefficients of $r > 0.1$, $P > 0.3$) or incidence of cankers (for both isolates, Spearman correlation coefficients of $r > 0.2$, $P > 0.4$).

*Phenotyping Assay 3 – Leaf symptoms (severity, incidence) and shoot lengths measured 6 weeks post-inoculation on plants propagated from rooted, dormant cuttings*

Many of the plants in this Assay did not develop shoots or roots (and were dead) before the 6-week post-inoculation period was reached, so disease data could not be determined for these plants. This contributed to low sample sizes of 13 to 32 across replicate experiments. There were high levels of mortality among all three inoculation treatments for 'Merlot' (49%), 'Black Corinth' (39%), and 'Muscat Hamburg' (17%).

Incidence of leaf symptoms (% symptomatic plants) varied among cultivars ($P = 0.0003$) and between isolates

**Table 5.** Assay 3. Mean incidence of leaf symptoms (% symptomatic plants of total inoculated), leaf symptom severity (median, relative treatment effects), and mean lengths of shoots from dormant cuttings, at 6 weeks post-inoculation, for grapevine plants inoculated with either *Eutypa lata* isolate BX1-10 or isolate M14. Each value is the mean of 13 to 32 observations, summed across two replicate experiments, with two blocks per experiment. Means for each inoculation treatment, in each column, accompanied by different letters are significantly different [based on Tukey's test ($P > 0.01$, $\alpha = 0.05$) for mean incidences of leaf symptoms and mean shoot lengths; based on no overlap of 95% confidence intervals for relative treatment effects].

| Inoculation treatment | Cultivar | Mean incidence of leaf symptoms (% symptomatic plants)[a] | Leaf symptom severity | | Mean shoot length (mm)[b] |
|---|---|---|---|---|---|
| | | | Median[a] | Relative treatment effect (RTE) | |
| BX1-10 | Primitivo | 9.4 a | 0 | 0.35 a | 74.1 bc |
| | Carignane | 26.8 ab | 0 | 0.43 ab | 53.7 abc |
| | Husseine | 28.6 ab | 0 | 0.44 ab | 86.9 c |
| | Thompson Seedless | 35.0 ab | 0 | 0.46 ab | 89.0 c |
| | Palomino | 37.5 ab | 0 | 0.49 bc | 49.0 ab |
| | Peloursin | 55.2 bc | 2.5 | 0.62 bc | 70.9 bc |
| | Muscat Hamburg | 67.9 bc | 2 | 0.64 c | 54.9 abc |
| | Black Corinth | 73.3 bc | 2.5 | 0.68 c | 29.9 a |
| | Merlot | 79.2 c | 3 | 0.66 c | 47.2 ab |
| M14 | Primitivo | 18.8 a | 0 | 0.40 a | 64.6 bc |
| | Thompson Seedless | 24.6 a | 0 | 0.43 a | 62.9 bc |
| | Palomino | 25.9 a | 0 | 0.43 a | 48.4 ab |
| | Carignane | 32.7 a | 0 | 0.48 ab | 53.7 ab |
| | Husseine | 33.5 a | 0 | 0.45 a | 91.6 c |
| | Peloursin | 40.6 ab | 0 | 0.52 ab | 77.6 bc |
| | Merlot | 44.9 ab | 0 | 0.53 ab | 62.0 abc |
| | Muscat Hamburg | 47.2 ab | 0 | 0.56 ab | 56.7 abc |
| | Black Corinth | 71.3 b | 4 | 0.71 bc | 26.3 a |

[a] Leaf symptom severity was assessed visually using a 0 to 5 scale, where 0 = no symptoms and 5 = no shoot growth or stunted dead shoot (See Figure 1).
[b] Mean lengths of the new green shoots that grew from the top of rooted, dormant cuttings were measured after 6 weeks.

($P = 0.04$), although the cultivar × inoculation treatment interaction was not significant ($P = 0.2$). There was a significant effect of cultivar on RTE ($P > 0.0001$), with no effects of inoculation treatment ($P = 0.2$) or cultivar × inoculation treatment ($P = 0.5$). 'Primitivo' had the least RTE and incidence of leaf symptoms, regardless of isolate (Table 5). 'Black Corinth' and 'Muscat Hamburg' had the greatest RTEs and incidences of leaf symptoms, regardless of isolate, and similarly for 'Merlot' plants inoculated with BX1-10. Plants inoculated with BX1-10 had greater incidences than those inoculated with M14 for six of nine assessed cultivars.

Comparing RTEs measured 1 year post-inoculation in Assay 1 (from shoots that were 8 weeks old) with those measured at 6 weeks post-inoculation in Assay 3 (Figure 4), these values were similar for 'Primitivo' and 'Black Corinth'. These two cultivars also ranked similarly in both assays, although at opposite extremes. 'Primitivo' had the lowest RTE from isolate M14 in Assay 1 (Table 3) and the lowest RTEs, regardless of isolate, in

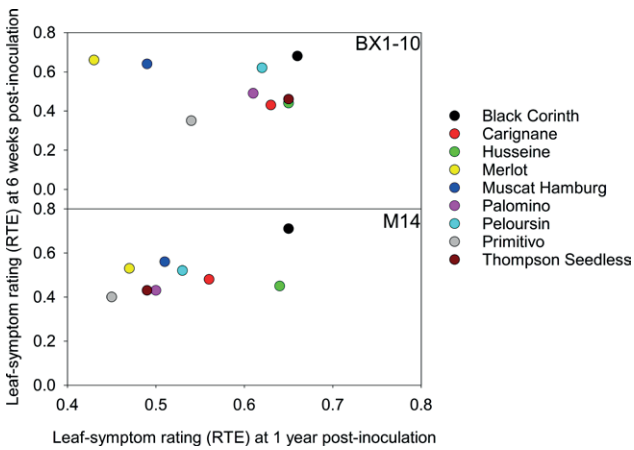


**Figure 4.** Relationships for nine grapevine cultivars, between mean leaf symptom ratings [relative treatment effect (RTE)], at 1 year post-inoculation (Assay 1), versus mean leaf symptom ratings, 6 weeks post-inoculation (Assay 3). Plants in both assays were inoculated with either *Eutypa lata* isolate BX1-10 or isolate M14.

Assay 3 (Table 5). 'Black Corinth' had the greatest RTEs, regardless of isolate, in Assay 1 (Table 3), and the greatest RTE from M14 in Assay 3 (Table 5). In contrast, 'Merlot' and 'Muscat Hamburg' were ranked very differently in the two assays (Figure 4). 'Merlot' had the least RTE from BX1-10 in Assay 1 (Table 3), but the greatest RTE from BX1-10 in Assay 3 (Table 5). 'Muscat Hamburg' had low to intermediate RTEs, depending on isolate, in Assay 1 (Table 3), but high RTEs, regardless of isolate, in Assay 3 (Table 5).

Mean shoot lengths were different ($P$ = 0.001) among the cultivars, but not between inoculation treatments ($P$ = 0.7), and the cultivar × inoculation treatment interaction was not significant ($P$ = 0.4). There was a trend (though not statistically significant) for shorter shoot lengths of inoculated plants compared to those of the non-inoculated controls for all cultivars except 'Primitivo'. 'Black Corinth' had the shortest shoot lengths, regardless of pathogen isolate, and this corresponded with the greatest RTEs (Table 5). However, the strength of the association between mean shoot lengths and the RTEs, when considered among all the cultivars, was not statistically significant for plants inoculated with either isolate.

## DISCUSSION

The grapevine cultivar 'Primitivo' was consistently the most resistant of the nine grapevine culivars assessed, to Eutypa dieback, or among the most resistant cultivars, depending on *E. lata* isolate. This was true for results from Assay 1 (short lesion lengths, low RTE, low incidence of leaf symptoms), from Assay 2 (short lesion lengths, low incidence of cankers), and from Assay 3 (low RTE, low incidence of leaf symptoms). These results of minimal symptoms on different plant organs (stems and leaves), for different tissue inoculations (green stems and woody stems), between two isolates (BX1-10 and M14), for different incubation periods (6 weeks, 4 months, or 1 year), and for plants propagated from different types of cuttings (green or dormant), indicate that 'Primitivo' is resistant to Eutypa dieback. The resistance of 'Primitivo' (aka 'Zinfandel') to Eutypa dieback possibly explains why this cultivar is surviving in Northern California, where vineyards planted decades ago are still in production.

The cultivar 'Merlot' ranked differently between Assays 1 and 3. 'Merlot' was most resistant, or among the most resistant depending on the isolate, in Assay 1 (short lesion lengths, low RTE, low incidence of leaf symptoms) and in Assay 2 (short lesion lengths, low incidence of cankers), but was very susceptible, regardless of isolate, in Assay 3 (high RTE, high incidence of leaf symptoms). 'Merlot' has been reported as resistant to Eutypa dieback, based on little to no leaf symptoms in separate field surveys conducted in Europe and Australia (Dubos 1987; Sosnowski *et al.,* 2022). Although the differences were not as great as for 'Merlot', 'Muscat Hamburg' also ranked differently between Assays 1 and 3. Consistently for the two *E. lata* isolates, 'Muscat Hamburg' was moderately resistant, based on the results from Assays 1 and 2 (short lesion lengths in woody and green stems), and based on the results from Assay 2 (intermediate RTE, intermediate incidence of leaf symptoms), but was susceptible, based on the results from Assay 3 (high RTEs, high incidence of leaf symptoms). Although Assay 3 was convenient, with no plant rooting necessary and a short (4 to 6 weeks) incubation period, the very different results for 'Merlot' (and to a lesser extent for 'Muscat Hamburg') for leaf symptoms compared to Assay 1 make it difficult to rely on the results obtained from Assay 3. Cuttings are not callused or rooted before inoculation in Assay 3, and the mortality rate of especially 'Merlot' was high, with no roots developing among a high proportion (49%) of cuttings. With low sample sizes among the non-inoculated and inoculated plants, it was difficult to evaluate whether the incidence of leaf symptoms and RTEs were representative of the host responses to infection of 'Merlot' and 'Muscat Hamburg'. Because cuttings for Assays 1 and 3 were obtained in different years, it is possible the low viability of 'Merlot' and 'Muscat Hamburg' cuttings for Assay 3 was specific to the field conditions in the year the cuttings were collected, possibly caused by low carbohydrate reserves. Regardless, Assay 3 did not provide results as definitive as obtained from Assays 1 and 2.

Consistently between the two *E. lata* isolates, 'Black Corinth' was moderately susceptible (intermediate lesion lengths in woody and green stems), based on the results from Assays 1 and 2, but was highly susceptible (high RTEs, high incidence of leaf symptoms), based on Assays 1 and 3 results. Wood symptoms probably result, in part, from pathogen enzymatic activities at infections (Rolshausen *et al.,* 2008; Blanco-Ulate *et al.,* 2013; Morales-Cruz *et al.,* 2015). Leaf symptoms may be affected by translocation of pathogen metabolites and polypeptides from the infected wood to shoots (Mahoney *et al.,* 2003; Smith *et al.,* 2003; Octave *et al.,* 2006a). These compounds modify mitochondrial, plastid and plasma membranes of grapevine cells (Deswarte *et al.,* 1996; Amborabé *et al.,* 2001; Octave *et al.,* 2006b), affect chloroplast structure (Deswarte *et al.,* 1994), alter cell nutrient uptake by inhibiting proton flux at the

plasma membranes (Octave *et al.,* 2006b), and inhibit photosynthesis and respiration in leaf tissues by decreasing energy charge (Amborabé *et al.,* 2001; Octave *et al.,* 2006b). Therefore, the mechanisms of host resistance to wood and leaf symptoms may differ. Resistance to wood colonization may be associated with high lignin and suberin deposition in wood of some cultivars (Munkvold and Marois 1995). Resistance to leaf symptoms may be associated with tolerance/detoxification of fungal secondary metabolites *in planta* (Guillén *et al.,* 1998). Detoxification of eutypine, one of many secondary metabolites produced by *E. lata* (Mahoney *et al.,* 2005), into its corresponding alcohol eutypinol, by transgenic grapevines genetically engineered to do so, has been associated with reduced severity of leaf symptoms (Guillén *et al.,* 1998; Legrand *et al.,* 2003).

Assay 1 was the most time-consuming of the three assays, but lesion length in woody stems was a repeatable measure between the two *E. lata* isolates, as has been previously demonstrated (Sosnowski *et al.,* 2007a). Given the repeatability of the results for isolate BX1-10 and the strong correlation between wood and leaf symptoms, Assay 1 was the most robust of the three assays for differentiating the nine grapevine cultivars evaluated. Propagation from green cuttings, which is the only way to root some grapevine germplasm, allowed the development of lesions in Assay 2 that were comparable in size to those of Assay 1, but in a shorter 4 month timeframe. Assay 2 may therefore be suited for preliminary screening of large germplasm collections (e.g., progeny from crosses, different *Vitis* species, or hybrids with resistance to other grapevine diseases). Assay 2 could then be followed by Assay 1 for secondary screening of subsets of the most resistant germplasm. Plants in Assay 2 also developed visible, external stem cankers, the incidence of which was positively correlated with lengths of internal stem lesions. A practical disadvantage of Assay 2, however, was the need for a greenhouse mist system, to propagate plants from green cuttings. Although Assay 2 was more rapid than Assay 1, *E. lata* ascospores do not directly infect green host stems in the field, so measuring lesions in green stems may not measure resistance to Eutypa dieback. Nonetheless, based on lesion lengths in woody or green stems, both Assays 1 and 2 identified the same resistant cultivars as 'Merlot', 'Primitivo', and 'Muscat Hamburg', and some of the same susceptible cultivars as 'Husseine' and 'Carignane'.

Previous authors have emphasized the importance of evaluating lesion lengths and the points at which, beyond the lesion margins, *E. lata* is undetectable (Moisy *et al.,* 2017; Sosnowski *et al.,* 2022). Results from Assay 1 of greater distance of detection beyond the visible lesion margins (e.g., 15 cm beyond the margin in the very susceptible 'Thompson Seedless') are consistent with this pattern. Measuring only lesion lengths may not reflect a pathogen's ability to colonize apparently healthy wood (Sosnowski *et al.,* 2007a). Assuming the pathogen will eventually rot asymptomatic wood from which it is recovered beyond the lesion margin, the extent of wood colonization may therefore be an appropriate (though more time-consuming) measure of host susceptibility.

Phenotyping assays that use the same isolates and pre- and post-inoculation growing conditions may help to standardize screening protocols that can accommodate the diversity of grapevine germplasm. The present study used two 'reference' isolates from previous research (Péros and Berger 1999; Camps *et al.,* 2010; Travadon *et al.,* 2013; Moisy *et al.,* 2017; Cardot *et al.,* 2019). Isolate BX1-10 has previously been characterized as virulent (Péros and Berger 1994; Moisy *et al.,* 2017), whereas isolate M14 was associated with repeatable wood symptoms in a previous study (Travadon *et al.,* 2013). For the majority of cultivars inoculated with BX1-10 compared to M14, the present results of lesion sizes in Assays 1 and 2, and the RTEs and incidences of leaf symptoms in Assays 1 and 3, indicate that isolate BX1-10 was more virulent that isolate M14. Differences in virulence among *E. lata* isolates inoculated onto replicate plants of the same cultivar have been previously reported (Péros *et al.,* 1997; Péros and Berger 2003; Sosnowski *et al.,* 2007a; Travadon and Baumgartner 2015), and these may correspond to high genetic variation in *E. lata* populations (Péros and Berger 1999; Travadon *et al.,* 2012; Onetto *et al.,* 2022), and/or to differences in secondary metabolite production (Mahoney *et al.,* 2003; Lardner *et al.,* 2006). Previous studies have shown that severity of leaf symptoms in some cultivars correlates to severity of wood symptoms (Sosnowski *et al.,* 2007a), or to *E. lata* biomass as quantified by qPCR (Moisy *et al.,* 2017). Inconsistencies have been reported between isolates, with no relationships between leaf and wood symptoms (Sosnowski *et al.,* 2007a). Similar results were recorded here from Assay 1, with positive correlation between lesion length and RTE for all nine cultivars inoculated with BX1-10, but not for M14. When inoculated with M14, 'Thompson Seedless' did not have severe leaf symptoms, despite large wood lesions. For future host germplasm studies that include any of the three Assays, isolate BX1-10 is probably more suitable than M14.

Knowledge of grapevine cultivar resistance to *E. lata* informs fundamental research on molecular mechanisms of this resistance (Rotter *et al.,* 2009; Camps *et al.,* 2010). For example, high expression of gene families associated with host defense responses (e.g., enzymes of

the jasmonic acid, salicylic acid and phenylpropanoid pathways) among resistant 'Merlot' and 'Cabernet-Sauvignon', compared to highly susceptible 'Ugni blanc', indicates similar genetic determinants of resistance, even among different cultivars (Cardot *et al*., 2019). The development of phenotyping methods for measuring grapevine resistance to Eutypa dieback, as those outlined in the present study, is important for future genetics studies, which may identify genomic regions associated with resistance to this economically important grapevine disease (Dry *et al.,* 2019).

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Research Papers – 12th Special issue on Grapevine Trunk Diseases

# Prevalence and pathogenicity of fungi associated with grapevine trunk diseases in Jordan

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**Summary.** Grapevines (*Vitis vinifera*) are important fruit producers in Jordan, and grapevine trunk diseases (GTDs) are suspected to cause problems in many Jordanian vineyards. This study aimed to estimate GTDs incidence and severity in selected vineyards, and to isolate and identify the causal agents associated with GTDs in this country. Field surveys were carried out and representative samples of diseased vines showing GTDs symptoms were collected to isolate and identify the causal organisms. Molecular analyses of DNA sequences of the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) were used to confirm the morphological identifications of the fungal isolates. GTDs were present in all the surveyed vineyards. Mean GTD incidence was 44% across all the fields evaluated, ranging from 9 to 69% in individual vineyards. Disease severity ranged from 52–74% (mean = 62%) across all vineyards and locations. A total of 325 fungal isolates were recovered from infected grapevines. The most prevalent pathogens identified were those associated with Botryosphaeria dieback, including *Diplodia seriata*, *Lasiodiplodia theobromae* and *Neofusicoccum parvum*, followed by the Esca disease pathogens *Phaeoacremonium parasiticum*, *P. aleophilum*, *P. rubrigenum*, and *Fomitiporia* spp. *Ilyonectria liriodendri* and *I.* spp., known to be associated with black foot of grapevines, were also isolated. Plant pathogens causing vascular wilts and root rots, including *Fusarium proliferatum*, *F. oxysporum*, *Verticillium* spp., and *Rhizoctonia solani*, were also identified from diseased plant samples, and were found in mixed infections with GTDs pathogens. Most of the identified pathogens, except those associated with vascular wilt and root rot, are reported for the first time in Jordan. Results of this study indicate that GTDs are widespread in Jordan, and that there is urgent need to adopt a "national strategy" for GTD management in this country.

**Keywords.** GTDs, Botryosphaeria dieback, Esca, black foot.

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## INTRODUCTION

Grapevine (*Vitis vinifera*. L) is an important fruit crop in Jordan and grape production ranks third among all fruit trees planted. The total area of cultivated grapevines in Jordan is 3,057.4 ha with total annual grape production of

53,886 tons (Department of Statistics, 2019). The AL-Mafraq region, with approx. 40% of the Jordanian production, is the leading grape-producing region, so this region was selected to conduct the present study.

During recent years, symptoms of grapevine decline were frequently observed in most grapevine production areas in Jordan. Several samples from farmers' vines from the Al Mafraq region were also sent to the Phytopathology laboratory at the Jordan University of Science and Technology (JUST), for diagnoses of unknown grapevine diseases that were causing decline and death of young and mature grapevines. Initial diagnoses from these samples suggested that most were infected with fungi known to cause the grapevine trunk diseases (GTD) complex.

GTDs are the most common fungal diseases of grapevines, and cause several symptoms in foliage and vascular tissue of these plants. Fungi causing GTDs primarily infect grapevines through pruning wounds, subsequently colonizing the vascular tissues (Bertsch *et al*., 2013; Mondello *et al*., 2018). GTDs are limiting factors that adversely affect production and longevity of vineyards, causing important economic losses (Agusti-Brisach *et al*., 2013; Gramaje and Dimarco, 2015). Major symptoms of GTDs include dieback, death of cordons or trunks, canker formation in vascular tissues, and necrotic wood (Mugnai *et al*., 1999; Úrbez-Torres *et al*., 2006). Over time, these infections gradually progress and spread within affected vines. When infection occurs, overall vine health begins to slowly deteriorate, making them susceptible to a wide range of other pathogens. Poor early growth and reduced vigour are prominent symptoms associated with these diseases (Trouillas *et al*., 2010; Van Niekerk *et al*., 2011; Fontaine *et al*., 2016). There are six major GTDs, each caused by different fungi, and include the Esca complex, Eutypa dieback, Botryosphaeria dieback, and *Phomopsis* dieback. The black-foot and Petri diseases affect primarily young grapevine (<5 years old) (Glawe and Rogers, 1982; Mostert *et al*., 2005; Van Niekerk *et al*., 2005; Bruez *et al*., 2013; Gramaje *et al*., 2018).

GTDs cause economic losses to grape growers in most regions where grapes are produced. In Jordan, GTDs are becoming serious diseases affecting most vineyards, but etiology of these diseases has not been studied or identified. The identification of the fungi causing GTDs is important for implementing appropriate disease management strategies. The aims of the present study were to examine the extent of GTDs in grapevine growing areas of Jordan, and to identify the associated fungi and test their pathogenicity to grapevine.

## MATERIALS AND METHODS

### *Field surveys, sample collection, and estimation of GTD incidence and severity*

Field surveys were conducted in the spring of 2019, 4 to 6 weeks after grapevine bud break. Eleven vineyards were included in this surveys. Farms were selected to include the main grapevine cultivars grown in Jordan, including Black magic, Zeiny, Halawani, and Superior. Other cultivars, including Cabernet Sauvignon, Grenache, Gewurztraminer, Syrah, and Chardonnay used for processing purposes, were also included. At each farm, one or more fields were selected to estimate disease incidence. The number of vines showing typical symptoms of GTDs was counted in different vine rows to estimate the disease incidence, and these assessments covered 15 to 20% the total number of vines in each field. Disease incidence generally indicates prevalence in each area or host plant population. Disease severity for each infected vine was estimated based on the appearance of different known GTD symptoms, and each symptom was given a relative weighting in the overall severity score, as shown in Table 1. Representative samples (approx. 5%) from diseased vines showing GTDs symptoms in each field were collected, to isolate and identify the causal organisms. These samples were labeled, kept in paper bags, placed in ice boxes, and transferred to the plant pathology laboratory at Jordan University of Science and Technology (JUST) for analyses.

### *Fungal isolations*

Tissue pieces (wood tissue, about 5.0 $mm^2$ diam.) from collected diseased samples were cut from the margins of necrotic wood tissues. Each wood tissue chip was disinfected in 70% ethanol solution for 2 min, soaked in sterile distilled water, then transferred to 1% sodium hypochlorite solution for 2–5 min, rinsed twice in sterile distilled water, and allowed to dry on Whitman no. 2 filter paper for 10 min inside a laminar flow cabinet (Cortesi *et al*., 2000; Larignon and Dubos, 1997). The tissue pieces were then placed into Petri plates containing either potato dextrose agar (PDA) or malt extract agar (MEA). The media in these plates had been amended with ampicillin and streptomycin (each at 50 ppm) after autoclaving to inhibit the growth of saprophytic bacteria (Abreo *et al*., 2013). The plates were then incubated at 25°C in the dark until the fungal mycelium growth was observed from wood sections. Pure cultures were obtained by transferring single fungal hyphal tips from

**Table 1.** Descriptions of different grapevine trunk disease symptoms, and scores and sub-scores used to calculate disease severity values for each grapevine examined in the present study.

| Categories of symptoms | Weight (%) for total severity score, and symptom description | Sub-score (%) for each symptom in the total severity score |
|---|---|---|
| *Foliar symptoms (20%)* | | |
| Slight yellowing | All or part of foliage showing slight yellowing. | 7 |
| Moderate yellowing | All or part of foliage showing moderate yellowing and stunting. | 10 |
| Severe yellowing | All or part of foliage showing severe yellowing and stunting. | 15 |
| Reddening | Foliage showing reddening symptoms | 5 |
| Tiger stripes | All or some leaves showing tiger stripes | 5 |
| Total defoliation | Most leaves on vines are absent (defoliated), or no leaves emerged after bud break. | 20 |
| *Cordon symptoms (50%) (in trellis training method)* | | |
| One cordon with dieback symptoms or death | 25% of the spurs on one cordon are affected | 12.5 |
| Two cordons with dieback symptoms or death | 26 to 50% of the spurs on two cordons affected by the disease | 25 |
| Three cordons with dieback symptoms or death | >50% of the spurs on three cordons were affected. All primary cordons affected by the disease | 37.5 |
| Four cordons with dieback symptoms or death | All cordons dead, and buds did not break | 50 |
| *Cordons symptoms (50%) (for two-cordon training systems)* | | |
| A few buds did not break in part of the cordon | 25% of the spurs on one cordon are affected. | 12.5 |
| One main cordon with dieback symptoms or death | More than 25% but less than 50% of the spurs on the two cordons of the vine are significantly impacted by the disease, | 25 |
| Two main cordons with dieback symptoms or death | More than 50% of the spurs on two cordons affected. All primary cordons affected by the disease. | 37.5 |
| All main cordons with dieback symptoms or death | All cordons dead, and buds did not break | 50 |
| *Crack Symptoms in the Main trunk (15%)* | | |
| No trunk cracks | No cracks present in the main vine trunk | 0 |
| Trunk with slight to moderate cracks | Some wood cracking extends downward and upward along the trunk | 10 |
| Trunk with severe cracks | Severe wood cracking extends downward and upward along the trunk | 15 |
| *Cross-section discolouration inside trunk (15%)* | | |
| No discolouration | No vascular tissue showing dark discolouration | 0 |
| Partial discolouration | Some vascular tissues dark brown, black vascular streaking when the trunk is cut | 10 |
| Total discolouration | Vascular tissues with dark discolouration and dead woody tissue | 15 |

each colony to individual plates of MEA.

### *Morphological identification of fungi*

Isolates having colony morphology representative of pathogenic species were examined microscopically. Different techniques were employed to identify the isolated microorganisms including direct examination of Petri plates with a stereo microscope and a compound microscope (Olympus Corp.). Initial identification of isolates to the genus level was determined using appropriate mycological identification keys. Culture features, including colony texture, colony colour, conidium morphology (shape, colour, presence or absence of septa), conidiophore morphology, and conidiation characteristics were recorded. General and specific mycological identification keys were used to determine the identity of each fungal culture. For *Botryosphaeriaceae* spp. the identification keys of Crous *et al*. (2006) and Phillips *et al*. (2006) were used. For *Phaeoacremonium* spp., keys of Mostert *et al*. (2006) and Essakhi *et al*. (2008) were used. Isolated and identified fungi were recorded, and each isolate was stored at 4°C in long-term storage for further identification using molecular techniques and for testing of pathogenicity on grapevine seedlings.

*Molecular identification of fungal isolates*

DNA extraction, PCR amplification and sequencing of amplification products

Fungal isolates were cultured on MEA for 2 weeks for DNA extraction. The DNeasy Plant Mini Kit (CAS NO. 69104; Qiagen group) was used to extract DNA from mycelium of different fungal isolates, following the manufacturer's instructions. The DNA concentration in each sample was estimated using NanoDrop 2000 (Thermo Scientific) from a 2 µL DNA sample. DNA from fungal isolates were used to amplify the Internal Transcribed Spacers (ITS region) by the primers ITS1 and ITS4 (White *et al*., 1990). PCR reactions were each carried out in a total final volume of 25 µL, using FIREPOL® Master mix ready to load 5x (Solis BioDyne), containing 3 ng of DNA template and 1µM of each of forward and reverse primers. The PCR was conducted using T100™ Thermal cycler (Biorad) at the following parameters: 95°C for 3 min, then 34 cycles each of 95°C for 30 sec, 60°C for 1 min, and 72°C for 1 minute, followed by 72°C for 5 min and 4°C. PCR reaction products were stored at -20°C before being electrophoresed on a 2% agarose gel with a Tris-Borate-EDTA (TBE) buffer. Amplification products were visualized using an ultraviolet light box.

The identification of each fungal isolate was confirmed by sequencing of the PCR products of approx. 300-750 bp (depending on the genus) by Genetics Company. Sequences were uploaded to SnapGene v5.0 software and to the GenBank database (http://www.ncbi.nlm.nih.gov). The BLAST tool was used to compare these sequences with published sequences of previously identified fungi in the database (Kaliterna *et al*., 2012).

*Pathogenicity tests of isolated fungi*

The pathogenicity of 88 fungal isolates was confirmed by inoculating each isolate onto 1-year-old self-rooted grapevine canes (two canes per isolate), and recoding the observed disease symptoms. Self-rooted canes from different grapevine cultivars (Zeiny, Halawani, Baladi, or Cabernet Sauvignon) were inoculated with the fungi identified in this study. Each isolate was grown on PDA or MEA plates for 2 weeks at 25C in the dark. Each cane was wounded using a 5 mm cork borer, and the wound was then inoculated with a 5 mm diam. isolate colonized PDA plug from a 2-week-old culture. Control treatments were inoculated with non-colonized PDA plugs. Each inoculation point was covered with Parafilm to prevent inoculum desiccation. The grapevine canes were then covered with plastic bags (to maintain humidity) and were placed in a growth chamber at 25°C, 90% relative humidity and a 12 h light 12 h dark cycle for 3 weeks. The plants were then moved to a greenhouse bench and were monitored for disease development.

A final pathogenicity assessment was made 1 to 3 months' post-inoculation. Development of GTD symptoms on plant foliage and stems were monitored and recorded twice each week, and the final assessment was carried out by removing the bark of each cane and observing wood tissue discolouration and necrosis. All the inoculated canes were subjected to re-isolation and identification of the inoculated pathogens, to determine fulfilment of Koch's postulates.

*Assessments of disease development in inoculated self-rooted grapevine canes*

To assess the extent of disease development within woody tissues of grapevine self-rooted canes, 200 inoculated canes from previous pathogenicity tests were evaluated. The aim of this assessment was to determine the development of necrotic wood tissue and discolouration during 3 months period after inoculation. Inoculated canes showing typical GTD symptoms (yellowing, stunting, leaf tiger stripes, stem cracking) were selected and cut above the soil surface line. Three sections, each approx. 10 mm thick, were taken from each inoculated self-rooted cane, at 1 cm intervals above and below the inoculation point (i.e., a total of six sections). The sections were then photographed using a dissecting microscope equipped with a digital camera.

Digital images of the wood sections were analyzed using the Image J program, provided by the National Institutes of Health (NIH) (Schneider *et al*., 2012). The image analysis software measured different levels of section darkening, which were converted to disease severity values on a scale from 0 to 100% relative to the total area of each section (Table 4). Colour of the wood tissue was assessed by measuring the reflective light intensity of each section. The change in light intensity was then used to calculate the disease severity relative to the light intensity of healthy sections, as reported by Niemira *et al*. (1999). The resulting values were reported in pixels, and included the total area of each section, total darkened area, and mean darkened area. These values were used to calculate disease severity in the grapevine sections using the following formula:

Disease severity relative to area of healthy section = (Total area of section darkened tissue / Total area of section) × 100.

## RESULTS

### *Field surveys, sample collection, and GTD incidence and severity*

GTD symptoms were observed and recorded in all the surveyed vineyards. Symptoms included leaf chlorosis, stunting, wedge-shaped cankers in trunks and cordons, discolouration and browning of the vascular tissues in the main trunks and cordons, and trunk cracks (Figures 1 and 2). The main Esca-complex symptoms were observed in most fields and included: general decline, reduced foliage and leaf size, tiger stripe symptoms on leaves (chlorosis and necrosis), dark brown to black streaking in trunk cross-sections, pathogen signs including black pycnidia in vascular tissues.

Wedge-shaped cankers and discolouration of wood tissues were the most common vascular symptoms observed, and these were associated with foliar symptoms, including lack of spring growth in the spur positions. Browning and black streaking of wood tissues were the second most common vascular wood discolouration symptoms (Figure 2), followed by light brown discolouration of wood and central stem necrosis. Black streaking of wood tissues was often found in vines showing characteristic Esca symptoms and in samples collected from vines showing general dieback with no characteristic foliar symptoms.

A total of 38,600 vines in the 11 fields were surveyed in this study. Among these, 7558 vines (approx. 20%) were evaluated for the presence of GTDs symptoms. The mean overall GTD disease incidence (11 fields), based on visual observation of symptoms, was 43.6%. Mean GTD incidence ranged from 9.5% to 68.8% in the 11 fields (Table 2).

The greatest mean GTD incidence (68.8%) recorded at Sama Al-Sarhan, in the grapevine cultivar Grenache, followed by Zamlat Al-Amir Ghazi, North Badia location (64.9%) in Chardonnay, and at Thagrat Al-Gubb (62.5%) in Zeiny. Incidence at the other locations and fields was generally less than 60% (Table 2). GTD severity ranged from 52 to 74% (overall mean = 62.3%) across all fields and locations (Table 2). The greatest mean GTD severity was 74% for Chardonnay at Zamlat Al-Amir Ghazi, North Badia, and least severity (52.5%) was in the cultivar Zeiny at Thagrat Al- Gubb (Table 2).

### *Morphological identification of fungal isolates*

Morphological identification of fungal isolates was carried out using several steps. Fungal isolates were divided into groups. The first group had dark green fast-growing mycelium on PDA and MEA. The cultures developed single or clustered black, globose pycnidia from which two types of conidia (pigmented or hyaline) were identified. These characteristics were consistent with descriptions of species of *Botryosphaeriaceae* (Phillips, 2002; Úrbez-Torres *et al*., 2006). Fungal cultures with pigmented conidia were preliminarily divided into two categories, *Lasiodiplodia* spp., and *Diplodia* spp. Cultures with hyaline conidia were similarly identified as *Neofusicoccum* spp., (Table 3) (Crous *et al*., 2006; Phillips *et al*., 2006; Alves *et al*., 2008).

The second group of isolates developed mycelial bundles, simple or branched conidiophores, slender phialides in three size types and bearing narrow funnel-shaped collarettes, and conidia aggregated in slimy heads and that were oblong-ellipsoidal to allantoid in shape on PDA and MEA. These isolates were typical of the *Phaeoacremonium* spp. Some cultures had white colour and cottony texture, producing dense aerial hyphae after 2 to 3 d incubation and developing white to yellowish mycelia after 10 d, and later becoming yellow or brown. These isolates were typical of the *Fomitiporia* spp. (Table 3) (Mostert *et al*., 2006; Essakhi *et al*., 2008).

Identification of fungal isolates from samples collected from different grapevine cultivars and details of isolates associated with different GTD symptoms are presented in Table 3.

Vascular wilt pathogens, including *Verticillium* spp., and *Fusarium* spp. (Table 3), known to cause wilt and decline of fruit tree crops, were also isolated. Other fungi unrelated to GTDs were also identified, including *Alternaria* spp., *Rhizoctonia* spp., *Penicillium* spp., and *Aspergillus* spp. Many other cultures were difficult to identify based on morphological characteristics, and were kept for further identification using molecular methods.

### *Molecular identification of fungal isolates*

Among the 325 isolates obtained from all the sampled plants, 216 were classified as fungi associated with GTDs, 139 (42.8%) were associated with Botryosphaeria dieback [*Diplodia seriata* (51 isolates); *Lasiodiplodia theobromae* (42 isolates); and *Neofusicoccum* spp. (46 isolates)], 47 isolates (14.5%) were associated with the Esca disease complex [*Phaeoacremonium* spp. (41 isolates); *Ilyonectria liriodendri* (four isolates); *Fomitiporia* spp. (two isolates). Seventy-nine isolates associated with vascular wilt and root rot pathogens were obtained, including *Fusarium* spp. (19.7%), *Verticillium* spp. (1.5%) and the root rot pathogen *Rhizoctonia* sp. (1.2%). The other

**Figure 1.** Foliar Symptoms of grapevine trunk diseases observed in mature vines in Jordan including, stunted shoots with chlorotic leaves and necrotic margins, stunted shoots, tiger-stripe symptoms on leaves of a red cultivar and apoplectic symptom which is a sudden wilting of the plant or one arm or several shoots.

**Figure 2.** Vascular symptoms of grapevine trunk diseases in cross sections cut from symptomatic vines. The different pattern of dark brown to black discoloration.

49 fungal isolates (15.1%) included many saprophytic fungi (e.g. *Aspergillus niger*, *Penicillium* sp.), endophytes, or potential biological control agents.

*Pathogenicity tests of isolated fungi*

Results of pathogenicity tests for selected isolates (14 isolates of *Phaeoacremonium* spp., eight of *Diplodia seriata*, 58 of *Fusarium* spp; six of *Lasiodiplodia theobromae*, nine of *Neofusicoccum parvum*, and five isolates of *Rhizoctonia solani*) (Figure 3) showed that all the tested isolates were pathogenic on self-rooted grapevine canes. These inoculations produced symptoms typical of GTDs. These symptoms varied depending on isolate and grapevine cultivar source, and include, chloroses, leaf yellowing, tiger stripes, and discolouration (purple, light tan, dark tan, brown), stunting, stem cracking, and different patterns of vascular tissue discolouration in stem cross sections (Figure 3). None of the control plants inoculated with plugs of non-colonized PDA showed any symptoms, and these plants continued to grow normally until the end of the experiment (Figure 3).

*Disease development in inoculated grapevine canes*

Analyses of cross sections cut from each inoculated cane showed that thickness of the cross sections did not affect light intensity measurements using image analysis software (Figure 3). Therefore, a section thickness of 10 mm was used for the analysis of discolouration caused by the different fungal isolates (Table 4). The mean disease severity values of stem cross sections varied among the different fungal isolates used in this experiment, ranging from 27.9 to 100% (Table 4).

The greatest mean disease severities occurred in canes inoculated with *L. theobromae*, *D. seriata*, *P. parasiticum*, *P. rubrigenum*, *I. liriodendra*, *N. parvum*, or two isolates of *F. oxysporum*. Disease severity values for these fungi were either 100% or close to 100%, indicating complete discolouration of stem sections above and

**Table 2.** Disease incidence (DI) and mean disease severity of grapevine trunk diseases (GTDs) in different locations and vineyards surveyed in Al Mafraq, Jordan.

| Field number | Vineyard location | Cultivar | Total No. of vines in the vineyard | No. of observed vines | No. of symptomatic vines | Mean incidence (DI %) | Mean severity (%) |
|---|---|---|---|---|---|---|---|
| 1. | Sama Sirhan | Gewurztraminer | 10,000 | 3000 | 284 | 9.5 | 66.0 |
| 2. | Sama Sirhan | Grenache | 5000 | 800 | 550 | 68.8 | 57.0 |
| 3. | Ad-Dafyanah | Superior seedless | 2450 | 700 | 224 | 32.0 | 67.0 |
| 4. | Sabha | black magic | 800 | 250 | 94 | 37.6 | 60.0 |
| 5. | Sabha | Zeiny | 4300 | 572 | 165 | 28.9 | 59.2 |
| 6. | Zamlat Al-Amir Ghazi | Cabernet Sauvignon | 4600 | 550 | 138 | 25.1 | 70.0 |
| 7. | Zamlat Al-Amir Ghazi | Syrah | 2500 | 490 | 238 | 48.6 | 63.0 |
| 8. | Zamlat Al-Amir Ghazi | Chardonnay | 3000 | 330 | 214 | 64.9 | 74.0 |
| 9. | Thagrat Al- Gubb | Zeiny | 2000 | 160 | 100 | 62.5 | 52.0 |
| 10. | Umm Al-Jimal | Zeiny | 1450 | 216 | 114 | 52.8 | 62.0 |
| 11. | Umm Al-Jimal | Zeiny | 2500 | 490 | 243 | 49.6 | 59.0 |
| Total | | | 38,600 | 7558 | 2364 | | |
| Overall mean | | | | | | 43.6 | 62.3 |

**Table 3.** Fungi associated with different grapevine trunk diseases symptoms, and numbers of plant samples from which each fungus was isolated.

| Fungus | Grapevine vascular symptoms [a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Wedge-shape canker [b] | Black wood streaking | Yellowish soft wood | Brown discolouration | Central necrosis | Black spots | Total | Proportion (%) of total isolates |
| *Diplodia seriata* | 23[b] | 17 | | | 11 | | 51 | 15.7 |
| *Lasiodiplodia theobromae* | 14 | 11 | | 10 | 7 | | 42 | 12.9 |
| *Neofusicoccum parvum* | 16 | 8 | | 13 | 9 | | 46 | 14.2 |
| *Fusarium oxysporum* | 18 | | 9 | | 20 | | 47 | 14.5 |
| *Fusarium proliferatum* | | | | | 15 | | 15 | 4.6 |
| *Fusarium perseae* | | | | 2 | | | 2 | 0.6 |
| *Phaeoacremonium* spp. | | 19 | 11 | | | 10 | 41 | 12.6 |
| *Ilyonectria liriodendra* | | | | | | 4 | 4 | 1.2 |
| *Fomitiporia* spp. | | | 2 | | | | 2 | 0.6 |
| *Verticillium* spp. | | | | 5 | | | 5 | 1.5 |
| *Rhizoctonia* spp. | | | | 10 | | | 10 | 3.07 |
| *Simplicillium obclavatum* | | | 1 | | | | 1 | 0.31 |
| *Nectria* spp. | | 7 | | | | 3 | 10 | 3.1 |
| Others | | | | | | | 49 | 15.1 |
| Total | | | | | | | 325 | 100 |

[a] Description of the typical GTD symptom observed in vineyards.
[b] Number of samples that had typical GTD symptoms.

below the inoculation points (Figure 3). The least stem cross section disease severity values were in plants inoculated with *F. oxysporum*, *F. perseae*, *F. proliferatum*, or *R. solani*, with disease severity values less than 50% (Table 4).

## DISCUSSION

This study was the first survey of grapevines affected by GTDs in Jordan, carried out to determine the prevalence of these diseases in vineyards, and to iden-

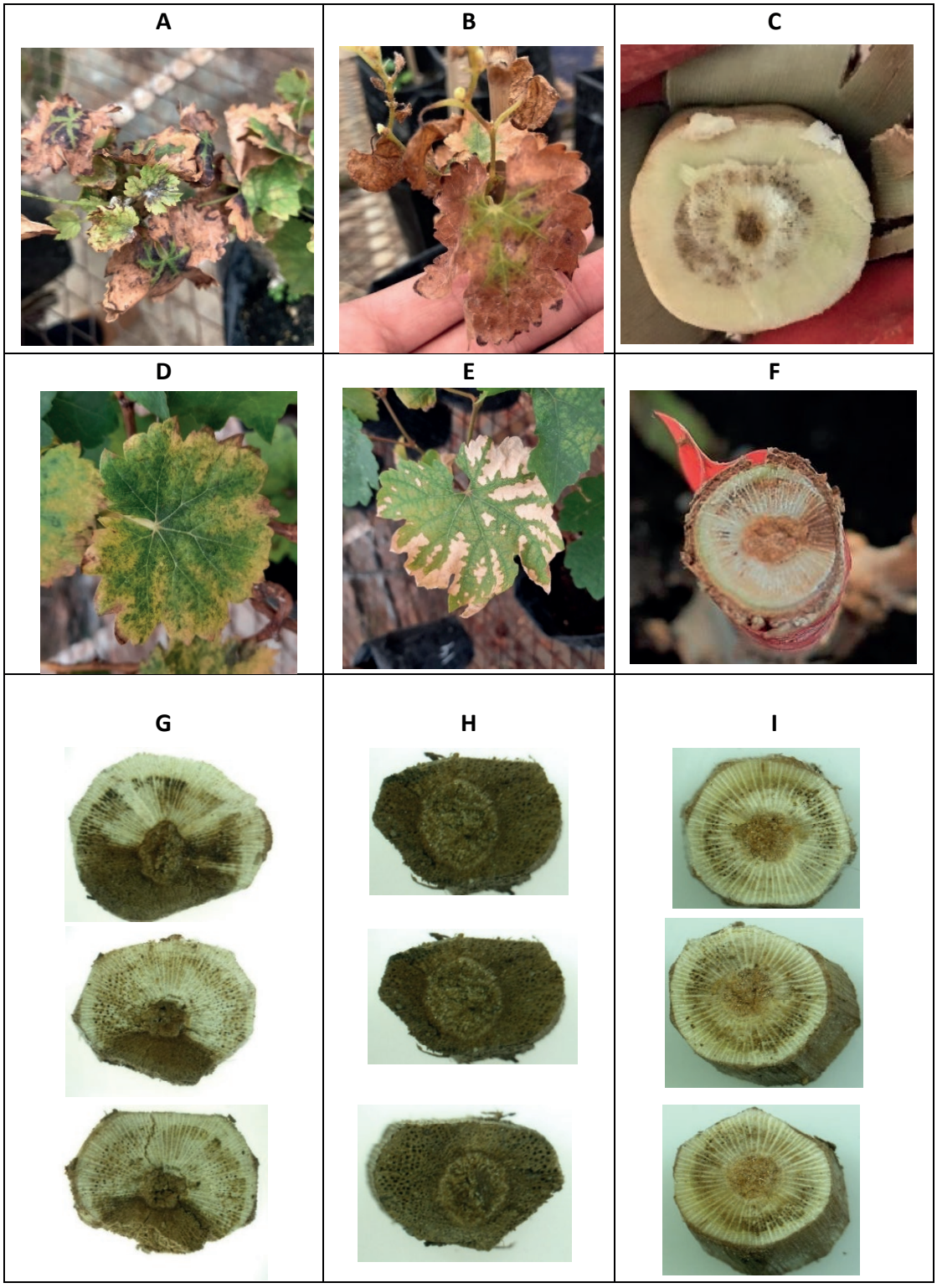


**Figure 3.** Grapevine self-rooted canes inoculated with GTDs pathogens. Panels **A**, **B**, and **C** display foliar and wood symptoms of grapevine canes after inoculation with *Phaeoacremonium parasiticum* isolate. Panels **D**, **E**, and **F** show foliar and wood symptoms of grapevine canes after inoculation with *Diplodia seriata* isolate. Panels **G**, **H**, and **I** present cross sections cut at 1.0 - 3.0 cm (top to bottom) below the inoculation point from grapevine canes inoculated with different fungal isolates. Panel **G** represents canes inoculated with *Fusarium oxysporum* isolate, panel **H** with *Lasiodiplodia theobromae* isolate, and panel **I** with *Fusarium proliferatum* isolate.

**Table 4.** Mean disease severities (MDS) of grapevine trunk diseases caused by isolates of different fungi, estimated using digital image analyses of cross sections from inoculated grapevine self-rooted canes. The sections were cut from locations, 1, 2 or 3 cm above or below each inoculation point.

| Fungus isolate | MDS [b] (%) above inoculation point | | | MDS (%) below inoculation point | | | MDS % (all sections) |
|---|---|---|---|---|---|---|---|
| | 3 cm | 2 cm | 1 cm | 1 cm | 2 cm | 3 cm | |
| *Lasiodiplodia theobromae* (n = 4)[a] | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| *Diplodia seriata* (n = 2) | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| *Phaeoacremonium parasiticum* (n = 5) | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| *Phaeoacremonium rubrigenum* (n = 1) | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| *Ilyonectria liriodendra* (n = 1) | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| *Neofusicoccum parvum* (n = 2) | 97.6 | 98.4 | 100.0 | 97.5 | 96.6 | 96.6 | 97.8 |
| *Fomitopsis* spp. (n = 1) | 67.5 | 70.9 | 74.8 | 72.2 | 70.3 | 69.8 | 70.9 |
| *Fusarium oxysporum* (n = 40) | 40.3 | 42.7 | 45.0 | 40.3 | 37.1 | 35.6 | 40.2 |
| *Fusarium proliferatum* (n = 15) | 38.8 | 41.2 | 43.3 | 38.8 | 36.5 | 33.6 | 38.7 |
| *Rhizoctonia solani* (n = 8) | 35.4 | 37.3 | 40.1 | 34.9 | 32.0 | 30.3 | 35.0 |
| *Valsa sordida* (n = 2) | 29.8 | 31.9 | 34.0 | 29.9 | 28.7 | 27.5 | 30.3 |
| *Fusarium perseae* (n = 2) | 28. 0 | 30.3 | 32.8 | 27.0 | 25.3 | 23.8 | 27.9 |

[a] Number of isolates of each fungus included in the pathogenicity tests.
[b] Mean disease severities (DS) were calculated using the following formula: Disease severity relative to the healthy sections (%) = (total area of darkened tissue in a section / total area of the section) × 100.

tify the fungal pathogens present in affected plants. This one-year survey found that average GTD incidence was 44%, observed and recorded in eleven commercial vineyards in the Al Mafraq region, the main grape production area in Jordan. Plants were visually assessed, based on symptoms that are internationally associated with GTDs. Overall mean disease severity of 63% was estimated, based on visual observation of foliar symptoms and wood tissue discolouration in the grapevine trunks and affected cordons. The disease severity scale used in this field survey was detailed, and considered all previously described symptoms of all GTDs. Symptoms were divided into major and minor categories (including foliar, cordon, and main trunk symptoms, and vascular tissue discolourations). Each category was given a specific weight from the total severity value, which reflected progression of GTDs in affected vines. The purpose of this detailed scale was to give accurate estimations of overall disease severity in each assessed vine.

Results of the field survey demonstrated that GTD incidence and severity varied among different cultivars in the different vineyards. Average disease incidences were 74% for the cultivar 'Chardonnay' and 70% for 'Cabernet Sauvignon'. On these cultivars, A 2-year survey of vineyards of these cultivars in central Italy by Quaglia *et al*. (2009) showed that GTD incidence on 'Cabernet Sauvignon' was greater than 50%, and was 15% for 'Chardonnay'. Marchi *et al*. (2006) reported 10 to 25% incidence of GTDs in 'Cabernet Sauvignon' Italian vineyards, depending on the region and age of the vineyards. Lesser incidences than those recorded in the present study were reported by Andreini *et al*. (2014), where incidence of symptoms in vineyards in Tuscany (Italy) during 6 years averaged 45% for 'Cabernet Sauvignon' and 8% for 'Chardonnay'. These studies were made for emerging GTD situations in the surveyed regions, so the reported incidence levels probably did not reflect the situation throughout Italy.

In the present study, mean GTD incidences of 63% in 'Syrah' grapevines and 66% in 'Gewurztraminer' were recorded. These values are similar to those recorded by Chacón-Vozmediano *et al*., (2021), who reported these two grape cultivars among those with the most severe GTD symptom and with the greatest numbers of infected plants. The local grapevine cultivar "Zeiny" had an incidence of 58%, which was less than most of the imported grapevine cultivars surveyed in the present study. In this survey, most 'Baladi' grapevine fields were recently established in Mafraq (less than 5 years old), and disease symptoms were not common in most fields visited. Therefore, and although only a few samples from this cultivar indicated presence of GTD pathogens, further surveys are required to assess these diseases in this important cultivar. Furthermore, Pathogenicity tests conducted with this cultivar using fungal isolates identified in the present study as GTD causal agents demonstrated high susceptibility to most isolates tested. Cultivar susceptibility based on visual assessments of external symp-

toms, mainly foliar symptoms associated with GTDs, has the limitation that the causative pathogens often occur in mixed infections with those causing other diseases such as vascular wilts and root rots. Fungi causing vascular wilts and root rots, including *F. proliferatum*, *F. oxysporum, F. perseae, Verticillium* spp.*,* and *R. solani*, were frequently isolated and identified in diseased plant samples showing GTD symptoms. These pathogens were found in mixed infections with other GTD pathogens, and they made up approx. 25% of the identified fungal pathogens.

Among different fungal pathogens identified in this study, several genera are known to cause GTDs, including *Diplodia* spp., *Lasiodiplodia* spp., *Neofusicoccum* spp., *Phaeoacremonium* spp., *Ilyonectria* spp., and *Fomitiporia* spp. (Table 3).

The most prevalent fungal pathogens identified in the present study are those associated with *Botryosphaeriaceae* dieback, including *D. seriata*, *L. theobromae*, and *N. parvum*. These pathogens made up 43% of the total isolates identified in this study. *Diplodia seriata*, *L. theobromae,* and *N. parvum* have been reported as main causal agents of *Botryosphaeriaceae* dieback of grapevines in many countries, including Tunisia (Chebil *et al*., 2014), Iran (Mohammadi *et al*., 2013a), Turkey (Akgul *et al*., 2014), Spain (Elena, and Luque, 2016), China (Yan *et al*., 2013), France, Italy, Portugal, Egypt, India, Mexico, Chile, and Brazil (Úrbez-Torres, 2011, Alves *et al*., 2008).

In the present study, different *Botryosphaeriaceae* species were isolated from grapevines showing similar vascular symptoms but diverse foliar symptoms. Additionally, these fungi were isolated along with other pathogens from the same or different symptoms in individual vines. This observation has been documented by other researchers working with GTDs (Úrbez-Torres *et al*., 2006; Luque *et al*., 2009; Úrbez- Torres, 2011). Consequently, diagnoses of grapevine diseases caused by *Botryosphaeriaceae* can be difficult based only on observations of vascular and external symptoms, so accurate diagnosis utilizing isolations and/or molecular techniques are required to confirm the presence of these species.

The second most prevalent fungal pathogens isolated from grapevines in this study were fungi known to cause the Esca disease complex, including *Phaeoacremonium* spp., which made up approx. 13% of all isolates identified. Three species of *Phaeoacremonium* were identified, morphologically and using molecular methods, and these were *P. parasiticum, P. aleophilum, and P. rubrigenum*. Both *P. parasiticum* and *P. aleophilum* have been frequently reported as causal agents of Esca in mature grapevines (Bertelli *et al*., 1998; Scheck *et al*., 1998; Mugnai *et al*., 1999; Aroca and Raposo, 2009; Halleen *et al*., 2007), while *P. rubrigenum* has been recently reported from Croatia as a cause of Esca (Essakhi *et al*., 2008). In the present study, *P. parasiticum, P. rubrigenum, and P. aleophilum* were almost exclusively isolated from dark streaked or soft-yellowish wood. This observation is consistent with previous reports of association of these pathogens with discoloured softwood vascular tissue (Mugnai *et al.,* 1999; Úrbez-Torres *et al.,* 2006; Luque *et al.,* 2009; Úrbez-Torres *et al.,* 2009; Trouillas *et al*.2010). *Fomitiporia* spp., which was also identified in the present study associated with Esca, has been reported by many authors as a cause of Esca in association with *Phaeoacremonium* spp. (Fischer *et al.,* 2005; Cobos and Martin 2007; Luque *et al.,* 2009; Mohammadi *et al* 2013b; Cloete *et al.,* 2015).

*Ilyonectria liriodendri* was identified in this study as one of the GTD pathogens in Jordan. This fungus was reported as a causal agent of black foot of grapevines in California (Petit and Gubler 2007), Australia (Whitelaw-Weckert *et al*., 2007) Spain (Alaniz *et al*., 2009), Iran (Mohammadi *et al.,* 2013a), and Uruguay (Abreo *et al*., 2010). Similarly, the *Ilyonectria* sp., which was isolated at low frequency in the present study, was also previously reported from grapevines affected by black foot in British Columbia (Urbez-Torres *et al*., 2014), China (Parkinson *et al*., 2017), Uruguay (Abreo *et al*., 2010), and Turkey (Savas *et al*., 2015).

Relationships were observed between grapevine vascular symptoms and particular fungal infections. Species of *Botryosphaeriaceae* (*D. seriata, L. theobromae, N. parvum*) were mostly isolated from wedge-shaped cankers, while *P. parasiticum, P. aleophilum,* and *P. rubrigenum,* were almost exclusively isolated from dark streaked and soft-yellowish wood. These observations agree with those in previous studies, where these fungi were elsewhere isolated from the same types of vascular symptoms (Mugnai *et al.,* 1999; Úrbez-Torres *et al.,* 2006; Luque *et al.,* 2009, Úrbez-Torres *et al.,* 2009; Trouillas *et al*., 2010). Some samples collected in the present study were hypothesized to have *Eutypa* infections, because they showed symptoms of Eutypa dieback, mainly wedge-shaped wood cankers. However, *Eutypa lata* was not isolated from collected samples which had wood discolouration symptoms resembling those caused by *Eutypa lata*.

Results of the pathogenicity assessments of different isolates on different grapevine cultivars did not provide insights into cultivar susceptibility, since no significant differences in disease development or final assessments of stem cross-sections were observed among the different cultivars examined. More research is required, probably using different methodology for inoculum delivery, to accurately assess susceptibility in host cultivars to different fungal GTD pathogens.

Most of the fungal pathogens identified in this study, except for those causing vascular wilt and root rots, are reported here from Jordan for the first time. It is likely that these pathogens have recently been introduced into this country on infected transplants or on rootstocks imported from different countries, especially from Europe. This hypothesis is based on questions directed to vineyard owners about the sources of their transplants, which showed that they imported wine grapevine and some table grape cultivars from different European countries during the last 10 years. This hypothesis conclusion is supported by results from the present study, which have demonstrated the presence of many GTD pathogens previously reported from European countries.

Jordanian grape producers in the Al Mafraq area and other locations are currently experiencing severe losses due to GTDs. Currently, there are no estimates of these losses, but future studies should investigate their extent and magnitude. Results of the present study suggest that GTDs are widespread in Jordan. Most grapevine nurseries are located in Al Mafraq region, and these enterprises distribute transplants to most grape growing areas in Jordan.

Effective management of GTDs is difficult, and there are no single and simple control methods for grape trunk diseases (Mondello *et al*., 2018). The present results confirm the urgency for adopting a "national strategy" for the management of GTDs that is applied in all Jordanian grapevine production areas. Strategies using different methods can be applied by nurserymen and grape growers to limit the economic impacts of GTDs in Jordan.

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Research Papers – 12th Special issue on Grapevine Trunk Diseases

# Potential role of *Fusarium* spp. in grapevine decline

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**Summary.** Despite studies associating *Fusarium* spp. with grapevine decline since late 1970s, no consensus has been reached regarding the roles these fungi play in grapevine health. Recent studies in British Columbia, Canada, assessed prevalence of grapevine trunk diseases (GTDs) in young and mature vineyards, and the presence of GTD fungi in ready-to-plant nursery material sold in Canada. This study characterized the *Fusarium* spp. isolated from grapevines in BC by sequencing part of the translation elongation factor 1-alpha (*TEF1*) gene, and carried out pathogenicity studies to determine whether *Fusarium* plays a role in grapevine decline. *Fusarium* spp. were isolated from 9.8% of samples collected from young vines (≤ 8-year-old), and 7.3% from mature vines (> 8-year-old), showing decline symptoms in commercial vineyards. *Fusarium* was also isolated from 43.9% of ready-to-plant dormant grapevines analyzed from four nurseries. *Fusarium* incidence varied between plants within the same nursery and between plants from the different nurseries. DNA sequences of *TEF1* allowed identification of *Fusarium oxysporum*, *F. proliferatum*, *F. ramigenum*, and a *Fusarium* sp. Pathogenicity studies were conducted in 1-year-old dormant rooted 'Chardonnay' plants grafted onto '3309C' rootstock, and treatments included: i) whole plant, ii) trimming of roots, iii) cut at rootstock basal ends, and iv) trimming of roots plus cut at rootstock basal ends. Plants were inoculated using standardized methods, and were then planted in a greenhouse. *Fusarium* was compared with *Dactylonectria macrodidyma*, *D. pauciseptata* and *Ilyonectria liriodendri* used as positive controls. The *Fusarium* spp. caused necroses in rootstock roots and basal ends that were similar to those caused by black-foot fungi. *Fusarium* spp. and black-foot fungi reduced root and shoot dry weights when compared with non-inoculated controls, but no statistically significant differences were recorded for most treatments. This study is the first in Canada to identify *Fusarium* spp. from grapevines. Though *Fusarium* was common in these grapevines, pathogenicity tests suggest that the identified *Fusarium* spp. were weakly pathogenic to '3309C' rootstock grapevines.

**Keywords.** *Fusarium*, grapevine trunk diseases, *Ilyonectria*, Petri disease, translation elongation factor 1-α, *Vitis vinifera*.

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## INTRODUCTION

Grapevine trunk diseases (GTDs) are major causes of grapevine decline, resulting in economic losses to grape and wine production industries (Wicks and Davies, 1999; Siebert, 2001; Kaplan *et al*., 2016). These complex diseases are associated with many fungal species, belonging to more than 30 genera, and include black-foot and Petri disease, responsible for grapevine decline and mortality in young vines, and Botryosphaeria dieback, Eutypa dieback, Esca, and Phomopsis dieback, which reduce yields and limit the lifespan of vines in mature vineyards (Gramaje *et al*., 2018). Most GTD fungi infect grapevines through pruning wounds, but those causing black-foot are soilborne (Agustí-Brisach and Armengol, 2013). Several of these fungi are also introduced into vineyards via contaminated grapevine nursery material (Gramaje and Armengol, 2011; Billones-Baaijens *et al*., 2013; Hrycan *et al*., 2023). Some GTD fungi are known to naturally occur in the vascular systems of asymptomatic grapevines, possibly as latent pathogens (González and Tello, 2011; Kraus *et al*., 2019; Hrycan *et al.* 2020).

*Fusarium* is a species-rich genus, which includes important plant pathogens causing root and crown rots, wilts, blights, and/or cankers in a wide range of annual and perennial crops (Gordon, 2017). Marais (1979) reported *Fusarium* spp. to be commonly isolated from roots and rhizosphere soil of stunted, dying or dead grapevines in South Africa. Grasso (1984) associated death of young grapevines with *Fusarium oxysporum* and *'Cylindrocarpon' destructans* in Sicily, Italy. Highet and Nair (1995) reported *F. oxysporum* to be prevalent in roots of declining 'Semillon' grapevines in the Hunter Valley, Australia. Omer *et al.* (1995), and Granett *et al.* (1998) isolated *F. oxysporum*, *F. roseum* and *F. solani* in high frequencies from roots damaged by grape phylloxera in self-rooted 'Chardonnay' and 'AXR#1' rootstock vines collected from commercial vineyards in California (CA), United States of America. Luque *et al.* (2009) isolated *Fusarium* spp. from wedge-shape cankers, central necroses and wood decay in declining vines from mature vineyards in Spain. Studies from different countries have continued to report *Fusarium* spp. as prevalent in roots, cordons, and/or trunks of grapevines showing decline symptoms (Ziedan *et al*., 2011; Abdullah *et al.* 2015; Chehri, 2017; Bustamante *et al*., 2022; Zhang *et al*., 2023).

*Fusarium* spp. have been associated with declining grapevines from young and mature vineyards and in grapevine nursery plants. Halleen *et al.* (2003) reported *Fusarium* spp. as the most frequently isolated fungi from asymptomatic rooted cuttings collected 3 months after planting from different commercial nurseries in South Africa. A survey in South Africa by van Coller (2004) identified up to ten *Fusarium* spp. from roots and crowns of declining nursery grapevines. Studies across several nurseries in Spain reported six different *Fusarium* spp. isolated from plants showing decline or vascular wilt symptoms (Pintos *et al*., 2018; Astudillo-Calderon *et al*., 2019). Akgül *et al.* (2023) confirmed *Fusarium* spp. to be commonly found in asymptomatic nursery-produced plants from different geographical regions in Türkiye.

Despite all these studies, the role of *Fusarium* as a true pathogen of grapevines remains unclear. Pathogenicity studies have shown *F. annulatum*, *F. commune*, *F. decemcellulare*, *F. equiseti*, *F. oxysporum*, and *F. solani* are virulent on grapevines, and cause similar symptoms to those in declining young and/or mature vines in nurseries or commercial vineyards (Highet and Nair, 1995; Omer et al., 1999; Ziedan *et al*., 2011; Chehri, 2017; Astudillo-Calderon *et al*., 2019; Bustamante *et al*., 2022; Zhang *et al*., 2023). In contrast, other studies have concluded that *Fusarium* spp. to not play important roles in grapevine decline, and refer to these fungi as common endophytes in *Vitis vinifera* (Marais, 1980; Casieri *et al*., 2009; Pancher *et al*., 2012). Therefore, *Fusarium* spp. found in many studies have not been included in completion of Koch's postulates (Rumbos and Rumbou, 2001; Halleen *et al*., 2003; Luque *et al*., 2009; Moreno-Sanz *et al*., 2013).

Grapevine trunk diseases and consequent vine decline and mortality have been identified as one of the main threats to long-term economic sustainability of the grape and wine industries in British Columbia (BC), Canada. To date, over 40 different fungal species have been identified from young and mature vines showing GTD symptoms (Úrbez-Torres *et al*., 2014a; 2014b; 2015a). These studies have routinely isolated *Fusarium* spp. from declining grapevines in the field or from ready-to-plant nursery vines. Accordingly, the objectives of this study were: i) to characterize the different *Fusarium* spp. isolated from grapevines from commercial vineyards and nursery plants in BC using molecular methods; and ii) to complete pathogenicity studies to determine the role of *Fusarium s*pp. in grapevine health.

## MATERIALS AND METHODS

### Fusarium *isolates used in this study*

*Fusarium* isolates were obtained from grapevine samples collected from young and mature commercial vineyards in BC during field surveys conducted between 2010 and 2013 (Úrbez-Torres *et al*., 2014a; 2014b). In

total, 215 and 248 vine samples (entire or part of the vine) showing decline symptoms were collected from 90 young vineyards (≤ 8-years-old) and 101 mature vineyards (> 8-years-old). Fungal isolations were conducted as described by Úrbez-Torres *et al.* (2014a). In addition, 362 ready-to-plant dormant rooted vines, including different scion-rootstock combinations and self-rooted cultivars, were collected between 2014 and 2021 from four nurseries selling grapevines in Canada. Isolations to identify different fungi were conducted from plant roots, basal ends of the rootstocks, graft unions, and scions, with the exception of self-rooted plants, from which samples were taken from roots, scion basal ends, and scions, as described by Hrycan *et al.* (2023). Wood pieces from the different host sections were plated on potato dextrose agar (PDA) (Difco) in Petri plates, amended with tetracycline hydrochloride (0.01%) (Sigma-Aldrich) (PDA-tet), and then incubated at 22°C in the dark until fungal colonies were observed. *Fusarium*-like colonies obtained from sample wood were sub-cultured on PDA. Hyphal tip subculturing onto PDA was then conducted from actively growing colonies, and these pure subcultures were held at 22°C in the dark. Based on colony morphology, a number of isolates representing different geographical locations, host cultivars and nurseries were chosen for molecular and pathogenicity studies. Fifteen to 20 mycelium plugs (0.5 cm diam.) were obtained from each selected pure culture isolate and placed in clear screw-cap glass vials containing autoclaved micropore filtered water. The vials were then maintained at 4°C in the Plant Pathology fungal collection at the Summerland Research and Development Centre (SuRDC), Summerland, BC, Canada until used.

### *Molecular identification of* Fusarium *isolates*

*Fusarium* isolates selected for DNA extraction were retrieved from the SuRDC fungal collection and revived on PDA at 22°C in the dark. DNA was extracted from actively growing colonies using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc.). Oligonucleotide primers EF1 and EF2 were used to amplify part of the translation elongation factor 1-α (*TEF1*) (O'Donnell *et al.*, 1998). Each polymerase chain reaction (PCR) contained 2 µL of 10× PCR buffer, containing 15 mM $MgCl_2$, 1.6 µL of 25 mM MgCl2, 1 µL of 10 mM dNTPs, 0.5 µL of 0.5 mM of each primer, 0.1 µL of Ultra Therm DNA polymerase (BocaScientific), and 1 µL of DNA template, adjusted with micropore filtered water to a final volume of 20 µL. Amplification reactions were carried out on a GeneAmp 2700 thermal cycler (Applied Biosystems), with an annealing temperature (T) of 53°C (Geiser *et al.*, 2004). Amplified products were purified using a QIAquick PCR purification Kit (QIAGEN Inc.), and both forward and reverse strands of the *TEF1* were sequenced using a 8-capillary 3500 Genetic Analyzer (Applied Biosystems) at the SuRDC. Sequences were edited and assembled using DNASTAR SeqMan™ Ultra version 17.4.1 (DNASTAR Inc.). Consensus sequences were then subjected to BLASTn queries in the GenBank database to determine species identifications. *Fusarium* sequences collected in this study were deposited into GenBank, and representative isolates are maintained in the SuRDC fungal collection.

### *Pathogenicity study*

Four isolates representing the *Fusarium* spp. identified in this study were selected to inoculate dormant rooted 'Chardonnay' vines grafted onto '3309C' rootstocks. *Dactylonectria macrodydima* (SuRDC-1207), *Dactylonectria pauciseptata* (SuRDC-1248) and *Ilyonectria liriodendri* (SuRDC-1203) isolates, previously identified from black-foot symptomatic vines in BC (Úrbez-Torres *et al.*, 2014a), were included in the study as positive controls. Conidium suspensions from the different *Fusarium* spp. and black-foot fungi were prepared as described by Úrbez-Torres *et al.* (2014a). Plants were inoculated by immersion of the roots and basal end of each plant in a suspension ($10^5$ conidia $mL^{-1}$) of each fungus in a separate bucket, and left overnight. Four different treatments were included in this study. These were: 'whole plant' (WP, no cut roots and basal end not cut), 'roots cut' (RC), 'basal end cut' (BC), and 'roots and basal end cut' (RBC). Six plants per fungus/treatment combination were used. Six plants for each treatment were immersed in separate buckets containing non-inoculated autoclaved distilled water as negative controls. After incubation (for approx. 18 h), the plants were retrieved from the buckets and planted into pots (22 L capacity) containing doubled-autoclaved standard potting mix soil, in a greenhouse. All plants were watered and fertilized equally and as needed. Six months after planting, the plants were uprooted and a small portion of roots and rootstock basal end of each plant were collected for fungus re-isolations. These were carried out from the roots (R), basal end (BE) and 1 cm above the basal end (1-BE) of each plant. Fungal isolations and shoot and root dry weights were recorded from each plant using the methods of Úrbez-Torres *et al.* (2014a).

Data analyses were carried to determine effects of fungal isolate and experimental treatments on shoot and root dry weights. These data were square root transformed to normalize residuals. Normality was confirmed

using inspection of residuals and Shapiro-Wilk tests. Shoot and root weights were analyzed using analysis of variance and Tukey's Honest Significant Difference test in R Software version 4.3.0 (R Core Team, 2023).

## RESULTS

### *Incidence of* Fusarium *in young and mature vineyards in BC, and in nursery grapevines.*

*Fusarium* was detected in 21 of 215 samples (9.8%) collected from young grapevines (≤ 8 years old) showing decline symptoms in commercial vineyards, and *Fusarium* isolates were mostly obtained from necrotic roots and vascular necroses observed around the stem pith at the bases of rootstocks or self-rooted cultivars (Figure 1). *Fusarium* was detected from 18 of 248 of samples (7.3%) collected from mature vines (> 8 years old) showing dieback in commercial vineyards, and was isolated from different host symptoms, including necrotic roots, wedge-shape cankers, vascular necroses and wood decay in cordons and trunks (Figure 1). Incidence of *Fusarium* in ready-to-plant nursery grapevines is shown in Table 1. *Fusarium* was isolated from 159 of 362 (43.9%) of the ready-to-plant dormant grapevines analyzed from all the assayed nurseries. Overall, the greatest infection was recorded from the graft-union sections of plants, followed by the base of the rootstocks or self-rooted cultivar roots and scions. Nursery C-2021 had the greatest level of infections, with 86.7% of plants yielding *Fusarium*. Nurseries A-2019 and D-2021 followed with, respectively, 70% and 78% of their plants infected with *Fusarium*. Nurseries A-2014 (31.8%) and B-2014 (20%) had the least *Fusarium* infection proportions (Table 1). *Fusarium* incidence also varied depending on the assessment year. Nursery A was sampled in 2014 and 2019. Though the number of plants analyzed in 2019 was less than in 2014, *Fusarium* incidence in 2019 was more than double than in 2014 (Table 1). Incidence of *Fusarium* in the different parts of the plants varied between plants within each nursery, and between nurseries. For example, roots and rootstock bases yielded, respectively, the greatest (73.1%) and least (15.4%) *Fusarium* incidence in nursery C-2021. In contrast, roots and rootstock bases yielded, respectively, the least (4.3%) and greatest (44.9%) *Fusarium* incidence nursery A-2014 (Table 1).

### *Molecular identification of* Fusarium *isolates*

In total, 311 *Fusarium* isolates were obtained in this study, 39 from commercial vineyards and 272 from ready-to-plant dormant rooted nursery plants. Eight isolates from commercial vineyards and 49 isolates from nursery plants were selected for molecular identifications. PCR amplification of the *TEF1* gave products between 650 and 750 bp. BLASTn analyses of the consensus sequences identified four species, including *F. oxysporum*, *F. proliferatum*, *F. ramigenum*, and a *Fusarium* sp. (Table 2). All four species were identified from samples collected from declining vines in commercial vineyards, and *F. oxysporum* and *F. proliferatum* were identified from nursery plants. *Fusarium proliferatum* was the most commonly isolated species from nursery plants (39 of 49 isolates). All *F. oxysporum* and *F. proliferatum* isolates from BC had 100% identity (100% query cover) with previously identified and published sequences in GenBank. Similarly, *F. ramigenum* isolates PARC425 had 99.84% similarity (100% query cover) to GenBank sequences. Isolate PARC428 had 95.40% similarity to *Fusarium napiforme* Genbank, so these isolates were classified as *Fusarium* sp. (Table 2).

### *Pathogenicity studies*

*Fusarium* spp. caused root necroses in '3309C' in all treatments, including when roots were not cut, and the symptoms were similar to those caused by the black-foot fungi used as positive controls. Statistical analyses showed that effects on mean root dry weights, of isolate ($F$ = 5.813, $P$ = 7.24E$^{-06}$), treatment ($F$ = 2.784, $P$ = 0.04354), and the isolate by treatment interaction ($F$ = 2.069, $P$ = 0.00728) were statistically significant. The effect of isolate on mean shoot dry weight ($F$ = 4.868, $P$ = 6.81E$^{-06}$) was significant, but this was not the case for treatment ($F$ = 0.582, $P$ = 0.628) or the isolate by treatment interaction ($F$ = 1.35, $P$ = 0.156). Though not significantly different from the control plants, most inoculated isolates reduced plant root dry weights compared with the non-inoculated controls (Table 3). *Ilyonectria liriodendri* was the only fungus that increased mean shoot dry weight for inoculated plants with the basal ends cut (Table 3). Among all the isolates and treatments, only *D. macrodidyma* and *F. proliferatum* significantly reduced root dry weight when inoculated onto plants with cut roots or basal ends. Similarly, *Fusarium* sp. isolate PARC428 significantly reduced root dry weight when the inoculated plants had cut ends (Table 3). A similar trend was observed for shoot dry weights. With few exceptions, where the inoculated plants had greater shoot dry weights than the non-inoculated controls, most isolates reduced mean shoot dry weights after all of the treatments, but none of these were statistically different from the controls (Table 3).

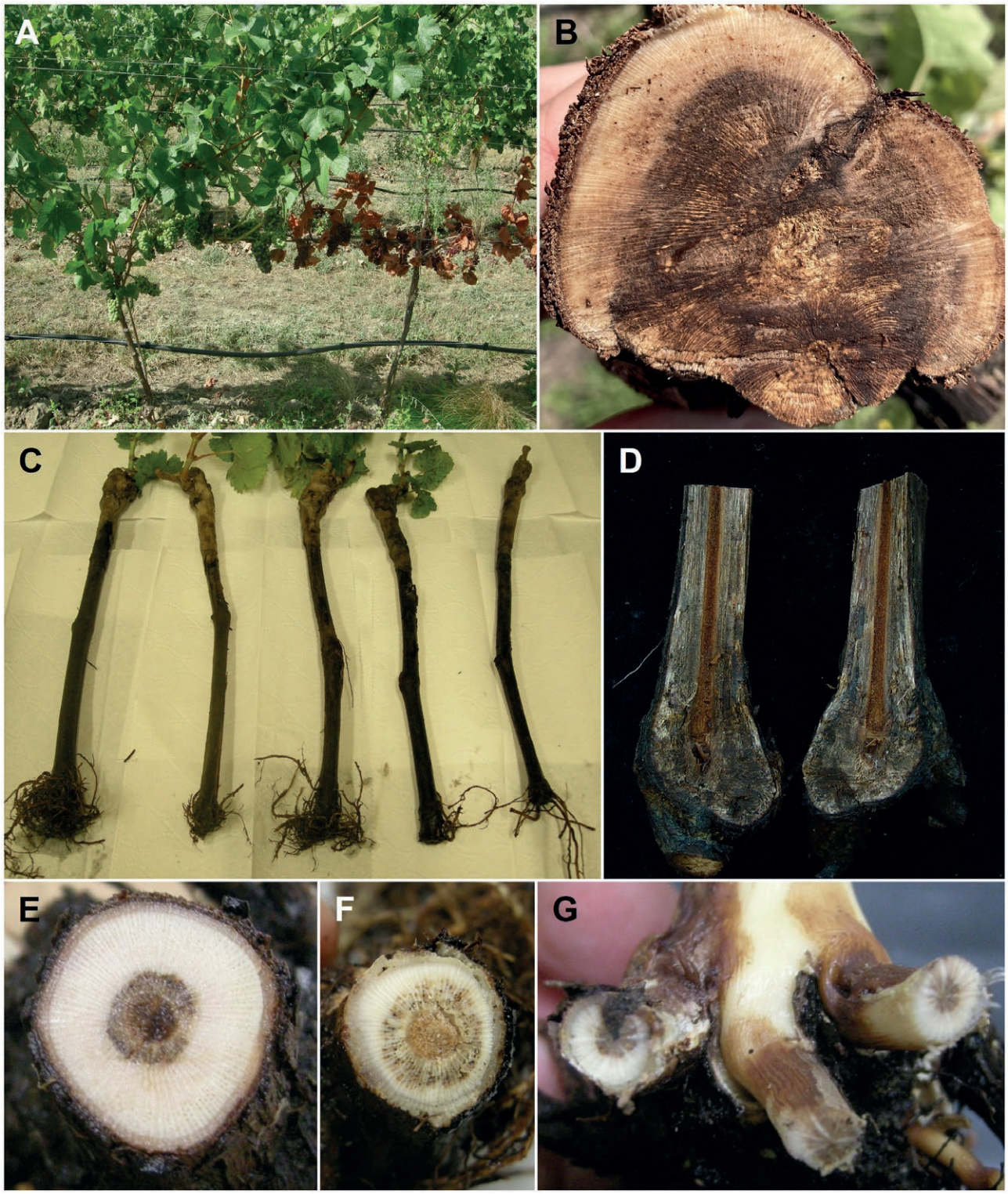


**Figure 1.** Grapevine decline symptoms from which *Fusarium* spp. were isolated in British Columbia, Canada. A, Decline symptoms and collapse observed in a young 'Merlot' vine in a commercial vineyard. B, Perennial canker, necrosis and soft wood symptoms observed at the base of the trunk of a mature 'Siegerrebe' vine in a commercial vineyard. C, Young vines showing poor shoot growth and root development. D, Vascular necrosis at a rootstock basal end. E and F, Vascular necrosis around the pith of a ready-to-plant nursery vine. **G**, Root necrosis and discolouration in a young vine from a commercial vineyard.

**Table 1.** Total numbers (and percentages) of plants and plant parts infected with *Fusarium*, that were analyzed from four grapevine nurseries.

| Nursery[a] | Plants | | Roots | Base | Graft-Union | Scion |
|---|---|---|---|---|---|---|
| | Total | Inf. (%)[b] | Inf. (%)[c] | Inf. (%)[c] | Inf. (%)[c] | Inf. (%)[c] |
| A-2014 | 217 | 69 (31.8) | 3 (4.3) | 31 (44.9) | 27 (39.1) | 16 (23.2) |
| A-2019 | 45 | 35 (77.8) | 11 (31.4) | 20 (57.1) | 23 (65.7) | 11 (31.4) |
| B-2014 | 40 | 8 (20.0) | 4 (50) | 3 (37.5) | - | 3 (37.5) |
| C-2021 | 30 | 26 (86.7) | 19 (73.1) | 4 (15.4) | 12 (46.2) | 5 (19.2) |
| D-2021 | 30 | 21 (70.0) | 12 (57.1) | 4 (19.0) | 14 (66.7) | 3 (14.3) |
| TOTAL | 362 | 159 (43.9) | 58 (35.6) | 62 (39.0) | 76 (47.8) | 38 (23.9) |

[a] Different letters correspond to different nurseries, and different numbers indicate the years when samples were analyzed from each nursery.
[b] Number (and percentage) of the total analyzed plants that were infected with *Fusarium*.
[c] Number (and percentage) of different plant parts infected with *Fusarium*.

Re-isolation proportions of the different inoculated fungi depended on experimental treatment (Table 4). None of the fungal pathogens were re-isolated from the non-inoculated controls. *Fusarium solani*, *Ilyonectria robusta* and *I. torresensis* were isolated from roots of some control plants, from the 'whole plant' and 'roots trimmed' treatments. Black-foot pathogens and *Fusarium* spp. were re-isolated from between 20 and 100% from the roots of 'whole plants'. Re-isolation percentages from roots were greater for all fungi in the 'roots trimmed' treatment (60 to 100%), and were even greater (80 to 100%) from the 'roots and basal end cut' treatment (Table 4). Only *I. liriodendri*, *D. macrodidyma* and *Fusarium* sp. PARC428 were re-isolated from the basal ends of 'whole plants', though at low proportions (20 to 40%). Re-isolation percentages were greater from the basal ends from the 'roots trimmed' (20 to 60%), 'basal end cut' (40 to 100%) and 'roots and basal end cut' (80 to 100%) treatments (Table 4). No fungal pathogens were re-isolated from 1 cm above the basal ends from the 'whole plant' treatment, and very re-isolation was recorded for *D. pauciseptata*, *F. oxysporum* and *F. proliferatum* from the 'roots trimmed' treatment (Table 4). Re-isolation of all fungi from 1 cm above the basal ends increased from the 'basal end cut' (20 to 60% isolation) or 'roots and basal end cut' (20 to 80%) treatments (Table 4).

## DISCUSSION

Seventeen different *Fusarium* species have been isolated and identified from asymptomatic and symptomatic grapevines (Highet and Nair, 1995; van Coller, 2004; Chehri, 2017; Kraus *et al.*, 2019; Bustamante *et al.*, 2022; Li *et al.*, 2023). The present study identified *F. oxysporum*, *F. proliferatum*, *F. ramigenum*, and a potentially novel *Fusarium* sp., from young and mature grapevines with GTDs symptoms, from vineyards in BC, Canada. Previous studies have identified more than 40 fungal species belonging to 14 genera, from declining vines in BC (Úrbez-Torres *et al.*, 2014a; 2014b; 2015a). The present study adds *Fusarium* spp. to the group of fungi associated with GTD symptoms in Canada.

Molecular identification of *Fusarium* spp. in this study was achieved using amplification of part of the *TEF1* using primers EF1 and EF2 (O'Donnell *et al.*, 1998), since this approach has been reported to be the most informative for identification and discrimination among *Fusarium* spp. (Geiser *et al.*, 2004). In addition, the use of short sequences obtained from other *TEF1* primers has been shown wrongly identify *Fusarium* spp. (Torres-Cruz *et al.*, 2022). Other loci, such as the RNA polymerase largest (*RPB1*) and the second largest subunit (*RPB2*) have been shown to effectively discriminate among *Fusarium* spp. in single or multi-loci phylogenetic analyses (O'Donnell *et al.*, 2013). Identification of isolates in the present study as belonging to the *F. oxysporum* species complex and as *F. proliferatum* and *F. ramigenum* using the *TEF1* is likely to be accurate, but further research including the *RPB1* and/or *RPB2* would allow further characterization of isolate PARC428, and determining if it is a novel *Fusarium* sp. or belong to an already known species.

*Fusarium* was isolated from 8.4% of samples collected from young and mature grapevines showing different vascular symptoms in roots, trunks, cordons, and spurs. Previous studies investigating fungi associated with GTDs symptoms or diversity of fungal communities in grapevine wood have identified *Fusarium* spp. at different levels of abundance. Marais *et al.* (1979), Granett *et al.* (1998), Abdullah *et al.* (2015), Moreno-Sanz *et al.* (2013), and Li *et al.* (2023) found *Fusarium* spp. to be among the most prevalent fungi isolated from mature grapevines showing decline symptoms. In contrast, other studies have reported incidence of *Fusarium* to be low in grapevines with characteristic GTD symptoms (Luque *et al.*, 2009). The incidence of *Fusarium* isolated from vines with GTD symptoms in vineyards in BC was low, and these results were similar to those reported by Luque *et al.* (2009). Since *Fusarium* spp. are primarily soilborne, the low incidence of *Fusarium* in the present study could be because most of the samples analyzed were symptomatic aerial plant parts, such as cordons and spurs. Incidence of *Fusarium* in the present study was greater

**Table 2.** *Fusarium* isolates from grapevines from British Columbia identified in this study.

| Isolate[a] | Species | Host | Source[b] | *TEF1*[c] |
|---|---|---|---|---|
| SuRCD-1207* | *Dactylonectria macrodydima* | *V. riparia* x *V. rupestris* | 3309C (base)[f] | KF511989 |
| SuRDC-1248* | *Dactylonectria pauciseptata* | *V. riparia* x *V. rupestris* | 3309C (base)[f] | KF511982 |
| PARC420* | *Fusarium oxysporum* | *V. riparia* x *V. rupestris* | 3309C (base)[f] | OR398349 |
| PARC422 | *Fusarium oxysporum* | *Vitis vinifera* | Riesling (trunk)[f] | OR398350 |
| SuRDC-1271 | *Fusarium oxysporum* | *Vitis riparia* | Riparia Gloire (base)[n] | OR398356 |
| SuRDC-1282 | *Fusarium oxysporum* | *V. riparia* x *V. rupestris* | 101-14 (base)[n] | OR398353 |
| SuRDC-1283 | *Fusarium oxysporum* | *V. riparia* x *V. rupestris* | 101-14 (base) [n] | OR398354 |
| SuRDC-1296 | *Fusarium oxysporum* | *V. riparia* x *V. rupestris* | 101-14 (base) [n] | OR398358 |
| SuRDC-1298 | *Fusarium oxysporum* | *V. riparia* x *V. rupestris* | 101-14 (graft-union)[n] | OR398359 |
| SuRDC-1300 | *Fusarium oxysporum* | *V. riparia* x *V. rupestris* | 3309C (base)[n] | OR398355 |
| SuRDC-1306 | *Fusarium oxysporum* | *V. berlandieri* x *V. riparia* | SO4 (base)[n] | OR398351 |
| SuRDC-1307 | *Fusarium oxysporum* | *V. berlandieri* x *V. riparia* | SO4 (base)[n] | OR398352 |
| SuRDC-1308 | *Fusarium oxysporum* | *V. berlandieri* x *V. riparia* | SO4 (base)[n] | OR398357 |
| PARC40 | *Fusarium proliferatum* | *Vitis vinifera* | Chardonnay (roots)[f] | OR398360 |
| PARC45 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (cordon)[f] | OR398361 |
| PARC64 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (roots)[f] | OR398362 |
| PARC416* | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[f] | OR398367 |
| SuRDC-1256 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | SO4 (base)[n] | OR398390 |
| SuRDC-1258 | *Fusarium proliferatum* | *Vitis riparia* | Riparia Gloire (base)[n] | OR398379 |
| SuRDC-1260 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (scion)[n] | OR398386 |
| SuRDC-1261 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[n] | OR398402 |
| SuRDC-1262 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (base)[n] | OR398389 |
| SuRDC-1263 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (scion)[n] | OR398392 |
| SuRDC-1264 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[n] | OR398394 |
| SuRDC-1265 | *Fusarium proliferatum* | *Vitis vinifera* | Pinoy Noir (scion)[n] | OR398383 |
| SuRDC-1266 | *Fusarium proliferatum* | *Vitis vinifera* | Chardonnay (scion)[n] | OR398363 |
| SuRDC-1267 | *Fusarium proliferatum* | *Vitis riparia* | Riparia Gloire (base)[n] | OR398364 |
| SuRDC-1268 | *Fusarium proliferatum* | *Vitis vinifera* | Chardonnay (scion)[n] | OR398368 |
| SuRDC-1269 | *Fusarium proliferatum* | *Vitis riparia* | Riparia Gloire (base)[n] | OR398369 |
| SuRDC-1270 | *Fusarium proliferatum* | *Vitis riparia* | Riparia Gloire (base)[n] | OR398381 |
| SuRDC-1272 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | SO4 (graft-union)[n] | OR398370 |
| SuRDC-1273 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (scion)[n] | OR398393 |
| SuRDC-1274 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | SO4 (graft-union)[n] | OR398371 |
| SuRDC-1275 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (scion)[n] | OR398372 |
| SuRDC-1276 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | SO4 (base)[n] | OR338399 |
| SuRDC-1277 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 101-14 (graft-union)[n] | OR398373 |
| SuRDC-1278 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 101-14 (graft-union)[n] | OR398365 |
| SuRDC-1280 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (scion)[n] | OR398374 |
| SuRDC-1281 | *Fusarium proliferatum* | *Vitis riparia* | Riparia Gloire (base)[n] | OR398382 |
| SuRDC-1285 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | SO4 (graft-union)[n] | OR398380 |
| SuRDC-1286 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | SO4 (base)[n] | OR398385 |
| SuRDC-1287 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (scion)[n] | OR398395 |
| SuRDC-1288 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[n] | OR398396 |
| SuRDC-1289 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[n] | OR398387 |
| SuRDC-1290 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 101-14 (base)[n] | OR398388 |
| SuRDC-1291 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[n] | OR398375 |
| SuRDC-1292 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[n] | OR398376 |
| SuRDC-1293 | *Fusarium proliferatum* | *Vitis vinifera* | Chardonnay (scion)[n] | OR398984 |
| SuRDC-1294 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (scion)[n] | OR398377 |

(Continued)

**Table 2.** (Continued).

| Isolate[a] | Species | Host | Source[b] | *TEF1*[c] |
|---|---|---|---|---|
| SuRDC-1295 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 101-14 (graft-union)[n] | OR398378 |
| SuRDC-1297 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 101-14 (graft-union)[n] | OR398366 |
| SuRDC-1299 | *Fusarium proliferatum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[n] | OR398391 |
| SuRDC-1301 | *Fusarium proliferatum* | *Vitis vinifera* | Pinot Noir (scion)[n] | OR398400 |
| SuRDC-1302 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | 420A (graft-union)[n] | OR398397 |
| SuRDC-1303 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | SO4 (graft-union)[n] | OR398398 |
| SuRDC-1304 | *Fusarium proliferatum* | *V. berlandieri* x *V. riparia* | SO4 (base)[n] | OR398401 |
| PARC425* | *Fusarium ramigenum* | *V. riparia* x *V. rupestris* | 3309C (graft-union)[f] | OR398403 |
| PARC428* | *Fusarium* sp. | *V. riparia* x *V. rupestris* | 3309C (roots)[f] | OR398404 |
| SuRDC-1205* | *Ilyonectria liriodendri* | *V. riparia* x *V. rupestris* | 3309C (roots)[f] | KF511985 |

[a] PARC: Pacific Agri-Food Research Centre. SuRDC: Summerland Research and Development Centre. CBS: Centraal Bureau voor Schimmelcultures. * Isolates selected for the pathogenicity assessments.
[b] f: isolate obtained from commercial vineyard. n: isolate obtained from nursery .
[c] *TEF1*: Translation elongation factor 1-α.

in young vines (≤ 8 years old) primarily showing characteristic black-foot symptoms, including root rot and vascular necrosis surrounding pith tissues at the rootstock bases and in self-rooted plants. This agrees with most studies that have shown high abundance of *Fusarium* in samples from symptomatic roots and basal areas of trunks (Marais *et al*., 1979; Grannet *et al*., 1998; Highet and Nair, 1995). Therefore, in order to better understand the associating between *Fusarium* spp. and vine decline in BC, further research should include assessments of the belowground status of symptomatic plants.

*Fusarium* incidence has been reported to be usually greater in ready-to-plant nursery grapevines than in young and/or mature vines from commercial vineyards. The present study showed that 43.9% of all analyzed nursery plants were infected by *Fusarium*, and in one nursery up to 87% incidence of infection was detected. The high prevalence of *Fusarium* spp. isolated from nursery material sold in Canada agrees with previous research in other countries (Halleen et al., 2003; van Coller, 2004; Pintos *et al*., 2018; Astudillo-Calderon *et al*., 2019; Akgül *et al*., 2023).

Only two species, *F. oxysporum* and *F. proliferatum* (i.e. low species diversity) were identified from nursery stocks in the present study. These results are of pertinent because the nursery plants came from different geographical regions, so greater species diversity was expected. Previous studies have identified greater *Fusarium* species diversity from nursery plants, including *F. oxysporum* and *F. proliferatum* (van Coller, 2004; Pintos *et al*., 2018). In contrast and similarly to the present study, other investigations have identified one *Fusarium* sp. from surveyed nursery plants (Astudillo-Calderon *et al*., 2019; Zhang *et al*., 2023). Several factors could explain these differences, but since morphological identification of *Fusarium* spp. is challenging, possible loss of diversity may have occurred in the present study as a result of the initial morphological classifications. Previous studies have shown molecular detection to be more informative than traditional culturing when determining the incidence of GTD fungi from grapevine nursery material (Úrbez-Torres *et al*., 2015b; Hrycan *et al*., 2023). Only traditional plating was conducted in the present study, so *Fusarium* incidence in nursery plants may have been greater if a molecular method was used. *Fusarium* incidence varied between plants within each nursery, between sections within the individual plants, and between plants from the different nurseries. These results were similar to those of Hrycan *et al.* (2023).

Results from the present study add to those that have reported high incidence of *Fusarium* spp. in ready-to-plant nursery material (Halleen *et al*., 2003; Pintos *et al*., 2018; Astudillo-Calderon *et al*., 2019; Akgül *et al*., 2023). Previous studies have investigated GTD fungi during nursery propagation processes, and the health status of nursery plants. (Gramaje and Armengol, 2011; Billones-Baaijens *et al*., 2013; Pintos *et al*., 2018; Hrycan *et al*., 2023; Akgül *et al*., 2023). Therefore, the high incidence of *Fusarium* confirmed in nursery material in several countries should be further investigated, to determine the main sources of infections during nursery propagation processes. Furthermore, the role of *Fusarium* as a common grapevine endophyte should be investigated.

Despite the numerous reports of *Fusarium* spp. as fungal pathogens of grapevines, there is no consensus regarding the role of *Fusarium* in grapevine health and

**Table 3.** Mean root and shoot dry weights for 'Chardonnay' grapevine plants grafted onto '3309C' rootstock and inoculated with spore suspensions of different *Fusarium* and black-foot fungi, in four experimental treatments.

| Isolate / Species | Mean root dry weight (g) | | | | Mean shoot dry weight (g) | | | |
|---|---|---|---|---|---|---|---|---|
| | WP[a] | RC[b] | BC[c] | RBC[d] | WP[a] | RC[b] | BC[c] | RBC[d] |
| Control | 10.30 ± 6.84 a | 9.00 ± 4.25 a | 6.28 ± 5.27 ab | 7.66 ± 3.35 a | 6.98 ± 3.15 a | 7.47 ± 1.43 a | 4.98 ± 1.77 a | 7.62 ± 5.85 a |
| SuRDC-1205 / *I. liriodendri* | 4.02 ± 3.59 a | 5.64 ± 5.50 ab | 12.36 ± 3.90 a | 4.62 ± 2.01 a | 3.85 ± 0.81 a | 6.54 ± 2.44 a | 9.05 ± 4.18 a | 5.82 ± 1.39 a |
| SuRCD-1207 / *D. macrodydima* | 6.62 ± 1.69 a | 3.35 ± 1.16 b | 3.52 ± 0.63 b | 3.78 ± 1.60 a | 4.45 ± 1.12 a | 5.77 ± 3.11 a | 4.58 ± 0.42 a | 7.74 ± 1.38 a |
| SuRDC-1248 / *D. pauciseptata* | 3.48 ± 2.50 a | 4.38 ± 1.16 ab | 4.14 ± 2.08 ab | 5.80 ± 2.98 a | 4.74 ± 0.50 a | 5.08 ± 1.80 a | 3.05 ± 2.19 a | 3.29 ± 2.94 a |
| PARC420 / *F. oxysporum* | 6.80 ± 1.69 a | 4.04 ± 1.42 ab | 5.38 ± 2.62 ab | 4.68 ± 2.72 a | 6.65 ± 1.54 a | 8.40 ± 4.39 a | 6.82 ± 2.55 a | 7.47 ± 3.66 a |
| PARC416 / *F. proliferatum* | 5.06 ± 0.63 a | 3.76 ± 0.63 b | 3.56 ± 1.15 b | 2.42 ± 1.24 a | 6.67 ± 0.66 a | 5.60 ± 1.29 a | 5.69 ± 1.50 a | 5.17 ± 1.76 a |
| PARC425 / *F. ramigenum* | 9.46 ± 3.93 a | 4.16 ± 1.48 ab | 7.50 ± 1.43 ab | 5.60 ± 3.76 a | 7.66 ± 2.31 a | 5.42 ± 1.04 a | 7.28 ± 2.65 a | 6.75 ± 4.15 a |
| PARC428 / *F usarium* sp. | 8.70 ± 3.44 a | 7.98 ± 3.62 ab | 3.65 ± 0.91 b | 6.26 ± 2.26 a | 5.98 ± 1.59 a | 5.66 ± 2.78 a | 4.68 ± 1.37 a | 6.15 ± 1.64 a |

Means in each column accompanied by the same letter are not significantly different ($P = 0.05$), Tukey's Honest Significant Difference test.
[a] WP: 'whole plant' untouched roots and basal end of the rootstock.
[b] RC: roots cut and basal end of the rootstock untouched.
[c] BC: basal end of the rootstock cut and roots untouched.
[d] RBC: roots cut and basal end of the rootstock cut.

**Table 4.** Percentage reisolations of *Fusarium* and black-foot fungi from different parts of grapevine plants after inoculations with different pathogen isolates.

| Isolate / Species | WP[a] | | | RC[b] | | | BC[c] | | | RBC[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R[e] | BE[f] | 1 cm BE[g] | R[e] | BE[f] | 1 cm BE[g] | R[e] | BE[f] | 1 cm BE[g] | R[e] | BE[f] | 1 cm BE[g] |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| SuRDC-1205 / *I. liriodendri* | 80 | 20 | 0 | 100 | 40 | 0 | 100 | 80 | 20 | 100 | 100 | 80 |
| SuRCD-1207 / *D. macrodydima* | 60 | 20 | 0 | 80 | 40 | 0 | 60 | 100 | 20 | 100 | 100 | 40 |
| SuRDC-1248 / *D. pauciseptata* | 40 | 0 | 0 | 60 | 60 | 20 | 60 | 100 | 60 | 100 | 80 | 40 |
| PARC420 / *F. oxysporum* | 100 | 0 | 0 | 100 | 20 | 20 | 80 | 80 | 20 | 80 | 80 | 20 |
| PARC416 / *F. proliferatum* | 100 | 0 | 0 | 100 | 60 | 20 | 80 | 40 | 20 | 80 | 100 | 40 |
| PARC425 / *F. ramigenum* | 20 | 0 | 0 | 60 | 0 | 0 | 80 | 100 | 40 | 80 | 100 | 40 |
| PARC428 / *Fusarium* sp. | 20 | 40 | 0 | 60 | 0 | 0 | 60 | 40 | 20 | 80 | 80 | 40 |

[a] WP: 'whole plant' untouched roots and basal end of the rootstock.
[b] RC: roots cut and basal end of the rootstock untouched.
[c] BC: basal end of the rootstock cut and roots untouched.
[d] RBC: roots cut and basal end of the rootstock cut.
[e] R: roots.
[f] BE: basal end of the rootstock.
[g] 1 cm BE: one cm above the cut done at the basal end of the rootstock.

its association with GTD symptoms. This could be due to lack of consistency in results from pathogenicity tests conducted with *Fusarium*. Marais *et al.* (1979) reported that three isolates of *Fusarium* (species unknown) caused root rot when inoculated onto three different grape rootstocks. That study also showed no statistically significant differences on root or shoot masses between *Fusarium* inoculated plants and non-inoculated controls. These results convinced the authors that *Fusarium* was not a pathogen of grapevines, but was an endophyte,

so *Fusarium* was not included in pathogenicity studies investigating effects of other soilborne fungi on grapevine health (Marais *et al.*, 1980). In contrast, Highet and Nair (1995) showed that *F. oxysporum* caused root rot on 99% of inoculated self-rooted 'Semillon' plants, although no other plant health parameters were measured. Similarly, Ziedan *et al.* (2011) fulfilled Koch's postulates when all the *F. oxysporum* isolates used in that study caused root rot and wilting of self-rooted 'Crimson' plants. Pathogenicity studies have also confirmed *F. equiseti* as a cause of root rot and wilting on 'Tempranillo' grafted onto '110R' (Astudillo-Calderon *et al.*, 2019), *F. annulatum* to cause vascular necroses on 'Chardonnay' (Bustamante *et al.*, 2022), and *F. commune* to cause root necroses and yellowing of leaves on 'Marselan' (Zhang *et al.*, 2023), but no other plant health parameters were considered in these studies.

In the present study, all four assessed *Fusarium* spp. caused root necroses in '3309C' plant, from all the experimental treatments including when roots were not cut. These symptoms were similar to those caused by the black-foot isolates used as positive experimental controls. Plants inoculated with *Fusarium* and black-foot isolates had reduced root and shoot dry weights when compared with negative controls, no matter the experimental treatment. However, with very few exceptions, data analyses gave no statistically significant differences between inoculated and non-inoculated plants. The results are similar to those reported by Marais (1979), and similar results were obtained with the well-known pathogens *D. macrodidyma*, *D. pauciseptata*, and *I. liriodendri*. *Fusarium* and black-foot fungi were re-isolated from roots from the 'whole plant' treatment, suggesting that these fungi do not need wounds for entry into host root tissues. However, results from the present study showed that fungal colonization of roots and rootstock basal ends increased when plants were wounded. Fungi were also capable of colonizing up to 1 cm above the basal ends when wounded. These results are similar to those reported by Grannett *et al.* (1998), where *Fusarium* spp. were recovered in greater incidence from roots damaged by the grape-phylloxera in CA, than from undamaged roots.

*Fusarium* may be a secondary pathogen on grapevines, as reported by Marais (1979), and wounded host tissues would facilitate vascular colonization. This is an important result, because poor quality nursery material with weak roots or poor callusing at the rootstock bases could be susceptible to *Fusarium* colonization. The differences observed among pathogenicity studies could be due to differences in host susceptibility to *Fusarium*. Omer *et al.* (1999) assessed effects of *Fusarium* on eight grapevine rootstocks, and concluded that rootstock type played a significant role in infection. 'Self-rooted' 'Carignan' and AXR#1 rootstocks were the most susceptible to root necrosis, while *Fusarium* infections did not cause necroses on '3309C', '420A', '5C', and 'Freedom'. In the present study, *Fusarium* spp. caused root necrosis on '3309C'. Based on available studies, including the present one, *Fusarium* spp. were more successful at causing root necrosis and plant wilt on 'self-rooted' *V. vinifera* than on rootstocks. This could explain why *Fusarium*, though present in grapevines, may not be a primary cause of grapevine decline, as most vineyards have vines grafted onto phylloxera resistant rootstocks. Self-rooted vines from nursery B-2014 showed the least *Fusarium* infection when compared with the rootstock material from the other nurseries. This could have given low *Fusarium* inoculum levels in nursery B-2014 during the propagation process, or could have resulted from effective sanitation strategies applied by that nursery (Gramaje and Armengol, 2011). However, since graft unions were the plant parts in the other three nurseries from which *Fusarium* was most isolated, lack of graft-union wounding in self-rooted material most likely resulted in the low incidence observed.

It is well-known that several GTD fungi occur in asymptomatic grapevines, so it has been suggested that these fungi may be latent pathogens transitioning from endophytic to pathogenic states under abiotic and/or biotic host stress conditions. Initial inoculum concentrations of these fungi may also affect this transition (Hrycan *et al.*, 2020). Results from the present study showed *Fusarium* to occur in declining field-grown vines, and at high incidence in ready-to-plant nursery material. However, the pathogenicity studies indicated that *Fusarium* was an opportunist or weak pathogen of grapevines. The same conclusion could be made from the pathogenicity tests with the three black-foot fungi used as positive controls. Abiotic and biotic factors have been associated with disease severity and fungal growth in other pathosystems. Light soil types increased severity of soybean Fusarium root rot symptoms compared with symptoms developing in sandy loam and silt loam soils (Yan and Nelson, 2022). *Fusarium pseudograminearum* biomass increased in drought stressed barley seedlings (Liu and Liu, 2016). For biotic factors, Li *et al.* (2023) reported that *Fusarium* spp. were more prevalent in GTD-symptomatic grapevine roots and rhizospheres than in for asymptomatic plants. That study also showed that disease indices were increased when *Fusarium* spp. were co-inoculated with *D. macrodidyma* than with individual inoculations, suggesting that *Fusarium* could enhance disease severity when in the presence of other GTD

fungi. Further research should investigate *Fusarium* inoculum thresholds in ready-to-plant nursery grapevine material and determine correlation of this information with studies assessing effects of abiotic and/or biotic factors on *Fusarium* infected grapevines. Also, there is no information from *Fusarium* pathogenicity studies conducted under natural field conditions. These types of research will increase understanding of the roles of *Fusarium* spp. in grapevine health.

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# Grapevine dieback caused by *Botryosphaeriaceae* species, *Paraconiothyrium brasiliense, Seimatosporium vitis-viniferae* and *Truncatella angustata* in Piedmont: characterization and pathogenicity

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**Summary.** Grapevine Trunk Diseases (GTDs) are major threats in Mediterranean countries, causing economic losses due to reduced grape yields and long-term vine productivity, as well as death of grapevines. A survey was conducted in Piedmont (Northern Italy) during 2021-2022 to investigate the species diversity and distribution of GTD pathogens in this important Italian wine region. Morphological and multilocus phylogenetic analyses (based on ITS, *tef1*, *tub2*, *act* and *rpb2*) identified species of *Botryosphaeriaceae* at high frequency, including *Botryosphaeria dothidea*, *Diplodia mutila, Diplodia seriata* and *Neofusicoccum parvum*. Other pathogens commonly associated with GTDs, including *Eutypa lata*, *Fomitiporia mediterranea* and *Phaeomoniella chlamydospora*, were also isolated. Less commonly isolated species included *Neocucurbitaria juglandicola, Paraconiothyrium brasiliense*, *Seimatosporium vitis-viniferae* and *Truncatella angustata*. Pathogenicity tests with two representative isolates of each species were carried out using one-year-old potted grapevine cuttings ('Barbera'). All isolates (except *N. juglandicola*) caused brown wood necrotic vascular discolourations on inoculated plants and were successfully re-isolated. Effects of temperature on colony growth were also assessed. For all tested isolates there was no growth at 5°C, only four isolates (*Botryosphaeriaceae*) grew at 35°C, and optimum growth temperatures were between 20 and 25°C. This is the first record of *Paraconiothyrium brasiliense* and *Neocucurbitaria juglandicola* associated with symptomatic grapevines in Italy.

**Keywords.** Botryosphaeria dieback, *Neofusicoccum, Neocucurbitaria juglandicola,* pathogenicity, Grapevine Trunk Diseases.

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## INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an important cultivated crop, with a worldwide vineyard area of 6.73 million ha (FAOSTAT, 2021), mainly grown for wine and table grape production. Reports have increased of diseases caused by grapevine trunk diseases (GTDs) associated fungi causing severe economic and yield losses as a result of reduced grape quality and early plant death. Grapevine trunk diseases are severely destructive in Europe and Mediterranean countries (Guerin-Dubrana *et al*., 2019), representing major threats to vineyard productivity.

Several factors, including climate change and rapid expansion and industrialization of viticulture, are related to increased incidence and severity of GTDs (Graniti *et al*., 2000; Surico *et al*., 2004). Up to 133 pathogens belonging to nine families have been associated with GTDs (Luque *et al.,* 2009; Carlucci *et al*., 2015; Gramaje *et al*., 2018; Mondello *et al*., 2018). These xylem-colonizing fungi are predominantly found in the grapevine framework (spurs, cordons and trunk), but also in root (Gramaje *et al*., 2018).

Symptoms of GTDs vary among the different diseases, and include sectorial wood necroses (cankers), black-brown streaking (discolourations or blackening of xylem vessels), central brown-red discolouration, and white rot, while external symptoms include leaf necrosis and chlorosis with typical tiger-stripes, reduced vine vigour and stunted growth, dieback of shoots and leaf drop (apoplexy). These symptoms generally lead to death of affected vines, caused by the Esca complex of diseases, decline and dieback or stunted shoots growth, including witch's broom symptoms, as in Eutypa dieback (Mugnai *et al*., 1999; Luque *et al.,* 2009; Úrbez-Torres, 2011; Bertsch *et al*., 2013; Carlucci *et al*., 2015; Gramaje *et al*., 2018; Mondello *et al*., 2018; Billones-Baaijens and Savocchia, 2019; Guerin-Dubrana *et al*., 2019; Reis *et al*., 2019; Claverie *et al*., 2020).

Grapevine trunk diseases are associated with different vascular xylem-colonizing pathogenic fungi. *Phaeomoniella chlamydospora* and several *Phaeoacremonium* spp. are responsible for Petri disease and Esca complex, the major GTDs reported in all European and Mediterranean countries. *Fomitiporia mediterranea*, in the same areas, is the most common lignin-degrading Basidiomycete fungus responsible for white rot (Surico *et al*., 2004). Different dieback diseases such as Botryosphaeria dieback, Diaporthe dieback and Eutypa dieback are associated with different species of related fungal families (Claverie *et al*., 2020).

Fungi in the Botryosphaeriaceae have cosmopolitan distribution, and have been associated with many host plants (Úrbez-Torres, 2011; Carlucci *et al*., 2015; Guarnaccia *et al*., 2022). Several species are reported to be plant pathogens while others are endophytes or saprophytes of annual or perennial plants. Over the last decade, interest in species in Botryosphaeriaceae has increased, due to importance of Botryosphaeria dieback of grapevines, where at least 26 species have been associated with this disease (Úrbez-Torres, 2011; Carlucci *et al*., 2015; Gramaje *et al*., 2018; Arkam *et al*., 2021). Botryosphaeria dieback, has been associated with different fungal genera, including *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria* and *Spencermartinsia* (Yang *et al*., 2017; Gramaje *et al*., 2018). Typical symptoms occur on grapevine trunk and shoot wood, and include wedge-shaped cankers, dark V-shaped wood necroses, and elongated black lesions in vessels. Vascular streaking, foliar discolouration, bud necrosis and severe decline can cause death of plants (Úrbez-Torres, 2011; Billones-Baaijens and Savocchia, 2019; Úrbez-Torres *et al*., 2020). Botryosphaeria dieback symptoms can discontinue but are commonly observed on mature vineyards (> 8 years), but have also been reported on 3- to 5-years-old vines (Úrbez-Torres *et al*., 2008). All GTDs pathogens can cause asymptomatic infections as latent pathogens (González and Tello, 2011; Bruez *et al*., 2014).

Among Fungi commonly associated with GTDs, several recent studies have highlighted the association of other wood-degrading fungi with symptomatic plants (Raimondo *et al*., 2019; Bekris *et al*., 2021). Numerous fungi belonging to *Neopestalotipsis*, *Truncatella*, *Seimatosporium* and *Sporocadus* have recently been reported as part of the grapevine microbiome, while their roles as causes of symptoms need to be clarified (Maharachchikumbura *et al*., 2017; Geiger *et al*., 2022; Vanga *et al*., 2022).

With more than 718,000 ha of wine and table grapevines (O.I.V, data 2022, https://www.oiv.int/it/what-we-do/country-report?oiv), Italy is leading grapevine production worldwide and represent the fourth largest vineyard acreage after Spain, France and China. Among all Italian regions, Piedmont (Northern Italy, with the Langhe area included in UNESCO's World Heritage list), is a renowned wine production region. In the last 30 years, incidence of GTDs has increased in all Italian regions, and several fungi have been reported associated with grapevines showing various symptoms (Surico *et al*., 2000; Guerin-Dubrana *et al*., 2019). Since the 1990s, studies have reported high disease incidence and mortality of plants in the first year of planting. The Esca complex, including apoplexy, is frequent and widespread in all grape growing Italian regions (Guerin-Dubrana *et al*., 2019), and reaches high incidence in climatical-

ly favourable seasons, up to 80% in mature vineyards (Romanazzi *et al*., 2009).

Botryosphaeria dieback associated with different pathogens, including *Diplodia seriata*, *Neofusicoccum parvum* and *Lasiodiplodia theobromae,* was reported in Apulia, Marche, Molise, Tuscany, Sardinia and Sicily (Burruano *et al*., 2008; Romanazzi *et al*., 2009; Spagnolo *et al*., 2014; Carlucci *et al*., 2015; Linaldeddu *et al*., 2015; Mondello *et al*., 2020), but no investigations have been carried out in Piedmont. Due to limited information on distribution of GTD related pathogens in Piedmont, the present research aims were: 1) to investigate the species diversity and distribution of GTD pathogens in Piedmont, focusing on canker agents and wood-degrading fungi associated with dead cordons, independently from detected foliar symptoms; 2) to characterize obtained isolates; and 3) to test representative isolates for pathogenicity to healthy grapevine plants.

## MATERIALS AND METHODS

### *Field sampling and isolation of fungi*

Surveys were carried out from July 2021 to November 2022, in five vineyards in the Alba and Alessandria areas of Piedmont, Northern Italy. Wood samples were collected from necrotic cordons, and from trunk portions of declining vines of 12 different grapevine cultivars (Table 1). Sampled vines were aged between 10 and 25 years, and showed dieback symptoms, including foliar discolourations, dieback and internal dark V-shaped wood necroses. The sampling method was destructive; vines were cut and transverse sections of the affected trunk and branches of each plant were examined to check for wood necroses. Each sample was reduced into small fragments, including the necrotic zone, and was sterilized in a sodium hypochlorite solution (1%) for 1 min and then rinsed in sterilized distilled water for 30 sec. Excess water was removed using sterilized filter paper. The wood samples were cut into small pieces from the margins of the necrotic zones. Five wood fragments from each sample were plated onto the surface of Potato Dextrose Agar (PDA, Merck) in a Petri plate, supplemented with streptomycin sulphate (25 ppm $L^{-1}$, PDA-S), and incubated at 25 ± 1°C. After 5 d the plates were examined. From the margins of resulting fungal colonies, single hyphal tips were cut and placed on new PDA plates, to obtain pure cultures.

### *DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing*

For recurring fungal colonies, mycelium from each 10-d-old pure culture on PDA was scraped and collected into a 2 mL capacity centrifuge tube. Total DNA was extracted directly from fresh mycelium using the

**Table 1.** Information on the vineyards surveyed and sampled in Piedmont, with respective fungi from different grapevine cultivars.

| Vineyard | | No. plants sampled | Isolated fungi | | | | |
|---|---|---|---|---|---|---|---|
| Grapevine cultivar | Location | | *Botryosphaeriaceae* spp. | *N. juglandicola* | *P. brasiliense* | *S. vitis* | *T. angustata* |
| Alba rossa | Carpeneto (AL) | 2 | + | - | - | + | - |
| Barbera | Dogliani (CN) | 2 | + | - | + | + | + |
| | Castiglione Falletto (CN) | 3 | + | - | - | - | - |
| | Fubine (AL) | 3 | + | - | - | + | - |
| Cabernet | Fubine (AL) | 2 | + | - | - | - | - |
| Cortese | Carpeneto (AL) | 2 | + | - | - | - | - |
| Erbaluce | Carpeneto (AL) | 2 | - | - | - | - | - |
| Grignolino | Carpeneto (AL) | 1 | + | - | - | - | - |
| Merlot | Fubine (AL) | 2 | + | - | - | - | - |
| Moscato | Carpeneto (AL) | 2 | + | - | - | - | - |
| Nebbiolo | Dogliani (CN) | 3 | + | - | - | - | + |
| | Monforte d'Alba (CN) | 2 | + | + | + | - | - |
| Rossese bianco | Monforte d'Alba (CN) | 1 | + | - | - | - | - |
| | Fubine (AL) | 1 | + | - | - | - | - |
| Sauvignon blanc | Carpeneto (AL) | 2 | - | - | - | - | - |
| Timorasso | Carpeneto (AL) | 2 | - | - | - | - | - |

E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions. DNA amplification and sequencing of different loci were carried out to achieve species identification. *Botryosphaeriaceae*-like isolates were characterized through DNA amplification and sequencing of the partial translation elongation factor-1α (*tef1*) gene, using the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999). For the remaining isolates, the nuclear ribosomal internal transcribed spacer (ITS) region was amplified using universal primers ITS1 and ITS4 (White *et al*., 1990), while the primers T1 and Bt2b (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997), fRPB2-5f and fRPB2-7cr (Liu *et al*., 1999), and ACT512f and ACT783r (Carbone and Kohn, 1999), were used to amplify, respectively, genes for partial beta-tubulin (*tub2)*, the fragment of the RNA polymerase II subunit 2 (*rpb2*), and γ-actin (*act*). The PCR reactions and conditions adopted for all the loci were described in the above respective cited studies. Polymerase chain reaction assays were carried out in a final 25 µL volume, using a Taq DNA polymerase kit (Qiagen) and 25 ng of DNA. Five microliters of each PCR reaction product were analyzed by electrophoresis at 100V in a 1% agarose (VWR Life Science AMRESCO® biochemicals) gel stained with GelRed™ in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA; pH 8.0). Amplified PCR fragments were sequenced by Eurofins Genomics Service (Cologne). Obtained sequences were trimmed in Geneious v. 11.1.5 (Auckland, New Zealand), and the blast function of NCBI's GenBank nucleotide database was used to determine the closest relatives of the studied isolates.

*Phylogenetic analyses*

To give an overview of isolated genera, an initial phylogenetic analysis was conducted with sequences of the partial translation elongation factor-1α (*tef1*) gene for *Botryosphaeriaceae*-like isolates, and with the nuclear ribosomal internal transcribed spacer (ITS) gene for other isolates. Subsequently, a subset of representative isolates was then selected based on the previous results, to distinguish the isolates at species level. A multilocus phylogenetic analysis was conducted using the following locus combinations: ITS and *tef1* for members of *Botryosphaeriaceae* (Pintos *et al*., 2018; Guarnaccia *et al*., 2020); ITS, *tub2* and *act* were amplified for *Paraconiothyrium* (Verkley *et al*., 2004); ITS, *tub2* and *rpb2* for *Neocucurbitaria* (Jaklitsch *et al*., 2018); and ITS, *tub2* and *tef1* for isolates related to the family *Sporocadaceae* including the genera *Truncatella* and *Seimatosporium* (Raimondo *et al*., 2019). Isolate sequences, including references downloaded from GenBank, were aligned with the software MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley, 2013), and were then manually adjusted in MEGA v.7 (Kumar *et al*., 2016). Multi-locus analyses, based on Maximum Parsimony (MP) were performed using Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 (Cummings, 2004), while MrModeltest v. 2.3 (Nylander, 2004) and MrBayes v. 3.2.5 (Ronquist and Huelsenbeck, 2003) were used for the Bayesian Inference (BI) analyses. The best nucleotide substitution model for each gene was estimated using MrModeltest. Based on obtained results for optimal setting criteria for each locus, BIs were performed using the Markov Chain Monte Carlo (MCMC) method. Four simultaneous Markov Chains were run for 1,000,000 generations starting from a random tree topology. Trees were saved each 1000 generation, while preburn and heating parameters were set, respectively, to 0.25 and 0.2. Based on burn-in fraction, the remaining trees were used to calculate the majority rule consensus tree and posterior probability (PP). The analyses stopped once the average standard deviation of split frequencies fell below 0.01. For Maximum Parsimony, phylogenetic relationships were estimated using the heuristic search option with 100 random addition sequences. Tree bisection reconnection (TBR) was used with branch swapping option as "best trees"; characters were treated as equally weighted, and gaps as fifth base. Parsimony and the bootstrap analyses were based on 1,000 replications, and tree lengths (TL), consistency indices (CI), retention indices (RI) and rescaled consistence indices (RC) were calculated. Resulting trees were visualized with FigTree version 1.4.4 (Page, 1996). Sequences generated in this study were deposited in GenBank (Table 2).

*Morphological analyses*

Two isolates of each species identified using molecular analyses were selected for the morphological observation. Mycelium plugs (each 6 mm diam.) were taken from each 10-d-old fungal colony growing on PDA and were transferred to Petri dishes containing different media. To enhance sexual sporulation or conidium production, for *Botryosphaeriaceae*-like isolates, 2% water agar supplemented with sterile pine needles (Pine Needle Agar or PNA, Smith *et al*., 1996) was used, with incubation at 25°C under near-UV light (Crous *et al*., 2006). For *Neocucurbitaria*, malt extract agar (MEA) and PDA were used, with incubation at 20°C and alternating light-dark periods (Jaklitsch *et al*., 2018). Corn meal agar (CMA), oat agar (OA) and MEA were used for *Paraconiothyrium*, incubated at 25°C under UV light. PDA, MEA

**Table 2.** List of isolates used for phylogenetic analyses, and their GenBank accession numbers.

| Species | Isolate code | Country | Host | GenBank accession number [a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | tef1 | tub2 | act | RPB2 |
| *Alloconiothyrium aptrootii* | CBS 980.95 | Papua Nuova Guinea | Soil | JX496121 | - | JX496460 | JX496347 | - |
| *Allocucurbitaria botulispora* | CBS 142452 | USA | Superficial tissue | LT592932 | - | LT593001 | - | LT593070 |
| *Bartalinia robillardoides* | CBS 122705 | Italy | *Leptoglossus occidentalis* | LT853104 | LT853202 | LT853252 | - | - |
| *Botryosphaeria corticis* | CBS 119047 | USA: New Jersey | *Vaccinium corymbosum* | DQ299245 | EU017539 | EU673107 | - | - |
| *Botryosphaeria dothidea* | CBS 110302 | Portugal | *Vitis vinifera* | AY259092 | AY573218 | EU673106 | - | - |
| *Botryosphaeria dothidea* | CBS 115476 | Switzerland | *Prunus* sp. | AY236949 | AY236898 | AY236927 | - | - |
| *Botryosphaeria dothidea* | CBS 145971 | Australia | *Grevillea* sp. | MT587332 | MT592034 | MT592470 | - | - |
| *Botryosphaeria dothidea* | CVG 1582* | Italy | *Vitis vinifera* | *OQ612689* | *OQ680488* | *OQ680508* | - | - |
| *Botryosphaeria dothidea* | CVG 1615 | Italy | *Vitis vinifera* | *OQ612691* | *OQ680490* | *OQ680509* | - | - |
| *Botryosphaeria fabicerciana* | CGMCC 3.20320 | Unknown | Unknown | MW642163 | MW651965 | MW651966 | - | - |
| *Botryosphaeria fusispora* | MFLUCC 10-0098 | Thailand | *Entada* sp. | JX646789 | JX646854 | JX646839 | - | - |
| *Botryosphaeria kuwatsukai* | KUMCC 20-0106 | Unknown | Unknown | OP714405 | - | - | - | - |
| *Botryosphaeria pseudoramosa* | CERC 2001 | China | *Eucalyptus* hybrid | KX277989 | KX278094 | KX278198 | - | - |
| *Botryosphaeria ramosa* | CBS 122069 | Australia | *Eucalyptus camaldulensis* | EU144055 | EU144070 | KF766132 | - | - |
| *Botryosphaeria wangensis* | CERC 2298 | China | *Cunninghamina deodara* | KX278002 | KX278107 | KX278211 | - | - |
| *Cucurbitaria berberidis* | CBS 142401 | Austria | *Berberis vulgaris* | MF795756 | - | MF795886 | - | MF795798 |
| *Diplodia africana* | CBS 120835 | South Africa | *Prunus persica* | MH863094 | KF766397 | KF766129 | - | - |
| *Diplodia agrifolia* | CBS 132778 | California, U.S.A. | *Quercus agrifolia* | MH866051 | MT592036 | MT592472 | - | - |
| *Diplodia corticola* | CBS 112548 | Portugal | *Quercus suber* | AY259099 | KX464559 | KX464789 | - | - |
| *Diplodia fraxini* | CBS 136012 | Portugal | *Fraxinus angustifolia* | MT587345 | MT592050 | MT592492 | - | - |
| *Diplodia intermedia* | CBS 124462 | Portugal | *Malus sylvestris* | MH863374 | GQ923826 | MT592503 | - | - |
| *Diplodia mutila* | CBS 136014 | Portugal | *Populus alba* | KJ361837 | KJ361829 | MZ073932 | - | - |
| *Diplodia mutila* | CBS 112553 | Portugal | *Vitis vinifera* | MW810279 | MZ073947 | MZ073931 | - | - |
| *Diplodia mutila* | CVG 1739 | Italy | *Vitis vinifera* | *OQ612693* | *OQ680492* | *OQ680512* | - | - |
| *Diplodia mutila* | CVG 1741* | Italy | *Vitis vinifera* | *OQ612695* | *OQ680494* | *OQ680513* | - | - |
| *Diplodia pseudoseriata* | CBS 124906 | Uruguay | *Blepharocalyx salicifolius* | EU080927 | EU863181 | - | - | - |
| *Diplodia pyri* | CBS 121862 | Netherlands | *Pyrus* sp. | KX464093 | KX464567 | KX464799 | - | - |
| *Diplodia rosulata* | CBS 116470 | Ethiopia | *Prunus africana* | EU430265 | EU430267 | EU673132 | - | - |
| *Diplodia sapinea* | CBS 114864 | Unknown | Unknown | KX464096 | KX464570 | KX464803 | - | - |
| *Diplodia scrobiculata* | CBS 118110 | Wisconsin, U.S.A. | *Pinus banksiana* | KF766160 | KF766399 | - | - | - |
| *Diplodia seriata* | CBS 112555 | Italy | *Vitis vinifera* | AY259094 | AY573220 | DQ458856 | - | - |
| *Diplodia seriata* | CBS 119049 | Italy | *Vitis vinifera* | DQ458889 | DQ458874 | DQ458857 | - | - |
| *Diplodia seriata* | CVG 1577* | Italy | *Vitis vinifera* | *OQ612688* | *OQ680487* | *OQ680515* | - | - |
| *Diplodia seriata* | CVG 1753 | Italy | *Vitis vinifera* | *OQ612694* | *OQ680493* | *OQ680514* | - | - |
| *Diplodia subglobosa* | CBS 124133 | Spain | *Lonicera nigra* | GQ923856 | GQ923824 | MT592576 | - | - |

(Continued)

**Table 2.** (Continued).

| Species | Isolate code | Country | Host | GenBank accession number [a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | tef1 | tub2 | act | RPB2 |
| *Discosia artocreas* | CBS 124848 | Germany | *Fagus sylvatica* | MH553994 | MH554420 | MH554662 | - | - |
| *Heterotruncatella proteicola* | CBS 144020 | South Africa | *Protea acaulos* | MH554077 | MH554512 | MH554751 | - | - |
| *Heterotruncatella spartii* | CBS 143894 | USA | *Pinus edulis* | MH554134 | MH554569 | MH554807 | - | - |
| *Lecanosticta acicola* | LNPV252 | France | *Pinus attenuata* x *Pinus radiata* | JX901755 | JX901639 | JX902213 | - | - |
| *Massarina eburnea* | CBS 473.64 | Switzerland | *Fagus sylvatica* | AF383959 | - | - | - | GU371732 |
| *Neocucurbitaria acanthocladae* | CBS 142398 | Greece | *Genista acanthoclada* | MF795766 | - | MF795894 | - | MF795808 |
| *Neocucurbitaria acerina* | CBS 142403 | Austria | *Acer pseudoplatanus* | MF795768 | - | MF795896 | - | MF795810 |
| *Neocucurbitaria cava* | CBS 257.68 | Germany | Wheat-field soil | JF740260 | - | KT389844 | - | LT717681 |
| *Neocucurbitaria hakeae* | CBS 142109 | Australia | *Hakea* sp. | KY173436 | - | KY173613 | - | KY173593 |
| *Neocucurbitaria irregularis* | CBS 142791 | USA | Subcutaneous tissue | LT592916 | - | LT592985 | - | LT593054 |
| *Neocucurbitaria juglandicola* | CBS 142390 | Austria | *Juglans regia* | MF795773 | - | MF795901 | - | MF795815 |
| *Neocucurbitaria juglandicola* | CVG 1779 | Italy | *Vitis vinifera* | *OQ612696* | - | *OQ680503* | - | *OQ884601* |
| *Neocucurbitaria quercina* | CBS 115095 | Italy | *Quercus robur* | LT623220 | - | LT623237 | - | LT623277 |
| *Neocucurbitaria rhamni* | CBS 142391 | Austria | *Rhamnus frangula* | MF795775 | - | - | - | MF795817 |
| *Neocucurbitaria rhamnicola* | CBS 142396 | Spain | *Rhamnus lycioides* | MF795780 | - | MF795906 | - | MF795822 |
| *Neocucurbitaria ribicola* | CBS 142394 | Austria | *Ribes rubrum* | MF795785 | - | MF795911 | - | MF795827 |
| *Neofusicoccum algeriense* | CBS 113072 | Unknown | Unknown | KX464150 | KX464645 | KX464920 | - | - |
| *Neofusicoccum arbuti* | CBS 116131 | Washington, U.S.A. | *Arbutus menziesii* | AY819720 | KF531792 | KF531793 | - | - |
| *Neofusicoccum batangarum* | CBS 127348 | USA: Florida | *Schinus terebinthifolius* | MH864533 | KX464674 | KX464952 | - | - |
| *Neofusicoccum eucalypticola* | CBS 115679 | Australia | *Eucalyptus grandis* | KF766201 | - | - | - | - |
| *Neofusicoccum italicum / parvum* | MFLUCC 15-0900 | Italy | *Vitis vinifera* | KY856755 | KY856754 | MT592684 | - | - |
| *Neofusicoccum kwambonambiense* | CBS 123639 | South Africa | *Syzygium cordatum* | MH863317 | - | - | - | - |
| *Neofusicoccum occulatum* | CBS 128008 | Australia | *Eucalyptus grandis* | EU301030 | EU339509 | EU339472 | - | - |
| *Neofusicoccum parvum* | CBS 123650 | South Africa | *Syzygium cordatum* | KX464182 | KX464708 | KX464994 | - | - |
| *Neofusicoccum parvum* | CBS 145623 | Italy | *Ficus carica* | MN611180 | MN623347 | MN623344 | - | - |
| *Neofusicoccum parvum* | CVG 1588* | Italy | *Vitis vinifera* | *OQ612690* | *OQ680489* | *OQ680510* | - | - |
| *Neofusicoccum parvum* | CVG 1731 | Italy | *Vitis vinifera* | *OQ612692* | *OQ680491* | *OQ680511* | - | - |
| *Neofusicoccum protearum* | CBS 114176 | South Africa | *Leucadendron laureolum* | AF452539 | KX464720 | KX465006 | - | - |
| *Neofusicoccum ribis* | CBS 122553 | Panama | *Theobroma cacao* | EU683673 | EU683654 | MT592742 | - | - |
| *Neofusicoccum umdonicola* | CBS 123645 | South Africa | *Syzygium cordatum* | MH863318 | - | KF766145 | - | - |
| *Neofusicoccum vitifusiforme* | CBS 110887 | South Africa | *Vitis vinifera* | AY343383 | AY343343 | KX465061 | - | - |
| *Paraconiothyrium africanum* | CBS 121166 | South Africa | *Prunus persica* | EU295650 | - | JX496142 | JX496255 | - |
| *Paraconiothyrium brasiliense* | CBS 100299 | Brazil | *Coffea arabica* | AY642531 | - | JX496350 | JX496237 | - |
| *Paraconiothyrium brasiliense* | CBS 159.60 | Unknown | Unknown | JX496044 | - | JX496383 | JX496270 | - |
| *Paraconiothyrium brasiliense* | CBS 587.84 | Italy | *Vitis vinifera* | JX496099 | - | JX496438 | JX496325 | - |

(Continued)

**Table 2.** (Continued).

| Species | Isolate code | Country | Host | GenBank accession number [a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | tef1 | tub2 | act | RPB2 |
| *Paraconiothyrium brasiliense* | CVG 1579* | Italy | *Vitis vinifera* | *OQ612697* | - | *OQ680500* | *OQ680507* | - |
| *Paraconiothyrium brasiliense* | CVG 1736 | Italy | *Vitis vinifera* | *OQ612698* | - | *OQ680499* | *OQ680506* | - |
| *Paraconiothyrium cyclothyrioides* | CBS 432.75 | Sri Lanka | *Hevea brasiliensis* | JX496088 | - | JX496427 | JX496314 | - |
| *Paraconiothyrium fuckelii* | CBS 508.94 | Italy | *Rosa* sp. | JX496096 | - | JX496435 | JX496322 | - |
| *Paraconiothyrium hawaiiense* | CBS 120025 | Hawaii | *Sophora chrysophylla* | JX496027 | - | JX496366 | JX496253 | - |
| *Paraconiothyrium hawake* | CBS 142521 | Australia | *Hakea* sp. | KY979754 | - | KY979920 | - | - |
| *Paraconiothyrium minitans* | CBS 286.81 | Venezuela | *Solanum tuberosum* | JX496063 | - | JX496402 | JX496289 | - |
| *Paraconiothyrium sporulosa* | CBS 105.76 | Norway | *Picea abies* | JX496014 | - | JX496353 | - | - |
| *Paraconiothyrium variabile* | CBS 112.72 | Italy | *Dianthus* sp. | JX496019 | - | JX496358 | JX496245 | - |
| *Parafenestella pseudoplatani* | CBS 142392 | Austria | *Acer pseudoplatanus* | MF795788 | - | MF795914 | - | MF795830 |
| *Paraphaeosphaeria neglecta* | CBS 124076 | Italy | *Actinidia chinensis* var. Hort16A | JX496037 | - | JX496376 | JX496263 | - |
| *Phlogicylindrium eucalypti* | CBS 120080 | Australia | *Eucalyptus globulus* | NR_132813 | MH704607 | MH704633 | - | - |
| *Pseudopyrenochaeta lycopersici* | CBS 306.65 | Germany | *Lycopersicon esculentum* | NR103581 | - | LT717674 | - | LT717680 |
| *Robillarda africana* | CBS 122.75 | South Africa | Unknown | KR873253 | MH554414 | MH554656 | - | - |
| *Robillarda roystoneae* | CBS 115445 | Hong Kong | *Roystonea regia* | KR873254 | KR873310 | KR873317 | - | - |
| *Seimatosporium botan* | NBRC 104200 | Japan | *Paeonia suffruticosa* | AB594799 | - | LC047770 | - | - |
| *Seimatosporium luteosporum* | CBS 142599 | USA | *Vitis vinifera* | KY706284 | KY706334 | KY706259 | - | - |
| *Seimatosporium physocarpi* | CBS 139968 | Russia | *Physocarpus opulifolius* | KT198722 | MH554434 | MH554676 | - | - |
| *Seimatosporium vitifusiforme* | CBS 142600 | USA | *Vitis vinifera* | KY706296 | KY706346 | KY706271 | - | - |
| *Seimatosporium vitis* | MFLUCC 14-0051 | Italy | *Vitis vinifera* | KR920363 | - | - | - | - |
| *Seimatosporium vitis* | Napa774 | Napa County, U.S.A. | *Vitis vinifera* | - | KY706326 | KY706251 | - | - |
| *Seimatosporium vitis-viniferae* | CVG 1681 | Italy | *Vitis vinifera* | *OQ612700* | *OQ680496* | *OQ680502* | - | - |
| *Seimatosporium vitis-viniferae* | CVG 1682* | Italy | *Vitis vinifera* | *OQ612699* | *OQ680495* | *OQ680501* | - | - |
| *Seimatosporium vitis-viniferae* | CBS 123004 | Spain | *Vitis vinifera* | MH553992 | MH554418 | MH554660 | - | - |
| *Seimatosporium vitis-viniferae* | CBS 116499 | Iran | *Vitis vinifera* | MH553984 | MH554402 | MH554643 | - | - |
| *Sporocadus biseptatus* | CBS 110324 | Unknown | Unknown | MH553956 | MH554374 | MH554615 | - | - |
| *Sporocadus incanus* | CBS 123003 | Spain | *Prunus dulcis* | MH553991 | MH554417 | MH554659 | - | - |
| *Truncatella angustata* | CBS 144025 | France | *Vitis vinifera* Prunelard | MH554112 | MH554546 | MH554785 | - | - |
| *Truncatella angustata* | CBS 113.11 | Germany | *Picea abies* | MH553966 | MH554384 | MH554625 | - | - |
| *Truncatella angustata* | CBS 165.25 | Unknown | *Prunus armeniaca* | MH554010 | MH554444 | MH554686 | - | - |
| *Truncatella angustata* | CVG 1601 | Italy | *Vitis vinifera* | *OQ612701* | *OQ680497* | *OQ680504* | - | - |
| *Truncatella angustata* | CVG 1631* | Italy | *Vitis vinifera* | *OQ612702* | *OQ680498* | *OQ680505* | - | - |
| *Truncatella restionacearum* | CBS 118150 | South Africa | *Restio filiformis* | DQ278914 | MH554407 | MH554649 | - | - |

[a] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; act: actin; tef1: translation elongation factor 1-α gene; rpb2: RNA polymerase second largest subunit; tub2: beta-tubulin. Sequences generated in this study indicated in italics. Ex-type and ex-epitype isolates are indicated in bold font; Genbank accession numbers generated in this study are indicated in italic font. CVG strains marked with * were selected to investigate effects of temperature on fungal radial growth.


**Figure 1.** Grapevine trunk disease symptoms observed in Piedmont. A. Symptoms attributed to Botryosphaeria dieback on a grapevine shoots, with complete branches dissection, drying, and fall of affected leaves. B. Cross section of a cordon with internal necrotic wood cankers (wedge-shaped) characteristic of Botryosphaeria dieback.

and OA, with incubation at 25°C in dark, were used for *Seimatosporium* isolates (Kanetis *et al.*, 2022). For *Truncatella* isolates, CMA and MEA were used, and colonies were incubated at 21°C with alternating light-dark periods (Liu *et al.*, 2019).

*Effects of temperature on fungal colony radial growth*

To investigate the effect of temperature on the colony radial growth of selected fungal isolates (Table 2), each isolate was grown on PDA amended with streptomycin sulphate (25 ppm $L^{-1}$) for 7 d in the dark at 25°C. Mycelium plugs were taken from the margins of 10-d-old colonies using a cork borer (0.6 cm diam.) and were placed upside down at the centers of 9 cm diam. Petri dishes, each containing 10 mL of PDA-S medium. All plates were then incubated for 7 d at 5, 10, 15, 20, 25, 30 or 35°C, and each isolate was tested using seven replicate plates per temperature. Following incubation, the Petri plates were examined without being opened, and the mean colony diameters (minus the diameter of the initial inoculation plugs) of each growing mycelial colony were measured in two perpendicular directions at the end of the 4th and 7th day. Radial growth rates (mm $d^{-1}$) were calculated for each temperature. The variations in mycelium growth rates at different temperatures were analyzed using the generalized Analytics Beta model (López-Moral *et al.*, 2017). Based on this analysis, optimum growth temperature and the corresponding maximum growth rate were calculated for each isolate. Box-Cox transformation was applied to optimum growth temperature data. To satisfy ANOVA assumptions, normality and homogeneity of variance were evaluated with, respectively, Shapiro-Wilk and Levene's tests. One-way ANOVA was carried out, followed by Tukey's test for evaluation of statistically significance differences between means (at $P < 0.05$), as both ANOVA assumptions were satisfied for the growth rate data. Welch's ANOVA was performed on optimum growth temperature data because only the normality assumption was satisfied. Statistical differences ($P < 0.05$) were analyzed with the Games-Howell *post hoc* test. All statistical analyses were carried out using R (https://www.R-project.org/).

*Pathogenicity tests*

Fifteen representative isolates from seven identified fungal species were used to inoculate one-year-old potted 'Barbera' grapevine cuttings grafted on K5BB rootstock (Table 2). Ten plants were inoculated with each isolate. The inoculations were carried out in May 2022. Cuttings were inoculated above the grafting point by forming a slit (1.0–1.5 cm long) using a sterile scalpel as described by Carlucci *et al.* (2015) and Bezerra *et al.* (2021). Agar plugs (6 mm diam.) were taken from 10-d-old fungal cultures grown on PDA and plugs were placed with mycelium in contact with plant tissues, under the stem bark. Each inoculated wound was wrapped with wet sterile cotton wool soaked in sterilized distilled water and was then sealed firmly with Parafilm® (American National Can) to maintain high humidity at the inoculation point. Control plants were inoculated with sterile agar plugs. Inoculated plants were the placed in a greenhouse at 25 ± 3°C, from May to November 2022. After 180 d. from inoculation, the plants were examined after bark removal and lengths of any visible necrotic wood lesions were measured from the inoculation points. Small tissue pieces (0.5 cm) from the necrotic area were placed on PDA supplemented with streptomycin sulphate (25 ppm $L^{-1}$), and incubated at 25 ± 1°C. To fulfil Koch's postulates, resulting colonies were identified based on their morphological characteristics. Data of necrotic lesion lengths were subjected to statistical analysis. Shapiro-Wilk (W) tests were used to determine if the data followed normal distributions. Levene's tests were carried out to assess the homogeneity of the variances of the dataset. A Welch's ANOVA was performed because the dataset was normally distributed, but data were not homoscedastic. The Games-Howell *post hoc* test was used to evaluate statistically significant differences among mean lesion lengths caused by the different fungal isolates (at $P \leq 0.05$). All statistical analyses were carried out using R (https://www.R-project.org/).

## RESULTS

### *Sampling, isolation and morphological identification of isolates*

In sampled vineyards, more than 30% of the plants showed Botryosphaeria dieback related symptoms. Approximately 5–10% of plants showed decline with severe dieback and death. Sampled grapevines showed typical dieback symptoms, primarily associated with Botryosphaeria dieback, such as defoliation and wedge-shaped cankers of internal wood tissues and dark streaking of wood.

A total of 248 fungal isolates were obtained from a total of 32 symptomatic vines of 12 cultivars. The first screen and identification of isolates was based on their morphological and cultural characteristics. A group of isolates identified as *Botryosphaeriaceae*-like showed high isolation frequency (80 isolates, 37% of total isolates obtained) compared with other common GTDs fungi, such as *P. chlamydospora* (38 isolates), *E. lata* (5 isolates) and *F. mediterranea* (12 isolates) that were occasionally present.

Other less frequently isolated genera were also detected using morphological characteristics, including *Diaporthe* (5 isolates), *Kalmusia* (3 isolates), *Neocucurbitaria* (2 isolates), *Paraconiothyrium* (3 isolates), *Seimatosporium* (7 isolates), and *Truncatella* (4 isolates) species. Some common saprophytes, not considered to be associated with observed symptoms, were also isolated, including *Alternaria*, *Epicoccum, Cladosporium*, and *Dydimella* spp. Table 1 shows details of the fungal species isolated from each vineyard surveyed in Piedmont.

### *Phylogenetic analyses*

The multi-locus analyses conducted on all isolates confirmed the genera obtained with the initial phylogenetic analysis of the *tef1* and ITS regions. The combined locus analysis of *Botryosphaeriaceae*-like isolates consisted of 35 sequences and *Lecanosticta acicola*, which was chosen as the outgroup. A total of 995 characters (ITS: 1–660 and *tef1*: 666-995) were included in the phylogenetic analyses of *Botryosphaeriaceae*-like isolates. A total of 1405 characters (ITS, 1–628; *tub2*, 633–1123; *tef1*, 1128–1405) were included in the *Paraconiothyrium* analyses where, for a total of 15 sequences, *Alloconiothyrium aptrootii* was chosen as the outgroup. For *Neocucurbitaria*, *Pseudopyrenochaeta lycopersici* was chosen as outgroup, and a total of 1810 characters (ITS, 1–507; *tub2*, 511–896; *rpb2*, 901-1810) were included in the phylogenetic analyses performed with 16 sequences. The combined phylogenetic session for *Seimatosporium* and *Truncatella* had a total of 1823 characters (ITS, 1–579; *tef1*, 583–1071; *tub2*, 1076–1823) with 25 sequences. *Discosia artocreas* was chosen as the outgroup. For each session, a tree was created based on a maximum of 1000 equally most parsimonious trees. Bootstrap support values for all MP trees obtained are shown in Figures 2, 3, 4 and 5.

In the *Botryosphaeriaceae*-like analyses, two isolates (CVG15777 and CVG1753) clustered with *D. seriata* reference strains, and isolates CVG1582 and CVG1615 clustered with *B. dothidea* strains. Isolates CVG1588 and CVG1731 were grouped with *Neof. parvum*, and isolates CVG1739 and CVG1714 clustered with *D. mutila* reference strains. For *Paraconiothyrium*, both strains clustered with reference strains of *P. brasiliense*. In the phylogenetic tree from the *Truncatella* and *Seimatosporium* analysis, two isolates (CVG1601, CVG1631) were identified as *T. angustata*, and two isolates (CVG1681, CVG1682) were grouped with *S. vitis-viniferae* reference strains. Isolate CVG1779 of *Neocucurbitaria* clustered in the *Neoc. juglandicola* clade. The recommended evolutionary model, unique site patterns, number of generations, and tree produced and sampled for each partition of the Bayesian analyses are reported in Table 3, as well as other parameters produced by MB analyses, including tree lengths, consistency, retention, and rescaled consistency indices. Data obtained from the multi-locus analyses carried out on the 15 selected representative isolates gave four *Botryosphaeriaceae* species, including *B. dothidea, D. mutila, D. seriata* and *Neof. parvum*. Among other less frequently isolated taxa, *Neocucurbitaria juglandicola*, *P. brasiliense*, *S. vitis-viniferae* and *T. angustata* were identified.

### *Morphology*

Morphological observations were performed for all the selected species. Colonies characteristic, including edges shape, colony front and reverse color, mycelia appearance and conidia morphology of *B. dothidea*, *D. mutila, D. seriata* and *Neof. parvum* were congruent with previous descriptions of species belonging to *Botryosphaeriaceae* family (Phillips *et al.* 2013). Different conidia were observed (cylindrical to fusiform, hyaline to dark brown) and all isolate showed fast growth mycelia, becoming dark with age starting from the center, spreading to the whole colony.

Colonies of *Neoc. juglandicola* on PDA and MEA showed slow growth with uneven margins. Colony upper surfaces were brown to dark brown with dense zonate mycelium. Pycnidia appeared as dots, which were numerous and centrally located. Reverse colony sides

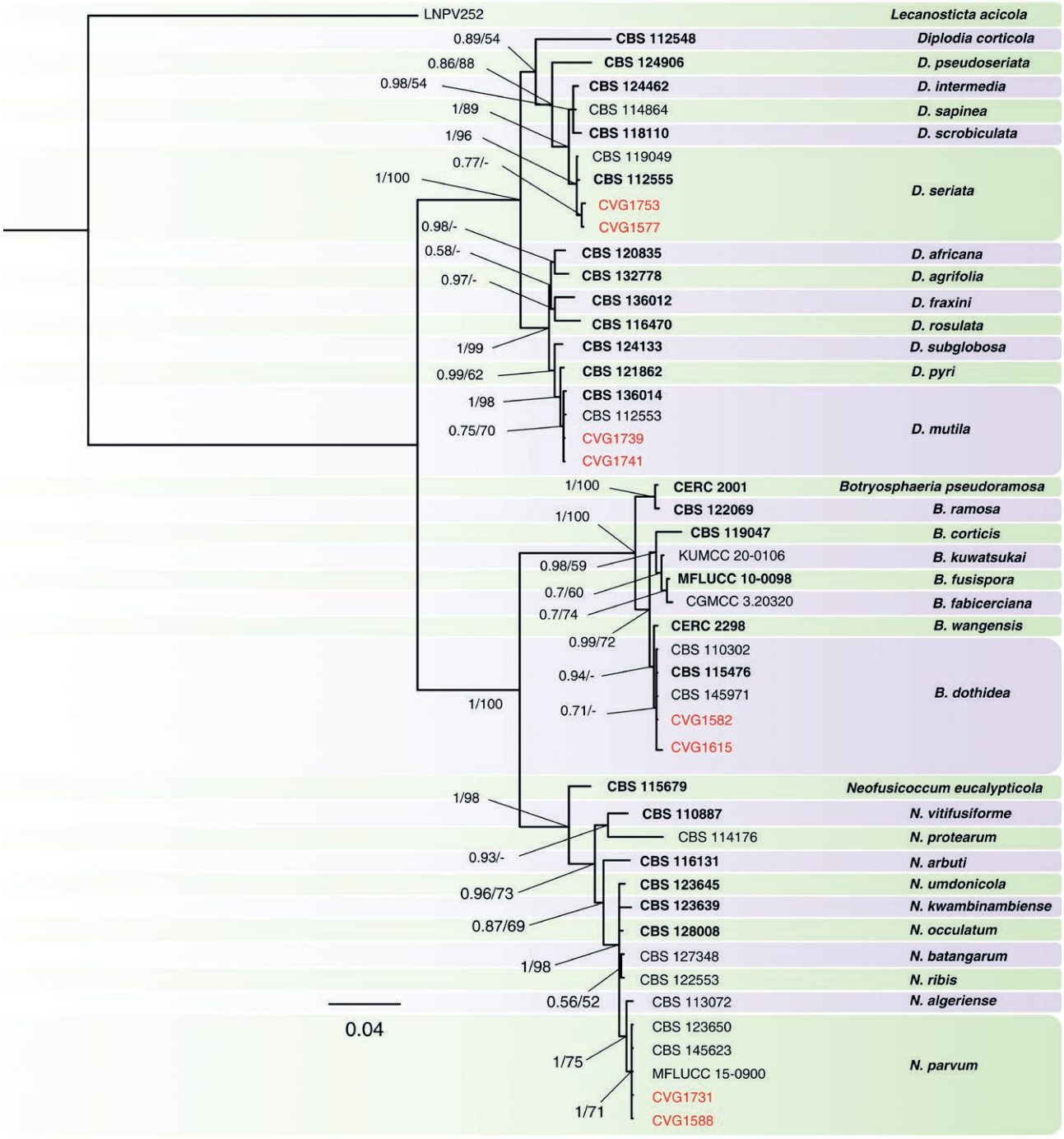


**Figure 2.** Phylogenetic tree for *Botryosphaeriaceae*, resulting from a Bayesian analysis of the combined ITS, *tef1* and *tub2* sequence alignment. Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type strains are indicated in bold font, and species are delimited with coloured blocks. Isolates collected in the present study are indicated in red font. The tree was rooted to *Lecanosticta acicola* (LNPV252).

were dark brown. Conidia, produced on PDA measured 2.0–3.1 × 1.3–1.5 µm, mean (± S.D) = 2.5 ± 0.5 × 1.4 ± 0.1 µm, and were unicellular with smooth surfaces, hyaline, and ellipsoid with rounded apices. Based on morphological features, colonies had similar characteristic to those reported by Jaklitsch *et al.* (2018).

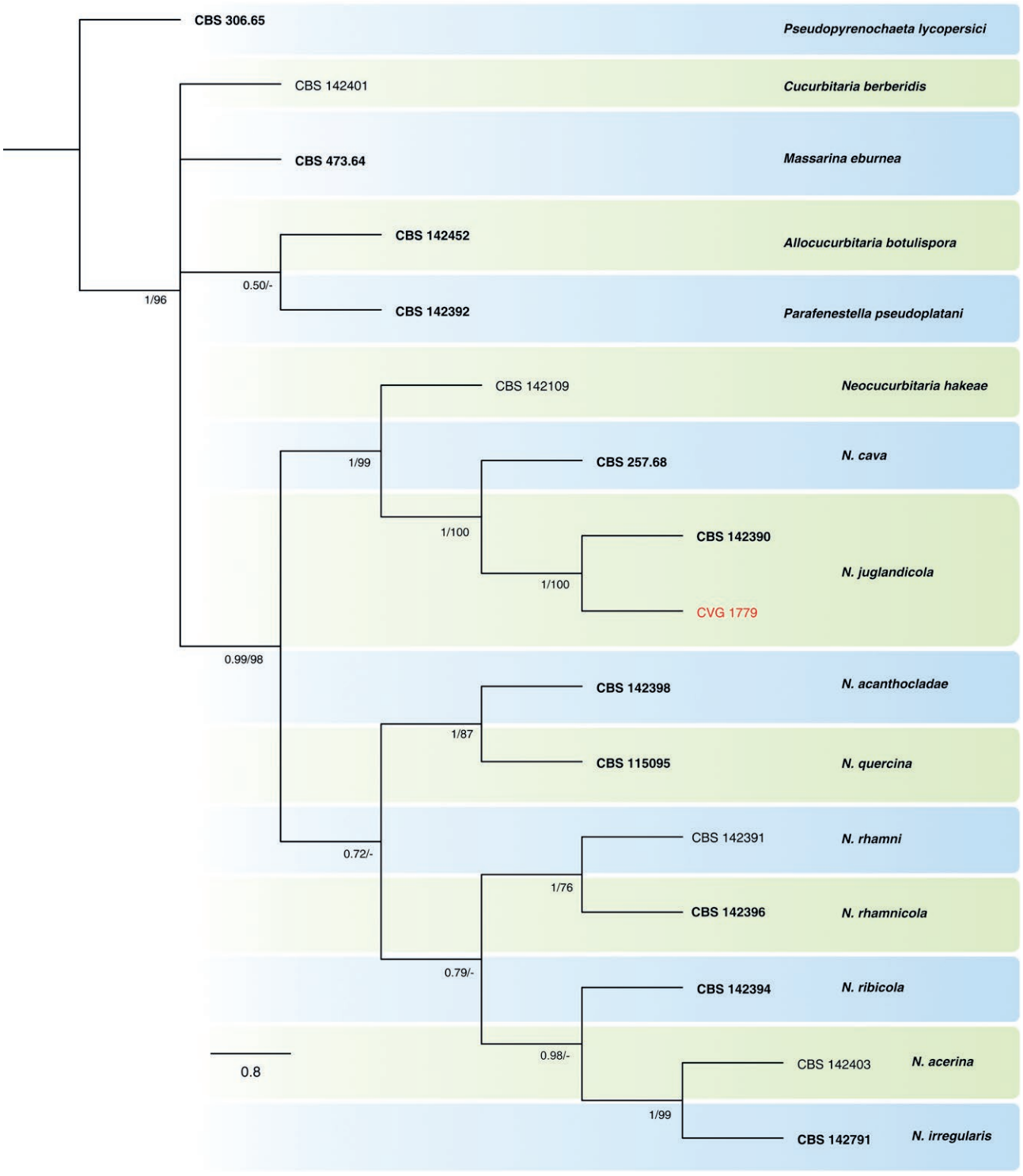


**Figure 3.** Phylogenetic tree of *Neocucurbitaria* sp., resulting from a Bayesian analysis of the combined ITS, *tub2* and *rpb2* sequence alignment. Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type strains are indicated in bold font, and species are delimited with coloured blocks. The isolate collected in the present study is indicated in red font. The tree was rooted to *Pseudopyrenochaeta lycopersici* (CBS 306.65).

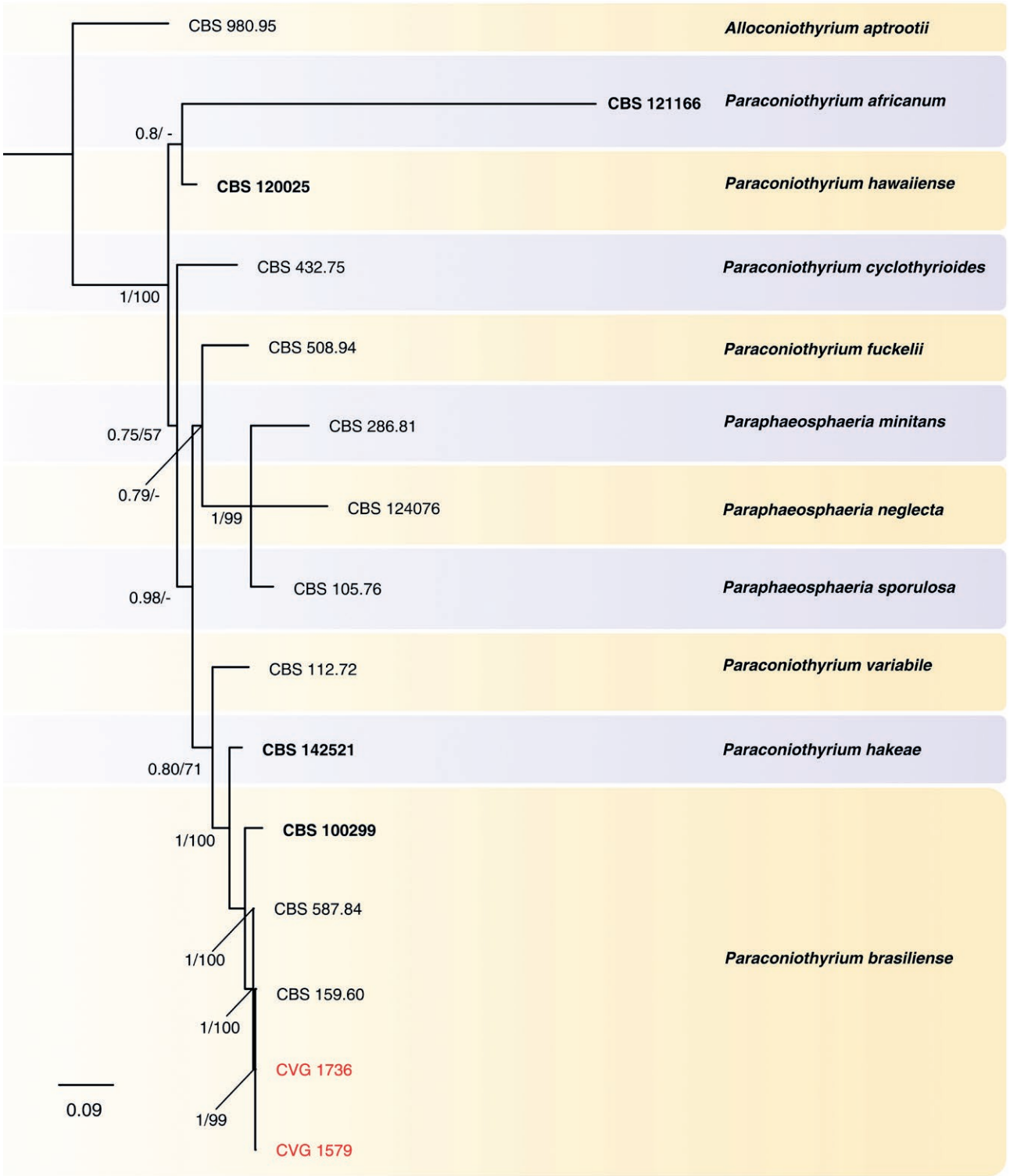


**Figure 4.** Phylogenetic tree of *Paraconiothyrium* sp. resulting from a Bayesian analysis of the combined ITS, *tef1* and *act* sequence alignment. Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type strains are indicated in bold font and species are delimited with coloured blocks. Strains collected in this study are indicated in red. The tree was rooted to *Alloconiothyrium aptrootii* (CBS 980.95).

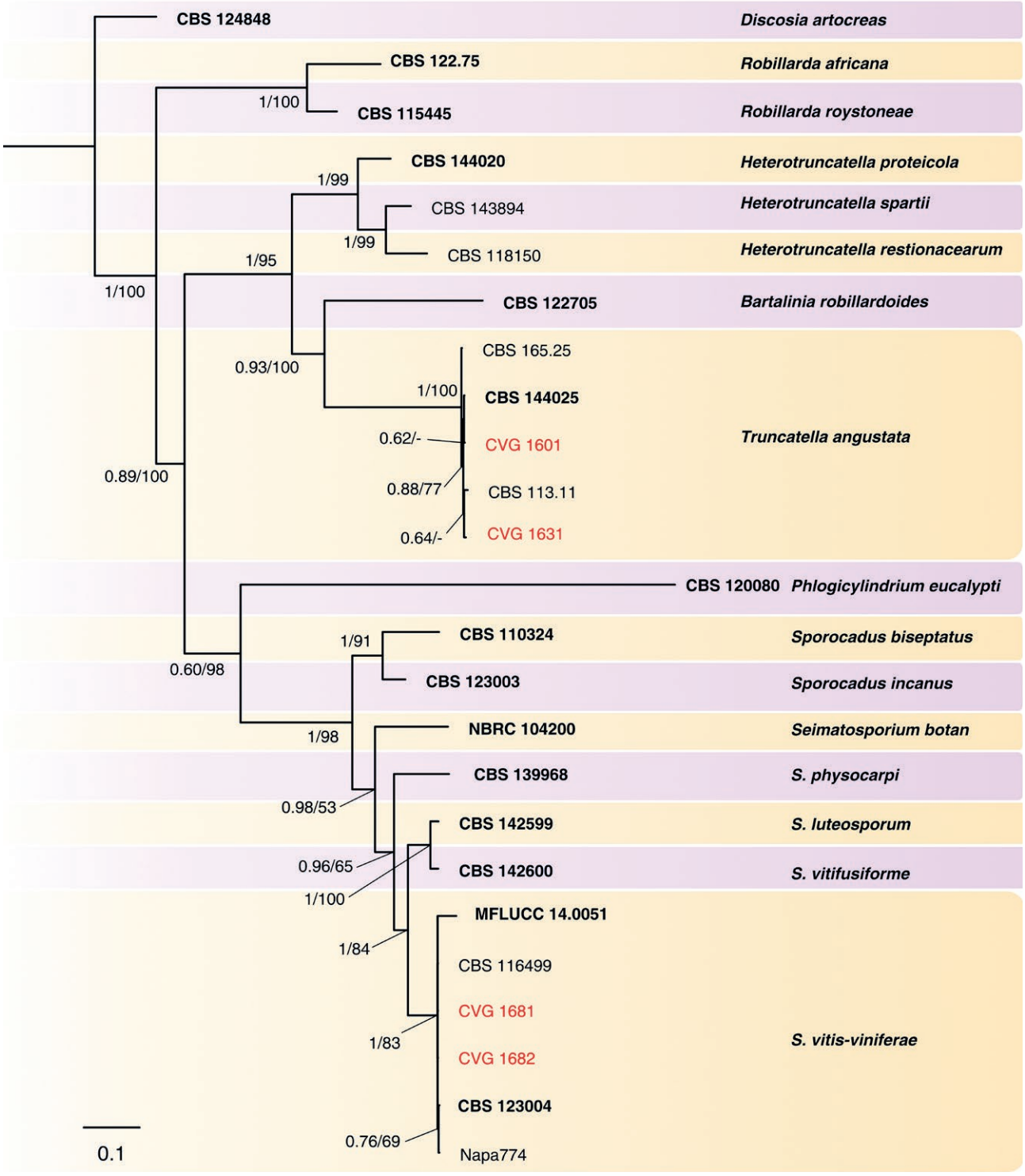


**Figure 5.** Phylogenetic tree of *Seimatosporium* sp. and *Truncatella* sp. resulting from a Bayesian analysis of the combined ITS, *tef1* and *tub2* sequence alignment. Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type isolates are indicated in bold font and species are delimited with coloured blocks. Isolates collected in the present study are indicated in red font. The tree was rooted to *Discosia artocreas* (CBS 124848).

**Table 3.** Parsimony and Bayesian parameters for each phylogenetic analysis to identify different fungi.

| Species | Locus(i) | Bayesian analysis | | | | Parsimony analysis | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Evolutionary model | Unique site pattern | Generation ran | Generated trees | Sampled trees | Total sites | Constant sites | Variable sites | Parsimony Informative sites | Tree length | Consistency index | Retention index | Rescaled consistence index |
| *Botryosphaeraceae* | ITS | SYM+G | 242 | 1140000 | 2282 | 1712 | 1395 | 763 | 263 | 369 | / | 0.787 | 0.946 | 0.744 |
| | *tef1* | HKY+I+G | 242 | | | | | | | | | | | |
| | *tub2* | GTR+G | 121 | | | | | | | | | | | |
| *Neocucurbitaria* sp. | ITS | GTR+I+G | 145 | 805000 | 1612 | 206 | 1803 | 1063 | 324 | 416 | 1666 | 0.628 | 0.495 | 0.311 |
| | *tef1* | SYM+G | 148 | | | | | | | | | | | |
| | RPB2 | HKY+G | 334 | | | | | | | | | | | |
| *Paraconiothyrium* sp. | ITS | GTR+I+G | 167 | 305000 | 612 | 93 | 1397 | 734 | 372 | 291 | 1273 | 0.742 | 0.612 | 0.454 |
| | *tub2* | SYM+G | 238 | | | | | | | | | | | |
| | *act* | HKY+G | 132 | | | | | | | | | | | |
| *Seimatosporium* sp. and *Truncatella* sp. | ITS | HKY+G | 178 | 705000 | 1412 | 711 | 1929 | 762 | 261 | 906 | 3376 | 0.68 | 0.803 | 0.546 |
| | *tef1* | GTR+I+G | 404 | | | | | | | | | | | |
| | *tub2* | HKY+I+G | 512 | | | | | | | | | | | |

Colonies of *P. brasiliense* on MEA were white-gray, had regular margins with rapid growth and darker aerial mycelium in the centre. Reverse colony sides were light brown-honey to light yellow amber. On OA the colonies were light gray, each with a darker area. On CMA the colonies were white, with mycelium development in the centres showing concentric and radiating patterns. Conidia were cylindrical to ellipsoid with rounded apices, and measured 2.5-4.4 × 1.3-2.8 µm, mean (± S.D) = 3.3 ± 0.9 × 1.9 ± 0.7 µm. They were hyaline and unicellular, with smooth walls, and granular contents. Based on morphological features, colonies had similar characteristic to those reported by Kanetis *et al.* (2022).

Colonies of *S. vitis-viniferae* on PDA and MEA had entire edges and were light brown to reddish with wooly aerial mycelium with smooth whitish margins. On OA the colonies were slightly to light brown with off-white wooly margins. Conidia were fusiform, each with three septa, and were constricted at each septum, measuring 15.8-22.7 × 4.2-6.1 µm, mean (± S.D) = 18.9 ± 3.5 × 5.1 ± 1.0 µm. The conidia were pale to dark brown, and each basal cell had an appendage while the apical cell had a rounded apex.

Colonies of *T. angustata* on PDA had entire edges, had pale gray to white fuzzy mycelium from above and grayish to white on the reverse sides, and were fast-growing. Black pycnidia were observed at the centre of each colony after 7 d. On MEA the colonies had entire edges, and grew slowly, with cottony white to light brown mycelia. Conidia were fusiform (17.5–19.7 × 6.1-7.3 µm, mean (± S.D) = 18.6 ± 1.1 × 6.7 ± 0.6 µm), mostly with three cells and were transversally septate without septal constrictions, and with truncate bases and several appendices. Based on morphological features of colonies and conidia, isolates studied have similar characteristic to those reported by Raimondo *et al.* (2019).

### *Effects of temperature on fungal growth*

None of the tested isolates grew at 5°C, growth was slow between 10 to 15°C, and was optimum at 20 to 25°C. Four isolates, CVG1577 (*D. seriata*), CVG1582 (*B. dothidea*), CVG1588 (*Neof. parvum*) and CVG1741 (*D. mutila*), grew at 35°C. A generalized Analytics Beta model was used to describe the relationship between mycelial growth and selected temperatures (Figure 7) and optimum growth temperature, and the corresponding maximum growth rates were calculated. Coefficients of determination ($R^2$) for the Analytics Beta model ranged between 0.88 and 0.99. Analysis of variance (ANOVA) was carried out on data of mycelial growth rates and optimum growth temperatures.

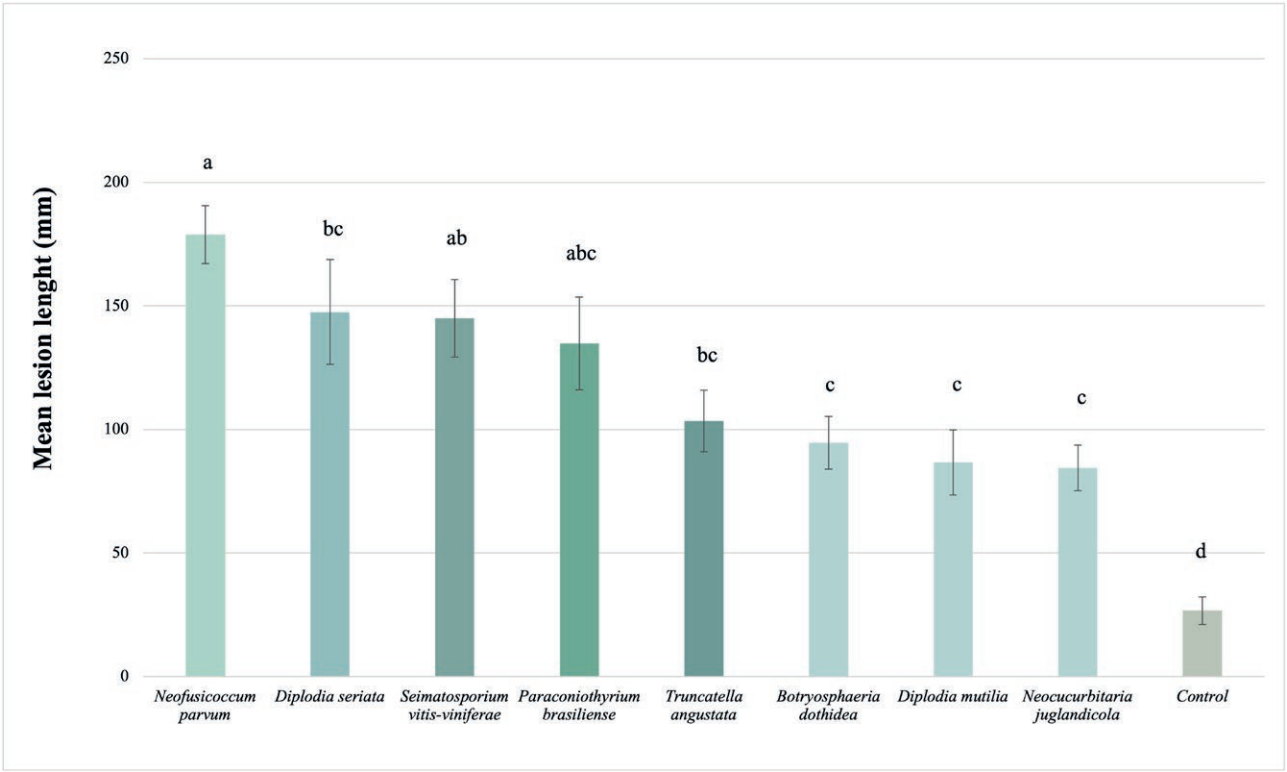


**Figure 6.** Mean necrosis lengths (mm) in grapevine stems resulting from inoculations with different fungi. A Games-Howell *post hoc* test was performed and means accompanied by different letters are significantly different ($P < 0.05$).

Statistically significant differences ($P < 0.05$) obtained with Games-Howell *post hoc* tests for optimum growth temperatures and Tukey's test for mycelial growth rates are shown in Figure 8. Among the *Botryosphaeriaceae* species, optimum mean growth temperatures ranged from 23.2°C for *D. seriata* and 23.9°C for *D. mutila*, to 25.5°C for *Neof. parvum* and 27.3°C for *B. dothidea*. *Neocucurbitaria juglandicola* did not grow 35°C. At the respective optimum temperatures, *D. mutila* grew the most rapidly at 10.9 mm $d^{-1}$, followed by *D. seriata* (10.5 mm $d^{-1}$), *B. dothidea* (10.0 mm $d^{-1}$) and *Neof. parvum* (8.7 mm $d^{-1}$). Mean optimum temperatures for mycelial growth were 24.8°C for *S. vitis-viniferae* and 24.6°C for *Neoc. juglandicola*. Both the species showed slow growth at, respectively, 0.83- and 0.54-mm $d^{-1}$. Maximum growth for *T. angustata* (3.8 mm $d^{-1}$) was obtained at 20.8°C and for *P brasiliense* (1.69 mm $d^{-1}$) was at 22.8°C. Based on maximum growth rates, *Botryosphaeriaceae* isolates had the fastest growth rate (>8 mm $d^{-1}$), followed by *T. angustata* and *P. brasiliense* (< 5 mm $d^{-1}$). *Seimatosporium vitis-viniferae* and *Neoc. juglandicola* had the slowest growth rates (< 1 mm $d^{-1}$).

*Pathogenicity tests*

The fungal isolates used for pathogenicity tests caused brown necroses and vascular discolourations in the wood of inoculated grapevines, 180 d after inoculation. No lesions were observed on inoculation control plants. *Neocucurbitaria juglandicola* (CVG 1779) was not re-isolated from necrotic areas in the wood, while all the other respective inoculated fungi were successfully re-isolated from the grapevine plants, fulfilling Koch's postulates. Re-isolated identifications were confirmed through morphological and molecular analyses (partial *tub* gene sequencing) while frequencies of re-isolations of inoculated species ranged from 80% and 90%. A Shapiro-Wilk (W) test was used for data of necrotic lesion lengths on the inoculated plants to determine if they followed normal distributions with W = 0.9807 ($P$-value = 0.07556). Levene's test showed that the homogeneity of variance was not significant for the dataset ($P$ = 0.001825). Because data were normally distributed but not homoscedastic, a Welch's ANOVA was performed. This showed that statistically significant differences occurred among the inoculated fungi ($P$= 3.167e-15). Results of the Games-Howell *post hoc* test to evalu-

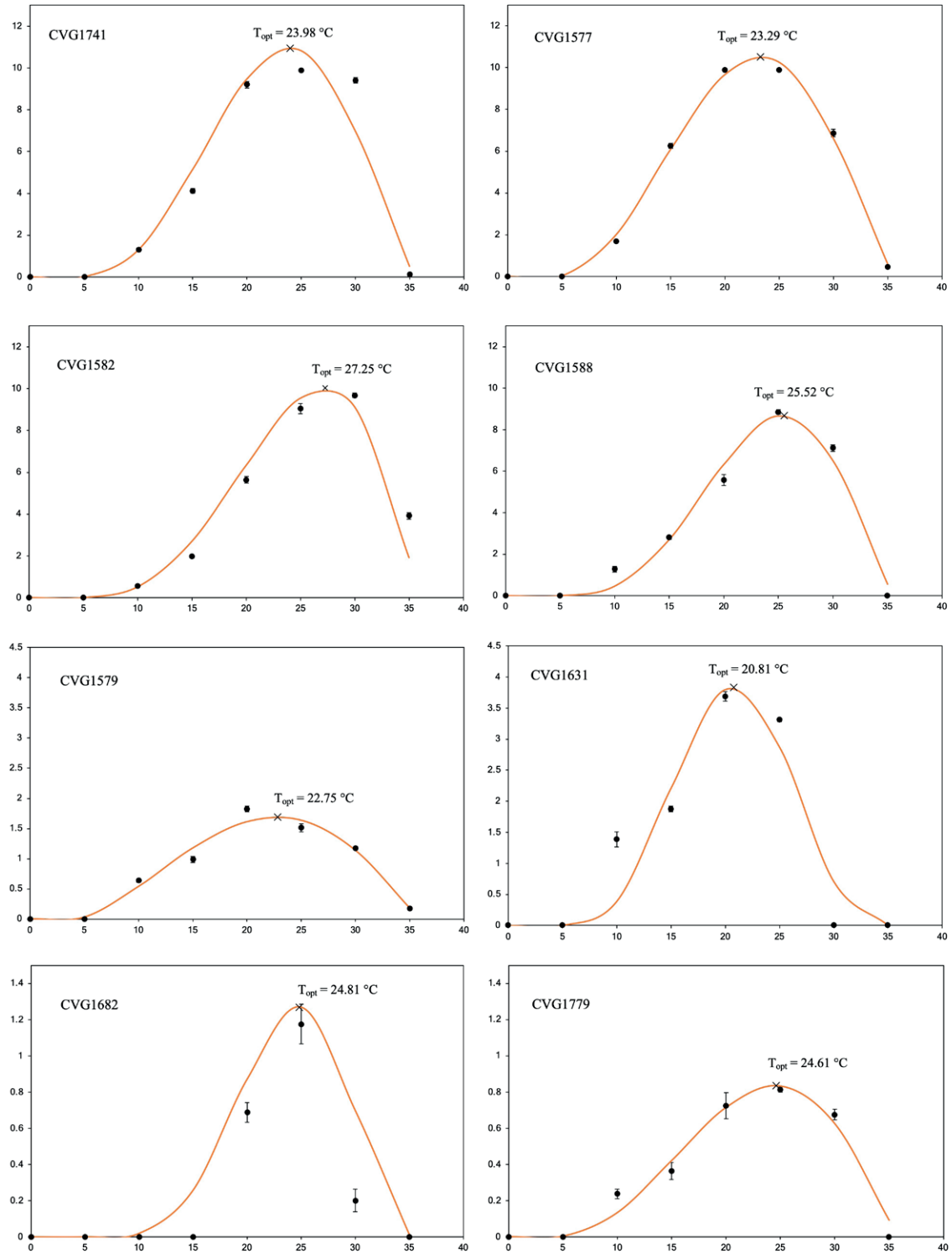


**Figure 7.** Mean mycelium growth rates (mm d-1) at different temperatures for *D. mutila* (CVG1741), *D. seriata* (CVG1577), *B. dothidea* (CVG1582), *N. parvum* (CVG1588), *P. brasiliense* (CVG1579), *T. angustata* (CVG1631), *S. vitis-viniferae* (CVG1682) and *N. juglandicola* (CVG1779). Strains were grown on PDA at 0, 5, 10, 15, 20, 25, 30, and 35°C for 4 days. A nonlinear regression curve based on Analytis Beta model was used to plot average growth rates over temperature. Data points are the means of seven independent replicated and standard error of the means is showed as vertical bars.

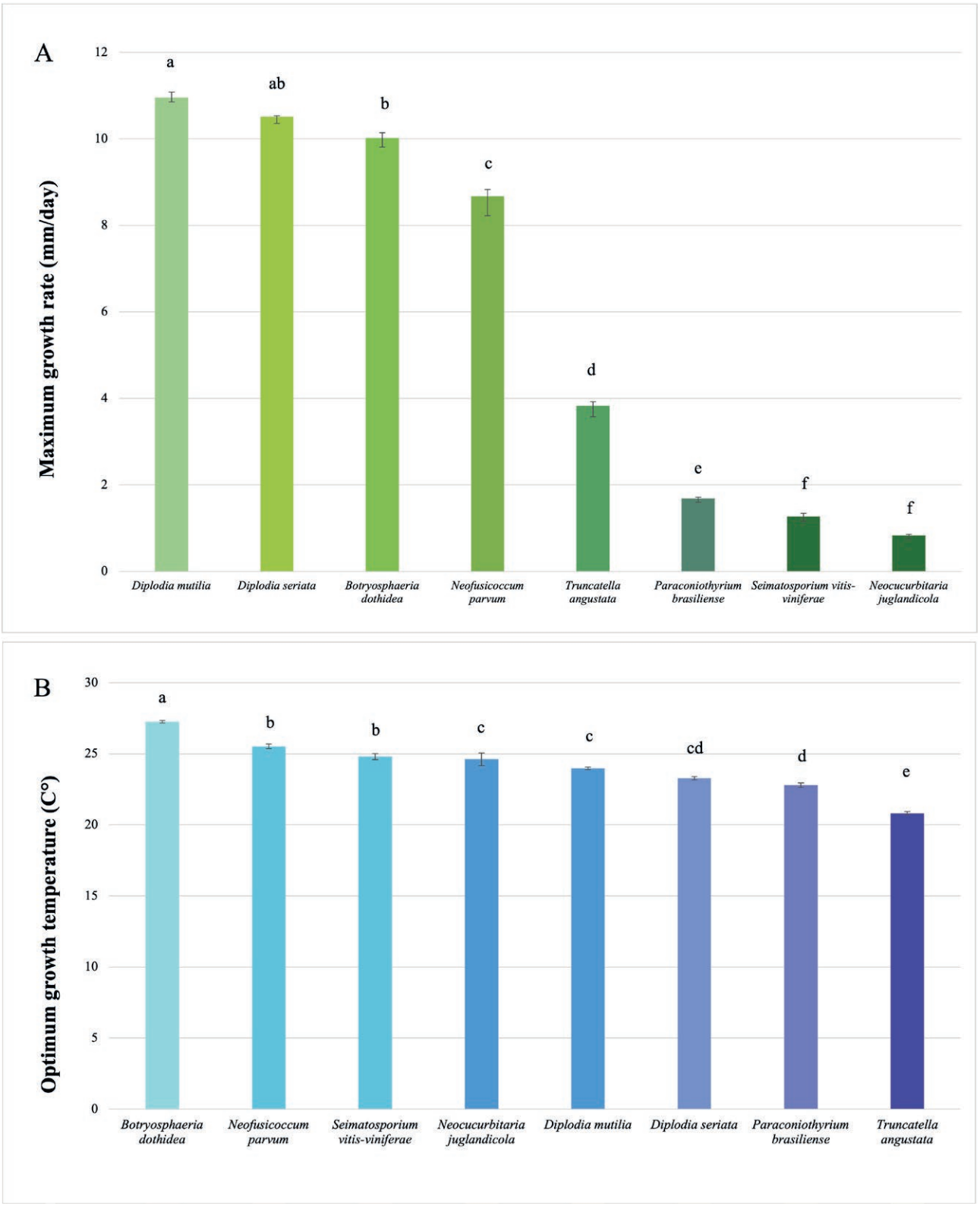


**Figure 8.** Mean maximum growth rates (A) at respective optimum growth temperature (B) for eight fungi, Vertical bars indicate standard errors. Means in each histogram accompanied by different letters are significantly different ($P = 0.05$).

ate differences among mean lesion lengths are shown in Figure 6. The longest necrotic lesions were produced by *Neof. parvum* (mean length = 178.8 mm). Aggressiveness of the other *Botryosphaeriaceae* was also confirmed: these strains produced variable vessel discolouration, with mean lesion lengths of 147.5 mm from *D. seriata*, 94.6 mm from *B. dothidea,* and 86.6 mm from *D. mutila*. *Paraconiothyrium brasiliense* and *S. vitis-viniferae* each showed similar aggressiveness compared to the *Botryosphaeriaceae* isolates, with, respectively, mean lesion lengths of 134.8 mm and 145.0 mm. *Neocucurbitaria juglandicola* (mean lesion length = 84.4 mm) and *T. angustata* (mean lesion length = 103.4 mm) were the least aggressive among the non-reported GTD pathogens.

## DISCUSSION

This study has characterized the different fungal species associated with dieback symptoms observed in representative vineyards in Piedmont, Italy. Some of these fungi are already known to be associated with diseases such as the Esca complex or Botryosphaeria dieback. Among all isolates collected, most were *Botryosphaeriaceae*, with *B. dothidea*, *D. seriata*, *D. mutila* and *Neof. parvum* identified through morphological characterization and confirmed by multi-locus phylogenetic analyses. No *Lasiodiplodia* isolates were found, which may be result because of these fungi is more prevalent in tropical and sub-tropical climatic regions. In Italy, *Lasiodiplodia* was only reported in Sicily (Burruano *et al*., 2008). Other pathogens commonly associated with the Esca complex, were sporadically recovered, including *Fomitiporia mediterranea* and *Phaeomoniella chlamydospora*. No isolates of *Phaeoacremonium* spp. were detected, which is another pathogen commonly associated with the Esca complex (Essakhi *et al*., 2008). *Eutypa lata*, the Eutypa dieback pathogen was also occasionally isolated. The pathogens commonly associated with the various symptoms observed on sampled grapevines were successfully isolated. The wedge-shape cankers, typically caused by *Botryosphaeriaceae*-like fungi or Eutypa infection, were the most common wood symptoms, but Esca-complex associated symptoms were also observed.

Some species less frequently isolated from affected grapevine wood were detected. These included *Neoc. juglandicola, P. brasiliense*, *S. vitis-viniferae* and *T. angustata*. For some of these isolates, association with grapevine woody tissues had been reported previously (Elena *et al*., 2018; Raimondo *et al*., 2019), and their respective pathogenicity related to GTDs was confirmed.

In different countries, including China (Yan *et al*., 2013), Iran (Arzanlou *et al*., 2012), Portugal (Phillips, 2002), Spain (Úrbez-Torres *et al*., 2006), Turkey (Akgul *et al*., 2014), France (Larignon *et al*., 2001), and the United States of America (Úrbez-Torres and Gubler, 2009; Trouillas *et al*., 2010). *Botryosphaeria dothidea* has been described as one of the species associated with typical V-shaped necrotic wood lesions and brown discolouration of the xylem vessels. In Italy, *B. dothidea* was reported on grapevine in the southern and central regions, on which its pathogenicity was confirmed (Carlucci *et al*., 2009). The two *B. dothidea* strains used in the present study produced dark streaks on inoculated cuttings, similar to those previously reported.

*Diplodia seriata* is known to be widespread in Europe, as it was described associated with symptomatic grapevine in Spain (Martin and Cobos, 2007), Portugal (Rego *et al*., 2009), France (Larignon *et al*., 2001), Turkey (Akgul *et al*., 2014) and Croatia (Kaliterna and Miličević, 2014). Reported as either pathogenic or saprophytic in different hosts, pathogenicity trials conducted by Taylor *et al*. (2005) and Carlucci *et al*. (2015) confirmed its role in causing necrotic wood lesions on *V. vinifera*. Based on the present results, isolates of *D. seriata* produced longer lesions than those caused by *D. mutila* and *B. dothidea*, confirming the variability in virulence among different isolates (Elena *et al*., 2015).

While *D. seriata* has been reported to be associated with grapevine in different Italian regions, including Apulia (Pollastro *et al*., 2000) and Tuscany (Spagnolo *et al*., 2011). However, *D. mutila* has already been reported associated with *Vitis vinifera* in Hungary (Lehoczky, 1974; Kovács *et al*., 2017), Spain, California, and Chile (Morales *et al*., 2012; Díaz *et al*., 2013) and with grapevine canker and dieback in Italy (Carlucci et al. 2015). In the present study, *D. mutila* produced wood discolourations after artificial inoculations, so the association with symptomatic grapevine in Italy was confirmed.

Furthermore, pathogenicity trials carried out in the present study showed *Neof. parvum* to be the most virulent species producing the longest necrotic lesions. This result is similar to those of Billones-Baaijens *et al*. (2013) and Úrbez-Torres and Gubler (2009), who, after pathogenicity trials conducted in, respectively, New Zealand and California, reported *Neof. parvum* as one of the most aggressive species associated with Botryosphaeria dieback. In Italy, Carlucci *et al*. (2015) came to the same conclusion after testing its pathogenicity on two grapevine cultivars. *Neofusicoccum parvum* was usually reported as an aggressive wood pathogen, able to infect many hosts. This fungus was also described in association with Botryosphaeria dieback symptoms on grape-

vine in France (Larignon *et al*., 2015), Algeria (Berraf-Tebbal *et al*., 2014), Spain (Luque *et al*., 2009), Portugal (Phillips, 2002), and Turkey (Akgul *et al*., 2014). Likewise, it was isolated from symptomatic grapevines in the Italian regions of Apulia and Tuscany (Carlucci *et al*., 2009; Spagnolo *et al*., 2011).

The association of *Seimatosporium* with grapevine is known, as well as its wide distribution and ability to colonize many hosts (Raimondo *et al*., 2019). Among all *Seimatosporium* species, recent studies have reported the association of *S. vitis* with GTD symptoms, where it was isolated from necrotic tissues and dead cordons in California (Lawrence *et al*., 2018) and Hungary (Váczy, 2017). In Italy, *S. vitis* was the first *Seimatosporium* species to be described in association with dead stem of *V. vinifera* (Senanayake *et al*., 2015) and Camele and Mang (2019) described it for the first time causing GTDs. In 2022, Kanetis *et al*. reported another species, *S. vitis-viniferae,* associated with lesion and wood discolouration on grapevine. In Italy, Raimondo *et al*. (2019) tested its pathogenicity, confirming its association with GTD symptoms. This agrees with results in the present study, which showed *S. vitis-viniferae* as causing wood necroses after artificial inoculation, with similar severity to necroses caused by *D. seriata*. *Truncatella* genera, which is phylogenetically close to *Seimatosporium*, has been revised by Liu *et al*. (2019) and includes only one species, *T. angustata*, while other *Truncatella* species have been reallocated to other genera. *Truncatella angustata* has been reported in association with grapevine, isolated as an endophyte in Spain (González and Tello, 2011) and Switzerland (Casieri *et al*., 2009). As a pathogen, the involvement of *T. angustata* with GTDs has been demonstrated, isolation from symptomatic grapevines in France (Pintos *et al*., 2018) and Iran (Arzanlou *et al*., 2013), and in pathogenicity tests (Úrbez-Torres *et al*., 2009). This fungus is considered an opportunistic pathogen on grapevine which is not primarily involved in GTDs. In Italy, *T. augustata* was first reported by Raimondo *et al*. (2019), who after isolation from symptomatic grapevines, confirmed its pathogenicity and involvement in GTDs. In accordance with the above studies, the present study confirmed its weak pathogenicity on grapevine, causing necrotic discolourations (mean length = 103.4 mm) after artificial inoculations.

*Paraconiothyrium brasiliense* was also less frequently isolated from woody tissues, and the role of *Paraconiothyrium* spp. on grapevine requires clarification. Pathogenicity on fruit trees and other woody hosts has been demonstrated for different species (Damm *et al*., 2008), while, *P. brasiliense* has been isolated from symptomatic and non-symptomatic grapevine tissues from Spain (Elena *et al*., 2018) and the United States of America (DeKrey *et al*., 2022). In Italy, *P. brasiliense* was also recently reported associated with dieback of apple trees (Martino *et al*., 2023). Pathogenicity trials conducted in the present study showed that *P. brasiliense* produced wood streaking with similar lesion length (mean = 134.8 mm) to lesions produced by *S. vitis-viniferae* (mean = 145.0 mm), confirming pathogenicity of *P. brasiliense* on grapevine. This study has demonstrated the role of *P. brasiliense* as a weak woody pathogen, and this is the first report of this fungus as a grapevine pathogen in Italy. *Neocucurbitaria juglandicola* has also been identified in this study. *Neocucurbitaria quercina* was reported from grapevine in the United States of America (DeKrey *et al*., 2022), while *Neoc. juglandicola* has been reported in association with *Juglans regia* and *Quercus rubra* (Jaklitsch *et al*., 2018). In the present study, after artificial inoculations, necrotic discolouration was visible, but it was not possible to re-isolate the fungus from necrotic areas. The presence of *Neoc. juglandicola* demonstrates its association with grapevine in Italy, while the pathogenicity tests did not prove its virulence or its association with GTDs. This is the first report of *Neoc. juglandicola* associated with grapevine, however.

Optimum growth temperatures of tested isolates ranged between a maximum of 27°C (for *Neof. parvum*) to a minimum of 21°C (for *T. angustata*). The respective virulences, assessed as lesion lengths, had no relationships with optimum growth temperatures in cultures. Several abiotic factors, including plant drought stress or water excess after climate events, or increases in average temperatures, can play roles in disease development, and may influence pathogen wood colonization and virulence. It is well known that global warming and climate change can increase plant stress and generate favourable conditions for the development of many diseases, including grapevine trunk disease (Guarnaccia *et al*., 2023).

Several fungi, especially *Botryosphaeriaceae*, are known to be able to switch from endophytic to pathogenic behaviors as a result of triggers connected with environmental stresses, such as drought, extreme temperatures and nutrient deficits (Slippers and Wingfield, 2007). These fungi may therefore benefit from the ongoing global warming scenario. The high percentage of isolation of these pathogens from vineyards located in Piedmont suggests a shift of these fungi may also be occurring in northern regions, as has occurred in Mediterranean areas. This expansion may be related to climatic changes. Factors such as prolonged drought, high summer temperatures, and changes in agronomic practices could favor development, spread, and pathogenicity of these fungi.

Results from the present study have demonstrated the presence of well-known GTD pathogens in Piedmont, one of the most important wine-production regions in Italy. The diversity and virulence of these pathogens in Piedmont was previously unexplored. Association of *P. brasiliense* and *N. juglandicola* with grapevine wood in Italy has been reported for the first time.

This first survey in Piedmont aimed to determine the presence and distribution of Botryosphaeria dieback pathogens, and to investigate the occurrence of other fungi associated with symptomatic grapevines. Further research is required to better clarify the distribution of grapevine pathogens in Northern Italy, especially species of *Botryosphaeriaceae*, and to determine which are the GTDs pathogens present in this region and monitor their possible shifts following climate changes.

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# Phenolic compounds inhibit viability and infectivity of the grapevine pathogens *Diplodia seriata*, *Eutypa lata*, *Fomitiporia mediterranea*, and *Neofusicoccum parvum*

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**Summary.** Many fungal pathogens are associated with grapevine trunk diseases (GTDs), which cause important yield and economic losses in grape production. There are no effective control methods against GTDs once plants are infected, so research is aimed at preventive measures to avoid infections in nurseries and vineyards. Inhibitory activities of the phenolic compounds eugenol, epigallocatechin–3–O–gallate (EGCG) and thymol against the GTD fungi *Diplodia seriata, Eutypa lata, Fomitiporia mediterranea* and *Neofusicoccum parvum* were assessed *in vitro*, and *in planta* as grapevine pruning wound treatments. Greatest inhibition of pathogen mycelium growth was observed with eugenol (fungistatic at 1,500 μg $mL^{-1}$, fungicidal at 2,500 μg $mL^{-1}$). No inhibitory activity against GTD fungi was observed with EGCG. Minimum concentrations with *in vitro* inhibitory effects on *D. seriata* and *N. parvum* spore germination were 360 μg $mL^{-1}$ for thymol and 750 μg $mL^{-1}$ for eugenol. In the grapevine wound protection tests, thymol was effective against *N. parvum* at 360 μg $mL^{-1}$, but eugenol was not.

**Keyword.** Grapevine trunk diseases, thymol, eugenol, epigallocatechin-3-O-gallate, mycelium growth, spore germination, wound protection.

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## INTRODUCTION

Grapevine trunk diseases (GTDs) are major threats to the sustainability of vineyards, and are widespread in all wine-producing countries (Songy *et al.*, 2019). As no curative treatments for these diseases is available, infected grapevine plants must be replaced, resulting in financial losses of approx. $1.5 billion annually (Fischer *et al.*, 2019). In the Czech Republic, damage caused by GTDs is estimated at CZK 150 million p.a. (approx. € 6 million) (Baránek *et al.*, 2017). In the 2000s, GTDs reduced potential wine produc-

tion in France by 13% (Bruez *et al*., 2013). More than 130 species of fungi in 34 genera have been associated with GTDs, and are the largest group of pathogenic fungi that infect one host species (Gramaje *et al*., 2018).

GTDs include the six diseases, the ESCA complex, Eutypa dieback, Botryosphaeria dieback, and Phomopsis dieback occurring especially in mature vineyards, and Petri disease and black foot in young grapevines (Fussler *et al*., 2008; Bertsch *et al*., 2009; Hofstetter *et al*., 2012; Fontaine *et al*., 2016; Gramaje *et al*., 2018; Mondello *et al*., 2018a). Effective management of GTDs is difficult, especially after sodium arsenite was banned, due to its human and animal toxicity (Mondello *et al*., 2018b). Because complete eradication is not possible, GTD control is primarily focused on disease prevention and alleviation (Úrbez–Torres, 2011).

In addition to chemical control attempts (Mondello *et al*., 2018a), recent studies have focused on developing sustainable management strategies against GTD fungi (Compant and Mathieu, 2016). Many organic extracts, such as chitosan, *Evernia prunastri* lichen extract, garlic extract, lemon peel extract, vanillin, and propolis, have been shown to inhibit GTD pathogens such as *Botryosphaeria dothidea*, *Diaporthe ampelina Diplodia seriata*, *Eutypa lata, Ilyonectria macrodidyma, Phaeoacremonium minimum*, and *Phaeomoniella chlamydospora* (Cobos *et al*., 2015, Mondello *et al*., 2018b).

With the increasing implementation of integrated pest management (IPM) systems, natural products are of considerable importance. These substances are obtained from biological sources and include essential oils (phenolic compounds), and natural plant protection products are considered more environmentally friendly than synthetic pesticides (Raveau *et al*., 2020). The use of biological agents instead of synthetic pesticides is strongly encouraged in Europe by Directive 2009/128/EC, which aims to reduce the pesticide use and thus bring agriculture in line with sustainable development.

The present study aimed to evaluate the potential of phenolic compounds for natural resource protection of grapevines. Phenolic compounds are secondary metabolites ubiquitous in most plant tissues (de la Rosa *et al*., 2019). Eugenol ($C_{10}H_{12}O_2$; 2-methoxy-4-(2-propenyl) phenol) is a major component of clove oil (*Syzygium aromaticum, Myrtaceae*), and is also occurs basil, cinnamon, lemon balm, and nutmeg (Dable-Tupas and Egbuna, 2022). It is an allyl chain–substituted guaiacol, clear, light yellow, and greasy liquid, that is slightly soluble in water and soluble in organic solvents (Pramod *et al*., 2010). The antifungal effects of eugenol have been demonstrated on *Aspergillus* spp. and *Cladosporium* spp. (Abbaszadeh *et al*., 2014), and inhibition of the pathogens *Botrytis cinerea* (Hastoy *et al*., 2023; Wang *et al*., 2010), *Fusarium oxysporum* f. sp. *vasinfectum* (Abd–Elsalam and Khokhlov, 2015), and *Rhizoctonia solani* (Zhao *et al*., 2021).

The polyphenolic compound (-)-epigallocatechin-3-O-gallate, $C_{22}H_{18}O_{11}$ (EGCG), is the main catechin detected in green tea (*Camellia sinensis* (L.) Kunze). This compound is an ester of epigallocatechin and gallic acid (Nagle *et al*., 2006), and has antiviral (Calland *et al*., 2012; Steinmann *et al*., 2013; Kaihatsu *et al*., 2018), antibacterial (Kanagaratnam *et al*., 2017; Lee *et al*., 2017), and antifungal effects (Li *et al*., 1999; Navarro-Martinez *et al*., 2006; Park *et al*., 2006) which have been demonstrated in *in vitro* studies.

Thymol ($C_{10}H_{14}O$; 2-isopropyl-5-methylphenol) is the main monoterpene phenol present in essential oils isolated from plants in *Lamiaceae* (*Thymus, Ocimum, Origanum,* and *Monarda*). Thymol can also be isolated from other plants in *Verbenaceae, Scrophulariaceae, Ranunculaceae* and *Apiaceae* (Marchese *et al*., 2016). Thymol had marked *in vitro* inhibitory activity against the phytopathogens *Alternaria* spp., *Botrytis sp., Fusarium* spp., *Phytophora capsici* (Kordali *et al*., 2008), *Colletotrichum acutatum*, *Lasiodiplodia theobromae* (Numpaque *et al*., 2011), and *Rhizoctonia solani*. In *in planta* tests, thymol promoted the emergence of cucumber seedlings (Chauhan *et al*., 2017).

Based on current knowledge of antifungal activities of the phenolic substances, the compounds eugenol, thymol, and EGCG were tested *in vitro* and *in planta* (eugenol and thymol) against GTD fungi. *Diplodia seriata, Eutypa lata, Fomitiporia mediterranea,* and *Neofusicoccum parvum* were selected for these experiments. The aim of this study was to determine whether selected phenolic substances have inhibitory effects against these selected GTD fungi that could be utilized for sustainable management of GTDs in nurseries and vineyards. Inhibitory effects on mycelium growth and spore germination of the three phenolic compounds were tested *in vitro*, and then *in planta* as pruning wound treatments of grapevine cuttings.

## MATERIALS AND METHODS

### *Phenolic substances and chemicals*

The phenolic compounds and chemicals used in this study are listed in Table 1.

In the *in vitro* tests where the effects of phenolic substances on the growth of fungal mycelia were assessed, eugenol was used at concentrations of 1.5 μL $mL^{-1}$ (effective concentration (e.c.) 1,500 μg $mL^{-1}$) and 2.5 μL $mL^{-1}$

**Table 1.** Phenolic compounds and chemicals used in this study.

| Name | Form | Chemical formula | Stock solution concentration (mg mL$^{-1}$) | Concentration used (µg mL$^{-1}$) | Supplier |
|---|---|---|---|---|---|
| Ethanol absolute | liquid | $C_2H_6O$ | - | 2,250.0<br>13,500.0<br>18,000.0 | Charbonneaux Bradant, France |
| Eugenol (100%) | liquid | $C_{10}H_{12}O_2$ | - | 375.0<br>750.0<br>1,500.0<br>2,500.0 | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Epigallocatechin-3-O-gallate (EGCG) | powder | $C_{22}H_{18}O_{11}$ | 1 | 7.5<br>45.0 | Merck KGaA, Darmstadt, Germany |
| Thymol | powder | $C_{10}H_{14}O$ | 1 | 7.5<br>45.0<br>90.0<br>180.0<br>360.0 | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Benomyl. 1-(butylcarbamoyl)-1*H*-1,3-benzimidazol-2-yl methylcarbamate | powder | $C_{14}H_{18}N_4O_3$ | 10 | 20.0 | Sigma Aldrich, Saint Louis, MO, USA |

**Table 2.** Details of the four selected grapevine trunk pathogens examined in this study.

| Pathogen | Isolate | Geographical origin | Sampling date | Grape cultivar | Accession number | Literature source |
|---|---|---|---|---|---|---|
| *Diplodia seriata* | Ds 99-7 | Perpignan, France | 1998 | Syrah | MSZU00000000 | Robert–Siegwald *et al*., 2017 |
| *Eutypa lata* | Bx1.10 | Gironde, Bordeaux, France | 1990 | Cabernet-Sauvignon | – | Péros and Berger, 1994 |
| *Fomitiporia mediterranea* | CO36 | Saint–Preuil, France | 1996 | Ugni-Blanc | – | Laveau *et al*., 2009 |
| *Neofusicoccum parvum* | Bt 67 | Portugal | – | Aragonez | CBS140888 | Rego *et al*., 2009 |

(e.c. 2,500 µg mL$^{-1}$), according to Chauhan *et al*. (2017). For thymol, a stock solution of 1.0 mg mL$^{-1}$ was first prepared using 70:30 water:absolute ethanol solvent (Scoralik *et al*., 2012). Thymol was used at concentrations of 7.5 µL mL$^{-1}$ (e.c. 7.5 µg mL$^{-1}$) and 45 µL mL$^{-1}$ (e.c. 45 µg mL$^{-1}$) (Chauhan *et al*., 2017). Effects of ethanol on fungal growth were assessed at of 2.25 µL mL$^{-1}$ (e.c. 2,500 µg mL$^{-1}$) and 13.5 µL mL$^{-1}$ (e.c. 13,500 µg mL$^{-1}$). An EGCG stock solution of 1.0 mg mL$^{-1}$ was prepared in water (Bartosikova and Necas, 2018). The tested EGCG concentrations were 7.5 µL mL$^{-1}$ (e.c. 7.5 µg mL$^{-1}$) and 45 µL mL$^{-1}$ (e.c. 45 µg mL$^{-1}$) (Chauhan *et al*., 2017).

In the *in vitro* tests for effects on spore germination, thymol solution was prepared at 1.0 mg mL$^{-1}$ in 5% ethanol and then diluted with sterile deionized water to obtain final concentrations of 7.5, 45, 90, 180, or 360 µg mL$^{-1}$. Eugenol solutions were prepared in 1% DMSO at final concentrations of 375, 750, 1,500, or 2,500 µg mL$^{-1}$. To exclude inhibitory effects of ethanol and DMSO on spore germination, concentrations of 18,000 µg mL$^{-1}$ of ethanol and 1% of DMSO were tested. Benomyl (1-(butylcarbamoyl)-1*H*-1,3-benzimidazol-2-yl methylcarbamate; Sigma Aldrich) at 20 µg mL$^{-1}$ was used as a synthetic fungicide for comparisons.

The lowest concentrations that inhibited spore germination were selected to evaluate the potential protective effects of the phenolic compounds on pruning wounds. Thymol solution was used at 360 µg mL$^{-1}$, and eugenol solution was used at 750 µg mL$^{-1}$ with the addition of 1% DMSO.

### *Fungal isolates*

The selected pathogens are shown in Table 2. These fungi were isolated from grapevine wood, and the isolates are available at the collection at Unité Résistance Induite et Bioprotection des Plantes, Université de Reims Champagne-Ardenne, France. Four GTD fungi were tested. These were *D. seriata* Ds 99-7 (Larignon *et al*.,

**Table 3.** Details of the phenolic compounds assessed in this study for their effects on grapevine trunk diseases pathogens. The tests were carried out in PDA culture plates.

| Phenolic compound | Concentration (µL mL$^{-1}$) | Amounts of stock solution per 20 mL PDA | Effective concentration (µg mL$^{-1}$) | Pathogen | Number of repetitions | Total |
|---|---|---|---|---|---|---|
| Ethanol | 2.25 | 45 µL | 2,250.0 | *Diplodia seriata* | 4 | 8 |
| | | | | *Eutypa lata* | 4 | 8 |
| | 13.5 | 270 µL | 13,500.0 | *Fomitiporia mediterranea* | 4 | 8 |
| | | | | *Neofusicoccum parvum* | 4 | 8 |
| Eugenol | 1.5 | 30 µL | 1,500.0 | *Diplodia seriata* | 4 | 8 |
| | | | | *Eutypa lata* | 4 | 8 |
| | 2.5 | 50 µL | 2,500.0 | *Fomitiporia mediterranea* | 4 | 8 |
| | | | | *Neofusicoccum parvum* | 4 | 8 |
| EGCG | 7.5 | 150 µL | 7.5 | *Diplodia seriata* | 4 | 8 |
| | | | | *Eutypa lata* | 4 | 8 |
| | 45.0 | 900 µL | 45.0 | *Fomitiporia mediterranea* | 4 | 8 |
| | | | | *Neofusicoccum parvum* | 4 | 8 |
| Thymol | 7.5 | 150 µL | 7.5 | *Diplodia seriata* | 4 | 8 |
| | | | | *Eutypa lata* | 4 | 8 |
| | 45.0 | 900 µL | 45.0 | *Fomitiporia mediterranea* | 4 | 8 |
| | | | | *Neofusicoccum parvum* | 4 | 8 |
| Control | – | – | – | *Diplodia seriata* | 4 | 4 |
| | | | | *Eutypa lata* | 4 | 4 |
| | | | | *Fomitiporia mediterranea* | 4 | 4 |
| | | | | *Neofusicoccum parvum* | 4 | 4 |
| Total Petri dishes | | | | | | 140 |

2001; Robert-Siegwald *et al*., 2017), *E. lata* Bx1.10 (Péros and Berger, 1994; Laveau *et al*., 2009), *F. mediterranea* CO36 (Laveau *et al*., 2009) and *N. parvum* Np Bt 67 (Rego *et al*., 2009; Reis *et al*., 2016).

### In vitro *assessments of inhibition of mycelium growth*

Inhibitory effects of the selected substances against the four GTD fungi were tested *in vitro* using the potato dextrose agar (PDA; Difco™, Becton, Dickinson and Company) supplemented with different concentrations of phenolic compounds (Table 3). The enriched media were pipetted onto Petri dishes (20 mL per dish). After solidification, a mycelium plug (5 mm diam.) taken from the edge of a 7-d-old colony of the assessed GTD fungus was placed into the centre of the prepared Petri plate. The plates (four replicates per treatment) were then incubated at 25°C in the dark and observed until the mycelium of the control treatment plates reached the plate edges, or for a maximum of 9 days post inoculation (dpi). Two orthogonal diameters of each fungi colony were measured, and the mean colony diameter was calculated. The mycelial plugs that did not show any growth in the enriched PDA were transferred onto nonamended PDA to verify fungistatic or fungicidal effects of the tested phenolic compounds. The same method was used to test effects of ethanol on the growth of *D. seriata* and *N. parvum*, because ethanol was used to prepare the thymol stock solutions.

Statistical analyses of the data obtained were carried out using Statistica 12 CZ StatSoft Prague CZ. Analysis of variance (ANOVA) was performed (at $P$ = 0.01), and post hoc tests (Tukey's HSD tests: $P$ = 0.01) were then applied. The percentage inhibition was calculated according to the formula $I=[(r_c-0.5)-(r_t-0.5)]/[(r_c-0.5)\times100]$, where I = percentage of inhibition, $r_c$ = average of the measured values for the experimental controls, and $r_t$ = average of the measured values for the treated condition. The value 0.5 cm was subtracted as the disc size (Chauhan *et al*., 2017).

The $EC_{50}$ were determined for the phenolic compounds that gave statistically significant inhibition. Concentrations and percentage inhibition values were used for linear regression analyses. Regression equations were determined, from which the $EC_{50}$ value for each compound was calculated. This experiment was carried out twice.

In vitro *assessments of inhibition of spore germination*

Based on the results from the mycelium growth assessments, thymol and eugenol were selected for *in vitro* treatments of *D. seriata* and *N. parvum* spore suspensions. *Eutypa lata* and *F. mediterranea* were discarded due to the lack of sporulation of both fungi in laboratory conditions, difficulty in collecting *E. lata* ascospores from infected grapevine wood, and the low germination rates of *F. mediterranea* basidiospores (Živković *et al*., 2012; Moretti *et al*., 2021).

Isolates of *D. seriata* Ds 99-7 and *N. parvum* Bt 67 were maintained on PDA at 25°C. Pycnidia of *D. seriata* were observed after an approx. 4 week incubation period. For *N. parvum,* conidia were obtained according to Úrbez-Torres and Gubler (2009). Mycelial plugs from 7- to 10-day-old cultures were placed on 2% water agar containing sterile pine needles. Petri dishes were incubated at 25°C in a 12 h light:12 h dark cycle, until pycnidia were formed. Pycnidia of the two isolates were collected separately, and were then each crushed in sterile demineralized water in a 1.5 mL sterile microcentrifuge tube, using a sterile plastic pestle. Each spore suspension was filtered through sterile cheesecloth, and the spore concentrations were adjusted with potato dextrose broth (PDB, Difco Laboratories) to $2 \times 10^4$ conidia $mL^{-1}$ using haemacytometer spore counts.

In the first phase of testing, inhibitory effects of thymol and eugenol on *D. seriata* spore germination were assessed at the same concentrations that were used in the *in vitro* tests on mycelium growth, thymol at 7.5 and 45 μg $mL^{-1}$, and eugenol at 1,500 and 2,500 μg $mL^{-1}$. The fungicide benomyl at 20.0 μg $mL^{-1}$, and water with 1% DMSO were also tested, respectively, as positive and negative experimental controls. In the second phase of testing against *D. seriata*, double concentrations of thymol (90, 180 and 360 μg $mL^{-1}$) and half doses of eugenol (375 and 750 μg $mL^{-1}$) were used. The effects of ethanol (at 18,000 μg $mL^{-1}$), which was used for the preparation of thymol solutions, and 1% DMSO, which was used to increase the dispersion of eugenol in water, were also assessed. For *N. parvum*, the two greatest concentrations of thymol (180 and 360 μg $mL^{-1}$), and the two lowest concentrations of eugenol (375 and 750 μg $mL^{-1}$) were tested. Effects of benomyl (20.0 μg $mL^{-1}$) were also assessed. Spore suspension (100 μL) was mixed with 100 μL of each tested solution in each well of 96-well microplates, and these were incubated at 25°C with shaking (180 rpm) in the dark. Optical density measurements were carried out at 0 and 72 h at 600 nm wavelength, using a spectrophotometer (MP96 Safas). This experiment was carried out twice.

In planta *treatment of grapevine wounds with phenolic compounds*

Evaluation of the potential protective effects of phenolic compounds on grapevine wounds was carried out using the method described by Lecomte and Dewasme (2004), with some modifications. Cuttings from *Vitis vinifera* 'Chardonnay' plants were collected from 15-year-old pruned canes growing in established vineyards, after winter pruning (January). The three-node long segments were cut and kept in a cold chamber at 4°C for 1 month. These segments were then surface sterilized with 0.05% cryptonol (8-hydroxyquinoline sulfate), rinsed with tap water, and placed back at 4°C until used. The cuttings were cut into 1-node segments, surface sterilized by wiping with cotton wool swabs of 70% ethanol, and the tips were each trimmed to 1 cm lengths with scissors, to produce fresh wounds. The cuttings were then placed vertically in plastic boxes containing moistened sand to maintain high humidity. On each fresh wound spray applications were made with approx. 650 μL of thymol solution (360.0 μg $mL^{-1}$) or eugenol solution (750 μg $mL^{-1}$ with 1% DMSO). For experimental controls, the same volumes of water containing 1% DMSO were used. Each plastic box was covered with a lid, and the samples were incubated at 25°C for 24 h. Subsequently, 50 μL of spore suspension of *N. parvum* or *D. seriata* in sterile deionized water (prepared as described above), containing approx. 200 conidia were pipetted onto each wound, and the samples were left at room temperature until the conidium suspension drops were absorbed. Plastic boxes (one for each tested condition) were covered with lids and incubated at 25°C for 7 d. Ten cuttings were used for each treatment.

After incubation, each cutting was removed from the sand, and the surface was sterilized by cotton wool wiping with 70% ethanol. The cutting tip was briefly sterilized in the flame, and the bark was removed using a sterile scalpel. Five wood chips (approx. 2 mm thick) were cut using sterilized scissors that were flamed before each cut. The five wood chips (levels) cut from the same cane were placed onto one Petri dish containing PDA, noting their position with regard to the inoculated wound zone, and the dishes were then incubated at 25°C for 5 d.

Development of *N. parvum* mycelium was evaluated on every wood chip, to determine how deeply the germinating conidia of the pathogens were able to colonize the woody tissues of each cane, and, inversely, the efficacy of phenolic compound treatment to prevent pathogen colonization. The percentage of colonized chips was calculated using the formulae $\%_{col.} = x \times 100/n$, and the percentage of treatment efficiency was calculated as $\%_{ef.} = y \times 100/n$, where $\%_{col.}$ = colonization percentage, $x$ = number of chips in which pathogen mycelium growth

was observed, $\%_{ef.}$ = efficiency percentage, $y$ = number of chips in which no mycelial growth was observed, and n = 10 (the number of replicates). The efficiency percentages were calculated for each level of wood section (1 to 5). The test results were considered valid if the experimental controls gave at least 60% colonized chips in the first two wood levels. The treatments were considered efficient if the colonized chips did not exceed 30% in the first two wood levels (= efficiency at > 70%).

## RESULTS

### In vitro *inhibitory effects on mycelium growth*

The results of the *in vitro* assays are reported in Table 4. Among the studied phenolic compounds, eugenol and thymol inhibited all of the tested GTD fungi, while no significant inhibition was detected from the EGCG treatments.

Eugenol at both concentrations had strong antifungal activity. Complete growth inhibition (100%) was observed for all four GTD pathogens after treatment with 2,500 μg mL$^{-1}$, due to an ascertained fungistatic effect, and only a slightly lower effect was observed at 1,500 μL mL$^{-1}$, where *D. seriata* growth was reduced by 98%, *E. lata* by 99%, *F. mediterranea* by 100%, and *N. parvum* by 99%.

Thymol at a concentration of 45 μg mL$^{-1}$ inhibited the growth of all the tested pathogens, with greatest activity (89%) against *D. seriata* and least (67%) against *E. lata*. Concentration of 7.5 μg mL$^{-1}$ thymol was less effective and reduced the mycelium growth of three of the four pathogens, by 23% for *F. mediterranea,* 16% for *N. parvum* and 12% for *D. seriata*. Ethanol, regardless of concentration, did not affect ($P > 0.01$) growth of *D. seriata* or *N. parvum* growth, so ethanol did not affect the efficacy of thymol solution. For *E. lata* and *F. mediterranea*, the effect of ethanol was not tested due to the low percentages of thymol inhibition found for the four fungi.

$EC_{50}$s for phenolic compounds with statistically significant inhibitory activity were calculated using linear regression analyses (Table 5). For eugenol, the $EC_{50}$ were

**Table 4.** Mean percent (%) inhibition of mycelial growth of four grapevine trunk disease pathogens measured in *in vitro* tests of phenolic compounds. (* indicates treatments giving differences ($P = 0.01$) from experimental controls).

| Substance | Concentration (μL mL$^{-1}$) | Effective concentration (μg mL$^{-1}$) | *Diplodia seriata* | *Eutypa lata* | *Fomitiporia mediterranea* | *Neofusicoccum parvum* |
|---|---|---|---|---|---|---|
| Ethanol | 2.25 | 2,250.0 | 0% | - | - | 2% |
| | 13.5 | 13,500.0 | 0% | | | 19% |
| Eugenol | 1.5 | 1,500.0 | 98%* | 99%* | 100%* | 99%* |
| | 2.5 | 2,500.0 | 100%* | 100%* | 100%* | 100%* |
| EGCG | 7.5 | 7.5 | 0% | 9% | 11% | 8% |
| | 45.0 | 45.0 | 0% | 3% | 0% | 6% |
| Thymol | 7.5 | 7.5 | 12%* | 4% | 23%* | 16%* |
| | 45.0 | 45.0 | 89%* | 67%* | 74%* | 88%* |

**Table 5.** Linear regression analyses, and determination of the $EC_{50}$ concentrations, for two phenolic compounds (eugenol or thymol) with statistically significant inhibitory activities when tested against four grapevine trunk pathogens.

| Treatment | Pathogen | Regression equations | $R^2$ | $EC_{50}$ (μg mL$^{-1}$) |
|---|---|---|---|---|
| Eugenol | *Diplodia seriata* | $y = 41.707x + 10.139$ | 0.8492 | 960.0 |
| | *Eutypa lata* | $y = 41.758x + 10.379$ | 0.8435 | 950.0 |
| | *Fomitiporia mediterranea* | $y = 42.061x + 10.438$ | 0.8440 | 940.0 |
| | *Neofusicoccum parvum* | $y = 41.571x + 8.544$ | 0.8659 | 1,000.0 |
| Thymol | *Diplodia seriata* | $y = 2.0062x - 1.0627$ | 0.9977 | 25.45 |
| | *Eutypa lata* | $y = 1.5565x - 3.3607$ | 0.9469 | 34.28 |
| | *Fomitiporia mediterranea* | $y = 1.5472x + 5.0982$ | 0.9728 | 29.02 |
| | *Neofusicoccum parvum* | $y = 1.9466x + 0.6071$ | 0.9961 | 25.37 |

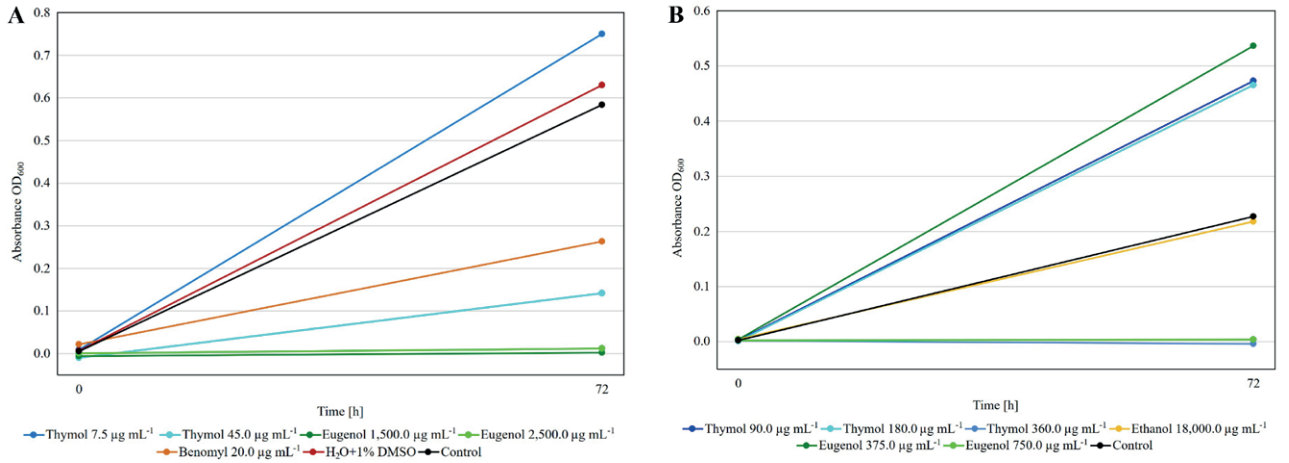


**Figure 1.** Absorbances measured for *Diplodia seriata* conidium suspensions treated with different phenolic compounds or chemicals (benomyl or DMSO). A) Tests of different concentrations of thymol and eugenol in *in vitro* tests for effects on conidium germination. B) Tests of doubled concentrations of thymol and halved concentrations of eugenol.

from 940 to 1,000 µg mL$^{-1}$, and for thymol, were from 25.4 to 34.3 µg mL$^{-1}$, depending on the pathogen.

### In vitro *effects of phenolic compounds on conidium germination*

Thymol and eugenol, at the same concentrations as those for the mycelium inhibition assays, were used to evaluate their activities against conidia of *D. seriata*. Eugenol at 1,500 or 2,500 µg mL$^{-1}$ prevented germination of conidia (Figure 1A). Thymol at 7.5 µg mL$^{-1}$ did not inhibit spore germination, while at 45 µg mL$^{-1}$ conidium germination was reduced. Similar results were obtained for the fungicide benomyl at 20 µg mL$^{-1}$, which also suppressed *D. seriata* conidium germination.

To determine the minimum effective concentrations against *D. seriata* conidia, the eugenol concentration was decreased, and the thymol concentration increased. Eugenol completely inhibited spore germination at 750 µg mL$^{-1}$, while thymol was similarly efficient at 375 µg mL$^{-1}$ (Figure 1B). Conidia treated with ethanol at 18,000 µg mL$^{-1}$ germinated at a similar rate as the control, showing that ethanol did not affect the thymol assay, even at the greatest thymol concentration used.

For treatments of *N. parvum* conidia, thymol and eugenol concentrations were selected based on results obtained for *D. seriata*. No increased absorbance was observed for thymol at 360 µg mL$^{-1}$, or eugenol at 375 or 750 µg mL$^{-1}$, indicating inhibition of conidium germination (Figure 2). For *N. parvum*, the fungicide benomyl completely inhibited conidium germination.

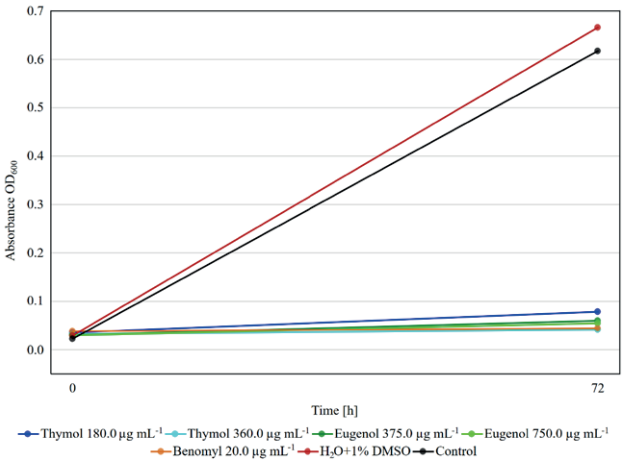


**Figure 2.** Absorbances measured for *Neofusicoccum parvum* spore suspensions treated with different phenolic compounds each at two concentrations, or chemicals (benomyl or DMSO).

Water plus 1% DMSO had no effect on the germination of *D. seriata* or *N. parvum* conidia, indicating that use of DMSO to facilitate formation eugenol emulsions did not affect the results (Figure 1 A and B, and Figure 2).

### In planta *treatments of grapevine wounds with phenolic compounds*

Thymol at 360 µg mL$^{-1}$ or eugenol at 750 µg mL$^{-1}$ were assessed on grapevine canes as wound protection agents against *N. parvum* and *D. seriata*. The results for *N. parvum* can be considered valid, as the colonization

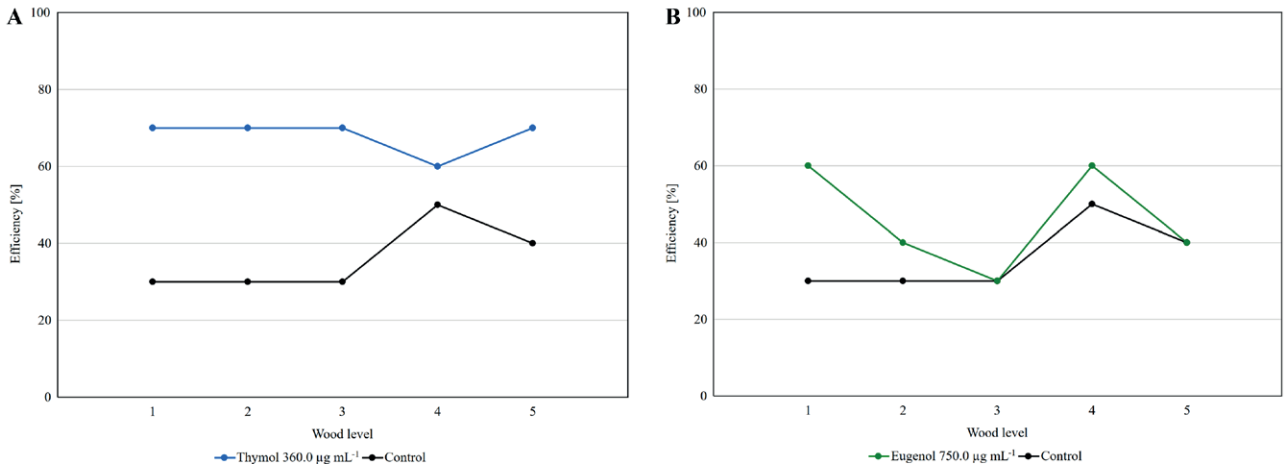


**Figure 3.** Efficiency (%) of grapevine wound protection from *Neofusicoccum parvum* infection, with: A) thymol, and B) eugenol.

of wood at the first two levels exceeded 60% in the controls (Figure 3 A and B). In contrast, *D. seriata* did not reach the minimum of colonized fragments in untreated wood under the same conditions as *N. parvum*, despite repetition of the experiment (observed colonization rates were 35% in the first experiment and 40% in the second). The thymol treatment reduced *N. parvum* colonization to 30% in the first three wood chips, which confirms efficiency of this compound against *N. parvum*. In contrast, eugenol showed maximum efficacy of 60%; so it is unlikely to be an effective grapevine wound treatment for against *N. parvum*.

## DISCUSSION

Previous studies have confirmed the antimicrobial effects of phenolic compounds produced and purified from plants. Substances that inhibit fungi, bacteria, or viruses include eugenol, thymol, and EGCG (Li *et al.*, 1999; Kordali *et al.*, 2008; Wang *et al.*, 2010; Calland *et al.*, 2012; Kanagaratnam *et al.*, 2017; Hakalová *et al.* 2022; Hastoy *et al.,* 2023), and these were selected for the present study. The *in vitro* tests demonstrated the antifungal activity of thymol and eugenol towards mycelium growth and conidium germination of the tested GTD fungi. In addition, thymol was also effective for pruning wound protection against *N. parvum*.

These results confirm previous reports demonstrating that eugenol and thymol reduced mycelium growth of fungal plant pathogens. Eugenol limited *in vitro* growth of the postharvest apple pathogens *Phlyctema vagabunda, Penicillium expansum, Botrytis cinerea* and *Monilinia fructigena* by 73% to 90% at a concentration of 1,000 µg $mL^{-1}$, depending on the pathogen species and incubation temperature, and 100% at 2,000 µg $mL^{-1}$ for all these fungi and at 4 and 20°C (Amiri *et al.*, 2008). This is similar to the present study results where greater than 98% growth decreases were obtained after treatment with 1,500 µg $mL^{-1}$ eugenol, and complete inhibition with 2,500 µg $mL^{-1}$. Lower concentration (30 µg $mL^{-1}$) of eugenol added to liquid medium inhibited growth of *Botrytis fabae* by 73% (Oxenham *et al.*, 2005).

The efficiency of thymol for suppression of GTD pathogen mycelium growth in the present study was comparable to or greater than that of other pathogenic fungi, according to some reports. Numpaque *et al.* (2011) showed that thymol, at 50 µg $mL^{-1}$, reduced mycelium growth of *Lasiodiplodia theobromae*, a pathogen associated with grapevine dieback, by approx. 25%. The present study measured approx. 90% decreases for *D. seriata* and *N. parvum* at 45 µg $mL^{-1}$ of thymol. Complete inhibition of *L. theobromae* growth was achieved only after treatment with 150 µg $mL^{-1}$. The same concentration completely inhibited the mycelium growth of *Fusarium moniliforme, Rhizoctonia solani,* and *Sclerotinia sclerotiorum* (Müeller-Riebau *et al.*, 1995). Zhang *et al.* (2019) reported 100% effectiveness of thymol at 65 µg $mL^{-1}$ against *B. cinerea*.

For EGCG (concentrations 7.5 and 45.0 µg $mL^{-1}$), no inhibitory effects against the tested GTD fungi were measured. EGCG has exhibited antifungal activity in other studies, at concentrations similar to those used in the present study experiment. Li *et al.* (1999) measured an EGCG MIC of 12.5 µg $mL^{-1}$ against the human pathogen *Cryptococcus neoformans*. This result was different from the MIC determined by Matsumoto *et al.* (2012) (64 µg $mL^{-1}$) for the same species. Only 0.31 µg

$mL^{-1}$ EGCG was sufficient to inhibit the growth of *Candida glabrata*, while for *C. albicans* and *C. parapsilosis*, 5 μg $mL^{-1}$ was necessary (Chen *et al*., 2015). Hirasawa and Takada (2004) reported that antifungal activity of catechin was pH dependent. pH strongly influenced EGCG efficiency against *C. albicans* strains, as demonstrated by yeast growth reduction of 90% from 2,000 μg $mL^{-1}$ at pH 6.0, and from 15.6 to 250 μg $mL^{-1}$ at pH 7.0.

The $EC_{50}$ determined for eugenol in the present study was greater than those established for other pathogens. The $EC_{50}$s ranged from 940 μg $mL^{-1}$ for *F. mediterranea* to 1,000 μg $mL^{-1}$ for *N. parvum,* which contrast with the published $EC_{50}$ of 3.6 μg mL−1 for *Rhizoctonia solani* (Zhao *et al*., 2021). Lower $EC_{50}$ values than those for the fungi examined in the present study were also reported for *B. cinerea*, at 36.62 μg $mL^{-1}$) by Wang *et al.* (2010) and 240 and 263 μg $mL^{-1}$ by Hastoy *et al*. (2023), depending on the isolate. Other *Botryosphaeriaceae* pathogens were found to be sensitive to lower eugenol concentrations than *D. seriata* and *N. parvum*, such as *Botryosphaeria kuwatsukai* ($EC_{50}$ = 65.07 μg $mL^{-1}$; Wang *et al*., 2010) and *B. dothidea* ($EC_{50}$ = 114.43 μL $mL^{-1}$/120.08 μg $mL^{-1}$; Li *et al*., 2021).

The $EC_{50}$ values determined for thymol in the present study were 40 times less than those for eugenol, and are similar to those reported in other studies. Ding *et al.* (2023) evaluated thymol activity against the postharvest blueberry pathogens *Aspergillus niger*, *Neopestalotiopsis* sp., *Alternaria alternata*, *Penicillium* sp., *Cladosporium xylophilum* and *B. cinerea*, which indicated a mean $EC_{50}$ of 38.52 μg $mL^{-1}$ for the six fungi. This is similar to the $EC_{50}$ observed here for *E. lata* (34.28 μg $mL^{-1}$). The thymol $EC_{50}$ for the remaining pathogens tested in the present study was from 25.37 μg $mL^{-1}$ for *N. parvum* to 29.02 μg $mL^{-1}$ for *F. mediteranea*, which correspond to reports for *Fusarium graminearum* (26.3 μg $mL^{-1}$; Gao *et al*., 2016) and for *F. oxysporum* (26.4 μg $mL^{-1}$; Liu *et al*., 2022).

Compared to other research, the present study indicates that eugenol can exhibit diverse efficacy for suppressing conidium germination, depending on the fungal species. Conidium germination of *Diplodia seriata* and *N. parvum* was completely inhibited by eugenol at concentration of 750 μg $mL^{-1}$. Abbaszadeh *et al.* (2014) tested eugenol against propagules of important plant and food pathogens, and determined MIC values from 350 μg $mL^{-1}$ for *Aspergillus ochraceus* to 500 μg $mL^{-1}$ for *Alternaria alternata*, while Wang *et al.* (2010) indicated that 40,000 μg $mL^{-1}$ did not inhibit conidium germination of *B. cinerea*. The concentration of eugenol was greater (2,000 μg $mL^{-1}$) than the $EC_{50}$s determined in the present study for *D. seriata* and *N. parvum*, and reduced germination of *Phytophthora vagabunda* and *P. expansum* by less than 60% (Amiri *et al.,* 2008). Thymol had greater efficacy than eugenol, and at 360.0 μg $mL^{-1}$, thymol completely inhibited germination of *N. parvum* and *D. seriata* conidia. A lower thymol concentration (100.0 μg $mL^{-1}$) was sufficient to reduce *F. oxysporum* spore germination by 80% (Liu *et al*., 2022), and 100% inhibition was achieved by Oh *et al.* (2022) for *Botrytis aclada* conidia. Their study also reported that *Aspergillus awamori*s conidia were resistant to thymol, and only treatment with 500 μg $mL^{-1}$ completely inhibited germination. These results indicate that the two tested GTD fungi are susceptible to thymol at similar concentrations to those of other investigated fungi.

Of the two pathogens inoculated onto grapevine wounds, only *N. parvum* colonized the wood of control canes at sufficient depths (70% in the first two wood levels) to give valid results. *Diplodia seriata* colonization was only 40% at maximum, which could be due to reduced aggressiveness of the isolate assessed, or to low conidium viability. Grapevine wound treatments with eugenol against *N. parvum* were not effective.

No previous studies have focused on the application of eugenol to wood wounds for management of fungal pathogens. Thymol treatment (360 μg $mL^{-1}$) was efficient against *N. parvum*. According to Jankura (2012), thymol at 500 μg $mL^{-1}$ inhibited *in planta Phaeomoniella chlamydospora* on the ends of woody grapevine segments.

Commercial antifungal preparations containing phenolic compounds are approved in some European countries. For example, BIOXEDA (clove (*Syzygium aromaticum*) essential oil), which can be used as a fungicide or bactericide for apple and pear storage pathogens. PREV-AM and ESSEN' CIEL (sweet orange (*Citrus sinensis*) essential oil) is approved for use against mealybug, potato blight, powdery mildew and tobacco thrips on vegetables, fruit, ornamental crops, and tobacco. BIOXEDA has certified approval in Cyprus, France, Greece, and Spain. PREV-AM is approved in Belgium, France, German, Italy, Romania, and Spain. ESSEN' CIEL is approved for use in France, Italy, and Spain. The use of these products can therefore lead to reductions in the use of chemical pesticides in agriculture (Raveau *et al*., 2020). According to Torre *et al*. (2014), BIOXEDA can also be used in vineyards to protect against downy mildew (*Plasmopara viticola*). Application of the product to grapevine leaves reduced symptom incidence by 30%. According to Lasorello *et al*. (2018), applying PREV-AM in vineyards reduced incidence of powdery mildew (*Erysiphe necator*) by 92% on leaves and 79% on grapes. Applications of preparations based on phenolic substances that inhibit grapevine pathogens is therefore likely to reduce application of chemical pesticides, especially in vineyards with ecological management systems.

## CONCLUSIONS

This study examined the antifungal effects of three phenolic compounds on the GTD fungi *D. seriata, E. lata, F. mediterranea*, and *N. parvum*. In the *in vitro* mycelial growth tests, the inhibitory effects were demonstrated for eugenol and thymol, but no impacts of EGCG on mycelium growth were detected. Based on the inhibitory effects observed for eugenol and thymol against GTD fungi, their efficacy was further evaluated by treating conidium suspensions and grapevine pruning wounds under controlled conditions. Protection with thymol at 360 µg $mL^{-1}$ was effective against conidium germination of these fungi *in vitro*, and against *N. parvum* development on pruning wounds. Eugenol at 750 µg $mL^{-1}$, prevented conidium germination *in vitro* but did not prevent development of *N. parvum* on pruning wounds. These results show that thymol has potential for use in vineyards as a natural agent for use against the *Botryosphaeriaceae* pathogen *N. parvum*.

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Short Notes – 12th Special issue on Grapevine Trunk Diseases

# Dual labelled probe assays for differentiation of *Botryosphaeria dothidea*, *Neofusicoccum mediterraneum* and *Neofusicoccum parvum*, based on polymorphisms in the MAT1-2-1 gene

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**Summary.** Botryosphaeriaceous fungi are widespread, and cause serious diseases in many economically important crops. *Botryosphaeria dothidea*, *Neofusicoccum mediterraneum* and *N. parvum* are the most important members of this family in the Mediterranean region. These fungi are frequently isolated from the same host, which together with their extensive and increasing host range necessitates development of rapid and reliable diagnostic tools. Species boundaries within the *Botryosphaeriaceae* have been defined based on phylogenetic analyses of multiple gene sequences, including those of mating type genes. The MAT1-2-1 gene displayed high sequence variability between Botryosphaeriaceous species, so was selected as the target for development of a definitive diagnostic tool. This paper outlines a new and robust molecular tool, composed of three TaqMan assays based on polymorphisms located in the MAT1-2-1 gene of *B. dothidea*, *N. mediterraneum* and *N. parvum*. Each assay differentiated the target species from other *Botryosphaeriaceae*, and from non-target fungi.

**Keywords.** Diagnosis, mating type genes, molecular marker, real time PCR.

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Fungal pathogens in the *Botryosphaeriaceae* have been reported as the causal agents of severe losses in a wide range of hosts, including agricultural, horticultural and forest plants (Slippers *et al.*, 2007; Vakalounakis *et al.*, 2019; Aiello *et al.*, 2020; Guarnaccia *et al.*, 2020; Batista *et al.*, 2021; Luna *et al.*, 2022; Guarnaccia *et al.*, 2023). Symptoms caused by these pathogens include cankers and dieback in twigs and branches, leaf necroses, blight, and fruit rots (Chen et al., 2014; Marsberg et al., 2017). Important hosts of these pathogens are pistachio (*Pistacia vera* L.), grapevine (*Vitis vinifera*) and citrus. The main species causing high yield losses in Greece and other Mediterranean countries, where pistachio and grapevine are cultivated, are *Botryosphaeria dothidea*, *Diplodia seriata, Neofusicoccum vitifusiforme*, *N. mediterraneum* and *N. parvum* (Lazzizera *et al.*, 2008; Chen *et al.*, 2015; Stempien *et al.*, 2017; Bezerra *et al.*, 2021).

Members of the *Botryosphaeriaceae* are known to reproduce sexually, through ascospores formed in perithecia, and asexually from conidia produced in pycnidia. *Botryosphaeria dothidea* and *N. parvum* are homothallic, bearing the MAT1-1-1 and MAT1-2-1 genes in single strains, whereas *N. vitifusiforme*, and *N. mediterraneum* are heterothallic. For *N. vitifusiforme*, only MAT1-1-1 strains have been reported, whereas for *N. mediterraneum*, strains bearing MAT1-1-1 or MAT1-2-1 genes have been described (Lopes *et al*., 2017; Marsberg *et al*., 2017).

Species differentiation within the *Botryosphaeriaceae* has been mainly based on phylogenetic analyses of multiple gene sequences, such as the internal transcribed spacer region (ITS), the elongation factor 1-alpha, (TEF1-a), the beta tubulin, and the RNA polymerase II subunit (RPB2) (Pavlic *et al*., 2009; Chen *et al*., 2015). Although an RFLP analysis tool based on the ITS region could distinguish the main Botryosphaeriaceous species, this could not differentiate five species, including *N. parvum* (Slippers *et al*., 2007). More rapid molecular tools, such as PCR primers, have been developed for detection of Botryosphaeriaceous species only at the genus level, or for differentiation of *Botryosphaeria dothidea* from *Neofusicoccum* spp. (Ridgway *et al*., 2011; Palavouzis *et al*., 2022). Pathogen phylogenies based on mating type genes were comparable to multigene analyses for species differentiation. Notably, the MAT1-2-1 gene displayed high sequence variability between Botryosphaeriaceous species (Lopes *et al*., 2017).

With many species causing disease in an increasing range of hosts (Vakalounakis *et al*., 2019; Batista *et al*., 2020), rapid and accurate detection at the species level is important for the effective management of these pathogens, including quarantine measures and epidemiological studies. The present study reports development of robust and dual labelled probe molecular tools ("TaqMan" technology), targeting the MAT1-2-1 gene, for differentiation among *B. dothidea*, *N. mediterraneum* (MAT1-2-1 strains) and *N. parvum*, from fungal cultures and *in planta*.

Initially, gene sequences of MAT1-2-1 from *N. mediterraneum*, *N. parvum* and *B. dothidea* retrieved from the NCBI database, and sequences for each species derived from isolates of our own collection (deposited in GenBank under the Accession Numbers OQ632937 for *B. dothidea*, OQ632936 for *N. parvum* and OQ596433 for *N. mediterraneum*), were aligned using Clustal Omega (Madeira *et al*., 2019). Differences between sequences of the three species were detected using the MEGA software (Kumar *et al*., 2018). Based on sequence polymorphisms, dual labelled probes and primers were designed for MAT1-2-1 genes of *B. dothidea* (homothallic), *N. mediterraneum* MAT1-2-1 strain and *N. parvum* (homothallic). Primers were designed so that nucleotide polymorphisms specific for the target sequence were positioned at the 3' end of each primer, and PCR amplicons were smaller than 200 bp. Dual labelled probes were designed to have a Tm 8–10°C higher than the primers with a sequence length of 15–30 bp, avoiding a G nucleotide at the 5' end of the probe so that quenching of the 5' fluorophore was prevented. Nucleotide polymorphisms conferring probe specificity were placed close to the middle of the probe sequence. Primers and probes were screened for self-dimers, heterodimers and hairpins, using primer 3 plus software. To check target specificity, blast search against non-target *Diplodia seriata* MAT 1-2-1 sequences showed low homology with designed primers and probes. Primers and probes were then synthesized by eurofins genomics, labelling the 5' end of all probes (for *B. dothidea*, *N. mediterraneum* and *N. parvum*) with fluorescein (FAM) and the 3' end with the Black Hole Quencher-1 (BHQ1) (Table 1).

**Table 1.** List of primers and probe sequences for TaqMan qPCR assays, developed for detection and quantification of *Botryosphaeria dothidea, Neofusicoccum mediterraneum* and *Neofusicoccum parvum*, based on polymorphisms in the corresponding MAT1-2-1 genes. Polymorphisms of primers and probes between the different species are indicated with nucleotides letters in bold font.

| Primer/probe name | Sequence (5'->3') | Target Gene | Product size (bp) |
|---|---|---|---|
| Botdo2-1_295F | TCGCATCCTCTTCCCTCC**TG** | Botryosphaeria dothidea MAT1-2-1 | 86 |
| Botdo2-1_380R | AGGCCAAGACCTGCTGA**AGT** | | |
| Tqm-Botdo2-1_316pb | **ATA**C**GTC**GC**ACCC**G**CTCC**CAAC | | |
| Neomed2-1_260F | GTCCGCGCTCCAGTCA**TC** | Neofusicoccum mediterraneum MAT1-2-1 | 114 |
| Neomed2-1_373R | AGGCTGAGGAGTGGAA**ACC** | | |
| Tqm-Neomed2-1_324pb | CGA**CCC**TCC**AT**GC**TGA**CGG**CG** | | |
| Neopar2-1_292F | CTGACCTTGTCCAGCA**CG** | Neofusicoccum parvum MAT1-2-1 | 133 |
| Neopar2-1_424F | GCTGAGAAGCCGAA**GGTG** | | |
| Tqm-Neopar2-1_377pb | CGA**ACT**TCC**GC**GC**CA**A**T**GG**TA**TCAAC | | |

**Table 2.** Mean Ct values (and standard deviations) for different fungi in three dual labelled probe assays targeting the MAT1-2-1 gene for diagnosis of *Botryosphaeria dothidea, Neofusicoccum mediterraneum* (MAT 1-2-1 strains) and *Neofusicoccum parvum*.

| MAT1-2-1 gene target | Template | Mean Ct | St. Dev. (3 reps) |
|---|---|---|---|
| *B. dothidea* | *B. dothidea* 8 ng $\mu L^{-1}$ | 20.6 | 0.2 |
| | *B. dothidea* 2 ng $\mu L^{-1}$ | 21.1 | 0.2 |
| | *B. dothidea* 200 pg $\mu L^{-1}$ | 24.6 | 0.3 |
| | *B. dothidea* 200 pg $\mu L^{-1}$ & *Pistacia vera* 8 ng $\mu L^{-1}$ | 23.5 | 0.1 |
| | *B. dothidea* 20 pg $\mu L^{-1}$ | 28.1 | 0.3 |
| | *B. dothidea* 2 pg $\mu L^{-1}$ | 31.3 | 0.3 |
| | *B. dothidea* 200 fg $\mu L^{-1}$ | 35.7 | 0.3 |
| | *N. parvum* 8 ng $\mu L^{-1}$ | 35.6 | 1.1 |
| | *N. mediterraneum* "58" (MAT1-2-1) 8 ng $\mu L^{-1}$ | undetermined | |
| | *N. mediterraneum* "28" (MAT1-1-1) 8 ng $\mu L^{-1}$ | undetermined | |
| | *P. chlamydospora 8* ng $\mu L^{-1}$ | undetermined | |
| | *Diplodia seriata* 8 ng $\mu L^{-1}$ | undetermined | |
| | *Pistacia vera* 8 ng $\mu L^{-1}$ | undetermined / 35.5 | NA |
| *N. mediterraneum* | *N. mediterraneum* "58" (MAT1-2-1) 8 ng $\mu L^{-1}$ | 21.7 | 0.1 |
| | *N. mediterraneum* "28" (MAT1-1-1) 8 ng $\mu L^{-1}$ | undetermined | |
| | *N. mediterraneum* 2 ng $\mu L^{-1}$ | 24.7 | 0.0 |
| | *N. mediterraneum* 200 pg $\mu L^{-1}$ | 28.3 | 0.3 |
| | *N. mediterraneum* 200 pg $\mu L^{-1}$ & *Pistacia vera* 8 ng $\mu L^{-1}$ | 26.0 | 0.3 |
| | *N. mediterraneum* 20 pg $\mu L^{-1}$ | 31.9 | 0.3 |
| | *N. mediterraneum* 2 pg $\mu L^{-1}$ | 35.2 | 1.2 |
| | *B. dothidea* 8 ng $\mu L^{-1}$ | 36.5 | 0.4 |
| | *N. parvum* 8 ng $\mu L^{-1}$ | 35.8 | 0.7 |
| | *P. chlamydospora* 8 ng $\mu L^{-1}$ | undetermined | |
| | *Diplodia seriata* 8 n $\mu L^{-1}$ | undetermined | |
| | *Pistacia vera* 8 ng $\mu L^{-1}$ | 37.5 | 1.2 |
| *N. parvum* | *N. parvum* 8 ng $\mu L^{-1}$ | 22.4 | 0.3 |
| | *N. parvum* 2 ng $\mu L^{-1}$ | 24.2 | 0.1 |
| | *N. parvum* 200 pg $\mu L^{-1}$ | 27.9 | 0.1 |
| | *N. parvum* 200 pg $\mu L^{-1}$ & *Pistacia vera* 8 ng/ $\mu L^{-1}$ | 27.7 | 1.0 |
| | *N. parvum* 20 pg $\mu L^{-1}$ | 31.3 | 0.4 |
| | *N. parvum* 2 pg $\mu L^{-1}$ | 34.5 | 0.7 |
| | *B. dothidea* 8 ng $\mu L^{-1}$ | 36.3 | 0.5 |
| | *N. mediterraneum* "58" (MAT1-2-1) 8 ng $\mu L^{-1}$ | undetermined | |
| | *N. mediterraneum* "28" (MAT1-1-1) 8 ng $\mu L^{-1}$ | undetermined | |
| | *P. chlamydospora* 8 ng $\mu L^{-1}$ | undetermined | |
| | *Diplodia seriata* 8 n $\mu L^{-1}$ | undetermined | |
| | *Pistacia vera* 8 ng $\mu L^{-1}$ | 36.0 | 0.1 |

NA: not applicable - the target was detected in one replicate.

For the three dual labelled probe assays, the PCR mixture contained 1× KAPA Probe Fast Universal qPCR kit (KK4701) with fungal DNA of different concentrations as template, 300 nM of each of the forward and

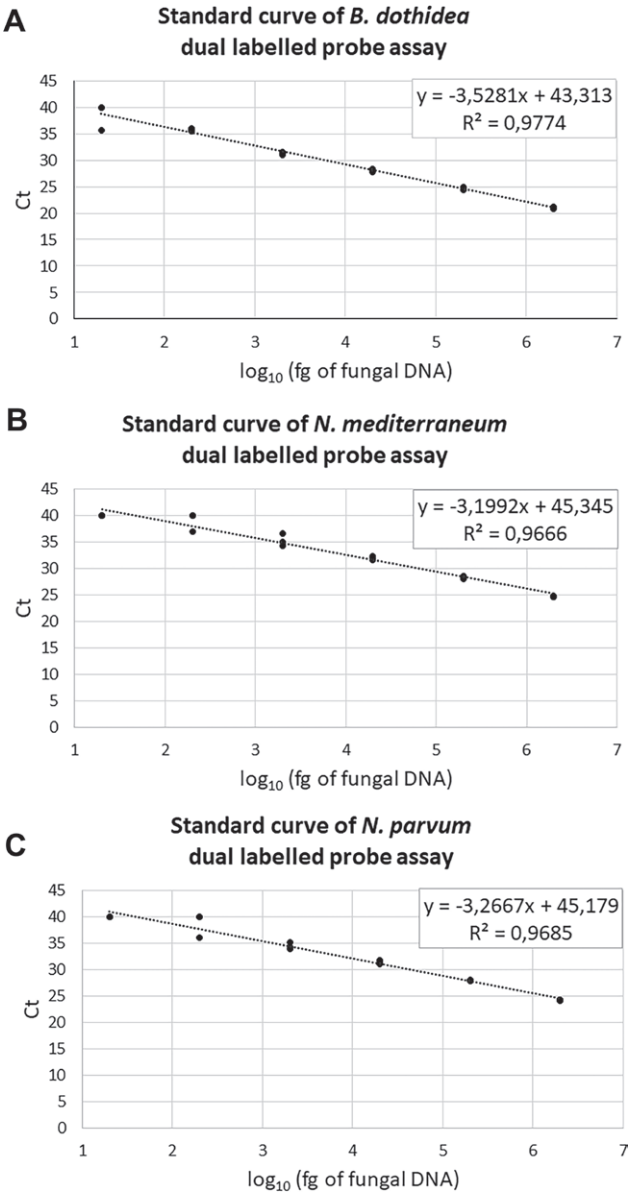


**Figure 1.** Standard curves for *Botryosphaeria dothidea* (A), *Neofusicoccum mediterraneum* (B) and *N. parvum* (C) specific dual labelled probe (TaqMan) assays. Mean cycle threshold (Ct) values of DNA dilution series for each pathogen were plotted against the log of the DNA concentration (three replicates per concentration of fungal DNA tested). Reactions of undetermined values were set at 40.

reverse primers and probe, and 1× Rox dye. The qPCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles each of 3 sec at 95°C and 30 sec at 60°C, without a final extension period. All reactions were performed in a Step One Plus (Applied Biosystems) real time PCR machine cycler.

As a first step, the quality of all genomic DNA samples was assessed using universal primers ITS4 and ITS5. Amplification was performed with KAPA Taq polymerase using the manufacturer's instructions. PCR conditions consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles each of 30 s at 95°C, 30 s at 55°C, and 60 s at 72°C, and a final extension period of 10 min at 72°C. DNA from the following species was used: *B. dothidea*, *N. parvum, N. mediterraneum* MAT1-2-1 strain, while *Diplodia seriata*, *P. chlamydospora* and *N. mediterraneum* MAT1-1-1 strain were included as non-target species.

All three TaqMan assays differentiated the target species from the other two species under study, with no cross-reactions. In particular, the *B. dothidea* dual labelled probe assay reliably detected target DNA up to 2 pg $\mu L^{-1}$, at mean Ct 31.3. The *N. mediterraneum* MAT1-2-1 specific assay detected target DNA up to 20 pg $\mu L^{-1}$, at mean Ct 31.9. Non-target DNA at 8 ng $\mu L^{-1}$ from *Diplodia seriata, P. chlamydospora* and *N. mediterraneum* MAT1-1-1 strain was not detected, while *N. parvum* and *B. dothidea* DNA occasionally produced amplification signal at Ct above 35. The *N. parvum* specific assay detected target DNA up to 2 pg $\mu L^{-1}$ at mean Ct 34.5. Non-target DNA at 8 ng $\mu L^{-1}$ from *Diplodia seriata*, *P. chlamydospora* or *N. mediterraneum* was not detected, while *B. dothidea* produced a non-specific signal at Ct 36 (Table 2). For all assays, linear relationships were observed between log of DNA concentrations (serial dilution) and Ct values (Figure 1). Target DNA at 200 pg $\mu L^{-1}$ for all three assays was detected at similar Ct, even if spiked with 8 ng $\mu L^{-1}$ DNA from pistachio (average Ct = 24 for *B. dothidea* and 27 for *N. mediterraneum* and *N. parvum* assays). Pistachio DNA at 8 ng $\mu L^{-1}$ was detected over Ct 35 for all assays.

Due to the high number of species in the *Botryosphaeriaceae* causing similar symptoms, molecular tools that enable rapid and accurate detection at the pathogen species level are considered to be important for disease management. The present study developed a diagnostic tool to differentiate among *B. dothidea*, *N. mediterraneum* and *N. parvum*. For detection of *N. mediterraneum*, the assay described here allowed detection only of MAT1-2-1 strains. Another diagnostic tool targeting the MAT1-1-1 gene has yet to be designed. The present study focused on detection of these pathogens because they prevail among species isolated from pistachio, grapevine, citrus (Rumbos and Rumbou, 2001; Vakalounakis *et al.*, 2019; Gusella *et al.*, 2021), and other hosts (including olive, pomegranate, and white willow) (personal communications, Dr Tsopelas; Dr E.J. Paplomatas).

The present study developed three TaqMan assays based on sequence variation of the MAT1-2-1 gene (Lopes *et al.*, 2017) that enabled differentiation between

**Table 3.** List of MAT1.2.1 sequences (Accession number, fungal strain information, host, origin and reference) from species of *Botryosphaeriaceae*, that were aligned using Clustal Omega, in order to detect polymorphisms unique for each of *Botryosphaeria dothidea, Neofusicoccum mediterraneum* (MAT1-2-1 strains) and *Neofusicoccum parvum* to design species-specific dual labelled probe qPCR assays.

| | Fungal strain information | Accession Number | Host | Origin | Reference |
|---|---|---|---|---|---|
| 1 | *Botryosphaeria dothidea* | Genome database* | | | |
| 2 | *Botryosphaeria dothidea* MAT1-2-1 | OQ632937 | *Pistacia vera* | Greece | This study |
| 3 | *Neofusicoccum mediterraneum* MAT1-2-1 | OQ596433 | *Pistacia vera* | Greece | This study |
| 4 | *Neofusicoccum parvum* MAT1-2-1 | OQ632936 | *Pistacia vera* | Greece | This study |
| 5 | *Neofusicoccum mediterraneum* strain CAA001 MAT1-2-1 | KX505884.1 | *Pistacia vera* | Portugal | Lopes *et al.*, 2017 |
| 6 | *Neofusicoccum parvum* strain CMW14085 MAT1-2-1 | KX766044.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 7 | *Neofusicoccum parvum* strain CMW9080 MAT1-2-1 | KY612508.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 8 | *Neofusicoccum parvum* strain CMW9081 MAT1-2-1 | KX505872.1 | *Populus nigra* | South Africa | Lopes *et al.*, 2017 |
| 9 | *Neofusicoccum parvum* strain CBS 110301 MAT1-2-1 | KX505873.1 | *Populus nigra* | The Netherlands | Lopes *et al.*, 2017 |
| 10 | *Neofusicoccum eucalyptorum* strain CAA511 MAT1-2-1 | KX505881.1 | *Eucalyptus globulus* | Portugal | Lopes *et al.*, 2017 |
| 11 | *Neofusicoccum mangiferae* strain CBS 118531 MAT1-2-1 | KX505889.1 | *Mangifera indica* | The Netherlands | Lopes *et al.*, 2017 |
| 12 | *Neofusicoccum luteum* strain CMW9076 MAT1-2-1 | KY775143.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 13 | *Neofusicoccum algeriense* strain CBS 137504 MAT1-2-1 | KX505876.1 | *Vitis vinifera* | The Netherlands | Lopes *et al.*, 2017 |
| 14 | *Neofusicoccum algeriense* strain CAA322 MAT1-2-1 | KX505877.1 | *Eucalyptus globulus* | Portugal | Nagel *et al.*, 2018 |
| 15 | *Neofusicoccum cordaticola* strain CMW14124 MAT1-2-1 | KX766043.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 16 | *Neofusicoccum cordaticola* strain CMW13992 MAT1-2-1 | KY612506.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 17 | *Neofusicoccum kwambonambiens*e strain CAA755 MAT1-2-1 | KX505878.1 | *Eucalyptus globulus* | Portugal | Lopes *et al.*, 2017 |
| 18 | *Neofusicoccum kwambonambiens*e strain CMW14155 MAT1 | KX766045.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 19 | *Neofusicoccum kwambonambiens*e strain CMW14023 MAT1 | KY612507.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 20 | *Neofusicoccum ribis* strain CMW7054 MAT1-2-1 | KX766041.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 21 | *Neofusicoccum ribis* strain CMW7772 MAT1-2-1 | KY612509.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 22 | *Neofusicoccum ribis strain* CBS 115475 MAT1-2-1 | KX505879.1 | *Ribes* sp. | The Netherlands | Lopes *et al.*, 2017 |
| 23 | *Neofusicoccum umdonicola st*rain CMW14106 MAT1-2-1 | KX766042.1 | N.A. | South Africa | Nagel *et al.*, 2018 |
| 24 | *Neofusicoccum umdonicola st*rain MAT1-2-1 | KY612510.1 | N.A. | South Africa | Nagel *et al.*, 2018 |

N.A.: not applicable.

*B. dothidea*, *N. mediterraneum* (MAT1-2-1 strains), and *N. parvum*. The developed diagnostic tool is superior to other differentiation methods for *Botryosphaeriaceae*, as it requires no time-consuming steps such as RLFP-PCR (Slippers *et al.*, 2007) or polyacrylamide electrophoresis for SSCP analyses (Ridgway *et al.*, 2011). Furthermore, its practical application will be important, as it is possible to quantify species within infected plant tissues, potentially contributing to studies of pathogen prevalence and species interactions, and epidemiology of the diseases they cause.

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## AVAILABILITY OF DATA AND MATERIAL

Sequence data that support part of the findings of this study are available at the GenBank database under the accession numbers listed in Table 3.

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Short Notes – 12th Special issue on Grapevine Trunk Diseases

# Grapevine histological responses to pruning: the influence of basal buds on tissue defence reactions

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**Summary.** Grapevines require pruning procedures to maintain plant morphology and ensure productivity, and these procedures cause wounds that induce physical and biological host defence mechanisms. Grapevine tissue reactions to wounding resulting from four different pruning methods were assessed. Rapid (immediate) defence reactions were detected in 1-year-old canes with preserved basal buds. Formation of tyloses (≈ 90% of xylem vessels) was observed 1 month later on canes where the basal buds were maintained and no short stubs were left (i.e. the pruning cuts preserved the buds). At 2 months after pruning, lignin was slightly increased in cortical parenchyma after pruning of 3-year-old grapevine wood. Neither callose nor suberin production was observed in healing wounds, as is known in other fruit or broadleaf trees. In 3-year-old canes, fungal hyphae were observed in the non-active wood below the pruning cut surfaces. Preliminary observations of desiccation cones within canes confirmed that the basal buds preserved the canes from desiccation, after comparing different pruning procedures on canes of the same age. After 9 months, the desiccation cones were greater in 3- than 1-year-old wounds.

**Keywords.** Pruning, wood anatomy, tyloses, early tissue defence reactions.



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## INTRODUCTION

High-quality grapevine production depends on the phytosanitary state of vineyards, which includes the management of pruning strategies (Palliotti *et al.*, 2014). Pruning is required to maintain the vine size and shape, through control of shoot numbers and positions (Deloire, 2012), and to remove necrotic plant parts. Reduction of excessive and tangled shoots leads to improved sunlight exposure and air circulation (Palliotti *et al.*, 2014).

Adjusting bud numbers also regulates crop production (Keller *et al*., 2005; Keller, 2020), creating a balance between vegetative growth and grape yields. When optimum balance is achieved, grape quality is enhanced (Kliewer and Dokoozlian, 2005).

Cane pruning and removal produces wounds, and the amount of exposed surface is related to the diameter of the pruning cuts and the pruning method (Dal *et al*., 2008; Dal, 2013). The size of the wounds is related to the age of the cane; pruning an older cane (e.g., more than 2-years-old) creates larger wounds compared with a young cane cut at the same distance from the cane base. In canes of the same age, wound size depends on where the pruning cut is made relative to the shoot base (Sun *et al*., 2006); wound size is smaller if the cuts are made close to the cane apex compared to cuts near the cane base. Faúndez-López *et al*. (2021) and Henderson *et al*. (2021) demonstrated that cutting at distance from cane bases exposes wounds to potential airborne pathogens.

In the last 30 years reports of damage due to fungal wood pathogens in grapevines, i.e. Grapevine Trunk Diseases (GTDs) (Bertsch *et al*., 2013; Guérin-Dubrana *et al*., 2019; Mondello *et al*., 2018) have increased, and research has shown that wounds are their main infection sites for wood pathogens (Úrbez-Torres, 2011; Úrbez-Torres *et al*., 2013; Travadon *et al*., 2015, 2016; Lecomte *et al*., 2018). This has raised concerns about the roles of training systems and pruning methods, which may increase wood exposure to pathogen colonization, infection, and wood degradation by GTD pathogens (Sicavac, 2022).

There is little information on grapevine wood histological reactions to pruning, but this could be important for understanding wound colonization by pathogens. Wound sealing reactions in grapevines consist of tyloses development in xylem vessels. During each growing season, tyloses are early tissue responses near the cut surfaces, but when plants are dormant, wounds induce gel formation that will partially occlude vessels (Sun *et al*., 2006, 2008). Tyloses observed in 1-year-old canes pruned on active vines appeared under the cut surfaces 1 day after pruning, and developed rapidly, occluding the vessels up to 10 mm from the cuts (Sun *et al*., 2006). Following tyloses occlusion, the regions below the cut surfaces showed reduced water flow in vessels, and sap flow rate was negatively correlated with increased tyloses that limited pathogen entrance and impaired xylem function (Zhao *et al*., 2014). As a result, dehydration from reduced water translocation induced formation of necrotic dry areas just below the cut surfaces. These areas have been described as "desiccation cones" (Faúndez-López *et al*., 2021), due to the tapered shapes of the dry wood from the cut surfaces to the inner central wood. The desiccation area, with low tissue water content, is a physical barrier discouraging proliferation of invading microorganisms. The extent of the affected regions is variable (Faúndez-López *et al*., 2021), depending on the diameter, age or location of the removed part, or the grapevine cultivar (Bruez *et al*., 2022).

In summary, grapevines display specific reactions to wounds made during the growing season, consisting of tyloses occlusions in xylem vessels and formation of 'desiccation cones', as consequences of natural dehydration due to vessel deactivation (Faúndez-López *et al*., 2021). As well, "summer pruning wounds" (in actively growing grapevines) do not induce callus production or resin secretion to seal the cut surfaces, as occurs in fruit trees (Brown, 1995).

Considerable research and a recent metadata study (Rosace *et al*., 2023) on effects of winter pruning have focused on factors that most affect the period of grapevine pruning wound susceptibility to fungal colonization, especially timing to reduce wound infections. Late pruning may reduce susceptibility to colonization by increasing defence response in relation to pathogen activity in spring, based on the occurrence of rain, as has been reported in Italy, Spain, and California (Larignon and Dubos, 2000; Serra *et al*., 2008; Rolshausen *et al*., 2010; Úrbez-Torres and Gubler, 2011; Elena and Luque, 2016).

Histological research is required to investigate grapevine reactions in woody tissues to different pruning practices. This could assist selection of efficacious techniques for reducing pathogen infections and increase host defence reactions, and, therefore, wound protection efficacy (Martínez-Diz *et al*., 2021; Di Marco *et al*., 2022) to minimize damage and losses following infections. As a practice to protect pruning wounds, up to now technical operators report that pruning cuts made over the basal buds on canes prevent wood necroses, especially if a stub ("legno di rispetto" in Italian, or "chicot" in French) is left at each pruning site (Simonit, 2013). However, no histological observations have been made on grapevine tissues react to produce physical barriers or active defence substances (i.e., tannins and phenols; Falsini *et al*., 2022), that potentially prevent pathogen entry and colonization.

The present study focused on the early response to pruning wounds in the *V. vinifera* L. 'Trebbiano Toscano', a white grape variety that is widely planted in central and southern Italy. This cultivar displays moderate to very high susceptibility to the Esca complex of diseases (Mugnai *et al*., 1999; Andreini *et al*., 2013; Borgo *et al*., 2016). This study investigated how grapevine tissues reacted during the 9 months after wounding in late pruning (March) on 1-year-old canes. The purpose was

to consider histological reactions within one host phenological cycle, without interfering with the possible interfering carry-over effects into the following season. The main parameters compared were removal or retention of cane basal buds, and the basal portions of the canes (i.e. leaving cane stubs). A preliminary investigation was carried out on the different tissue reactions depending on cane age, comparing 1- and 3-year-old canes.

The aims of this study were: (i) to examine responses in grapevine vessels with tyloses within the first month after pruning; (ii) to document synthesis of defence compounds at the end of the second month after pruning; and (iii) to make preliminary observations of desiccation areas induced 9 months after pruning with four different procedures.

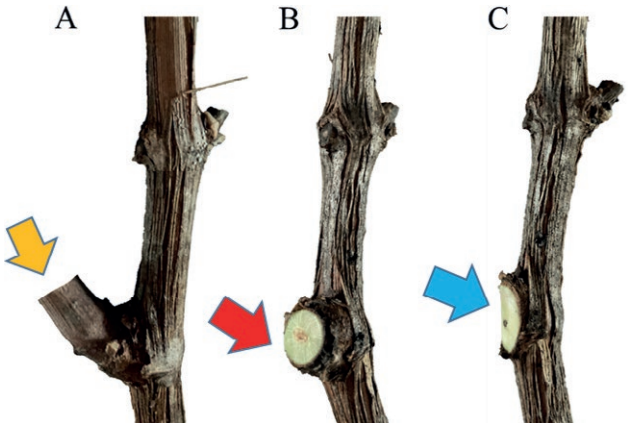


**Figure 1.** A) Cut on a 1-year-old grapevine cane preserving a short stub (treatment designated 1ySS+BB; see text); B) cut on a 1-year-old cane taking off the short stub (designated 1yNoSS+BB); C) cut on a 1-year-old cane removing the basal bud (designated 1yNoSS-NoBB).

## MATERIALS AND METHODS

### *The vineyard*

An experiment was conducted between March and December 2019, in a vineyard of the Azienda Agricola Montepaldi s.r.l., located in the northern part of the Chianti Classico production area of Tuscany (San Casciano in Val di Pesa, Florence, Italy) (43°39'46.8"N, 11°09'16.0"E). The vineyard has a plant density of 5200 plants $ha^{-1}$, the soil is medium textured, and vineyard was managed under integrated agricultural practices with no artificial irrigation. The selected vines were *V. vinifera* 'Trebbiano Toscano', and were 18-years-old and trained to cordons with spur pruning.

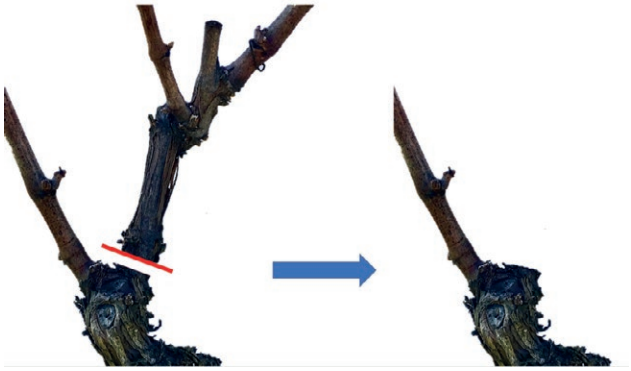

**Figure 2.** Cut on a 3-year-old grapevine spur taking off the short stub but leaving the basal bud (treatment designated 3yNoSS+BB).

### *Pruning methods, sampling and trial set-up*

To obtain information about how wounds react to pruning, four different pruning methods were applied to grapevines in the trial. These were: i) cuts on 1-year-old canes preserving short stubs of lengths twice the their diameters, and therefore preserving the basal buds, (designated 1ySS+BB; Figure 1A); ii) cuts on 1-year-old canes taking off the short stubs but leaving the basal buds (1yNoSS+BB; Figure 1B); iii) cuts on 1-year-old canes removing short stubs and basal buds (1yNoSS-NoBB; Figure 1C); and iv) cuts on 3-year-old spurs taking off the short stubs but leaving the basal buds (3yNoSS+BB; Figure 2). The mean diameters of 1-year-old canes were 0.8 ± 0.2 cm (wound area ≈ 0.5 ± 0.2 $cm^2$), and of 3-year-old canes were 2.1 ± 0.1 cm (wound area ≈ 3.5 ± 0.3 $cm^2$).

Pruning was carried out in March 2019, at the end of the winter season, when the average temperature was 10.5°C. Samplings were carried out either on the same day as pruning (experimental control, T0); or after 1 (T1), 2 (T2), or 9 (T9) months after pruning. Three replicates from different plants (biologically independent) were examined at each sampling time ($n$ = 3), following the methods described by Battiston *et al.* (2022). For each of the four pruning methods (described above), at T0 and T1 the percentages of stem vessels occluded by tyloses were recorded, at T2, light microscopy observations of histological responses were carried out, and at T9, the desiccation areas below the wounds were described.

### *Histological analyses: sample preparation, chemicals and data collection*

Histological studies were carried out on transverse or longitudinal sections (thickness 30–40 μm) made

with a cryo-microtome (Cryo-cut, American Optical), from 1 cm long cane samples. The sections were then mounted on glass microscope slides and stained, following several protocols. Toluidine blue O 0.5% (w/v) in distilled water added sodium carbonate (to give pH = 11.1; Feder and O'Brien, 1968) was used to resolve tissue sections into cell-type components by different colour gradations. Sudan III and IV (Backer, 1946) were used to detect the presence of suberin. Phloroglucinol-HCl (Johansen, 1940) was used to indicate presence of lignin. Tannic and phenolic compounds were stained with Vanillin-HCl (Gardner, 1975). Cellulose and chitin of fungal hyphae were revealed using fluorescence of Calcofluor white staining (Hughes and McCully, 1975).

A Zeiss stereomicroscope equipped with an Optika digital camera and a Leitz D.M.-R.B. Fluo Optic microscope (Wetzler, Germany) equipped with a Nikon DS-Fi3 digital camera were used for qualitative and quantitative analyses of stained tissue samples. Percentage (%) of xylem vessels partially or totally occluded by tyloses (per $mm^2$ of each tissue section) were determined. For this purpose, three 0.5 cm length grapevine canes were sampled from the different plants receiving each of the four pruning methods. The cut surfaces were observed using a Zeiss stereomicroscope equipped with an Optika digital camera.

Tyloses formation was measured at two different times, either immediately after pruning (T0) or 1 month after pruning (T1), and the data obtained were analyzed as indicated below. Histological observations of defence compounds in the tissues immediately under the cut surfaces were made 2 months after pruning (T2), using light microscopy.

To evaluate the morphological features of the desiccation areas resulting from each pruning method, three 2.5 cm long cane cuttings were sampled at T9. The longitudinal section of each short cutting was photographed using a Canon Power Shot SX100 IS camera.

### *Trial design and statistical analyses*

A completely randomized design was used for the field experiment. For each histological observation, three biologically independent replicates were considered ($n$ = 3). Data distributions were checked using the Kolmogorov-Smirnov test, and homoscedasticity was determined using the Brown-Forsythe test. The percentages of xylem vessels occluded with tyloses were analyzed using one-way ANOVA followed by Tukey's multiple-comparison test ($P < 0.05$) to separate groups of means. Diameters of xylem vessels (occluded and non-occluded) underwent Kruskal-Wallis nonparametric analyses, followed by Dunn's multiple-comparison test ($P < 0.05$) to separate mean ranks. The nonparametric test was chosen because the data distribution was not normal (Kolmogorov-Smirnov test, $P \leq 0.05$). Statistical analyses were carried out using Prism8 (GraphPad Software).

## RESULTS

### *Histological analyses*

The overall anatomical regions under the wounded tissue were investigated to describe tissue reaction at different times (T0, T1, T2 and T9) after pruning.

In general, tyloses appeared rapidly within 1 month after pruning cuts had been made, and the tyloses extended up to several mm from the cuts, but there were differences among the pruning methods. Other common responses to multiple stresses such as callose production and cell wall suberification were not detected.

Figure 3 shows transverse sections of the four cut types immediately after cutting (T0) and 1 month later (T1), stained with Toluidine blue O. At T0, in all types of pruning cuts, some vessels already had tyloses at different stages of development. Most of the vessels were partially occluded, and only a small number were totally occluded (Figure 3, A, C, and G). Only vessels from treatment 1yNoSS-NoBB were mostly free from tyloses (Figure 3E).

Plant defence responses included differences in tyloses formation among the four treatments after 1 month. Tyloses development increased particularly from treatment 1yNoSS+BB, where this was increased at T1 (Figure 3D) compared to T0 (Figure 3C). In the 1ySS+BB (Figure 3B) and 3yNoSS+BB (Figure 3H) treatments, the increases in tyloses were less evident compared to 1yNoSS+BB. At T1 from 1yNoSS-NoBB, no appreciable differences were detected compared to T0 (Figure 3F). To support the histological observations, a statistical analysis regarding the numbers of xylem vessels that were partially and totally occluded by tyloses were also assessed, as described below.

Observations of histological responses carried out after 2 months (T2) showed that thin necrotized layers had started to develop below the stem cut surfaces in all the four pruning methods. At the edges of these areas, the host defence responses were investigated using different staining procedures, as shown in Table 1 and in the representative images in Figure 4. Sudan III-IV positive stained tissues were observed in all samples (Figure 4 A), showing suberin deposition only on the cell walls of the cork tissues, but never on the wound surfaces, to protect the living tissues from the exter-

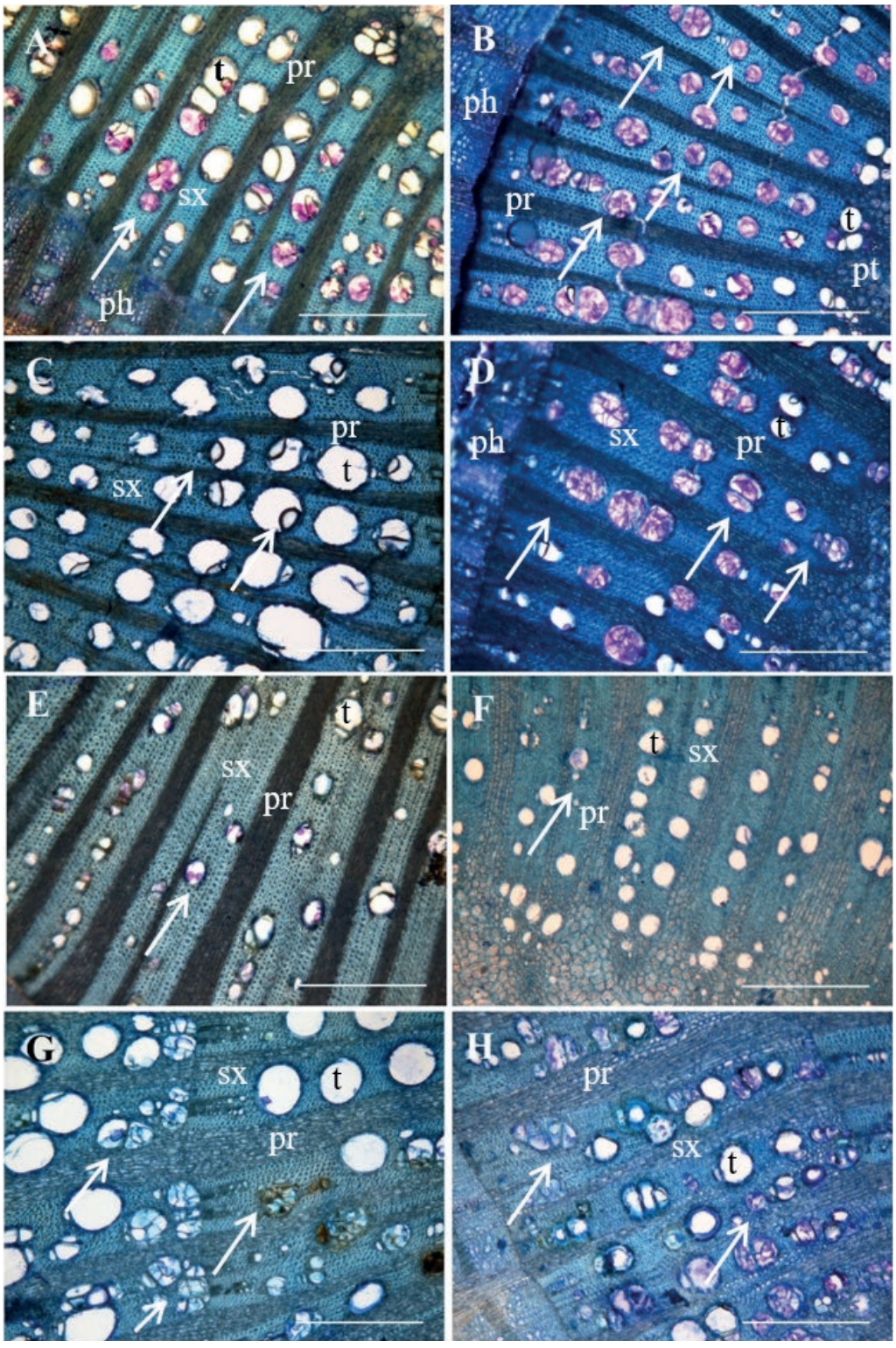


**Figure 3.** Micrographs of pruned grapevine cane cross sections observed after different pruning methods at different times (T, months) after pruning. (A) is for the pruning method designated (see text) as 1ySS+BB at T0, (C) designated 1yNoSS+BB, (E) designated 1yNoSS-NoBB, and (G) designated 3yNoSS+BB; at T1: (B) is from treatment 1ySS+BB, (D) from treatment 1yNoSS+BB, (F) from treatment 1yNoSS-NoBB, and (H) from treatment 3yNoSS+BB at T1. Sections were stained with Toluidine blue. Scale bars: (A, B, D, E, F, G, H) = 250 μm; (C) = 200 μm. White arrows indicate vessels, t trachea, sx secondary xylem, pt pith, ph phloem, and pr parenchyma rays.

**Table 1.** Results from different staining methods to show defence mechanisms of defence observed in the four pruning techniques, including (see text) 1ySS+BB, 1yNoSS+BB, 1yNoSS-NoBB or 3yNoSS+BB, and presence of living fungal hyphae in the wounded tissues.

| Pruning techniques (see text) | Sudan III-IV for suberin | Phloroglucinol HCl for lignin | | Vanillin for tannin | Calcofluor for cellulose and chitin | |
|---|---|---|---|---|---|---|
| | | Cortical parenchyma | Tyloses | | Plant cell walls | Fungal hyphae |
| 1ySS+BB | -* | ++ | - | + | +++ | + |
| 1yNoSS+BB | - | ++ | - | + | +++ | + |
| 1yNoSS-NoBB | - | ++ | - | + | +++ | + |
| 3yNoSS+BB | - | +++ | + | + | ++ | + |

* - = not detected; +, ++, +++ = presence at different levels.

nal stress factors. Although suberin deposition was not detected, other protection mechanisms were observed as physical barriers (Danti *et al*., 2018). For example, lignin deposition was observed onto the cellulose frameworks of the primary walls in the cortical parenchyma. This, in addition to tyloses, produced physical obstacles to pathogen penetration (Sun *et al*., 2006), and is an early response that appears soon after the pruning cuts are made (at T0 and T1). At the boundaries of necrotized regions, lignification (shown from Phloroglucinol-HCl staining), involved longitudinal, continuous layers of parenchyma cells in the cortical cylinders. Thickness of the lignified tissues differed for the different pruning methods (Figure 4, B and C). The most extended and deepest parenchymatic lignified tissues close to the necrotic areas were those formed in treatment 3yNoSS+BB (Figure 4B), and a representative example of a pruning wound in a 1-year-old cane is shown in Figure 4C. The treatment 3yNoSS+BB gave cellular walls modified by lignin deposition in parenchyma, but this thickening process was also observed in tylosis walls (Figure 4D).

In the cortical regions at the borders between dead and living tissues, another defence response was represented by tannin biosynthesis, as a biochemical mechanism for host cell protection in addition to physical barriers created through lignification. Vanillin staining (Figure 4E) showed that tannin accumulation regions were more extended than the lignified areas in parenchyma. Tannin compounds accumulated either in the cell walls or in the vacuoles. Similar tannin production was detected from all the four pruning techniques applied.

Calcofluor reactions confirmed what was shown from Phloroglucinol-HCl treatment, since these are complementary stains. The cellulose components (e.g. in the tylosis walls) were detected in the living cell whereas chitin of fungal hyphae was found in living and dead tissues (Table 1). The experimental treatment 3yNoSS+BB showed dead wood colonized by fungal hyphae (Figure 4F). Thus, in large pruning cuts, the superficial wound tissues (> 2-years-old) underwent dehydration to form large dead areas.

*Descriptions of wood anatomy traits*

The numbers of partially and totally occluded vessels after the four different pruning cut treatments at T0 were compared to the numbers at T1 (Figure 5). In general, the proportions of occluded vessels increased within 1 month after treatment, for all the types of cuts (Figure 5). Presence of basal buds influenced the plant defence reactions, as these were activated more efficiently in 1yNoSS+BB treatment compared to 1yNoSS-NoBB. The proportions of xylem vessels with tyloses at T1 was close to 93% for 1yNoSS+BB compared to 5% for 1yNoSS-NoBB, which was similar to the proportion (2 %) recorded at T0.

Regardless of time after application of treatments (T0 and T1), 3-year-old grapevine cane samples receiving the 3yNoSS+BB pruning method always had xylem vessels with the greatest diameters (Figure 6).

At T0, mean diameters of occluded and non-occluded vessels followed similar trends, i.e. the vessels showed large diameters in the 3yNoSS+BB treatment, but these were smaller for 1yNoSS-NoBB, where the cuts were at the cane nodes and the basal bud were removed. At T1, influence of the different cane cutting methods was apparent. Among all the 1-year-old canes, more occluded vessels were of bigger sizes. Only in treatment 3yNoSS+BB were the non-occluded (mean = 104 μm) and occluded (mean = 93 μm) vessel diameters closely similar (Figure 6).

*Desiccation cones*

The stem desiccation cones were evaluated at the end of the experiment (T9), as indicated by necrotized dry zones at the edges of the cut surfaces of the sampled grapevine canes. The means cone sizes were not significantly different ($P > 0.05$), but these indicated deeper necrotic zones were formed in larger than smaller wounds (3yNoSS+BB), and in wounds with no basal buds and no short stubs. Differences were detected in the sizes and morphologies of the desiccation areas, depending on the pruning method. The necrot-

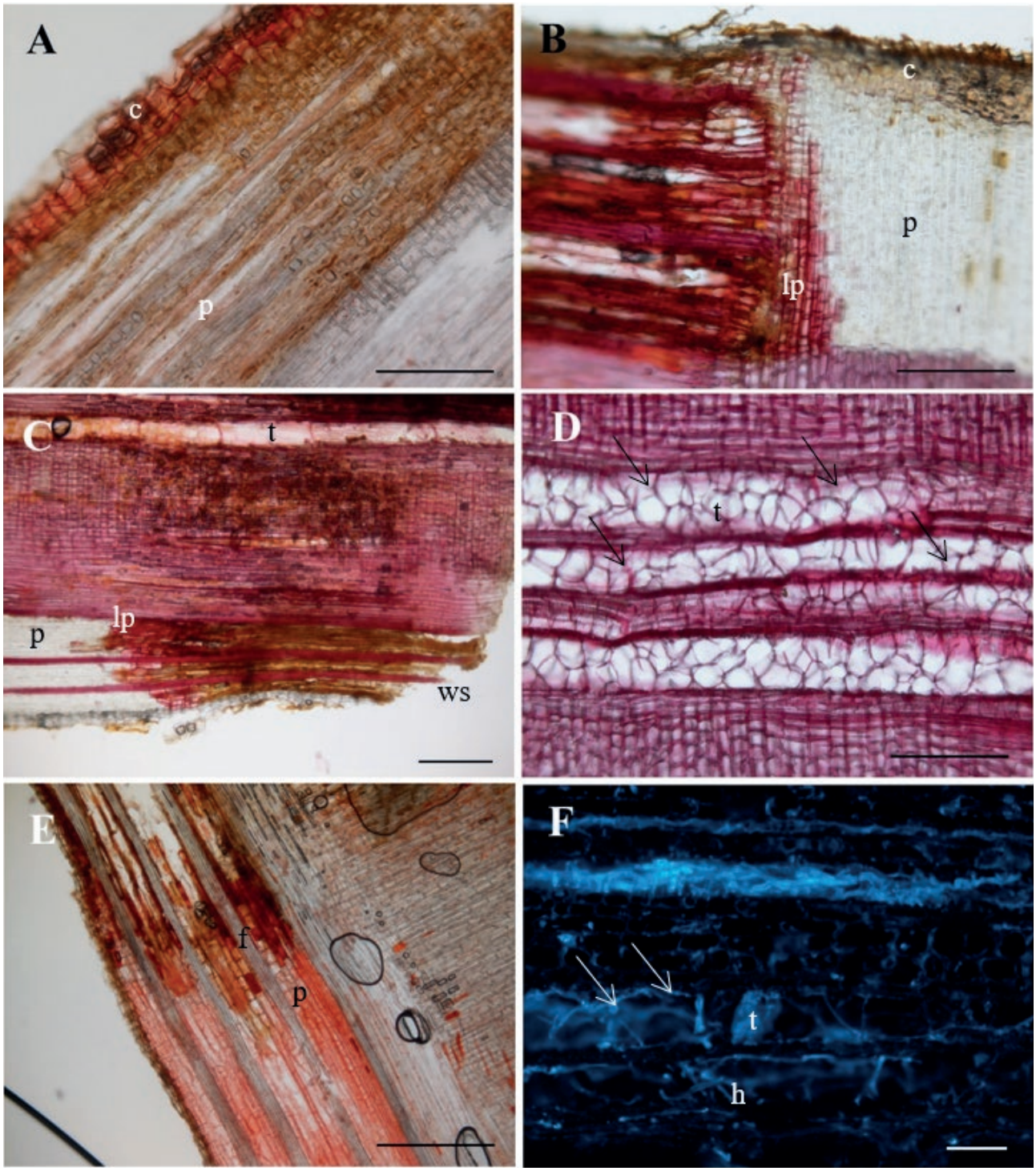


**Figure 4.** Wound tissues of the pruned grapevine canes, from the cork to the depth of vascular cambium at T2 (see text), as representative images of the four different pruning techniques used in this study. The sections were stained with: (A) Sudan III-IV for suberin; (B) Phloroglucinol-HCl for lignin observed after experimental treatment (see text) in 3yNoSS+BB, and (C) in a 1-year-old cane; (D) Phloroglucinol-HCl staining for lignified cell walls of tyloses; (E) Vanillin staining for tannins as a representative image for all four pruning techniques; (F) Calcofluor staining for cellulose and chitin after treatment 3yNoSS+BB; c = cork tissue; p = parenchymatic tissue; lp = lignified parenchymatic tissue; t = trachea; ws = wound surface; white or black arrows = lignified tyloses; h = fungal hyphae.

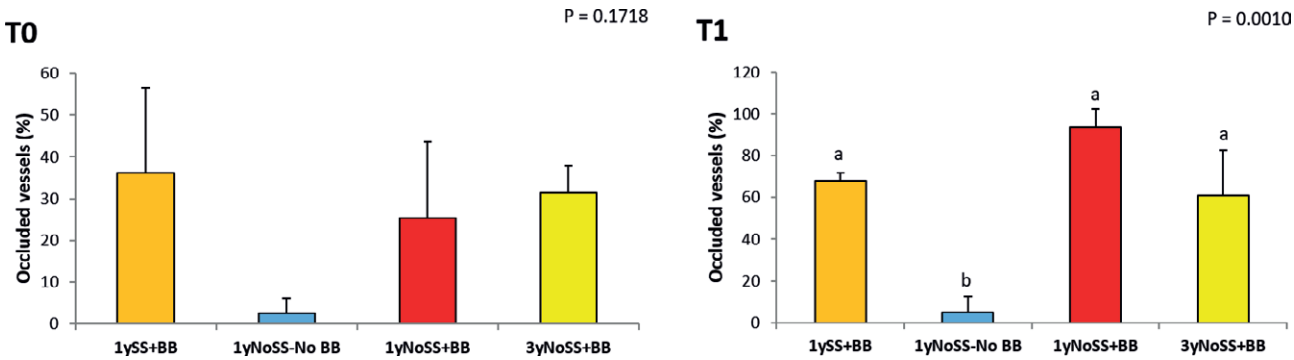


**Figure 5.** Mean percentages of partially and totally occluded grapevine stem vessels found after four different pruning techniques: 1ySS+BB, 1yNoSS-NoBB, 1yNoSS+BB or 3yNoSS+BB at T0 and T1 (see text). The values are means + standard deviations ($n$ = 3). Results for each time point were analyzed using one-way ANOVA, and groups of means were separated by Tukey's multiple comparison test. Different letters indicate differences ($P$ < 0.05) indicated by post-hoc tests.

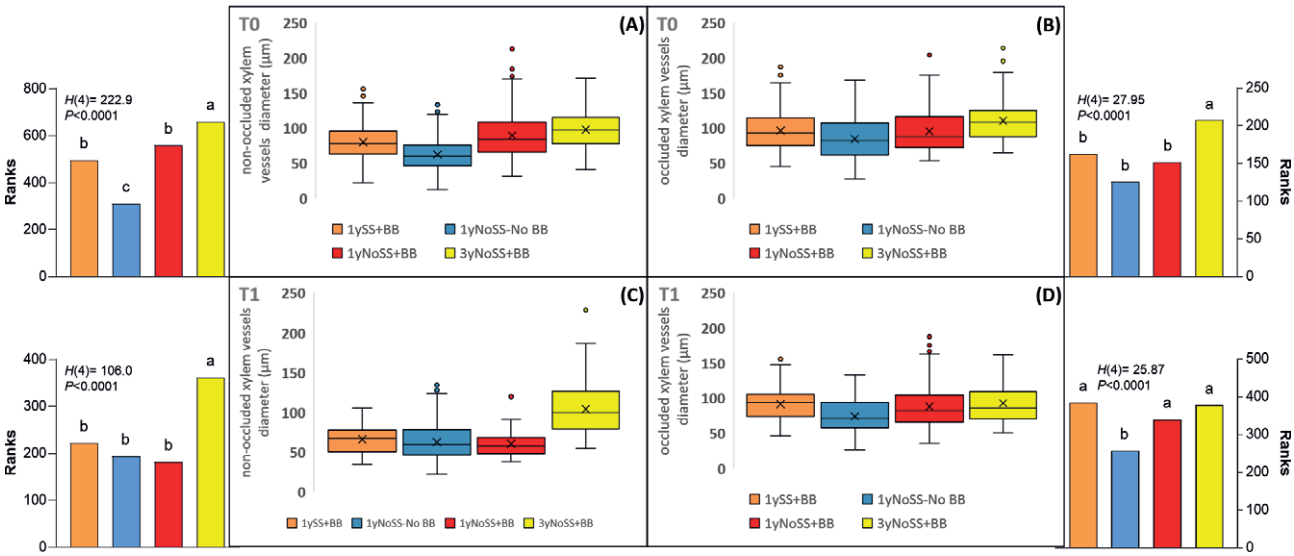


**Figure 6.** Boxplots (A, B, C and D), and respective mean rank analyses (barchart external panels) of mean diameters (µm) of non-occluded and occluded grapevine xylem vessels. Analyses were conducted before (T0) and one month after pruning (T1), using four different pruning techniques: 1ySS+BB, 1yNoSS-NoBB, 1yNoSS+BB or 3yNoSS+BB. In each boxplot, the Tukey whiskers represent maximum and minimum values ($n$ = 3) excluding outliers (represented by dots). Horizontal black lines inside the boxes are the median values, while the crosses represent the treatment means. Results for each time point for both conditions (occluded and non-occluded vessels) were analyzed using Kruskal-Wallis tests, and mean ranks were separated according to Dunn's multiple comparison tests ($P$ < 0.05). Results of mean ranks are plotted on the barchart external panels. Different letters indicate differences ($P$ < 0.05) indicated by post-hoc tests.

ic areas from treatment 3yNoSS+BB had overall mean depth of 4.1 mm, while treatment 1yNoSS-NoBB gave mean depth of 1.2 mm. In contrast in the 1-year-old canes where the basal buds were maintained (treatments 1ySS+BB and 1yNoSS+BB), the necrotized zones developed a few millimeters below the cut surfaces without producing deep desiccation regions in the tissues around the pith, as was observed from treatments 3yNoSS+BB and 1yNoSS-NoBB.

## DISCUSSION AND CONCLUSIONS

In *V. vinifera*, a species with creeping habit, pruning wounds do not heal as in fruit trees, where the stem cambium usually develops new tissues such as callose (Nakashima *et al.*, 2003; Câmpu, 2009; Grünwald *et al.*, 2002; Battiston *et al.*, 2022). Neither does the plant produce suberin in comparison to other trees (Rittinger *et al.*, 1987; Hawkins and Boudet, 1996) to protect the internal living tissues against the external environment.

Different defence mechanisms were adopted by grapevines to seal wounds to prevent the entry of pathogens. The grapevine survival strategy is based on producing new substitution basal shoots, rather than allocating energy to wound healing. The histochemical analysis carried out in the present study has shown the formation of physical barriers to external stressors, consisting of tylosis development in xylem vessels and lignin deposition in living tissues immediately below cut surfaces in grapevine canes.

Biochemical responses of tannins and suberin have been studied as important factors in protecting living tissues (Danti *et al*., 2018; Falsini *et al*., 2022). In the present trials, tannins and suberin highlighted by specific staining reactions were found to be similar in the different pruning cut methods examined. The production of tannins was widely extended in living tissues under the necrotized regions as discussed by other authors also for histopathological studies (Al-Saadoon *et al*., 2012).

The present study has demonstrated that, in 'Trebbiano Toscano' grapevines, winter pruning, applied on 1- or 3-year-old canes at the end of winter (in March) but close to commencement of vegetative growth, caused tissue activation and the rapid development of tyloses, as previously shown by Sun *et al*. (2006) in current year shoots during the growing season. The present study has shown that tylosis initiation was clearly visible 1 month after wounding, with the exception of canes where no basal buds were preserved (treatment 1yNoSS-NoBB). It was anticipated at the onset of this research that, under field conditions, some tyloses were already present at T0 in each type of pruning method, but, at T1, tylosis presence rapidly increased, especially in the samples where the basal buds were retained. Thus, this study allows development an hypothesis that the basal buds are involved in activating the processes of tylosis occlusion of xylem vessels. At T1, the occlusion process increased as from the 1yNoSS+BB treatment where up to 90% of the vessels contained tyloses.

Results from the present study also support, with histological data, observations from previous studies. Faúndez-López *et al*. (2021) showed that pruning cuts over nodes can preserve the basal buds and diaphragms and prevent wood necrosis in the permanent wood structures. Bruez *et al*. (2022) demonstrated that leaving 2–3 cm pruning stubs stopped desiccation cone at the diaphragm, leaving unaffected sap flow. These results were confirmed also by preliminary observations from treatment 1yNoSS-NoBB, where the desiccation areas were deeper than from the 1yNoSS+BB and 1ySS+BB treatments, both of which retained the basal buds. In treatment 3yNoSS+BB, the desiccation cones were even deeper than for 1yNoSS-NoBB, confirming that wounds in older and larger diameter wood than in young canes produces large necrotic areas, as from treatment 1yNoSS+BB, as described by Faúndez-López *et al*. (2021). Thus, comparing 1-year-old samples, we hypothesize that the basal buds on grapevine canes prevent extensive wood necroses. Moreover, comparing the 1yNoSS+BB samples with those from the 3yNoSS+BB treatment (both with basal buds but with different cane ages), the necrotic area depths were influenced by cane age, and thus by wound size.

The light microscopy observations indicated that the more occluded vessels by tyloses were the larger vessels, but no gels were found. Gels entangled between tyloses may increase wound susceptibility to pathogens, by providing substrate for growth and routes to escape occluded vessels (Pouzoulet *et al*., 2017).

Grapevine pruning needs to start with appropriate management of pruning wounds. Even if wound protection reduces pathogen infections (Mounier *et al*., 2016; Di Marco *et al*., 2022), the cutting methods can also influence vine reactions to wounding, and these differences will influence the efficiency and amount of fungal colonization by wood pathogens (Pouzoulet *et al*., 2020, 2022).

Future research should include confirmation of the relationship between fungal colonization from artificial and natural infections in grapevine cuts, applied with different methods, and host histological defensive reactions that impact pathogen colonization and activity. This information could then be used to provide specific guidelines to growers for reducing pathogen entry to, and infection of, their grapevine hosts.

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Research Papers

# *Fusarium oxysporum* f. sp. *lycopersici* biomass variations under disease control regimes using *Trichoderma* and compost

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**Summary.** A comprehensive understanding of population dynamics of pathogens and bioagents in plant rhizospheres is important for improving organic farming. *Fusarium oxysporum* f. sp. *lycopersici* (FOL30) causes Fusarium wilt of tomato. In this study, we compared biomass variations of FOL30 under different disease control regimes, using *Trichoderma asperellum* TA23 strain, compost, or their combination. Biomass variations of FOL30 and TA23 were observed for 13 weeks using quantitative real-time PCR. Separate applications of TA23, compost, and their combination all reduced FOL biomass when compared to experimental controls. Regression analyses of the qPCR data showed that FOL populations fitted curvilinear polynomial order 3 regression models ($R^2$ = 0.87 to 0.95). Areas under the population dynamic curves (AUPDCs; $\log_{10}$ ng DNA week$^{-1}$ g$^{-1}$ soil) were: 43.8 from FOL30 alone, 36.6 from FOL30 plus TA23, 25.4 from FOL30 plus compost, and 25.5 from FOL30 plus TA23 plus compost. These results indicate that the individual applications of TA23 or compost, or their combination, decreased the FOL biomass. The negative correlation between TA23 and FOL30 populations showed that the compost and biocontrol agent reduced FOL pathogen populations. This study demonstrates that compost fortified with *T. asperellum* TA23 decreased FOL populations and reduced disease, and that their use is a promising strategy for managing Fusarium wilt of tomato in organic farming.

**Keywords**. Area Under Population Dynamic Curve (AUPDC), biological control, quantitative PCR.

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## INTRODUCTION

*Fusarium oxysporum* f. sp. *lycopersici* (FOL) is an important soil-borne pathogen, causing serious wilt disease of tomato (*Lycopersicon esculentum*) plants (Srinivas *et al*., 2019). The pathogen is difficult to control with standard cultural and chemical methods. Wilt resistant varieties of tomato are avail-

able, but that resistance can be overcome by the development of new FOL races due to ability of the fungus to evolve in different ways under selection pressure (Biju *et al*., 2017). Growing awareness of the potential hazards from the use of agrochemicals has also led to increased research on alternative methods for effective disease control, including the use of biological control agents. The antagonistic activities of many soil microorganisms against plant pathogens, including *Trichoderma*, *Clonostachys*, *Bacillus* spp., and fluorescent *Pseudomonas* spp., can offer alternative approaches to manage many plant diseases, including Fusarium wilt of tomato (Alabouvette *et al*., 1993; Larkin and Fravel 1998; De Cal *et al*., 1999; Sánchez-Montesinos *et al*., 2021). Biological control is ecologically safe and compatible with different agricultural practices including organic and integrated pest/pathogen management programs (Baker *et al*., 2020).

Organic farming is considered a sustainable and climate-friendly agriculture system, with a possibility to feed the world with organic products. Saudi Arabia is one of 181 countries that strongly promote and adopt organic farming, to reduce water usage, save environments and alleviate negative consequences of chemical-based agriculture (IFOAM, 2020). Application of organic matter such as compost and manure can improve soil quality by increasing water-holding capacity and organic content, along with maintaining exchangeable cations (Reeves, 1997; Etana *et al*., 1999; Balesdent *et al*., 2000; De Corato, 2023). A major challenge with implementing biocontrol strategies is how to maintain stable populations of biocontrol agents throughout crop growing seasons (Lewis and Papavizas, 1984; Waage and Greathead, 1988; Chammem *et al*., 2022). Amendment of organic substrates with biocontrol agents offers a promising solution, where organic substrates have been shown to support the survival of biocontrol agents in soil near plant roots (Hoitink and Boehm, 1999). Composts are naturally suppressive of plant diseases, especially if the composts are amended with biocontrol microorganisms (Abbasi *et al*., 2002; Spadaro and Gullino, 2005).

Understanding the ecology and population dynamics of bioagents and pathogens in the host plant rhizospheres/rhizoplanes provides insights on significance of bioagents for management of crop diseases (Gangwar *et al*., 2013). DNA-based assays are used to monitor populations of microorganisms in soil (Zhang *et al*., 2017), and quantitative real-time PCR (QPCR) can be used to detect, characterize and quantify nucleic acids for numerous applications. QPCR has been widely used to study population dynamics of microorganisms, including pathogenic fungi (Moya-Elizondo *et al*., 2011; Sui *et al*., 2022), bacteria (Hu *et al*., 2013), and to monitor spatial and temporal responses of soil microorganisms to abiotic stresses (Pereira e Silva *et al*., 2012).

Mathematical descriptions of microbial population dynamics can be used to reduce the amount of measured data, to explain observed patterns, to compare growth rates and patterns, and to predict population growth (Karkach, 2006). These descriptions can be modeled using techniques such as empirical, mechanistic and polynomial regression statistics. Empirical models are derived from measures of population size and age, while mechanistic models are derived from differential equations relating growth rates to population size (France and Thornley, 1984). Modelling the development of pathogens, establishing thresholds, and monitoring pest populations facilitate the implementation of integrated disease management in greenhouse crop production systems (Marchand *et al*., 2020).

The present study has used QPCR to investigate biomass variations of the pathogenic FOL30 strain and the biocontrol strain *T. asperellum* TA23, under different soil regimes. Polynomial regression and population dynamic rate models were used to determine the effects of biocontrol agent and compost on FOL populations in soil. The correlation between FOL biomass variations and disease intensity was also measured.

## MATERIALS AND METHODS

### *Fungal strains and composting material*

A pathogenic FOL30 strain was recovered from naturally infected roots of tomato plants showing wilt symptoms, and was morphologically and molecularly characterized by Khan *et al*. (2020). The strain was maintained on Petri plates containing potato dextrose agar (PDA; Difco). The *T. asperellum* strain TA23, originally isolated from soil samples from Riyadh region, Saudi Arabia (El_Komy *et al*., 2015), was obtained from cryogenic storage in the fungal collection at the Fungal and Bacterial Plant Diseases Laboratory, Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, and was maintained on PDA plates.

Compost material used in this study was the commercial compost Al-Reef (Al-Reef Organic Fertilizers Co., Riyadh, Kingdom of Saudi Arabia), with composition of 80% cow manure and 20% vegetable materials.

### *Preparation of fungal inocula for soil infestation*

FOL30 inoculum was prepared by inoculating 500 mL capacity Erlenmeyer flasks each containing 100 mL

of potato dextrose broth (PDB; Difco) with mycelial discs from 10-d-old FOL30 cultures. Inoculated flasks were fitted on a shaker set at 200 rpm and incubated at 25°C for 7 d. Mycelial/conidial suspensions were filtered through a sterile sintered glass funnel (pore size 100 µm) to separate mycelia from conidia. The resulting conidial suspension was then centrifuged at 2000 × *g* for 15 min. The resulting conidial pellets were washed twice with sterile distilled water, vortexed and then re-centrifuged. The final conidial pellets were suspended in sterile distilled water, and suspensions were adjusted to $10^5$ conidia $mL^{-1}$ using a haemocytometer (Hawkley Ltd). This inoculum was applied to infest soils to achieve a final concentration of $10^5$ conidia $g^{-1}$ soil.

*Trichoderma asperellum* strain TA23 was grown on PDA in 90 mm diam. Petri dishes, which were incubated at 25°C for 10 d. Erlenmeyer flasks (500 mL capacity), each containing 100 mL of PDB, were then inoculated with 5 mm diam. mycelium plugs from these 10-d-old cultures. Inoculated flasks were fitted on a shaker set at 200 rpm and incubated at 25°C for 7 d. The contents of the flasks were filtered through four layers of sterile cheesecloth, and the resulting conidial suspensions were centrifuged at 2000 × *g* for 15 min. Conidial pellets were washed twice with sterile distilled water, vortexed and re-centrifuged. After washing, the pellets were suspended in sterile distilled water, and conidial suspensions were adjusted to $10^6$ conidia $mL^{-1}$ using a haemocytometer. The inoculum was applied to soils to achieve a final concentration of $10^6$ conidia $g^{-1}$ soil.

*Soil infestation and plant material*

'Tristar' tomato seeds (Sorouh Agricultural Co.) were surface-sterilized for 30 s in 1% sodium hypochlorite and then rinsed three times with sterile distilled water. The surface-sterilized seeds were pre-germinated in germinating trays containing an autoclaved potting mix of soil, peat moss, and perlite (2:1:1, v:v:v). Then these seeds were incubated in a growth chamber with a 16 h day (24°C) and 8 h night (20°C) cycle at 70% relative humidity. The seedlings were irrigated as needed and fertilized twice each week with 1 g $L^{-1}$ of 20-20-20 (N-P-K) fertilizer (Alahmari Group). The subsequent experiments were carried out on 3-week-old tomato seedlings that had 3-5 fully expanded leaves. Plastic pots (16 cm diam.) were filled with either autoclaved sandy clay soil (1:1 v/v) or a mixture of autoclaved sandy clay soil and Al-Reef Ltd organic compost at a ratio of 4:1. Control pots were filled with 100% autoclaved sandy clay soil.

For soils inoculated with FOL30, the pots were infested by mixing conidial suspension of the fungus with soil at concentration of $10^5$ conidia $g^{-1}$ soil, and were then left for 1 week to allow establishment of the pathogen. The TA23 strain was applied at concentration of $10^6$ conidia $g^{-1}$ soil alone or in combination with compost, and also left for 1 week for the establishment of the fungus. Following the establishment period for FOL30 and TA23, three 3-week-old tomato seedlings were transplanted into each pot.

The experiments were conducted with completely randomized designs, each with five replicates for each treatment (15 plants per replicate). The treatments were: soil (designated T1), soil + compost (T2), soil + TA23 (T3), soil + FOL30 (T4), soil + FOL30 + TA23 (T5), soil + FOL30 + compost (T6), and soil + FOL30 + TA23 + compost (T7). The pot experiments were carried out in the greenhouse of the Plant Protection Department, College of Food and Agricultural Sciences, King Saud University. At the end of the 13th week post FOL-infestation, disease severity (DS) was assessed using the following scoring system of visual foliar symptoms: 1 = no symptoms (i.e., healthy plants with green leaves); 2 = light wilting, one or two yellow leaves; 3 =moderate wilting, three or more yellow leaves; 4 = extensive wilting, dead lower leaves with some wilted upper leaves and stunting; and 5 = dead plants (Horinouchi *et al*., 2007). Disease scores were converted to DS using the following formula: DS = [(A × 1) + (B × 2) + (C × 3) + (D × 4) + (E × 5)]/(total number of plants) × 100, where A, B, C, D, and E are the numbers of plants corresponding, respectively, to scores of 1, 2, 3, 4, and 5.

*Quantification of FOL30 and TA23 strains*

*Extraction of total DNA from fungal cultures*. Two 15 mL capacity Corning tubes, each containing 10 ml of PDB (one tube had $6 \times 10^9$ conidia of FOL30 and the other had $2 \times 10^9$ conidia of TA23) were centrifuged at 2300 × *g* in swing bucket centrifuge (Eppendorf, model 5810R). The pelleted conidia were then re-suspended in 300 µL Microbead solution buffer (MO BIO Laboratories Inc), and DNA isolation was carried out according to instructions for the MO BIO UltraClean® Microbial DNA Isolation Kit. At the final step, DNA was eluted in 50 µL of MD 5 solution. The DNA concentration was measured spectrophotometrically (Nanodrop 2000, Thermo Scientific), and also estimated using agarose gel stained with acridine orange. Each DNA sample was quantified three times and the average was used. Following the DNA quantification, 10-fold serial dilutions ($10^0$ to $10^{-5}$ ng $\mu L^{-1}$) were made of each DNA stock, using ultrapure molecular water (Genekam, Biotechnology).

*Isolation of total DNA from soil.* Soil samples (2-3 cm depth) were collected from tomato rhizospheres after 1, 2, 4, 6, 8, 11, or 13 weeks post tomato seedling planting in FOL-infested and non-infested soils. Three biological replicates were collected from each treatment for QPCR analyses. Total DNA was isolated from soil samples using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc.). Soil (250 mg) was transferred to each Power Bead Tube® and then 60 µL of C1® solution were added. The tubes were vortexed for 10 min, and were then centrifuged at 10,000 × *g* for 30 sec. Approx. 450 µL of supernatant were transferred to a 2 mL capacity clean tube. Two hundred and fifty µL of C2® solution were added to the supernatant. The mixtures were vortexed for 5 s and then incubated at 4°C for 5 min. The tubes were centrifuged at 10,000 × *g* for 1 min at room temperature. Six hundred µL of supernatant were transferred to a 2 mL capacity clean tube, and 200 µL of C3® solution were added to each tube. The tubes were briefly vortexed and incubated at 4°C for 5 min. The tubes were centrifuged at 10,000 × *g* for 1 min, and the supernatants were transferred to clean 2 mL capacity tubes and were each mixed with 1200 µL of C4® solution. Supernatant (650 µL) was then transferred to clean spin filter and centrifuged at 10,000 × *g* for 1 min, and the supernatant was discarded. This washing step was repeated three times by adding additional 650 µL of C4® solution each time. Five hundred µL of C5® solution were then added to the spin filter and centrifuged at 10,000 × *g* for 1 min. The supernatant was discarded, and the spin filter was centrifuged once more. The filter was carefully transferred to another clean 2 mL capacity collection tube. To elute DNA, 100 µL of C6® solution were added to the centre of each white filter membrane and the tube was centrifuged at room temperature for 30 sec at 10,000 × *g*. The flow through solution containing DNA was retained and the spin filter was discarded.

*QPCR reaction and program*. Standard curves were constructed based on cycle thresholds ($C_t$) of 10-fold dilution series ($12 \times 10^0$, $12 \times 10^{-1}$, $12 \times 10^{-2}$, $12 \times 10^{-3}$, $12 \times 10^{-4}$ and $12 \times 10^{-5}$ ng µL$^{-1}$) for *F. oxysporum* genomic DNA preparations, using primer pair Fef1F/Fef2R (Haegi *et al*., 2013), and dilution series ($1 \times 10^0$, $1 \times 10^{-1}$, $1 \times 10^{-2}$, and $1 \times 10^{-3}$ ng µL$^{-1}$) for *T. asperellum* genomic DNA preparations, using primer pair TGP4F/TGP4R (Kim and Knudsen, 2008). $C_t$ values were determined using the Applied Biosystem QPCR software program. The logarithm ($\log_{10}$) of the concentration of each 10-fold dilution series of fungal genomic DNA was plotted along the X axes and the respective $C_t$ values were plotted along the Y axes. The standard curves were constructed using the linear regression equation y = mx + b. Standard curves were established from at least four dilution factors, each with three replicates. Each QPCR reaction contained 3 µL DNA (4 ng µL$^{-1}$ - 40 fg µL$^{-1}$), 7.5 µL of 2′ SYBR green (Applied Biosystems), 0.15 µL of each 10 µM primer (Fef1F/Fef2R for *F. oxysporum* and TGP4F/TGP4R for *T. asperellum*), and 4.2 µL of ultrapure molecular water (Genekam). Negative control reactions contained the same mixtures, each with 2.0 µL of sterile water replacing the DNA template. The QPCR programs each consisted of one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec, and 56°C for 1 min. QPCRs were run using the 7500 Real-Time PCR system (Applied Biosystems). For quantifying FOL30 and TA23 in soil, each QPCR mixture contained 5 µL of DNA extracted from soil, 7.5 µL of 2′ SYBR green, 0.15 µL of each 10 µM primer, and 2.2 µL molecular grade water. The QPCR program was as described above. Three technical replicates were conducted for each biological replicate.

*Statistical analyses*

The data of DS collected from greenhouse experiments were analyzed using analysis of variance (ANOVA) in SAS (SAS Institute) at $P < 0.05$ significance, followed by the least significant difference (LSD) tests. QPCR data were analyzed with Statistix 8.1 analytical software to compute ANOVA for treatments, time (weeks) and treatments × time interactions. Mean separation was accomplished using LSD at $\alpha = 0.05$. Pearson correlation analyses were conducted using Statistix 8.1. In addition, simple polynomial regression for FOL populations was generated from the $\log_{10}$ DNA data using Microsoft Excel 2010. The areas under population dynamic curves (AUPDC) were calculated as $\int_1^{13} at^3 + bt^2 + ct$, and were expressed as population size (ng week$^{-1}$ g$^{-1}$ soil).

## RESULTS

*Effects of treatments on disease severity and FOL30 biomass*

In the pathogenicity experiments, none of the control plants (treatment T1) showed any disease symptoms throughout the experiments. In addition, tomato plants in treatments T2 (soil amended with compost) and T3 (soil amended with TA23) did not show any disease symptoms throughout the experiments (Table 1). However, plants in treatments T4 (soil infested with FOL30) and T5 (soil infested with FOL30 and amended with *T. asperellum* TA23) showed significant disease severity (Table 1). The T6 treatment (soil amended with compost)

**Table 1.** Mean disease severity scores and mean *Fusarium oxysporum* f. sp. *lycopersici* (FOL) biomasses for tomato plants inoculated with FOL and receiving different soil, compost or *Trichderma asperellum* treatments. The pathogenic FOL strain was FOL30 (Khan *et al.*, 2020), and the biocontrol agent *T. asperellum* strain was TA23 (El_Komy *et al.*, 2015).

| Treatment | Disease severity | Biomass of FOL[a] |
|---|---|---|
| T1 (soil) | 0.0 d [b] | 0 d* |
| T2 (soil + compost) | 0.0 d | 0 d |
| T3 (soil + *T. asperellum*) | 0.0 d | 0 d |
| T4 (soil + FOL) | 1.53 b | -1.7363 c |
| T5 (soil + FOL + *T. asperellum*) | 2.0 a | -2.4175 b |
| T6 (soil + FOL + compost) | 1.0 c | -3.205 a |
| T7 (soil + FOL + *T. asperellum* + compost) | 0.0 d | -3.355 a |

[a] Biomass of FOL30 was expressed as $\log_{10}$ ng DNA $g^{-1}$ soil.
[b] Means accompanied by the same letter within each column are not significantly different ($P < 0.05$).

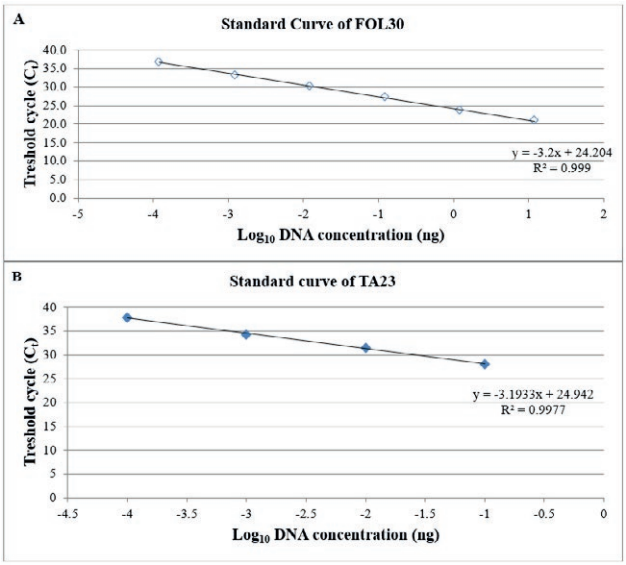


**Figure 1.** Standard curves of fungal DNA concentrations vs threshold cycles ($C_t$). $C_t$ values were plotted against log-transformed DNA amounts and the linear regression equation was calculated. (A) FOL30 DNA concentration standards ranged from 120 fg to 12 ng, and (B) TA23 DNA concentrations ranged from 10 fg to 1 ng.

reduced mean disease severity ($P < 0.05$), while no disease symptoms were recorded on tomato plants in T7 treatment (soil infested with FOL30 strain and amended with both compost and TA23) (Table 1). Individual applications of compost, TA23 or their combination reduced FOL biomass ($P < 0.05$). The greatest reductions were recorded from T6 and T7 treatments, with no significant difference between these treatments. Application of TA23 alone also reduced the FOL biomass compared with the T4 treatment (Table 1).

*Standard curves*

The standard curves for DNA of FOL30 and TA23 showed strong relationships ($R^2 = 0.99$) between $C_t$ and $\log_{10}$ DNA concentrations (Figure 1). These developed standard curves were suitable for detecting DNA at concentrations ranging from 10 to $10^{-4}$ ng. The standard curve slopes obtained for both FOL30 and TA23 were -3.2, with amplification efficiency (E) of 2.05 ($E = 10^{-(1/-3.2)}$), suggesting that the amounts of PCR products were probably doubled during each PCR cycle.

*Tracking of FOL30 and TA23 populations in soil*

Real-time QPCR showed that FOL30 was not detected in soil samples collected from treatments T1, T2 and T3, indicating that neither soil nor compost contained fungi related to *F. oxysporum*. However, FOL30 was detected in all FOL-infested soil samples collected from treatments T4 to T7 (Table 2). FOL30 populations at the first week after infestation were approx. 0.73 $\log_{10}$ DNA from T4 and T5, and 1.11 $\log_{10}$ DNA from T6 and T7. FOL30 decreased sharply at the second week after infestation, where $\log_{10}$ DNA amounts were -0.67 from T4 and T5 treatments, and -2.19 from T6 and T7 treatments. FOL30 populations continued to decease after 4 weeks post infestation, with $\log_{10}$ DNA values of -2.65 from T4, -1.95 from T5, -4.00 from T6, and -3.61 from T7. At the sixth week after infestation, FOL30 populations increased, with $\log_{10}$ DNA values of -1.47 from T4 and -2.92 from T6. However, for T5 and T7, FOL30 populations continued to decrease (-3.36 $\log_{10}$ DNA for T5 and -4.00 for T7). At the eighth week after infestation, FOL populations decreased in for T4 and T6, with $\log_{10}$ DNA values of -1.55 from T4 and -3.93 from T6. The FOL populations increased for T5 (-1.7 $\log_{10}$ DNA) and remained stable for T7 (Table 2). At the 11th week, FOL populations increased either slightly from T4 and T5, or considerably from T6 and T7. The FOL populations then decreased at the 13th week after infestation for treatments T4 to T7 (Table 2).

Populations of the biocontrol agent TA23 were established and detected from treatments T3, T5, and T7. The TA23 populations were relatively stable for T3 within the period of observation (Table 2). For T5, TA23 populations fluctuated, slightly increasing from the 4th week after infestation ($\log_{10}$ DNA -2.78) to the 6th week

**Table 2.** Tracking of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and *Trichoderma asperellum* populations in soil following different experimental treatments.

| Treatment[a] | Weeks after treatments applied [b] | | | | | | | Population |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 6 | 8 | 11 | 13 | |
| T1 (soil) | - | - | - | - | - | - | - | - |
| T2 (soil + compost) | - | - | - | - | - | - | - | - |
| T3 (soil + *T. asperellum*) | - | - | 32.39±1.51 B (-2.30±0.47) | 34.52±3.66 B (-2.96±1.13) | 34.22±1.40 A (-2.87±0.43) | 32.43±0.88 B (-2.32±0.27) | 33.28±3.74 A (-2.58±1.16) | TA23 |
| T4 (soil + FOL) | 21.86±7.04 a (0.73±2.20) | 26.34±2.55 a (-0.67±0.80) | 33.40±3.53 ab (-2.65±1.10) | 30.52±4.02 a (-1.47±1.26) | 29.15±4.37 a (-1.55±1.37) | 26.90±2.90 b (-0.84±0.90) | 28.83±2.75 a (-1.44±0.86) | FOL30 |
| T5 (soil + FOL + *T. asperellum*) | - | - | 33.91±2.01 B (-2.78±0.62) | 33.47±1.97 B (-2.64±0.61) | 35.59±1.04 A (-3.29±0.32) | 35.30±1.60 A (-3.20±0.50) | 33.09±2.15 A (-2.52±0.66) | TA23 |
| | 21.86±7.04 a (0.73±2.20) | 26.34±2.55 a (-0.67±0.80) | 30.45±3.93 b (-1.95±1.23) | 34.95±2.75 a (-3.36±0.86) | 29.63±2.50 a (-1.70±0.78) | 29.26±4.12 ab (-1.58±1.29) | 35.41±2.17 b (-3.50±0.68) | FOL30 |
| T6 (Soil + FOL30 + compost) | - | - | - | - | - | - | - | TA23 |
| | 20.66±4.98 a (1.11±1.56) | 31.22±3.66 a (-2.19±1.14) | 37.00±0.00 a (-4.00±0.00) | 33.53±3.88 a (-2.92±1.21) | 36.77±4.56 b (-3.93±1.43) | 32.10±2.69 a (-2.47±0.84) | 35.29±2.64 b (-3.46±0.83) | FOL30 |
| T7 (Soil + FOL+ *T. asperellum* + compost) | - | - | 37.09±2.03 A (-3.76±0.63) | 36.22±2.27 A (-3.49±0.70) | 35.19±0.74 A (-3.17±0.23) | 34.73±1.19 A (-3.03±0.37) | 35.55±1.97 A (-3.28±0.61) | TA23 |
| | 20.66±4.98 a (1.11±1.56) | 31.22±3.66 a (-2.19±1.14) | 35.76±2.47 a (-3.61±0.77) | 37.00±0.00 a (-4.00±0.00) | 37.00±0.00 b (-4.00±0.00) | 29.71±1.38 ab (-1.72±0.43) | 32.83±2.35 b (-2.69±0.74) | FOL30 |

[a] The pathogenic FOL strain was FOL30 (Khan *et al.*, 2020), and the biocontrol agent was *Trichoderma asperellum* strain TA23 (El_Komy *et al.*, 2015).

[b] Fungal populations were expressed as $C_t$ values and $\log_{10}$ DNA; Up: average of $C_t$ ± Std. dev.; Down: average of biomass ($\log_{10}$ DNA ± Std. dev.). Values accompanied with the same letter (capitals for TA23 and lower case for FOL30) within a column are not significantly different (LSD, α = 0.05).

($\log_{10}$ DNA -2.64), and then decreasing sharply at the 8th week ($\log_{10}$ DNA -3.29), and increasing again in the 11th and 13th weeks after infestation (Table 2). In T7, TA23 populations slightly increased through the 13 weeks of observations (Table 2).

Analysis of the average $C_t$ values of FOL30 and TA23 from the 4th to the 13th sampling weeks showed negative correlation for T5 but positive correlation for T7. For T5 (Soil + FOL30 + TA23), the correlation analysis showed negative correlation (r = -0.90; $P$ = 0.035), while for T7 (Soil + FOL30+ TA23 + compost), this analysis showed positive correlation (r = 0.54; $P$ = 0.35). The positive correlation for the T7 treatment indicates that the compost treatment acted as a substrate promoting and maintaining TA23.

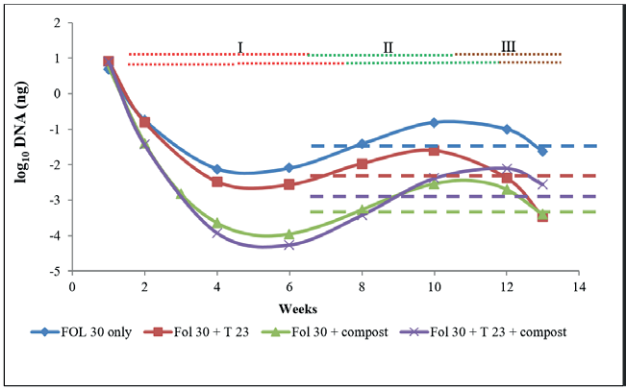


**Figure 2.** Polynomial regression model for FOL30 populations following different experimental treatments. Dash lines represent predicted pathogen capacity. Dotted lines represent three predicted phases: I adaptation (lag) phase, II log (growth) phase, and III stationary phase.

*Polynomial regressions of FOL30 populations*

Data collected from the T4, T5, T6 and T7 treatments of FOL30 populations fitted a third order polynomial regression model ($at^3 + bt^2 + ct + d$), with $R^2$ values of 0.87 for T4, 0.92 for T5, 0.90 for T6, and 0.95 for T7 (Table 3, Figure 2). According to the polynomial regression analysis, FOL30 populations from T4 decreased up to the 5th week from infestation, and increased from the 6th week and reached a maximum at the 10th week

**Table 3.** Mathematical descriptions of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) populations in soil receiving different treatments.

| Treatment [a] | Polynomial regression model [b] | | | | | | Population dynamic rate model [c] | |
|---|---|---|---|---|---|---|---|---|
| | Function (f(t)) | Y *max* | | Y *min* | | $R^2$ | AUPDC [d] | (*dy*/*dt*) | |
| | | Value | Time (week) | Value | Time (week) | | ng week/g soil | ng/week | Slope (m) |
| T4 (soil + FOL) | $= -0.015t^3 + 0.356t^2 - 2.3944t + 2.7361$ | -0.81 | 10 | -2.3 | 5 | 0.87 | 43.84 c | $-0.0456t^2 + 0.712t - 2.3944$ | 0.073 |
| T5 (soil + FOL + *T. asperellum*) | $= -0.019t^3 + 0.4273t^2 - 2.8712t + 3.3825$ | -1.59 | 10 | -2.6 | 5 | 0.92 | 36.59 b | $-0.057t^2 + 0.8566t - 2.8712$ | 1.057 |
| T6 (soil + FOL + compost) | $= -0.0196t^3 + 0.4767t^2 - 3.4323t + 3.7162$ | -2.4 | 11 | -3.9 | 5 | 0.90 | 25.41 a | $-0.0588t^2 + 0.9534t - 3.4323$ | 1.13 |
| T7 (soil + FOL + *T. asperellum* + compost) | $= -0.0202t^3 + 0.5104t^2 - 3.7371t + 4.1515$ | -2.08 | 11 | -4.0 | 5 | 0.95 | 25.46 a | $-0.0606t^2 + 1.0208t - 3.7371$ | 1.172 |

[a] The pathogenic FOL strain was FOL30 (Khan *et al.*, 2020), and the bioagent *Trichoderma asperellum* strain was TA23 (El_Komy *et al.*, 2015).
[b] Model was constructed from 63 data points of $\log_{10}$ DNA (ng) at 1, 2, 4, 6, 8, 11 or 13 weeks
[c] Plotted time value in differential equation (*dy*/*dt*) generated linear trend line for the period from 1st to 13th weeks (see Figure 3b).
[d] Area Under Population Dynamic Curve (AUPDC) was calculated as $\int_1^{13} at^3 + bt^2 + ct$, expressed as population size ($\log_{10}$ ng DNA week$^{-1}$ g$^{-1}$ soil), ($P < 0.05$).

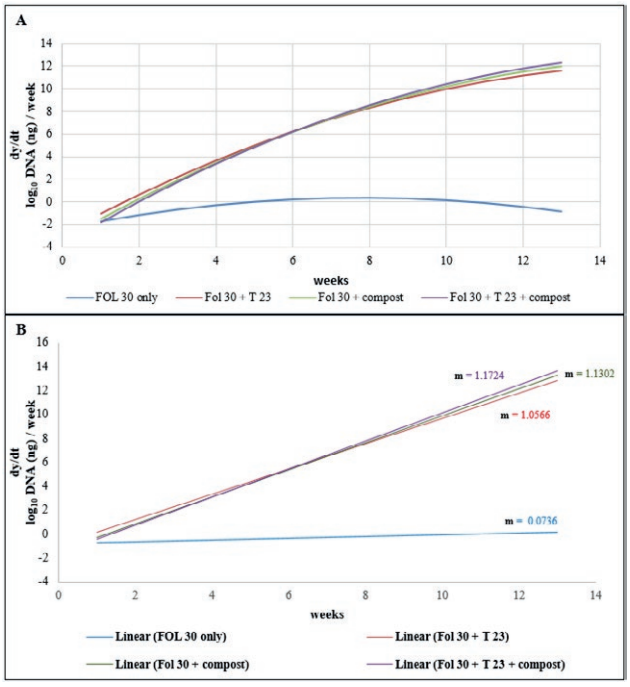


**Figure 3.** (A) Population dynamic rate model of FOL30 in different experimental treatments. Model was generated from differential ( of polynomial models. (B) Linear trend line analysis on the model of FOL30. Straight lines indicate the continuous increasing rate. m = slope values.

(Table 3). For T5, T6 and T7, FOL30 populations also decreased up to the 5th week, then increased from the 6th week and reached maximum at the 10th week for T5 and at the 11th week for T6 and T7 (Table 3). The AUPDC values representing FOL disease potentials were: 43.84 for T4; 36.59 for T5; 25.41 for T6; and 25.46 $\log_{10}$ ng DNA week$^{-1}$ g$^{-1}$ soil for T7 (Table 3). Biomass variation rates from treatments T5 to T7 were obtained by differentiating the polynomials of the regression models in Table 3 with time (dy/dt). The biomass change rates were described as second order polynomials in the form $y = at^2 + bt + c$ (a, b, and c are constants and t is time in weeks), and were plotted against time (week) to obtained the curves in Figure 3 A. To precisely clarify the rates of change of FOL biomass with time, data in Figure 3 A were fitted with linear trend lines (Figure 3 B). This gave different slope values of 0.07 for T4, 1.05 for T5, 1.13 for T6, and 1.172 for T7 (Table 3, Figure 3 B). Slope values reflected continuously increasing dynamic rates of FOL30 biomass with time. Lesser slope values probably indicated stable conditions of the biosystems compared to the greater slope values.

## DISCUSSION

QPCR is a powerful technique for detecting and quantifying nucleic acids in different environments (Filion *et al.*, 2003; Taberlet *et al.*, 2018). For example, Filion

*et al.* (2003) used QPCR to directly detect and quantify DNA of *F. solani* f. sp. *phaseoli* in different substrates, and found no significant differences between amounts of DNA extracted from spore suspensions or from soil infested with known concentrations of *F. solani* f. sp. *phaseoli* conidia. In the present study, biomass of the tomato pathogenic FOL30 strain and the biocontrol agent TA23 were estimated using QPCR. The standard curve constructed for the pathogen had an $R^2$ of 0.999, and that for the biocontrol agent had an $R^2$ of 0.997. The values confirmed the linearity of quantification between exponential increases of DNA concentrations and real-time PCR threshold cycles.

Our results confirmed that autoclaved soil contained very low amounts of DNA that could not be amplified by QPCR ($C_t$ was low and non-repeatable). These results gave confidence as to freedom of the soil used from contamination of non-degraded DNA. Previous studies (Neate *et al.*, 2004; Taberlet *et al.*, 2018) have shown that DNA molecules do not persist in soil, especially under high temperature conditions.

Application of TA23 as a biocontrol competitor, alone or in combination with compost reduced FOL30 biomass when compared to treatment T4 (soil infested only with FOL30). These results were similar to those from previous studies, that have shown *Fusarium* biomass in rhizospheres was reduced due to application of biocontrol agents, e.g. *Trichoderma* and *Bacillus subtilis* fortified with compost (Jangir *et al.*, 2019; Cucu *et al.*, 2020). Similarly, our results demonstrate a significant reduction of FOL30 populations. There was a significant reduction in disease severity of tomato plants with the preventive applications of TA23 in combination with compost, and with compost applied alone. Sawant *et al.* (2017) showed that *Trichoderma* isolates overgrew *Erysiphe necator* and reduced powdery mildew of *Vitis vinifera* by up to 53%. However, the disease severity of tomato plants in FOL-infested soils and treated with TA23 was greater compared to the control plants in FOL-infested soils. This result indicates that the biocontrol TA23 strain increased FOL pathogenicity under certain conditions, e.g. nutrient shortage. Previous studies have shown that nutrient shortage may trigger *Trichoderma*, such as *T. saturnisporum* and *T. viridae*, to become pathogenic to seedlings of cucumber, pepper and tomato (Menzies, 1993; Marín-Guirao *et al.*, 2016).

Strains of *F. oxysporum* have different abilities to colonize soils. These abilities depend on factors related to the strains or to the substrate environment (Couteaudier and Steinberg, 1990; Fravel *et al.*, 2003). In our study, FOL populations from treatment T4 fluctuated through the period of observation. The populations decreased by up to 50% until the 4th week post infestation, then later increased. The decreases in detectable FOL30 DNA indicated that the FOL30 populations adapted to the environment. However, FOL30 populations also decreased after application of treatments T5, T6 and T7. In the presence of the TA23 strain, FOL30 populations showed negative correlations with statistically significant reductions ($r = -0.90$; $P = 0.035$). In a previous study, strain TA23 had high antagonistic activity against FOL isolates under laboratory conditions (El_ Komy *et al.*, 2015). With compost, FOL30 populations also decreased. In general, amendments of soils with compost increases suppressiveness against soil-borne pathogenic fungi (Hoitink and Changa, 2004; Vida *et al.*, 2016). The greatest reduction in FOL30 populations recorded in our study was with the combination treatment of TA23 and compost. However, there were no significant differences in reduction of FOL30 populations between the application of a combination of compost with TA23 and the application of compost alone.

Besides increasing the soil suppressiveness, compost can also be a substrate to establish, promote and maintain biocontrol agents (Leandro *et al.*, 2007; Xu *et al.*, 2011; Gava and Pinto, 2016; Vida *et al.*, 2016). The use of *Trichoderma* strains as biocontrol agents may require formulated products and suitable substrates, e.g. compost, in order to establish and survive in field soils (Leandro *et al.*, 2007). In our study, populations of *T. asperellum* biocontrol strain TA23 fluctuated during all the experiments. Consequently, the use of *Trichoderma* strains as biocontrol agents may require appropriate formulated products and suitable substrates for establishment and survival in field soils (Leandro *et al.*, 2007).

Both mathematical modelling and description have been used to investigate population dynamics of plant pathogens and their biocontrol agents (Couteaudier and Steinberg, 1990; Jeger and Xu, 2015). In the present study, a model of FOL populations under different control regimes best fitted an order 3 polynomial regression model. Polynomial models have been widely used to summarize information from data sets, since these models are simple to fit to experimental data, and statistical distribution properties of the parameters are simpler to calculate when fitted to samples of individuals, than for logistic curves (Goldstein, 1979). In the present study, all the treatments gave similar curves of polynomial regressions, which could be interpreted into three phases. These were adaptation (lag phase), growth phase (exponential phase) and stationary phase. Using polynomial regression models, optimum populations of the FOL pathogen that can be sustained by a soil ecosystem could be predicted in particular time periods.

The present study showed that the pathogen capacity of FOL in the T4 was greater than from the other treatments (disease control regimes), where FOL capacity reduced. This result indicates that disease development was influenced by the pathogen "carrying capacity" of the environment or the host plants (Aylor, 2003; Savarya *et al*., 2018). Application of compost alone or in combination with TA23 may have prolonged the FOL30 growth period, indicating that both the compost and TA23 delayed and reduced the growth of the pathogen. The prolonged lag phase may be an indicator of cellular stress (Hamill *et al*., 2020). Based on the AUPDC values, the individual applications of TA23 or compost, and their combination, reduced FOL30 population size that was expressed as $\log_{10}$ ng DNA week$^{-1}$ g$^{-1}$ soil. Population size may reflect the potential for a pathogen to cause disease.

Dynamic rate models were also constructed from differential equations (dy/dt) of a polynomial regression model for FOL populations. The FOL populations after different potential disease control regimes (T5, T6 or T7) gave high slope values of dynamic rates compared to the control treatment (T4). High slope values of dynamic rate models could be positive indicators of the effectiveness of disease control regimes. The high slope value of the dynamic rate model was possibly achieved because of different factors, e.g. continuous population increase with time, large gaps between minima and maxima FOL30 biomasses, rapid fluctuations in FOL30 populations in certain periods, and/or impacts of the biocontrol agent and compost.

In conclusion, the locally available compost, applied alone or combined with TA23, decreased FOL biomass, and reduced disease severity caused by *F. oxysporum* on tomato plants. Use of the local compost and indigenous *Trichoderma* could therefore be promising environmentally friendly approaches for control of Fusarium wilt in tomato under organic farming systems in Saudi Arabia. The present study also showed that mathematical descriptions provided comprehensive understanding of the population dynamics of the *F. oxysporum* pathogen of tomato.

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Research Papers

# A synergic and compatible microbial-based consortium for biocontrol of Fusarium wilt of tomato

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**Summary.** Bioconsortia, based on *Chaetomium globosum* (isolate CgCG-2), *Pseudomonas putida* (PpTS-1), *Bacillus subtilis* (BsS2BC-1), and *Trichoderma harzianum* (ThS17TH), were designed to develop eco-friendly alternatives for biocontrol of vascular wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). *In vitro* compatibilities of microbes in these consortia were assessed for growth, antagonism, and biocontrol-related gene expression. In these bioassays, the biocontrol isolates had positive interactions for the tested parameters. In pot experiments, seed and soil applications of culture suspensions of five different isolate mixtures were assessed, in comparisons with individual isolates, for efficacy in vascular wilt control after challenge inoculations with *Fol* under polyhouse conditions. Compared to experimental controls, the biocontrol isolate mixtures reduced vascular wilt incidence and promoted plant growth. PpTS-1 + CgCG-2 + ThS17TH was the most effective microbial consortium, giving 71% reduction of Fusarium wilt incidence compared to non-treated controls. This reduced incidence increased plant growth by 135%. Upregulation of genes encoding for allene oxide cyclase, pathogenesis-related proteins 3, and 5, and β-1,3-glucanase in tomato plants indicated that the reduction in vascular wilt by the consortia could be partly plant-mediated. This study provides new insights into the development of microbial-based consortia for the biocontrol of vascular wilt in tomato.

**Keywords.** *Chaetomium*, bioconsortium, tomato, Fusarium wilt, management.

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## INTRODUCTION

Vascular wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* W. C. Snyder & H. N. Hans (*Fol*), is an important disease of tomato. This disease causes yield losses (25 to 55%) and tomato fruit quality reductions (Sidharthan *et al.,* 2018; Sidharthan *et al.,* 2019). This disease is currently being managed with fungicides, but without adequate control. The use of chemicals may affect the environment and cause development of fungicide-resistant pathogen isolates, and is expensive (Shanmugam and Kanoujia, 2011). The use of

resistant cultivars for disease management may be constrained by development of new physiological races of pathogens (McGovern, 2015, Shanmugam *et al*., 2011a). Due to problems associated with the use of chemicals and resistant cultivars (Shanmugam and Kanoujia, 2011), biomanagement of diseases using plant growth-promoting rhizobacteria (PGPR's), particularly fluorescent pseudomonads and *Bacillus* spp., or *Trichoderma* spp., may be an alternative disease management strategy (Aggarwal, 2015), also because these microbes can have growth-promoting activities in crop plants (Jetiyanon and Kloepper, 2002).

In biocontrol of plant diseases, PGPRs and *Trichoderma* spp. act directly on the pathogens by competing for nutrients, space and ecological niches, or by producing antimicrobial compounds, and/or indirectly, through induction of host plant defence mechanisms (induced systemic resistance). This has been reported to be broad-spectrum and long-lasting (Shanmugam and Kanoujia, 2011). Besides these microbes, species of *Chaetomium* (*Ascomycetes*) can have potential for the biocontrol of plant diseases (Aggarwal, 2015). *Chaetomium* spp. commonly exist in soil or organic compost, and are exploited by biotechnology industries due to their ability to produce enzymes such as cellulase and laccase. Among *Chaetomium* spp., *Chaetomium globosum* has been widely used for biocontrol of soilborne and foliar plant pathogens (Aggarwal, 2015; Aggarwal *et al.,* 2016; Darshan *et al*., 2020).

Biocontrol mechanisms of *Chaetomium* spp. are generally attributed to secretion of cell wall-degrading enzymes and antibiotics. They are also known for plant growth stimulation and induction of host resistance (Aggarwal, 2015). Induced resistance by single biocontrol agents of *Bacillus* spp., fluorescent pseudomonads, or *Trichoderma* has been reported, but mechanisms for biocontrol consortia have been less publicized (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2013). Induction of defence enzymes has mostly been implicated in plant-mediated resistance to pathogens (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2013; Aggarwal, 2015).

Fluorescent pseudomonads and sporulating Gram-positive bacteria (e.g. *Bacillus* spp.) have been extensively used to manage a broad spectrum of plant pathogens, including *Fusarium* in many plant species (van Loon *et al.,* 1998; Kloepper *et al.,* 2004). Likewise, *Trichoderma* spp. have also been commonly reported as potential biocontrol agents against *Fusarium* and other pathogens that incited diseases in several crop plants (Asad, 2022). In tomato, fluorescent pseudomonads and species of *Bacillus* or *Trichoderma* have been reported to manage Fusarium wilt (Shanmugam *et al.,* 2011b; 2015). In the biomanagment of crop diseases, deploying a single antagonist often results in inconsistent field performance, due to poor stability of the antagonist in different soil environments (Shanmugam and Kanoujia, 2011). Improved efficacy of biocontrol consortia compared with individual antagonists, has been reported, by employing combinations of bioagents (Shanmugam *et al.,* 2013). These bioagents exhibit different modes of action, and occupy different or complementary niches (Larkin and Fravel, 1998).

*Colletotrichum globosum* could be a candidate in a biocontrol consortium, as this fungus can produce several secondary metabolites. However, inappropriate use of antagonists in combinations may reduce their biocontrol efficacy (Shanmugam *et al.,* 2002; Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2013). Therefore, antagonists designated for isolate mixtures must be carefully evaluated to exploit their fullest potential (Shanmugam *et al.,* 2011b; Shanmugam *et al.,* 2013). Management of Fusarium wilt of tomato involving PGPRs (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2011b), or PGPRs and *Trichoderma* spp. (Shanmugam *et al.,* 2013), has been reported. *C. globosum,* despite being a potential biocontrol agent, has seldom been exploited for *Fol* management in tomato either individually or as a consortium.

The present study aimed to test the antagonistic activities of *Bacillus subtilis*, *Pseudomonas putida*, *Trichoderma harzianum* and *C. globosum* against *Fol*, and design a synergic and compatible microbial consortium exhibiting *in vitro* compatibilities for host plant growth promotion, antagonism and biocontrol gene expression, as attributes of a biocontrol agent for management of vascular wilt control in tomato. Expression of candidate defence-related genes in tomato were also profiled, to identify possible plant-mediated mechanisms of the effective *Chaetomium*-based biocontrol.

## MATERIALS AND METHODS

### *Fungal pathogen and biocontrol isolates*

A highly virulent *Fol* isolate, TOFOL-IHBT (Sidharthan *et al.,* 2018) was used as the pathogen. To prepare inoculum, the fungus was cultured on Potato Dextrose Agar (PDA) in Petri plates, for 7 d at 28°C. Conidium suspension of the isolate were prepared by pouring 20 mL of sterile distilled water into each Petri plate. The suspension was filtered through fine nylon mesh to remove large mycelial parts, was quantified using a haemocytometer, and then adjusted to $10^4$ conidia $mL^{-1}$.

These suspensions were used as inoculum after mixing with 0.05% Tween 80. Either mycelium maintained on the PDA or conidium suspension was used as the pathogen inoculum.

Potential rhizobacterial isolates, TEPF-Sungal-1 (PpTS-1, *P. putida*, GenBank No. MZ363827.1) and S2BC-1 (BsS2BC-1, *B. subtilis*, GenBank No. AM268039), and a *T. harzianum* isolate, S17TH (ThS17TH, GenBank No. GU048855) and a *C. globosum* isolate, CG-2 (CgCG-2, GenBank No. AY429049), elsewhere reported as effective biocontrol agents against soilborne and foliar diseases of ginger and wheat (Shanmugam *et al.,* 2013; Aggarwal *et al.,* 2016), were obtained from laboratory collections, and were assessed as biocontrol isolates for control of vascular wilt of tomato. Pure cultures of the rhizobacterial and fungal isolates were obtained by sub-culturing on nutrient agar/King's B agar for the rhizobacteria, or potato dextrose agar (PDA) for the fungi, and purified cultures were maintained at 28 ± 2°C.

*Evaluation of isolates for antagonism*

The isolates were tested *in vitro* for antagonism against the pathogen by dual culture (DC) assays on PDA plates, in triplicate. In each assay, inoculation of the antagonistic isolate was timed with respect to the growth rate of the pathogen. A 5 mm mycelial disc taken from 7-d-old *Fol* culture was inoculated to the near periphery of one half of a Petri dish containing PDA. A loopful of a 48 h bacterial culture was streaked perpendicular to the pathogen on the opposite side towards the edges of the plate. A mycelial disc (5 mm diam.) of fungal antagonist was placed at the edge of the opposite half of the Petri dish. PDA plates inoculated with the pathogen alone served as experimental controls. Antagonistic effects of the bacterial isolates and CgCG-2 were recorded by measuring the inhibition zones from the bacterial streaks or the edges of the antagonist mycelium to the edges of the pathogen mycelium. Growth inhibition by the *T. harzainum* isolate was assessed using the scale of Shanmugam *et al.* (2013).

The biocontrol isolates were also assessed for antagonism using a 10% cell-free culture filtrate (CFC) using the technique of Vaidya *et al.* (2004). To obtain the CFC filtrates, 50 mL of sterile Potato Dextrose Broth (PDB) in a 250 mL capacity conical flask was inoculated separately with a loopful of *Pseudomonas* grown on nutrient agar or *Bacillus* colonies from King's B agar medium. For *Trichoderma* and *Chaetomium*, 5 mm mycelial discs of actively growing young cultures grown on PDA were used. The flasks were then incubated in shake cultures, at 28 ± 2°C for 2 d for the bacteria, 7 d for *Trichoderma*, or 10 d for *Chaetomium*. These cultures were then coarse-filtered using Buckner flasks. Each filtrate was again filtered through a Millipore filter (pore size 450 nm), and the required quantity of the filtrate was aseptically mixed with a measured volume of PDA slurry to obtain the desired (10%) concentration. The medium was poured into sterile Petri plates (90 mm diam.) and solidified. Mycelial discs (5 mm diam.) were taken from a 7-d-old culture of *Fol*, and placed at the centre of each Petri plate. Plates with only the PDA medium (without the filtrate) were also inoculated with the pathogen as experimental controls. The plates were incubated at 28 ± 2°C, and mycelium growth was measured diametrically when growth in the control plates reached reached the plate perimeters. Mycelial growth inhibition was calculated using the following formula: inhibition of growth (%) = (C–T/C) × 100, where C is the average mycelial growth (diameter in mm) for three replicates in the control, and T is the average for three replicates of growth (diameter in mm) for the treatment plates.

*Antifungal effects of extracellular metabolites of biocontrol isolates*

Mutual inhibition among the biocontrol isolates was initially assessed by the DC and CFC filtrate assays in triplicate, as described earlier, to elucidate their compatibility. Later, each of the biocontrol isolates was evaluated in triplicate for their antagonistic efficacies against the fungal pathogen by the DC assay on PDA in the presence of CFC (10% v/v) of the other biocontrol isolates prepared as described earlier. PDA alone without CFC filtrate served as a control.

*Inhibitory effects of extracellular metabolites of biocontrol isolates*

Compatibility among the biocontrol isolates was also assessed by testing CFC filtrates (10% v/v) of each isolate for their inhibitory effects on growth of the other isolates. For effects of isolates ThS17TH and CgCG-2 on the growth of rhizobacteria, the CFC filtrates of the fungi were prepared as described above, and were incorporated separately into nutrient broth in triplicate. The broth was then inoculated with bacterial cell suspensions grown for 24 h ($1.5 \times 10^8$ cfu $mL^{-1}$). Population of the rhizobacteria was then assessed after 48 h incubation in a rotary shaker, by observing the optical density of the broth in a colorimeter at 600 nm. The cultures of the rhizobacteria without the filtrate were used as experi-

mental controls. Either an increase or no change in the population densities of the treatments compared with the control was considered a positive interaction.

To test the effects of isolates PpTS-1 and BsS2BC-1 on the antagonistic fungi, their respective CFC filtrates, prepared as described above, were incorporated into PD broth (at 10% v/v), and the broth was inoculated with the respective fungal spore suspensions ($1 \times 10^5$ conidia $mL^{-1}$). After incubation at 28 ± 2°C for 7 d on a rotary shaker (180 rpm) in the dark, the resulting mycelium was filtered through pre-weighed filter paper, and the mycelium weight was recorded after drying at 65°C for 4 h. PDB alone was used as the experimental control.

*Effects of differentially treated rhizobacteria and* Trichoderma *isolates on mycelial growth and lytic enzyme production by isolate CgCG-2*

Cells of each isolate were differentially treated, and the differentially treated bacterial cells or spores of isolate ThS17TH were evaluated in triplicate for effects on the growth of isolate CgCG-2. An inductive fermentation broth consisting of cell suspensions (grown for 24 h) of either isolate BsS2BC-1 or PpTS-1 was amended with $1.5 \times 10^8$ cells $mL^{-1}$ into 100 mL of minimal synthetic broth (MSB) (0.2 g of $MgSO_4.7\ H_20$, 0.9 g of $K_2HPO_4$, 0.2 g of KCl, 1.0 g of $NH_4NO_3$, 2 mg of $FeSO_4.7\ H_20$, 2 mg of $ZnSO_4.7\ H_20$, 2 mg of $MnCl_2.7\ H_20$, supplemented with 0.1% glucose; pH 5.6), and was then incubated on a rotary shaker (200 rpm) for 48 h. For live cells, optical density of the broth was adjusted to 0.7 at 600 nm ($1.5 \times 10^8$ cells $mL^{-1}$) with sterile distilled water. For isolate ThS17TH, a conidium spore suspension was prepared as described above for *Fol*, and was amended to 100 mL with MSB. The cell/conidium suspensions were then each inoculated with 1 mL of conidial suspension ($1 \times 10^5$ conidia $mL^{-1}$) prepared from a 5-d-old isolate CgCG-2 culture, and then incubated at 28±2°C for 5 d in shake cultures. The resulting mycelium was collected by centrifugation (5000 rpm, 15 min) and was weighed on sterile Whatman No. 1 filter paper. The weighed mycelia were fine-ground with liquid nitrogen, and were then used for biocontrol gene expression analyses. For heat-inactivated cells, the bacteria-amended MSB was sterilized, and the mycelium was collected as described above. Non-inductive ferment broths without the bacterial suspensions/ThS17TH conidia were similarly prepared as experimental controls.

The CFC filtrates of isolate CgCG-2 grown in MSB alone or MSB amended with heat-inactivated or live cells/conidia were also obtained, and were used as enzyme sources for estimating extracellular enzyme activities. Activity of chitinase (*N*-acetyl-β-D-glucosaminidase, NAGase) of the crude extracellular filtrate was assessed using a chitinase assay kit (Sigma), following the manufacturer's protocol. One unit of chitinase was defined as the amount of the enzyme that released 1.0 µmole $min^{-1}$ of *p*-nitrophenol from 4-nitrophenyl- *N*-acetyl-b-D-glucosaminide, at pH 4.8 and 37°C. For β-1,3-glucanase, the reaction mixture contained 500 µL of 5.0% (w/v) laminarin (Hi-media) in 50 mM acetate buffer (pH 4.8) and 200 µL supernatant from each culture filtrate. The mixture was incubated at 45°C for 30 min, and the reducing sugar produced was determined using dinitrosalicylic acid (El-Katatny *et al.,* 2000). The amount of reducing sugars released was calculated from standard curves recorded for glucose, and enzyme activity was expressed as nkat (nmoles $sec^{-1}$) of glucose released per mL of culture filtrate. One unit of β-1,3-glucanase liberated 1 mg $min^{-1}$ of reducing sugar (measured as glucose) from laminarin, at pH 5.0 and 37°C.

*Effects of differentially treated potential biocontrol isolates on chitinase gene expression in isolate CgCG-2*

Total RNA was isolated from 100 mg of isolate CgCG-2 mycelium using Tri-reagent (Life Tech), as per the manufacturer's instructions. The concentration and purity of RNA were quantified using a nano-Drop ND-100 (Nano-Drop Technologies). The total RNA was treated with DNase (Thermo Scientific), according to the manufacturer's guidelines. First-strand cDNA synthesis was carried out using 1 µg of total RNA and 1 µL of oligo (dT)18 (0.5 µg or 100 pmol), using a first-stand cDNA synthesis kit (Thermo Scientific), following the manufacturer's protocol. The qRT-PCR assay was conducted in triplicate along with a non-template control in a BioRad iCycler, with an initial denaturation for 180 s at 95 °C, 40 cycles of 95°C for 15s, 58°C for 20 s, and 72°C for 20 s, followed by melt curve analysis**.** Alpha-tubulin was used for the normalization of chitinase gene expression. Each 20 µL reaction mixture included 2 µL of the cDNA template, 10 µL of SYBR Green PCR Master Mix (Thermo Fisher) and 250 nM final concentrations of each forward and reverse primer specific to the chitinase gene (Table 1). Comparison of relative gene expression fold changes between the treatments was determined according to the ΔΔCt method (Livak and Schmittgen 2001).

*Preparation of biocontrol inoculum for pot experiments*

Bacterial inoculum was prepared by growing each bacterial isolate in an Erlenmeyer flask (250 mL capac-

**Table 1.** qRT-PCR primers used in this study.

| Organism | Gene | Primer sequence (5'-3') | Reference |
|---|---|---|---|
| *Solanum lycopersicum* | Cyclopilin (*CYP*) | GAGTGGCTCAACGGAAAGCA<br>CCAACAGCCTCTGCCTTCTTA | Shavit *et al.* (2013) |
| | Pathogenesis-related protein 5 (*PR 5*) | AAACGGTGAATGCCCTGGTTCA<br>AGGACCACATGGACCGTGATTA | |
| | Pathogenesis-related protein 3b (*PR 3b*) | GCCCAAACTTCCCATGAAAC<br>CAAGGCCATTGACTACTTGGTG | Sotoyama *et al.* (2015) |
| | β-1,3-glucanase (*glu B*) | CGAGATGGTGGGTACAGAAGAAC<br>CAAGATTGGAAGTGCCAGTAACAGG | Martínez-Medina *et al.* (2013) |
| | Allene oxide cyclase (*AOC*) | GCACGAAGAAGAGAAGAAAGGAGAT<br>CGGTGACGGCTAGGTAAGTTTC | Uppalapati *et al.* (2005) |
| *Chaetomium globosum* | Endochitinase (*chi 46*) | AGGTGCTGGCGGATTATGACG<br>CACATTCCCAATCAGACTCTCG | Liu *et al.*, (2008) |
| | α-tubulin | CCTACGCGCCCGTTGTCTC<br>GAACTGGATGGTGCGCTTGG | |

ity) containing 150 mL of King's B (for isolate PpTS-1) or nutrient broth (for BsS2BC-1) for 48 h at 28 ± 2°C on a rotary shaker. The resulting cells were removed by centrifugation at 6000 g for 12 min at 4°C, and then washed in sterile distilled water. The pellet was resuspended in 0.2 M sodium phosphate buffer (pH 7.0), and the population was adjusted to $3 \times 10^9$ cfu $mL^{-1}$ (0.5 OD at 600 nm = $1 \times 10^8$ cfu $mL^{-1}$). To prepare the inoculum of each fungal biocontrol isolate, a 5 mm mycelial disc was inoculated into 150 mL of PDB, and incubated for 10 d at 28 ± 2°C on a rotary shaker. The mycelium was then removed after passing the culture through muslin cloth, and the spore (or conidium) suspension was used as inoculant after adjusting the concentration to $3 \times 10^7$ cfu $mL^{-1}$. For bioconsortia, the biocontrol isolates designated for mixtures were grown separately on their respective media at 28 ± 2°C, and equal volumes (v/v) of the isolates were used as inoculants.

*Polyhouse evaluation of selected antagonists for Fusarium wilt management*

Experiments with tomato ('Pusa Rohini') were carried out over 90 d (from the day of sowing), in a polyhouse (75% RH and 18–20°C), using a completely randomized experimental design with six replicates of 12 plants per treatment. Single isolates or a designated mixture of the biocontrol isolates were applied as seed treatments or soil applications. For each seed treatment, the tomato seeds were surface sterilized with 1% sodium hypochlorite for 5 min, and were then soaked in the microbial inoculum ($10^4$ spores/conidia $mL^{-1}$). After 12 h, the suspension was drained off, and the seeds were dried for 30 min. The treated seeds were then sown (25 seeds per pot) in 30 cm diam. pots each containing 15 kg of steam-sterilized soil. After 30 d, seedlings were thinned to three seedlings per pot, and 15 d later, the remaining seedlings were inoculated with 50 mL of *Fol* conidium suspension. The microbial inoculants were also applied as soil applications, with the first application made 1 d after pathogen inoculation, and a second application at 15 d later. Non-microbe treated seeds treated with *Fol* served as pathogen experimental controls. Carbendazim (0.1%) applied as a seed treatment, or soil application at 0.2% (i.e., 2 g ai $kg^{-1}$ seed or pot) served as fungicide controls. Vascular wilt development on each tomato plant was rated at 14 d after pathogen inoculations, and a proportional (%) disease index (PDI) was calculated, as described by Shanmugam and Kanoujia (2011). Plant heights (root + shoot lengths) was recorded at the time of harvest to assess plant growth-promoting activity of the biocontrol isolates.

*Tissue collection and defence gene expression assay*

To understand the role of tomato defence genes in disease suppression by the biocontrol isolates, root samples (one per replication for each treatment; six plants per treatment) were collected at 3 or 6 d post-inoculation (dpi) with the pathogen. The samples were washed in running tap water, and were each homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The

homogenized root tissues were stored at −80°C. Total RNA extraction, DNase treatment, cDNA synthesis and qRT-PCR analyses were carried out, using the tomato primer pairs listed in Table 1. In the quantitative analyses, cyclophilin (*cyp*) was used as the housekeeping gene to balance the amount of cDNA in the samples.

*Statistical analyses*

All the experiments were replicated as described, and carried out in completely randomized designs. The pot culture experiments were repeated with similar results, so results from one representative trial are presented. Statistical analyses were carried out using the package IRRISTAT version 92-1, developed by the International Rice Research Institute Biometrics Unit, the Philippines. Differences between treatment mean values were determined following LSD tests (at $P = 0.05$).

## RESULTS

*Antagonism of potential biocontrol isolates against* Fusarium oxysporum

In the DC assays, the isolates BsS2BC-1, PpTS-1, ThS17TH and CgCG-2 all exhibited antifungal activity against *Fol* compared to the controls. Among them, isolate BsS2BC-1 gave mean mycelium growth inhibition of 47.3%, PpTS-1 gave 38.2%, and CgCG-2 gave 41.9% inhibition, and all these isolates displayed zones of inhibition, indicating antibiosis. Isolate ThS17TH exhibited mycoparasitic activity and was classified as producing class I antagonism by overgrowing the pathogen and completely covering the medium surfaces (Table 2 and Supplementary Figure 1).

In the CFC assays, similar to the DC assays, the CFC of antagonists inhibited mycelial growth of *Fol*, and the mean mycelial growth inhibition ranged from 28.7% (isolate ThS17TH) to 59.6% (isolate PpTS-1) compared with control, (data not shown; Supplementary Figure 2). Inhibition of the pathogen by isolate ThS17TH to a level comparable with that of the other biocontrol isolates indicated that this isolate exhibited antibiosis in addition to mycoparasitism (Table 2).

*Antifungal effects of extracellular metabolites from isolates*

In the DC assays for mutual inhibition of the potential biocontrol isolates, no inhibition of the antagonistic fungi was displayed by either of the two rhizobacterial isolates. No mutual inhibition was observed between the two fungal isolates ThS17TH and CgCG-2. Likewise, in the antagonism assay, none of the CFC filtrates from the potential biocontrol isolates inhibited mycelial growth of the other isolates (data not shown; Supplementary Figures 3 and 4). The lack of growth inhibition in the confrontation and antagonism assays indicated compatibility among the isolates. In further studies on the antagonistic effects of each of the biocontrol isolates against the pathogen in the presence and absence of CFC filtrate of each of the other antagonists, the four isolates all exhibited similar or greater efficacies on PDA amended with the CFC filtrate in comparison to their efficacies on the medium without the filtrate. In the presence of CFC filtrate, among the biocontrol isolates, BsS2BC-1 exhibited greatest efficiency (24.5% inhibition) compared with the control, on PDA amended with the CFC filtrate of isolate PpTS-1. In contrast, the antagonistic efficiency of isolate BsS2BC-1 was less (10.9%) on PDA amended with the CFC of isolate PpTS-1. However, isolate PpTS-1 displayed slightly lesser levels of the antagonism (2.9% to 6.7%) compared with the control on PDA amended with the CFC filtrates of isolates BsS2BC-1, ThS17TH and CgCG-2. Like the rhizobacteria, the antagonistic fungal isolate CgCG-2 generally displayed less (0.6%), or better efficacies (2.5% to 7.4%) on PDA amended with the CFC filtrate of the other antagonists in comparison to their efficacies on the medium without filtrate. However, isolate ThS17TH displayed similar levels of the antagonism (class I) on PDA in the presence and absence of the CFC filtrate (Table 3; Supplementary Figure 5). These experiments demonstrated the general non-inhibitory effects of CFC of any of the antagonists on the antifungal activity of the other potential biocontrol isolates.

*Extracellular metabolite effects biocontrol isolates on their growth*

In the colorimetric assays, growth of both rhizobacterial isolates, BsS2BC-1 and PpTS-1, in the MSB amended with the CFC filtrate of any of the other three biocontrol isolates, was significantly greater than that of the control treatment. Among the isolates, BsS2BC-1 exhibited greater population density ($5.7 \times 10^8$ cfu $mL^{-1}$) in the presence of CFCs of isolate PpTS-1, which was 1.48-fold greater than that of the control (MSB alone). In contrast, the population densities of isolate PpTS-1 were not significantly different in the MSB amended with CFC filtrate from any of the three biocontrol isolates. However, populations were greater (0.3- to 0.6-

**Table 2.** Mean colony diameters of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) when grown in association with different species of bacteria or fungi.

| Treatment | Species | GenBank Accession No. | Antagonism against *Fol*[a] | | |
|---|---|---|---|---|---|
| | | | Mycelial growth (mm) of *Fol* in dual culture assays | Class of antagonism[b] | Mycelial growth (mm) of *Fol* in cell free culture filtrate assays |
| PpTS-1 | *Pseudomonas putida* | GU048848 | 55.6 a | - | 36.4 d |
| BsS2BC-1 | *Bacillus subtilis* | AM268039 | 47.4 c | - | 57.7 b |
| CgCG-2 | *Chaetomium globosum* | AY429049 | 52.3 b | - | 44.0 c |
| ThS17TH | *Trichoderma harzianum* | GUO48855 | - | Class I | 64.2 a |
| LSD (*P*=0.05) | | | 3.2 | - | 0.8 |

[a] Values are means of three replications.
[b] Isolates that overgrew the pathogen, covering at least two-thirds of the medium surface in dual culture was considered antagonistic (Shanmugam *et al.,* 2013). *Fol* alone served as experimental controls. Treatment means followed by a common letter(s) are not significantly different from each other (LSD tests, $P = 0.05$).

**Table 3.** Mean population densities or mycelium weights for different potential biocontrol agents when grown in association with other antagonists.

| Treatment[a] | Optical densities (O.D.) and population densities for two rhizobacteria[b] | | | | Mycelial weights (g $L^{-1}$) of two antagonistic fungi[b,d] | |
|---|---|---|---|---|---|---|
| | BsS2BC-1 | | PpTS-1 | | ThS17TH | CgCG-2 |
| | O.D. (600 nm) | Population density ($\times 10^8$ cfu $mL^{-1}$)[c] | O.D. (600nm) | Population density ($\times 10^8$ cfu $mL^{-1}$)[c] | | |
| BsS2BC-1-CFC (10%) | - | - | 2.4 b | 4.7 c | 12.3 a | 8.5 a |
| PpTS-1-CFC (10%) | 2.9 a | 5.7 a | - | - | 11.8 b | 8.7 a |
| CgCG-2-CFC (10%) | 2.0 b | 4.0 b | 2.8 a | 5.5 b | 12.0 a | - |
| ThS17TH-CFC (10%) | 2.1 b | 4.2 ab | 2.9 a | 5.7 a | - | 8.2 b |
| Control (MSB alone) | 1.1 c | 2.3 c | 1.7 c | 3.5 d | 11.4 ab | 8.0 ab |
| LSD ($P = 0$ .05) | 1.7 | 3.2 | 1.3 | 3.2 | 2.2 | 1.9 |

[a]BsS2BC-1, PpTS-1, ThS17TH and CgCG-2, respectively, refer to the biocontrol isolates of *Bacillus subtilis* (GenBank No. AM268039), *Pseudomonas putida* (GenBank No. GU048848), *Trichoderma harzianum* (GenBank No. GU048855) and *Chaetomium globosum* (GenBank No. AY429049).
[b] Values are the means of three replications.
[c] 0.5 OD at 600 nm = $1 \times 10^8$ cfu $mL^{-1}$.
[d] No mutual antagonism was observed among them in dual plates or CFC filtrate assays. Treatment means followed by a common letter(s) are not significantly different from each other (LSD tests; $P = 0.05$).

fold) than the MSB alone (control). The increases in population densities of the rhizobacterial isolates in the MSB amended with CFC filtrate indicated positive interactions with metabolites from the other biocontrol isolates. Unlike the rhizobacterial isolates, though the mycelial weights of isolate ThS17TH in the MSB amended with the CFC filtrate of BsS2BC-1 and CgCG-2 were significantly greater than that of the control (MSB alone), no statistically significant change in mycelial weight was detected for isolate PpTS-1. Likewise, no difference in mycelial weight of isolate CgCG-2 was detected in MSB amended with any of the three other biocontrol isolates. Increased mycelial weight or no change in the mycelial growth indicated positive interactions of the fungal isolates with the metabolites of the other biocontrol isolates (Table 4).

**Table 4.** Growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) by antagonists upon interaction with other antagonists in cell free filtrate (10%) assays.

| Treatment[a] | Inhibition zone (mm)[b] | | Mycelial growth of Fol (mm)[b] | Class of antagonism[b] |
|---|---|---|---|---|
| | BsS2BC-1 | PpTS-1 | CgCG-2 | ThS17TH |
| BsS2BC-1-CFC | - | 9.8 ab | 50.2 b | I |
| PpTS-1-CFC | 13.7 a | - | 48.7 ab | I |
| ThS17TH-CFC | 9.8 d | 10.2 b | 52.6 a | - |
| CgCG-2-CFC | 11.9 b | 10.2 b | - | I |
| Control | 11.0 c | 10.5 a | 49.0 ab | I |
| LSD ($P$ = 0.05) | 1.9 | 1.3 | 1.6 | - |

[a] BsS2BC-1, PpTS-1, ThS17TH and CgCG-2, respectively, refer to the biocontrol isolates of *Bacillus subtilis* (GenBank No. AM268039), *Pseudomonas putida* (GenBank No. GU048848), *Trichoderma harzianum* (GenBank No. GU048855) and *Chaetomium globosum* (GenBank No. AY429049).
[b] Values are means of three replications. Treatment means followed by a common letter(s) are not significantly different from each other (LSD tests; $P$ = 0.05).

*Mycelial growth and lytic enzyme production by isolate CgCG-2, as affected by the potential biocontrol isolates*

In evaluating the effects of live or heat-treated cells of isolates BsS2BC-1 and PpTS-1, or conidia of isolate ThS17TH, mycelial weight of isolate CgCG-2 was similar or greater (0.1- to 0.4-fold) from the treatments than that for the control (MSB alone). Among the treatments, 0.3- to 0.4-fold greater mycelial growth than the control was detected for MSB amended with heat-treated cells of both the rhizobacteria, indicating that the bacterial cells were used as nutrient sources by the biocontrol fungus. No significant differences in mycelial weight over the control were noticed from the treatments of MSB amended with live cells or CFC filtrate of isolate BsS2BC-1 (Table 5). In the enzyme assay, similar to results for mycelial growth, isolate CgCG-2 had greater chitinase (0.5- to 3.0-fold) and β-1,3-glucanase (0.2- to 0.6-fold) activities in the CFC filtrates of MSB amended with the cells/conidia in relation to MSB alone (control). The enzyme activities were greater in the heat-killed cells, with 2.5- to 3.0-fold increases in chitinase activity, and 0.5- to 0.6-fold increases in β-1,3-glucanase activity compared with the MSB control. As observed for the mycelial weight assessments, no statistically significant differences in the enzyme activities were detected for the CFC filtrates of MSB amended with the live cells or the CFC (Table 5).

*Chitinase gene expression in isolate CgCG-2 after different treatments of potential biocontrol isolates*

In the qRT-PCR assays, expression of the endochitinase gene *chi46* was upregulated (1.1- to 5.1-fold) from all the treatments compared to the MSB control. Among the isolate BsS2BC-1 treatments, *chi46* expression was

**Table 5.** Mean mycelial growth and lytic enzyme production by *Chaetomium globosum* isolate CgCG-2 upon interaction with other antagonists.

| Treatment[a] | Mycelial weight (g) of CgCG-2 | Enzyme assays[b] | |
|---|---|---|---|
| | | Chitinase (N-acetyl-β-D glucosaminidase) activity (U/mL of culture filtrate)[c] | Beta-1,3- glucanase (U of glucose/mL of culture filtrate)[d] |
| BsS2BC-1-Live cells | 1.6 cd | 0.9 b | 10.2 b |
| BsS2BC-1-Heat killed cells | 1.9 ab | 1.6 a | 11.6 ab |
| PpTS-1-Live cells | 1.8 ab | 0.9 b | 9.8 bc |
| PpTS-1-Heat killed cells | 2.1 a | 1.4 a | 12.3 a |
| ThS17TH-CFC (10%) | 1.7 cd | 0.6 c | 10.8 ab |
| Control (MSB alone) | 1.5 cd | 0.4 d | 7.9 c |
| LSD ($P$ = 0.05) | 0.2 | 0.4 | 2.2 |

[a] BsS2BC-1, PpTS-1, ThS17TH and CgCG-2, respectively, refer to the biocontrol isolates of *Bacillus subtilis* (GenBank No. AM268039), *Pseudomonas putida* (GenBank No. GU048848), *Trichoderma harzianum* (GenBank No. GU048855) and *Chaetomium globosum* (GenBank No. AY429049).
[b] Values are means of three replications; [b] One unit of each enzyme was defined as the amount of enzyme that will release 1.0 μmole of p-nitrophenol from 4-nitrophenyl-N-acetyl-β-D-glucosaminide per min, at pH 4.8 at 37°C.
[c] One unit $min^{-1}$ of beta-1,3-glucanase liberated 1 mg of reducing sugar (measured as glucose) from laminarin, at pH 5.0 and 37°C. Treatment means followed by a common letter(s) are not significantly different (LSD tests ($P$ = 0.05).

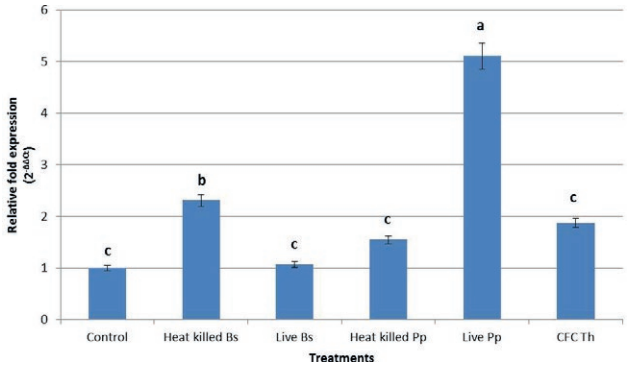


**Figure 1.** Relative expression of the biocontrol gene *chi46* in CgCG-2 on interaction with other antagonists. Control- MSB, Heat killed Bs- Heat killed cells of *Bacillus subtilis* BsS2BC-1, Live Bs- Live cells of *B. subtilis* BsS2BC-1, Heat killed Pp- Heat killed cells of *Pseudomonas putida* PpTS-1, Live Pp- Live cells of *P. putida* PpTS-1 and CFC Th- 10% CFC of *Trichoderma harzianum* ThS17TH. FOL mycelium (1%) was amended as a carbon source in MSB; Error bars indicate SD ($P < 0.05$). The amounts of cDNA in the samples were normalised by employing *α-tubulin* as an internal control.

greater (2.3-fold) in the MSB-containing heat-killed cells, whereas, among the isolate PpTS-1 treatments, expression was strongly upregulated (5.1-fold) in the MSB inoculated with the live cells, and the expression was greater than any of the other treatments. In the isolate ThS17TH treatment, though expression was upregulated (1.9-fold) in comparison to the control, expression was less (0.2- and 1.7-fold) than that of MSB with heat-killed isolate BsS2BC-1 or live cells of isolate PpTS-1 (Figure 1).

*Polyhouse assessments of biocontrol isolates for Fusarium wilt control*

In evaluation of cell suspensions of the potential biocontrol isolates for growth promotion and vascular wilt reduction in tomato, after inoculations with the fungal pathogen, the biocontrol treatments of seed or soil generally increases tomato plant height relative to pathogen controls by 3.5 to 135.4%. Among the biocontrol treatments, except for isolate PpTS-1, the consortia treatments comprising a maximum of three isolates gave the greatest plant heights (101 to 135% increases), and greatest vascular wilt reductions (57% to 71%) compared with the pathogen controls. The isolate CgCG-2 based consortium of PpTS-1 + CgCG-2 + ThS17TH increased plant height by 135%, and gave significantly less (71%) incidence of vascular wilt, compared with the pathogen control. Biocontrol performance of the consortium comprising all four isolates was less than that from the

**Table 6.** Polyhouse evaluation of biocontrol agents in management of Fusarium wilt of tomato.

| Treatment (challenged with *Fol*)[a] | RT+ST (cm)[b] | Vascular wilt (PDI) of tomato[c] |
|---|---|---|
| BsS2BC-1 | 40.7 c | 41.7 bc |
| PpTS-1 | 60.5 b | 25.0 ef |
| ThS17TH | 31.3 d | 39.6 bc |
| CgCG-2 | 31.1 d | 35.4 cd |
| BsS2BC-1+PpTS-1+CgCG-2 | 61.6 ab | 25.0 ef |
| BsS2BC-1+PpTS-1+ThS17TH | 60.3 b | 21.9 ef |
| BsS2BC-1+CgCG-2+ThS17TH | 63.3 ab | 22.9 ef |
| PpTS-1+CgCG-2+ThS17TH | 70.7 a | 16.7 f |
| BsS2BC-1+PpTS-1+ThS17TH+CgCG-2 | 59.5 b | 29.2 de |
| Carbendazim (0.1%) | 33.7 cd | 45.8 b |
| Pathogenic control | 30.1 d | 58.3 a |
| LSD ($P$=0.05) | 0.6 | 1.6 |

[a] BsS2BC-1, PpTS-1, ThS17TH and CgCG-2, respectively, refer to the biocontrol isolates of *Bacillus subtilis* (GenBank No. AM268039), *Pseudomonas putida* (GenBank No. GU048848), *Trichoderma harzianum* (GenBank No. GU048855) and *Chaetomium globosum* (GenBank No. AY429049).-

[b] Mean of 12 replications; RT + ST, root length + shoot length. No wilt incidence was observed in the uninoculated experimental controls. Treatment means followed by a common letter(s) are not significantly different (LSD tests, $P = 0.05$).

[c] The wilt development on each tomato plant was rated as described (Sidharthan *et al.,* 2018): 0 = no symptoms; 1 = <25% of leaves with symptoms; 2 = 26–50% of leaves with symptoms; 3 = 51–75% of leaves with symptoms; 4 = 76-100% of leaves with symptoms. The per cent disease index was calculated as follows: Disease index = (Σ (rating × number of plants rated)/Total number of plants × highest rating) × 100.

mixtures containing three isolates. Among the single-isolate treatments, PpTS-1 gave the greatest increase in plant height (101%) and greatest disease reduction (57%) compared with the pathogen control. However, the plant growth promotion and disease reductions were less than that of the best-performing isolates PpTS-1 + CgCG-2 + ThS17TH consortium. The fungicide treatment was less effective for vascular wilt control and plant growth promotion, giving 21% less wilt and 11% increase in plant height, than the pathogen control (Table 6; Supplementary Figure 6).

*Induction of resistance in tomato plants by the potential bioconsortium*

To elucidate induction of resistance to vascular wilt in tomato plants by the potential bioconsortia identified in the pot experiments, expressions were assessed of the

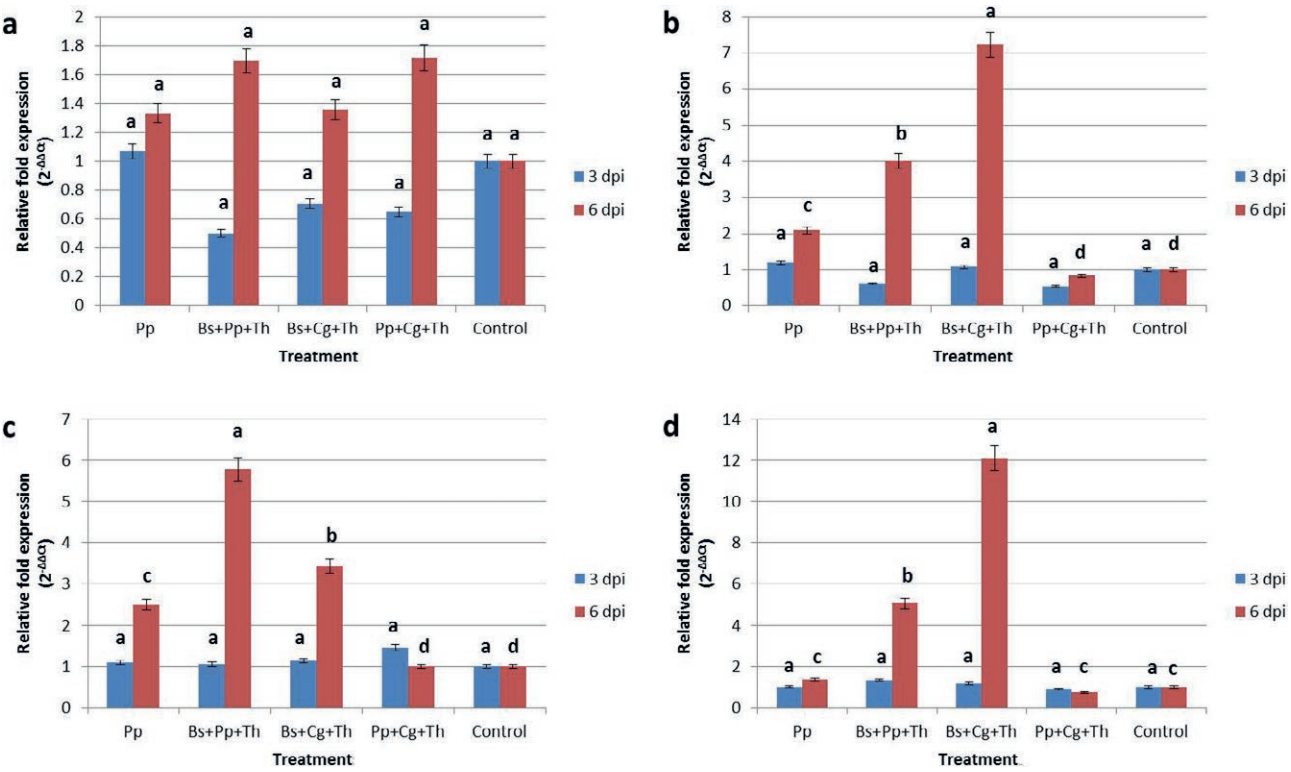


**Figure 2.** Relative expression of defence genes in tomato plants 'Pusa Rohini' (a) *AOC*, (b) *PR3b*, (c) *PR5* and (d) *gluB*. *AOC* is a gene involved in JA biosynthetic pathway (Uppalapati *et al.,* 2005); *PR3b* and *PR5* were identified as marker genes for Systemic Acquired Resistance, SAR (Shavit *et al.,* 2013; Sotoyama *et al.,* 2016); *gluB* was identified as a marker gene for ET modulated pathway (Martínez-Medina *et al.,* 2013). Bs-*Bacillus subtilis* BsS2BC-1, Pp-*Pseudomonas putida* PpTS-1, Th-*Trichoderma harzianum* ThS17TH, Cg-*Chaetomium globosum* CgCG-2 and Control- Pathogenic control; Error bars indicate SD ($P < 0.05$). The amounts of cDNA in the samples were normalised by employing cyclophilin (*cyp*) as an internal control. Blue and red bars indicate samples obtained at 3- and 6-days post pathogen inoculation, respectively.

JA pathway gene *AOC*, and three defence genes, *PR3B*, *PR5* and *gluB* using qRT-PCR assays. At 3 dpi, in comparison to the pathogen control, not significantly greater expressions of *AOC* were observed for any of the biocontrol treatments. However, at 6 dpi, strong expression (1.0- to 1.7-fold) of *AOC* in comparison to that from the control, was observed for all the bioconsortium treatments (Figure 2). Expressions of defence genes modulated by the SA and ET pathways were increased after the biocontrol treatments. Increases for the SA modulated pathway were 2.1- to 7.2-fold for *PR3b* and 2.5- to 5.8-fold for *PR* for treatments with isolate PpTS-1, or the consortia of isolates BsS2BC-1 + PpTS-1 + ThS17TH or isolates BsS2BC-1 + CgCG-2 + ThS17TH. On the contrary, the best-performing treatment PpTS-1 + CgCG-2 + ThS17TH at 6 dpi reduced expression. At 6 dpi, the consortia of isolates BsS2BC-1 + PpTS-1 + ThS17TH and BsS2BC-1 + CgCG-2 + ThS17TH gave greatest expression of the genes *PR3b* (7.2-fold) and *PR5* (5.8-fold). Similarly, *gluB*, the marker gene for the ET modulated pathway, was significantly overexpressed at 6 dpi from the consortia treatments with isolates BsS2BC-1 + CgCG-2 + ThS17TH or isolates BsS2BC-1 + PpTS-1 + ThS17TH, in comparison to the pathogen control. However, *gluB* was down-regulated from the best-performing treatment, isolates PpTS-1 + CgCG-2 + ThS17TH (Figure 2). Induction of the pathway or defence genes for induced resistance indicated that the reductions in vascular wilt by the biocontrol isolates could be partly plant mediated.

## DISCUSSION

In biocontrol of plant diseases, success depends on availability of consortia of effective isolates with varying modes of action and potential to colonise different niches. Both the rhizobacterial isolates BsS2BC-1 and PpTS-1, and the *T. harzianum* isolate ThS17TH used in this study were reported to produce lytic enzymes (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,*

2011b; Shanmugam *et al.,* 2013), whereas isolate CgCG-2 produced antibiotics (Aggarwal *et al.,* 2004; Rashmi *et al.,* 2007). Though the colonizing potential of the biocontrol isolates was not assessed in the present study, the rhizobacteria and fungus biocontrol agents are known to occupy different or complementary niches (Larkin and Fravel, 1998). The bacterial and the *T. harzianum* isolates are potential bioagents when used alone or in combinations for management of Fusarium wilt of tomato or in other crop plants (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2011b; Shanmugam *et al.,* 2013). Testing consortia of these bioagents with the *C. globosum* isolate CgCG-2, another potential bioagent (Aggarwal, 2015) but not previously assessed on tomato, would offer potential benefits, because of the mechanisms outlined above. However, development of a consortium for large-scale applications, needs careful selection and testing of the bioagents for their compatibility, particularly relating to mutual growth inhibition, antagonism against *Fol*, and biocontrol gene expression, as well as validating their biocontrol abilities. The *in vitro* studies outlined here demonstrated compatibility of the biocontrol isolates with the parameters assessed.

Ability of the candidate biocontrol isolates to inhibit *F. oxysporum* f. sp. *lycopersici* in the DC and the CFC filtrate assays indicated their potential efficacies against the pathogen. *In vitro* inhibition of the pathogen by rhizobacterial and *Trichoderma* isolates has been previously reported (Shanmugam *et al.,* 2011b; 2015). The lack of mutual antagonism among the candidate antagonists in either the DC or CFC assays in the present study indicated their compatibility, as well as the suitability of using their cells or metabolites as possible treatments to assess positive interactions for antagonism, host growth increases and antifungal gene or enzyme expressions.

The positive interactions among the biocontrol isolates were also reflected in their antagonistic abilities against *Fol*. Each of the candidate biocontrol isolates displayed greater or similar mycelial growth inhibition of the pathogen in the DC assays, even in the presence of the CFC filtrates of the other potential agents compared with controls without the CFC filtrate. Likewise, in the co-cultivation studies, the CFC filtrates of each of the biocontrol isolates exerted positive interactions with the other isolates, as the bacterial and fungal isolates increased population densities or increased mycelial masses compared to the untreated controls. These results indicate enhanced reliability of the isolates as consortia.

Chitinases and β-1,3-glucanases are potential lytic enzymes elaborated by biocontrol microbes. These enzymes play significant roles in controlling fungal diseases, by degrading chitin and β-1,3-glucan, the chief components of fungal cell walls (Shanmugam *et al.,* 2013; Sharma *et al.,* 2018). Because of the compatibilities identified among the biocontrol isolates, the rhizobacteria and the *Trichoderma* isolates were differentially treated and tested against the *C. globosum* isolate CgCG-2. The CgCG-2 isolate gave greater mycelial growth and lytic enzyme production than the MSB control. Synergism between metabolites of *Pseudomonas* with that of the lytic enzymes of *Trichoderma* in antifungal activity has been previously reported (Woo *et al.,* 2002; Dugassa *et al.,* 2021). Among the treatments, increased mycelial growth and lytic enzyme production were observed for MSB amended with heat-treated cells of both of the rhizobacteria. This indicates the degrading ability of isolate CgCG-2 to use the bacterial cells as its nutrients. Increased production of NAGases, proteases and muramidase to degrade heat-inactivated bacterial cells has been previously reported for different species of *Trichoderma* (Manczinger *et al.,* 2002; Shanmugam *et al.,* 2013). In comparison to heat-treated cells, enzyme production was reduced in the differentially treated bacterial treatments. From an ecological perspective, the CgCG-2 isolate is not supposed to produce high amounts of lytic enzymes to compete with the bacteria for nutrition on co-cultivation. Hence, the relatively lesser production of lytic enzymes from the bacterial treatment could be a positive interaction.

Gene expressions have been extensively studied during interactions of biocontrol isolates. In the co-cultivation of a wild *P. fluorescens* isolate with that of *T. atroviridae,* though a significant reduction in the growth of the fungus was observed, *P. fluorescens* had a positive biocontrol gene expression influence on *T. atroviridae* (Lutz *et al.,* 2004). Similarly, upregulation of secondary metabolite genes in *T. asperellum* was observed upon co-cultivation with *B. amyloliquefaciens* (Karuppiah *et al.,* 2019). The endochitinase gene, *chi46* has been shown to play a major role in the antagonistic ability of *C. globosum* (Liu *et al.,* 2008). Hence, its expression in isolate CgCG-2 isolate was assessed during its growth in differentially treated bacterial cells or fugus conidia. Upregulation of the gene to various levels from all treatments compared to the MSB control indicated positive interactions of the CgCG-2 isolate with that of the other biocontrol isolates. Therefore, the *in vitro* studies showed that each of the four candidate biocontrol isolates was compatible with the others, so these isolates were assessed for vascular wilt control in the polyhouse experiments.

In evaluating the biocontrol efficacies of the isolate mixtures designed based on the *in vitro* experiments, the suspension cultures of the consortia as seed soil treat-

ments reduced vascular wilt incidence and promoted tomato plant growth, in comparison to the pathogen controls. Among the treatments, the isolate CgCG-2 containing consortium of isolates PpTS-1 + CgCG-2 + ThS17TH increased plant height to a greater extent, and gave less vascular wilt incidence, than the pathogen controls. Since the polyhouse provided similar conditions to those applying in fields, this isolate mixture could be suitable for field studies, to assess its applicability for practical biocontrol of vascular wilt. Disease suppression and growth-promoting abilities of biocontrol consortia have been demonstrated for other diseases (Kamalakannan and Shanmugam, 2009; Senthilraja *et al.,* 2010). In the polyhouse experiments of the present study, since the isolate mixture gave better biocontrol under the pathogen challenge, the inoculated plants may have been primed to activate their defence mechanisms. For this reason and to understand the defence mechanisms underlying the biocontrol ability of isolate CgCG-2, the roles of marker defence genes were assessed.

In biocontrol of plant diseases, the bioagents act directly on pathogens and/or through induction of resistance in host plants. Resistances may be systemically induced in plants upon elicitation by pathogens. Specific isolates of non-pathogenic PGPR or fungi induce, respectively, Systemic Acquired Resistance (SAR) or Induced Systemic Resistance (ISR). ISR is characterized by defence priming, where the defence responses are rapidly induced than pathogen attack-inducing resistance, which is metabolically less costly to host plants. Induction of resistance in plants involves hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Pieterse *et al.,* 2009), to activate defence responses. Among the defence responses, the SA pathway commonly induces pathogenesis-related proteins during SAR. In contrast, JA/ET induces proteinase inhibitor (PI), polyphenol oxidase (PPO), arginase, threonine deaminase, leucine aminopeptidase and acid phosphatase during ISR (Shanmugam and Kanoujia, 2011). However, enhanced expression of SA pathway genes by biocontrol treatments, as well as JA/ET pathway genes, has also been reported (Morán-Diez *et al.,* 2012; Kamou *et al.,* 2020). Hence, to elucidate induction of resistance in tomato plants by the potential bioconsortia identified in the pot experiments of the present study, expression was assessed of the JA pathway gene *AOC*, the marker genes *PR3b*, and *PR5* for the SA modulated pathway, and an ET modulated pathway gene, *gluB*, for the best performing biocontrol treatments (Uppalapati *et al.,* 2005; Martínez-Medina *et al.,* 2013; Shavit *et al.,* 2013; Sotoyama *et al.,* 2016).

The SA pathway involves endogenous accumulation of SA and the defence regulatory protein NPR 1 to activate PR genes. Among the PR genes, chitinases accumulate in plants to degrade the cell wall components of fungal pathogens, as part of self defence mechanisms (Shanmugam, 2005). Among chitinases, *PR3b* has been shown to have fungitoxic action (Woloshuk *et al.,* 1991; Sun *et al.,* 2019). Like the chitinases, the *PR5* gene encoding thaumatin-like proteins is known to exhibit antimicrobial activity (Shavit *et al.,* 2013). In the present study, among the pathway genes, strong expression of *AOC* compared to that of for control treatments was observed for all the biocontrol treatments. In further studies on the relative expressions of *PR3b* and *PR5*, though the genes were strongly expressed in the treatments, the combination treatments with isolates BsS2BC-1 + CgCG-2 + ThS17TH and BsS2BC-1 + PpTS-1 + ThS17TH reduced expression compared with the experimental controls. The best performing treatment was that with isolates PpTS-1 + CgCG-2 + ThS17TH. Similar to *PR3b* and *PR5*, the combination treatments with isolates BsS2BC-1 + CgCG-2 + ThS17TH and BsS2BC-1 + PpTS-1 + ThS17TH showed strong expression of *gluB*, which was significantly greater than that from the pathogen controls. However, *gluB* was downregulated in the best-performing treatment with isolates PpTS-1 + CgCG-2 + ThS17TH. Despite the down-regulation of selected defence genes at 3 dpi and 6 dpi, involvement of other tomato defence genes is likely to have contributed to the enhanced performance of the PpTS-1 + CgCG-2 + ThS17TH combination.

Induction of the pathway or defence genes for induced resistance indicated that the reduction in vascular wilt control by the biocontrol isolates could be partly plant-mediated. Previous research has shown that induction of plant defence genes, chitinase, and β-1,3-glucanase can lead to decreased disease incidence (Shanmugam *et al.,* 2011b; 2015). In defence priming, the defence responses are more rapidly induced upon pathogen attack. The lack of early expression of genes (at 3 dpi) observed in the present study requires further investigation.

Beside disease suppression, the biocontrol treatments also promoted the tomato plant growth. This could be due to suppression of pathogens, increased availability of nutrients, or production of plant growth-promoting substances. Nevertheless, elicitation of the plant defence genes observed in this study indicated that the induction of host defence could also be a mechanism for growth promotion.

The *C. globosum*-based microbial consortia could be important for subsistence farming of tomatoes grown with limited rotations, where soilborne diseases can severe problems, fungicide treatments are unaffordable. The consortia identified in the present study have poten-

tial as inputs in integrated disease management systems, once they have been formulated and tested under field conditions. However, consortium efficacy in the field is often primarily determined by the ecological processes (Verma *et al*., 2007; Niu *et al*., 2020). Therefore, effects of the ecological factors on the bioagents in a consortium must be assessed for successful utilisation of isolate mixtures. Unlike most current biocontrol studies, which mainly assess disease incidence at a particular times, temporal dynamics must be evaluated. More research is also required to elucidate associations of biocontrol efficacy and variability with the microbial community structure where the biocontrol action takes place. Careful consideration of these factors is likely to favour field success of identified biocontrol consortia.

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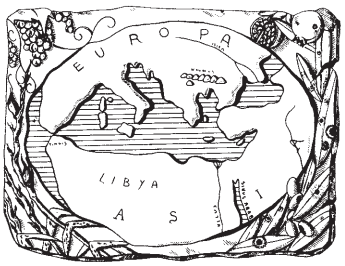


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